Metabolitos secundarios exudados por raíces de plantas de Estrategia I en respuesta a la deficiencia de hierro: caracterización, transporte y función

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Universitat de Lleida

TESI DOCTORAL

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Patricia Sisó Terraza

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida

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ABREVIATURAS

ABC	ATP-binding cassette / Cassete de unión a ATP
ACC	1-aminocyclopropane-1-carboxylic acid / Ácido 1-aminociclopropano-1-carboxílico
ADN	Deoxyribonucleic acid / Ácido desoxirribonucleico
ALMT	Aluminum-activated malate transporter / Transportador de malato activado por aluminio
AVA	Avénic acid A / Ácido avénico A
ATP	Adenosine triphosphate / Adenosín trifosfato
CCoAMT	Caffeoyl CoA-3-*o*-methyltransferase / Cafeoil CoA-3-*o*-metiltransferasa
COMT	Caffeic-*o*-methyltransferase / Cafeico-*o*-metiltransferasa
DMA	2'-deoxymuginic acid / ácido 2'-desoximugineico
DMRL	6,7-dimethyl-8-ribitil lumazine / 6,7-dimetil-8-ribitil lumazina
FAD	Flavin adenine dinucleotide / Flavina adenina dinucleótido
FCR	Ferric chelate reductase enzyme / Enzima quelato reductasa férrica
Fl	Oxidized flavin / Flavina oxidada
FlH·	Semiquinone flavin / Flavina semiquinona
FlH_2	Completely reduced flavin / Flavina completamente reducida
FMN	Flavin mononucleotide / Flavina mononucleótido
FRO	Ferric reductase / Reductasa de quelato férrico
FW / PF	Fresh weight / Peso fresco
F6'H1	Feruoyl-CoA *o*-hydroxylase / Feruoil-CoA *o*-hidroxilasa
GC / CG	Gas chromatography / Cromatografía de gases
GTP	Guanosine triphosphate / Guanosina trifosfato
HAVA	2'-hydroxyvenoic acid / ácido 2'-hidroxiavénico
HMA	3-hydroxy mugineic acid / ácidos 3-hidroximugineico
HPLC	High-performance liquid cromatography / Cromatografía líquida de alta eficacia
IAA	Indole acetic acid / Ácido indolacético
IBA	Indole-3-butyric acid / Ácido indol-3-butírico
IRT	Iron regulated transporter / Transportador regulado por hierro
LMWOA / AOBPM	Low molecular weight organic acids / Ácidos orgánicos de bajo peso molecular
MATE	Multidrug and toxin effux / Transportadores de múltiples fármacos y toxinas
MAs	Mugineic acids / Ácidos mugineicos
MFS	Major facilitator superfamily / Gran familia de transportadores
MS	Mass espectrometry / Espectrometría de masas
MS^n	Tandem mass spectrometry / Espectrometría de masas en tándem
m/z	Mass-to-charge ratio / Relación masa carga
NA	Nicotianamine / Nicotianamina
NADH	Nicotinamide adenine dinucleotide / Nicotinamida adenina dinucleótido
NBDs	Nucleotide binding domain / Dominio de unión a nucleótido
NMR / RMN	Nuclear magnetic resonance / Resonancia magnética nuclear
NO / ON	Nitric oxide / Óxido nítrico
PAL	Phenylalanine ammonia lyase / Fenilalanina amonio liasa
PCA	Protocatechuic acid / Ácido protocatecuico

PDR	Pleiotropic drug resistance / Proteínas resistentes a los medicamentos pleiotrópicos
PEZ	Phenolics efflux zero / Transportador de fenólicos
PM / MP	Plasmatic membrane/ Membrana plasmática
PS / FS	Phytosiderophores / Fitosideróforos
Q-TOF	Quadrupole-time of flight / Cuadrupolo-tiempo de vuelo
Rbfl	Riboflavin / Riboflavina
SAM	S-adenosyl-L-methionine / S-adenosil-L-metionina
TMD	Transmembrane domain / Dominio transmembrana
TOF	Time of flight / Tiempo de vuelo
TOM	Transporter of mugineic acids / Transportador de ácidos mugineicos
UV-VIS	Ultraviolet-visible spectroscopy / Espectroscopía ultravioleta-visible
WT	Wild specie / Especie salvaje
YSL	Yellow stripe-like

ÍNDICE

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RESUMEN / RESUM / SUMMARY

Una característica metabólica importante de las raíces de las plantas es la exudación a la rizosfera de una amplia variedad de compuestos. La regulación de la síntesis y exudación activa de éstos permite que su acción se produzca en el lugar, momento y tiempo adecuados y, por consiguiente, confiere a la planta la capacidad de reaccionar ante variaciones del entorno eludiendo posibles efectos adversos, como cambios osmóticos, ataque de patógenos o escasez de nutrientes, entre otros. Parte de los compuestos exudados son producto del metabolismo secundario y ha sido muy estudiado su papel en la regulación de la comunidad microbiana del suelo, en el fomento de simbiosis beneficiosas y en la inhibición del crecimiento de plantas competidoras. Menos investigada ha sido, sin embargo, la influencia de estos compuestos en la disponibilidad de nutrientes en la rizosfera. En esta Tesis Doctoral se investiga la exudación radicular de compuestos fenólicos y flavinas en respuesta a la deficiencia de hierro (Fe), micronutriente muy abundante en los suelos y generalmente poco disponible. Aunque esta respuesta se conoce desde hace décadas en especies no gramíneas, la identidad y la cantidad de compuestos exudados, los mecanismos de transporte y sus funciones en la rizosfera, son poco conocidos. Para este estudio se han utilizado la especie modelo *Arabidopsis thaliana*, y dos especies de interés agronómico, tomate (*Solanum lycopersicum*) y remolacha (*Beta vulgaris* var. saccharífera).

El estudio constituye el primer análisis exhaustivo y simultáneo de compuestos fenólicos y flavinas acumulados en raíces y exudados al medio de cultivo como respuesta de las plantas a la deficiencia de Fe. Se aplica una aproximación analítica basada en la separación de los compuestos por cromatografía líquida de alta eficacia y su detección por la combinación de espectroscopía de fluorescencia y de ultravioleta-visible con espectrometría de masas de diferentes modalidades. En raíces de *A. thaliana* y tomate, la deficiencia de Fe induce la síntesis y acumulación de compuestos fenólicos de tipo cumarina, y no afecta a la concentración de flavinas. Remolacha no responde a la deficiencia de Fe produciendo cumarinas sino que, de acuerdo a lo ya conocido, sintetiza y acumula flavinas en raíz, y las exuda al medio, principalmente como sulfatos de riboflavina. El tiempo de exposición a la deficiencia de Fe y/o el pH del medio de cultivo afecta a la producción de cumarinas en *A. thaliana* y tomate. Los perfiles de compuestos de tipo cumarina producidos por ambas especies también difieren. Aunque las dos producen cumarinas simples con las sustituciones oxigenadas -OH, -OCH_3 y -O-hexosil, el grado de oxigenación es diferente. Así, en tomate predominan las cumarinas tetra-oxigenadas como trihidroximetoxicumarina frente a las tri- y di-oxigenadas como fraxetina y escopoletina, respectivamente, mientras que en *A. thaliana* son di- y tri-oxigenadas y se producen junto con cumarinolignanos de tipo cleomiscosina. Las cumarinas se acumulan en raíz tanto en formas hexósido como aglicona, con la forma predominante dependiendo de cada cumarina y/o especie vegetal. El perfil de compuestos de tipo cumarina presentes en el medio de cultivo es diferente al observado en las raíces, presentándose en forma aglicona y siendo mayoritarias escopoletina y fraxetina. Esta exudación de cumarinas en *A. thaliana* está mediada por la proteína transportadora AtABCG37.

La retirada de las cumarinas y flavinas exudadas del medio de *A. thaliana* y de remolacha tiene un impacto negativo en la nutrición férrica de estas especies. La cumarina tipo catecol fraxetina y las flavinas en forma reducida permiten la disolución de óxido férrico -forma característica del Fe del suelo- a pHs relevantes desde el punto de vista agronómico. Además, se demuestra que las raíces de las plantas de remolacha deficientes en Fe pueden utilizar flavinas endógenas extracelulares para promover la disolución reductiva de óxidos férricos. El trabajo concluye que la exudación radicular de compuestos de tipo cumarina en plantas de *A. thaliana* y de flavinas en plantas de remolacha permite aumentar la concentración de Fe soluble en suelos con baja disponibilidad de este nutriente y, por lo tanto, mejora la nutrición férrica de estas especies.

Una característica metabòlica important de les arrels de les plantes és l'exsudació a la rizosfera d'una àmplia varietat de compostos. La regulació de la síntesi i exsudació activa d'aquests, permet que la seva acció es produeixi en el lloc, moment i temps adequats i, per tant, confereix a la planta la capacitat de reaccionar davant de variacions de l'entorn eludint possibles efectes adversos, com canvis osmòtics, atac de patògens o l'escassetat de nutrients, entre d'altres. Part dels compostos exsudats són producte del metabolisme secundari i el seu paper ha estat molt estudiat en la regulació de la comunitat microbiana del sòl, en el foment de simbiosis beneficioses i en la inhibició del creixement de plantes competidores. Menys investigada ha estat la influència d'aquests compostos en la disponibilitat de nutrients de la rizosfera. En aquesta Tesis Doctoral s'investiga l'exsudació radicular de compostos fenòlics i flavines en resposta a la deficiència de ferro (Fe), micronutrient molt abundant en els sòls i generalment poc disponible. Tot i que aquesta resposta es coneix des de fa dècades en espècies no gramínies, la identitat i la quantitat de compostos exsudats, els mecanismes de transport i les seves funcions a la rizosfera, són poc coneguts. Per aquest estudi s'ha utilitzat l'espècie model *Arabidopsis thaliana*, i dues espècies d'interès agronòmic, el tomàquet (*Solanum lycopersicum*) i la remolatxa (*Beta vulgaris* var. saccharífera).

L'estudi constitueix el primer anàlisi exhaustiu i simultani de compostos fenòlics i flavines acumulats a les arrels i exsudats al medi de cultiu com a resposta de les plantes a la deficiència de Fe. S'aplica una aproximació analítica basada en la separació dels compostos per cromatografia líquida d'alta eficàcia i la seva detecció per la combinació d'espectroscòpia de fluorescència i d'ultravioleta-visible amb espectrometria de masses de diferents modalitats. En arrels de *A. thaliana* i tomàquet, la deficiència de Fe indueix la síntesi i acumulació de compostos fenòlics de tipus cumarina, i no afecta la concentració de flavines. La planta de remolatxa no respon a la deficiència de Fe produint cumarines sinó que, d'acord al que ja s'ha conegut, sintetitza i acumula flavines a les arrels, i les exsuda al medi, principalment com a sulfats de riboflavina. El temps d'exposició a la deficiència de Fe i/o el pH del medi de cultiu afecta la producció de cumarines en *A. thaliana* i tomàquet. Els perfils de compostos de tipus cumarina produïts per les dues espècies també difereixen. Encara que les dues produeixen cumarines simples amb les substitucions oxigenades -OH, -OCH_3 i -O-hexosil, el grau d'oxigenació és diferent. Així, en tomàquet predominen les cumarines tetra-oxigenades com trihidroximetoxicumarina enfront de les tri- i di-oxigenades com la fraxetina i l'escopoletina, respectivament, mentre que a *A. thaliana* són di- i tri-oxigenades i es produeixen conjuntament amb cumarinolignans de tipus cleomiscosina. Les cumarines s'acumulen a les arrels tant en formes de hexòsid com aglicona, amb la forma predominant depenent de cada cumarina i/o espècie vegetal. El perfil de compostos de tipus cumarina presents en el medi de cultiu és diferent a l'observat a les arrels, presentant-se en forma aglicona i sent majoritàries l'escopoletina i la fraxetina. Aquesta exsudació de cumarines en *A. thaliana* està intervinguda per la proteïna transportadora AtABCG37.

La retirada de les cumarines i flavines exsudades del mitjà de *A. thaliana* i de remolatxa té un impacte negatiu en la nutrició fèrrica d'aquestes espècies. La cumarina tipus catecol fraxetina i les flavines en forma reduïda permeten la dissolució d'òxid fèrric -forma característica del Fe als sòls- a pHs rellevants des del punt de vista agronòmic. A més, es demostra que les arrels de les plantes de remolatxa deficients en Fe poden utilitzar les flavines endògenes extracel·lulars per promoure la dissolució reductiva d'òxids fèrrics. El treball conclou que l'exsudació radicular de compostos de tipus cumarina en plantes de *A. thaliana* i de flavines en plantes de remolatxa, permet augmentar la concentració de Fe soluble en sòls amb baixa disponibilitat d'aquest nutrient i, per tant, millora la nutrició fèrrica d'aquestes espècies.

A remarkable metabolic feature of plant roots is the ability to secrete a vast array of compounds into the rhizosphere. The regulation of the synthesis and active secretion of these compounds allows their action to occur at the precise site and time required, enabling plants the ability to cope with changes in the local environment, thus avoiding possible adverse effects such as osmotic changes, pathogen attack, low nutrient supply, etc. A part of the secreted compounds are produced by the secondary metabolism, and their role in the regulation of the soil microbial community, the promotion of beneficial symbiotic associations and the inhibition of the growth of competing plant species has been extensively investigated. However, the influence of these compounds in rhizosphere nutrient availability has been less studied. The aim of this study was to investigate the root exudation of phenolic compounds and flavins induced by the deficiency of iron (Fe), an abundant but generally poorly soluble micronutrient in soils. Although these root responses have been reported in different non-graminaceous species for decades, the identity of the compounds, their transport outside the roots and their role in the rhizosphere are largely unknown. The study used the model plant species *Arabidopsis thaliana* and two crop species, tomato (*Solanum lycopersicum*) and sugar beet (*Beta vulgaris* var. saccharífera).

The present Thesis includes the first thorough and systematic analysis of the phenolic compounds and flavins accumulated and secreted by roots in response to Fe deficiency. The analytical approach used is based on the separation of the compounds by high performance liquid chromatography and their detection by a combination of fluorescence and UV-visible spectroscopy and different types of mass spectrometry. In *A. thaliana* and tomato, Fe deficiency induced the synthesis and accumulation of coumarin-type phenolic compounds and did not affect the root production of flavins. Conversely, sugar beet did not produce phenolic compounds in response to Fe deficiency, but instead synthesizes and accumulates flavins -mainly riboflavin sulfates- in roots, and also exports them to the growth medium. The time of Fe deficiency exposure and/or the pH of the culture medium affect the production of coumarins in *A. thaliana* and tomato, and the coumarin profile was different in both species. Although the two species produce simple coumarins with the oxygen-containing substituents hydroxyl, methoxyl and -O-hexosyl in the benzene ring, the number of such substituents was different. In tomato, tetra-oxygenated coumarins such as trihydroxymethoxycoumarin were predominant over the tri- and di-oxygenated coumarins (such as fraxetin and scopoletin, respectively), whereas *A. thaliana* produced only tri- and di-oxygenated coumarins, along with cleomiscosin-type coumarinolignans. The root coumarins were in hexoside and aglycone forms, with the prevalent form being dependent on the coumarin and/or the plant species. The coumarin profile of the culture medium differed from that found in roots: the coumarins were in the aglycone form, and the predominant ones were scopoletin and fraxetin. This Thesis revealed that the transporter AtABCG37 is involved in the secretion of coumarins in *A. thaliana*.

This study also showed that the depletion of coumarin-type phenolics and flavins in the culture medium of Fe-deficient *A. thaliana* and sugar beet plants, respectively, has a negative impact on the plant Fe nutritional status. The catechol coumarin fraxetin and the reduced forms of flavins promoted the dissolution of ferric oxide –the major form of Fe in soils- at agronomically relevant pH values. In addition, other results of this study provide support for the view that roots of Fe-deficient sugar beet plants can use endogenous extracellular flavins to dissolve ferric oxide. This Thesis concludes that the root secretion of coumarin-type compounds by *A. thaliana* and of flavins by sugar beet allows for increasing the concentration of soluble Fe in soils with low Fe availability, therefore improving the Fe nutritional status in these species.

CAPÍTULO 1. INTRODUCCIÓN GENERAL

1.1. Deficiencia de hierro en plantas

El hierro (Fe) es un elemento esencial para los organismos vivos. Este micronutriente se caracteriza por su facilidad para cambiar de estado de oxidación ($Fe^{3+} \leftrightarrow Fe^{2+}$) de forma reversible y por su tendencia a formar complejos octaédricos con varios ligandos. Dependiendo del ligando, el potencial redox Fe(II/III) varía considerablemente y esta propiedad es la que explica la relevancia de los sistemas biológicos redox de Fe. Así, por ejemplo, en plantas el Fe está implicado en funciones tan importantes como fotosíntesis, respiración, fijación de nitrógeno (N), síntesis de ADN y síntesis de hormonas (Marschner, 2012).

En situaciones de limitada disponibilidad de Fe, las plantas suelen presentar un amarilleamiento internerval en las hojas jóvenes, conocido como clorosis férrica (Fig. 1). En los casos más severos de clorosis las hojas adquieren un color casi blanco, pudiendo presentar lesiones necróticas y causar la defoliación de los brotes jóvenes, y, como consecuencia final, la muerte prematura de las plantas (Sanz y col., 1992).

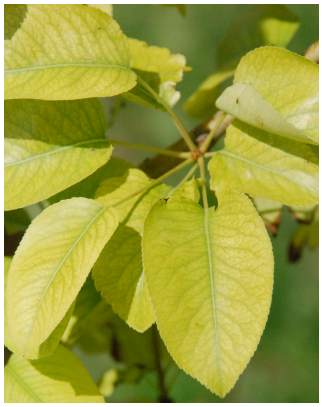

Figura 1 Clorosis en hojas de peral (*Pyrus communis*) afectado por deficiencia de Fe.

La clorosis férrica es un problema frecuente en muchos cultivos en todo el mundo, ya que aproximadamente un 30% de la superficie cultivada se encuentra en suelos calizos en los que el Fe está mayoritariamente en formas no disponibles para las plantas. La deficiencia de Fe ha sido descrita en un gran número de especies de plantas de interés agronómico. Se ven afectadas, entre otras, especies como arroz (*Oryza sativa*), maíz (*Zea mays*), trigo (*Triticum* spp), alfalfa (*Medicago sativa*), girasol (*Helianthus annuus*), tomate (*Solanum lycopersicum*) o guisante (*Pisum sativum*), ocasionando disminuciones en la calidad y en el rendimiento de los cultivos (Korcak, 1987; Hansen y col., 2006). En la cuenca del río Ebro la deficiencia de Fe tiene una gran incidencia en las plantaciones de árboles frutales, como, por ejemplo, en manzano (*Malus domestica*), peral (*Pyrus communis*), melocotonero (*Prunus persica*) (Fig. 2), ciruelo (*Prunus domestica*), cerezo (*Prunus avium*) o cítricos (*Citrus*) (Sanz y col., 1992).

La falta de corrección de este desorden nutricional conduce a grandes mermas en la producción, y en el caso de los cultivos frutales se reduce tanto el número como el tamaño de los frutos (Álvarez-Fernández y col., 2003; Álvarez-Fernández y col., 2006). Una de las formas más eficaces para corregir la clorosis férrica es la aplicación de Fe al suelo en forma de quelatos sintéticos y es ampliamente utilizada en cultivos de alto rendimiento económico. Este tratamiento conlleva importantes costes económicos, estimados en torno a 200 - 400 € ha^{-1} en el caso de árboles frutales (Rombolà y Tagliavini, 2006). Además, los quelatos sintéticos de Fe aplicados al suelo tienen una alta solubilidad, por lo que pueden ser lixiviados y llegar a causar problemas medioambientales, y, por ello, son importantes las investigaciones para corregir la deficiencia de este nutriente dirigidas hacia el desarrollo de nuevos métodos más amigables con el medio ambiente. Por otra parte, el uso actual de prácticas agrícolas intensivas -uso combinado de variedades de alto rendimiento con sistemas intensivos de riego y fertilización de macronutrientes- causa una acentuada disminución de nutrientes del suelo, y está originando que la deficiencia de Fe sea un problema cada vez más frecuente que llega a afectar a cultivos en los que antes no aparecía, como es el caso de las plantaciones intensivas de olivo (*Olea europaea*) (Sánchez-Rodríguez y col., 2013).

Figura 2 Plantación comercial de melocotonero afectada por clorosis férrica. La plantación se encuentra situada en el valle medio del Ebro, en una zona con predominancia de suelos calizos (Plasencia de Jalón, Zaragoza, España).

1.1.1. Hierro en el suelo

El Fe es el cuarto elemento más abundante de la corteza terrestre, después de oxígeno (O), silicio (Si) y aluminio (Al). Se estima que este elemento representa un 3,8% del suelo y se encuentra en sus dos estados de oxidación más comunes (Fe^{2+} o Fe^{3+}). El Fe en estado ferroso (Fe^{2+}) está presente mayoritariamente en minerales primarios y en filosilicatos secundarios. El Fe^{2+} liberado en la hidrólisis de estos minerales es oxidado a la forma férrica (Fe^{3+}) que se coordina con agua e hidroxilos formando una serie de compuestos de solubilidad reducida. Así pues, los suelos tienen óxidos, oxihidróxidos y óxidos hidratados de Fe y el conjunto de todos ellos se conoce con el término general de óxidos de Fe. En los suelos bien drenados y aireados las formas cristalinas de óxidos de Fe más comunes son goethita (α-FeOOH) y hematita (α-Fe_2O_3) mientras que en suelos poco aireados pueden existir otros óxidos entre los que se encuentran algunos cristalinos como lepidocrocita (γ-FeOOH), maghemita (γ-Fe_2O_3) y magnetita (Fe_3O_4), otros menos cristalinos como ferrihidrita y ferroxita, y algunos precipitados no cristalinos (Colombo y col., 2014). En condiciones reductoras también se pueden encontrar pequeñas cantidades de minerales de Fe como pirita (FeS_2) en suelos ácidos o siderita ($FeCO_3$) en suelos alcalinos. Además, los óxidos férricos forman agregados complejos con otros componentes del suelo como arcillas o materia orgánica y ambas fracciones a su vez poseen también ciertas cantidades de este nutriente, ya sea como elemento estructural o unido a la superficie. Es importante señalar que la fracción de materia orgánica de bajo peso molecular puede formar complejos solubles de Fe.

Dependiendo del potencial redox (es decir, de las condiciones de oxidación y reducción) y del pH del suelo, el Fe en disolución se puede encontrar formando mayoritariamente especies ferrosas (Fe^{2+}), disponibles para las plantas, o bien formando especies férricas (Fe^{3+}), en principio no directamente asimilables para muchas especies vegetales. Las especies férricas son características de los suelos bien aireados que presentan concentraciones de O_2 próximas a las atmosféricas. Cuando el pH de estos suelos es ácido la disolución de óxidos de Fe contribuye a incrementar la concentración de especies de Fe^{3+} en disolución, mientras que a pH neutros o alcalinos, como sucede en suelos calizos, la solubilidad de los óxidos de Fe alcanza valores mínimos (Colombo y col., 2014). Se estima que la concentración de Fe disponible en este tipo de suelos es, aproximadamente, de 10^{-10} M (Lindsay y Schwab, 1982), mientras que las plantas para desarrollar un crecimiento óptimo necesitan niveles de Fe superiores, alrededor de 10^{-7} M (Guerinot y Ying, 1994).

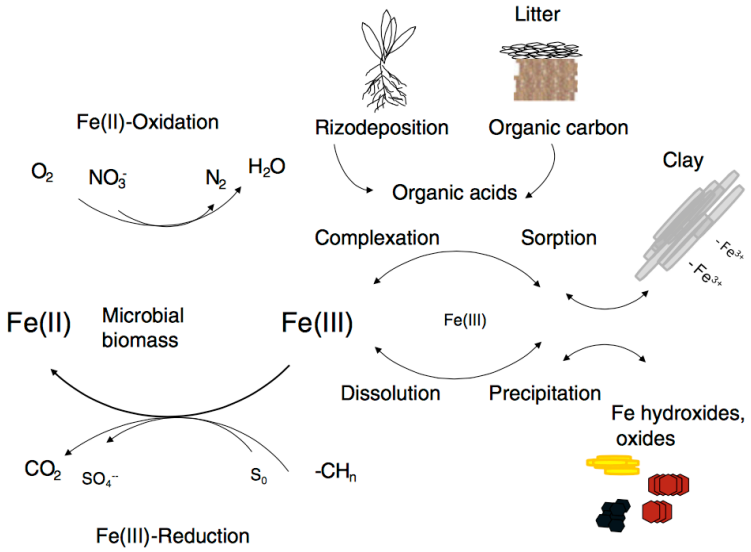

Figura 3 Disponibilidad de Fe en el suelo. En la Figura se muestran algunos de los diferentes procesos que condicionan la disponibilidad de Fe^{2+} y Fe^{3+} en el suelo (tomado de Colombo y col., 2014).

La disponibilidad del Fe en el suelo está también influenciada por otros procesos que junto con los ya descritos se indican esquemáticamente en la Figura 3 (tomada de Colombo y col., 2014). La actividad de los microorganismos es un factor que afecta fuertemente a la solubilidad del Fe en el suelo (Mimmo y col., 2014). En condiciones de anaerobiosis algunas bacterias como *Geothrix fermentans* reducen óxidos férricos para obtener energía (Lovley y col., 2004) y este proceso favorece el incremento de Fe^{2+} disponible para las plantas. En condiciones aeróbicas existen microorganismos como las bacterias acidófilas (p. ej. bacterias del género Acidothiobacillus) y neutrófilas (p. ej. las especies *Gallionella* y *Leptothrix*) que oxidan Fe^{2+} de las fuentes minerales primarias produciendo Fe^{3+} que a pH neutro rápidamente se hidroliza y precipita en forma de óxidos férricos (Colombo y col., 2014). Sin la contribución de estos microorganismos la liberación de Fe^{2+} de los minerales primarios es un proceso lento. Otros microorganismos liberan pequeñas moléculas orgánicas (<1.000 Da) con alta afinidad por Fe (sideróforos) que reaccionan con la superficie de los óxidos férricos liberando Fe mediante la formación de quelatos férricos que contribuyen a aumentar la concentración de este elemento en la disolución del suelo (Crumblis y Harrington, 2009). En una estrategia similar -que será explicada en detalle en secciones posteriores-, también las raíces de las plantas pueden liberar ácidos orgánicos como el ácido cítrico, compuestos fenólicos y/o aminoácidos, como el ácido muginéico, que promueven la liberación de Fe de las fases sólidas del suelo mediante procesos de reducción/quelación (Mimmo y col., 2014).

La degradación microbiana de la materia orgánica también afecta a la disponibilidad del Fe soluble en el suelo. Los climas cálidos y húmedos favorecen la actividad microbiana que mineraliza rápida y completamente la materia orgánica, reduciendo de esta manera la inmovilización de Fe en formas minerales poco cristalinas. Los climas fríos y templados ralentizan la descomposición de la materia orgánica lo que lleva a la formación de humus y/o sustancias húmicas de alto peso molecular que tienden a formar agregados con los óxidos férricos (Wiseman y Püttmann, 2005). Por consiguiente, los cambios en el clima y en la biogeoquímica también afectan a la movilidad de Fe y a otros metales presentes en el suelo.

1.1.2. Causas de la deficiencia de Fe

La deficiencia de Fe en las plantas es un problema complejo, cuyas causas se deben generalmente a la combinación de varios factores entre los que se incluyen la predisposición genética de la planta, la poca solubilidad y movilidad del Fe en el suelo, los problemas en la absorción del Fe por las raíces

y otros factores relacionados con el metabolismo de las plantas.

Se observa una amplia variabilidad genética entre especies de plantas y variedades en relación a su susceptibilidad a la clorosis férrica (Tabla 1) (Korcak, 1987). Así, una determinada planta puede desarrollar clorosis férrica en un determinado suelo, pero otra especie o incluso otro cultivar de la misma especie puede ser más tolerante en dicho suelo. Es poco común observar plantas afectadas de clorosis férrica en la naturaleza en zonas no cultivadas (Chen y Barak, 1982). Por ello, se considera que la toma de Fe del suelo es un factor importante en la adaptación de las plantas a sus hábitats naturales y podría ser un factor decisivo en su distribución natural (Schmidt y Fühner, 1998). La selección genética continua ejercida por el hombre durante la adaptación de las plantas para su cultivo podría explicar parte de la variabilidad genética encontrada en las plantas cultivadas.

La presencia en el suelo de Fe en formas oxidadas y cristalinas no disponibles para las plantas viene favorecida por un pH elevado, una buena aireación, tamaños de partícula grandes y poca concentración de agentes quelantes en la solución del suelo. Todas estas condiciones favorecen la aparición de la deficiencia de Fe en plantas (Loeppert, 1986). Asimismo, las cantidades excesivas de arcillas, altas humedades y elevadas cantidades de ion bicarbonato (HCO_3^-) y de fosfatos también llevan a una baja solubilización del Fe en el suelo (Lindsay y Schwab, 1982; Lucena, 2000). El ión HCO_3^- tampona el pH del suelo a valores entre 7,5 y 8,5 a los que los óxidos de Fe presentan la mínima solubilidad (Lindsay y Schwab, 1982). La fertilización fosfatada induce la aparición de clorosis férrica en diferentes cultivos algunos con una gran importancia económica en el área mediterránea, como en plantaciones de olivo (Sánchez-Rodríguez, 2013).

Existen factores que afectan a la absorción del Fe por las raíces y a su crecimiento, como son las bajas temperaturas, encharcamiento, suelos compactos, y presencia en el suelo de herbicidas y metales pesados (Chen y Barak, 1982; Chaney, 1984). También la presencia del ión HCO_3^- en el suelo disminuye la absorción de Fe, afectando al metabolismo de las raíces y al transporte de Fe hacia la parte aérea (Lucena, 2000). Diferentes estudios han demostrado que este ión tiene un efecto inhibidor a diferentes niveles: la reducción del Fe de las raíces (Nikolic y Römheld, 2002; Lucena y col., 2007), la expresión de genes implicados en la toma de Fe (Lucena y col., 2007), y la acción reguladora génica de la hormona etileno sobre los genes implicados en la toma de Fe (García y col., 2014). La absorción radicular de nitrato (NO_3^-) provoca un aumento del pH en la disolución del suelo afectando a la disponibilidad de Fe para la planta, mientras que con la forma de amonio (NH_4^+) ocurre lo opuesto (Kosegarten y col., 2001; Nikolic y Römheld, 2003).

La baja movilidad del Fe en los tejidos vegetales es otra posible causa de la deficiencia de Fe en las plantas, ya que hay que tener en cuenta que el 80% del Fe absorbido por las raíces se moviliza hacia las hojas. En hojas cloróticas deficientes en Fe de árboles frutales se encontraron concentraciones de Fe similares o incluso mayores que en hojas verdes (Römheld, 1997; Morales y col., 1998). Este fenómeno se conoce como "paradoja de la clorosis férrica". Los fosfatos y un elevado pH en el apoplasto podrían

Tabla 1. Susceptibilidad relativa de varios cultivos a la deficiencia de Fe. Variaciones en las características del suelo, condiciones de crecimiento o diferentes respuestas de una variedad de un cultivo determinado pueden ocasionar que algunos cultivos se enmarquen en dos categorías (modificada a partir de Korcak, 1987)

Susceptible	Moderadamente susceptible	Tolerante
Cacahuete	Alfalfa	Alfalfa
Cítricos	Algodón	Arroz
Nogal	Arroz	Cebada
Soja	Leguminosas forrajeras	Girasol
Sorgo	Maíz	Patata
Tomate	Soja	Remolacha azucarera
Vid	Trigo	Trigo

inducir la precipitación del Fe en el exterior de las células, acumulándose en forma no disponible para la planta e impidiendo su utilización (Mengel y Geurtzen, 1986). Estudios posteriores mostraron que el pH del fluido apoplástico tiene poca influencia en dicha inmovilización, mientras que los ácidos orgánicos u otras características de dicho fluido podrían ser factores determinantes en la distribución de Fe entre la parte soluble (fluido apoplástico) y la insoluble (paredes celulares) de dicho compartimento y podrían controlar la absorción de Fe por las células del mesófilo (Nikolic y Römheld, 2007).

1.1.3. Mecanismos de adquisición de Fe

Ante situaciones de poca disponibilidad de Fe las plantas pueden permanecer indiferentes (plantas ineficientes), o pueden desarrollar mecanismos de adaptación para la adquisición de Fe del suelo (plantas eficientes). Según el mecanismo de adaptación las estrategias de las plantas se pueden clasificar en dos grupos: Estrategia I o de reducción y Estrategia II o de quelación (Fig. 4).

La **Estrategia I o de reducción** ocurre en la mayoría de plantas dicotiledóneas y monocotiledóneas (con excepción de las gramíneas). Se basa en la inducción de la enzima quelato reductasa férrica (FCR), localizada en la membrana plasmática de las células de la raíz (FRO2; (Robinson y col., 1999), que reduce Fe^{3+} a Fe^{2+}. El transporte de Fe^{2+} al interior de la célula se realiza a través del transportador IRT1 (Eide y col., 1996; Vert y col., 2002). Una enzima H^+-ATPasa produce la secreción de protones (H^+) hacia la rizosfera para disminuir el pH, lo que permite una mayor disolución de formas insolubles de Fe del suelo, p. ej. de los óxidos (Santi y Schmidt, 2009). Muchas plantas con Estrategia I en condiciones de deficiencia de Fe acumulan y/o secretan compuestos orgánicos de bajo peso molecular, como compuestos fenólicos, carboxilatos o flavinas, que tienen propiedades redox y pueden reducir o acomplejar metales, con lo que podrían movilizar el Fe en la raíz y/o en la rizosfera (Cesco y col., 2010; Mimmo y col., 2014). Este aspecto se abordará con más detalle en secciones posteriores. Asimismo, en las raíces de muchas especies vegetales se producen cambios morfológicos a nivel macroscópico:

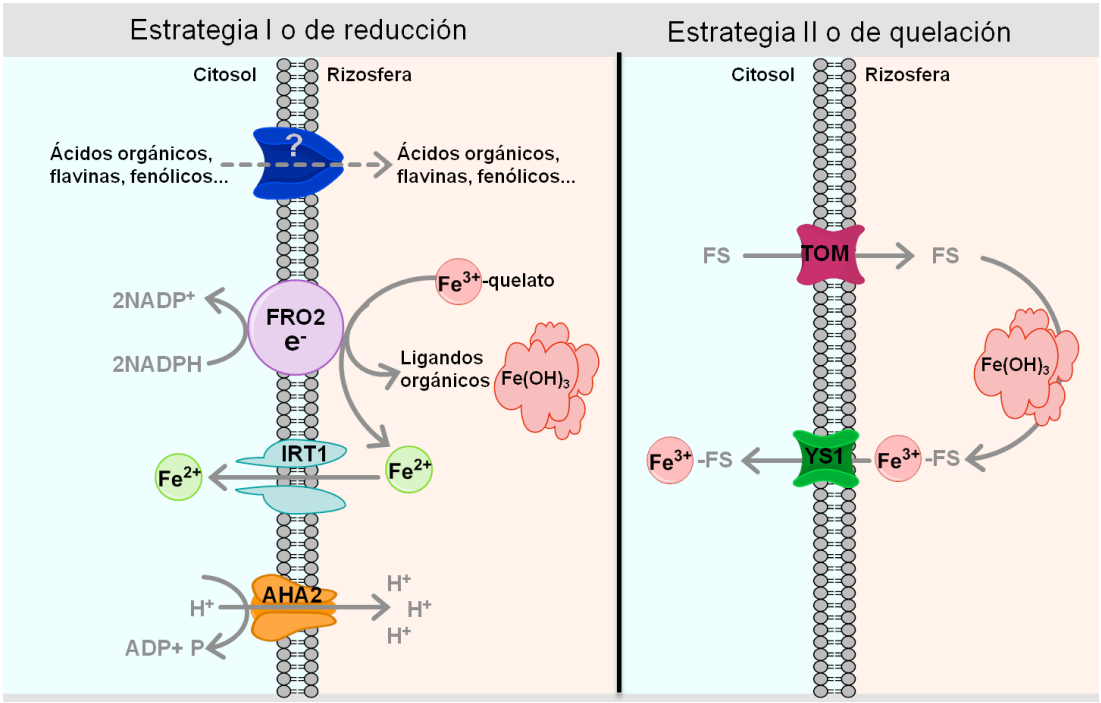

Figura 4 Estrategias desarrolladas por las plantas ante la deficiencia de Fe. La Estrategia I o de reducción se encuentra en plantas dicotiledóneas o monocotiledóneas no gramíneas y la Estrategia II se observa en plantas gramíneas.

se incrementan los pelos radiculares, se produce un engrosamiento en las zonas subapicales de la raíz y, con largos periodos de deficiencia de Fe, se llega a producir una disminución de la raíz primaria (Moog y col., 1995; López-Millán y col., 2000; Schmidt, 1999). Los cambios microscópicos de la raíz consisten en la formación de células de transferencia, tanto en la rizodermis como en la hipodermis (Welkie y Miller, 1993; Landsberg, 1994). Todas estas modificaciones de la raíz ante la deficiencia de Fe tienden a incrementar la superficie de contacto entre la raíz y el suelo y entre la pared celular y el citoplasma de las células de la raíz para aumentar la absorción de Fe.

La **Estrategia II o de quelación** es utilizada principalmente por las gramíneas, y se basa en la secreción de fitosideróforos (FS), compuestos de la familia del ácido mugineico. La liberación de FS está mediada por proteínas transportadoras de la familia MFS (OsTOM1 en arroz y HvTOM1 en cebada) cuya expresión aumenta con la deficiencia de Fe (Nozoye y col., 2011). Los FS tienen una alta afinidad por el Fe, solubilizan Fe en la rizosfera por quelación produciendo quelatos Fe(III)-FS, y se toman por la raíz a través de una proteína transportadora específica de la familia "yellow stripe-like" (YSL) que se localiza en las zonas apicales (Curie y col., 2001; Murata y col., 2006). Dentro de la planta se produce la liberación del Fe -proceso que podría ocurrir mediante la reducción del Fe(III)-FS facilitada por ascorbato a través de la formación de un complejo ternario (Weber y col., 2008)-, y posteriormente el FS se degrada o se secreta al exterior.

Algunas especies de la Estrategia II tienen elementos de la Estrategia I y *viceversa*. El arroz, especie de Estrategia II, puede tomar Fe^{2+} a través de un transportador IRT (Ishimaru y col., 2006). La toma de Fe^{2+} es fundamental cuando esta especie se cultiva en suelos inundados donde la concentración de O_2 es baja y, por consiguiente, se favorece la presencia de esta forma de Fe en la disolución suelo. El cacahuete (*Arachis hypogaea*), especie de Estrategia I, cuando se cultiva con maíz (*Zea mays*) puede tomar el Fe^{3+} como complejo Fe(III)-FS a través de transportador AhYSL1 que se expresa en raíz en dichas condiciones (Xiong y col., 2013).

1.1.4. Formas de aliviar la deficiencia de Fe en los cultivos

Como ya se ha comentado, la clorosis férrica se corrige habitualmente mediante la aplicación de quelatos sintéticos de Fe como Fe-EDDHA y otros al suelo, y es una práctica rutinaria empleada de manera incluso preventiva por los agricultores cuando los cultivos son de un importante valor económico. Estos tratamientos deben realizarse una o dos veces al año para mantener su eficiencia (Sanz y col., 1992; Rombolà y Tagliavini, 2006). La fertilización foliar con compuestos inorgánicos como $FeSO_4$, orgánicos incluyendo naturales como Fe (III)-citrato y Fe (III)-lignosulfonatos y sintéticos como Fe(III)-EDTA pueden también aliviar la deficiencia de Fe, aunque en los cultivos de frutales esta práctica aún no es muy común y podría ser un valioso complemento a la fertilización aplicada al suelo (Abadía y col., 2011; El-Jendoubi y col., 2014). El rendimiento de los esprays foliares de Fe se ve afectado por numerosas variables que no se conocen en profundidad, incluyendo factores ambientales, fisicoquímicos y otras relacionadas con el tipo de planta, y que conllevan una baja reproducibilidad de los resultados obtenidos (Fernández y col., 2009; Ríos y col., 2016). Otras técnicas menos utilizadas son la inyección con solución de fertilizantes de Fe en las raíces, ramas o troncos de árboles frutales (Liu y col., 2002; Larbi y col., 2003; Zhang y col., 2008) que también contribuyen eficazmente a la recuperación de la deficiencia de Fe, pero que sin embargo son difíciles de utilizar para tratar grandes extensiones. No obstante, siempre es interesante considerar técnicas agronómicas más económicas y menos lesivas para el medio ambiente.

El uso de estrategias agronómicas como el manejo del suelo y/o la gestión de cultivos pueden favorecer las condiciones de crecimiento y mejorar la movilización y la utilización del Fe. Por ejemplo, mediante la regulación del pH de la rizosfera a través del suministro de N, ya sea en forma de NO_3^- o de NH_4^+, se puede influir en la disponibilidad de Fe y reducir de forma sensible la deficiencia de este elemento (Zou y col., 2001; Silber y col., 2004). Asimismo, se ha demostrado que mediante la regulación del riego y un drenaje

adecuado se podría mitigar la deficiencia de Fe, pues, como ya se ha dicho, altos contenidos de humedad en el suelo aumentan la concentración del ión HCO_3^- (Zuo y col., 2007). La intercalación de cultivos con especies gramíneas también produce una mejora en la captación de Fe por plantas dicotiledóneas como cítricos (Cesco y col., 2006), cacahuete (Zuo y Zhang, 2008) y olivo (Cañasveras y col., 2014). Los exudados conteniendo FS de plantas de Estrategia II podrían tener importancia ecológica en los suelos calizos para satisfacer la demanda de Fe de la plantas de Estrategia I. También los sistemas de cubierta de tierra (por ejemplo: láminas de plástico o mediante una capa vegetal) han demostrado ser una forma de remediar la deficiencia de Fe, aunque la eficiencia de esta técnica depende de la especie considerada (Zuo y Zhang, 2011).

Otra práctica útil para la adquisición de Fe podría ser la fertilización indirecta mediante inoculantes en las raíces de las plantas. Así, la inoculación de bacterias promotoras del crecimiento da lugar a una mayor absorción de Fe en plantas eficientes con una alta capacidad para adquirir este elemento a partir de Fe(III)-sideróforos (Khan, 2005; Bakker y col., 2007).

El uso de variedades conocidas y eficientes en la toma de Fe es una práctica común entre los agricultores. Por ejemplo, en fruticultura se usan injertos de dos individuos en los que la raíz o patrón es tolerante a la clorosis férrica (Jiménez y col., 2008). También a través de técnicas de ingeniería molecular se pueden obtener nuevas variedades de mejor eficiencia en la adquisición de Fe. Por ejemplo, líneas de arroz transgénico con genes que codifican enzimas de la ruta biosintética de los FS son más productivas y más tolerantes a la baja disponibilidad de Fe que las líneas no transgénicas (Ishimaru y col., 2007).

A pesar de todas las técnicas mencionadas anteriormente, cada vez tiene mayor interés el uso de formas naturales y/o más amigables con el medio ambiente para la corrección de las deficiencias nutricionales. Así, una de las líneas de investigación de mayor desarrollo en este momento es el estudio de la exudación radicular de compuestos en respuesta a alteraciones nutricionales y su posible aplicación práctica en agricultura.

1.2. Exudación radicular en el contexto de la deficiencia de Fe

Las plantas al ser organismos sésiles no pueden escapar de su entorno, estando expuestas continuamente a las constantes variaciones y adversidades del medio que les rodea. Para poder sobrevivir a las situaciones desfavorables, como pueden ser la escasez de un nutriente, la limitación en la disponibilidad de agua o el ataque de patógenos, las plantas desarrollan mecanismos de adaptación, muchos de ellos activados en la raíz como órgano encargado de la absorción de los nutrientes minerales y el agua. Estos mecanismos no son sólo de tipo físico, como la modificación de la arquitectura de la raíz o la regulación del crecimiento, sino que también ponen en marcha mecanismos químicos como la liberación de compuestos realizada por células intactas que se conoce como exudación. La exudación (secreción, difusión y excreción) radicular puede suponer hasta un 20% del carbono (C) asimilado por la planta y su destino es la parte del suelo inmediata a las raíces vivas que está bajo la directa influencia de éstas y se denomina rizosfera (Neumann y Römheld, 2011). La composición de los exudados radiculares es muy compleja ya que incluye una gran variedad de compuestos de diferentes características químicas (Tabla 2) y en un intervalo de concentraciones muy amplio. Además, esta composición depende de muchos factores entre los que se encuentran la especie y la variedad, el estado nutricional, la etapa de desarrollo y las características fisicoquímicas del medio que rodea a la raíz entre otros (Neumann y Römheld, 2011). Los componentes de los exudados radiculares liberados activamente mediante una estrategia estrictamente controlada y que cumplen una función específica, se conocen bajo el término de secreciones y su liberación es inducida por la disponibilidad limitada de nutrientes como N, P y Fe entre otros factores (Dakora y Phillips, 2002; Carvalhais y col., 2011). El conocimiento de la actividad de estos compuestos en el ámbito de la nutrición vegetal es todavía limitado (Keuskamp y col., 2015).

En los siguientes apartados de esta sección se describen los principales compuestos

Tabla 2. Compuestos orgánicos exudados por las raíces de las plantas. Tomado de Dennis y col. (2010).

Clase	Compuestos
Azúcares	arabinosa, fructosa, galactosa, glucosa, maltosa, manosa, mucilagos de composición variada, oligosacáridos, rafinosa, ramnosa, ribosa, sacarosa, xilosa, desoxirribosa
Aminoácidos	α-alanina, β-alanina, ácido γ-aminobutírico, ácido α-aminoadípico, ácido aspártico, ácido desoximugineico, ácido glutámico, ácido 3-hidroximugineico, ácido mugineico, arginina, asparagina, citrulina, cistationina, cisteína, fenilalanina, glutamina, glicina, histidina, homoserina, isoleucina, leucina, lisina, metionina, ornitina, prolina, serina, treonina, triptófano, tirosina, valina
Ácidos orgánicos de bajo peso molecular	acético, aconítico, aldónico, ascórbico, benzoico, butírico, cafeico, cítrico, *p*-cumárico, eritrónico, ferúlico, fórmico, fumárico, glutárico, glicólico, glioxílico, láctico, málico, malónico, oxalacético, oxálico, *p*-hydroxybenzoíco, piscídico, propanoico, ácido pirúvico, succínico, siríngico, tartárico, tetronico, valérico, vanílico
Ácidos grasos	linoleico, linolénico, oleico, palmítico, esteárico
Esteroles	campesterol, colesterol, sitosterol, estigmasterol
Factores de crecimiento y vitaminas	ácido 4-aminobenzoico, ácido N-metilnicotina, ácido pantoténico, biotina, colina, niacina, tiamina, riboflavina, piridoxina
Enzimas	amilasa, fosfatasa alcalina, fosfatasa ácida, invertasa, peroxidasa, polifenol oxidasa, poligalacturonasa
Flavonoides y purinas/nucleótidos	adenina, flavanona, guanina, uridina, citidina
Varios	ácido cianhídrico, alcoholes, auxinas, betaina, compuestos del tipo myo-inositol, compuestos no identificados que dan positivo para ninhidrina, compuestos reductores, dihidroquinona, escopoletina, etanol, glicina, glucósidos, inositol, iones inorgánicos, isotiocianato, moléculas gaseosas (p. ej., CO_2, H_2), polipéptidos inducidos por Al, proteínas solubles no identificadas, sorgoleona y sulfuros de alquilo

exudados en respuesta a deficiencia de Fe, los mecanismos de secreción, y por último las funciones de dichos compuestos en la rizosfera.

1.2.1. Compuestos exudados

En el contexto de la deficiencia de Fe se pueden identificar tres importantes clases de compuestos en los exudados radiculares: ácidos orgánicos de bajo peso molecular (AOBPM), fitosideróforos (FS) y compuestos reductores. En *Arabidopsis thaliana*, a pesar de ser la especie modelo por excelencia de la Estrategia I, no se han realizado estudios de exudación en deficiencia de Fe. Este estudio es uno de los objetivos de esta Tesis y por consiguiente toda la información publicada al respecto dentro del presente trabajo queda recogida en los capítulos de Resultados y de Discusión General y no está comentada en las siguientes secciones de esta Introducción.

Ácidos orgánicos de bajo peso molecular

Los AOBPM se caracterizan por tener uno o más grupos carboxílicos (-COOH) en su estructura química. La exudación de AOBPM en respuesta a la deficiencia de Fe se ha estudiado en varias especies de plantas (Tabla 3), mayoritariamente de Estrategia I. Un análisis conjunto de los datos revela que se han determinado hasta 10 diferentes AOBPM (PM< 200 Da) incluyendo 3 monocarboxílicos (acético, ascórbico y láctico), 6 dicarboxílicos (fumárico, málico, malónico, oxálico, succínico y tartárico) y 1 tricarboxílico (cítrico) (Fig. 5). En la mayoría de los casos los AOBPM encontrados incrementan su

concentración con la deficiencia de Fe o son detectados sólo en los exudados de plantas deficientes en Fe (Tabla 3). Las especies estudiadas (12) pertenecen a 8 familias diferentes (Cucurbitaceae, Fabaceae, Poaceae, Rosaceae, Rutaceae, Solanaceae, Urticaceae y Vitaceae) y la familia Fabaceae es la única en la que se ha evaluado más de un miembro (*Cicer arietinum*, *Glycine max, Medicago ciliaris*, *Pisum sativum*). Los ácidos cítrico y málico son encontrados repetidamente. Así, estos dos ácidos se detectaron en dos genotipos de *Medicago ciliaris* y de guisante, en *Parietaria judaica*, y en un patrón de cítricos (Citrange Carrizo) (M'Sehli y col., 2008; Jelali y col., 2010; Donnini y col., 2012; Martínez-Cuenca y col., 2013). Las diferencias intraespecíficas observadas guardan relación con la eficiencia en la toma de Fe, siendo más acusada la exudación de estos ácidos en los genotipos más eficientes. El ácido cítrico fue el único AOBPM descrito en exudados de soja (*Glycine max*) y de fresa (*Fragaria×ananassa*) (Zocchi y col., 2007; Valentinuzzi y col., 2015). El ácido malónico fue el compuesto mayoritario encontrado en los exudados de garbanzo (*Cicer arietinum*), especie en la que también se encontraron en menor abundancia los ácidos cítrico, fumárico, y tartárico (Ohwaki y Sugahara, 1997). En uno de los dos estudios realizados con pepino (*Cucumis sativus*) se encontraron los ácidos cítrico y málico, aunque éstos junto con fumárico eran minoritarios con respecto al

Tabla 3. Ácidos orgánicos de bajo peso molecular (AOBPM) encontrados en exudados radiculares en estudios de deficiencia de Fe y técnicas analíticas utilizadas para su determinación. Los AOBPM marcados en negrita aumentan con la deficiencia de Fe y los subrayados son mayoritarios (>20% del total de los ácidos determinados).

Especie	AOBPM	Técnica de análisis	Ref.
Medicago ciliaris	**cítrico**, **málico**	Enzimática	[1]
Guisante (*Pisum sativum*)	**cítrico**, **málico**		[2]
Parietaria judaica	**cítrico**, **málico**		[3]
Soja (*Glycine max*)	**cítrico**		[4]
Garbanzo (*Cicer arietinum*)	**cítrico**, **fumárico**, **malónico**, **tartárico**	CG-ionización por llama	[5]
Tomate (*Solanum lycopersicum*)	cítrico, fumárico, málico, oxálico, succínico		[6]
Cebada (*Hordeum vulgare*)	**acético**, **fumárico**, **láctico**, **málico**, **succínico**		[7]
Pepino (*Cucumis sativus*)	**cítrico**, **fumárico**, **láctico**, **málico**, **oxálico, succínico**		[8]
Pepino (*Cucumis sativus*)*	**acético**, fumárico, oxálico, **succínico**	HPLC-UV	[9]
Fresa (*Fragaria×ananassa*)	**cítrico**		[10]
Patrón de viña (*Vitis berlandieri* x *Vitis rupestris*)	ascórbico, málico, oxálico, tartárico		[11]
Cítricos (*Citrus sinensis* Osbeck x *Poncirus trifoliata* Raf.)	**cítrico**, **málico**	HPLC-conductividad	[12]
Maíz (*Zea mays*)	**cítrico**, málico, succínico		[13]

[1] M'Sehli y col. (2008); [2] Jelali y col. (2010); [3] Donnini y col. (2012); [4] Zocchi y col. (2007); [5] Ohwaki y col. (1997); [6] Mozafar y col. (1992); [7] Fan y col. (1997); [8] El-Baz y col. (2004); [9] Pii y col. (2015); [10] Valentinuzzi y col. (2015); [11] López-Rayo y col. (2015); [12] Martínez-Cuenca y col. (2013); [13] Carvalhais y col. (2011).

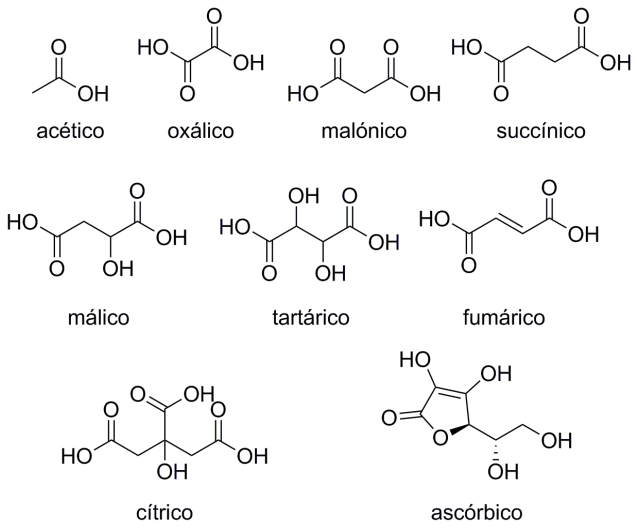

Figura 5 Estructuras químicas de los ácidos orgánicos de bajo peso molecular encontrados en exudados de raíces de plantas deficientes en Fe.

resto (láctico, oxálico y succínico) (El-Baz y col., 2004). En el segundo de los estudios llevado a cabo en esta especie, la deficiencia de Fe sólo aumentó la concentración de acético y succínico (Pii y col., 2015). Los exudados de plantas deficientes en Fe de un patrón de viña tolerante (*Vitis berlandieri* x *Vitis rupestris*), presentaron mayoritariamente ácido oxálico y cantidades menores de los ácidos málico, tartárico y ascórbico (López-Rayo y col., 2015). En este estudio se encontró que el aporte de Fe a plantas deficientes no tuvo efecto en la cantidad de AOBPM en los exudados lo que pudo ser debido a que la deficiencia de Fe no llegó a ser completamente corregida. Tampoco hubo diferencias entre las concentraciones de los 5 AOBPM encontrados en los exudados de tomate WT (FER) y su mutante *fer* (Mozafar y col., 1992), ineficiente en la toma de Fe por la falta del factor de transcripción FER que regula la expresión de genes clave en la Estrategia I. En este caso los exudados se obtuvieron en condiciones de esterilidad y los ácidos mayoritarios fueron fumárico y cítrico. En plantas de Estrategia II los estudios son escasos. En cebada (*Hordeum vulgare*) se han encontrado hasta 5 AOBPM diferentes y aunque la concentración de todos ellos aumenta con la deficiencia de Fe, los mayoritarios fueron acético y láctico (El-Baz y col., 2004). Sin embargo, en maíz (*Zea mays*) se encontraron 3 AOBPM y sólo cítrico aumentó con la deficiencia de Fe (Carvalhais y col., 2011).

El perfil de AOBPM encontrado en estos estudios (Tabla 3) puede estar afectado por el tipo de muestreo de exudados y las técnicas utilizadas para su análisis. En la mayoría de los casos se recogen los compuestos exudados por las raíces de plantas intactas en agua destilada (a veces con el pH ajustado en el intervalo de 6 a 7) en presencia o no de un agente químico bactericida y durante un periodo de tiempo que varía entre 2 a 24 horas. Recientemente comparando diferentes condiciones de cultivo y métodos de recolección de exudados radiculares, Oburger y col. (2013) han observado que la intensidad de la exudación fue del mismo orden de magnitud entre los diferentes sistemas a pesar de las diferencias cualitativas y cuantitativas encontradas y que la exudación de AOBPM fue menos afectada que la de aminoácidos. En cuanto a las técnicas de análisis, algunas son específicas para un ácido en particular (p. ej. los métodos enzimáticos utilizados en la determinación de cítrico y málico) sin considerar la posible presencia de otros ácidos o el sistema de detección es poco selectivo (p. ej. conductividad, absorbancia UV) lo que puede conllevar a identificaciones incorrectas o sobreestimaciones de las concentraciones de algunos ácidos. Cuando se emplean técnicas que permiten la detección simultánea de varios ácidos orgánicos se requieren técnicas de detección muy selectivas o procedimientos de extracción de los compuestos del medio de exudación que permitan descartar posibles interferentes así como una óptima separación

cromatográfica, y todos estos aspectos fueron poco o nada considerados en los estudios citados. Actualmente existen métodos selectivos y sensibles, específicamente desarrollados para el análisis de los AOBPM en exudados radiculares y que utilizan separaciones de HPLC y detección por espectrometría de masas (MS) (Chen y col., 2008; Erro y col., 2009).

La exudación de AOBPM también se incrementa en respuesta a una baja disponibilidad de Fe ante valores de pH próximos a la neutralidad y/o la presencia de $CaCO_3$ o bicarbonato en la solución nutritiva (El-Baz y col., 2004; M'Sehli y col., 2008; Jelali y col., 2010; Donnini y col., 2012; Martínez-Cuenca y col., 2013). En todas las condiciones descritas las concentraciones de AOBPM en exudados de plantas deficientes en Fe están en el intervalo de 0,1-200 nmol/g PF raíz y generalmente son de uno hasta tres órdenes de magnitud inferiores a las encontradas en las correspondientes raíces (M'Sehli y col., 2008; Jelali y col., 2010; Donnini y col., 2012). No obstante, todos estos estudios se han realizado con plantas cultivadas en hidroponía y podrían diferir de las concentraciones exudadas por plantas crecidas en suelo. Así, se ha visto que las condiciones ambientales y la presencia de un medio de enraizamiento sólido y de microorganismos son factores que pueden incrementar la exudación (Dakora y Phillips, 2002; Dennis y col., 2010). Sin embargo, se ha descrito que la adición de la bacteria benéfica *Pseudomonas fluorescens* a un medio estéril causó una disminución de la cantidad total de AOBPM exudados por raíces de tomate, principalmente de los ácidos fumárico, oxálico y málico, posiblemente porque éstos son consumidos por la bacteria (Mozafar y col., 1992). Por otra parte, la total ausencia de Fe impuesta por el uso de soluciones nutritivas nunca ocurre en el suelo, y, por tanto, las plantas pueden mantener activos los circuitos que regulan las estrategias de toma de Fe ya que éstos están en equilibrio con el Fe disponible (Oburger y col., 2014). Por ello es posible que las plantas cultivadas en suelo puedan mostrar menor exudación de compuestos capaces de movilizar Fe del suelo. Sin embargo, este aspecto ha sido muy poco estudiado en el contexto de la deficiencia de Fe. El único estudio que compara la exudación radicular de AOBPM en hidroponía y suelo realizado con plantas de pepino mostró que aunque en ambas condiciones aumentan la exudación en respuesta a la deficiencia de Fe -con un incremento mucho más acusado en suelo-, la exudación de las plantas deficientes fue del mismo orden de magnitud en hidroponía y suelo (aprox. 10-50 µg/g peso fresco) (Pii y col., 2015).

La exudación radicular de AOBPM en respuesta a la deficiencia de Fe ya había sido propuesta años antes, en plantas de Estrategia I y Estrategia II, en base al aumento de la concentración de estos compuestos en raíces y la acidificación del medio de cultivo (Venkat Raju y col., 1972; Landsberg, 1981). Posteriormente se constató que el aumento de la concentración de AOBPM ocurría también en otros tejidos y fluidos vegetales como respuesta a la deficiencia de Fe (ver revisión de Abadía y col., 2002) y que era más intenso en las de Estrategia I (López-Millán y col., 2012). Estos aumentos son consecuencia del incremento de la fijación de CO_2 en las raíces de las plantas deficientes en Fe, al incorporarse posteriormente el C en ácidos orgánicos (principalmente cítrico), amino-ácidos e hidratos de carbono (ver referencias en Abadía y col., 2002). En esta misma línea, numerosos estudios no sólo bioquímicos sino también metabolómicos, proteómicos y transcriptómicos han demostrado que las plantas deficientes en Fe incrementan varias de las enzimas del ciclo de los ácidos tricarboxílicos y también la enzima fosfo*enol*-piruvato carboxilasa, que cataliza la fijación de HCO_3^- (ión abundante en suelos calizos) sobre el fosfo*enol*piruvato permitiendo la reposición de intermediarios para dicho ciclo (Rabotti y col., 1995; López-Millán y col., 2000, 2012; Zocchi, 2006; M'Sheli y col., 2008; Rellán-Álvarez y col., 2010; Jelali y col., 2010; Donnini y col., 2012; Martínez-Cuenca y col., 2013). Esta actividad metabólica aporta el poder reductor (NADH) que las raíces de las plantas de Estrategia I necesitan para mantener una elevada actividad de la enzima FCR (Landsberg, 1994; López-Millán y col., 2000; Zocchi, 2006; Vigani, 2012).

Fitosideróforos

Los fitosideróforos (FS) son compuestos análogos al aminoácido no proteinogénico nicotianamina (NA) (Kobayashi y col., 2006) presente en todas las especies de plantas e involucrado en el movimiento de metales dentro de los tejidos y entre órganos (Schuler y col., 2012). Estos compuestos son ligandos multidentados con más de un grupo α-aminoarboxilíco en su estructura (Fig. 6) lo que les confiere afinidad por Fe y otros metales como zinc (Zn), cobre (Cu), manganeso (Mn), níquel (Ni) y cadmio (Cd). La NA tiene afinidad por Fe(III) y Fe(II), y los FSs tienen también un grupo de unión α-hidroxicarboxílico que les proporciona selectividad para Fe(III). La síntesis de NA y FSs aumenta en raíz en respuesta a la deficiencia de Fe y se produce mediante la trimerización del aminoácido L-metionina, que conducen a la formación de NA, reacción catalizada por la enzima nicotianamina sintasa (Kobayashi y col., 2006). Posteriormente actúa la enzima nicotianamina aminotransferasa que es específica de las especies de la familia Poaceae (las únicas que utilizan la Estrategia II) y cataliza la transaminación de NA para producir el precursor del FS ácido 2'-desoximugineico (DMA). El resto de los FSs (Fig. 6) se producen a partir de DMA en etapas sucesivas por hidroxilación y/o apertura del anillo azetidina de su estructura (Kobayashi y col., 2006; Ueno y col., 2007). Los FSs se sintetizan durante el periodo de luz (día) y se acumulan dentro de las células corticales para ser exudados durante un tiempo limitado al medio de crecimiento tras el periodo de oscuridad (noche), alcanzando un máximo en las primeras horas de luz que es variable entre especies y dependiente de la temperatura (Takagi, 1993; Ma y col., 2003; Ueno y Ma, 2009).

El primer FS descubierto fue el ácido mugineico (MA) en exudados de cebada cultivada en deficiencia de Fe (Takemoto y col., 1978). Todas las especies de la familia Poaceae investigadas hasta la fecha comparten la capacidad de producir y exudar FSs en respuesta a la deficiencia de Fe (Römheld, 1987; Takagi, 1993; Kobayashi y col., 2006; Ueno y col., 2007) y algunas de ellas (p. ej. cebada) en respuesta a la deficiencia de Zn, aunque en este caso la intensidad de la exudación es menor (Suzuki y col., 2006). Existe un correlación positiva entre la tolerancia a la deficiencia de Fe de diferentes especies y la capacidad de las plantas de

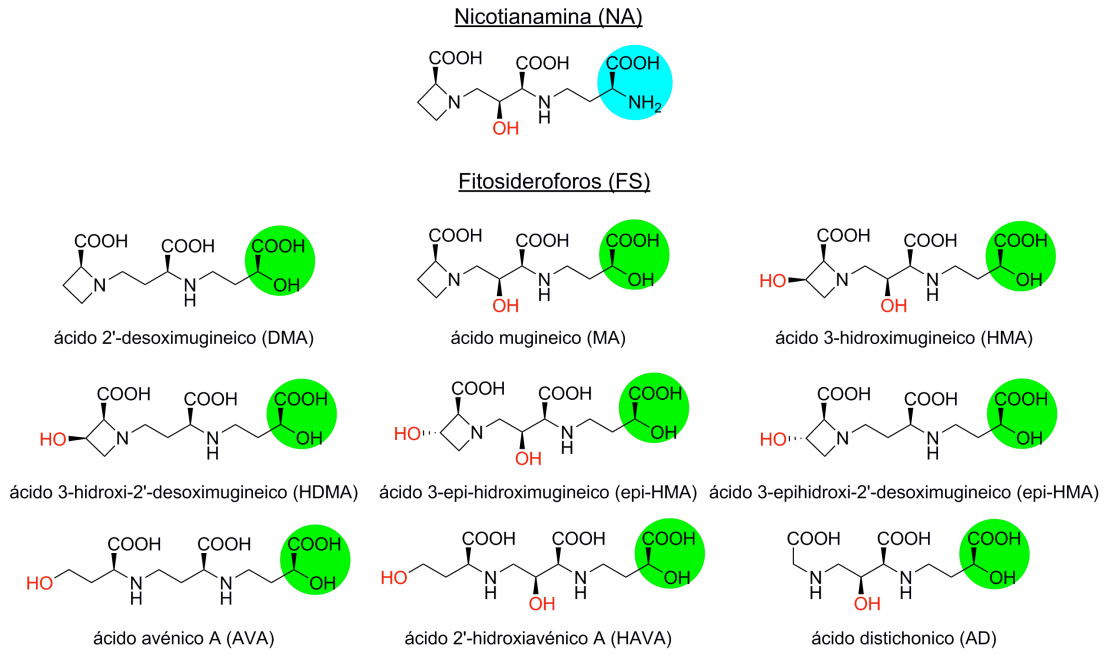

Figura 6 Estructuras químicas de los fitosideróforos encontrados en exudados de plantas deficientes en Fe y de su análogo estructural nicotianamina. Los círculos verde y azul resaltan el grupo α-hidroxicarboxílico y α-aminocarboxílico específicos de NA y FSs, respectivamente. Las hidroxilaciones adicionales de los FSs están indicadas en rojo

exudar FSs. Las especies más estudiadas presentan el siguiente orden de acuerdo con la intensidad de estas dos características (tolerancia y exudación): cebada > trigo, centeno (*Secale cereale*) > avena (*Avena sativa*) > maíz > sorgo (*Sorghum* spp.) > arroz (Takagi, 1993). Así, cebada exuda generalmente hasta 10 y 100 veces más FSs que maíz y sorgo, respectivamente. Para una misma especie, la exudación de FSs depende del genotipo y, en general, disminuye con la edad de la planta (calculada en base a peso fresco de raíz) (Oburger y col., 2014). Se observa una disminución clara de la exudación cuando aumenta la disponibilidad de Fe para la planta (White, 2011). Por otra parte, la exudación en la zona apical de la raíz es 5 veces más intensa que en la zona basal, patrón similar al observado con la toma de Fe a partir de ^{59}Fe-FS (Marschner y col., 1987).

El ácido 2'-desoximugineico (DMA) es el más frecuente de los FSs encontrados en deficiencia de Fe (Tabla 4), aunque se conocen un total de 9 FSs, la mayoría de los cuales conservan el anillo azetidina de NA (Fig. 6). Otra diferencia estructural es el grado de hidroxilación del FS, siendo MA el único no hidroxilado mientras que el resto son monohidroxilados (DMA y otros 4 FSs) o dihidroxilados (3 FSs incluyendo el ácido 3-epi-hydroxymugineico). El tipo y número de fitosideróforos biosintetizados varía entre especies y cultivares. La cebada exuda MA, ácido 3-epihidroxi-2'-desoximugineico (epiHDMA), ácido 3-epihidroximugineico (epiHMA), ácido avénico (AVA), ácido distichonico (AD), HAVA, además de DMA (Tabla 4). Sin embargo, existen diferencias importantes en el número de FSs exudados entre cultivares de cebada. Así, Ma y col. (1999) observaron que algunos sólo secretaban DMA mientras que en otros la exudación contenía DMA y MA, o bien DMA, epi-HMA y MA. El centeno secreta DMA, MA y el ácido 3-hidroxymugineico (HMA), mientras que la avena secreta DMA y AVA. Arroz, trigo, maíz y sorgo generalmente exudan sólo DMA, aunque algunos tipos de trigo ancestral también secretan algunos FSs desconocidos(Singh y col., 2000), y plantas adultas de arroz (cv. Honenwase cultivadas con Fe y posteriormente en condiciones de deficiencia) secretaron MA y epiHMA (Mori y col., 1991). La exudación de FSs hidroxilados, frecuente en algunos cultivares de cebada y centeno, parece estar asociada con una mayor tolerancia a la deficiencia de Fe. Las hidroxilaciones confieren al quelato Fe(III)-FS alta estabilidad no sólo a pHs alcalinos sino también a pHs ligeramente ácidos e incrementan la competitividad del FS para mantenerse unido al Fe ante la presencia de otros ligandos (otros FSs menos hidroxilados, sideróforos) y de los H^+ que pueden ser más abundantes en la zona más próxima a la raíz (von Wirén y col., 2000).

Tabla 4. Fitosideróforos encontrados en exudados radiculares de diferentes especies Poaceae. AVA, ácido avénico; AD, ácido distichonico; DMA, ácido 2'-desoximugineico; epi-HDMA, ácido 3-epi-hidroximugineico; epi-HDMA, ácido 3-epihidroxy-2'-desoximugineico; HAVA, ácido 2'-hidroxiavénico; HMA, ácido 3-hidroximugineico.

Especie	Fitosideróforo	Ref.
Avena (*Avena sativa*)	AVA, DMA	[1, 2]
Arroz (*Oryza sativa*)	DMA	[1, 2]
Cebada (*Hordeum vulgare*)	AVA, AD, DMA, HAVA, epi-HDMA, epi-HMA, MA	[1, 2, 3]
Centeno (*Secale cereale*)	DMA, HMA, MA	[1, 2]
Festuca rubra L.	DMA	[4]
Lolium perenne	DMA, epi-HDMA, HDMA	[5]
Maíz (*Zea mays*)	DMA	[1, 2]
Poa pratensis	AVA, DMA, HAVA	[5]
Trigo (*Triticum* spp)	DMA	[1, 2]
Sorgo (*Sorghum* spp)	DMA	[1, 2]

[1] Takagi (1993); [2] Kobayashi y col., 2006; [3] Tsednee y col. (2012); [4] Ma y col. (2003); [5] Ueno y col. (2007).

Como en el caso de los AOBPM, la mayoría de los estudios de exudación de FSs se han llevado a cabo con plantas crecidas en hidroponía, ya que se puede controlar el aporte de nutrientes y se facilita el muestreo y posterior análisis de los compuestos exudados por la raíz (Oburger y col., 2014). Sin embargo, como subrayan estos autores y se ha indicado anteriormente, las condiciones de crecimiento y/o de muestreo del exudado de las plantas en hidroponía difieren significativamente de las que crecen en su entorno natural (el suelo), no sólo en términos de disponibilidad de nutrientes, sino también en cuanto a concentración de O_2/CO_2, población microbiana e impedancia mecánica, lo que puede provocar cambios en la morfología y anatomía de la raíz. Todas estas variables podrían dar lugar a importantes diferencias respecto a la dinámica de la exudación radicular. Las metodologías de estudio de la rizosfera, incluyendo los desarrollos más avanzados para conocer la composición de exudados en condiciones próximas a las naturales, han sido recientemente revisados por Oburger y Schmidt (2016). Algunos de los últimos avances en el muestreo de exudados de plantas cultivadas en suelo han sido aplicados al estudio de FSs en trigo (Oburger y col., 2014): la exudación de DMA resultó ser 50 veces más baja en suelo que en solución nutritiva sin Fe (47 pmol/g y 2.200 pmol/g PF raíz y segundo, respectivamente).

El primer FS se descubrió ya hace casi 40 años (Takemoto y col., 1978) y desde entonces se han empleado diversas técnicas para la determinación de FSs en exudados. En las dos primeras décadas, los métodos consistían en: i) determinación de la capacidad de solubilizar hidróxido férrico coloidal en unas condiciones específicas, descritas en detalle por Takagi (1993); ii) determinación individual de FSs mediante cromatografía de capa fina o HPLC con derivatización post-columna con *o*-ftalaldehído y detección por fluorescencia o con derivatización pre-columna con fenilisotiocianato y detección por UV; y iii) determinación de la estructura por resonancia magnética nuclear (RMN). Posteriormente, se han utilizado técnicas que permiten el análisis individualizado de FSs y/o sus complejos metálicos con más selectividad y sensibilidad mediante separación por HPLC y detección por MS con ionización por fuente de electrospray (ESI) (Xuan y col., 2006; Tsednee y col., 2012; Dell'mour y col., 2012) o separación por electroforesis capilar y detección por UV (Xuan y col., 2007), ESI-MS (Dell'mour y col., 2010) o MS con plasma acoplado inductivamente (ICP) (Dell'mour y col., 2010).

Compuestos reductores: fenólicos y flavinas

Los compuestos reductores pueden donar uno o más electrones a otra especie química en lo que se conoce como reacción redox. En el contexto de la deficiencia de Fe en plantas, entre la gran variedad de compuestos inorgánicos y orgánicos que pueden ejercer como reductores tienen especial relevancia los compuestos fenólicos y las flavinas.

Compuestos fenólicos

Los fenólicos son compuestos orgánicos cuya estructura molecular contiene al menos un anillo aromático unido al menos a un grupo funcional. Muchos de estos compuestos son metabolitos secundarios, es decir, productos biosintetizados por las plantas que aparentemente no tienen una función directa o conocida en su crecimiento y desarrollo. Son un grupo amplio y heterogéneo de compuestos (en plantas existen alrededor de 10.000) que comprende desde moléculas simples con un solo fenol, como los ácidos benzoicos, hasta polímeros complejos como los taninos condensados (Fig. 7). El grupo más amplio de compuestos fenólicos en plantas son los flavonoides (se conocen más de 5.000), estando algunos de ellos presentes en exudados radiculares y con papeles relevantes en la nutrición vegetal (p. ej., fijación de nitrógeno) (Cesco y col., 2010). Otros dos grupos de compuestos fenólicos frecuentes en los exudados radiculares son los ácidos cinámicos y las cumarinas, muchos de ellos conocidos por su capacidad para producir alelopatía. Una característica química notable de los compuestos fenólicos es que son muy susceptibles a la oxidación y participan en numerosas reacciones como agentes antioxidantes. Cabe señalar que a pH $\leq$ 5 el Fe(III) puede oxidar los grupos catecol a quinonas que pueden reaccionar con otros catecoles para dar dímeros y en el caso de que el proceso de oxidación/dimerización se repita varias veces, se produce la formación de polímeros. Asimismo, los compuestos fenó-

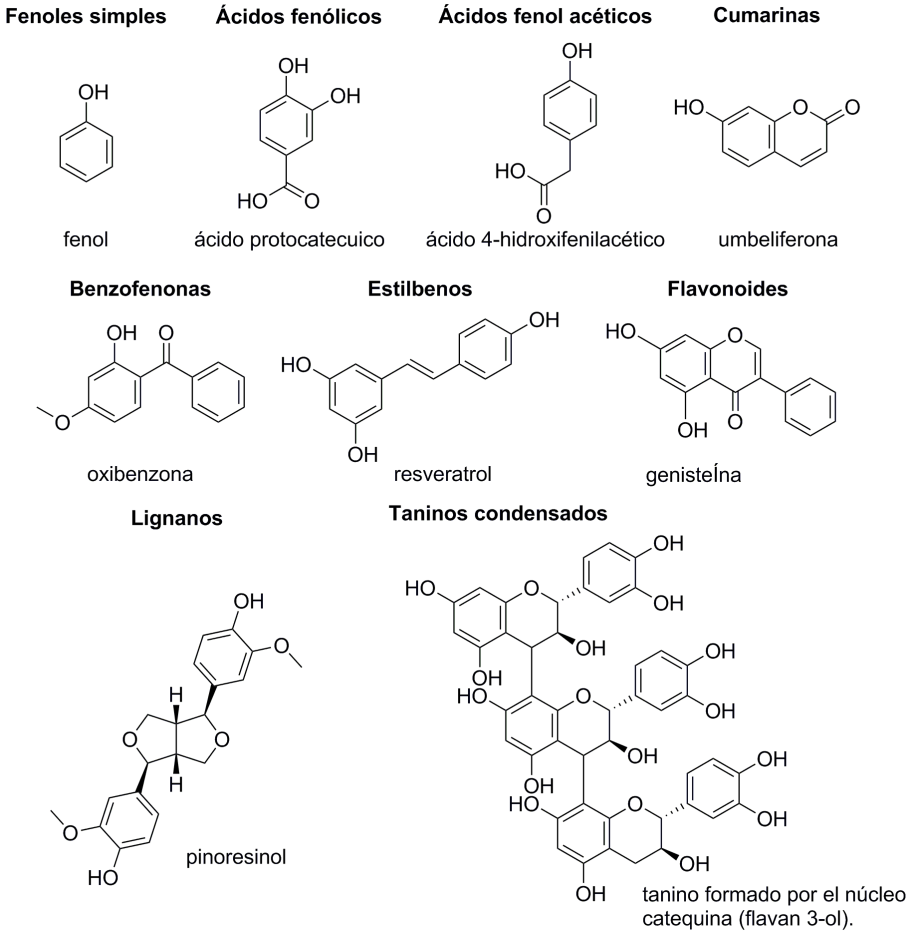

Figura 7 Estructuras químicas de algunos compuestos fenólicos producidos por las plantas.

licos de tipo catecol pueden formar con Fe(III) hasta tres complejos diferentes, con uno, dos, o tres ligandos catecolato según el pH del medio, dando lugar a disoluciones que difieren en su coloración (Fullenkamp y col., 2014) (Fig. 8).

La deficiencia de Fe o baja disponibilidad de Fe soluble en el medio de crecimiento aumenta la exudación de compuestos fenólicos de varias especies de plantas de Estrategia I (Tabla 5). La mayoría de las especies de esta Estrategia en las que se ha estudiado la exudación son de la familia Fabaceae (*G. max*, *M. ciliaris*, *Medicago sativa*, *P. sativum*, *Trifolium pratense* y *P. judaica*). Existen diferencias interespecíficas en la exudación de compuestos fenólicos, siendo destacadas las diferencias en función del pH del medio. Asi, en cacahuete (*Arachis hypogeae*), tomate, trébol rojo (*T. pratense*) y *M. ciliaris* la mayor exudación se observó a pH en el intervalo de 4 a 5 (Olsen y col., 1981; Römheld y Marschner, 1983; Hether y col., 1984; Jin y col., 2007; M'Sehli y col., 2008). Por el contrario, en *P. judaica* se produjo una mayor exudación y acumulación en las raíces de compuestos fenólicos cuando el pH del medio de cultivo fue próximo a la neutralidad (pH 8,3) (Donnini y col., 2012). Cuando se compararon diferentes genotipos de la especies *M. ciliaris* y guisante, se encontraron diferencias intraespecíficas relacionadas con la eficiencia en la toma de Fe, siendo más acusada la exudación de estos compuestos fenólicos en los genotipos considerados más eficientes (M'Sehli y col., 2008; Jelali y col., 2010). En un estudio más reciente se muestra que en plantas de guisante el estatus de Fe no influyó en la cantidad de compuestos fenólicos extraídos del suelo en contacto con las plantas, y fue 5 veces superior a la cantidad exudada por las raíces de plantas cultivadas en hidroponía (Pii y col., 2015). Cuando el suelo se inoculó con la rizobacteria promotora del crecimiento vegetal *Azospirillum brasilense* la cantidad de compuestos fenólicos extraídos del suelo aumentó con la deficiencia de Fe (Pii y col., 2015).

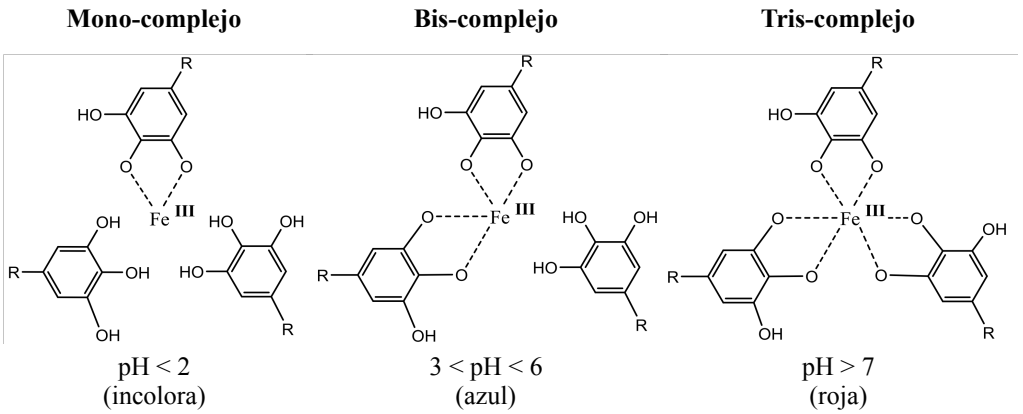

Figura 8 Complejos de Fe(III) con catecoles en función del pH y color de las soluciones resultantes. Tomada de Fullenkamp y col. (2014).

En muchos de estos estudios se analizaron compuestos fenólicos totales con el método de Folin-Ciocalteu (Tabla 5). Sin embargo, se sabe que este método es poco selectivo ya que el reactivo de Folin-Ciocalteu (mezcla de fosfomolibdato y fosfotungstato) reacciona con cualquier sustancia reductora que exista en la muestra (p. ej., ácido ascórbico) (Sánchez-Rangel y col., 2013). Por consiguiente, los datos presentados son más una medida de las sustancias reductoras que de los compuestos fenólicos liberados por las raíces de las plantas. Otros estudios utilizaron técnicas más selectivas que permitieron la identificación de compuestos fenólicos individuales en exudados radiculares de plantas deficientes en Fe. En exudados de plantas de cacahuete se identificó el ácido cafeico (Fig. 9) (Römhel y Marschner, 1983), mientras que en exudados de raíces de tomate se identificaron dos ácidos cinámicos: cafeico y clorogénico (Fig. 9) (Olsen y col., 1981; Hether y col., 1984). Estas identificaciones fueron realizadas separando los compuestos por HPLC y detectándolos por absorbancia en UV, tomando como referencia los tiempos de retención de estándares de fenólicos analizados en las mismas condiciones. Dado que todos los compuestos fenólicos absorben en la región del UV, los tiempos de retención no permiten la diferenciación entre la multitud de compuestos fenólicos existentes y por consiguiente los compuestos fenólicos identificados podrían ser esos o cualquier otro con un comportamiento cromatográfico similar en las condiciones utilizadas. Más recientemente, en exudados de alfalfa se identificó el isoflavonoide 2-(3′,5′-dihidroxiphenil)-5,6-dihidrobenzofurano (Fig. 8) utilizando análisis por MS y RMN del compuesto previamente aislado por HPLC-UV (Masaoka y col, 1993). Nuestro conocimiento actual adolece de estudios que, mediante tecnologías de análisis selectivas como HPLC-MS, permitan conocer la identidad de los fenólicos exudados por las raíces de algunas especies en respuesta a la deficiencia de Fe.

La síntesis y acumulación en la raíz de compuestos fenólicos inducida por deficiencia de Fe es consistente con la exudación de dichos compuestos. Así, en *M. ciliaris*, guisante y *P. judaica* la deficiencia de Fe incrementó la exudación y acumulación de compuestos fenólicos así como la actividad de la enzima fenilalanina-amonio liasa (PAL) y de otras enzimas de la ruta del ácido shikímico (M'Sehli y col., 2008; Jelali y col., 2010; Donnini y col., 2012; Tato y col., 2013). La ruta del ácido shikímico produce el amino-ácido aromático fenilalanina a partir del cual se sintetizan la mayoría de los compuestos fenólicos en plantas (Taiz y Zeiger, 2006). La PAL cataliza la reacción de eliminación de una molécula de amonio de la fenilalanina que produce ácido cinámico, sustrato de reacciones subsiguientes que adicionan grupos hidroxilo y otros sustituyentes dando lugar a ácidos, alcoholes y aldehídos hidroxi-cinámicos. Aunque esta parte de la ruta de biosíntesis de los compuestos fenólicos es general ya que da lugar a los productos a partir de los que se sintetizan los compuestos

Tabla 5. Especies de plantas que exudan compuestos fenólicos como respuesta a la deficiencia de Fe, compuestos encontrados y técnicas analíticas utilizadas.

Especie	Fenólicos	Técnica de análisis	Ref.
Guisante (*Pisum sativum*)	fenólicos totales	Método Folin-Ciocalteau (Espectroscopia VIS)	[1]
Medicago ciliaris			[2]
Parietaria difussa			[3-5]
Pepino (*Cucumis sativus*)			[6]
Soja (*Glycine max*)			[7,8]
Trébol rojo (*Trifolium pretense*)			[9]
Tomate (*Solanum lycopersicum*)	ácido cafeico, ácido clorogénico	HPLC-UV	[10, 11]
Cacahuete (*Arachis hypogaea*)	ácido cafeico		[12]
Alfalfa (*Medicago sativa*)	2-(3',5'-dihidroxiphenol)-5,6-dihidroxibenzofurano	HPLC, MS y RMN	[13]

[1] Jelali y col. (2010); [2] M'Sehli y col. (2008); [3] Dell'Orto y col. (2003); [4] Donnini y col. (2012); [5] Tato y col. (2013); [6] Pii y col. (2015); [7] Zocchi y col. (2007);. [8] Brown y Ambler (1973); [9] Jin y col. (2007); [10] Olsen y col. (1981); [11] Hether y col. (1984); [12] Römheld y Marschner (1983); [13] Masaoka y col. (1993).

fenólicos más complejos como flavonoides, cumarinas y otros (Fig. 7), la reacción catalizada por la PAL es la que separa una rama del metabolismo primario del secundario y representa un importante punto de regulación en la producción de muchos de los compuestos fenólicos. El análisis transcriptómico y proteómico de raíces de *A. thaliana* mostró que la deficiencia de Fe aumentaba la expresión de un conjunto de genes que codifican enzimas de varias de las reacciones de la parte general de la síntesis de los compuestos fenólicos (PAL, hidroxicinamoil CoA ligasas, cafeoil CoA-O-metiltransferasa) junto con la enzima feruloil-CoA 6'-hidroxilasa (F6'H1) (Yang y col., 2010; Lan y col., 2011) que cataliza la conversión de cafeoil-CoA a feruloil-CoA, clave en la síntesis de la cumarina escopoletina (Kai y col., 2008). Aunque los análisis mediante UPLC-MS/MS mostraron un aumento de escopoletina en raíz con la deficiencia de Fe en *A. thaliana* (3 días de tratamiento), dicha cumarina no llegó a ser detectada en los exudados radiculares (Yang y col., 2010).

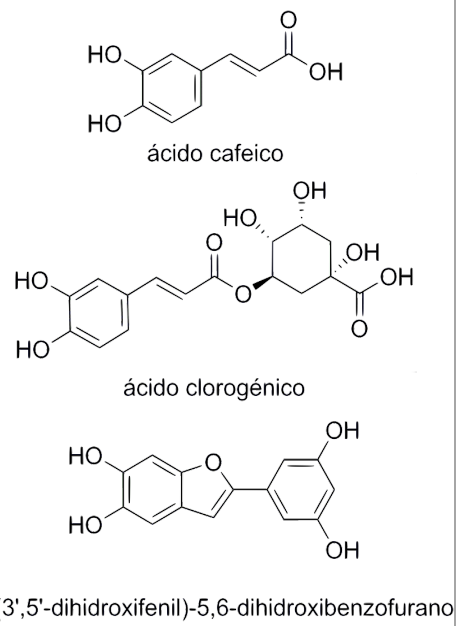

Figura 9 Estructuras químicas de compuestos fenólicos identificados en exudados de plantas deficientes en Fe.

Flavinas

Las flavinas son grupo pequeño de compuestos que, como los fenólicos, pueden actuar como reductores. La estructura básica de las flavinas es el heterocíclico nitrogenado de tres anillos y dos grupos oxo 7,8-dimetil-isoaloxacina, dibujada en color negro en la Figura 10 en la que se presentan las estructuras químicas de los nucleótidos de flavina biológicamente importantes (Fig. 10a) junto con los exudados por las raíces de las plantas en respuesta a la deficiencia de Fe (Fig. 10b). El anillo de isoaloxazina otorga una gran versatilidad química por lo que las flavinas intervienen en muchas reacciones metabólicas de oxidación-reducción en las que transfieren

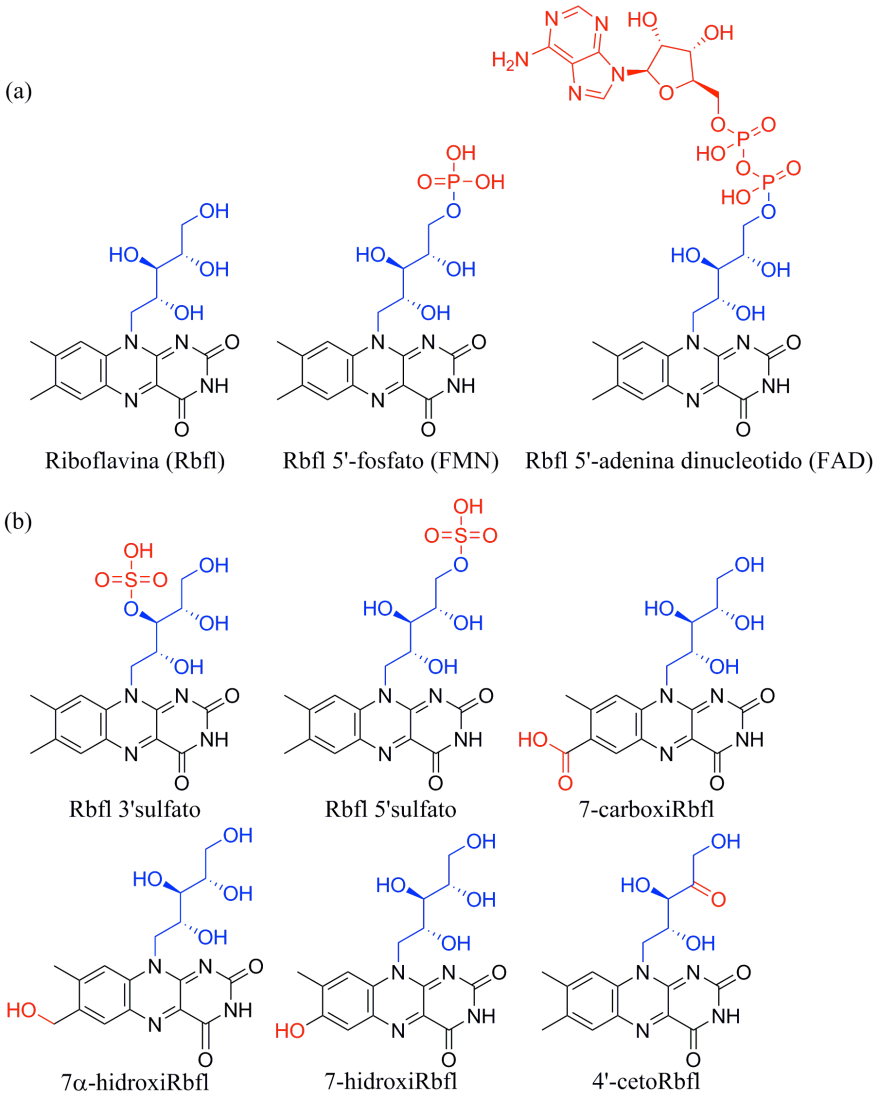

Figura 10 Estructuras químicas de flavinas identificadas en plantas: a) Rbfl, FMN y FAD; y b) derivados de Rbfl encontrados en exudados de plantas deficientes en Fe: Rbfl 3' sulfato y Rbfl 5´sulfato en *B. vulgaris*, 7-carboxi-Rbfl, 7-α-hidroxi-Rbfl y 7 hidroxi-Rbfl en *M. truncatula*, y 4´-ceto Rbfl en *C. sativus*. Se resaltan las modificaciones con respecto al triple anillo de 7,8-dimetil-isoaloxacina: unidad ribitol en azul y diferentes modificaciones químicas en rojo.

uno o dos electrones (Miura, 2001). Las flavinas pueden existir en tres estados redox diferentes: oxidado (Fl), semiquinona (FlH·) y completamente reducido ($RFlH_2$) (Fig. 11a). El estado semiquinona tiene afinidad por metales, incluyendo Fe, llegándose a formar complejos metálicos con un anillo de cinco miembros (Fig. 11b) (Hemmerich y Spence, 1966; Müller y col., 1968). El nucleótido riboflavina (Rbfl), o vitamina B2, es la flavina más abundante de la naturaleza y la 7,8-dimetil-isoaloxacina de su estructura está unida a una unidad ribitol (indicada en azul en la Fig. 10a), derivada del monosacárido ribosa. En tejidos vegetales la presencia de flavinas libres es escasa, y la mayoría de éstas se hallan unidas a proteínas (flavoproteínas), que utilizan las flavinas Rbfl-5'-fosfato (FMN) y Rbfl-5'-adenosina dinucleotido (FAD) como coenzimas (Fig. 10a).

Cuando se cultivan en deficiencia de Fe, muchas especies de Estrategia I secretan flavinas al medio de cultivo. Se han realizado estudios con un número significativo de especies y se puede decir que la exudación y/o acumulación de flavinas inducida por deficiencia de Fe es un fenómeno bastante difundido entre las especies dicotiledóneas. Se ha demostrado la presencia de flavinas

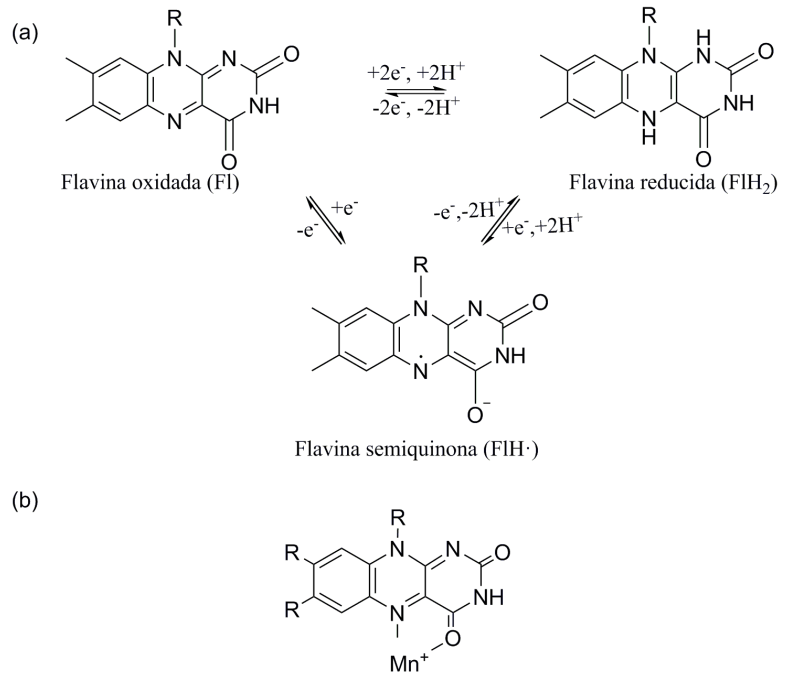

Figura 11 Estructura básica de las flavinas con sus estados redox y esquema de su quelación con metales. a) Estados redox de las flavinas: oxidada (Fl), semiquinona (FlH·) y completamente reducida (FlH_2); y b) Quelación de metales (en este caso Mn) mediante el anillo de isoaloxacina.

en exudados y/o raíces de al menos 29 especies (Tabla 6) de 7 familias distintas (Amaranthaceae, Amaryllidaceae, Apiaceae, Asteraceae, Curcubitaceae, Fabaceae y Solanaceae), mientras que no se hallaron en 41 especies de 13 familias distintas (Aizoceae, Apiaceae, Asteraceae, Brassicaceae, Boraginaceae, Caryophylllaceae, Fabaceae, Lamiaceae, Malvaceae, Papaveraceae, Poaceae, Portulaceceae y Solanaceae) (Welkie, 2000; Higa y col., 2010; Rodríguez-Celma y col., 2011b; Satoh y col., 2016). Parece que la exudación de flavinas no se rige por categorías taxonómicas obvias. Por ejemplo, se produce en las 7 especies de la familia Amaranthaceae investigadas (incluida *Beta vulgaris* var. Saccharífera y *Spinacia oleracea*), en 9 de las 14 especies de la familia Solanaceae y en sólo 1 especie (*Medicago truncatula*) de las 9 de la familia Fabaceae. Hasta el momento no se ha encontrado en ninguna especie de la familia de las Brassicaceae (incluida *A. thaliana*).

Todos los estudios de exudación radicular de flavinas en respuesta a deficiencia de Fe utilizan plantas crecidas en hidroponía y determinan las flavinas directamente en la solución nutritiva. La técnica más utilizada es espectroscopía de fluorescencia (Tabla 6), técnica que no permite diferenciar entre Rbfl y otras flavinas por lo que algunos estudios utilizan el término "Rbfl" en lugar de "flavinas totales" usado en la Tabla 6. Cuando se han empleado técnicas de separación como HPLC acoplada a detectores UV-VIS y fluorescencia se ha observado generalmente la presencia de Rbfl junto con otros compuestos de características espectrales similares que fueron identificados bien aislando los compuestos por cromatografía y analizando el aislado por RMN y MS o bien por HPLC acoplado a MS con analizadores de tiempo de vuelo (TOF) y cuadrupolo-TOF (Susín y col., 1993, 1994; Rodríguez-Celma y col. 2011b; Satoh y col., 2016). Así, la flavina más frecuentemente exudada es la Rbfl, minoritaria cuando se exudan también otras flavinas. Se han identificado dos sulfatos de Rbfl en exudados y en raíces de remolacha azucarera (*Beta vulgaris* var. Saccharífera) y espinaca (*Spinacia oleracea*) (Susín y col., 1993, 1994), dos derivados hidroxilados y uno carboxilado de Rbfl en *M. truncatula* (Rodríguez-Celma y col., 2011b), y más recientemente se ha identificado 4'-ceto Rbfl en exudados de pepino (Satoh y col., 2016) (ver estructuras en la Fig. 10b).

Tabla 6. Especies de plantas que exudan flavinas como respuesta a la deficiencia de Fe, flavinas encontradas y técnicas analíticas utilizadas. El asterisco indica que para esa especie sólo se ha observado que acumula flavinas en raíces pero se desconoce si las exuda al medio.

Especie	Flavina	Técnica de análisis	Ref.
Acelga silvestre (*Beta maritima)* Amaranto redondo (*Gomphrena globosa)* *Calabaza* (*Curcubita moschata*) Calabaza del peregrino (*Lagenaria siceraria*) Calabacín (*Curcubita pepo)* *Datura meteloides* *Datura stramonium* *Franseria dumosa* Girasol (*Hellianthus annuus*) *Luffa aegypticra* Lechuga (*Lactuca sativa*) *Nicotiana alata* *Nicotiana rustica* *Physalis alkekengi* *Physalis ixocarpa* Pimiento (*Capsicum annuum*) Quenopodio (*Chenopodium murale*) Quinua (*Chenopodium quinoa*) Sandía (*Citrullus lanatus*) Tabaco (*Nicotiana tabacum*)	flavinas totales	Espectroscopía de fluorescencia	[1]
Hyoscyamus albus	Rbfl	HPLC-UV/VIS	[2]
Amaranto (*Amaranthus caudatus*)*	Rbfl, Rbfl sulfatos	HPLC-UV/VIS-MS	[3]
Melón (*Cucumis melo*)*	Rbfl, flavina desconocida		
Perejil (*Petroselinum crispum*)*	Rbfl		
Medicago truncatula	Rbfl, 7α-hidroxiRbfl, 7-hidroxiRbfl, 7-carboxiRbfl	HPLC-UV/VIS-MS y HPLC-MS-MS	[3]
Espinaca (*Spinacea oleracea*)*	Rbfl, Rbfl sulfatos	HPLC-UV/VIS, MS, RMN y HPLC-UV/VIS-MS	[3,4]
Remolacha azucarera (*Beta vulgaris*)	Rbfl, Rbfl sulfatos		[3,4,5]
Pepino (*Cucumis sativus*)	Rbfl, 4-ceto Rbfl	HPLC-fluorescencia, HPLC-MS-MS y RMN	[6]

[1] Welkie (2000) y sus referencias; [2] Higa y col. (2008); [3] Rodríguez-Celma y col. (2011b); [4] Susín y col., 1993; [5] Susín y col., 1994; [6] Satoh y col. (2016).

Por lo tanto, no se descarta la posibilidad de identificar otros derivados de Rbfl en alguna de las especies ya estudiadas mediante el uso de tecnologías basadas en HPLC-MS solas o en combinación con RMN. Los grupos funcionales que diferencian la estructura química de la Rbfl y de las otras flavinas confieren más polaridad a la molécula (Fig. 10b) y probablemente más solubilidad. Por ejemplo, los sulfatos de Rbfl pueden alcanzar una solubilidad dentro del orden de mM, mientras que Rbfl tiene una solubilidad máxima de aproximadamente 140 μM (Susín y col., 1994).

En las especies remolacha azucarera y *M. truncatula* se ha observado que la exudación radicular de flavinas inducida por deficiencia de Fe es mucho mayor a pH ligeramente ácido que neutro (pH 5,5 frente a pH 7,5) y que las concentraciones de flavinas acumuladas en la raíz son mucho mayores a pH neutro que

a pH ligeramente ácido (Susín y col., 1993; Rodríguez-Celma y col, 2011b). En base a estos datos algunos autores han hipotetizado que la exudación de las flavinas esta mediada por un gradiente de protones, o por un transportador "antiporte" con protones. En estas dos especies, la deficiencia de Fe aumentó la expresión en raíz del gen que codifica la enzima 6,7-dimethyl-8-ribitillumazina sintasa (DMRL) y cataliza el paso previo a la última reacción que conduce a la biosíntesis de Rbfl (Andaluz y col., 2009; Rellán-Álvarez y col., 2010; Rodríguez-Celma y col., 2011a). Las flavinas se acumulan mayoritariamente en la parte distal de la raíz (0-10 mm desde el ápice) donde la actividad FCR es también mayor (Susín y col., 1993; López-Millán y col., 2000; Rodríguez-Celma y col., 2011b).

1.2.2. Mecanismos de exudación

A pesar de la importancia de la exudación radicular para la nutrición de la planta y sus relaciones con otros organismos, se conoce poco sobre el paso de cada uno de los metabolitos exudados a través de la membrana plasmática (MP) de las células de la raíz. El tamaño de los poros en las paredes celulares de las plantas es de alrededor de 2 nm (Berestovsky y col., 2001) por lo que la liberación de los metabolitos de tamaños menores no parece estar restringida por la pared celular. Dada la diversidad química de los metabolitos presentes en los exudados radiculares (Tabla 2), los mecanismos de transporte a través de la MP son también variados (Fig. 12). En general, la mayoría de los compuestos de bajo peso molecular se liberan al exterior de la célula sin aporte de energía, bien a través del transporte pasivo o a favor de gradientes de concentración o de carga eléctrica. El transporte se puede realizar ya sea a través de la bicapa lipídica de la membrana plasmática, o bien por canales iónicos, hasta conseguir el equilibrio entre el interior y exterior de la célula. Los metabolitos de carácter lipofílico se pueden transportar mediante difusión en la propia capa lipídica de la membrana plasmática, sin requerir el uso de canales o de proteínas transportadoras. Otro mecanismo sin necesidad de aporte energético es la difusión facilitada, que mediante el cambio de conformación de proteínas integrales de la membrana plasmática puede facilitar el transporte de algunos solutos. El transporte de metabolitos de mayor tamaño o la transferencia de sustancias polares o apolares en contra de gradiente de concentración o de carga eléctrica se realiza mediante transporte activo, que requiere un gasto de energía metabólica. Este tipo de transporte puede ser realizado mediante la secreción celular activa o exocitosis, en el que una vesícula de membrana se desplaza hasta la membrana plasmática y su contenido se vierte a la rizosfera. Asimismo, el transporte activo puede realizarse mediante proteínas transportadoras de membrana. Entre las proteínas transportadoras se incluyen las proteínas ABC (*del inglés* ATP-Binding Cassette transporters), proteínas de la familia MATE (*del inglés* Multidrug and Toxin Effux), proteínas MFS (*del inglés* Major Facilitator Superfamily) y proteínas ALMT (*del inglés* ALuminum-activated Malate Transporter) (Fig. 12).

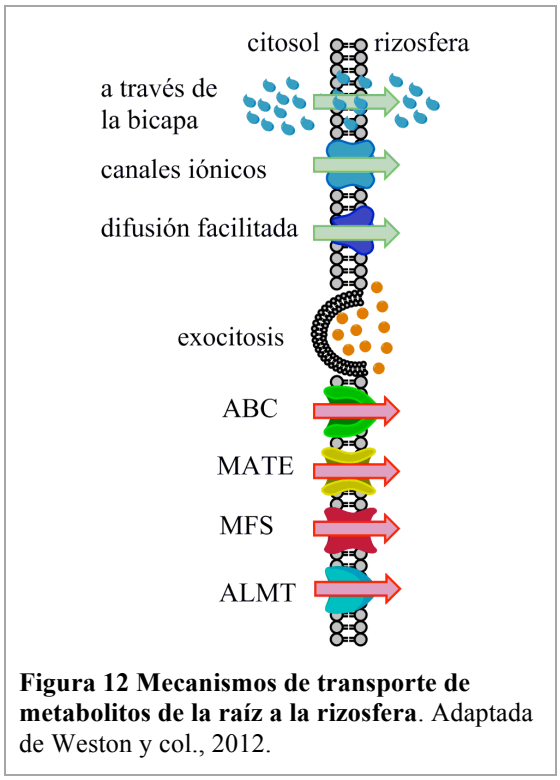

Figura 12 Mecanismos de transporte de metabolitos de la raíz a la rizosfera. Adaptada de Weston y col., 2012.

Transportadores ABC

Las proteínas ABC comprenden una gran familia de proteínas que es de dos a cuatro veces más numerosa en las plantas terrestres que en otros organismos (Hwang y col., 2016). En plantas modelo como arroz y *A. thaliana* se han identificado 133 y 130 genes *ABC*, respectivamente. La mayoría de estas

proteínas son transportadores que transfieren metabolitos utilizando la energía de la hidrólisis del adenosín trifosfato (ATP). Por lo tanto, los ABC son transportadores activos primarios, que pueden transportar metabolitos en contra del gradiente de concentración (Verrier y col., 2008). Un reciente análisis de la literatura sobre los ABC de plantas propuso que la gran diversidad de estos transportadores permite la supervivencia de las plantas en suelo (Hwang y col., 2016). Esta podría ser la razón por la que las plantas mantuvieron los transportadores ABC que se multiplicaron durante la evolución alcanzando un alto grado de diversificación funcional (Hwang y col., 2016).

Los transportadores ABC se caracterizan por transportar numerosos metabolitos de estructuras y características químicas muy diferentes, desde iones hasta macromoléculas. Es más, un mismo ABC transporta múltiples sustratos. Los ABC no sólo exportan sustratos desde el citosol hacia el exterior de la célula a través de la MP sino también los exportan hacia otros orgánulos internos tales como la vacuola. Proteínas ABC están involucradas en el transporte de: i) materiales para recubrimiento superficial de los células epidérmicas, como lípidos cuticulares; ii) metabolitos de defensa contra agentes patógenos (p. ej. el alcaloide berberina); iii) hormonas como citoquininas (p. ej., trans-zeatina), ácido abcísico (ABA), auxinas (p. ej. ácido indolacético; IAA) o estrigolactonas (p. ej. rac-GR24); y iv) compuestos tóxicos, como arsénico en forma de complejos con fitoquelatinas (Hwang y col., 2016). No se ha descrito que ninguna otra familia de transportadores sea capaz de transportar tal diversidad de metabolitos.

Los ABC poseen una estructura modular consistente en una o dos unidades de dos elementos estructurales básicos, un dominio transmembrana hidrófobo (TMD *del inglés* TransMembrane Domain), usualmente con seis α-hélices que se disponen en la membrana, y un dominio citosólico de unión al ATP (NBD *del inglés* Nucleotide Binding Domain) (Verrier y col., 2008). Se considera que las TMD forman la vía para el movimiento del soluto a través de la bicapa fosfolipídica y parecen determinar, al menos en parte, la selectividad por el/los sustrato/s del transportador. La clasificación de las proteínas ABC mediante alineaciones de las secuencias de aminoácidos y análisis filogenéticos han dado lugar a ocho subfamilias (A-H) (Verrier y col., 2008). Los grupos particularmente interesantes son las subfamilias ABCB y ABCG, que son numerosas en las plantas terrestres (Hwang y col., 2016). Así, en arroz existen 26 ABCBs y 50 ABCGs y en *A. thaliana* 30 y 43, respectivamente, por tanto estas dos subfamilias representan 56-57% de todos los ABCs presentes en estas especies. Transportadores de estas dos subfamilias fueron relevantes en el primer estudio que demostró de una forma directa la participación de esta familia de transportadores en la secreción radicular de metabolitos (Badri y col., 2008). Este estudio identificó 8 ABCGs, 4 ABCBs entre los 25 genes altamente expresados en células de raíz (células epidérmicas, caliptra y endodermis) de *A. thaliana* y compararon los perfiles analizados por GC-MS y HPLC-MS de los exudados recogidos de plantas silvestres y líneas mutantes "knock-out" de 7 de dichos transportadores incluidos 3 ABCGs. Las diferencias observadas en la composición del exudado evidencian por una parte la participación de estos transportadores en la secreción radicular de metabolitos estructuralmente diferentes, y por otra que más de un transportador podría estar implicado en la secreción de un metabolito específico.

La subfamilia G contiene dos grupos de proteínas, uno de ellos que comprende las que tienen sólo una unidad NBD-TMD y otro con las que contienen dos unidades TMD-NBD también llamados PDR (*del inglés* Pleiotropic Drug Resistance) o también ABCG de tamaño completo. Entre las funciones conocidas de los PDRs de las plantas se incluyen la resistencia a patógenos y el transporte de terpenoides antimicrobianos y herbicidas auxínicos. Las primeras proteínas PDR de plantas identificadas fueron NpABC1 en *Nicotiana plumbaginifolia* y SpTUR2 en *Spirodela polyrrhiza*, involucradas en el transporte del diterpeno esclareol, un agente anti-fúngico (Smart y Fleming, 1996; Jasinski y col., 2001). En *A. thaliana* existen 15 proteínas PDR, y se determinó que la proteína AtABCG40 (AtPDR12) era homóloga a NpABC1 y SpTUR2 por su alta resistencia a patógenos (van den Brûle y Smart, 2002).

Posteriormente, se demostró que en el mutante de *A. thaliana abcg30* (*pdr2*) aumenta la exudación de compuestos fenólicos, disminuye la de azúcares y lleva a cambios en los microorganismos de las proximidades de la raíz (Badri y col., 2009). Así, en el entorno de las raíces de *abcg30* aumenta la abundancia de bacterias potencialmente beneficiosas y bacterias eficientes en la detoxificación bien porque éstas son capaces de utilizar y/o neutralizar los compuestos fenólicos exudados o bien porque dichos fenólicos tienen actividad antimicrobiana frente a muchas otras especies bacterianas del suelo (Badri y col., 2009).

La primera referencia existente de cambios de expresión de ABCG inducidos por deficiencia de Fe fue el aumento de *NtPDR3* en cultivos de células de tabaco (Ducos y col., 2005). Posteriormente, se descubrió que su ortólogo en *A. thaliana*, *AtABCG37* (también conocido como *AtPDR9* o *PIS1*) aumenta su expresión en raíz con la deficiencia de Fe (Yang y col., 2010). Otros autores han descrito que AtABCG37 se expresa en la MP de las células epidérmicas de las puntas de las raíces permitiendo la salida del precursor de auxinas, ácido indol-3-butírico (IBA), y del análogo auxínico sintético, ácido 2,4-dicloro-fenoxi-acético (2,4-D) pero no del IAA (Ito y Gray, 2006; Růžička y col., 2010). Cuando se expresa en otros organismos se ha observado que los compuestos fenólicos, como ácidos cafeico, sinápico y benzoico, inhibían el transporte de IBA y 2,4-D mediado por AtABCG37 (Růžička y col., 2010) lo que conduce a plantear la hipótesis de que este transportador podría mediar el transporte de compuestos fenólicos a la rizosfera, especialmente en plantas dicotiledóneas creciendo en condiciones de deficiencia de Fe.

La mayoría de los estudios de transportadores ABC en plantas han descrito los fenotipos de las líneas mutantes pero no han identificado los sustratos del transportador. Los obstáculos para esta identificación son: i) la dificultad en el manejo de un grupo tan amplio de genes que además son difíciles de expresar en sistemas heterólogos; ii) la baja disponibilidad de metabolitos fluorescentes o marcados con isótopos radiactivos requeridos para el estudio de la actividad transportadora; iii) la dependencia del fenotipo del mutante de una mezcla compleja de metabolitos; y iv) la dificultad de analizar por métodos convencionales los sustratos de los ABCs que pueden ser compuestos hidrófobos o complejos (tales como compuestos conjugados con glutatión) y también metabolitos secundarios muchos de los cuales probablemente todavía no se conocen (Hwang y col., 2016). La utilización de técnicas avanzadas de análisis que permitan la identificación de los sustratos en combinación con el cribado a gran escala de sustratos y enfoques de biología de sistemas pueden ayudar a superar estos obstáculos.

Transportadores MATE

La familia de proteínas transportadoras MATE está presente en todos los organismos vivos incluyendo las plantas superiores y, al igual que la familia ABC, es más abundante en plantas que en otros organismos. En arroz y *A. thaliana* se han identificado 45 y 56 genes *MATE*, respectivamente (Wang y col., 2016). Los MATE utilizan un gradiente electroquímico de Na^+ o H^+ a través de la membrana como fuerza motriz para el transporte. Dado que los MATE transportan el sustrato en la dirección opuesta a la de Na^+ o H^+, la mayoría de estos transportadores exportan el sustrato desde el citoplasma al apoplasto o a las vacuolas. Las proteínas MATE contienen entre 400 y 700 aminoácidos dispuestos en 12 hélices que atraviesan la membrana (Takanashi y col., 2014). Aunque no parecen tener una secuencia de consenso conservada, todas las proteínas MATE comparten aproximadamente el 40% de similitud de secuencia. Estos transportadores están involucrados en muchos eventos biológicos durante el desarrollo de las plantas. Hasta la fecha se conoce que permiten la exportación de xenobióticos (p. ej. tetrametilamonio), la señalización hormonal a través del transporte de ABA y ácido salicílico, la acumulación de metabolitos secundarios (alcaloides y flavonoides) y la translocación de ligandos metálicos involucrados en la tolerancia a la toxicidad de Al y el transporte de Fe (Takanashi y col., 2014).

La mayoría de los MATE estudiados se expresan en raíz y algunos están localizados en la MP e involucrados en la exudación radicular de citrato hacia la rizosfera de varias especies vegetales tolerantes a toxicidad por Al (Takanashi y col., 2014). El citrato forma

quelatos con Al disminuyendo la especie tóxica de este elemento (Al^{3+}) en el apoplasto y en el entorno próximo a la punta de la raíz. Así, por ejemplo una alta expresión de *HvAACT1* y *SbMATE* es necesaria para la tolerancia a Al en cebada y sorgo, respectivamente (Furukawa y col., 2007; Sivaguru y col., 2013).

En relación con la nutrición férrica de las plantas se han descrito hasta 8 MATEs tanto en plantas de Estrategia I como de Estrategia II. Todos ellos se expresan en raíz, pero no exclusivamente, y todos están involucrados en el transporte de Fe en la planta. Los estudios en dos de ellos (AtFRD3 y OsFRDL1) mostraron su expresión en las células del periciclo y en células de los tejidos vasculares, cuando los genes de estos MATEs se expresan en oocitos de *Xenopus* transportan citrato y las líneas mutantes *Atfrd3* y *Osfrdl1* tienen concentraciones de citrato en el xilema menores que el WT y acumulan más Fe en raíz. Por tanto, estas proteínas permiten la exportación de citrato al xilema y facilitan la translocación de una importante fracción de Fe desde las raíces a las partes superiores de la planta (Durrett y col., 2007; Yokosho y col., 2009). Otros MATE que realizan la misma función se identificaron en soja (GmFRD3a y GmFRD3b), centeno (ScFRDL1), cebada (HvAACT1) y trigo (TaMATE1b) [ver referencias en la revisión de Takanashi y col., (2014)]. Otros dos MATE, OsPEZ1 y OsPEZ2, están involucrados en el transporte de Fe en arroz. Ambos transportadores aumentan su expresión en respuesta a la deficiencia de Fe inducida por Cd, los mutantes *Ospez1* y *Ospez2* tienen concentraciones en xilema de Fe y de los ácidos cafeico y protocatecuico menores que las plantas WT, y cuando *OsPEZ1* y *OsPEZ2* se expresan en oocitos de *Xenopus* transportan ácido protocatecuico (Ishimaru y col., 2011; Bashir y col., 2011). No obstante el análisis histoquímico mostró una diferencia notable entre OsPEZ1 y OsPEZ2: OsPEZ1 se encontró en las células próximas a los vasos del xilema, mientras que OsPEZ2 se encontró en las células de la región de elongación de la raíz. Esta localización es consistente con el papel de este MATE en la secreción de compuestos fenólicos hacia la rizosfera, ya que los ácidos cafeico y protocatecuico fueron detectados en los exudados de WT pero no en los de las plantas *Ospez2* (Bashir y col., 2011). Los cambios diurnos de expresión de *OsPEZ2*, pero no de *OsPEZ1*, son similares a los descritos para la secreción de fitosideróforos a la rizofera (ver en la sección 1.2.1. el apartado dedicado a fitosideróforos) y, por consiguiente, se podría esperar que la secreción de fitosideróforos y fenólicos en arroz fuese simultánea (Bashir y col., 2011).

Transportadores MFS

La superfamilia principal de transportadores facilitadores es la familia más numerosa de transportadores secundarios. En esta familia el transporte de un sustrato en contra del gradiente electroquímico está acoplado al transporte de otra especie química a favor de dicho gradiente. Incluye transportadores que funcionan como "uniporters" (transportan una sola molécula), "simporters" (transportan dos moléculas a la vez en la misma dirección), y "antiporters" (transportan dos moléculas en direcciones opuestas). Son proteínas que se caracterizan por tener entre 400 y 600 aminoácidos en 2 unidades estructurales de 6 dominios transmembrana (Reddy y col., 2012). La superfamilia MFS puede dividirse en varias familias, siendo las de mayor número de miembros las de azúcares, las de aminoácidos, y las de permeasas que confieren resistencia a fármacos (MDR).

Los pocos transportadores MFS de plantas caracterizados hasta la fecha están implicados esencialmente en el transporte de azúcar, oligopéptidos, auxina, nitrato, fosfato y ligandos de Zn o complejos orgánicos con Zn (Remy y col., 2013; Liu y col. 2016). Además, dos miembros de la familia MFS, OsTOM1 y HvTOM1, que transportan los fitosideróforos desde el citosol a la rizosfera, constituyeron el último componente molecular identificado de la maquinaria de adquisición de Fe de las plantas de Estrategia II (ver sección 1.1.3.) (Nozoye y col., 2011). En arroz y cebada *OsTOM1* y *HvTOM1* aumentan su expresión con la deficiencia de Fe en raíz (localizándose las proteínas Gus-OsTOM1 y Gus-HvTOM1 en epidermis, exodermis, cortex y cilindro central) y cuando los genes se expresan en oocitos de *Xenopus* transportan DMA y no NA (Nozoye y col., 2011). Los cambios diurnos de expresión de *OsTOM1* y *HvTOM1* son consistentes con la secreción radicular de

DMA de la raíz. Además, cuando las plantas se cultivan en deficiencia de Fe estas proteínas se expresan en la células del sistema vascular y en el floema de las hojas lo que sugiere que tienen un papel en el transporte de DMA al xilema y floema lo que facilitaría la translocación de Fe dentro de la planta (Nozoye y col., 2011).

Transportadores ALMT

Los genes *ALMT* son exclusivos de plantas, y codifican canales aniónicos que forman poros selectivos en membranas y permiten que los sustratos se muevan a través de la membrana de manera pasiva. Estos canales poseen un dominio denominado ALMT, todavía sin caracterizar, y entre cinco y siete dominios transmembrana (Kochian y col., 2015). En arroz y *A. thaliana* se han identificado 9 y 14 genes *ALMT*, respectivamente. Esta familia de transportadores lleva el nombre de la función del primer miembro caracterizado (*TaALMT1*). Este gen se expresa en los ápices de las raíces de plantas de trigo, confiere tolerancia a Al y cuando se expresó en oocitos de *Xenopus*, en plantas de arroz y en suspensiones celulares de tabaco indujo la secreción de malato en respuesta a la presencia de Al (Sasaki y col. 2004; Piñeros y col. 2008). Posteriormente, se identificaron los ortólogos *TaALMT1* que conferían tolerancia a Al en colza (*Brassica napus)*, *A. thaliana* y centeno (ver referencias en revisión de Kochian y col., 2015). También se han identificado y caracterizado genes de esta familia que no confieren tolerancia a Al en maíz (ZmALMT1 y ZmALMT2) y cebada (HvALMT1) y se localizan en la MP de la raíz. ZmALMT1 transporta aniones inorgánicos en lugar de malato. Como para las otras familias de transportadores, las funciones de los ALMTs se extienden más allá de la tolerancia a Al, e incluyen papeles en la nutrición mineral, la homeostasis iónica, la regulación de turgencia y la función de las células guarda (ver revisión de Kochian y col., 2015).

1.2.3. Funciones de los compuestos exudados

Las funciones de los exudados de raíz en la rizosfera son muy complejas, debido por una parte a la propia complejidad de la mezcla de metabolitos exudados (Tabla 2) y por otra a la diversidad de las posibles interacciones (físicas, químicas y biológicas) con el entorno de la raíz. Además, dichas interacciones están afectadas por multiples factores como temperatura, presiones parciales de oxígeno y CO_2, pH, suelo, microbioma, etc. A continuación se describen algunas funciones de las tres clases principales de metabolitos (AOBPM, fitosideróforos, compuestos reductores) que son exudados por las plantas en respuesta a la deficiencia de Fe. Aunque se hace especial énfasis en la influencia de estos compuestos en la disponibilidad de Fe y otros nutrientes en la rizosfera, también se detallan otras funciones conocidas de los citados compuestos.

Movilización de nutrientes

La interacción de los componentes de los exudados radiculares con las partículas del suelo puede promover la movilización de nutrientes como el Fe, desde las fases sólidas hacia la solución del suelo, y, por consiguiente, facilitar la absorción del nutriente por la raíz. Los nutrientes pueden ser movilizados a través de una serie de mecanismos que no son necesariamente específicos para una determinada clase de metabolitos (Keuskamp y col., 2015). La eficiencia de los metabolitos de la misma clase o de diferentes clases en la movilización es muy dependiente de la estructura química de los mismos, así como de un amplio conjunto de factores como la concentración exudada, el pH y las características físicas y químicas de los componentes del suelo que interaccionan con los exudados radiculares.

Ácidos orgánicos de bajo peso molecular

Los AOBPM pueden movilizar nutrientes del suelo mediante tres mecanismos distintos: disminución de la saturación del metal en la solución, disolución del mineral e intercambio de ligandos. El primero de estos mecanismos, la disminución del estado de saturación del metal en la solución del suelo, se produce cuando los AOBPM exudados reaccionan con el Fe presente en la solución del suelo y forman complejos Fe-AOBPM, disminuyendo la concentración del metal en la solución y promoviendo la disolución de óxidos de Fe. Así, la adición de oxalato, citrato o malonato promovió la extracción de Fe de la superficie de goethita (Reichard y col., 2007). La afinidad de los AOBPM, como

la de otros ligandos orgánicos, por cationes metálicos como el Fe^{3+} es muy dependiente de su estructura, particularmente de la denticidad (número de grupos funcionales que pueden formar unirse al metal). Los AOBPM se unen al metal mediante grupos carboxilo e hidroxilo, y la estabilidad del complejo formado es dependiente de la proximidad de estos grupos dentro de la estructura del ácido y de la presencia de unidades α-hidroxiácido. Así, los AOBPM con tres grupos de carboxilos, como el citrato, forman complejos más estables con Fe [constante de estabilidad (logK) de 10^{11}] que los dicarboxílicos como malato o oxalato (log K de 10^{7} y 10^{8}, respectivamente). Así, el citrato fue más efectivo que el oxalato en la movilización tanto de Fe como de otros nutrientes (Ca, Mn y Zn) en tres suelos calizos (Khademi y col., 2009). En general, cuanto mayor sea la concentración del AOBPM en la solución extractante, mayor es la cantidad de elementos extraídos del suelo. Las soluciones a pHs ácidos proporcionan mayor estabilidad a los complejos de Fe(III)-AOBPM que las de pHs alcalinos.

La exudación de AOBPM puede movilizar el Fe a través de un segundo mecanismo que consiste en la disolución de minerales. El AOBPM ataca la superficie de los minerales lo que comporta la meteo-rización de los mismos. El proceso ocurre en varias etapas y comienza con la adsorción específica del AOBPM a la superficie del mineral a través de la formación de un complejo de coordinación de esfera interna con los átomos de Fe contenidos en la estructura del mineral. Este complejo hace que se debiliten los enlaces del Fe al mineral y promueve la liberación del complejo Fe-AOBPM que finalmente es transportado a la disolución suelo aumentando el Fe soluble a disposición de la planta. La denticidad del ligando orgánico también afecta a la labilidad de los complejos de esfera interna, de forma que los ligandos bi- y tri-dentados, como los AOBPM di- y tri-carboxílicos, son más eficientes en la disolución de óxidos que los ligandos monodentados, como los AOBPM monocarboxílicos (Ludwig y col., 1995). Además, otra característica estructural que afecta a la eficiencia en la disolución es el número de miembros del anillo de quelato que se establece en la formación del complejo. Por ejemplo, cuando se compara entre AOBPM dicarboxílicos, el oxalato que forma un anillo de cinco miembros conduce a la disolución máxima de Fe a partir de los óxidos férricos seguida de malonato, con un anillo de seis miembros y, por último, de succinato, malato y tartrato que forman quelatos con anillos de siete miembros (Johnson y Loeppert, 2006). A pHs alcalinos la baja estabilidad de los complejos de Fe con AOBPM junto con la lenta disolución de los minerales de Fe hace que estos componentes de los exudados puedan no ser suficientes para suministrar Fe a la planta (Jones y col., 1996; Jones 1998).

Los AOBPM, en general, existen en su estado total o parcialmente desprotonado en la rizósfera y, por tanto, actúan como ligandos aniónicos y tienen la posibilidad de intercambiarse con otros ligandos aniónicos presentes en el suelo en lo que constituye un tercer mecanismo de estos ligandos para movilizar nutrientes del suelo. Por ejemplo, los AOBPM pueden sustituir a los aniones inorgánicos fosfato o sulfato que, a menudo, se encuentran adsorbidos a las superficies positivamente cargadas de los óxido férricos y de aluminio en los suelos calizos, y así aumentar la disponibilidad de P y S para la planta (Tejedor-Tejedor y Anderson, 1990; Obruger y col., 2009, 2011). El efecto del pH sobre la movilización promovida por AOBPM de fosfato y Fe ha sido estudiado en un sistema modelo con óxido férrico y fosfato adsorbido. Se observó un aumento de la liberación de ambos nutrientes con la disminución del pH de 7,0 a 4,0 (Johnson y Loeppert, 2006). El óxido férrico utilizado afectó a la cantidades de fosfato y Fe liberadas por interacción con AOBPM, siendo mayor con ferrihidrita que con goethita.

Mediante el mecanismo de intercambio de ligando los aniones de AOBPM también se puede liberar el fosfato adsorbido a sustancias húmicas complejas poco solubles, lo que aumenta la biodisponibilidad de este macro-nutriente. De hecho, en un estudio con citrato se observó que éste moviliza más eficazmente fosfato unido a los complejos Fe-húmicos que unido a óxidos metálicos (Gerke, 1993). En cuanto a la eficiencia relativa de citrato, malato, malonato y oxalato para movilizar fosfatos y Fe de suelos diferentes en la cantidad de sitios de unión a P y en el contenido de P lábil, el citrato resultó ser

generalmente el más eficaz (Obruger y col., 2011).

Otras reacciones de intercambio de ligando en las que pueden estar involucrados los AOBPM en la rizosfera son las que ocurren con complejos insolubles de sustancias húmicas y Fe, y que pueden conducir a la disolución de estos complejos. Así, la unión de AOBPM con el Fe de dichos complejos rompe enlaces del metal con la molécula húmica disminuyendo el tamaño y la polaridad del complejo húmico de Fe lo que conduce a aumentar su solubilidad (Gerke, 1993).

El comportamiento de los AOBPM en el suelo es complejo y pueden ocurrir una serie de procesos que restan eficiencia a la movilización de nutrientes. Uno de los factores más relevantes es la degradación de los AOBPM por los microorganismos, especialmente abundantes en las próximidades de la raíz. En estudios realizados con una amplia gama de suelos se observaron dos fases de descomposición de estos compuestos (Oburger y col., 2009). Una fase rápida debida a la absorción microbiana de los AOBPM de la solución del suelo y su uso inmediato en procesos catabólicos (p. ej. respiración), y una fase lenta debida a la toma y temporal inmovilización de los AOBPM por los microorganismos en formas metabólicas no respirables, que más tarde se pueden transformar en AOBPM. Otro factor que afecta a la eficacia en la movilización de nutrientes es la adsorción/desorción de los AOBPM en determinadas fase sólidas del suelo. La pérdida de un AOBPM de la solución del suelo aumenta cuando disminuyen las cantidades de sitios de unión a aniones, sugiriendo que la adsorción regula la disponibilidad de estos componentes de los exudados (Oburger y col., 2009). Existen otros factores como la formación de precipitados con otros elementos como el oxalato de Ca, la mineralización abiótica, y la lixiviación en el suelo que también reducen la movilización de nutrientes promovida por AOBPM (Jones, 1998).

Fitosideróforos

Los FSs exudados por las raíces de las plantas de Estrategia II también promueven la movilización de nutrientes del suelo, especialmente de micronutrientes metálicos. Los FSs poseen una afinidad elevada para la quelación del Fe(III), Cu(II), Zn(II), y Ni(II), y forman complejos de estabilidad comparable, decreciendo en el orden Fe(III)> Cu(II) > Ni(II) > Zn(II) (Murakami y col., 1989). Aunque los FSs difieren en la estructura química (Fig. 6), todos se coordinan con Fe(III) a través de los 2 grupos amino, 3 grupos carboxilato y 1 grupo hidroxilo, formando un complejo octaédrico (Kraemer y col., 2006). La alta afinidad de FSs por Fe(III) (logK = 18-19; Murakami y col., 1989) hace que estos ligandos reaccionen rápidamente con las formas iónicas de este elemento presentes en la solución del suelo reduciendo el estado de saturación de la solución y favoreciendo la disolución de los minerales de Fe (Kraemer y col., 2006). Sin embargo, cuando se investigó la disolución del óxido férrico goethita por FS, se encontró una relación lineal entre la disolución del óxido y el DMA adsorbido, que es consistente con un mecanismo de disolución promovido por la interacción directa del ligando con el óxido (Reichard y col., 2005). La disolución fue máxima a pH próximo a la neutralidad y a concentraciones bajas de FSs, y se incrementa con la presencia de un AOBPM (oxalato), por lo que la coexudación de AOBPM y FSs puede tener un efecto sinérgico sobre la movilización de este micronutriente. Los FSs en la rizosfera también pueden participar en reacciones de intercambio de ligando. Una reacción de este tipo parece ser la que se da cuando los complejos de Fe formados con sustancias humicas extraibles en agua interaccionan con el FS HMA (Cesco y col., 2000).

Los complejos Fe(III)-FS presentes en la solución del suelo pueden ser absorbidos como moléculas no disociadas a través de la membrana plasmática de las células de la corteza de la raíz de las plantas gramíneas, como se ha descrito en la sección 1.1.3. Las plantas dicotiledóneas también pueden adquirir el Fe de dicho complejo a través de la reducción del complejo Fe-FS por la acción de la enzima de membrana quelato reductasa férrica (Cesco y col., 2006). Esta es la razón por la que el cultivo intercalado con plantas gramíneas puede mejorar la disponibilidad de Fe en plantas dicotiledóneas (ver sección 1.1.4).

En recientes estudios, utilizando concentraciones del FS DMA de hasta 3 μmol kg^{-1} de suelo, más próximas a las esperadas en la rizosfera (Oburger y col., 2014), se observó una movilización de Fe, Zn, Mn y Cu de diferentes suelos (Schenkeveld y col., 2014a,b) inferior a la encontrada en estudios previos (Awad y Römheld, 2000; Takagi y col., 1988) en los que utilizaban concentraciones de 10.000 μmol kg de $suelo^{-1}$. Además, en los estudios de Schenkeveld y col. (2014a,b) se observó que la movilización de Fe por DMA está fuertemente afectada por la competencia con Cu y Zn. En el caso del Zn este hecho puede afectar a las plantas de Estrategia II, ya que la toma de Zn-DMA por plantas de arroz deficientes en este elemento condujo a un mayor transporte de este nutriente a la parte aérea (Suzuki y col., 2008).

La eficiencia del FS en la movilización de metales está limitada por su biodegradación y adsorción en las fases sólidas del suelo. Para contrarrestar el efecto de la biodegradación, las plantas exudan FS en unas horas concretas del día y por la parte apical de la raíz en donde la densidad de microorganismos es baja (von Wirén y col., 1993). La adsorción de DMA en el suelo llega a causar pérdidas de este FS en la solución de entre 25% y 62% dependiendo del tipo de suelo (Schenkeveld y col., 2014a).

Compuestos reductores

Los compuestos reductores secretados por las raíces también pueden movilizar nutrientes adsorbidos a las fases sólidas del suelo o contenidos en la estructura de los minerales. En este proceso, un reductor forma un complejo de esfera interna con el mineral, p. ej. un óxido férrico, permitiendo la transferencia de electrones entre el reductor y el nutriente del mineral que actúa como oxidante. Esto conlleva la formación de una especie reducida que se libera del mineral y pasa a la disolución del suelo. A su vez, mediante esta acción los compuestos reductores pueden incrementar indirectamente la disponibilidad de otros nutrientes precipitados junto a minerales de Fe como el P del fosfato férrico (Tomasi y col., 2008). En concreto los compuestos reductores de carácter fenólico exudados por plantas deficientes en Fe pueden movilizar este elemento de fases sólidas del suelo. Así, en deficiencia de Fe, el ácido cafeico exudado por las plantas de tomate solubilizó Fe de óxidos férricos (Olsen y col., 1981) y el isoflavonoide, 2-(3′,5′-dihidroxifenil)-5,6-dihidrobenzofurano exudado por las plantas de alfalfa condujo a la disolución de fosfato férrico (Masaoka y col., 1993). Mediante el mismo mecanismo, los compuestos fenólicos pueden movilizar Fe precipitado en el apoplasto de trébol rojo y arroz (Jin y col., 2007; Ishimaru y col., 2011).

La disolución de minerales promovida por compuestos reductores es mucho más rápida que la promovida por ligandos, aunque en presencia de oxígeno la especie reducida del nutriente liberada a la solución del suelo puede oxidarse rápidamente y ser de nuevo inmovilizada. Dos estudios recientes, han demostrado que la movilización de nutrientes como Fe o P es mayor cuando se utiliza una mezcla del reductor ácido ascórbico en combinación o con el fitosideróforo DMA o con el sideróforo desferrioxamina B o con AOBPM citrato (Wang y col., 2015; Schenkeveld y col, 2016). Por tanto, estos efectos sinérgicos podrían ser decisivos para la supervivencia de plantas de Estrategia I y II y microorganismos bajo condiciones de baja disponibilidad de Fe.

Otras funciones

Los exudados de raíz, además de movilizar nutrientes del suelo, también pueden desempeñar otras funciones en la rizosfera. Una parte importante de los exudados radiculares sirven como sustratos metabólicos para macro y microbiota del suelo que compiten por adquirirlos. Otros componentes de los exudados son mensajeros importantes para la comunicación química y regulan las interacciones entre las raíces y los organismos del suelo. Los exudados radiculares ejercen una fuerza selectiva que conforma la estructura de la comunidad microbiana rizosférica, modificando estructura y biodiversidad. Las plantas sometidas a estrés modifican rápidamente la composición de los exudados y esto repercute en los organismos con los que cohabitan.

Las plantas deficientes en Fe cambian la composición de los exudados radiculares lo que afecta a la población microbiana y conlleva beneficios para la planta en relación a la adquisición de Fe. Por ejemplo, se ha

comprobado que los compuestos fenólicos exudados por trébol rojo actúan de forma selectiva en los microorganismos del suelo favoreciendo el crecimiento de micro-organismos que producen y secretan auxinas y sideróforos (Jin y col., 2006, 2010). Las auxinas inducen un aumento en la expresión de los genes involucrados en la adquisición de Fe por la raíz como el que codifica la enzima reductasa férrica (Romera y col., 2017). Los sideróforos secretados a la rizosfera, movilizan el Fe del suelo y los quelatos Fe-sideróforo resultaron ser una fuente de Fe asimilable para plantas tanto de Estrategia I (soja, tomate y trébol rojo) como II (avena, cebada y maíz), especialmente cuando son deficientes en Fe (Crowley y col., 1988; Chen y col. 2000; Yehuda y col., 2000; Jin y col., 2010). Un aumento de la abundancia de este tipo de bacterias que secretan sideróforos se observó en un reciente estudio realizado con plantas de tomate y cebada crecidas en hidroponía en deficiencia y suficiencia de Fe y después trasladadas a un suelo calizo (Scagliola y col., 2016). Este estudio también mostró que la adición de algunas cepas concretas de las bacterias aisladas de cebada y tomate a plantas de pepino crecidas en deficiencia y/o suficiencia de Fe aumenta la actividad reductasa férrica de raíz. Otro ejemplo de la selección de microorganismos del suelo mediada por plantas crecidas en baja disponibilidad de Fe, se observó en una línea de tabaco transgénica que sobre-acumula Fe. Ésta redujo la biodisponibilidad de Fe en la rizosfera y favoreció el crecimiento de poblaciones de *Pseudomonas* fluorescentes eficientes en la toma de Fe (Robin y col., 2006; 2007). Varios estudios han mostrado una mejor nutrición férrica de las plantas cuando crecen con microorganismos. Así, cuando sorgo y colza son cultivadas en suelos estériles tuvieron menores concentraciones de Fe en raíces y hojas que las plantas cultivadas en suelos no estériles (Rroço y col., 2003). Se obtuvieron resultados parecidos en maíz y girasol, que mostraron síntomas de clorosis cuando son cultivados en suelos estériles, mientras que crecidos en suelos no estériles no mostraron clorosis y tuvieron mayores concentraciones de Fe en raíces (Masalha y col., 2000). En esta línea, en un estudio reciente se observó que cuando se trasladan a un suelo calizo plantas de pepino deficientes en Fe y se inoculan con *Azospirillum brasilense* aumenta la velocidad de recuperación de la deficiencia de Fe (Pii y col., 2015). Esta bacteria coloniza cultivos agronómicamente importantes como trigo, tomate y fresa, y promueve el crecimiento vegetal previniendo el desarrollo y/o la virulencia de los fitopatógenos, muy probablemente a través de la producción de sideróforos que secuestran Fe de la rizosfera en formas asimilables por la planta.

Los exudados de raíz pueden actuar también como un sistema de defensa para las plantas frente a agentes patógenos presentes en el suelo. Así, cuando existe la amenaza de un agente patógeno, las raíces pueden secretar hacia la rizosfera compuestos fitoquímicos antimicrobianos, creando una barrera defensiva contra la invasión de patógenos del suelo (Baetz y Martinoia, 2014). En este contexto varios estudios han demostrado que algunos componentes de los exudados radiculares de las plantas deficientes en Fe tienen efectos nocivos sobre el crecimiento de determinados microorganismos. El isoflavo-noide 2-(3′,5′-dihidroxiphenil)-5,6-dihidro-benzofurano exudado por plantas de alfalfa deficientes en Fe puede actuar como agente antifúngico (Masaoka y col., 1993). También se ha demostrado que altas concentraciones (del rango mM) de las flavinas secretadas por remolacha azucarera pueden inhibir el crecimiento de *Enterococcus faecalis* y *Staphylococcus aureus* (Susín, 1994).

Por último, los compuestos exudados por las raíces de determinadas plantas pueden establecer interacciones beneficiosas o perjudiciales con las especies de plantas vecinas cuando crecen en medios con baja disponibilidad de Fe. En concreto, el cultivo intercalado de especies gramíneas y dicotiledóneas establece una relación beneficiosa que mejora la captación de Fe. Un ejemplo de ello se demostró con el cultivo conjunto de maíz y cacahuete, ya que ambas especies tuvieron mejor nutrición férrica cuando fueron crecidas intercaladas que cuando se cultivaron en monocultivo (Zuo y Zhang, 2008, 2009). Sin embargo, la vía por la que las plantas de cacahuete toman el Fe en un cultivo intercalado de este tipo aún no está del todo clara. Existen estudios que apoyan la hipótesis de que el Fe se absorbe como Fe^{2+} a través de la reducción del complejo Fe-FS por la acción de la enzima quelato reductasa

férrica (Guo y col., 2014), mientras que otros estudios apuntan a que el complejo Fe(III)-FS [en concreto Fe(III)-DMA] es absorbido directamente por el transportador AhYSL1 expresado en las células de la epidermis de la raíz de cacahuete (Xiong y col., 2013). Otros ejemplos que ratifican la idea de que los FSs exudados por las gramíneas facilitan la toma de Fe a las plantas dicotiledóneas se obtuvieron con el intercalado en suelo calizo de cebada con cítricos y de cebada con olivo (Cesco y col., 2006; Cañasveras y col., 2014). Por el contrario, cuando los metabolitos liberados son fitotóxicos pueden causar reducción en el crecimiento y desarrollo de plantas vecinas, por lo que esta estrategia resultaría interesante para gestionar el control de malezas (Bhadoria, 2011).

1.3. Referencias

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CAPÍTULO 2. OBJETIVOS

En la Introducción se ha puesto de manifiesto la importancia en la toma de Fe de la síntesis y exudación radicular de compuestos orgánicos por las especies gramíneas (especies de Estrategia II). Esta Estrategia, dilucidada en las últimas décadas, está asociada a la tolerancia al estrés por deficiencia de Fe. También numerosas especies y/o cultivares de dicotiledóneas y monocotiledóneas no gramíneas (especies de Estrategia I) tolerantes a la deficiencia de Fe exudan compuestos orgánicos, aunque ni su identidad, ni los mecanismos de exudación radicular, ni su posible relevancia en la nutrición férrica han sido estudiados en profundidad.

La presente investigación se centra en el estudio de dos familias de compuestos orgánicos sintetizados y exudados por las raíces de plantas de Estrategia I en respuesta a la deficiencia de Fe, los compuestos fenólicos y las flavinas.

El objetivo general del trabajo es estudiar la acumulación, exudación y funciones de estos compuestos en la planta modelo *Arabidopsis thaliana* y en dos especies de interés agronómico, tomate (*Solanum lycopersicum*) y remolacha (*Beta vulgaris*), profundizando en aspectos relacionados con su identidad, las condiciones que determinan su síntesis y exudación, los mecanismos de esta exudación y sus posibles funciones.

Los objetivos específicos son:

1) El estudio de la síntesis y acumulación en las raíces, la exudación al medio de cultivo y la función de compuestos fenólicos y flavinas en *Arabidopsis thaliana* en respuesta a la deficiencia de Fe.
2) El estudio de la síntesis y acumulación en las raíces y la exudación al medio de cultivo de compuestos fenólicos y flavinas en tomate en respuesta a la deficiencia de Fe.
3) El estudio de la función de la exudación radicular de flavinas en remolacha en respuesta a la deficiencia de Fe.

CAPÍTULO 3. RESULTADOS

3.1. Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by *Arabidopsis* roots in response to iron deficiency

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Research

Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by *Arabidopsis* roots in response to iron deficiency

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Summary

- Studies of Iron (Fe) uptake mechanisms by plant roots have focussed on Fe(III)-siderophores or Fe(II) transport systems. Iron deficency also enhances root secretion of flavins and phenolics. However, the nature of these compounds, their transport outside the roots and their role in Fe nutrition are largely unknown.
- We used HPLC/ESI-MS (TOF) and HPLC/ESI-MS/MS (ion trap) to characterize fluorescent phenolic-type compounds accumulated in roots or exported to the culture medium of *Arabidopsis* plants in response to Fe deficiency. Wild-type and mutant plants altered either in phenylpropanoid biosynthesis or in the ABCG37 (PDR9) ABC transporter were grown under standard or Fe-deficient nutrition conditions and compared.
- Fe deficiency upregulates the expression of genes encoding enzymes of the phenylpropanoid pathway and leads to the synthesis and secretion of phenolic compounds belonging to the coumarin family. The *ABCG37* gene is also upregulated in response to Fe deficiency and coumarin export is impaired in *pdr9* mutant plants.
- Therefore it can be concluded that: Fe deficiency induces the secretion of coumarin compounds by *Arabidopsis* roots; the ABCG37 ABC transporter is required for this secretion to take place; and these compounds improved plant Fe nutrition.

Introduction

Iron (Fe) availability for plants depends on the physico-chemical properties of the soil (Lindsay & Schwab, 1982). Calcareous soils cover 30% of the earth surface (Vose, 1982) and favour the formation of scarcely soluble Fe^{3+} oxy-hydroxides. Consequently, plants grown on these soils are often Fe deficient and develop interveinal chlorosis. According to the plant family considered, that is, Graminaceae vs other species, plants have evolved two different mechanisms for mining Fe from the soil. One involves chelation of Fe^{3+} by small organic molecules belonging to the mugineic acids (MAs) family, and the other involves Fe^{3+} reduction to Fe^{2+} before transport across the plasmalemma of root epidermal cells (Morrissey & Guerinot, 2009). MAs are synthesized from S-adenosyl methionine, and are secreted into the rhizosphere by the TOM transporter (Nozoye *et al.*, 2011). The resulting Fe^{3+}–MAs complexes are taken up by the roots *via* YS1 transporters (Curie *et al.*, 2001). Nongrass plants respond to Fe deficiency with both morphological and physiological changes (Schmidt, 1999). The *AHA2* H^+-ATPase acidifies the rhizosphere of *Arabidopsis* iron (Fe) deficient plants, facilitating Fe solubilization (Santi & Schmidt, 2009). An enhanced Fe^{3+} reduction capacity of the roots (Yi & Guerinot, 1996) involves the FRO2 root Fe^{3+} chelate reductase (Robinson *et al.*, 1999). Then Fe^{2+} is transported across the root plasma membrane *via* the IRT1 divalent metal transporter (Eide *et al.*, 1996; Vert *et al.*, 2002).

In addition, it has been known for many years that plants also respond to Fe deficiency by enhancing root secretion of organic compounds, including flavins and phenolics (Römheld & Marschner, 1983; Susín *et al.*, 1993; Rodríguez-Celma *et al.*, 2011; Donnini *et al.*, 2012). The role of these molecules is still unclear. Flavins could participate by reducing or complexing extracellular Fe (González-Vallejo *et al.*, 1998; Cesco *et al.*, 2010) or bridging the electron flow to the root Fe^{3+} chelate reductase (López-Millán *et al.*, 2000; Higa *et al.*, 2010). Back in the seventies, phenolic compounds were already considered as putative external Fe reductants and/or chelators (Brown & Ambler, 1973). More recently, it was reported that phenolics secreted by

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red clover contribute to solubilization and utilization of apoplasmic Fe (Jin *et al.*, 2007), and that phenolics such as protocatechuic acid can solubilize and chelate Fe^{3+} and reduce it, both *in vitro* (Yoshino & Murakami, 1998) and within the plant (Bashir *et al.*, 2011; Ishimaru *et al.*, 2011).

Therefore, the chemical identification of these secreted compounds, the understanding of their biosynthetic pathways and the characterization of the root plasmalemma transporters responsible for export are of major interest to understand their possible roles in plant response to Fe deficiency. Transcriptome and proteome analyses of roots from *Arabidopsis* Fe-deficient plants have provided evidence that some enzymes of the phenylpropanoid pathway were upregulated, likely leading to an increase in scopoletin synthesis (Yang *et al.*, 2010; Lan *et al.*, 2011). Also, rice phenolics efflux transporters were characterized and shown to be essential for solubilizing apoplasmic Fe precipitated in the stele (Bashir *et al.*, 2011; Ishimaru *et al.*, 2011). In this paper, we characterize the fluorescent phenolic compounds accumulating in roots of Fe-starved *Arabidopsis* plants, as well as those secreted to the medium, and we show that their secretion is dependent upon the functionality of the *ABCG37* gene (previously named *PLEIOTROPIC DRUG RESISTANCE 9*: *PDR9*) belonging to the ATP-Binding Cassette family of transporters (Ito & Gray, 2006; Strader *et al.*, 2008; Strader & Bartel, 2009; Ruzicka *et al.*, 2010).

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh (ecotype Col0) seeds were surface sterilized and sown in 0.2-ml tubes containing 0.8% agar prepared in pH 5.5 Hoagland-based solution (in mM: 1 KH_2PO_4, 1 KNO_3, 1 $MgSO_4$, 5 $CaNO_3$; and in μM: 50 H_3BO_3, 0.05 $CoCl_2$, 0.05 $CuSO_4$, 15 $ZnSO_4$, 2.5 KI, 50 $MnSO_4$, 3 Na_2MoO_4). Iron was added as 50 μM Fe^{3+}-EDTA. After 3–4 d in the growth chamber, the tube bottoms were cut off and tubes placed in opaque plastic pipette tip racks (Starlab, Hamburg, Germany). Twelve plants were grown in boxes with 300-ml half-Hoagland solution (Terry, 1980) with 50 μM Fe^{3+}-EDTA. Growth conditions were 23°C, 70% relative humidity, 8 h : 16 h, light : dark photoperiod and 220 μE m^{-2} s^{-1} PPFD (PAR). Plants were grown for 4 wk before being subjected to Fe deficiency (zero Fe in the solution) for 7 d (final pH was *c.* 7.0). For *in vitro* experiments, sterilized seeds were sown on 0.8% (w/v) agarose-solidified Hoagland media. Mutant lines *pdr9-2* (SALK 050885) and *pdr9-3* (SALK 078574) were obtained from the Salk center, and seeds from the *comt* and *ccoamt-1* mutants were a generous gift of Dr Lise Jouanin (IJPB, Versailles, France; Do *et al.*, 2007). The *irt1 Arabidopsis* mutant was obtained previously in our laboratory (Vert *et al.*, 2002).

Brassica napus L. var. *napus* (cv Fantasio) seeds were a gift from Semences de France (*In vivo* Group, La Chapelle d'Armentière, France). Seeds were placed onto cheesecloth held with a plastic ring inside a Magenta vessel. Culture medium and growth conditions were identical to those of the *Arabidopsis* culture.

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from Arabidopsis roots using the RNeasy Plant Mini Kit (Quiagen). One microgram RNA was treated with RQ1 DNase (Promega) before use for reverse transcription (Goscript reverse transcriptase; Promega) with oligo (dT)18 and 0.4 mM dNTPs (Promega). The cDNAs were diluted twice with water, and 1 μl of each cDNA sample was assayed by qRT-PCR in a LightCycler 480 (Roche Applied Science) using Lightcycler 480 SYBR Green master I (Roche Applied Science). Expression levels were calculated relative to the housekeeping gene PP2 (*At1g13320*) using the ΔΔCT method to determine the relative transcript level. The primers used in this study are presented in Supporting Information Table S1.

Sample preparations for analytical measurements

Reagents, materials and standard solutions Reagents (eluents and phenolic standards) are shown in Table S2. For dilutions, we used analytical grade type I water (Ultramatic, Wasserlab, Pamplona, Spain). All standards were dissolved in methanol to concentrations in the range 1–5 mM and stored in darkness at −80°C until analysed. Working standard solutions were prepared before use by diluting the stock solutions with a solution 85 : 15 (v/v) of 0.1% (v/v) formic acid in methanol and 0.1% (v/v) formic acid in water.

Extraction of phenolic compounds from roots and nutrient solutions Roots frozen in liquid N_2 and stored at −80°C were extracted following the procedure described by Kai *et al.* (2006), with several modifications. Frozen roots (*c.* 100 mg) were ground in liquid N_2 using a Retsch M301 ball mill (Restch, Düsseldorf, Germany) for 3 min and then phenolic compounds were extracted from the tissue with 1 ml of 100% methanol by homogenization in the same mill for 5 min. The supernatant was recovered by centrifugation (12 000 ***g*** at 4°C and 5 min), stored at −20°C, and the pellet was resuspended in 1 ml of 100% methanol, homogenised again for 5 min and the supernatant recovered. The two supernatant fractions were pooled, vacuum concentrated and diluted in methanol to a final volume of 250 μl. Extracts were filtered through polyvinylidene fluoride (PVDF) 0.45 μm ultrafree-MC centrifugal filter devices (Millipore) and stored at −80°C until analysis.

In order to examine Fe-deficiency induced changes on the phenolic compounds secreted by *A. thaliana* roots, nutrient solutions from hydroponically grown *Arabidopsis* plants (12 plants grown in 300 ml-containers) were collected 7 d after the onset of Fe treatments and filtered using PVDF 0.45 μm membrane filters. Then, phenolic compounds were retained in a SepPack C18 cartridge (Waters, Mildford, MA, USA), eluted from the cartridge with 2 ml of 100% methanol and stored at −80°C until analysis.

Analytical procedures

Phenolic compound analysis by HPLC coupled to fluorescence detection Separations were performed with a binary HPLC

pump (Waters 1525) using an analytical HPLC column (Symmetry® C_{18}, 15 cm × 2.1 mm i.d., 5 μm spherical particle size, Waters) protected by a guard column (Symmetry® C18, 10 mm × 2.1 mm i.d., 3.5 μm spherical particle size; Waters), with a gradient mobile phase built with 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in methanol (B), and a flow rate of 0.2 ml min^{-1}. The gradient program started at 15% B for 2 min, then increased linearly to 55% B for 13 min. This proportion was maintained for 10 min and then returned linearly to initial conditions in 5 min. The column was then allowed to stabilise for 15 min at the initial conditions before the next injection. The injection volume was 20 μl. Phenolic compounds were detected with a scanning fluorescence detector (Waters 474) using λ_{exc} 365 and λ_{em} 460 nm. Empower software (build 2154; Waters) was used to control the HPLC system and process data.

Phenolic compound analyses by HPLC/ESI-MS(TOF) and by HPLC/ESI-MS/MS(ion trap) HPLC/ESI-MS(TOF) analysis was carried out using an Alliance 2795 HPLC system (Waters) coupled to a time-of-flight mass spectrometer (MicrOTOF, Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) source. Autosampler and column temperatures were 6 and 30°C, respectively, and the HPLC conditions were the same as those described above. The ESI-MS(TOF) operating conditions were optimized by direct injection of 50 μM solutions of phenolics standards at a flow rate of 250 μl h^{-1}. Mass spectra were acquired in positive ion mode (see settings in Table S3) in the range of 50–1000 mass-to-charge ratio (*m/z*) units. The mass axis was calibrated externally and internally using Li-formate adducts (10 mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol). The internal mass axis calibration was carried out by introducing the calibration solution with a divert valve at 1–3 and 40–43 min in each HPLC run. Bruker Daltonik (Bruker Biosciences Espanola, S.A. Madrid, Spain) software packages micrOTOF Control v2.2, HyStar v3.2 and Data Analysis v4.0 were used to control the ESI-MS(TOF) apparatus, interface the HPLC with the MS system and process data, respectively. Molecular formulae were assigned based on exact molecular mass with errors < 5 ppm (Bristow, 2006).

HPLC/ESI-MS/MS(ion trap) analysis was carried out with an 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an ion-trap mass spectrometer (Esquire 3000+; Bruker Daltonics) equipped with an ESI source. The HPLC conditions were those described above. ESI-ion trap-MS analysis was carried out in positive ion mode, the MS spectra were acquired in the standard/normal mass range mode and the mass axis was externally calibrated with a tuning mix (from Agilent Technologies) (see settings in Table S3). The protonated ions of interest $[M+H]^+$ were subjected to collision induced dissociation (CID; using the He background gas present in the trap for 40 ms) to produce a first set of fragment ions, MS/MS or MS^2. Subsequently, one of the fragment ions was isolated and fragmented to give the next set of fragment ions, MS^3 and so on. For each precursor ion, fragmentation steps were optimized by visualizing the intensity changes of the fragmented ions (see settings in Table S3).

Metal analyses by ICP-MS *Arabidopsis thaliana* roots and leaves were dried at 65°C for 2 d. Approximately 0.01–0.04 g of ground dry tissues were digested with 5.6 ml HNO_3 (4.6%) and 1.4 ml H_2O_2 (30%) in a vessel microwave oven (Milestone Model MLS 1200, Sorisole, Italy). Samples were diluted to 10 ml with Milli-Q water and total Fe, Mn, Cu and Zn was determined by ICP-MS (Agilent 7500ce equipped with an octapole collision cell to remove polyatomic interferences; Agilent Technologies, Tokyo, Japan).

Results

Iron deficiency induces the secretion of phenolic compounds by *Brassica napus* and *Arabidopsis thaliana* roots

The culture medium of *Brassica napus* grown without Fe for 7 d appeared fluorescent when exposed to 365 nm UV light (Fig. 1a). The culture medium fluorescence was detected after 3 d of Fe deficiency and increased up to 8 d (Fig. 1b). Roots and growth media (agar or nutrient solution) fluorescence was also observed when *Arabidopsis* plants were grown under low Fe concentrations (Fig. 1c,d), although *Arabidopsis* root density was lower than the one of *B. napus*. Furthermore, the increase in *Arabidopsis* root fluorescence was inversely proportional to the Fe concentration in the agar medium (Fig. 1c). The impact of Fe deficiency was evidenced by the red leaf chlorophyll fluorescence, which was much less pronounced at 0 Fe than at 50 μM Fe (Fig. 1c). Therefore, Fe deficiency induces the synthesis, root accumulation and secretion to the growth media of UV fluorescent compounds in *B. napus* and *A. thaliana* roots. Their maximum fluorescence emission, when excited at 365 nm, was observed at 420–460 nm indicating that they likely belong to the phenolic compounds family.

Arabidopsis-secreted phenolic compounds limit chlorosis

In order to know whether the compounds secreted to the growth medium impact plant Fe nutrition, we used the phenolic removal system described by Jin *et al.* (2007), which consisted of circulating the nutrient solution through a Sep-Pak C_{18} resin column. This system almost completely removed fluorescent compounds from the nutrient solution (Fig. S1). Removing these molecules from the hydroponic medium led to a marked chlorosis of the *Arabidopsis* leaves compared to the control in which the nutrient solution was re-circulated without placing the C_{18} resin in the circuit (Fig. 2). This simple experiment clearly demonstrates that the Fe deficiency-induced secretion of phenolics by *Arabidopsis* roots enhances Fe nutrition efficiency and limits leaf chlorosis.

In order to establish that the leaf chlorosis was the result of iron deficiency, and did not have any other cause, we measured Fe, Zn, Mn and Cu concentrations in young leaves and roots from *Arabidopsis thaliana* plants grown hydroponically with the nutrient solution circulated or not through the Sep-Pak C_{18} resin column. Young leaves from plants grown on the circulated medium had 60% more Fe than those of plants grown on

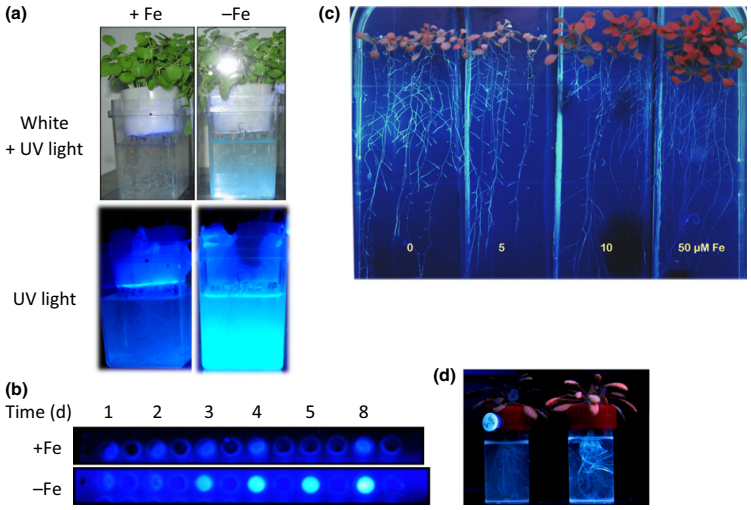

Fig. 1 Iron-deficiency induced synthesis and secretion of fluorescent compounds by plant roots. (a) 10-d-old *Brassica napus* plants grown for 7 d in hydroponic cultures in the presence (+Fe) or absence (−Fe) of iron. (b) Hydroponic culture medium after 1–8 d of *Brassica napus* plant growth with (+ Fe) or without (−Fe) iron placed in a 96-well plate under UV light at 365 nm. (c) 15-d-old *Arabidopsis thaliana* plants grown on agarose plates under different iron nutrition conditions (0, 5, 10 and 50 μM Fe(III)-EDTA), and observed under UV light (365 nm). (d) 15-d-old *Arabidopsis thaliana* plants grown in hydroponic cultures under +Fe (left) or −Fe (right) conditions.

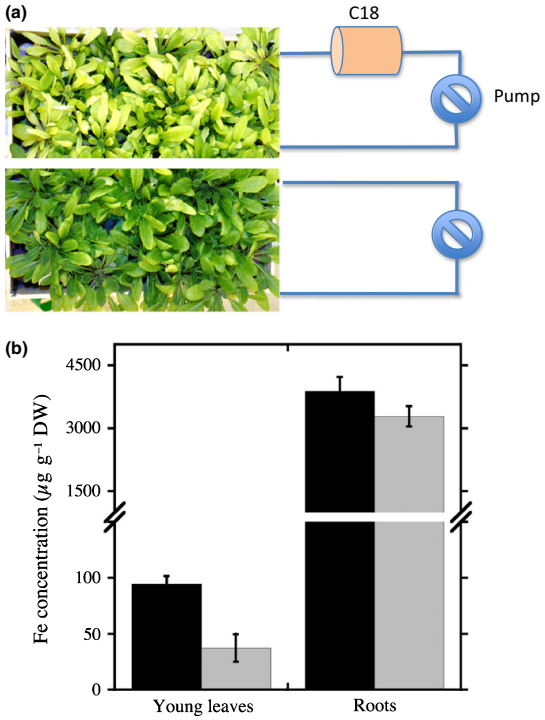

Fig. 2 Phenolic compounds secreted by *Arabidopsis* roots protect against leaf chlorosis and improve leaf iron (Fe) concentrations. *Arabidopsis* plants were grown in hydroponics for 4 wk in the presence of 50 μM Fe^{3+}-EDTA in the culture media. They were then transferred onto a medium without iron and continuously circulated with a peristaltic pump in the presence or absence of a C_{18} Sep-Pak cartridge. (a) Photo taken after 20 d of recirculation of the medium, representative of three independent experiments. (b) Iron concentrations in young leaves and roots sampled from plants after 20 d of recirculation of the medium. Data are means ± SE of 8 independent measurements. –Fe, black bars; −Fe, recirculated, grey bars.

medium passed through a Sep-Pak C_{18} column, supporting that the observed chlorosis was the result of Fe deficiency (Fig. 2 b). A lower (only 15%) decrease in root Fe concentration was found in plants where the culture medium was circulated through the column. The changes in the concentrations of other metals when using the column are shown in Table S4.

Involvement of the phenylpropanoid pathway and of the ABC transporter ABCG37 in the *Arabidopsis* Fe deficiency responses

Our above observations are consistent with the output of recent transcriptomes (Yang *et al.*, 2010; Rodríguez-Celma *et al.*, 2013) and proteome (Lan *et al.*, 2011) studies in roots of Fe-deficient *Arabidopsis* plants, which indicated that Fe shortage induced the phenylpropanoid pathway in roots.

Also, Fe deficiency led to an increase in abundance of the *Arabidopsis* ABCG37 transporter transcript (Yang *et al.*, 2010), known to be expressed predominantly in the lateral root cap and in epidermal cells of the root tip (Ito & Gray, 2006). ABCG37 has been described as a plasma membrane exporter of the auxin storage precursor indole-3-butyric acid (Strader *et al.*, 2008; Strader & Bartel, 2009; Ruzicka *et al.*, 2010). As stated by Yang *et al.* (2010) the role of ABCG37 in Fe homeostasis remains elusive, but its ability to transport the synthetic auxin analogue 2,4-dichlorophenoxyacetic acid suggests an alternative role in the export of phenolic compounds such as caffeic acid or chlorogenic acid.

As already reported by Yang *et al.* (2010), and more recently by Rodríguez-Celma *et al.* (2013) we observed an increased expression of *ABCG37* and *F6′H1* (encoding an oxidoreductase involved in scopoletin synthesis; Kai *et al.*, 2008) genes in *Arabidopsis* roots in response to Fe deficiency (Fig. 3), both in Col0 and *irt1* mutant genetic backgrounds (Fig. 3). These results indicate that the functionality of the major Fe^{2+} high affinity root

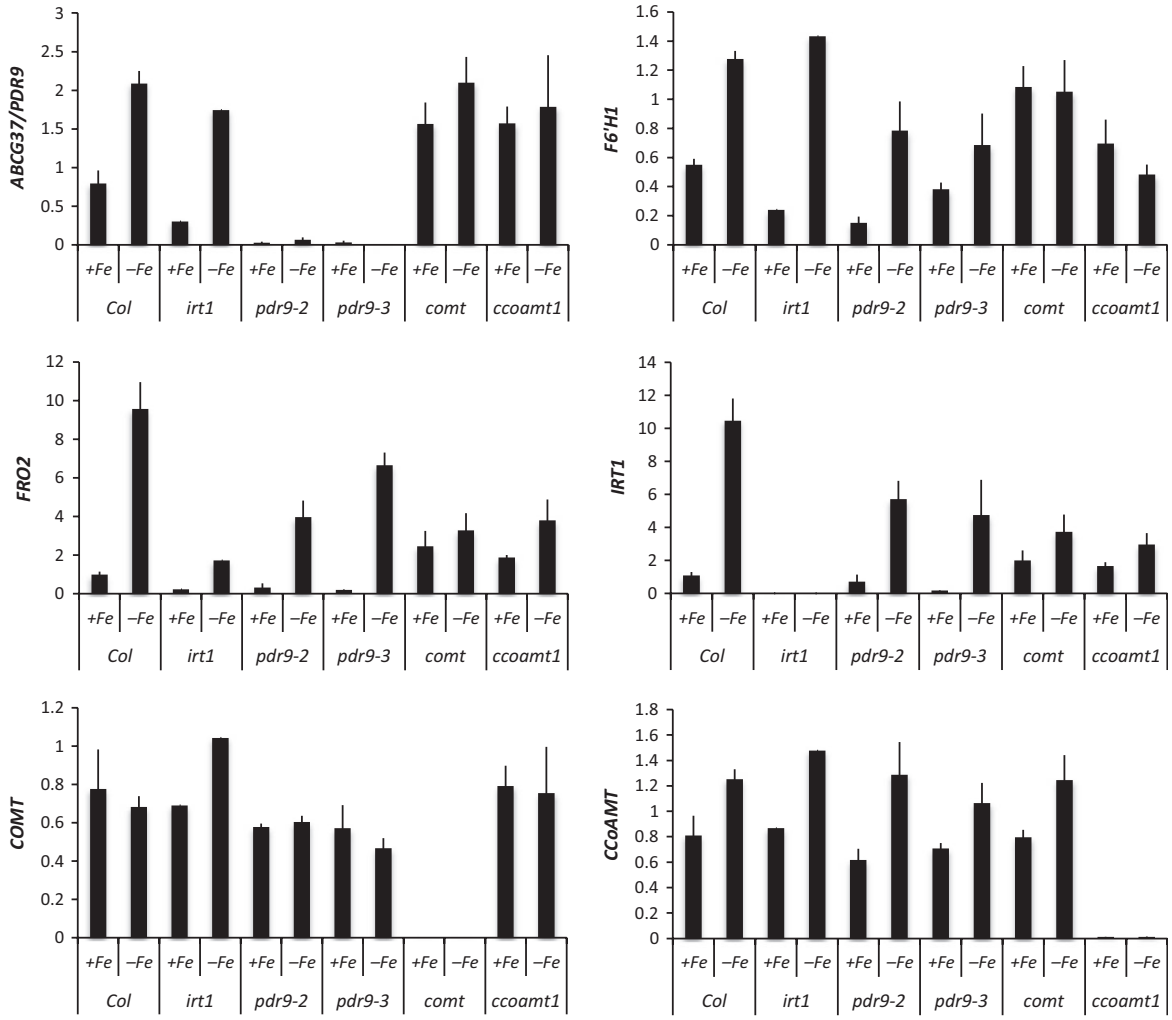

Fig. 3 Abundance of the *ABCG37* (*PDR9*), *F6'H1*, *FRO2*, *IRT1*, *COMT* and *CCoAMT* transcripts in various Arabidopsis genetic backgrounds. Plants were grown in the presence (+) or absence (−) of iron, and RNAs from roots were extracted and analysed by qRT-PCR, with *PP2* (At1g13320) as the housekeeping gene. The ΔΔCT method was used to determine the relative transcript level. Error bars indicate + SE of three replicates. Col0, wild-type Arabidopsis, ecotype Columbia 0; *irt1*, T-DNA insertion mutant in the Arabidopsis *IRT1* gene encoding the major high affinity Fe^{2+} root transporter; *pdr9-2* and *pdr9-3*, two nonallelic T-DNA insertion mutants in the Arabidopsis *ABCG37* (*PDR9*) gene; *comt*, T-DNA insertion mutant in the Arabidopsis *COMT* gene encoding the caffeic acid/5-hydroxyferulic acid O- methyltransferase; *ccoamt1*, T-DNA insertion mutant in the Arabidopsis *CCoAMT1* gene encoding the trans-caffeoyl-CoA 3-O-methyltransferase 1.

transporter is not required for the regulation of *ABCG37* and *F6′H1* in response to Fe shortage. *IRT1* and *FRO2* are among the best documented genes induced in response to Fe deficiency (Robinson *et al.*, 1999; Vert *et al.*, 2002; Morrissey & Guerinot, 2009). *IRT1* and *FRO2* gene expression was slightly lower in *pdr9* lines than in Col independently of the Fe conditions, but the induction factor in response to Fe deficiency was roughly the same, (Fig. 3). *CCoAMT1* (encoding trans-caffeoyl-CoA 3-O-methyltransferase) gene expression was Fe-deficiency responsive in all the genotype tested whereas *COMT* (encoding caffeic acid/5-hydroxyferulic acid O-methyltransferase) gene expression was Fe-deficiency independent. The upregulation of *F6′H1* in response to Fe deficiency was lost in the *comt* and *ccoamt1* mutant plants, and was strongly decreased in one of the *pdr9 allele*. Whether or not the regulation of *ABCG37* expression was indicative of its role in Fe acquisition was tested by comparing the growth of *Arabidopsis* Col0 plants with that of the *pdr9-2* T-DNA KO mutant, in the presence or absence of Fe at two different pH values (Fig. 4a). In the presence of Fe at pH 5.5, no differences were observed between the two genotypes. By

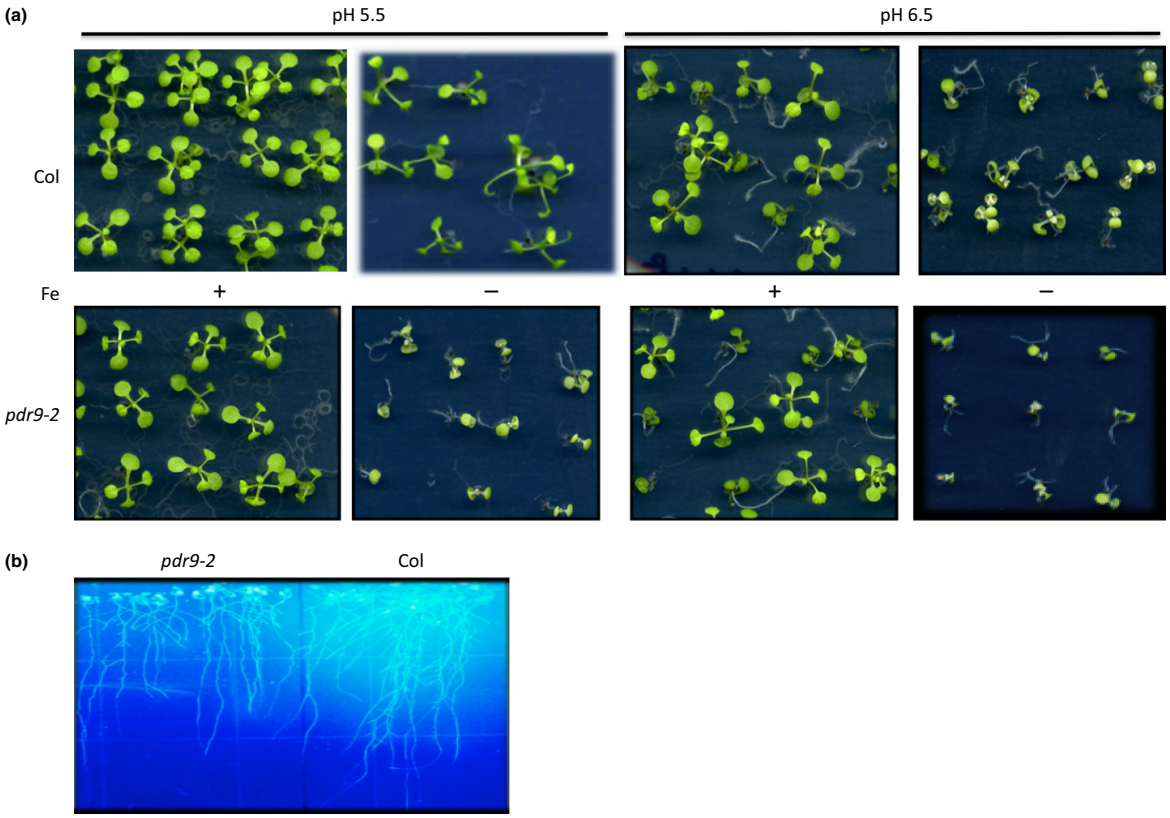

Fig. 4 Phenotypic characterization of the *pdr9-2 Arabidopsis* mutant. Comparison of 20-d-old Col0 and *pdr9-2* plantlets grown on agarose plates containing 50 μM Fe^{3+}-EDTA (+) or no iron (Fe) (−) at two pH values (a). Fifteen-day-old *Arabidopsis thaliana* plants grown on agarose plates under Fe-deficient conditions (no Fe added in the medium), and observed under UV light (365 nm) (b). Col0, wild-type Arabidopsis from the Columbia 0 ecotype; *pdr9-2*, T-DNA insertion null mutant in the Arabidopsis *ABCG37* gene.

contrast, at pH 6.5 and in the absence of Fe the *pdr9-2* seedlings were smaller and more chlorotic when compared to Col0 plants under the same conditions. At pH 6.5 Fe is less available than at pH 5.5. Consequently Col0 plants in the absence of Fe were more chlorotic at this pH than at pH 5.5, and it was even more pronounced for the *pdr9-2* plants (Fig. 4a). Interestingly, when using 365 nm UV light the medium, and to a lesser extent the roots, of the *pdr9-2* plantlets grown on agarose without Fe appeared much less fluorescent than those of Col0 plants (Fig. 4b). These observations establish a link, therefore, between a defect in the ABCG37 transporter, a decrease in fluorescent compounds in the medium, and the development of leaf chlorosis, in particular at acidic pH. The above observations prompted us to investigate in more detail the nature of the fluorescent compounds accumulated in roots and secreted to the medium of Col0 and *pdr9 Arabidopsis* plants in response to Fe deficiency. As a control we also included the *Arabidopsis* mutant *comt*. The rationale for using this mutant in our analyses was based on the fact that the enzyme COMT is involved in the methylation of several substrates in the phenylpropanoid biosynthesis pathway. The production of ferulate from cafeate is altered in *comt*, compromising the synthesis of feruloyl-CoA, which is *o*-hydroxylated by the pivotal enzyme F6′H1 for the biosynthesis of scopoletin.

ABCG37 exports scopoletin and derivatives

Accumulation and secretion of a range of fluorescent compounds by Arabidopsis roots Methanolic extracts were obtained from both roots and compounds occurring in the nutrient solutions, and then analysed by reverse phase C_{18} HPLC coupled to fluorescence (with λ_{exc} 365 and λ_{em} 460 nm). Each of the peaks in the chromatograms (Fig. 5) can contain one or more fluorescent compounds (see sections below for identification).

In Col0, the chromatograms of all root extracts showed two peaks, at *c.* 10 and 15 min, whereas those from growth media showed an additional peak at 19 min (Fig. 5). Under Fe deficiency, the area of the 15 min peak increased in the root extracts, whereas in the growth media large increases were observed for the 15 min peak, and to a lower extent for the 19 min peak.

The chromatograms from *pdr9-2* and *pdr9-3* root extracts also showed two peaks at 10 and 15 min, along with three additional

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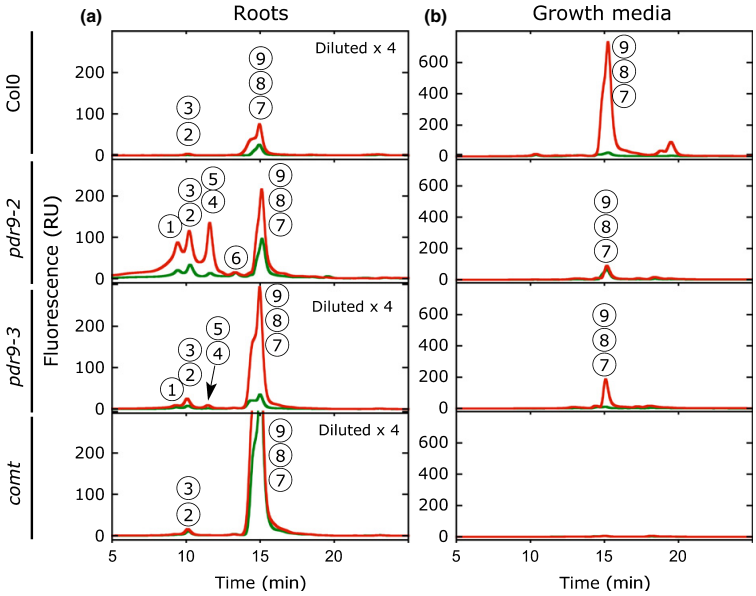

Fig. 5 Separation of a range of fluorescent compounds synthesized and secreted by *Arabidopsis* in response to iron (Fe) deficiency. Typical fluorescence chromatograms obtained using λ_{exc} 365 and λ_{em} 460 nm, respectively, for root (a) and growth media extracts (b) of Col0, *pdr9-2*, *pdr9-3* and *comt* 21-d-old *Arabidopsis* plants grown in hydroponic cultures (12 plants in 300 ml containers). All plants received Fe as 50 μM of Fe^{3+}-EDTA during the pre-treatment period, whereas during the 7 d of the treatment period they received either 0 (red, −Fe) or 50 μM Fe^{3+}-EDTA (green, +Fe). The encircled numbers above each peak correspond to the phenolic compounds listed in Table 1. RU, relative units.

peaks at 8, 11.5 and 13 min (Fig. 5). Under Fe deficiency, the area of all these peaks increased, with a larger increase for the peak at 15 min in *pdr9-3*. These observations indicate that the *pdr9* mutation caused an increase in abundance of several fluorescent compounds in roots, some of them with higher polarity than those present in Col0, and that this increase in abundance was further enhanced by Fe deficiency. In the growth media extracts, only a minor peak at 15 min was observed in *pdr9-2* and *pdr9-3* that was much smaller than that found at the same retention time (RT) in Col0. Therefore, the *pdr9* mutation markedly impairs the secretion of the fluorescent compounds eluting at 15 min that occurs in Col0 in response to Fe deficiency.

Chromatograms of *comt* root extracts showed two peaks at 10 and 15 min. Although these RT were similar to those of the peaks present in Col0, peak areas were much larger in *comt*. Furthermore, the area of both peaks was only slightly affected by Fe deficiency. These results indicate that the *comt* mutation results in the accumulation in the roots of fluorescent compounds, and that Fe deficiency did not result in major changes in their abundance. No fluorescent peaks were observed in the *comt* growth media, indicating that the step catalysed by COMT could be involved in the synthesis of either the precursors or the actual fluorescent compounds excreted by the Col0 roots in response to Fe deficiency. Nevertheless, so far we do not understand why the *comt* mutant failed to secrete these fluorescent compounds, including scopoletin.

Identification of the Arabidopsis fluorescent compounds as scopoletin and derivatives The identity of the fluorescent compounds found in the *Arabidopsis* root extracts and secreted to the medium was studied in detail by HPLC coupled to two MS techniques, ESI-MS(TOF) and ESI-MS/MS (ion trap), with the same HPLC conditions used in the fluorescence analysis. The HPLC/ESI-MS(TOF) analysis provides highly accurate *m/z* measurements (mass accuracy < 5 ppm) that allows for accurate elemental formula assignments (Bristow, 2006). The identification of the nine unknown compounds behind the fluorescence peaks observed by HPLC analysis (Fig. 5) was tackled first by using TOF data (RT, exact *m/z* values and elemental formulae) (Table 1). Three of the nine compounds – *6*, *7* and *8* in Fig. 5 – matched the RT and *m/z* values of the known standards fraxetin, scopoletin and isofraxidin, respectively (three fluorescent coumarins) (Tables 1, 2; Fig. S2). We confirmed these identifications using the MS^2 spectra, which matched with those obtained for the standards, both in terms of *m/z* values and relative intensities of the major fragment ions (Tables 1 and 2). Compound *9* had the same *m/z* value and elemental formula as isofraxidin (8-metoxy scopoletin or methylated fraxetin; see structures in Table 2), but eluted 0.8 min later, supporting the hypothesis that it could be a less polar isofraxidin isomer. It was identified as 5-metoxy scopoletin (see notes S1 for more details).

The MS(TOF) spectra of compounds *1–5* showed additional ions of high intensity at *m/z* 225.0393, 193.0498, 195.0646, 239.0547 and 225.0386, respectively, all of them consistent with the loss of a glucosyl moiety (−162.0523 Da, with an error below 5 ppm; see notes S1 for more details). The identity of glucosides *1–5* was further deciphered by MS^3 using as a model the fragmentation of esculin (see Fig. S3). Compounds *2, 1* and *5, 4* and *3* were identified as glucoside of scopoletin (scopolin), as two different glucosides of dihydroxyscopoletin, as a glucoside of hydroxymethoxyscopoletin, and as a glucoside of ferulic acid, respectively (see Notes S1 for more details).

Changes in scopoletin and derivatives in roots and growth media in response to Fe deficiency TOF ion chromatograms

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Table 1 Retention times (RT) and $[M+H]^+$ exact mass-to-charge ratios (*m/z*), molecular formulae and error of the compounds secreted and accumulated by roots of Col0, *pdr9*-2, *pdr9-2* and *comt Arabidopsis* roots in response to iron (Fe) deficiency

#	RT (min)	Measured *m/z*	Molecular formula	Calculated *m/z*	Error *m/z* (ppm)	Name	ESI-MS^n *m/z* (Relative intensity%)
1	8.4	387.0921	$C_{16}H_{19}O_{11}^+$	387.0922	−0.3	Glucoside of dihydroxy scopoletin	MS^2 [387]: 225 (100), 210 (8), 182 (0.3), 179 (0.2), 165 (1), 154 (0.5), 147 (0.3), 86 (0.1) MS^3 [387→225]: 210 (100), 165 (12), 140 (8), 74 (28)
2	9.8	355.1021	$C_{16}H_{19}O_9^+$	355.1024	−0.8	Glucoside of scopoletin	MS^2 [355]: 337 (11), 245 (3), 193 (100), 149 (1), 165 (1), 133 (12), 105 (5) MS^3 [355→193]: 178 (11), 165 (83), 133 (100)
3	10.0	379.0987	$C_{16}H_{20}O_9Na^+$	379.0999	−3.2	Glucoside of ferulic acid	MS^2 [357]: 297 (12), 195 (76), 194 (100) MS^3 [357→195]: 177 (100), 153 (5), 107 (2) MS^4 [357→195→177]: 145 (100), 117 (5), 91 (6)
4	11.3	401.1073	$C_{17}H_{21}O_{11}^+$	401.1078	−1.2	Glucoside of hydroxyl methoxy scopoletin	MS^2 [401]: 382 (6), 365 (5), 287 (8), 239 (100), 224 (9), 206 (3), 186 (2), 175 (3), 156 (2), 122 (1) MS^3 [401→239]: 224 (100), 206 (59), 193 (3), 179 (6)
5	11.5	387.0917	$C_{16}H_{19}O_{11}^+$	387.0922	−1.3	Glucoside of dihydroxy scopoletin	MS^2 [387]: 257 (4), 225 (100), 210 (6), 179 (0.3), 157 (1), 114 (1) MS^3 [387→225]: 210 (100), 192 (16), 179 (27), 165 (18)
6	13.0	209.0443	$C_{10}H_9O_5^+$	209.0444	−0.5	Fraxetin	MS^2 [209]: 194 (30), 181 (47), 177 (18), 163 (83), 153(6), 149 (100), 135 (23), 107 (27)
7	14.5	193.0494	$C_{10}H_8O_4^+$	193.0495	−0.5	Scopoletin	MS^2 [193]: 178 (8), 165 (31), 149 (12), 137 (12), 133 (100), 117 (2), 105 (3), 89 (3), 63 (6)
8	14.8	223.0600	$C_{11}H_{11}O_5^+$	223.0601	−0.4	Isofraxidin	MS^2 [223]: 208 (100), 195 (11), 190 (40), 179 (6), 163 (54), 135 (19), 107 (39), 91 (4)
9	15.6	223.0594	$C_{11}H_{11}O_5^+$	223.0601	−0.4	Methoxy scopoletin	MS^2 [223]: 208 (100), 195 (11), 190 (40), 179 (6), 163 (55), 135 (19), 107 (39), 91 (4)

The *m/z* ratios of parent and fragment ions were determined from the data in the HPLC/ESI-MS(TOF) and HPLC/ESI-MS/MS(ion trap) chromatograms, respectively. In the case of compound *3*, the MS(TOF) *m/z* included in the table is the one measured for the Na adduct ($[M+Na]^+$) because it was more intense than the $[M+H]^+$; the $[M+H]^+$ (at 357 *m/z*) ion was subsequently used as precursor in the MS/MS(ion trap) analyses.

extracted at the corresponding exact *m/z* values of each individual phenolic compound for the different root extract and nutrient solution samples (sampled 7 d after imposing the treatments) are shown in Fig. 6 (note some panels have different *y*-axes). Roots of +Fe Col0 contained minor amounts of scopoletin (*7*; Fig. 6f), and only traces of other derivatives, including isofraxidin and metoxyscopoletin (*8*, *9*; Fig. 6g), glucoside of scopoletin (*2*; Fig. 6b), and glucoside of ferulic acid (*3*; Fig. 6c). The growth media of +Fe Col0 plants contained almost exclusively minor amounts of glucoside of scopoletin (*2*; Fig. 6b), with only traces of scopoletin being present (*7*; Fig. 6f). When grown in the absence of Fe, the peak areas of all the phenolics already present in +Fe Col0 roots increased several-fold (up to 20-fold), whereas those of the phenolics present in the growth medium increased markedly: large amounts of scopoletin derivatives, including isofraxidin and methoxyscopoletin (*8–9*; Fig. 6g) and fraxetin (*6*; Fig. 6e) were found. On the other hand, the amount of scopoletin also increased (*7*; Fig. 6f), and no changes were found for the glucoside of scopoletin (*2*; Fig. 6b).

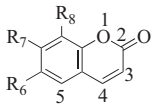
Roots of +Fe *pdr9-2* and *pdr9-3* contained glucosides of dihidroxyscopoletin (*1* and *5*; Fig. 6a) and some glucoside of scopoletin (in *pdr9-3*; *7*; Fig. 6f), as well as minor amounts of other derivatives, including the glucosides of hydroxymethoxyscopoletin (*4*; Fig. 6d) and scopoletin (*2*; Fig. 6b), and traces of glucoside of ferulic acid (*3*); *pdr9-2* also contained traces of isofraxidin (*8*; Fig. 6g). The growth media of Fe-sufficient *pdr9-2* and *pdr9-3* plants contained only minor traces of phenolic compounds. When grown in the absence of Fe, the peak areas of all the phenolics already present in +Fe *pdr9-2* and *pdr9-3* increased several-fold, and fraxetin was in large amounts in *pdr9-2* (*6*; Fig. 6e). In the growth medium, the phenolics peak areas were low in comparison with Col0, specially in *pdr9-2* (in *pdr9-3* the small increase was mainly due to isofraxidin and metoxyscopoletin; Fig. 6g).

Roots of +Fe *comt* contained large amounts of scopoletin (*7*; Fig. 6f) and minor amounts of glucosides of scopoletin (*2*; Fig. 6b) and glucoside of ferulic acid (*3*; Fig. 6c) and isofraxidin (*8*; Fig. 6g). The *comt* mutation fully abolished phenolics export to the medium. When grown in the absence of Fe, the concentrations of root phenolics did not change, except for the glucoside of ferulic acid (*3*; Fig. 6c), which increased, whereas the secretion of phenolics to the medium was also abolished.

Therefore, phenolics secreted to the growth media in Col0 consisted mainly of coumarins structurally analogous to scopoletin (*7*), including fraxetin (*6*) and the isomers isofraxidin and methoxyscopoletin (*8* and *9*), and export of these compounds to the medium in the *pdr9* plants was very low. The glucosides of coumarins were never found in the growth media, with the sole exception of minor amounts of glucoside of scopoletin in Col0. Phenolics in roots of Col0 and *comt* consisted of scopoletin (*7*), isofraxidin and methoxyscopoletin (*8* and *9*), whereas in the case of the *pdr9* mutants the amounts of coumarin glucosides were also large.

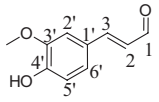
Table 2 Chemical structures, retention times (RT) and $[M+H]^+$ exact mass-to-charge ratios (*m/z*), molecular formula and error of a selected group of phenolic compound standards; esculin, esculetin, and fraxetin

Coumarin

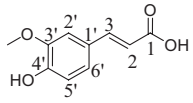


Esculin: R6: O-Glucose, R7: OH, R8: H
Esculetin: R6, R7: OH, R8: H
Fraxetin: R6: OMe, R7, R8: OH
Isofraxidin: R6, R8: OMe, R7: OH
Scopoletin: R6: OMe, R7: OH, R8: H

Coniferyl aldehyde



Ferulic acid



Name	RT (min)	Measured *m/z*	Molecular formula	Calculated *m/z*	Error *m/z* (ppm)	ESI-MS^n *m/z* (Relative intensity%)
Esculin	8.8	341.0860	$C_{15}H_{17}O_9^+$	341.0867	−2.0	MS^2 [341]: 179 (100), 151 (1.5), 135 (0.4), 133 (3), 123 (2), 105 (0.2) MS^3 [341→179]: 151 (53), 135 (19), 133 (100), 123 (48), 107 (3), 89 (3)
Esculetin	11.6	179.0334	$C_9H_7O_4^+$	179.0338	−2.2	MS^2 [179]: 151 (79), 135 (24), 133 (100), 123 (64), 107 (10), 89 (7)
Fraxetin	13.0	209.0441	$C_{10}H_9O_5^+$	209.0444	−1.4	MS^2 [209]: 194 (27), 181 (34), 177 (12), 163 (75), 153(6), 149 (100), 135 (16), 107 (29)
Scopoletin	14.6	193.0494	$C_{10}H_9O_4^+$	193.0495	−0.5	MS^2 [193]: 178 (13), 165 (19), 149 (15), 137 (8), 133 (100), 117 (2), 105 (3), 89 (1)
Isofraxidin	14.8	223.0604	$C_{11}H_{11}O_5^+$	223.0601	1.3	MS^2 [223]: 208(100), 195 (12), 190 (55), 179 (7), 163 (76), 135 (23), 107(86), 91 (9)
Coniferyl aldehyde	16.1	179.0708	$C_{10}H_{11}O_3^+$	179.0703	2.8	MS^2 [179]: 161 (93), 147 (100), 146 (57), 133 (11), 123 (8), 105 (8), 56 (7) MS^3 [179→161]: 144 (44), 133 (100), 115 (14), 105 (13)
Ferulic acid	15.6	195.0653	$C_{10}H_{11}O_4^+$	195.0652	0.5	MS^2 [195]: 177 (100), 153 (2) MS^3 [195→177]: 163 (5), 145 (100), 117 (6)

The *m/z* ratios of parent and fragment ions were determined from the data in the HPLC/ESI-MS(TOF) and HPLC/ESI-MS/MS(ion trap) chromatograms, respectively.

In general, the changes in the peak areas of the coumarins found in Fig. 6 were in line with the changes observed in the HPLC-fluorescence chromatogram in Fig. 5. Exceptions were the large amounts of fraxetin (*6*) seen in roots of -Fe *pdr9-2* and nutrient solutions of -Fe Col0, which did not result in major fluorescence changes in the chromatograms at the corresponding RT. This is likely due to the low fluorescence yield caused by the hydroxylation (Crosby & Berthold, 1962).

Discussion

Studies on reduction and chelation-based mechanisms for Fe uptake by plant roots in response to Fe deficiency have mainly focussed on the FRO/IRT (Vert *et al.*, 2002) and YS1/YSL (Curie *et al.*, 2001) systems. However, Fe-efficient plants also respond to Fe deficiency by enhancing the root secretion of organic compounds, such as flavins or phenolics (Römheld & Marschner, 1983; Susín *et al.*, 1993). The role of secreted red clover phenolics in increasing Fe efficiency was recently documented (Jin *et al.*, 2006, 2007). Consistent with this report we observed that both *Brassica napus* and *Arabidopsis thaliana* roots produced fluorescent compounds, the amount of which increased in response to Fe deficiency. Part of these compounds were secreted to the nutrient growth medium, and this secretion was enhanced by Fe shortage (Fig. 1). Furthermore, passing the culture medium of *Arabidopsis* plantlets through a Sep-Pak C_{18} column led to enhanced leaf chlorosis (Fig. 2a), and a 60% decrease in leaf Fe concentration (Fig. 2b). Scopoletin and derivatives found in the culture medium of Fe-deficient plants almost disappeared when the medium was re-circulated through the Sep-Pak C_{18} column (Fig. S1), and they were recovered from the column by elution with methanol (Fig. S4). These data strongly suggest that the secretion of phenolics is part of the plant response to Fe shortage, and that they participate in protection against Fe-deficiency induced chlorosis.

Recent transcriptome (Yang *et al.*, 2010; Rodríguez-Celma *et al.*, 2013) and proteome (Lan *et al.*, 2011) studies of Fe-deficient *Arabidopsis* roots revealed the upregulation of a set of genes encoding enzymes of the phenylpropanoid pathway. Phenylalanine ammonia-lyase, one of the most upstream enzymes of this pathway, and the coumarate:CoA ligases 4CL1 and 4CL2, mediating its last step, were induced by Fe deficiency, as well as caffeoyl-CoA O-methyltransferase and F6′H1, which mediate the conversion of caffeoyl-CoA to feruloyl-CoA and the subsequent production of 6′hydroxy-feruloyl-CoA, respectively. The expression of the *ABCG37* transporter gene was also upregulated under such conditions, and Yang *et al.* (2010) suggested that it could export phenolics compounds such as caffeic or chlorogenic acids. ABCG37 is a member of the G-subgroup of ABC transporters (Verrier *et al.*, 2008) able to transport auxinic compounds (Ito & Gray, 2006; Ruzicka *et al.*, 2010). The promoter sequence of the *ABCG37* gene contains several putative

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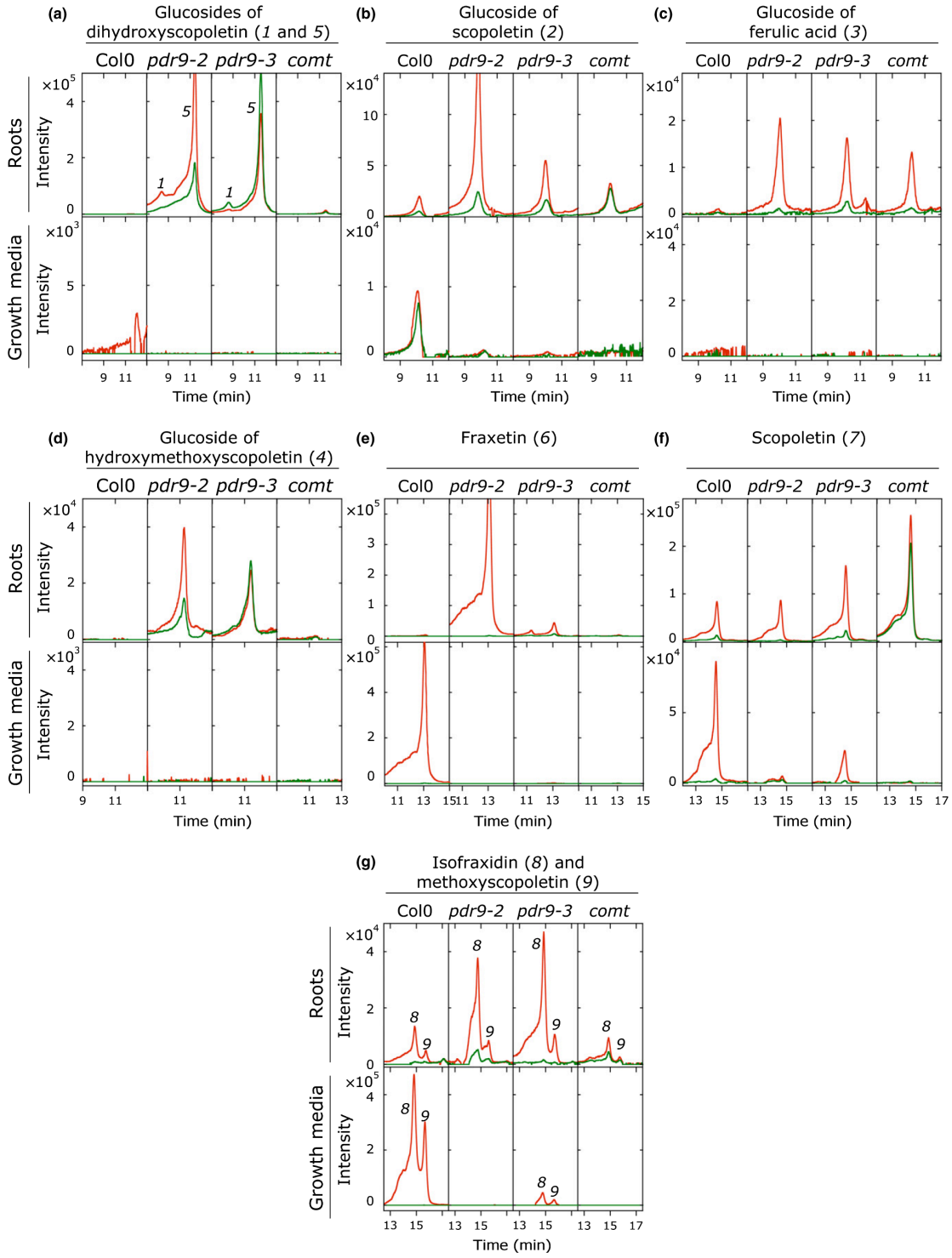

WRKY transcription factor binding sites (Ito & Gray, 2006), suggesting a role of ABCG37 in biotic stress defence and in transport of metabolites, including ferulic or coumaric acids (Walker *et al.*, 2003).

Involvement of specific transporters for phenolics efflux in response to Fe deficiency was recently documented. In rice the PEZ1 transporter controls the concentration of protocatechuic acid in the xylem sap, and is essential for the utilization of precipitated Fe in the stele apoplast (Ishimaru *et al.*, 2011). PEZ2 is closely homologous to PEZ1 and seems to perform a very similar function in rice plants (Bashir *et al.*, 2011).

More recently, comparative transcriptional profiling by RNA sequencing of roots of *Medicago* and *Arabidopsis* plants grown under Fe deficiency conditions revealed a massive upregulation of genes encoding enzymes involved in riboflavin biosynthesis in *Medicago* and in phenylpropanoid synthesis in *Arabidopsis* (Rodríguez-Celma *et al.*, 2013). Coexpression and promoter analysis provided evidence of a tight link between these biosynthetic pathways and a co-regulation with other genes encoding proteins involved in Fe uptake. This paper also reported that mutations in the Fe(II)- and 2-oxoglutarate-dependent dioxygenase family gene *F6′H*, and defects in the expression of *ABCG37*, abolished the presence of phenolic compounds in the culture medium and compromised Fe uptake from a low bioavailability Fe source. Our data presented in Figs 1, 2 and 4, although obtained under slightly different Fe nutrition conditions (no Fe added in our study vs Fe added as a low available source in Rodriguez-Celma and co-workers study), fully confirms these findings. However, the chemical nature of the phenolic compounds accumulated in Arabidopsis roots and/or secreted in response to Fe deficiency was not investigated in that study. We also confirmed that Fe deficiency led to increase the transcript abundances of *F6′H1* and *ABCG37* (Fig. 3; Yang *et al.*, 2010). This is also consistent with the report that the expression of the closest homologue of *AtABCG37* in tobacco (*NtPDR3*) is also induced in response to Fe deficiency (Ducos *et al.*, 2005). In our case, the role of ABCG37 in phenolic secretion by *Arabidopsis* roots was suggested by the fact that the culture medium of plantlets, grown without Fe added, appeared much more fluorescent than the one of the *pdr9-2* mutant allele (Fig. 4b), as previously reported by Rodríguez-Celma *et al.* (2013). This was confirmed by HPLC analysis of these compounds (Fig. 5). The *pdr9* mutation led to a Fe-deficiency dependent increase in abundance of several fluorescent compounds in roots, some of them of higher polarity than those present in Col0. In the growth media extracts of the *pdr9-2* and *pdr9-3* mutant plants only a minor peak eluting at 15 min was observed, much smaller than the one at the same retention time in growth media extracts of Col0 wild-type plants (Fig. 5). Therefore, the *pdr9* mutation markedly impaired the root excretion of the fluorescent compounds eluting at 15 min that occurs in Col0 in response to Fe deficiency. As shown in Fig. 2, these compounds participate in Fe nutrition. Because the ABCG37 transporter is required for their secretion, it was therefore consistent to observe that *pdr9-2* plantlets were more sensitive to Fe deficiency than wild-type Col0 plants (Fig. 4a), in agreement with the recent findings of Rodríguez-Celma *et al.* (2013). Therefore, it can be concluded that: Fe deficiency induces the secretion of fluorescent phenolic compounds by *Arabidopsis* roots; the ABCG37 transporter is required for this secretion to take place; and these compounds improved plant Fe nutrition.

The chemical characterization of these compounds is a prerequisite to the understanding of their function in Fe nutrition, and no such characterization has been reported so far (Rodríguez-Celma *et al.*, 2013). Their in-depth analysis by mass spectroscopy revealed that the compounds belong to the coumarin family, and more precisely are consistent with scopoletin and several derivatives (Table 2). Are these phenolics the elusive 'chelate' bringing Fe(III) to the root plasmalemma ferric chelate reductase, or do they have a role independent of Fe reduction and IRT1 uptake? Their chemical characterization reported in this study should help to adress these questions. Coumarins can interact *in vitro* with Fe either by chelating or reducing it. For instance some *o*-dihydroxy-coumarins chelate Fe^{3+} with the same affinity than deferoxamine at neutral pH, and may reduce Fe^{3+} at acidic pH (Mladenka *et al.*, 2010). Also, coumarins could play an allelopathic role (Jin *et al.*, 2006) similar to riboflavin derivatives (Susín *et al.*, 1993; Rodríguez-Celma *et al.*, 2011). They are toxic for bacteria and fungi (reviewed in Gnonlonfin *et al.*, 2012), and their secretion could decrease competition for Fe in the rhizosphere. It was known that mutations in the genes encoding F6′H1 and caffeoyl-CoA O-methyltransferase reduced the concentrations of scopoletin and its ß-glucoside scopolin in the roots (Kai *et al.*, 2008). Indeed, analysis of Fe-deficient *Arabidopsis* root extracts by ultra-performance liquid chromatography-MS/MS revealed that scopoletin, but not scopolin, accumulated under these conditions (Lan *et al.*, 2011). However, in that study these two compounds were not found in the root exudates of Fe-sufficient or Fe-deficient plants. This can be explained by the fact that the plant medium was analysed after 3 d of Fe-deficiency, a time point at which fluorescent compounds are not fully accumulated (Fig. 1). We also observed that scopoletin accumulated in the roots of Col0 in response to Fe deficiency, but in contrast to Lan *et al.* (2011) we also found it in the growth media, with its presence being dependent on the functionality of the ABCG37

Fig. 6 Effect of iron (Fe) deficiency on scopoletin and derivatives occurring in roots and growth media of Col0, *pdr9-2*, *pdr9-3* and *comt Arabidopsis* plants. HPLC/ESI-MS(TOF) typical chromatograms of root and growth media extracts from Col0, *pdr9-2*, *pdr9-3* and *comt Arabidopsis* plants. Twenty-one-day old plants, were hydroponically grown, and treated with (green; +Fe) and without (red; −Fe) 50 μM Fe^{3+}-EDTA supply for 7 d before sampling. The chromatograms were zoomed to show the peaks corresponding to glucosides of dihydroscopoletin (a), glucoside of scopoletin (b), glucoside of ferulic acid (c), glucoside of hydroxymethoxyscopoletin (d), fraxetin (e), scopoletin (f) and isofraxidin and methoxyscopoletin (g). The chromatograms were extracted at the *m/z* (± 0.02) ratios corresponding to $[M+H]^+$ ions, with the exception of glucosides, for which the *m/z* ratios corresponding to both $[M+H]^+$ and $[M\text{-glucose}+H]^+$ ions were used. The numbers in italics shown after the compound names refer to the labels used for each compound in Table 1 and Fig. 5.

transporter. Not only scopoletin, but also four glucosylated/hydroxylated coumarins and a glucosylated coumarin precursor accumulated in Fe-deficient roots of *pdr9* plants. Coumarins synthesis in the roots in response to Fe deficiency is likely coupled to their secretion to the external medium through ABCG37 activity. When this activity is impaired, they accumulate inside the roots. The glucosylated coumarin forms are adequate substrates to be detoxified within the vacuoles, suggesting the existence of an unidentified vacuolar transporter likely upregulated in Fe-deficient Arabidopsis roots of *pdr9* plants. However, at this stage it cannot be deduced whether ABCG37 excretes glucosylated and unglucosylated phenolics or whether the glucosylated phenolics are deglucosylated once excreted. Challenging these hypotheses will be the aim of future experiments.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Removal of scopoletin and derivatives by Sep-Pak C_{18} column from the *Arabidopsis* nutrient solution.

Fig. S2 Analysis of phenolic compound standards by HPLC/ESI-MS(TOF).

Fig. S3 MS^2 spectra of coumarin standards and MS^3 spectra of compounds 1, 4 and 5. Common losses from the precursors are marked in each spectrum.

Fig. S4 Scopoletin and derivatives eluted from the C18 resin column.

Table S1 Primers used for semi-quantitative RT-PCR

Table S2 List of eluents and standards used

Table S3 Operating conditions of HPLC/ESI-MS(TOF) and HPLC/ESI-MS/MS(ion trap)

Table S4 Manganese, Cu and Zn concentrations (μg g^{-1} DW) in young leaves and roots from *Arabidopsis thaliana* plants

Notes S1 Detailed information on the identification of the Arabidopsis fluorescent compounds as scopoletin and derivatives.

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Supplementary Material

Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by *Arabidopsis* roots in response to iron deficiency

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The following Supplementary Data is available for this article:

Notes S1 Detailed information on the identification of the Arabidopsis fluorescent compounds as scopoletin and derivatives.

Fig. S1 Analysis of phenolic compound standards by HPLC/ESI-MS(TOF).

Fig. S2 MS^2 spectra of coumarin standards and MS^3 spectra of compounds 1, 4, and 5. Common losses from the precursors are marked in each spectra.

Fig. S3 The Sep-Pak C18 column removes scopoletin and derivatives from the growth medium where Fe-deficient Arabidopsis plants were grown.

Fig. S4 Scopoletin and derivatives eluted from the C18 resin column.

Table S1 Primers used for semi-quantitative RT-PCR.

Table S2 List of eluents and standards used.

Table S3 Operating conditions of HPLC/ESI-MS(TOF) and HPLC/ESI-MS/MS(ion trap).

Table S4 Manganese, Cu and Zn concentrations ($\mu g\ g^{-1}$ DW) in young leaves and roots from *Arabidopsis thaliana* plants. The medium of the Fe-deficient plants was continuously circulated with a peristaltic pump in presence (-Fe+C18 resin column) or absence (-Fe) of a C18 Sep-Pak cartridge.

Notes S1 Detailed information on the identification of the Arabidopsis fluorescent compounds as scopoletin and derivatives.

High-resolution spectra were acquired in the 50-1000 *m/z* range during the whole HPLC run to obtain a three-dimensional dataset (time, *m/z*, and intensity). The RT of the phenolics standards ranged from 4.6 (L-phenylalanine) to 21.5 min (quercetin), and the ion chromatograms for each standard were extracted at the *m/z* value of the $[M+H]^+$ ion (Supporting information Fig. S2). The raw datasets from the richest materials in fluorescent compounds, -Fe Col0 growth media and -Fe *pdr9-2* root extracts, were processed, along with the corresponding +Fe counterparts, with the DISSECT algorithm (Data Analysis 4.0, Bruker). From all the analyzed spectra (approximately 100), only nine were chosen that: i) eluted at the RT where fluorescence was found, and ii) either occurred only in the Fe-deficient samples or increased in peak area with Fe-deficiency. Then, these MS spectra were examined in detail in the Fe-deficient samples, ions coming from adducts (formed with salts or solvents), dimers and trimers were generally discarded, and the ion chromatograms of all major remaining ions (that included non-fragmented $[M+H]^+$ ions and fragment ions produced in the ESI source) were extracted with a precision of ±0.02 *m/z*. From these, we selected major ions showing large changes in peak areas with Fe deficiency (Table 1 includes the RT, exact *m/z* and assigned elemental formulae, all of them with *m/z* errors <5 ppm). Fragments and minor ions were not considered. To obtain further structural information, HPLC/ESI-MS-MS(ion trap) was used, allowing for selection and isolation of specific ions and the subsequent fragmentation and detection of product ions. This was done in multiple MS stages (MS^2, MS^3, etc.), increasing progressively the amount of structural information gained. A comprehensive study of the MS^n of phenolics standards was used to build a fragmentation template (see Table 2 for the *m/z* values and relative intensities of the fragments).

Annotations proposed for compound *9* are in agreement with the RTs, which indicate relative polarities in the order fraxetin > scopoletin > isofraxidin > *9*. Compound *9* produced MS^2 fragment ions matching well with that of isofraxidin (Tables 1 and 2), indicating that the methoxy group must be in the benzenic ring as it occurs in the standard, since a methoxy group in the heterocyclic ring would yield a different MS^2 fragmentation pattern. The 5- position is the only one possible in the benzenic ring apart from the 8- occurring in the standard.

A similar glucosyl moieity loss was also found in the standard glucoside of esculetin (esculin), which produced an ion at *m/z* of 179.0343 corresponding to the aglycone (esculetin). This, along with the fact that *1-5* eluted at RTs close to that of esculin (8.8 min), much lower than those of all non-glucosylated coumarins (13-16 min), supports a putative annotation as glucosides of coumarins. In fact, the MS^2 of *1-5* (Table 1) show that the isolation and subsequent fragmentation of the parent ions (387, 355, 357, 401 and 387 *m/z*, respectively) produced generally just one major fragment ion in each case (at 225, 193, 195, 239 and 225 *m/z*, respectively), all consistent

with the loss of the glucosyl moiety, with the rest of fragments having generally relative intensities below 10%. A similar MS^2 fragmentation pattern was found for esculin (*m/z* of parent and major fragment ions of 341 and 179, respectively, with a loss of -162 Da; Table 2). Therefore, compounds *1-5* were confirmed to be glucosides, and their identity was further deciphered by MS^3 using as a model the fragmentation of esculin. The esculin MS^3 spectrum 341→179 produced the same fragment ions than those present in the aglycone esculetin MS^2 (Table 2), confirming that esculin is initially fragmented in MS^2 in a very abundant ion corresponding to the aglycone after the loss of the glucosyl moiety. Compound *2* was identified as glucoside of scopoletin (scopolin), since the MS^3 spectrum 355→193 produced several fragment ions matching with those present in the scopoletin MS^2 (Tables 1 and 2). Compounds *1* and *5* were annotated as two different glucosides of dihydroxyscopoletin. First, their MS^3 spectra, in both cases 387→225, produced several *m/z* losses similar to those observed in the MS^2 spectra of the coumarins studied (see Supporting information Fig. S3), indicating that they can be two glucosides of a coumarin with a $[M+H]^+$ 225 *m/z*. Taking advantage of the fact that ions with *m/z* 225.0393 and 225.0386 were observed in the TOF analysis, we were able to assign the elemental formula $C_{10}H_9O_6$ to the 225 *m/z* coumarin (with an error <4 ppm). Both this *m/z* and the elemental formula difference with scopoletin are consistent with the addition of two hydroxyl (-OH) groups to scopoletin, resulting in a change of +31.99 Da. Compound *4* was annotated as a glucoside of hydroxymethoxyscopoletin. The MS^3 spectra 401→239 also produced several *m/z* losses similar to those of the coumarins MS^2, indicating that it can be a glucoside of a coumarin with a $[M+H]^+$ 239 *m/z*. In TOF, *4* showed a major ion at *m/z* 239.0547, which permitted to assign the formula $C_{11}H_{11}O_6$ to the 239 *m/z* (with an error of 1.3 ppm). Both this *m/z* and the elemental formula difference with scopoletin are consistent with the addition of a hydroxyl (-OH) and a methoxy group ($-OCH_3$) to scopoletin, resulting in a change of +46.00 Da. Compound *3* was annotated as a glucoside of ferulic acid. The MS^3 357→195 and MS^4 357→195→177 spectra showed ions common to those observed in the MS^2 and MS^3 (195→177) spectra of ferulic acid (see Tables 1 and 2). Additionally, compound *3* showed a major ion at *m/z* 195.0646 in TOF, which permitted to assign the formula $C_{10}H_{11}O_4$ (with an error of -3.1 ppm). This elemental formula corresponds to two different compounds involved in coumarin synthesis (Yang *y col.*, 2010), ferulic acid and 6'-hydroxyconiferyl aldehyde. However, the fragmentation pattern of compound *3* matches well with that of ferulic acid, and differs from that of coniferyl aldehyde (Table 2).

Fig. S1 Removal of scopoletin and derivatives by Sep-Pak C_{18} column from the *Arabidopsis* nutrient solution. HPLC/ESI-MS(TOF) chromatograms of growth media extracts from Fe-deficient *Arabidopsis* plants. Plants were grown in hydroponics for four weeks in presence of 50 µM Fe(III)-EDTA in culture media, and then transferred on a nutrient solution without Fe. The nutrient solution was re-circulated continuously using a peristaltic pump in the presence (blue) or absence (red) of a C_{18} resin column. The nutrient solution was sampled at day 20 after imposing the treatments. The chromatograms were zoomed to show the peaks corresponding to fraxetin (a), scopoletin (b) and isofraxidin and methoxyscopoletin (c). Chromatograms were extracted at the m/z (± 0,02) ratios corresponding to $[M+H]^+$ ions. The numbers in italics shown after the compound names refer to the labels used for each compound in Table 1. The concentrations of isofraxidin and methoxyscopoletin found in the nutrient solutions at 20 d were lower that those found at 7 d (Fig. 3).

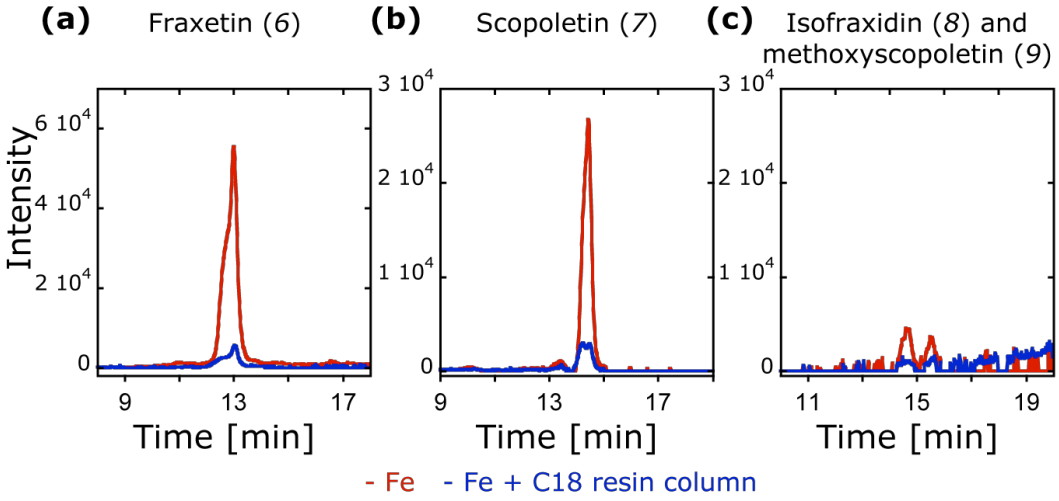

Fig. S2 Analysis of phenolic compound standards by HPLC/ESI-MS(TOF). Typical chromatograms of phenolic standard solutions extracted at the *m/z* (± 0.02) ratio corresponding to the $[M+H]^+$ ions. The chromatograms corresponding to cinnamic, *o*-coumaric acid and *p*-coumaric acids were extracted at the *m/z* ratios of both $[M+H]^+$ and $[M-H_2O+H]^+$ ions.

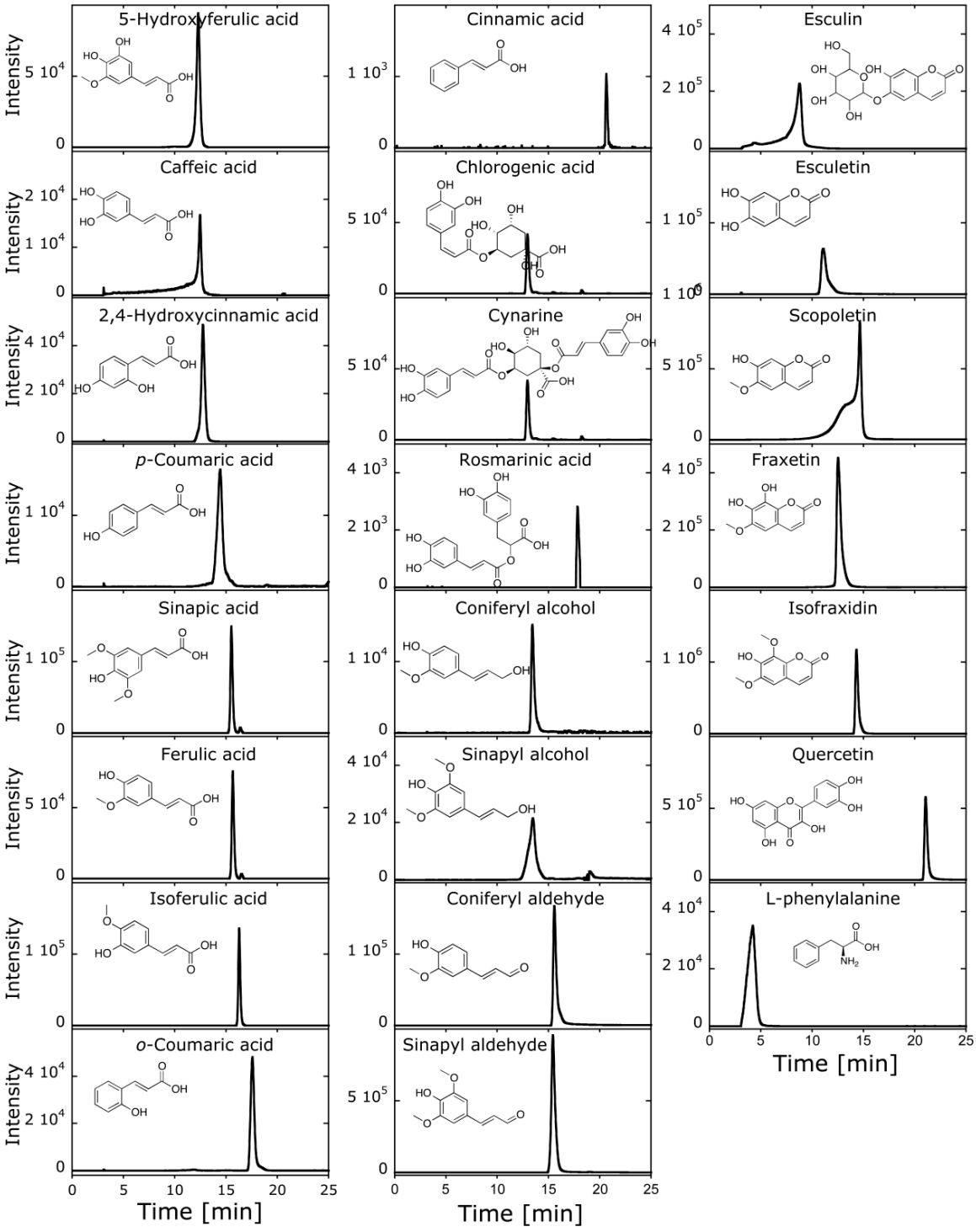

Fig. S3 MS^2 spectra of the coumarin standards scopoletin (a), fraxetin (b) and isofraxidin (c), and MS^3 spectra of the compounds *1* (d), *5* (e) and *4* (f) occurring in *Arabidopsis* roots. Common mass-to-charge (*m/z*) losses from the precursor ion are indicated with arrows.

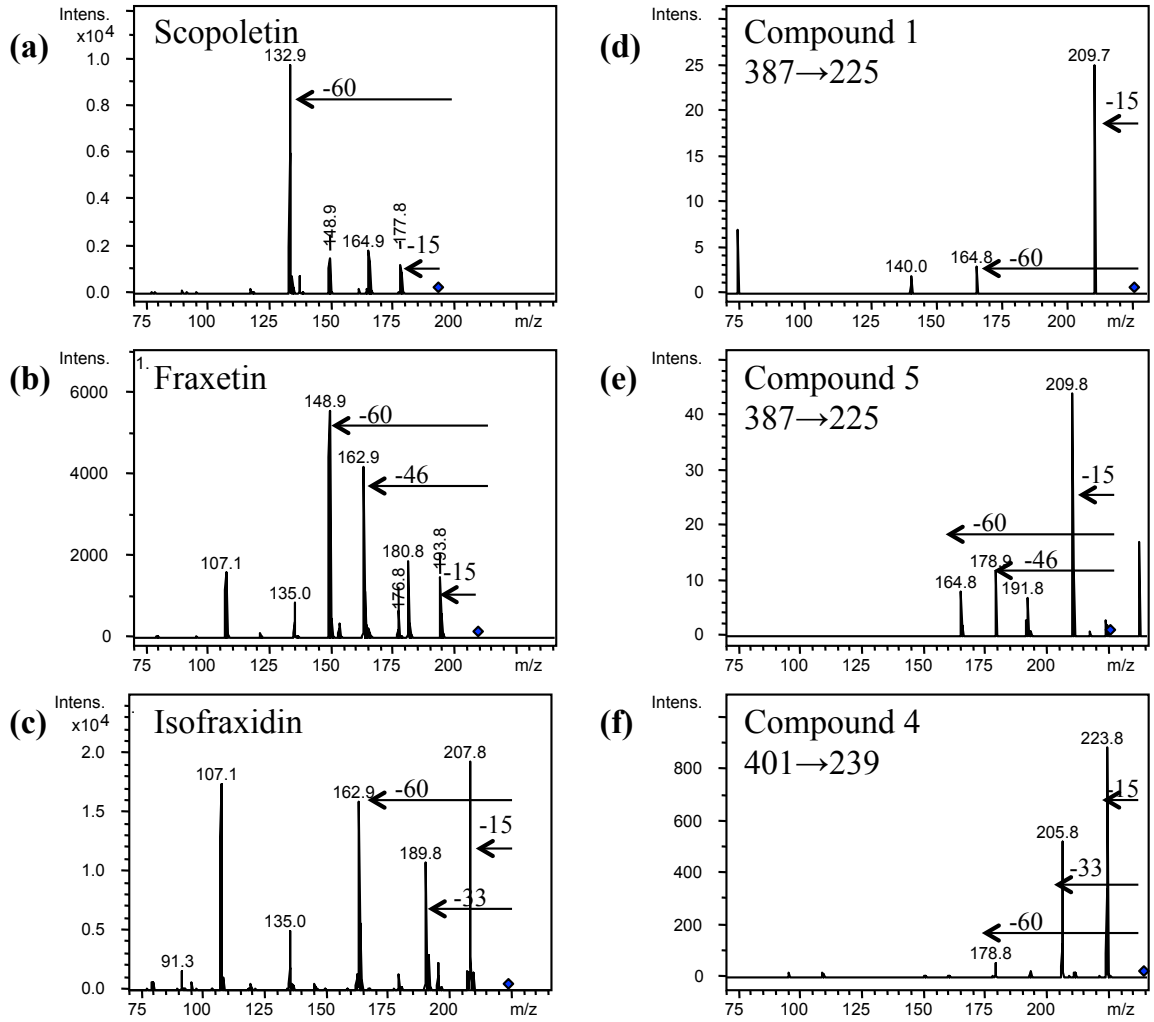

Fig. S4 Elution of scopoletin and derivatives trapped in the C_{18} resin column. HPLC/ESI-MS(TOF) chromatograms of growth media extracts from *Arabidopsis* plants. Plants were grown in hydroponics for four weeks in presence of 50 µM Fe(III)-EDTA in culture media, and then transferred on a nutrient solution without Fe. The nutrient solution was re-circulated continuously using a peristaltic pump in the presence or absence of the C_{18} resin column. At day 7 after imposing the treatments, the phenolics trapped in the C_{18} resin column were eluted three times with 10 ml of metanol. The chromatograms correspond to the second elution step. The chromatograms were zoomed to show the peaks corresponding to fraxetin (a), scopoletin (b) and isofraxidin, methoxyscopoletin (c). Chromatograms were extracted at the m/z (± 0,02) ratios corresponding to $[M+H]^+$ ions. The number in italics shown after the compound names refer to the labels used for each compound in Table 2.

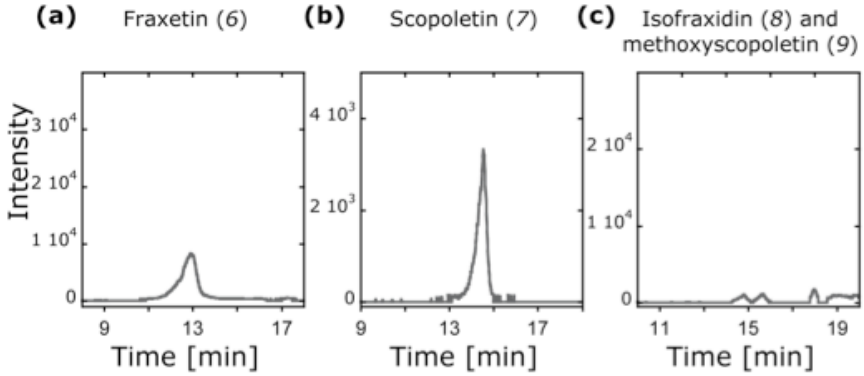

Table S1 Primers used for qRT-PCR

gene	ID	orientation	sequence
PP2	At1g13320	FW	TAACGTGGCCAAAATGATGC
		REV	GTTCTCCACAACCGCTTGGT
CCoAMT1	At4g34050	FW	GCTCCTCCTGATGCACCAAT
		REV	CCATCACCAACAGGGAGCAT
COMT	At5g54160	FW	TGCTCCTTCTCATCCTGGTAT
		REV	CACGCAATGTTCGTCACTCC
F6'H1	At3g13610	FW	GCCTGATATCTGCAGGAATGAAA
		REV	ACTCTAGAAGCCTCCTCACCA
FRO2	At1g01580	FW	GCGACTTGTAGTGCGGCTATG
		REV	CGTTGCACGAGCGATTCTTG
IRT1	At4g19690	FW	CGGTTGGACTTCTAAATGC
		REV	CGATAATCGACATTCCACCG
ABCG37 (PDR9)	At3g53480	FW	GCGAAACTCAGAGCTTGTGA
		REV	AGTGCGCCGAAGATCAAAGA

Table S2 List of chemicals (solvent and phenolic standards) used for analytical measurements.

Name	Quality grade	Manufacturer
lithium hydroxide monohydrate	99.995%	Aldrich
formic acid	50%	Fluka
methanol	LC-MS grade	Riedel-de-Haën
2-propanol	LC-MS grade	Riedel-de-Haën
caffeic acid	≥99%,	Fluka
2,4-dihidroxycinnamic acid	97%	Aldrich
5-hidroxyferulic acid	≥95%	Sigma
ferulic acid	99%	Aldrich
isoferulic acid	99%	Aldrich
o-coumaric acid	97%	Aldrich
p-coumaric acid	≥98%	Fluka
sinapic acid	≥98%	Aldrich
trans-cinnamic	99%	Aldrich
chlorogenic acid	≥95%	Aldrich
cynarine	≥95%	Fluka
rosmarinic acid	96%	Aldrich
coniferyl alcohol	98%	Aldrich
sinapyl alcohol	80%	Aldrich
coniferyl aldehyde	98%	Aldrich
sinapic aldehyde	98%	Aldrich
esculetin	≥98%	Aldrich
esculin	≥98%	Fluka
scopoletin	≥98.5%	Fluka
fraxetin	98%	Biorbyt
isofraxidin	95%	Fluka
quercitin	≥98%	Sigma
L-phenylalanine	certified reference material	Fluka

All phenolic standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), with the exception of fraxetin, which was purchased from Biorbyt (St. Francisco, CA, USA).

Table S3 Operating conditions of the time-of-flight (TOF) and ion trap mass spectrometers (MS) used for analytical measurements.

MS(TOF)	
Source	Electrospray
Polarity	Positive
Endplate voltage	-0.5 kV
Spray tip voltage	4.5 kV
Orifice voltage	100 V
Nebulizer gas	N_2
Nebulizer gas pressure	2.7 bar
Drying gas	N_2
Drying gas (N_2) flow rate	9.0 l min^{-1}
Drying gas temperature	200 ºC
MS/MS(ion trap)	
Nebulizer gas	N_2
Nebulizer gas pressure	40 psi
Drying gas	N_2
Drying gas (N_2) flow rate	9.0 l min^{-1}
Drying gas temperature	350 ºC and 200 ºC
Operation mode	Full scan and Multiple Reaction Monitoring (MRM)
Target for full scan mode	30,000
Maximum accumulation time for full scan mode	200 ms
Mass-to-charge ratio (*m/z*) range for full scan mode	50-1200 u
Target for MRM mode	30,000
Maximum accumulation time for MRM mode	200 ms
Mass-to-charge ratio (*m/z*) range for MRM mode	50-500 u
Fragmentation amplitude for MS^2 and MS^3	1.0 V
Isolation width for MS^2 and MS^3	1.0 and 2.0 u
Cutoff selection to precursor mass for MS^2 and MS^3	27.0%

Table S4 Manganese, Cu and Zn concentrations (µg g^{-1} DW) in young leaves and roots from *Arabidopsis thaliana* plants.

	Mn	Cu	Zn
		Young leaves	
-Fe+C_{18} resin column	795±82	14.6±1.3	358±21
-Fe	616±54	22.7±1.4	300±9
		Roots	
-Fe+C_{18} resin column	2,290±171	137±8	4,071±129
-Fe	7,708±417	110±14	3,608±307

Plants were grown in hydroponics for 4 weeks in presence of 50 µM Fe^{3+}-EDTA in the culture media. They were then transferred on a medium without Fe (Fe-deficient; -Fe). The medium of the Fe-deficient plants was continuously circulated with a peristaltic pump in presence (-Fe+C_{18} resin column) or absence (-Fe) of a C_{18} Sep-Pak cartridge. Leaf and root material were sampled from plants after 20 days of recirculation of the medium. Data are means ± SE of 8 independent measurements.

3.2. Accumulation and secretion of coumarinolignans and other coumarins in *Arabidopsis thaliana* roots in response to iron deficiency at high pH

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Accumulation and Secretion of Coumarinolignans and other Coumarins in *Arabidopsis thaliana* Roots in Response to Iron Deficiency at High pH

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[‡] *This paper is dedicated to the Memory of Pierre Fourcroy, a CNRS researcher, to largely contributed to initiate this research.*

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Root secretion of coumarin-phenolic type compounds has been recently shown to be related to *Arabidopsis thaliana* tolerance to Fe deficiency at high pH. Previous studies revealed the identity of a few simple coumarins occurring in roots and exudates of Fe-deficient *A. thaliana* plants, and left open the possible existence of other unknown phenolics. We used HPLC-UV/VIS/ESI-MS(TOF), HPLC/ESI-MS(ion trap) and HPLC/ESI-MS(Q-TOF) to characterize (identify and quantify) phenolic-type compounds accumulated in roots or secreted into the nutrient solution of *A. thaliana* plants in response to Fe deficiency. Plants grown with or without Fe and using nutrient solutions buffered at pH 5.5 or 7.5 enabled to identify an array of phenolics. These include several coumarinolignans not previously reported in *A. thaliana* (cleomiscosins A, B, C, and D and the 5′-hydroxycleomiscosins A and/or B), as well as some coumarin precursors (ferulic acid and coniferyl and sinapyl aldehydes), and previously reported cathecol (fraxetin) and non-cathecol coumarins (scopoletin, isofraxidin and fraxinol), some of them in hexoside forms not previously characterized. The production and secretion of phenolics were more intense when the plant accessibility to Fe was diminished and the plant Fe status deteriorated, as it occurs when plants are grown in the absence of Fe at pH 7.5. Aglycones and hexosides of the four coumarins were abundant in roots, whereas only the aglycone forms could be quantified in the nutrient solution. A comprehensive quantification of coumarins, first carried out in this study, revealed that the catechol coumarin fraxetin was predominant in exudates (but not in roots) of Fe-deficient *A. thaliana* plants grown at pH 7.5. Also, fraxetin was able to mobilize efficiently Fe from a Fe(III)-oxide at pH 5.5 and pH 7.5. On the other hand, non-catechol coumarins were much less efficient in mobilizing Fe and were present in much lower concentrations, making unlikely that they could play a role in Fe mobilization. The structural features of the array of coumarin type-compounds produced suggest some can mobilize Fe from the soil and others can be more efficient as allelochemicals.

Keywords: *Arabidopsis*, cleomiscosin, coumarin, fraxetin, iron nutrition, mass spectrometry, root secretion

INTRODUCTION

Iron (Fe) is required for many crucial biological processes, and is therefore essential for all living organisms. A sufficient supply of Fe is necessary for optimal plant productivity and agricultural produce quality (Briat et al., 2015). Iron is the fourth most abundant element in the earth's crust, but its availability for plants is influenced by pH and redox potential, as well as by the concentration of water-soluble Fe-complexes and the solubility of Fe(III)-oxides and oxyhydroxides (Lindsay, 1995). In calcareous soils, which cover more than 30% of the earth surface, the high soil pH and low soil organic matter content lead to Fe concentrations in the bulk soil solution far below those required for the optimal growth of plants and microbes (10^{-4}–10^{-9} and 10^{-5}–10^{-7} M, respectively; Guerinot and Ying, 1994). Since plants and microbiota have evolved in soils poor in available Fe, they have active mechanisms for Fe acquisition, often relying on the synthesis and secretion of an array of chemicals that modify the neighboring environment and reduce competition for Fe (Crumbliss and Harrington, 2009; Jin et al., 2014; Mimmo et al., 2014; Aznar et al., 2015). Some of these chemicals are capable to mine Fe from the soil *via* solubilization, chelation and reduction processes, whereas others can serve as repellants and/or attractants that inhibit or promote the growth of concomitant organisms.

In plants, two different Fe uptake mechanisms have been characterized (Kobayashi and Nishizawa, 2012). *Graminaceae* species use a chelation-type strategy (Strategy II) based on the synthesis of phytosiderophores (PS), metal-chelating substances of the mugineic acid family: PS are released by roots *via* specific transporters, mine Fe(III) from the soil by forming Fe(III)-PS complexes, and then complexes are taken up by transporters of the Yellow Stripe family. Non-graminaceous species such as *Arabidopsis thaliana* use a reduction-type strategy (Strategy I), based on the reduction of rhizospheric Fe(III) by a Fe(III) chelate reductase (FRO, ferric reduction oxidase) and the uptake of Fe(II) by root plasma membrane transporters (IRT, iron-regulated transporter). Other items of the Strategy I toolbox are an enhanced H^+-ATPase activity, an increased development of root hairs and transfer cells and the synthesis and secretion into the rhizosphere of a wide array of small molecules, including flavins, phenolic compounds and carboxylates (Cesco et al., 2010; Mimmo et al., 2014). Recent studies have unveiled direct roles in root Fe acquisition for flavin secretion in *Beta vulgaris* (Sisó-Terraza et al., 2016) and phenolics secretion in *Trifolium pratense* (Jin et al., 2006, 2007) and *A. thaliana* (Rodríguez-Celma et al., 2013; Fourcroy et al., 2014, 2016; Schmid et al., 2014; Schmidt et al., 2014).

The phenolic compounds category, including *ca.* 10,000 individual compounds in plants (Croteau et al., 2000), has been long considered to be one of the major components of the cocktail of small molecules secreted by roots of Fe-deficient plants (Cesco et al., 2010). In particular, the coumarin compounds class (O-containing heterocycles with a benzopyrone backbone; **Figure 1A**), which includes at least 1,300 compounds in plants (Borges et al., 2005) has been the focus of recent studies with *A. thaliana*. Upon Fe deficiency, there is a transcriptional up-regulation in roots both of the central phenylpropanoid pathway (from phenylalanine ammonia lyase, one of the upstream enzymes in the pathway, to the coumarate:CoA ligases 4CL1 and 4CL2 that mediate its last step) and of a crucial step of a phenylpropanoid biosynthetic branch, the 2-oxoglutarate-dependent dioxygenase enzyme feruloyl-CoA 6′-hydroxylase1 (F6′H1) (García et al., 2010; Yang et al., 2010; Lan et al., 2011; Rodríguez-Celma et al., 2013; Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014), which is responsible for the synthesis of the highly fluorescent coumarin scopoletin (Kai et al., 2008). Up to now, a total of five coumarins, esculetin, fraxetin, scopoletin, isofraxidin and an isofraxidin isomer have been described in Fe-deficient *A. thaliana* roots in both glycoside and aglycone forms (**Figure 1A**, Supplementary Table S1; Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014).

Root exudates from Fe-deficient *A. thaliana* plants contain the same coumarins that are found in root extracts, with the aglycone forms being more prevalent (Supplementary Table S1; Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014). These exudates have been shown to solubilize 17-fold more Fe from an Fe(III)-oxide (at pH 7.2) when compared to exudates from Fe-sufficient plants, and this was ascribed to the formation of Fe(III)-catechol complexes (Schmid et al., 2014). It is noteworthy that the catechol moiety in two of the five coumarins found to increase with Fe deficiency (esculetin and fraxetin) confers affinity for Fe(III) at high pH and therefore capability for Fe(III) chelation in alkaline soils. In the remaining three coumarins found so far (scopoletin, isofraxidin and its isomer), the catechol moiety is capped *via* hydroxyl (-OH) group methylation (**Figure 1A**), whereas in the glycoside forms of esculetin (esculetin 6-*O*-glucoside, known as esculin) and fraxetin (fraxetin 8-*O*-glucoside, known as fraxin) the catechol is capped *via* hydroxyl group glycosylation (**Figure 1A**). When coumarin synthesis is impaired, as in the *A. thaliana f6′h1* mutant, plants are unable to take up Fe from insoluble Fe sources at high pH (Rodríguez-Celma et al., 2013; Schmid et al., 2014; Schmidt et al., 2014), root exudates are unable to solubilize Fe from insoluble Fe sources, and supplementation of the agarose growth media with scopoletin, esculetin or esculin restores the Fe-sufficient phenotype (Schmid et al., 2014). However, in *in vitro* tests only esculetin (with a catechol moiety), was found to mobilize Fe(III) from an Fe(III) oxide source at high pH (Schmid et al., 2014).

The secretion of coumarins by Fe-deficient roots involves an ABC (ATP-binding cassette) transporter, ABCG37/PDR9, which is strongly over-expressed in plants grown in media deprived of Fe (Yang et al., 2010; Fourcroy et al., 2014, 2016) or containing insoluble Fe(III) at high pH (Rodríguez-Celma et al., 2013). The export of scopoletin, fraxetin, isofraxidin, and an isofraxidin isomer was greatly impaired in the mutant *abcg37* (Fourcroy et al., 2014), which, as it occurs with *f6′h1*, is inefficient in taking up Fe from insoluble Fe(III) at pH 7.0 (Rodríguez-Celma et al., 2013). The root secretion of fluorescent phenolic compounds in *A. thaliana* also requires the Fe deficiency-inducible β-glucosidase BGLU42 (Zamioudis et al., 2014). On the other hand, the IRT1/FRO2 high-affinity root Fe uptake system is necessary for the plant to take up Fe once mobilized, since *irt1* and *fro2* plants grown with unavailable Fe and in presence

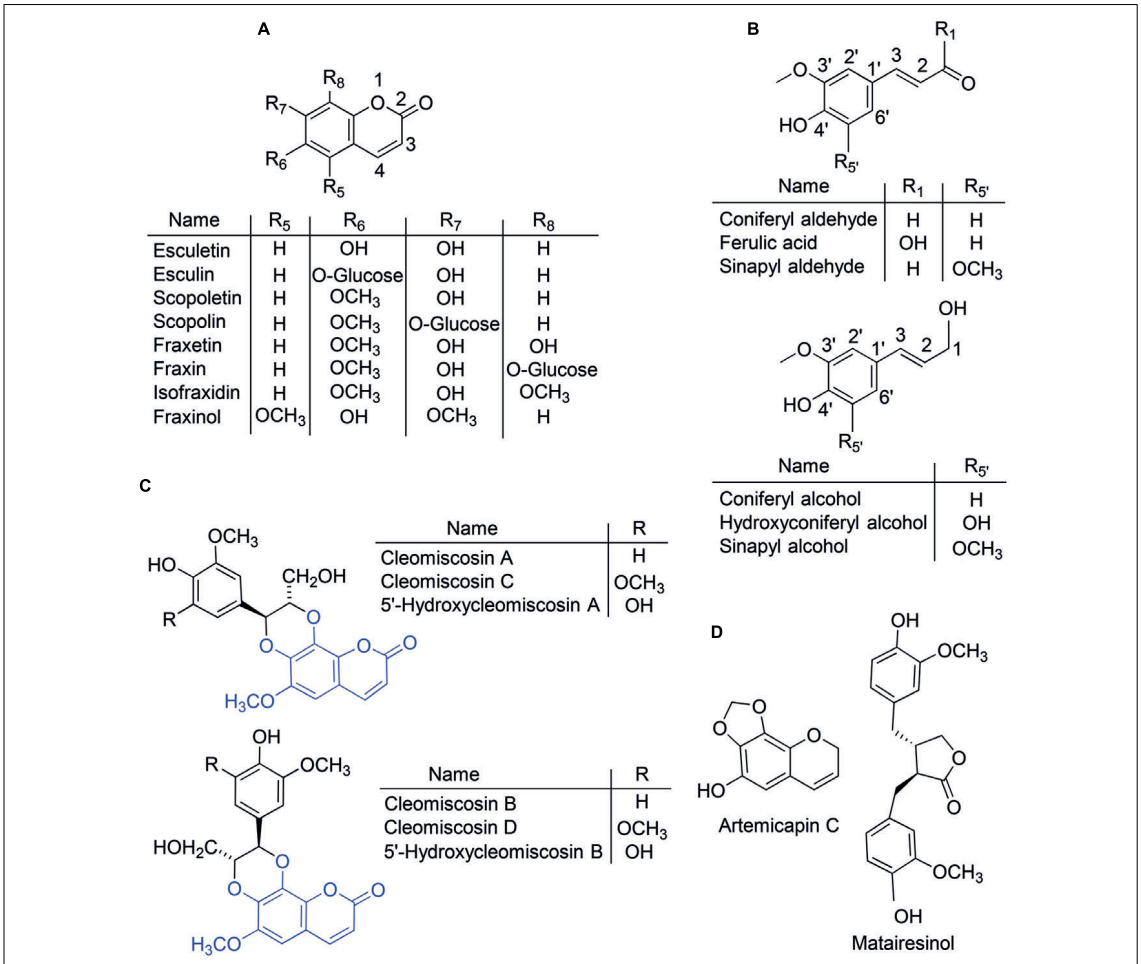

FIGURE 1 | Chemical structures of some of the phenolic compounds cited in this study. The plant compounds include coumarins and their glucosides **(A)**, coumarin precursors and monolignols **(B)** and coumarinolignans derived from the coumarin fraxetin **(C)**. The fraxetin moiety is highlighted in blue in the coumarinolignan structures. Compounds used as internal standards **(D)** include a methylenedioxy-coumarin and a lignan.

of phenolics develop chlorosis (Fourcroy et al., 2016). The co-regulation of *ABCG37* and coumarin synthesis genes with *FIT*, *IRT1*, *FRO2* and *AHA2* (Rodríguez-Celma et al., 2013) as well as the requirement of *FIT* for *F6'H1* up-regulation upon Fe deficiency (Schmid et al., 2014) support that all these components act in a coordinated mode.

Limitations inherent to the analytical procedures used and/or difficulties in compound structure elucidation have prevented the full characterization of the changes in coumarin composition promoted by Fe deficiency. First, HPLC coupled to fluorescence detection and mass spectrometry (MS and MS^n) identification was used, therefore focusing only on fluorescent coumarin compounds changing in response to Fe deficiency (Fourcroy et al., 2014); a similar approach was taken later on by Schmid et al. (2014). In a second approach, the use of full chromatographic MS profiles permitted the detection of dozens of compounds changing with Fe deficiency, but only the same coumarins already found with the fluorescence detection approach could be identified (Schmidt et al., 2014).

The aim of this study was to gain insight into the phenolic composition of *A. thaliana* root exudates in response to Fe deficiency, a necessary step for a thorough understanding of the function of phenolics in plant Fe acquisition. Root extracts and exudates from Fe-sufficient and Fe-deficient *A. thaliana* plants grown at pH 5.5 and 7.5 have been analyzed by HPLC coupled to five different detectors: fluorescence, photodiode array, MS-time of flight (TOF), MS-ion trap and MS-MS tandem quadrupole (Q)-TOF, and identification and quantification of phenolics was

carried out in roots and exudates. Up to now, quantification of coumarins in roots and exudates from Fe-deficient *A. thaliana* plants had been done only for the two fluorescent compounds esculetin and scopoletin (Schmid et al., 2014). We report herein the identification and quantification of coumarinolignans, coumarin precursors and additional coumarin glycosides, among an array of phenolics accumulated and/or secreted by *A. thaliana* roots in response to Fe deficiency. The root accumulation and secretion of coumarins and coumarinolignans was much higher in plants grown at pH 7.5 than those grown at pH 5.5, and the catechol coumarin fraxetin was predominant in nutrient solutions but not in root extracts. These findings demonstrate the inherent chemical complexity involved in the survival of *A. thaliana* in conditions of high competition for Fe, and give clues for the possible roles of some of the phenolic compounds found.

MATERIALS AND METHODS

Plant Culture and Experimental Design

Arabidopsis thaliana (L.) Heynh (ecotype Col0) seeds were germinated, pre-grown and grown as indicated in Fourcroy et al. (2014) with several modifications. Germination and plant growth took place in a controlled environment chamber (Fitoclima 10000 EHHF, Aralab, Albarraque, Portugal), at 21°C, 70% relative humidity and a photosynthetic photon flux density of 220 μmol m^{-2} s^{-1} photosynthetic active radiation with a photoperiod of 8 h light/16 h dark. Seeds were sown in 0.2 ml tubes containing 0.6% agar prepared in nutrient solution 1/4 Hoagland, pH 5.5. Iron was added as 45 μM Fe(III)-EDTA. After 10 d in the growth chamber, the bottom of the tubes containing seedlings was cut off and the tubes were placed in opaque 300-ml plastic boxes (pipette tip racks; Starlab, Hamburg, Germany), containing aerated nutrient solution 1/2 Hoagland, pH 5.5, supplemented with 20 μM Fe(III)-EDTA. Plants were grown for 11 d and nutrient solutions were renewed weekly. After that, plants (12 plants per rack) were grown for 14 days in nutrient solution 1/2 Hoagland with 0 or 20 μM Fe(III)-ethylendiaminedi(*o*-hydroxyphenylacetate) [Fe(III)-EDDHA; Sequestrene, Syngenta, Madrid, Spain]. Solutions were buffered at pH 5.5 (with 5 mM MES) or at 7.5 (with 5 mM HEPES) to maintain a stable pH during the whole treatment period. Nutrient solutions were renewed weekly. Two batches of plants were grown and analyzed. Pots without plants, containing only aerated nutrient solution (with and without Fe) were also placed in the growth chamber and the nutrient solutions sampled as in pots containing plants; these samples were later used as blanks for root exudate analyses.

Roots were sampled 3 days after the onset of Fe deficiency treatment, immediately frozen in liquid N_2, and stored at −80°C for RNA extraction. Nutrient solutions were sampled at days 7 and 14 after the onset of Fe deficiency treatment, and immediately stored at −20°C until extraction of phenolic compounds. Shoots and roots were sampled separately at the end of the experimental period. Leaf disks (0.1 cm × 0.1 cm) were taken from young leaves and stored at −20°C for photosynthetic pigment analysis. Roots were washed with tap water and then with type I water, dried with filter paper, and then frozen immediately (in aliquots of approximately 300 mg) in liquid N_2 and stored at −80°C until extraction of phenolic compounds. Roots and shoots from 12 plants per treatment and replication were processed for mineral analysis as in Fourcroy et al. (2014).

Photosynthetic Pigment Composition

Leaf pigments were extracted with acetone in the presence of Na ascorbate and stored as described previously (Abadía and Abadía, 1993). Pigment extracts were thawed on ice, filtered through a 0.45 μm filter and analyzed by HPLC-UV/visible as indicated in Larbi et al. (2004), using a HPLC apparatus (600 pump, Waters, Mildford, MA, USA) fitted with a photodiode array detector (996 PDA, Waters). Pigments determined were total chlorophyll (*Chl a* and *Chl b*), neoxanthin, violaxanthin, taraxanthin, antheraxanthin, lutein, zeaxanthin and β-carotene. All chemicals used were HPLC quality.

Mineral Analysis

Plant tissues were ground and digested as indicated in Fourcroy et al. (2014). Iron, Mn, Cu, and Zn were determined by flame atomic absorption spectrometry using a SOLAAR 969 apparatus (Thermo, Cambridge, UK).

Extraction of Phenolic Compounds from Roots and Nutrient Solutions

Phenolic compounds were extracted from roots and nutrient solutions as described in Fourcroy et al. (2014), with some modifications. First, extraction was carried out without adding internal standards (IS) to identify relevant compounds, including those increasing (or appearing) with Fe deficiency. This extract was also used to check for the presence of the compounds used as IS and other endogenous isobaric compounds that may co-elute with them, since in both cases there will be analytical interferences in the quantification process. The extraction was then carried out adding the following three IS compounds: artemicapin C (**Figure 1D**), a methylenedioxy-coumarin, for quantification of the coumarins scopoletin, fraxetin, isofraxidin and fraxinol; esculin (**Figure 1A**), the glucoside form of the coumarin esculetin, for quantification of coumarin glycosides; and the lignan matairesinol (**Figure 1D**), for quantification of coumarinolignans.

Frozen roots (*ca.* 100 mg) were ground in liquid N_2 using a Retsch M301 ball mill (Restch, Düsseldorf, Germany) for 3 min and then phenolic compounds were extracted with 1 ml of 100% LC-MS grade methanol, either alone or supplemented with 20 μl of a IS solution (37.5 μM artemicapin C, 50 μM esculin and 37.5 μM matairesinol) by homogenization in the same mill for 5 min. The supernatant was recovered by centrifugation (12,000 × *g* at 4°C and 5 min), and stored at −20°C. The pellet was re-suspended in 1 ml of 100% methanol, homogenized again for 5 min and the supernatant recovered. The two supernatant fractions were pooled, vacuum dried in a SpeedVac (SPD111V, Thermo-Savant, Thermo Fisher Scientific, Waltham, Massachusetts, MA, USA) and dissolved with 250 μl of a solution containing 15% methanol and 0.1% formic acid. Extracts

were filtered through poly-vinylidene fluoride (PVDF) 0.45 μm ultrafree-MC centrifugal filter devices (Millipore) and stored at −80°C until analysis.

Phenolic compounds in the nutrient solutions (300 ml of solution used for the growth of 12 plants) were retained in a SepPack C_{18} cartridge (Waters), eluted from the cartridge with 2 ml of 100% LC-MS grade methanol, and the eluates stored at −80°C. Samples were thawed and a 400 μl aliquot was dried under vacuum (SpeedVac) alone or supplemented with 10 μl of a IS solution (80 μM artemicapin C and 150 μM matairesinol). Dried samples were dissolved in 15% methanol and 0.1% formic acid to a final volume of 100 μl, and then analyzed by HPLC-MS. No determinations could be made in nutrient solutions of Fe-sufficient plants due to the presence of Fe(III)-EDDHA, that causes the overloading of C_{18} materials.

Extraction of Cleomiscosins from *Cleome viscosa* Seeds

Cleomiscosins were extracted from *Cleome viscosa* seeds (B & T World Seeds, Paguignan, France) as described by Chattopadhyay et al. (2008). Seeds were ground using a Retsch M400 ball mill and 25 g of the powder was defatted by homogenization with 50 ml petroleum ether at 25°C for 48 h. The defatting procedure was repeated three times. The solid residue was extracted with 50 ml methanol for 48 h at 25°C, and the extraction was repeated three times. The methanolic extracts were pooled, dried with a rotavapor device and the residue dissolved in 15% methanol and 0.1% formic acid.

Phenolic Compounds Analysis by HPLC-Fluorescence and HPLC-UV/VIS/ESI-MS(TOF)

HPLC-fluorescence analyses were carried out using a binary HPLC pump (Waters 125) coupled to a scanning fluorescence detector (Waters 474) as in Fourcroy et al. (2014). Separations were performed using an analytical HPLC column (Symmetry® C_{18}, 15 cm × 2.1 mm i.d., 5 μm spherical particle size, Waters) protected by a guard column (Symmetry® C_{18}, 10 mm × 2.1 mm i.d., 3.5 μm spherical particle size, Waters) and a gradient mobile phase built with 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in methanol (Elution program 1; Supplementary Table S2). The flow rate and injection volume were 0.2 ml min^{-1} and 20 μl, respectively. Phenolic compounds were detected using λ_{exc} 365 and λ_{em} 460 nm.

HPLC-UV/VIS/ESI-MS(TOF) analysis was carried out with an Alliance 2795 HPLC system (Waters) coupled to a UV/VIS (Waters PDA 2996) detector and a time-of-flight mass spectrometer [MS(TOF); MicrOTOF, Bruker Daltonics, Bremen, Germany] equipped with an electrospray (ESI) source. Two HPLC protocols were used, the one described above and a second one with a different elution program (Elution program 2; Supplementary Table S2) designed to improve the separation of the phenolic compounds of interest. The ESI-MS(TOF) operating conditions and software used were as described in Fourcroy et al. (2014). Mass spectra were acquired in positive and negative ion mode in the range of 50–1000 mass-to-charge ratio (*m/z*) units. The mass axis was calibrated externally and internally using Li-formate adducts [10 mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol]. The internal mass axis calibration was carried out by introducing the calibration solution with a divert valve at the first and last 3 min of each HPLC run. Molecular formulae were assigned based on exact molecular mass with errors <5 ppm (Bristow, 2006). Phenolic standards used are shown in Supplementary Table S3. Concentrations of phenolic compounds were quantified using external calibration with internal standardization with the exception of ferulic acid hexoside and the cleomiscosins. Ferulic acid hexoside was quantified as fraxin because there is no commercially available authenticated standard. The levels of the cleomiscosins are expressed in peak area ratio, relative to the lignan matairesinol used as IS. For quantification, analytes and IS peak areas were obtained from chromatograms extracted at the *m/z* (±0.05) ratios corresponding to $[M+H]^+$ ions, with the exception of glycosides, where the *m/z* ratios corresponding to $[M\text{-hexose}+H]^+$ ions were used.

Phenolic Compounds Analysis by HPLC/ESI-MS(Q-TOF) and by HPLC/ESI-MS(Ion Trap)

Phenolic compounds were also analyzed by HPLC/ESI-MS(Q-TOF) using a 1100 HPLC system (Agilent Technologies) coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF; MicroTOF-Q, Bruker Daltonics) equipped with an ESI source. The HPLC conditions were described in Fourcroy et al. (2014) (see above and Supplementary Table S2). The ESI-MS(Q-TOF) operating conditions were optimized by direct injection of 50 μM solutions of phenolic compound standards at a flow rate of 250 μl h^{-1}. Mass spectra (50–1000 *m/z* range) were acquired in positive ion mode, with capillary and endplate offset voltages of 4.5 and −0.5 kV, respectively, and a collision cell energy of 100–2000 eV. The nebulizer (N_2) gas pressure, drying gas (N_2) flow rate and drying gas temperature were 1.0 bar, 4.0 L min^{-1} and 200°C, respectively. The mass axis was calibrated externally and internally as indicated above for the HPLC/ESI-MS(TOF) analysis. Molecular formulae for the product ions were assigned based on exact molecular mass with errors <5 ppm (Bristow, 2006).

HPLC/ESI-MS(ion trap) analysis was carried out with an Alliance 2795 HPLC system (Waters) coupled to an ion-trap mass spectrometer (HCT Ultra, BrukerDaltonics) equipped with an ESI source. The HPLC conditions were as described in Fourcroy et al. (2014) and Supplementary Table S2 (Elution program 2). ESI-ion trap-MS analysis was carried out in positive and/or negative ion mode, the MS spectra were acquired in the standard mass range mode and the mass axis was externally calibrated with a tuning mix (Agilent). The HCT Ultra was operated with settings shown in Supplementary Table S4. The ions of interest were subjected to collision induced dissociation (CID; using the He background gas present in the trap for 40 ms) to produce a first set of fragment ions, MS/MS or MS^2. Subsequently, some of the fragment ions were isolated and fragmented to give the next set of fragment ions, MS^3 and so on. For each precursor

ion, fragmentation steps were optimized by visualizing fragment intensity changes.

RNA Extraction and Quantitative RT-PCR Analysis

Total RNA was extracted from roots using the RNeasy Plant Mini Kit (Quiagen). One microgram RNA was treated with RQ1 DNase (Promega) before use for reverse transcription (Goscript reverse transcriptase; Promega) with oligo (dT)18 and 0.4 mM dNTPs (Promega). The cDNAs were diluted twice with water, and 1 μl of each cDNA sample was assayed by qRT-PCR in a LightCycler 480 (Roche Applied Science) using Lightcycler 480 SYBR Green master I (Roche Applied Science). Expression levels were calculated relative to the housekeeping gene PP2 (At1g13320) using the ΔΔCT method to determine the relative transcript level. The primers used for qRT-PCR were those described in Fourcroy et al. (2014) and indicated in Supplementary Table S5.

Dissolution of Fe(III)-oxide Using Coumarins

Ten milligrams of poorly crystalline Fe(III)-oxide was incubated (in the dark at 25°C and 300 ppm in a Eppendorf Thermomixer Comfort, Eppendorf AG, Hamburg, Germany) for 6 h with 1.5 ml of an assay solution containing appropriated concentrations (in the range of 0–100 μM) of different coumarins (fraxin, fraxetin, scopoletin, and isofraxidin) and 600 μM of bathophenanthrolinedisulphonate (BPDS) -as Fe(II) trapping agent- and buffered at pH 5.5 (with 5 mM MES-KOH) or pH 7.5 (with 5 mM HEPES-KOH). Afterward, the assay medium was filtered through PVDF 0.22 μm centrifugal filters (Millipore) at 10,000 *g* for 1 min. Absorbance was measured at 535 nm in the filtrates and then the Fe(II) concentration determined as Fe(II)-$BPDS_3$ using an extinction coefficient of 22.14 mM^{-1} cm^{-1}. The filtrates were also measured for total Fe by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7500ce, Santa Clara, CA, USA) after diluting a 50 μl aliquot with 65% ultrapure HNO_3 (TraceSELECT Ultra, Sigma–Aldrich).

Statistical Analyses

Statistical analysis was carried out with SPSS for PC (v.23.0, IBM, Armonk, NY, USA), using ANOVA or non-parametric tests ($p \leq 0.05$), and a Levene test for checking homogeneity of variances. *Post hoc* multiple comparisons of means corresponding to each one the four different treatments were carried out ($p \leq 0.05$) using Duncan test when variances were equal and Games–Howell's test when variances were unequal.

RESULTS

Changes in Leaf Photosynthetic Pigment Concentrations, Fe Contents and Biomass with Fe Deficiency and High pH

Arabidopsis thaliana plants grown for 14 days in zero-Fe nutrient solution, buffered at either pH 5.5 or pH 7.5, had visible symptoms of leaf chlorosis (**Figure 2A**). The Chlorophyll (*Chl*) concentration in young leaves decreased by 56% in response to Fe deficiency, but was unaffected by the nutrient solution pH (**Figure 2B**). The concentrations of other photosynthetic pigments (neoxanthin, violaxanthin, lutein and β-carotene) in young leaves also decreased upon Fe deficiency (in the range of 48–60%) and were unaffected by the plant growth pH (Supplementary Table S6).

Iron deficiency decreased shoot biomass by 32% only when plants were grown at pH 7.5, whereas root biomass did not change significantly (**Figure 2C**). Shoot Fe content decreased significantly with Fe deficiency only in plants grown at pH 5.5 (by 61%; **Figure 2C**), whereas root Fe content was markedly decreased by 92% in plants grown at both pH values (**Figure 2C**). Iron deficiency also affected the contents of other micronutrients in plants, and this occurred mainly in shoots (Supplementary Table S7). The largest change found was a sixfold increase over the control value in the shoot Cu content of plants grown at pH 5.5.

Changes in the Expression of Genes Involved in Fe Root Uptake and the Phenylpropanoid Pathway with Fe Deficiency and High pH

The transcript levels of *IRT1*, *FRO2*, *ABCG37*, *F6'H1*, the caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (*COMT*) and the trans-caffeoyl-CoA 3-*O*-methyltransferase (*CCoAMT*) were assessed by quantitative RT-PCR in control (Fe-sufficient) and Fe-deficient roots from both plants grown at pH 5.5 or at pH 7.5 3 days after treatment onset (**Figure 2D**). Under high Fe supply, the only pH effect observed was for *FRO2*, whose transcript abundance was 12-fold higher in plants grown at pH 7.5 than in those grown at pH 5.5. Under Fe deficiency conditions, *IRT1* and *FRO2* gene expression increased in plants grown both at pH 5.5 and pH 7.5; the increases were ninefold for *IRT1* and 15-fold for *FRO2* in plants grown at pH 5.5, and 20-fold for *IRT1* and 5-fold for *FRO2* in plants grown at pH 7.5. Other genes studied, *ABCG37* and *F6'H1*, also showed increases in their expression in response to Fe deficiency when compared to the Fe-sufficient controls, although they were smaller than those observed for *IRT1* and *FRO2*. The increases in *ABCG37* gene expression were 2-(although this change was not statistically significant) and 4-fold in plants grown at pH 5.5 and pH 7.5, respectively, whereas those of *F6'H1* were 4- and 8-fold in plants grown at pH 5.5 and pH 7.5. On the other hand, *COMT* and *CCoAMT* gene expression in roots was only increased by Fe deficiency at pH 7.5 (twofold).

Arabidopsis Roots Accumulate and Secrete an Array of Fluorescent and Non-fluorescent Phenolic-Type Compounds with Fe Deficiency and High pH

Methanolic extracts of roots of *A. thaliana* plants and their nutrient solutions were analyzed using the reverse phase C_{18} HPLC-based method used in Fourcroy et al. (2014) (Elution

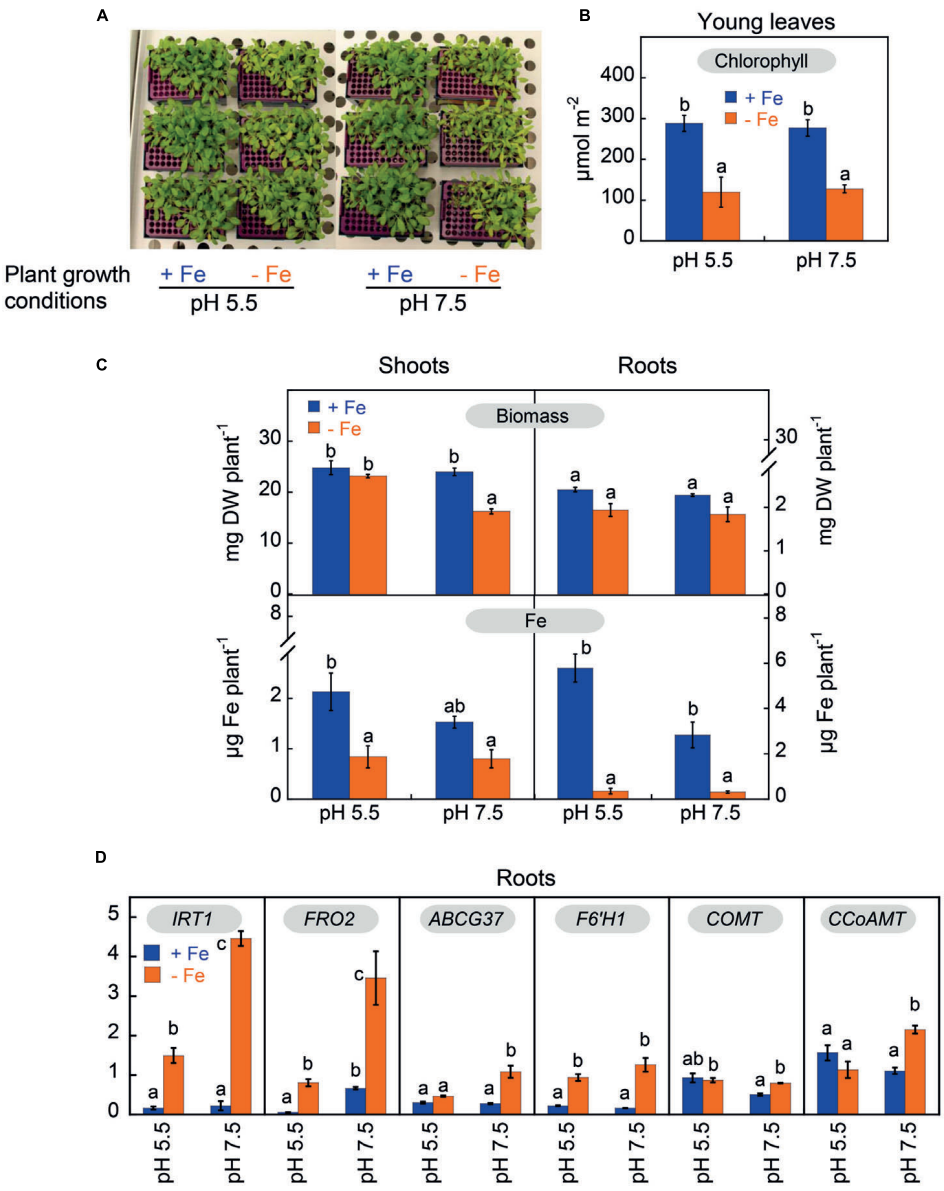

FIGURE 2 | Effects of Fe deficiency and high pH on plant Fe status, root Fe uptake machinery and phenylpropanoid pathway components in *Arabidopsis thaliana*. Plants were pre-grown for 11 days in the presence of 20 μM Fe (III)-EDTA at pH 5.5, and then grown for 14 days in a medium with 0 (−Fe) or 20 μM (+Fe) Fe(III)-EDDHA in nutrient solutions buffered at pH 5.5 (with 5 mM MES-NaOH) or 7.5 (with 5mM HEPES-NaOH). **(A)** Plants at day 14 after imposing treatments. **(B)** Leaf chlorophyll concentration in young leaves of plants at day 14 after imposing treatments; data are means ± SE ($n = 3$) and significant differences among treatments (at $p < 0.05$) are marked with different letters above the columns. **(C)** Dry weights and Fe contents in shoots and roots at day 14 after imposing treatments. Data are means ± SE for biomass ($n = 5$) and for Fe contents ($n = 2$–5), and significant differences among treatments (at $p < 0.05$) are marked with different letters above the columns. **(D)** Abundance of *IRT1*, *FRO2*, *ABCG37* (*PDR9*), *F6'H1*, *COMT* and *CCoAMT* transcripts in roots at day 3 after imposing treatments. RNAs were extracted from roots and analyzed by qRT-PCR, using PP2 (At1g13320) as housekeeping gene. The ΔΔCT method was used to determine the relative transcript level. Data are means ± SE ($n = 3$–5). For each gene, significant differences among treatments (at $p < 0.05$) are marked with different letters above the columns.

program 1), using both UV/VIS detection in the range 200–600 nm and fluorescence detection at λ_{exc} 365 and λ_{em} 460 nm (only the latter was used in the original study). Fluorescence alone cannot detect all phenolic compounds, since many of them emit little or no fluorescence. However, all phenolic compounds absorb light in the UV region; coumarins, their derivatives and precursors (e.g., ferulic and other cinnamic acids) have absorption maxima in the range 290–330 nm.

This is illustrated by the absorbance chromatograms of *A. thaliana* root extracts and growth media at 320 nm, which show many additional peaks to those found in fluorescence chromatograms obtained with the same samples (**Figure 3**). Each of the peaks in the chromatogram may contain one or more compounds (either fluorescent and/or non-fluorescent; see sections below for identification). In the control root extracts, fluorescence chromatograms showed only two peaks at approximately 10 and 15 min, whereas the absorbance chromatograms show several small peaks at two retention time (RT) ranges, 9–16 and 19–24 min, as well as a large peak at approximately 18 min (**Figure 3**). In the root extracts from Fe-deficient plants, increases were found in fluorescence in the area of the 15 min peak and in absorbance in the 18 min peak. In the control nutrient solution, the fluorescence chromatogram showed peaks at 10, 15, and 19 min, whereas the absorbance chromatogram showed peaks at 18 and 19 min (**Figure 3**). Iron deficiency caused large increases in the areas of all these peaks, with further absorbance ones appearing at 13, 14, 15, 16, and 17 min. This shows that Fe deficiency induces the synthesis, root accumulation and secretion to the growth media not only of fluorescent coumarins, as described by Fourcroy et al. (2014) and Schmid et al. (2014), but also of a number of previously unreported non-fluorescent phenolic compounds.

Identification of Phenolic Compounds Induced by Fe Deficiency as Coumarins, Coumarin Precursors and Coumarinolignans

To identify the compounds found in the *A. thaliana* root extracts and growth media, samples were analyzed using four different HPLC-UV/VIS/ESI-MS(TOF) protocols, including two Elution programs (1 and 2; Supplementary Table S2) and two electrospray (ESI) ionization modes (positive and negative). The newly designed Elution program 2 led to a better separation of phenolic compounds than that obtained with the original Elution program 1 used in Fourcroy et al. (2014). With the new elution program, RTs for a selected set of phenolics standards ranged from 8.4 (for esculin, the glucoside form of the coumarin esculetin) to 51.7 min (for the flavone apigenin) (Supplementary Figures S1 and S2). These HPLC/ESI-MS(TOF) analyses provided highly accurate (error below 5 ppm) measurements of the mass-to-charge (*m/z*) ratio of the detected ions, therefore allowing for accurate elemental formulae assignments (Bristow, 2006).

Raw MS(TOF) datasets (time, *m/z* and ion intensity) from the root extracts and nutrient solutions from Fe-deficient and Fe-sufficient plants were first analyzed with the DISSECT algorithm (Data Analysis 4.0; Bruker) to obtain mass spectral features attributable to individual compounds. From a total of approximately 180 possible mass spectral features analyzed per run and sample, only 18 complied with the following two requirements: (i) occurring at chromatographic RTs where absorbance at 320 nm was observed, and (ii) showing peak area increases (or appearing) with Fe-deficiency. Then, associated ions coming from adducts (with salts or solvents), dimers and trimers were discarded (with some exceptions, see below), and the ion chromatograms of all major remaining ions (including non-fragmented ones as well as fragment ions produced in the ESI source) were extracted with a precision of ±0.02 *m/z*. From these, we selected major ions showing large changes in peak areas in response to Fe deficiency, without considering fragments and minor ions. The localization in the chromatograms of the 18 selected compounds is depicted in **Figure 3**, and the RT, exact *m/z* and assigned elemental formulae are shown in **Table 1**. These 18 compounds were never detected in nutrient solutions of pots without plants, and include some coumarins already known to occur and others not previously reported, as explained in detail below.

Coumarins and Related Compounds Previously Reported in *A. thaliana* upon Fe-Deficiency

As expected, some compounds (five out of 18) have RTs and *m/z* values matching with those of coumarins previously found in roots and exudates from Fe-deficient *A. thaliana* plants (Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014). These include compounds *1, 7–9,* and *11* (**Figure 3**; **Table 1**), and were assigned to scopoletin hexoside, fraxetin, scopoletin, isofraxidin and fraxinol (an isofraxidin isomer), respectively (Supplementary Table S1). These annotations were further confirmed using the RT and *m/z* values of standards (**Table 1** vs. **Table 2**). A sixth compound, *2*, was assigned to ferulic acid hexoside based on the presence of a major ion at *m/z* 195.0656 in its positive MS(TOF) spectrum, which is consistent with the elemental formula of ferulic acid $[M+H]^+$ ion (**Table 2**) and with the neutral loss of a hexosyl moiety (162.0528 Da, $C_6H_{10}O_5$) from the $[M+H]^+$ ion (with an absolute error of 1.2 ppm). We could not confirm the identity using a ferulic acid hexoside standard because to the best of our knowledge no such standard is commercially available.

The remaining 12 compounds were subjected to further MS-based analyses to obtain structural information. First, low resolution HPLC/ESI-MS(ion trap) analyses were carried out, including MS^2 and/or MS^3 experiments with the $[M+H]^+$ or $[M-H]^-$ ions.

Coumarins and Coumarin-Precursor Hexosides Not Previously Reported in *Arabidopsis* upon Fe-Deficiency

Three of the compounds (*10*, *12,* and *13*) were identified as ferulic acid, coniferyl aldehyde and sinapyl aldehyde (three phenylpropanoid precursors; **Figure 1B**), respectively, by comparing the MS spectra of the analytes and those of standards: there was a good match of the RT values and exact *m/z* ratios of the $[M+H]^+$ and $[M-H]^-$ ions (**Tables 1** and **2**) as well as of the MS^2 spectra of the $[M+H]^+$ ions (**Tables 2** and **3**).

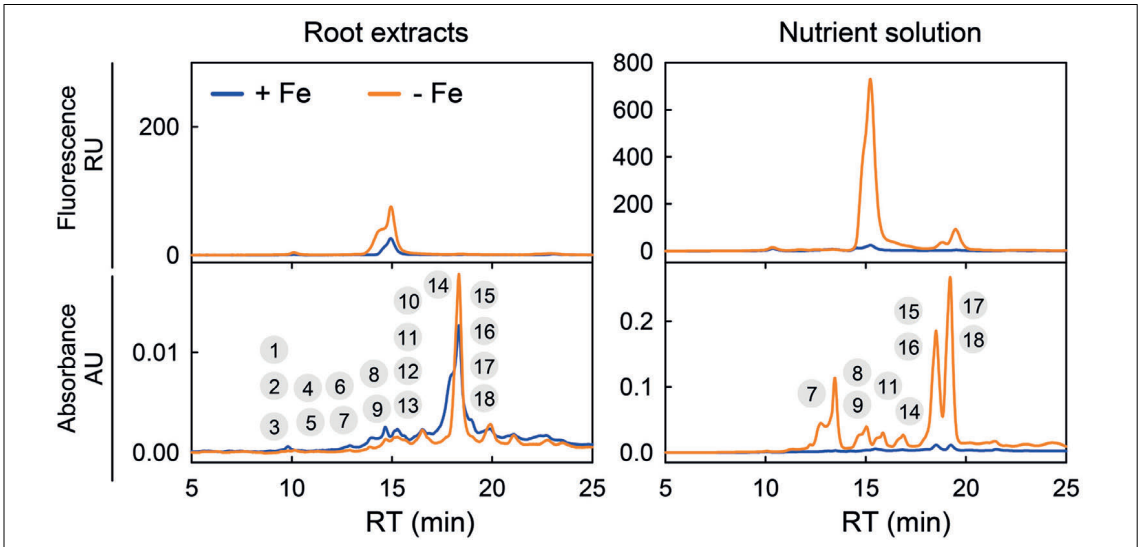

FIGURE 3 | Chromatographic separation of a range of phenolic-type compounds produced in response to Fe deficiency by *Arabidopsis thaliana* roots. Typical fluorescence (at λ_{exc} 365 and λ_{em} 460 nm) and absorbance (at 320 nm) chromatograms for root and growth media extracts from plants grown as described in Fourcroy et al. (2014): plants were pre-grown for 29 days in the presence of 45 μM Fe (III)-EDTA at pH 5.5, and then grown for 7 days in a medium with 0 (−Fe) or 45 μM Fe (III)-EDTA (+Fe) (the pH was not readjusted to 5.5, with the final pH being *c.* 7.0 in all pots). Chromatograms were obtained using Elution program 1. The encircled numbers above each peak correspond to the phenolic compounds listed in **Table 1**. RU, relative units, AU, absorbance units, and RT, retention time.

Four more compounds (*3–6*) were first confirmed to be hexoside-type compounds from the RT, exact *m/z* values and MS^2 spectra of the $[M\text{-}H]^-$ ions. The RT values of these compounds (12.3–14.9 min) were close to those of known coumarin glucosides (10.3 and 13.0 min for scopolin and fraxin, respectively), and lower than those of coumarin aglycones (16.4–25.1 min for fraxetin, scopoletin, isofraxidin and fraxinol), phenylpropanoids (e.g., 23.0 and 25.1 min for ferulic acid and sinapyl aldehyde), and glycoside and aglycone forms of other phenolics (e.g., 27–52 min for flavonoids, stilbenes and lignans) (Supplementary Figures S1 and S2). Therefore, the RTs indicate that compounds *3–6* are likely to be polar (i.e., hexoside) forms of coumarins and/or phenylpropanoids. Furthermore, in the MS(TOF) spectra, ions (positive/negative) at *m/z* 179.0707/177.0544, 209.0450/207.0289, 223.0600/221.0447 and 209.0801/207.0648 for *3*, *4*, *5*, and *6*, respectively, were consistent with the loss of a hexosyl moiety (162.05 Da) from their corresponding $[M+H]^+/[M\text{-}H]^-$ ions (see *m/z* values in **Table 1**). This was confirmed using the low resolution MS^2 spectra obtained with the ion trap: major fragment ions (100% relative intensity at *m/z* 177, 207, 221 and 207 in the MS^2 spectra of *3–6*, respectively; **Table 3**) corresponded to the $[M\text{-}H]^-$ ions (*m/z* 339, 369, 383 and 369 for *3*, *4*, *5*, and *6*, respectively) after a mass loss of 162 Da. The same mass loss was also observed in the MS^2 spectra of authenticated standards of the coumarin glucosides scopolin and fraxin described above, with major ions at *m/z* 193/191 (scopolin) and 209/207 (fraxin), corresponding with the *m/z* of their aglycones, scopoletin and fraxetin, respectively (**Table 2**). The rest of ions in the MS^2 spectra of compounds *3–6*, scopolin and fraxin showed significantly lower relative intensities (<40%), indicating the hexosyl loss is favored.

The aglycon moieties of compounds *3–6* were identified taking advantage of having the dehexosylated ions in the MS(TOF) spectra and also carrying out low resolution MS^3 experiments on the ion trap. First, from the positive and negative MS(TOF) spectra, the *m/z* values for dehexosylated ions (see above) of *3*, *4*, *5*, and *6* were assigned to the elemental formulae $C_{10}H_{10}O_3$, $C_{10}H_8O_5$, $C_{11}H_{10}O_5$ and $C_{11}H_{12}O_4$, respectively (with absolute errors <4 ppm). Two of these elemental formulae, $C_{10}H_{10}O_3$ and $C_{11}H_{12}O_4$, were consistent with coniferyl and sinapyl aldehydes, involved in coumarin synthesis (Kai et al., 2008) (**Table 2**), whereas the other two, $C_{10}H_8O_5$ and $C_{11}H_{10}O_5$, were consistent with two coumarins already identified in the samples (compounds *7* and *9*, respectively) (**Table 1**). Finally, compounds *3–6* were confirmed as the hexoside forms of coniferyl aldehyde, fraxetin, isofraxidin and sinapyl aldehyde, respectively (**Table 1**) from the good fit between the MS^3 ion trap spectra of *3-6* (339→177, 369→207, 383→221 and 369→207, respectively) (**Table 3**) and the MS^2 spectra of the corresponding aglycone standards (**Table 2**).

Coumarinolignans: Newly Identified Compounds Synthesized in Response to Fe-Deficiency

The last five compounds (*14–18* in **Table 1**) are very hydrophobic, since they elute later (RTs 31–39 min) than compounds *1–13* (RTs

TABLE 1 | Phenolic compounds secreted and accumulated by *Arabidopsis thaliana* roots in response to Fe deficiency: retention times (RT), exact mass-to-charge ratios (*m/z*), molecular formulae and error *m/z* (in ppm).

Compound #	RT (min) program 1	RT (min) program 2	Measured *m/z*	Molecular formula	Calculated *m/z*	Error *m/z* (ppm)	Annotation
1	9.8	10.3	355.1028	$C_{16}H_{19}O_9^+$	355.1024	1.1	7-hydroxy-6-methoxycoumarin hexoside
			353.0877	$C_{16}H_{17}O_9^-$	353.0867	2.8	(scopolin, scopoletin hexoside)
2	10.0	10.6	357.1182	$C_{16}H_{21}O_9^+$	357.1180	0.6	Ferulic acid hexoside
			355.1030	$C_{16}H_{19}O_9^-$	355.1024	1.7	
3	10.4	12.3	363.1055	$C_{16}H_{20}O_8Na^+$	363.1050	1.4	Coniferyl aldehyde hexoside
			339.1079	$C_{16}H_{19}O_8^-$	339.1074	−1.5	
4	11.3	13.0	371.0975	$C_{16}H_{19}O_{10}^+$	371.0973	0.5	7,8-dihydroxy-6-methoxycoumarin hexoside
			369.0827	$C_{16}H_{17}O_{10}^-$	369.0816	3.0	(fraxetin hexoside)
5	12.1	14.7	407.0949	$C_{17}H_{20}O_{10}Na^+$	407.0949	0.0	7-hydroxy-6,8-dimethoxycoumarin hexoside
			383.0992	$C_{17}H_{19}O_{10}^-$	383.0973	5.0	(isofraxidin hexoside)
6	12.3	14.9	409.0893	$C_{17}H_{22}O_9K^+$	409.0895	−0.5	Sinapyl aldehyde hexoside
			369.1194	$C_{17}H_{21}O_9^-$	369.1180	3.8	
7	13.0	16.4	209.0446	$C_{10}H_9O_5^+$	209.0445	0.5	7,8-dihydroxy-6-methoxycoumarin (fraxetin)
			207.0282	$C_{10}H_7O_5^-$	207.0288	−2.9	
8	14.5	20.0	193.0502	$C_{10}H_9O_4^+$	193.0495	3.6	7-hydroxy-6-methoxycoumarin (scopoletin)
			191.0341	$C_{10}H_7O_4^-$	191.0339	1.0	
9	14.8	21.6	223.0604	$C_{11}H_{11}O_5^+$	223.0601	1.3	7-hydroxy-6,8-dimethoxycoumarin (isofraxidin)
			221.0442	$C_{11}H_9O_5^-$	221.0445	−1.4	
10	15.6	23.0	195.0649	$C_{10}H_{11}O_4^+$	195.0652	−1.5	Ferulic acid
			193.0504	$C_{10}H_9O_4^-$	193.0495	4.7	
11	15.6	23.8	223.0604	$C_{11}H_{11}O_5^+$	223.0601	1.3	6-hydroxy-5,7-dimethoxycoumarin (fraxinol)
			221.0442	$C_{11}H_9O_5^-$	221.0445	−1.4	
12	16.1	24.6	179.0708	$C_{10}H_{11}O_3^+$	179.0703	2.7	Conyferyl aldehyde
			177.0551	$C_{10}H_9O_3^-$	177.0546	2.8	
13	16.5	25.1	209.0809	$C_{11}H_{13}O_4^+$	209.0808	0.5	Sinapyl aldehyde
			207.0660	$C_{11}H_{11}O_4^-$	207.0652	3.9	
14	16.5	30.7	403.1018	$C_{20}H_{19}O_9^+$	403.1024	−1.5	5′-hydroxycleomiscosins A and/or B
			401.0877	$C_{20}H_{17}O_9^-$	401.0867	2.5	
15	18.0	35.5	417.1175	$C_{21}H_{21}O_9^+$	417.1180	−1.2	Cleomiscosin D
			415.1022	$C_{21}H_{19}O_9^-$	415.1024	−0.5	
16	18.5	37.0	417.1173	$C_{21}H_{21}O_9^+$	417.1180	−1.7	Cleomiscosin C
			415.1022	$C_{21}H_{19}O_9^-$	415.1024	−0.5	
17	18.5	37.0	387.1073	$C_{20}H_{19}O_8^+$	387.1074	−0.3	Cleomiscosin B
			385.0930	$C_{20}H_{17}O_8^-$	385.0918	3.1	
18	19.0	38.6	387.1073	$C_{20}H_{19}O_8^+$	387.1074	−0.2	Cleomiscosin A
			385.0922	$C_{20}H_{17}O_8^-$	385.0918	1.0	

The m/z ratios for $[M+H]^+$ and $[M-H]^-$ were determined from the HPLC/ESI-MS(TOF) data obtained in positive and negative mode, respectively. For compounds 3, 5, and 6 in positive mode, the m/z shown are those measured for the Na ($[M+Na]^+$) or K ($[M+K]^+$) adducts, because they were more intense than those for $[M+H]^+$. Common names for coumarins are also indicated in brackets.

10–25 min), and have *m/z* values supporting elemental formulae with a high number of C atoms (20–21 vs. 10–17 for compounds *1–13*). In fact, the RTs of *14–18* are in line with those of phenolics bearing either C_{15} (C_6-C_3-C_6; as in flavonoids and stilbens) or C_{18} (C_6-C_3-C_3-C_6; as in lignans) skeletons (27–52 min; Supplementary Figures S1 and S2), whereas compounds *7–13* (coumarins and phenylpropanoids) share a C_9 (C_3–C_6) skeleton and compounds *1–6* (hexose conjugates of *7–13*) share a C_{15} (C_3-C_6-C_6) skeleton (**Table 1**).

The MS(TOF) spectra show that compounds *15–18* are two pairs of isomers, with elemental formulae $C_{21}H_{20}O_9$ for *15–16* and $C_{20}H_{18}O_8$ for *17–18*, with the difference between formulae being consistent with a single methoxy ($-OCH_3$) group. The elemental formula of compound *14*, $C_{20}H_{18}O_9$, is consistent with the addition of both a hydroxyl ($-OH$) group to *17–18* or the addition of a methyl ($-CH_3$) group to *15–16*. The presence of these structural differences are common among phenolics, since part of the phenylpropanoid biosynthesis proceeds *via* a series of ring hydroxylations and *O*-methylations. The low resolution MS^2 spectra from *14* to *18* (**Figure 4A**) indicate that these five compounds have highly related chemical structures: (i) the spectra of *15–16* show the same ions with only some differences in their relative intensity, and the same was also observed for *17–18*; (ii) most of the ions in the *15–18* spectra were either common (*m/z* 263, 233, 209, 161) or consistent with common mass losses from the $[M+H]^+$ ion (e.g., *m/z* 367 and 337 in the *15–16* and *17–18* MS^2 spectra, corresponding to a mass loss of 50 Da; Supplementary Table S8), and (iii) the spectrum of *14*

TABLE 2 | Phenolic compound standards used for identification purposes: retention times (RT), exact mass-to-charge ratios (*m/z*), molecular formulae and error *m/z* (in ppm).

Name	RT (min) program 2	Measured *m/z*	Molecular formula	Calculated *m/z*	Error *m/z* (ppm)	ESI-MS^n *m/z* (Relative intensity, in %)
7-hydroxy-6-methoxycoumarin 7-glucoside (scopolin, scopoletin 7-*O*-glucoside)	10.3	355.1021	$C_{16}H_{19}O_9^+$	355.1024	−0.8	MS^2 [355]: 337 (11), 245 (3), **193 (100)**, 149 (1), 165 (1), 133 (12), 105 (5) MS^3 [355→193]: 178 (16), 165 (21), 149 (11), 137 (6), **133 (100)**
		353.0876	$C_{16}H_{17}O_9^-$	353.0867	2.5	MS^2 [353]:**191 (100)**, 176 (9) MS^3 [353→191]: **176 (100)**
7,8-dihydroxy-6-methoxycoumarin 8-glucoside (fraxin)	13.0	371.0956	$C_{16}H_{19}O_{10}^+$	371.0973	−4.6	MS^2 [371]: 368 (11), 362 (13), 357 (12), 355 (66), 353 (35), 340 (13), 327 (23), 326 (25), 325 (195), 309 (15), 300 (17), 288 (10), 269 (19), 268 (11), 265 (11), 262 (14), 261 (17), 221 (12), **209 (100)**, 187 (19), 177 (14), 170 (19), 156 (15), 133 (24) MS^3 [371→209]: **194 (100)**
		369.0825	$C_{16}H_{17}O_{10}^-$	369.0816	2.4	MS^2 [369]: **207 (100)**, 192 (20) MS^3 [369→207]: **192 (100)**, 163 (0.2)
7,8-dihydroxy-6-methoxycoumarin (fraxetin)	16.4	209.0444	$C_{10}H_9O_5^+$	209.0445	−0.5	MS^2 [209]: 194 (31), 181 (52), 177 (15), 165 (7), 163 (80), 153(9), **149 (100)**, 135 (13), 107 (18)
		207.0291	$C_{10}H_7O_5^-$	207.0288	1.4	MS^2 [207]: **192 (100)**, 163 (0.3)
7-hydroxy-6-methoxycoumarin (scopoletin)	20.0	193.0494	$C_{10}H_9O_4^+$	193.0495	−0.5	MS^2 [193]: 178 (8), 165 (31), 149 (12), 137 (12), **133 (100)**, 117 (2), 105 (3), 89 (3), 63 (6)
		191.0346	$C_{10}H_7O_4^-$	191.0339	3.7	MS^2 [191]: **176 (100)**, 148 (0.4)
7-hydroxy-6,8-dimethoxycoumarin (isofraxidin)	21.6	223.0594	$C_{11}H_{11}O_5^+$	223.0601	−3.1	MS^2 [223]: **208 (100)**, 207 (7), 195 (14), 191 (8), 190 (49), 179 (7), 163 (72), 162 (6), 135 (19) 107 (45)
		221.0443	$C_{11}H_9O_5^-$	221.0445	−0.9	MS^2 [221]: **206 (100)**, 209 (0.5), 191 (5), 162 (0.8)
Ferulic acid	23.0	195.0657	$C_{10}H_{11}O_4^+$	195.0652	2.6	MS^2 [195]: **177 (100)**, 153 (4), 145 (3)
		193.0504	$C_{10}H_9O_4^-$	193.0495	4.7	MS^2 [193]: 178 (70), **149 (100)**, 139 (80)
6-hydroxy-5,7-dimethoxycoumarin (fraxinol)	23.8	223.0594	$C_{11}H_{11}O_5^+$	223.0601	−3.1	MS^2 [223]: **208 (100)**, 195 (11), 190 (40), 179 (6), 163 (54), 135 (19), 107 (39), 91 (4)
		221.0440	$C_{11}H_9O_5^-$	221.0444	−1.8	MS^2 [221]: **206 (100)**, 191 (5), 209 (0.5), 162 (0.2)
Coniferyl aldehyde	24.6	179.0706	$C_{10}H_{11}O_3^+$	179.0703	1.7	MS^2 [179]: **161 (100)**, 147 (97), 133 (18), 119 (7), 105 (10)
		177.0554	$C_{10}H_9O_3^-$	177.0546	4.5	MS^2 [177]: **162 (100)**, 163 (1), 158 (0.3)
Sinapyl aldehyde	25.1	209.0810	$C_{11}H_{13}O_4^+$	209.0808	1.0	MS^2 [209]: 191 (47), 181 (10), **177 (100)**, 153 (7), 149 (20), 145 (15), 131 (12), 121 (17), 103 (5)
		207.0662	$C_{11}H_{11}O_4^-$	207.0652	4.8	MS^2 [207]: **192 (100)**, 191 (0.3), 177 (2), 147 (0.2), 133 (0.2)

The m/z ratios of parent and fragment ions were determined from the data in the HPLC/ESI-MS(TOF) and HPLC/ESI-MS(ion trap) chromatograms, respectively, working in both positive and negative mode. Common names for coumarins and their glucosides are indicated in brackets. The parent ion m/z ratios correspond to $[M+H]^+$ and $[M-H]^-$. The major ion of the MS^2 and MS^3 spectra is indicated in bold.

also has some of these features, including an ion at *m/z* 209 and a mass loss of 30 Da from the $[M+H]^+$ ion (Supplementary Table S8). When the MS^2 spectra of *14–18* were obtained on a high resolution Q-TOF mass analyzer, which allows for an accurate mass determination of fragment ions, all spectra showed a common fragment ion at *m/z* 209.0435, consistent with the elemental formula $C_{10}H_9O_5^+$ (with an error of −4.7 ppm) (Supplementary Figure S3) of the dihydroxymethoxycoumarin fraxetin (compound *7*). The presence of a fraxetin moiety in compounds *14–18* was further confirmed by their MS^3 spectra (403→209, 417→209, 417→209, 387→209 and 387→209 for *14*, *15*, *16*, *17* and *18*, respectively; **Figure 4B**), which match perfectly with the fraxetin MS^2 spectrum.

Among the plant-derived fraxetin derivatives known so far (Begum et al., 2010; Zhang et al., 2014), six coumarinolignans have elemental formulae consistent with those of compounds *14–18*, including cleomiscosins A, B, C (also known as aquillochin) and D, first isolated and identified in seeds of *Cleome viscosa* (a common weed of the *Capparidaceae* family), and 5′-hydroxycleomiscosins A (also known as 5′-demethylaquillochin) and B, first isolated from *Mallotus apelta* roots and *Eurycorymbus cavaleriei* twigs, respectively. Cleomiscosins C and D (regioisomers -also called constitutional isomers- arising from the fusion of fraxetin and the monolignol sinapyl alcohol through a dioxane bridge; **Figure 1C**) have a formula identical to that of *15–16* ($C_{21}H_{20}O_9$), cleomiscosins A and B (regioisomers arising from the fusion of fraxetin and the monolignol coniferyl alcohol through a dioxane bridge; **Figure 1C**) have a formula identical to that of *17-18* ($C_{20}H_{18}O_8$), whereas 5′-hydroxycleomiscosins A and B (regioisomers arising

TABLE 3 | MS/MS data for some of the compounds secreted and accumulated by *Arabidopsis thaliana* roots in response to Fe deficiency: *m/z* ratios of the fragment ions and their relative intensity.

Compound #	Annotation	Parent ion *m/z*	Ion type	ESI-MS^n *m/z* (Relative intensity, in %)
3	Coniferylaldehyde hexoside	339.1	$[M-H]^-$	MS^2 [339]: 295 (6), 275 (8), 250 (6), 249 (3), 188 (3), **177 (100)**, 162 (3) MS^3 [339→177]: **162 (100)**
4	7,8-dihydroxy-6-methoxycoumarin hexoside (fraxetin hexoside)	369.1	$[M-H]^-$	MS^2 [369]: 325 (7), 323 (5), 223 (11), 215 (8), **207 (100)**, 193 (5), 192 (20) MS^3 [369→207]: **192 (100)**
5	7-hydroxy-6,8-dimethoxycoumarin hexoside (isofraxidin hexoside)	383.1	$[M-H]^-$	MS^2 [383]: 365 (13), 347 (24), 341 (12), 339 (10), 337 (22), 323 (24), 322 (18), 303 (14), 270 (20), 268 (25), 266 (18), 252 (9), 251 (30), **221 (100)**, 215 (38), 207 (7), 206 (11), 203 (11), 199 (15), 187 (8), 177 (20), 173(8), 156 (11), 131 (17), 129 (30), 125 (6), 114 (24) MS^3 [383→221]: **206 (100)**
6	Sinapyl aldehyde hexoside	369.1	$[M-H]^-$	MS^2 [369]: 351 (33), 325 (11), 289 (10), 254 (5), 253 (6), 246 (11), 245 (8), 239 (9), 237 (11), 217 (6), **207 (100)**, 192 (18), 159 (11), 128 (10) MS^3 [369→207]: **192 (100)**
10	Ferulic acid	193.1	$[M-H]^-$	MS^2 [193]: 178 (70), **149 (100)**, 134 (72)
12	Coniferyl aldehyde	179.1	$[M+H]^+$	MS^2 [179]: 161 (86), **147 (100)**, 133 (17), 119 (10), 105 (8)
13	Sinapyl aldehyde	209.1	$[M+H]^+$	MS^2 [209]: 191 (41), 181 (17), **177 (100)**, 149 (22), 145 (13), 131 (5), 121 (18)

*Numbers in italics (Compound #) refer to the labels used for each compound in **Table 1**. All data were taken from the HPLC-ESI-MS/MS(ion trap) analysis. The major ion of the MS^2 and MS^3 spectra is also indicated in bold.*

from the fusion of fraxetin and the monolignol hydroxyconiferyl alcohol, Cheng and Chen, 2000, **Figure 1C**), have a formula identical to that of compound *14* ($C_{20}H_{18}O_9$). The structural differences among these coumarinolignans -corresponding to the monolignol moiety (**Figure 1B**)- are identical to those found among the elemental formulae of *14–18*: (i) a methoxy group differentiates coniferyl from sinapyl alcohols and the elemental formula of *17–18* from that of *15–16*; (ii) a hydroxyl group differentiates hydroxyconiferyl from coniferyl alcohols and the elemental formula of *14* from that of *17–18*; and (iii) a methyl group differentiates hydroxyconiferyl and sinapyl alcohols and the formula of *14* from those of *15–16*.

To confirm the identification of *15–18* as cleomiscosins, we isolated coumarinolignans from *C. viscosa* seeds. The seed isolate was analyzed by both HPLC-UV/VIS/ESI-MS(TOF) and HPLC/ESI-MS(ion trap) using Elution program 2 and positive ESI ionization. The HPLC/ESI-MS(TOF) chromatogram for *m/z* 417.12 ± 0.02, corresponding to the cleomiscosins C and D $[M+H]^+$ ions, showed only two peaks, at 35.4 and 37.0 min, matching with the RTs of *15* and *16* (**Figure 4C**; **Table 1**). Similarly, the HPLC/ESI-MS(TOF) chromatogram for *m/z* 387.11 ± 0.02, corresponding to the cleomiscosins A and B $[M+H]^+$ ions, showed only two peaks, at 37.0 and 38.4 min, matching with the RTs of *17–18* (**Figure 4C**; **Table 1**). Peaks were assigned to cleomiscosin isomers according to the elution order reported in the literature (Chattopadhyay et al., 2008; Kaur et al., 2010). These annotations were confirmed by the full match between the MS^2 spectra of the cleomiscosins D, C, B, and A, and those of compounds *15*, *16*, *17* and *18*, respectively (**Figure 4C**). Compound *14* eluted at shorter times than the cleomiscosins (30.7 vs. 35.5–38.6 min), as expected from the structural differences between 5′-hydroxycleomiscosin A and B and cleomiscosins (see above). Furthermore, compound *14* shares elemental formula and the presence of a fraxetin moiety with 5′-hydroxycleomiscosins A and B, and its MS^2 spectrum showed a loss of 18 Da from the $[M+H]^+$ ion (**Figure 4B**; Supplementary Table S8), which was previously reported for 5′-hydroxycleomiscosin A (Cheng and Chen, 2000) but does not occur in cleomiscosins. Therefore, *14* was putatively annotated as 5′-hydroxycleomiscosin A and/or B (**Table 1**).

Coumarin and Coumarinolignan Concentrations in Root Extracts

Quantification of phenolic compounds was carried out using the $[M+H]^+$ and $[M\text{-hexoside}+H]^+$ signals in the HPLC/ESI-MS(TOF). Coumarins and their hexosides were quantified using authenticated standards, whereas coumarinolignan concentrations were estimated using peak/area ratios relative to that of the IS lignan matairesinol (**Figure 1D**), because of the lack of commercially available authenticated standards.

The phenolic compound profiles in root extracts included coumarins and coumarinolignans, and were markedly dependent on the plant growth pH (**Figure 5**); no phenolics of the flavonoid and stilbene families were found. Under sufficient Fe supply, root extracts from plants grown at pH 5.5 had mainly scopoletin hexoside (scopolin) and its aglycone (scopoletin) as well as the coumarin precursor hexoside of ferulic acid. When Fe-sufficient plants were grown at pH 7.5, no significant changes were found for ferulic acid hexoside, scopolin, scopoletin and fraxetin and isofraxidin hexosides, and the coumarinolignans cleomiscosins A, B, C, and D, whereas other coumarins increased (including fraxetin and isofraxidin).

Iron deficiency changed markedly the coumarin/-coumarinolignan profiles in root extracts (**Figure 5**). In plants grown at pH 5.5 the profiles were similar under Fe deficiency or sufficiency conditions, with moderate increases

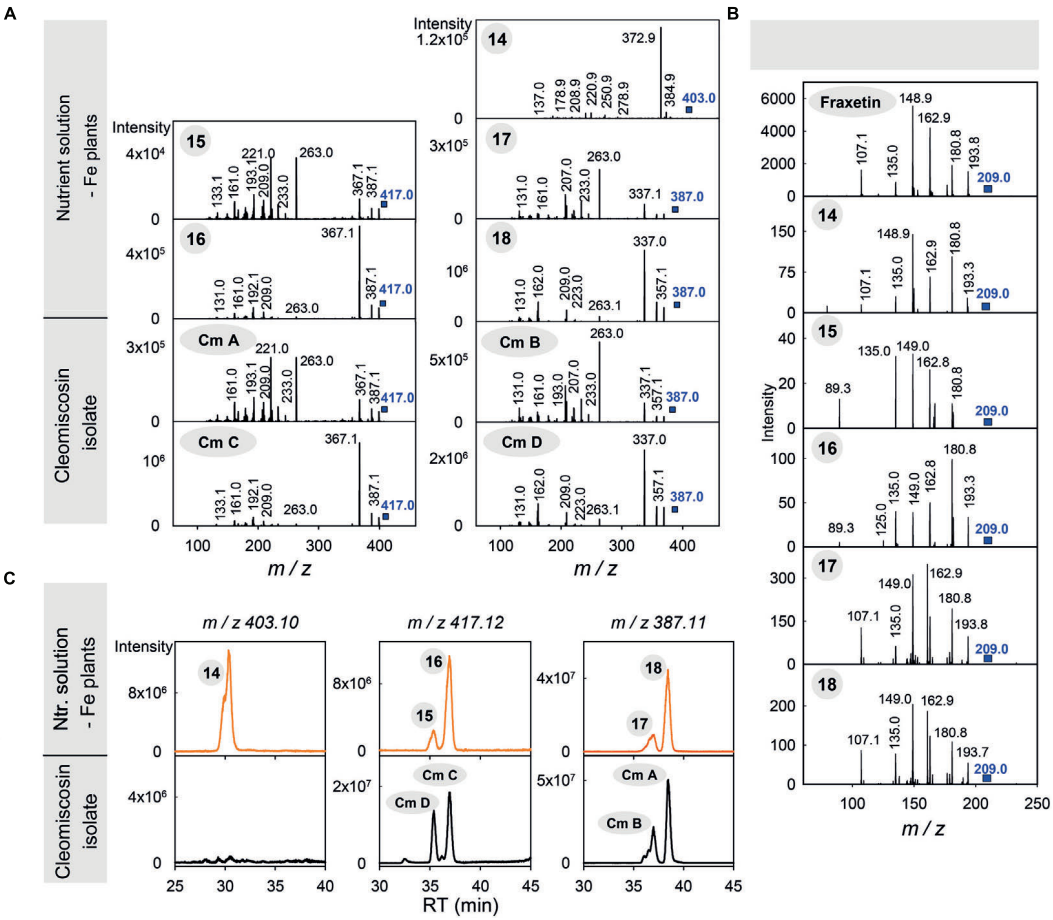

FIGURE 4 | Identification of compounds *14–18,* produced by Fe-deficient *Arabidopsis thaliana* roots, as coumarinolignans derived from fraxetin. **(A)** MS^2 spectra of compounds *14–18* and the cleomiscosins A (Cm A), B (Cm B), C (Cm C) and D (Cm D) isolated from *Cleome viscosa* seeds. **(B)** MS^2 spectra of fraxetin and MS^3 spectra of *m/z* 209 ion from the corresponding $[M+H]^+$ ions of compounds *14–18*. Spectra were obtained from the HPLC/ESI-MS(ion trap) analyses of growth media extracts from Fe-deficient plants and a cleomiscosin isolate. **(C)** Typical HPLC-ESI-MS(TOF) chromatograms for growth media extracts from Fe-deficient plants and for the cleomiscosin isolate, extracted at *m/z* 403.10, 417.12 and 387.11 and with a precision of ± 0.02 *m/z* units. The encircled numbers in the spectra and above each chromatographic peak correspond to the phenolic compounds listed in **Table 1**.

(not always significant) in fraxetin and isofraxidin hexosides and their aglycones (fraxetin, isofraxidin and fraxinol), as well as of the cleomiscosins A, B, C and D. However, in plants grown at pH 7.5 Fe deficiency caused a marked increase of all coumarin hexosides, their aglycones and all coumarinolignans. When compared to their concentration in Fe-sufficient plants at pH 7.5, the largest increase was 18-fold for cleomiscosin D, followed by 13-fold for isofraxidin, 12-fold for fraxinol and the cleomiscosins A, B, and C, 9-fold for the hexoside of isofraxidin, 7-fold for the hexoside of fraxetin and the aglycone fraxetin, 5-fold for scopoletin, and 2-fold for both scopolin and ferulic acid hexoside.

The most abundant coumarin in root extracts, irrespective of the growth conditions, was scopoletin (**Figure 6A**). Summing up the two forms detected, the hexoside and aglycone, scopoletin was 90–100% of the total coumarins, depending on the root conditions, with the aglycone form being always predominant (85–93%) (Supplementary Figure S4B). In the case of fraxetin, the aglycone was also the predominant form (at least 73–76%) in root extracts from plants grown at pH 7.5, whereas in plants grown in absence of Fe at pH 5.5, only 24% of the total fraxetin occurred in the aglycone form. In the case of isofraxidin the hexoside form was predominant, with the aglycone accounting for 23–46% of

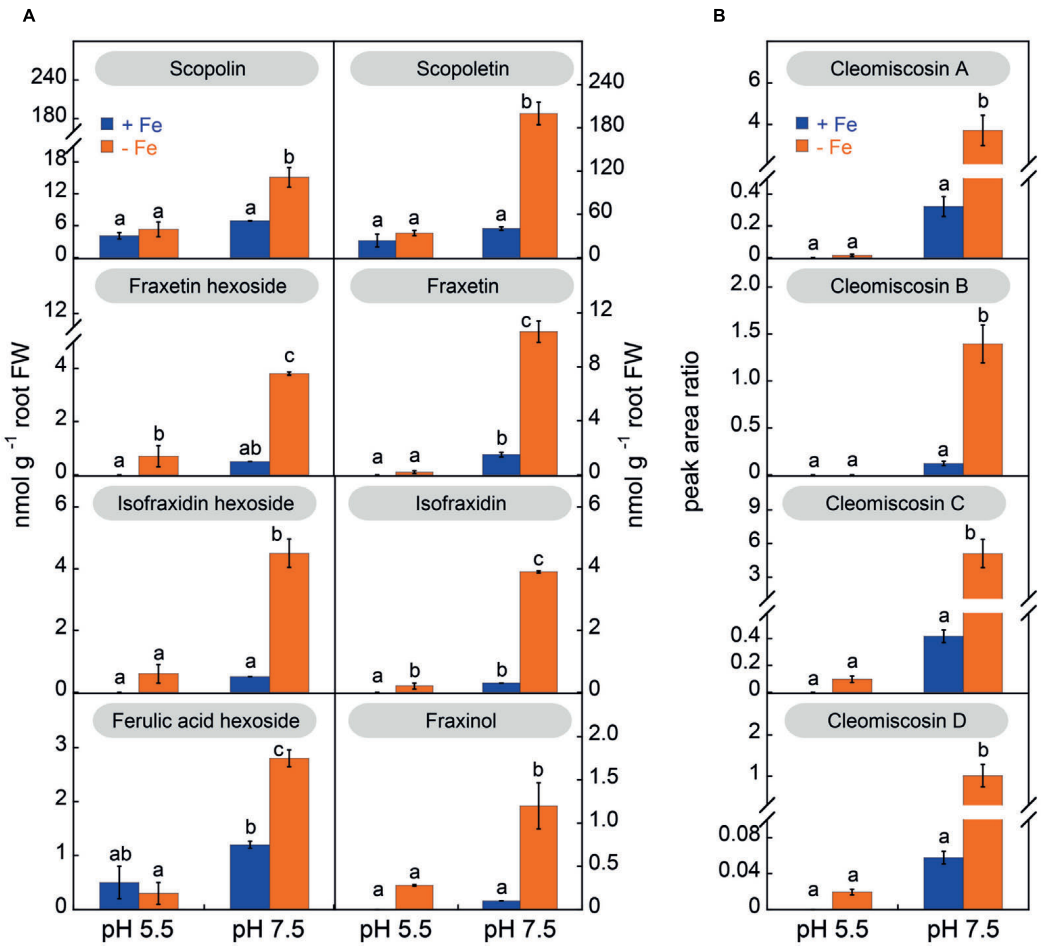

FIGURE 5 | Effects of Fe deficiency and high pH on the concentrations (in nmol g^{-1} root FW) of coumarins (A) and coumarinolignans (B) in *Arabidopsis thaliana* roots. Plants were pre-grown as indicated in **Figure 2** and grown for 14 days with 0 (−Fe) or 20 μM Fe (+Fe) in nutrient solution buffered at pH 5.5 (with 5 mM MES-NaOH) or 7.5 (with 5 mM HEPES-NaOH). Ferulic acid hexoside was quantified as fraxin. The levels of the cleomiscosins are expressed in peak area ratio, relative to the lignan matairesinol used as internal standard. Data are means ± SE (n = 3–5). For each compound, significant differences among treatments (at $p < 0.05$) are marked with different letters above the columns.

the total depending on the growth conditions (Supplementary Figure S4B).

Coumarin and Coumarinolignan Concentrations in the Nutrient Solution

The concentrations of coumarins and coumarinolignans were determined in the nutrient solution of Fe-deficient plants after 7 and 14 days after imposing Fe deficiency (nutrient solutions were renewed on day 7) (**Figure 7**). No determinations could be made in nutrient solutions of Fe-sufficient plants due to the presence of Fe(III)-EDDHA, which causes the overloading of C_{18} materials. Coumarin hexosides were only occasionally detected at trace levels (data not shown). When plants were grown at pH 5.5, the growth media at day 7 contained low concentrations of aglycones (scopoletin, fraxetin, isofraxidin, and fraxinol; **Figure 7**) and coumarinolignans (cleomiscosins A, C, and D as well as the putative 5′- hydroxycleomiscosin; **Figure 7**). After 14 days of Fe deficiency no significant changes were observed. In contrast, when plants were grown at pH 7.5, the concentration of coumarins and coumarinolignans in the nutrient solution were much higher than that found in the culture medium of plant grown at pH 5.5 (**Figure 7**). When compared to the concentrations found with Fe-deficient plants at pH 5.5, increases were large for scopoletin (6- and 12-fold at days 7 and 14, respectively) and very large for the rest of phenolics (in the range from 17- to 537-fold). In addition, when

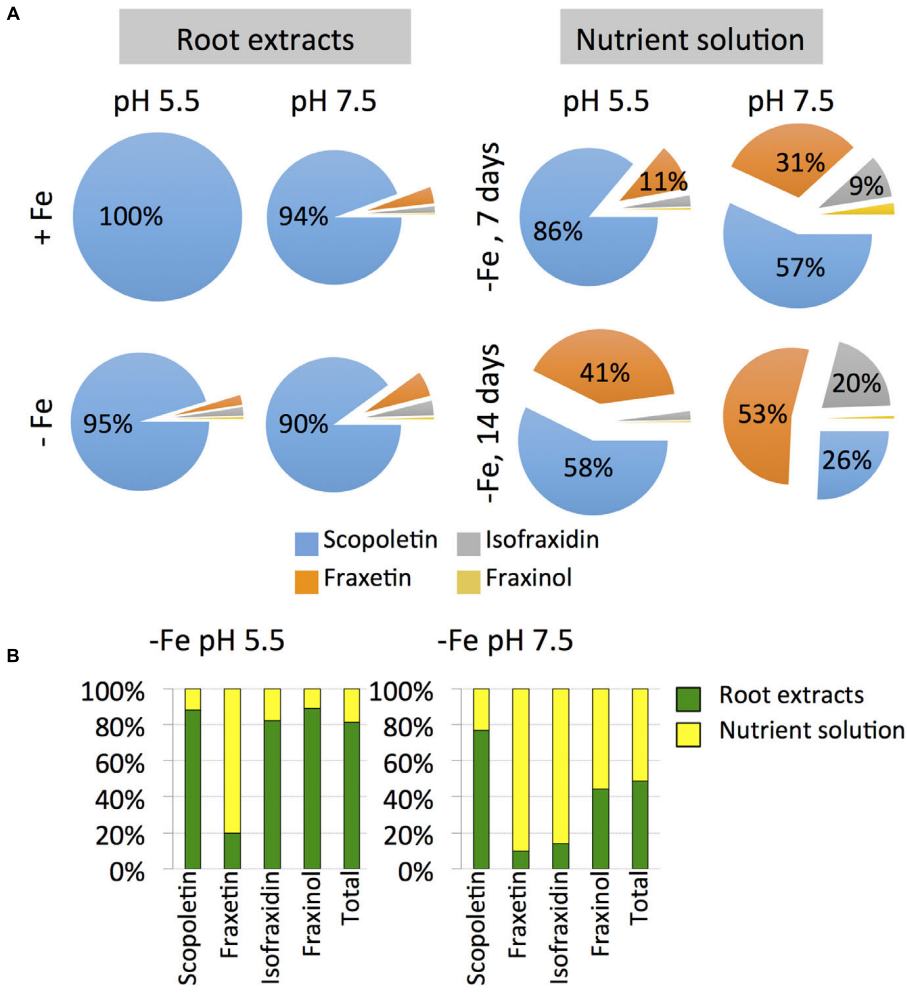

FIGURE 6 | Effects of Fe deficiency, high pH and/or time on the relative concentrations of coumarins (scopletin, fraxetin, isofraxidin, fraxinol and total coumarins) in root extracts and nutrient solution (A) and on the allocation of coumarins to the roots and the nutrient solutions of *Arabidopsis thaliana* (B). Plants were pre-grown as indicated in **Figure 2** and grown for 7 or 14 days with 0 (−Fe) or 20 μM Fe (+Fe) in nutrient solution buffered at pH 5.5 (with 5 mM MES-NaOH) or 7.5 (with 5 mM HEPES-NaOH). Data are means of $n = 3$–5. The absolute values are shown in **Figures 5** and **7**.

Fe-deficient plants were grown at pH 7.5, the concentrations of coumarins (with the exception of fraxinol) and coumarinolignans in the nutrient solution increased with time. When compared to the concentrations at day 7, increases at d 14 were 12-fold for isofraxidin, 9-fold for fraxetin, 5-fold for cleomiscosin A, 3-fold for 5′-hydroxycleomiscosins and the cleomiscosins B and D, and 2-fold for scopoletin and cleomiscosin C.

Scopoletin was the predominant coumarin only at pH 5.5 after 7 days of Fe deficiency (86% of the total coumarins), whereas at 14 days scopoletin and fraxetin accounted for 58 and 41% of the total, respectively (**Figure 6A**). At pH 7.5 scopoletin and fraxetin were the major coumarins at day 7 (57 and 31%, respectively), whereas at d 14 scopoletin, fraxetin and isofraxidin accounted for 26, 53, and 20% of the total, respectively.

Allocation of Coumarins to the Roots and the Nutrient Solutions

The allocation of coumarins produced by Fe-deficient plants was affected by the growth media pH. In plants grown at pH 5.5, only 19% of the total amount of coumarins was allocated to the nutrient solution, whereas for plants grown at pH 7.5 coumarins were allocated equally between nutrient solutions

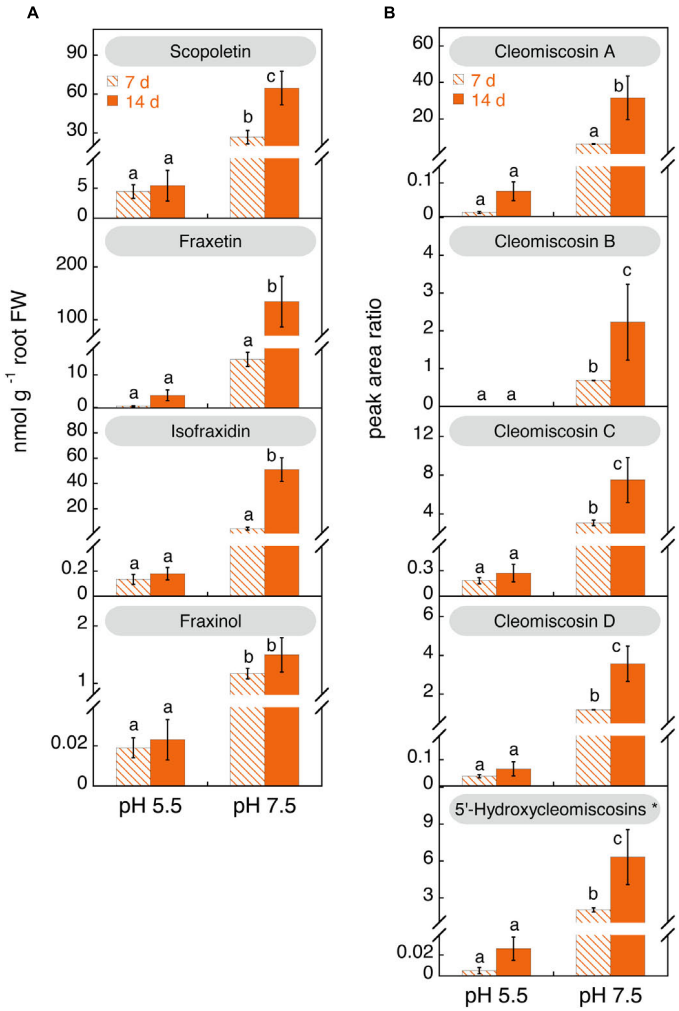

FIGURE 7 | Effects of time of Fe deficiency and high pH treatments on the concentrations (in nmol g^{-1} root FW) of coumarins (A) and coumarinolignans (B) in the nutrient solution of iron (Fe)-deficient *Arabidopsis thaliana*. Plants were pre-grown as indicated in **Figure 2** and grown for 7 or 14 days with 0 μM Fe in nutrient solution buffered at pH 5.5 (with 5 mM MES-NaOH) or 7.5 (with 5 mM HEPES-NaOH). The levels of the cleomiscosins are expressed in peak area ratio, relative to the lignan matairesinol used as internal standard. Data are means ± SE ($n = 3$–5). For each compound, significant differences among treatments (at $p < 0.05$) are marked with different letters above the columns. *5′-Hydroxycleomiscosins A and/or B should be considered since separation of these isomer compounds might have not been achieved.

(51% of the total per plant) and roots (49%) (**Figure 6B**). Fraxetin was preferentially allocated to the nutrient solution at both pH values, whereas isofraxidin and fraxinol did only so at pH 7.5.

Mobilization of Fe from Fe(III)-Oxide Promoted by Coumarins

In order to understand the role that coumarins could play in Fe plant nutrition, their ability to mobilize Fe from Fe(III)-oxide was measured in *in vitro* incubation assays. The experiments were carried out with a poorly crystaline Fe(III)-oxide and 1.5 ml of an assay medium containing 0 (blank) or 100 μM of coumarin and buffered at pH 5.5 or 7.5. Three out of the four coumarins assayed (scopoletin, isofraxidin and fraxin) have a catechol moiety capped *via* hydroxyl group methylation or hydroxyl group glucosylation, whereas the fourth coumarin, fraxetin, bears an available catechol moiety (see structures in **Figure 1A**). Coumarolignans could not be used in these

experiments because of the lack of commercial authenticated standards. Assays were run in the presence of the Fe(II) trapping agent BPDS to monitor the reductive dissolution of Fe(III)-oxide, and the concentration of Fe(II)-$BPDS_3$ was termed Fe(II). The overall mobilization of Fe was assessed by determining the total Fe in solution using ICP-MS (**Figure 8**). The Fe mobilized by the buffer solutions (blanks) was on the average 0.2 nmol Fe g^{-1} Fe(III)-oxide min^{-1}. When the assay medium contained the non-catechol coumarins fraxin, scopoletin and isofraxidin, the total Fe mobilized was in the range 0.9–1.2 nmol Fe g^{-1} Fe(III)-oxide min^{-1} (depending on the coumarins and the assay pH) and statistically significant differences were found when compared to the blank (**Figure 8A**). However, when the assay medium contained the catechol coumarin fraxetin, the amounts of Fe mobilized (5.8 and 9.4 nmol Fe g^{-1} Fe(III)-oxide min^{-1} for the assays at pH 5.5 and pH 7.5, respectively) were significantly higher than the rest (**Figure 8A**). Furthermore, the total mobilization of Fe promoted by fraxetin at pH 7.5 increased linearly when the concentration of fraxetin increased from 10 to 100 μM. A relevant fraction (40–44%) of the mobilized Fe was trapped by BPDS and this fraction also increased linearly when the concentration of fraxetin increased from 10 to 100 μM (**Figure 8B**).

DISCUSSION

Arabidopsis thaliana plants produce and secrete an array of phenolics in response to Fe deficiency when the pH of the nutrient solution is high. Phenolics found in this study include several coumarinolignans not previously reported in *A. thaliana* (cleomiscosins A, B, C, and D and the 5′-hydroxycleomiscosins A and/or B), as well as other previously reported coumarins (scopoletin, fraxetin, isofraxidin and fraxinol) and some coumarin precursors (ferulic acid and coniferyl and sinapyl aldehydes). The identification of all these phenolic compounds was achieved through an integrative interpretation of analytical data, including exact molecular mass-to-charge ratios (*m/z*), low and high-resolution MS^n spectra, chromatographic RTs and fluorescence/UV-VIS data. Furthermore, we report here for the first time on the quantification of all identified coumarins, revealing that Fe deficiency mainly induced the root accumulation and exudation of the non-catechol coumarin scopoletin and the catechol coumarin fraxetin, with the exudation of fraxetin being more prominent when Fe chlorosis was intense. Also, we show for the first time that fraxetin, but not scopoletin, was effective to mobilize Fe from an scarcely soluble Fe(III)-oxide.

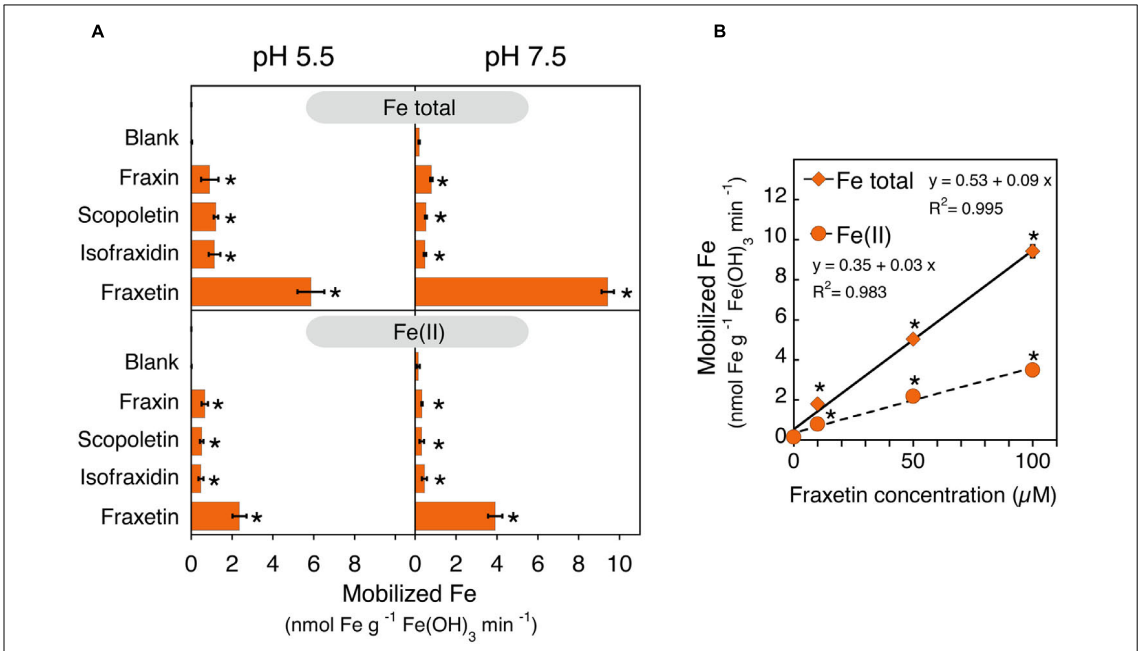

FIGURE 8 | Iron mobilization from an scarcely soluble Fe(III)-oxide as affected by coumarins. (A) Structure-activity relationship of coumarins on Fe mobilization activity. The assays consisted in the incubation of 10 mg of Fe(III)-oxide with a solution of 0 (blank) or 100 μM of the indicated coumarins and 600 μM BPDS at two different pH values, 5.5 and 7.5. Total Fe and Fe(II)-$(BPDS)_3$ in solution were determined by ICP-MS and spectrophotometry, respectively. **(B)** Effects of the fraxetin concentration on the Fe mobilization activity at pH 7.5. Scatter plot of the concentration of fraxetin vs. the total Fe mobilized and the Fe(II), with linear regression lines in black and their corresponding equations. In all cases **(A,B)**, data are means ± SE (n = 3–12) and asterisks denote a statistically significant difference between blank and a coumarin-containing assay medium as determined by Student's t- test ($p < 0.05$).

This is the first time cleomiscosins and 5′-hydroxycleomiscosins have been reported in *A. thaliana*. Cleomiscosins were found in both roots and nutrient solutions, whereas 5′-hydroxycleomiscosins were found only in nutrient solutions (**Figures 5B** and **7B**). All coumarinolignans found have a fraxetin moiety linked to different phenylpropanoid units (**Figure 1C**). Non-conventional lignans, including coumarinolignans and other hybrid ones, harbor a single phenylpropanoid unit, whereas conventional ones consist in phenylpropanoid dimers. The common coumarin moiety in the coumarinolignans found, fraxetin, has been consistently reported to increase with Fe deficiency in roots and growth media of *A. thaliana* (**Figures 5** and **7**; Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014). The phenylpropanoid units found are the primary lignin precursors coniferyl (in cleomiscosins A and B) and sinapyl alcohols (in cleomiscosins C and D), and the non-canonical monolignol 5-hydroxyconiferyl alcohol (in 5′-hydroxycleomiscosins A and B) (Begum et al., 2010) (**Figure 1C**). Previously, two other coumarinolignans, composed of esculetin and either coniferyl alcohol or sinapyl alcohol, were tentatively identified in *A. thaliana* root exudates (Strehmel et al., 2014). Until now, cleomiscosins have been only reported in seeds and stem wood and bark of various plant species, whereas 5′-hydroxycleomiscosins A and B were found in *Mallotus apelta* roots (Xu et al., 2008) and *Eurycorymbus cavaleriei* twigs (Ma et al., 2009), respectively. Cleomiscosin A has been reported in 22 plant species belonging to 12 families (e.g., Sapindaceae and Simaroubaceae), whereas cleomiscosins B, C, and D, although less common, have been found in 6–10 plant species belonging to 5–9 families (Begum et al., 2010).

Besides coumarinolignans, ferulic acid and other related metabolites were found to accumulate in roots of Fe-deficient *A. thaliana* plants when grown at high pH (**Table 1**; **Figure 5A**). This is consistent with Fe-deficient *A. thaliana* root transcriptomic (Rodríguez-Celma et al., 2013), proteomic (Lan et al., 2011) and metabolite data (Fourcroy et al., 2014): (i) ferulic acid can be converted to feruloyl-CoA by the action of 4-coumarate:CoA ligases (4CL1 and 4CL2), two enzymes that have been found to be robustly induced by Fe deficiency (Lan et al., 2011; Rodríguez-Celma et al., 2013), (ii) feruloyl-CoA is a key precursor in the biosynthesis of scopoletin (Kai et al., 2008), which accumulates in roots of Fe-deficient plants (**Figures 5A** and **7A**; Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014), and (iii) ferulic acid hexoside has been reported to occur in Fe-deficient roots (Fourcroy et al., 2014). Also, two other metabolites, coniferyl and sinapyl aldehydes, were occasionally found in Fe-deficient roots (in the aglycone and hexoside forms, **Tables 1** and **3**). Coniferyl aldehyde can either lead to scopoletin biosynthesis *via* oxidation to ferulic acid (Kai et al., 2008) or be reduced to coniferyl alcohol (Fraser and Chapple, 2011), a precursor of lignin and lignans (Barros et al., 2015), including cleomiscosins A and B. Sinapyl aldehyde is an intermediate metabolite in the synthesis of lignin and lignans such as cleomiscosins C and D (Barros et al., 2015), and may (assuming that isofraxidin synthesis is analogous to that of scopoletin, as proposed by Petersen et al., 1999) be a precursor of the coumarin isofraxidin, which accumulates consistently in Fe-deficient roots (**Figure 5A**).

Coumarins also accumulate in *A. thaliana* roots along with coumarinolignans and are secreted to the growth media in response to Fe deficiency, especially when pH was high. Four coumarins (scopoletin, fraxetin, isofraxidin and the isofraxidin isomer fraxinol) were found in both root extracts and nutrient solutions (**Tables 1** and **2**) confirming previous results (Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014) (Supplementary Table S1). We could identify fraxinol (annotated in a previous study as methoxyscopoletin; Fourcroy et al., 2014), using an authenticated standard. Aglycones and hexose conjugates of the four coumarins were found in roots (**Figure 5**; Supplementary Figure S4B), whereas only the aglycone forms were quantifiable in nutrient solutions, with hexoside forms being detected only occasionally and in low amounts (**Figure 7**). We did not detect three more coumarins, esculetin, isofraxetin and dihydroxyscopoletin, previously found as aglycones and/or glycoside forms by Schmid et al. (2014) and/or Schmidt et al. (2014) in roots or exudates of Fe-deficient *A. thaliana*. This could be due to differences in protocols for exudate collection and isolation of organic compounds from the growth/exudation media or plant growth conditions. In any case, from the published data it seems that the relative amount of these three coumarins was very low: in the only study where quantification of some coumarins was carried out, the amount of esculetin was 0.1% (roots) and <1% (exudates) when compared to those of scopoletin (Schmid et al., 2014). Assuming similar ratios in our study, the concentration of esculetin would be approximately 0.2–0.5 nmol g^{-1} root FW in roots and nutrient solutions, respectively, values still lower than those of fraxinol, the least abundant of the coumarins detected in this work (**Figures 5** and **7**). Regarding the other two coumarins not detected in this study, isofraxetin and dihydroxyscopoletin, they were only detected in Schmid et al. (2014) and Schmidt et al. (2014), respectively, indicating that their occurrence in Fe-deficient plants is not consistent.

High pH induces by itself a certain Fe stress that results in the synthesis of phenolics in roots. The increase in the production of some phenolic compounds was already observed in Fe-sufficient plants grown at high pH (**Figure 5**; Supplementary Figure S4A), along with decreases in root and shoot Fe contents (**Figure 2C**) and increases in *FRO2* expression (**Figure 2D**), even when leaf Chl and biomass were not affected (**Figures 2A–C**). It was already known that high pH compromises the root Fe acquisition from Fe(III)-chelates, with FCR activities being much lower at pH 7.5 than at the optimal pH range of 5.0-5.5 (in *A. thaliana* and other species; Moog et al., 1995; Susín et al., 1996), and FCR rates are known to be especially low with highly stable chelates such as Fe(III)-EDDHA (Lucena, 2006). When plants were grown in absence of Fe at pH 7.5 the Fe stress was much more intense and the synthesis of phenolics in roots was fully enhanced (when compared with Fe-sufficient plants grown either at high or low pH): concentrations of all phenolics in roots were much higher (**Figure 5**; Supplementary Figure S4A), the concentration of phenolics in the nutrient solution increased markedly with time (**Figure 7**; Supplementary

Figure S4A), and there were marked decreases in leaf Chl (**Figures 2A,B**), shoot biomass and shoot and root Fe contents (**Figure 2C**). The high pH/zero Fe effect is rapid, since only after 3 days roots already showed an increased expression of genes coding for root coumarin synthesis (*COMT*, *CCoAMT* and *F6′H1*) and Fe acquisition components (*IRT1* and *FRO2*) (when compared with Fe-sufficient plants grown either at high or low pH) (**Figure 2D**). In contrast, when plants were grown in absence of Fe at pH 5.5, there was no effect on biomass (**Figure 2C**) and the decreases in leaf Chl and shoot and root Fe contents (when compared with Fe-sufficient plants grown either at high or low pH) were as large as those found at high pH (**Figures 2A–C**), and only moderate effects were found with respect to phenolics, including: (i) increases of some phenolics in roots (fraxetin, isofraxidin, fraxinol, cleomiscosins A, C, and D) (**Figure 5**; Supplementary Figure S4A); (ii) time dependent increases in the concentration of all phenolics in the nutrient solution, although concentrations were always lower than those found at high pH (**Figure 7**; Supplementary Figure S4A), and (iii) a rapid (at 3 days) root increased expression of genes for Fe root uptake, although to a much lower extent than at high pH, without any change in the expression of genes involved in coumarin synthesis (**Figure 2D**).

Iron-supply and nutrient solution pH affect the relative coumarin concentrations in root extracts and growth media. Whereas the non-catechol coumarin scopoletin was initially the most abundant coumarin in root extracts and growth media, the catechol coumarin fraxetin was progressively more abundant with time in the growth media of plants grown with zero Fe (**Figure 6**). When other authors used HPLC-fluorescence for quantification, scopoletin was found to be the most abundant coumarin in the growth media of Fe-deficient *A. thaliana* (Schmid et al., 2014); fraxetin was not quantified in that study, possibly due to the very low fluorescence rate of this compound. The extremely low fluorescence of fraxetin in comparison with those of other coumarins (scopoletin, isofraxidin and esculetin) in the growth media of Fe-deficient *A. thaliana* plants is shown in Supplementary Figure S5. Interestingly, in the roots of Fe-deficient plants grown at pH 7.5 the coumarins that have a larger aglycone fraction (scopoletin and fraxetin; Supplementary Figure S4B), likely due to the action of a glucosidase, were also the prevalent ones in the growth media, supporting that the aglycone forms are likely to be the substrate for the plasma membrane transporter ABCG37. In this respect, the β-glucosidase BGLU42 is induced by Fe deficiency in roots (García et al., 2010; Yang et al., 2010; Lan et al., 2011; Rodríguez-Celma et al., 2013), and the roots of Fe-deficient *bglu42 A. thaliana* mutant plants apparently fail to secrete coumarins (Zamioudis et al., 2014). However, coumarin glucosides such as scopolin have been reported to occur in the exudates of Fe-deficient *A. thaliana* in other studies (Schmid et al., 2014; Schmidt et al., 2014).

The structural features of each coumarin-type compound may confer specific roles that contribute to the adaptation of *A. thaliana* to low Fe availability in alkaline conditions. The catechol moiety enable coumarins to mobilize efficiently Fe from an Fe(III)-oxide (**Figure 8A**). Fraxetin, a coumarin bearing a catechol moiety and a methoxy substituent, mobilized much more Fe than any of the non-catechol coumarins tested at the same concentration (100 μM; scopoletin, isofraxidin and fraxin) at physiologically relevant pH values (5.5 and 7.5). Specific structural features of the non-catechol coumarins tested, such as the *O*-glucosyl moiety (in fraxin) and one or two methoxy groups (in scopoletin/fraxin and ixofraxidin, respectively) do not appear to affect to the Fe mobilization ability of the coumarin, since these three coumarins mobilized similar amounts of Fe (**Figure 8A**). This confirms what has been reported previously (at pH 7.2) with the catechol coumarin esculetin (no methoxy substituent) and the non-catechol coumarins scopoletin (one methoxy and one hydroxy substituents) and esculin (one *O*-glucosyl and one hydroxy substituents) (Schmid et al., 2014). In addition, the present study revealed that the mobilization of Fe from Fe(III)-oxide promoted by fraxetin involves a significant reduction of Fe(III) to Fe(II) and appears to be controlled by the fraxetin concentration and the medium pH. Approximately 42% of the Fe mobilized by fraxetin was trapped by BPDS, regardless of the assay pH and the fraxetin concentration (**Figure 8**). The Fe(II) produced may be directly taken up by root cells, chelated by other natural ligands and/or re-oxidized to Fe(III). The amount of Fe mobilized by fraxetin was 1.6-fold higher at pH 7.5 -typical of calcareous soils- than at pH 5.5 (**Figure 8A**). Also, increases in fraxetin concentration (from 10 to 100 μM) led to a marked enhancement in Fe mobilization rates (**Figure 8B**). Most of the fraxetin produced by Fe-deficient plants (80–90%) was allocated to the nutrient solution regardless of the growth media pH, in contrast with the small amount of the non-catechol coumarin, scopoletin, allocated to the nutrient solution (12–23%) (**Figure 6B**). Taking also into account the concentrations estimated for scopoletin (21 μM), fraxetin (43 μM), isofraxidin (14 μM) and fraxinol (0.5 μM) in the soil solution surrounding the root (apex) of *A. thaliana* growing without Fe at pH 7.5 (calculated as in Römheld, 1991, for phytosiderophores), it seems likely that fraxetin could play a role as an Fe mobilizer in natural conditions. A catechol group is also present in the coumarinolignans 5′-hydroxycleomiscosins A and B (**Figure 1C**) that were found only in exudates (**Table 1**; **Figure 7**). Therefore, not only fraxetin but also 5-hydroxycleomiscosins A/B may have a role in mining Fe from soil Fe sources at high pH, providing soluble Fe for plant uptake. Unfortunately, no authenticated standards exist in the market for these compounds. On the other hand, coumarins, having or not catechol groups, play a well-established role in plant defense, serving as allelochemicals against a broad array of organisms (e.g., bacteria, fungi, nematodes, insects, etc), with their synthesis being activated in plants after infection (Weinmann, 1997; Bourgaud et al., 2006). Therefore, the array of coumarin-type compounds found in the growth media could play multiple roles, achieving different benefits for Fe-deficient plants.

Accumulating experimental evidences suggest that the Fe deficiency-elicited production of coumarin-type phenolics allows *A. thaliana* plants interacting with the rhizosphere microbiome, including beneficial and pathogen organisms. On one hand, Fe-deficient *A. thaliana* plants display reduced susceptibility

to infection with the necrotrophic fungus *Botrytis cinerea* and the bacterial plant pathogen *Dickeya dadantii*, with an Fe supplementation restoring symptoms severity (Kieu et al., 2012). On the other hand, the activation of immunity toward broadly diverse pathogens and even insects and herbivores in *A. thaliana* elicited by the beneficial rhizobacteria *Pseudomonas fluorescens* WCS417 and mediated by the root-specific transcription factor MYB72 (Van der Ent et al., 2008; Segarra et al., 2009), also required for the induction of Fe deficiency responses (Palmer et al., 2013), involves not only the production of F6'H1-dependent coumarins but also their secretion (Zamioudis et al., 2014). In fact, two *Arabidopsis* mutants failing in the production and/or secretion of coumarins, *myb72* and *bglu42*, did not show, when grown in the presence of WCS417, enhanced resistance against two biotrophic pathogens (the Gram-negative bacterium *Pseudomonas syringae* pv. tomato DC3000 and the pseudo-fungus *Hyaloperonospora arabidopsidis*; Zamioudis et al., 2014). Also, BGLU42 overexpression led to a significantly enhanced resistance against *B. cinerea*, *H. arabidopsidis* and *P. syringae* pv. tomato DC3000 (Zamioudis et al., 2014). The enhanced disease resistance of *A. thaliana* against different pathogens can be associated with the structure of the coumarin-type compounds produced, since different substituents in the backbone of coumarins and lignans can influence biological activity (Weinmann, 1997; Apers et al., 2003; Borges et al., 2005; Zhang et al., 2014; Pilkington and Barker, 2015).

Certain structural features of coumarins and coumarinolignans produced by roots of Fe-deficient *A. thaliana* plants may confer specific roles in shaping the rhizosphere microbiome. In fact, the existence of differences in inhibitory potential against specific microorganisms may be expected in Fe deficiency-induced coumarins. First, all coumarins detected in Fe-deficient *A. thaliana* root extracts and exudates are highly oxygenated and with hydroxyl/methoxy substituents: scopoletin and esculetin are di-oxygenated and fraxetin, fraxetin isomer, isofraxidin and fraxinol are tri-oxygenated (**Figure 1A**). A high number of oxygen-containing substituents in the benzopyrone coumarin backbone (**Figure 1A**) appears to be determinant for broadening the antibacterial spectrum (Kayser and Kolodziej, 1999), whereas the presence of simple substituents (e.g., hydroxy, methoxy) instead of bulkier chains may aid bacterial cell wall penetration. Second, an oxygenation pattern consisting in two methoxy substituents and at least one additional hydroxyl substituent is present in the minor tri-oxygenated coumarins isofraxidin and fraxinol produced by Fe-deficient *A. thaliana* roots. This oxygenation pattern seems to confer to tri-oxygenated coumarins a strong and wide inhibitory activity against Gram-positive and Gram-negative bacteria (Kayser and Kolodziej, 1999; Smyth et al., 2009). Furthermore, the estimated concentrations of scopoletin, fraxetin, isofraxidin and fraxinol in the soil solution surrounding the root (apex) of *A. thaliana* growing without Fe at pH 7.5 (see above) are close or above the minimum inhibitory concentration of di- and tri-oxygenated coumarins against Gram-positive and Gram-negative bacteria (1.3-11.2 and 0.9-4.5 μM, respectively; Kayser and Kolodziej, 1999).

Regarding plant coumarinolignans, the current knowledge on their biological activities is mostly pharmacological, derived from the ethno-medical utilization of some plant species (Begum et al., 2010; Zhang et al., 2014; Pilkington and Barker, 2015). Known activities of cleomiscosins include liver protection, cytotoxicity against lymphocytic leukemia cells, immunomodulation, and others. In plants, the defense roles for conventional lignans have been studied, and certain structural features appear to affect the activities against specific organisms. First, coumarinolignans are more aromatic than conventional lignans, suggesting they may have a higher effectiveness. For instance, increased antifungal activities were observed when the phenyl ring in a monomeric phenylpropanoid derivative was replaced by naphthyl or phenanthryl rings, whereas no or very low antifungal activity is associated to the monomeric phenylpropanoid moieties in conventional lignans (Apers et al., 2003). Second, the occurrence of methoxy substituents in lignans appear confer stronger insecticide and fungicide activities, whereas the presence of polar substituents, especially hydroxy or glycoside groups, sometimes reduced them (Harmatha and Nawrot, 2002; Harmatha and Dinan, 2003; Kawamura et al., 2004). Since cleomiscosin structures differ in the methoxy and hydroxy substituents (**Figure 1C**), their possible insecticide and fungicide activities is likely to be different.

Results presented here highlight that Fe deficiency elicits the accumulation in roots and secretion into the growth media of an array of coumarin-type compounds, including coumarinolignans (cleomiscosins A, B, C, and D and the 5′-hydroxycleomiscosins A and/or B) and simple coumarins (scopoletin, fraxetin, isofraxidin and fraxinol) in *A. thaliana*. The phenolics response was much more intense when the plant accessibility to Fe was decreased and Fe status deteriorated, as it occurs when plants are grown in the absence of Fe at pH 7.5. The structural features of the array of coumarins and lignans produced and their concentrations in roots and growth media suggest that they may play dual, complementary roles as Fe(III) mobilizers and allelochemicals. Fraxetin, a catechol coumarin, was the most prominent coumarin found in the growth media of Fe-deficient *A. thaliana* plants grown at high pH and was especially effective in mobilization of Fe from an Fe(III)-oxide. In contrast, the rest of coumarins were non-catechols and were present in much lower concentrations, and therefore their role in mobilizing Fe is unlikely, although they can still be efficient as allelochemicals. Therefore, the production and secretion of phenolics by roots in response to Fe deficiency would promote an overall decrease in the competition for Fe in the immediate vicinity of roots, resulting in improved plant Fe nutrition. Results also suggest that Fe deficiency could be a good experimental model to understand the ecological dynamics of the biotic interactions in the plant rhizosphere.

AUTHOR CONTRIBUTIONS

AA-F, PF, and AA conceived and designed the experiments, PS-T conducted experiments, collected data, and drafted the manuscript, AL-V quantified phenolics, carried out Fe mobilization studies and made figures, AA, FG, J-FB, JA, and

AA-F wrote, reviewed and edited the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01711/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material I

Accumulation and Secretion of Coumarinolignans and other Coumarins by *Arabidopsis thaliana* Roots in Response to Iron Deficiency at High pH

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The following Supplementary Data is available for this article:

Fig. S1 Analysis of standards of a selected group of coumarin glucosides, coumarins and phenylpropanoids, and a lignan by HPLC-ESI-MS(TOF) using Elution program 2.

Fig. S2 Analysis of standards of a selected group of flavonoids and a stilbene by HPLC-ESI-MS(TOF) using Elution program 2.

Fig. S3 High-resolution MS^2 spectra of compounds *14-18*.

Fig. S4 Total concentrations of coumarins in root extracts and nutrien solutions, and relative concentrations of glycosides and aglycones in root extracts.

Fig. S5 Emission spectra of scopletin, fraxetin, isofraxidin and other coumarins**.**

Table S1 Coumarins produced by *Arabidopsis thaliana* roots in response to iron deficiency as reported in previous studies.

Table S2 Elution programs used for HPLC-based separation.

Table S3 List of eluents and standards used.

Table S4 Operating conditions of HPLC-ESI-MS/MS(HCT Ultra ion trap).

Table S5 Primers used for qRT-PCR.

Table S6 Changes in young leaf photosynthetic pigment concentrations with Fe deficiency and growth media pH in Arabidopsis.

Table S7 Changes in shoot and root Mn, Cu and Zn contents with Fe deficiency and growth media pH in Arabidopsis.

Table S8 Mass losses, from the precursor ion, observed in the MS^2 spectra of compounds *14-18*.

Fig. S1 Analysis of a selected group of coumarin glucosides (a, b, and d), coumarins (c, f, i, j, k, o, and q) and phenylpropanoids (e, g, h, l, m, n, and p), and a lignan (r) by HPLC-ESI-MS(TOF). The standards were separated using Elution program 2. Chromatograms were extracted at the *m/z* (± 0.05) ratio corresponding to the $[M+H]^+$ ions with the exception of those corresponding to glucosides (esculin, scopolin and fraxin) and the phenylpropanoid acids (caffeic and ferulic acid) for which the *m/z* (± 0.05) ratio corresponding to the $[M\text{-glucose}+H]^+$ and $[M\text{-}H_2O+H]^+$ ions were used, respectively.

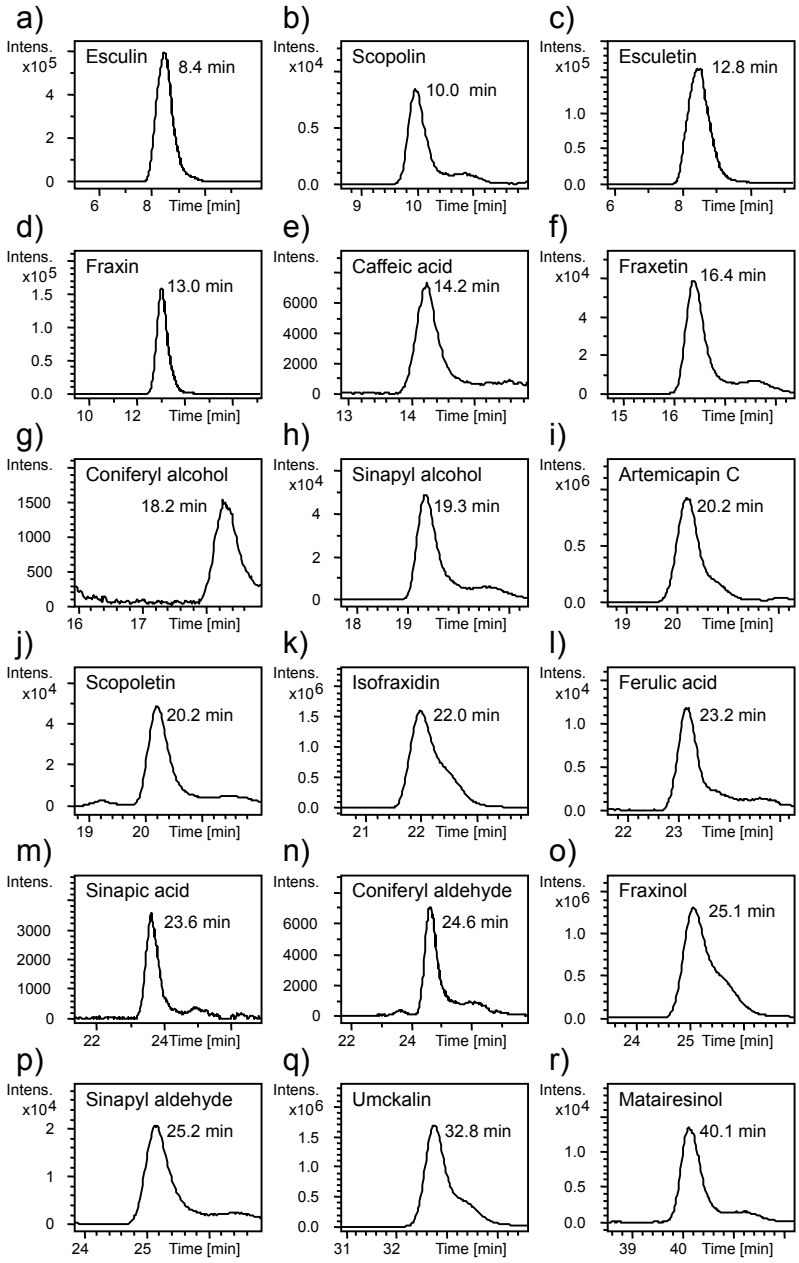


Fig. S2 Analysis of standards of a selected group of flavonoids (a, b, d, e, f, g, h, i) and a stilbene (c) by HPLC-ESI-MS(TOF). The flavonoids selected correspond to different classes: i) two flavonols (d, g) and their rhamnosides (b, e), ii) a flavanone (f), iii) a isoflavone (h) and its glucoside (a), iv) flavone (i). The standards were separated using Elution program 2. Chromatograms were extracted at the *m/z* (± 0.05) ratio corresponding to the $[M+H]^+$ ions with the exception of those corresponding to the glucoside myricitrin and the rhamnosides (myricitrin and quercetrin) for which the *m/z* (± 0.05) ratio corresponding to the $[M\text{-glucose}+H]^+$ and $[M\text{-rhamnose}+H]^+$ions were used, respectively.

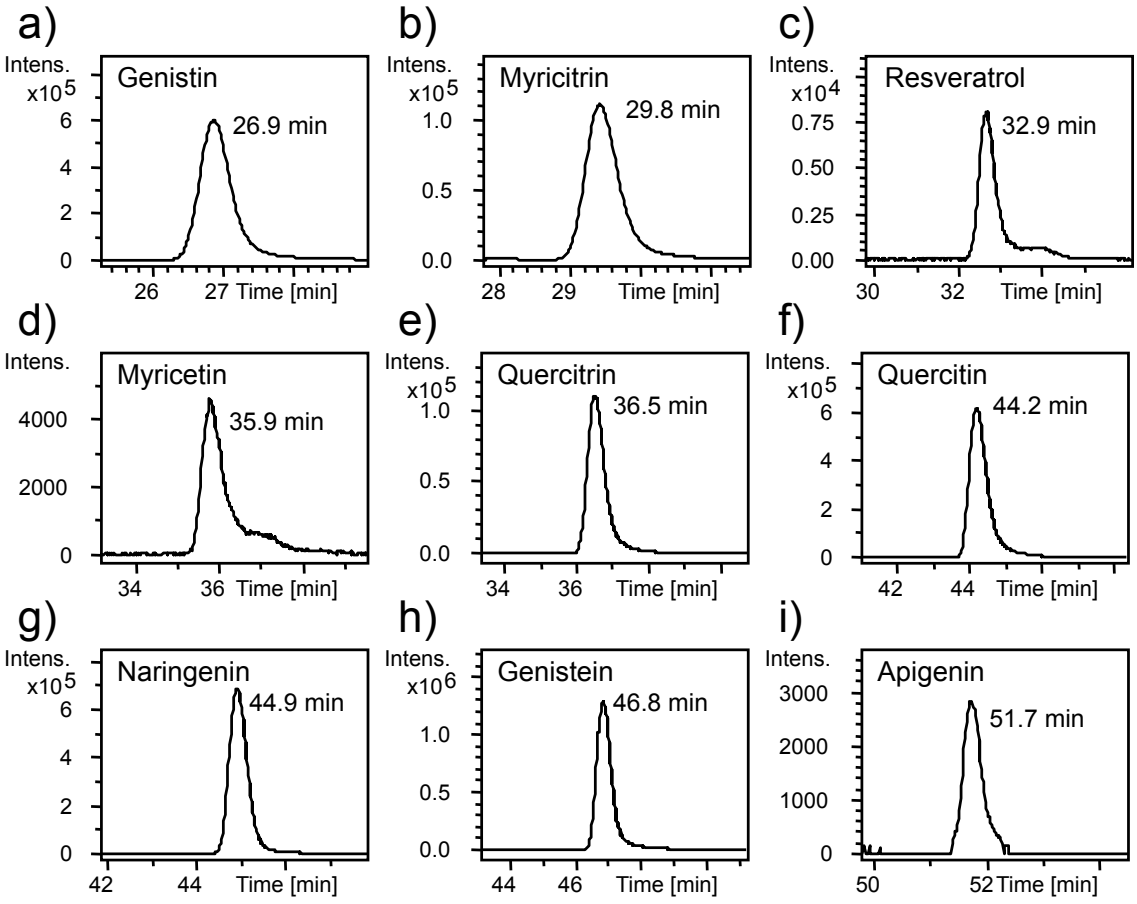


Fig. S3 High-resolution MS^2 spectra of compounds *14-18* obtained on the HPLC-ESI-Q-TOF mass spectrometer in positive ion mode. The encircled numbers in each spectra correspond to the phenolic compounds listed in Table 1. In each spectra, the parent ion and the product ion used in later MS^3 (Figure 4b) experiments are in bold blue colour and bold black colour, respectively.

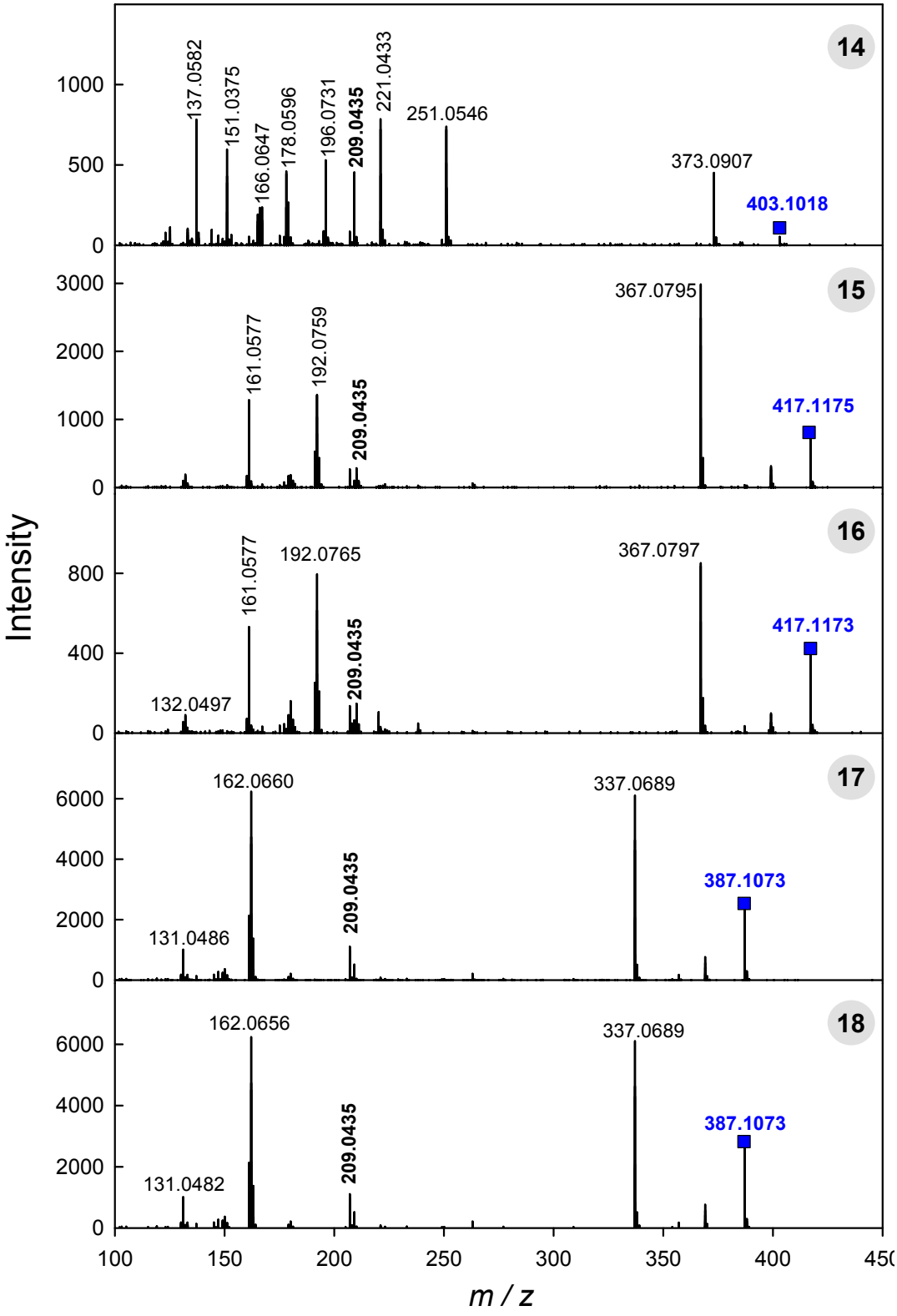


Fig. S4 Effects of Fe deficiency, high pH and/or time on the total coumarin concentrations in *Arabidopsis thaliana* root extracts and nutrient solutions (a) and on the relative concentration of the aglycone and glycoside (hexoside) form for each coumarin in root extracts (b). Plants were pre-grown as indicated in Figure 2 and grown for 7 or 14 d with 0 (-Fe) or 20 µM Fe (+Fe) in nutrient solution buffered at pH 5.5 (with 5 mM MES-NaOH) or 7.5 (with 5 mM HEPES-NaOH). Data are means of n=3-5. The absolute values are shown in Figures 5 and 7.

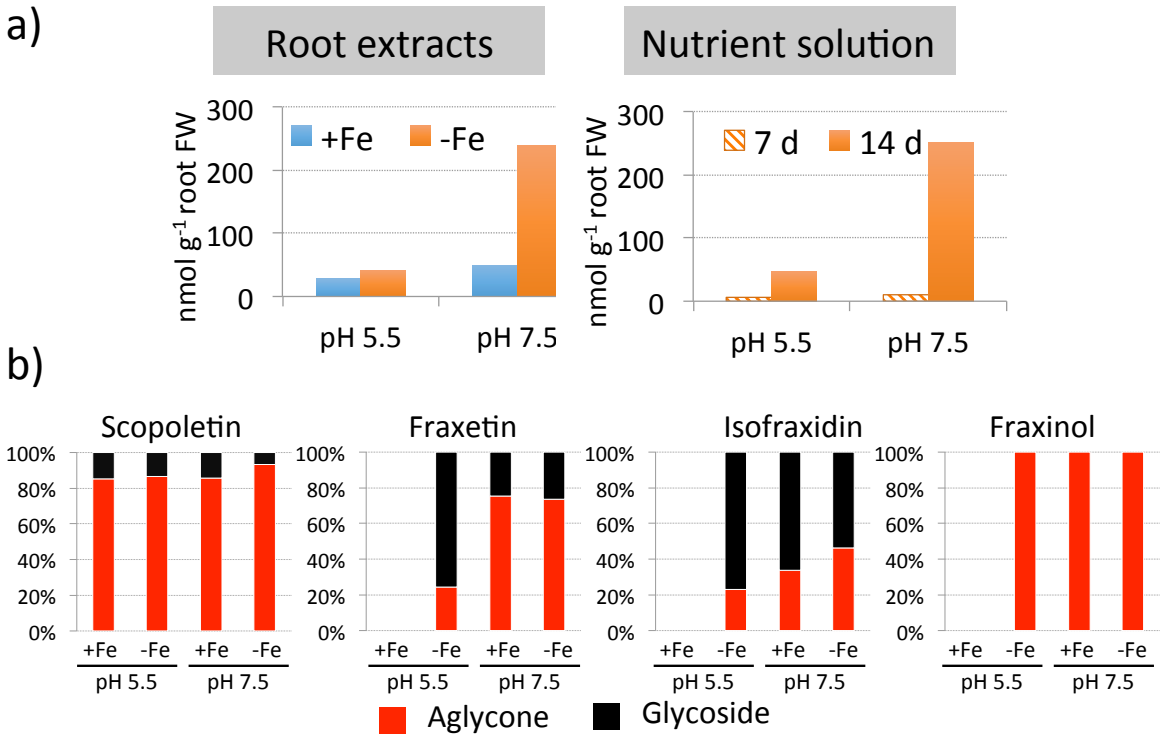


Fig. S5 Emission spectra of a selected group of coumarins at wavelength of excitation of 360 nm. Solutions were prepared in water at pH 5.3. The spectra were recorded using a Perkin Elmer Luminescence Spectrometer Model LS-50B.

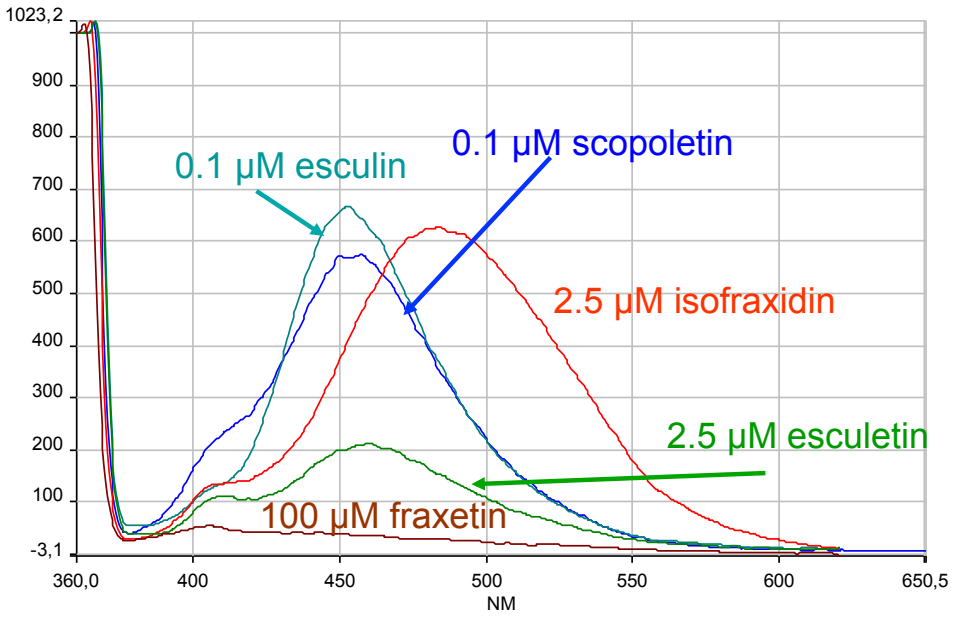


Table S1 Coumarins accumulated and/or released into the growth media by roots of *Arabidopsis thaliana* (Col0, *pdr9-2*, *pdr9-3* and *comt*) in response to Fe deficiency as reported in previous studies ([1] Fourcroy y col. (2014), [2] Schmidt y col. (2014) and [3] Schmid y col. (2014)). N.D. means not detected. Table footnotes denote other names used for the cited coumarins. Col0, wild-type Arabidopsis, ecotype Columbia 0; *pdr9-2* and *pdr9-3*, two nonallelic T-DNA insertion mutants in the Arabidopsis *ABCG37* (*PDR9*) gene; *comt*, T-DNA insertion mutant in the Arabidopsis *COMT* gene encoding the caffeic acid/5-hydroxyferulic acid O-methyltransferase.

Coumarin	Roots	Growth media	Reference
dihydroxyscopoletin hexoside[1]	*pdr9-2*	Col0	[1], [3]
fraxin[2]	Col0	N.D.	[2]
scopolin[3]	Col0, *pdr9-2*, *pdr9-3*	Col0	[1], [2], [3]
ferulic acid hexoside[4]	Col0, *pdr9-2*, *pdr9-3*, *comt*	N.D.	[1]
hydroxymethoxyscopoletin hexoside[5]	*pdr9-2*	N.D.	[1]
fraxetin[6]	Col0, *pdr9-2*, *pdr9-3*	Col0	[1], [2], [3]
isofraxetin[7]		Col0	[3]
scopoletin[8]	Col0, *pdr9-2*, *pdr9-3*	Col0, *pdr9-3*	[1], [2], [3]
isofraxidin[9]	Col0, *pdr9-2*, *pdr9-3*	Col0, *pdr9-3*	[1], [3]
fraxinol[10]	Col0, *pdr9-2*, *pdr9-3*	Col0, *pdr9-3*	[1], [3]
esculetin[11]	Col0	Col0	[2], [3]

[1]Trihydroxymethoxycoumarin hexoside; [2]fraxetin glucoside or 8-hydroxyscopoletin glucoside; [3]scopoletin glucoside; [4]precursor of scopletin; [5]dihydroxydimethoxycoumarin hexoside; [6]7,8-dihydroxy-6-methoxycoumarin; [7]dihydroxymethoxycoumarin; [8]7-hydroxy-6-methoxycoumarin; [9]methoxyscopoletin isomer or 7-hydroxy-6,8-dimethoxycoumarin; [10]methoxyscopoletin isomer or 6-hydroxy-5,7-dimethoxycoumarin; [11]6,7-dihydroxycoumarin.

Table S2 Elution programs used for HPLC based separation of phenolics. The mobile phase was built with two solvents: 0.1% of formic acid in water (A) and 0.1% formic acid in methanol (B). The elution program 1 was designed by Fourcroy y col. (2014).

Elution program 1		Elution program 2	
Time (min)	% solvent B	Time (min)	% solvent B
0	15	0	15
3	15	45	50
15	55	48	55
25	55	50	55
30	15	53	15
45	15	60	15

Table S3 List of chemicals used.

Name	Quality grade	Manufacturer
2-propanol[1]	LC-MS grade	Fluka
apigenin[1]	≥95%	Sigma
artemicapin C[2]	≥95%	Phytolab
caffeic acid[1]	≥99%,	Fluka
coniferyl alcohol[1]	98%	Aldrich
coniferyl aldehyde[1]	98%	Aldrich
esculetin[1]	98%	Aldrich
esculin[1]	≥98%	Fluka
ferulic acid[1]	99%	Aldrich
formic acid[1]	98%	Fluka
fraxetin[1]	≥98%	Aldrich
fraxin[2]	≥98%	Phytolab
fraxinol[2]	≥99%	Phytolab
genistein[1]	≥98%	Sigma
genistin[1]	≥97.5%	Sigma
isofraxidin[1]	≥95%	Sigma
lithium hydroxide[1]	≥98%	Sigma
matairesinol[1]	≥85%	Fluka
methanol[1]	LC-MS grade	Fluka
myricetin[1]	≥96%	Sigma
myricitrin[1]	≥99%	Sigma
naringenin[1]	≥95%	Aldrich
nitric acid[1]	TraceSELECT®	Fluka
quercetin[1]	≥98%	Sigma
quercitrin[3]	91.7%	HWI Analytik
resveratrol[1]	≥99%	Sigma
scopoletin[1]	≥99%	Sigma
scopolin[2]	≥95%	Phytolab
sinapic acid[1]	≥98%	Aldrich
sinapic aldehyde[1]	98%	Aldrich
sinapyl alcohol[1]	80%	Aldrich
umckalin[2]	≥95%	Phytolab

[1]Chemicals purchased from Sigma-Aldrich (St. Louis, MO, USA). [2]Chemicals purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). [3]Chemical purchased from HWI Analytik GmbH (Ruelzheim, Germany).

Table S4 Operating conditions of the HCT Ultra ion trap mass spectrometer used for identification of phenolics.

Source	Electrospray
Nebulizer gas	N_2
Nebulizer gas pressure	40 psi
Drying gas	N_2
Drying gas (N_2) flow rate	9.0 l min^{-1}
Drying gas temperature	360 ºC
Polarity	negative or positive
Endplate voltage	-0.5 kV
Spray tip voltage	4 kV (for negative) or -4 kV (for positive)
Skimmer voltage	40 V (in negative) or -40 V (for positive)
Orifice voltage	117.3 V (in negative) or -117.3 V (for positive)
Operation mode	Multiple Reaction Monitoring (MRM)
Target for MRM mode	70,000
Maximum accumulation time for MRM mode	200 ms
Mass-to-charge ratio (*m/z*) range for MRM mode	100-1000 u
Fragmentation amplitude for MS^2 and MS^3	0.5-1.0 V
Isolation width for MS^2 and MS^3	1.0
Cutoff selection to precursor mass for MS^2 and MS^3	27.0%

Table S5 Primers used for qRT-PCR.

gene	ID	orientation	sequence
PP2	At1g13320	FW	TAACGTGGCCAAAATGATGC
		REV	GTTCTCCACAACCGCTTGGT
CCoAMT1	At4g34050	FW	GCTCCTCCTGATGCACCAAT
		REV	CCATCACCAACAGGGAGCAT
COMT	At5g54160	FW	TGCTCCTTCTCATCCTGGTAT
		REV	CACGCAATGTTCGTCACTCC
F6'H1	At3g13610	FW	GCCTGATATCTGCAGGAATGAAA
		REV	ACTCTAGAAGCCTCCTCACCA
FRO2	At1g01580	FW	GCGACTTGTAGTGCGGCTATG
		REV	CGTTGCACGAGCGATTCTTG
IRT1	At4g19690	FW	CGGTTGGACTTCTAAATGC
		REV	CGATAATCGACATTCCACCG
ABCG37 (PDR9)	At3g53480	FW	GCGAAACTCAGAGCTTGTGA
		REV	AGTGCGCCGAAGATCAAAGA

Table S6 Effects of Fe deficiency and high pH on the photosynthetic pigment concentrations (in µmol m^{-2}) in *Arabidopsis thaliana* young leaves. Plants were pre-grown for 11 d in the presence of 20 µM Fe (III)-EDTA at pH 5.5, and then grown for 14 d in a medium with 0 (-Fe) or 20 µM (+Fe) Fe(III)-EDDHA in nutrient solutions buffered at pH 5.5 (with 5 mM MES-NaOH) and 7.5 (with 5mM HEPES-NaOH). Data are means ± SE (n=2-3). For each compound, significant differences among treatments (at $p<0.05$) are marked with different letters in the same row.

	+Fe	-Fe	+Fe	-Fe
	pH 5.5		pH 7.5	
Neoxanthin	8.5 ± 0.6 b	3.4 ± 0.9 a	8.3 ± 0.2 b	4.3 ± 0.5 a
Violaxanthin	10.4 ± 1.6 b	4.4 ± 0.9 a	10.0 ± 0.2 b	4.7 ± 0.6 a
Antheraxanthin	0.00 ± 0.00 a	0.13 ± 0.08 a	0.00 ± 0.00 a	0.26 ± 0.17 a
Lutein	39.6 ±1.7 b	18.4 ± 4.4 a	38.2 ± 1.4 b	20.0 ± 1.5 a
β-carotene	16.4 ± 1.0 b	7.4 ± 2.4 a	16.8 ± 0.6 b	7.2± 0.9 a
Carotenoids / Chl total	0.26 ± 0.01 a	0.33 ± 0.04 a	0.27 ± 0.01 a	0.28 ± 0.01 a

Table S7 Effects of Fe deficiency and high pH on the Mn, Cu, and Zn contents (in µg metal $plant^{-1}$) in *Arabidopsis thaliana* shoots and roots. Plants were pre-grown for 11 d in the presence of 20 µM Fe (III)-EDTA at pH 5.5, and then grown for 14 d in a medium with 0 (-Fe) or 20 µM (+Fe) Fe(III)-EDDHA in nutrient solutions buffered at pH 5.5 (with 5 mM MES-NaOH) and 7.5 (with 5mM HEPES-NaOH). Data are means ± SE (n=2-5). For each metal, significant differences among treatments (at $p<0.05$) are marked with different letters in the same row.

	+Fe	-Fe	+Fe	-Fe
	pH 5.5		pH 7.5	
SHOOTS				
Mn	3.36 ± 0.42 ab	4.62 ± 0.80 b	2.03 ± 0.16 a	2.3 ± 0.10 a
Cu	0.19 ± 0.00 a	1.22 ± 0.17 b	0.18 ± 0.01 a	0.45 ± 0.10 a
Zn	2.90 ± 0.22 ab	4.29 ± 0.56 b	1.71 ± 0.06 a	1.23 ± 0.09 a
ROOTS				
Mn	0.36 ± 0.11 a	1.98 ± 0.92 ab	5.09 ± 2.25 c	4.02± 1.94 bc
Cu	0.05 ± 0.01 a	0.66 ± 0.42 a	0.04 ± 0.00 a	0.36 ± 0.20 a
Zn	2.18 ± 0.70 a	1.79 ± 0.25 a	1.19 ± 0.28 a	0.88 ± 0.02 a

Table S8 Mass losses of the MS^2 ion trap product ions, from their corresponding precursor $[M+H]^+$ ions, for the compounds *14-18* produced by *Arabidopsis thaliana* roots in response to Fe deficiency. The numbers in italics (#) refer to the labels used for each compound in Figure 3 and Table 1. Common product ions and mass losses among the MS^2 spectra of *15-18* are indicated in bold.

#	*14*		*15*		*16*		*17*		*18*	
Annotation	5'-Hydroxycleomiscosins A and/or B		Cleomiscosin D		Cleomiscosin C		Cleomiscosin B		Cleomiscosin A	
Parent ion *m/z*	403		417		417		387		387	
	Product ions *m/z*	Mass loss (Da)	Product ions *m/z*	Mass loss (Da)	Product ions *m/z*	Mass loss (Da)	Product ions *m/z*	Mass loss (Da)	Product ions *m/z*	Mass loss (Da)
	385	18	387	**30**	387	**30**	357	**30**	357	**30**
	373	30	367	**50**	367	**50**	337	**50**	337	**50**
	279	124	263	154	263	154	263	124	263	124
	251	152	233	184	233	184	233	154	233	154
	221	182	221	196	221	196	**209**	178	**209**	178
	209	194	**209**	208	**209**	208	161	226	223	164
	151	252	193	224	192	225	131	**256**	162	225
	137	266	161	**256**	161	**256**			131	**256**
			133	284	131	286				

Supplementary Material II

Accumulation and Secretion of Coumarinolignans and other Coumarins by *Arabidopsis thaliana* Roots in Response to Iron Deficiency at High pH

This Supplementary Material II includes results of the preliminary experiments performed to obtain the plant growth conditions used in Chapter 3.2. Different pre-growth and Fe deprivation periods and Fe supplies were tested to obtain chlorotic plants.

Fig. SII.1 Experimental designs used for growing *Arabidopsis thaliana* plants.

Fig. SII.2 Shoot and root biomass of *Arabidopsis thaliana* plants.

Fig. SII.3 Pictures of *Arabidopsis thaliana* plants.

Fig. SII.4 Leaf chlorophyll concentrations in young leaves of *Arabidopsis thaliana* plants

Fig. SII.5 Coumarins, coumarin precursors, and coumarinolignans levels in roots of *Arabidopsis thaliana* plants.

Fig. SII.6 Coumarins and coumarinolignans levels in the nutrient solution of iron-deficient of *Arabidopsis thaliana* plants.

Fig. SII.1 Experimental designs used for growing *Arabidopsis thaliana* plants. Plants were germinated for 10 days (marked in a gray box) as described in Materials and Methods in Chapter 3.1 and pre-growth (marked with a solid black line) with 45 µM Fe (III)-EDTA at pH 5.5 for 29 d (experiments I and II) or 17 d (experiment III). Then, plants were treated (marked with a dashed black line) with 0 (-Fe) or 45 µM (+Fe) Fe (III)-EDDHA for 7 d (I), 14 d (II) or 21 d (III). During the treatment period (for both Fe supply regimes) the nutrient solutions were pH 5.5 unbuffered (I and II), pH 5.5 buffered (with 5 mM MES-NaOH; I, II and III) or pH 7.5 buffered (with 5mM HEPES-NaOH; II and III). For a given experimental design, a filled rectangle indicates the nutrient solution pHs used, whereas an empty rectangle indicates the pH did not used.

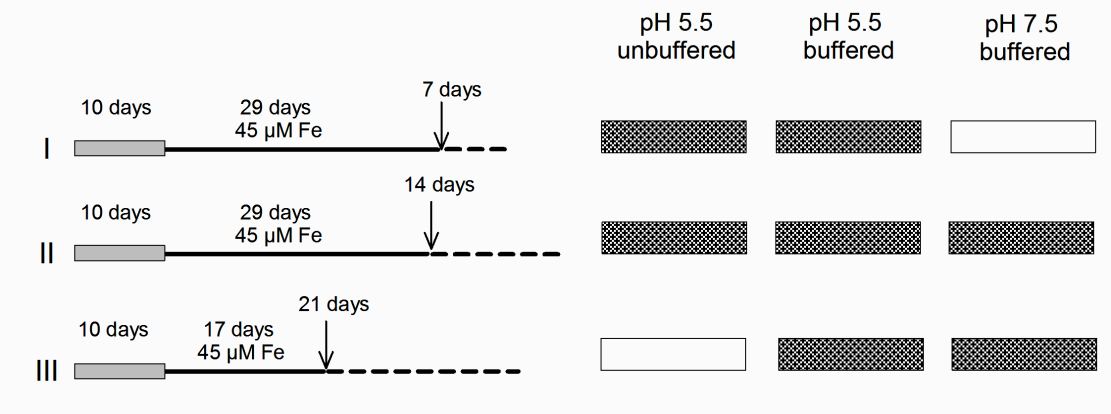


Fig. SII.2 Shoot and root biomass (fresh weight in g per plant) of control (+Fe) and Fe-deficient (-Fe) *Arabidopsis thaliana* plants of experiments I, II and III. Plants were sampled at the end of the treatment period. The plant growth conditions are described in Figure SII.1. Data are means ± SE (n=2-3). Significant differences among treatments (at $p < 0.05$) are marked with different letters above the columns.

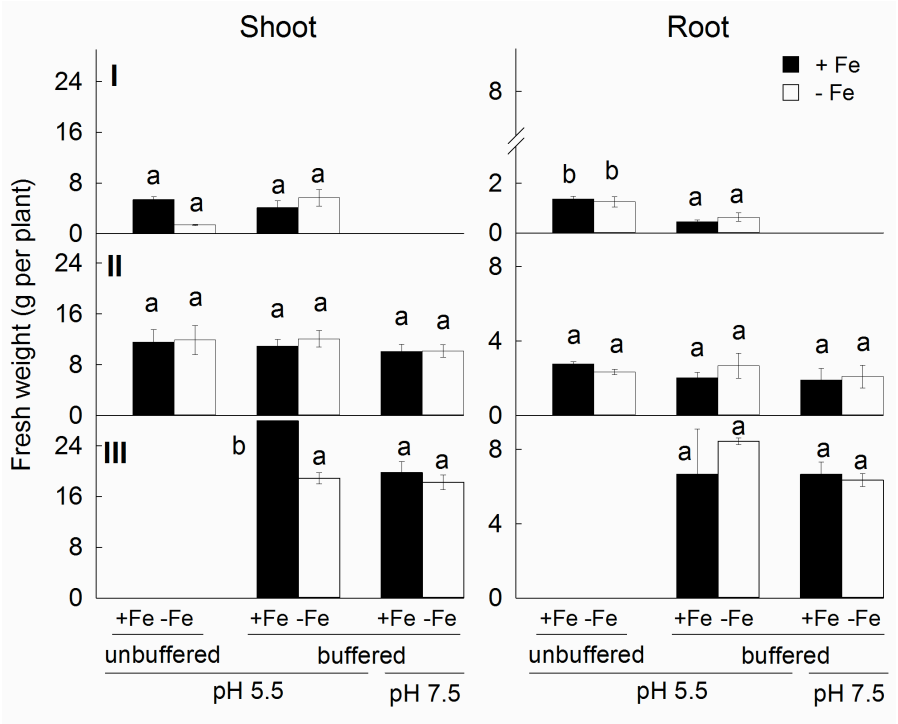


Fig. SII.3 Pictures of control (+Fe) and Fe-deficient (-Fe) *Arabidopsis thaliana* plants of experiments I, II and III. Pictures were taken at the end of the treatment period. The plant growth conditions are described in Figure SII.1.

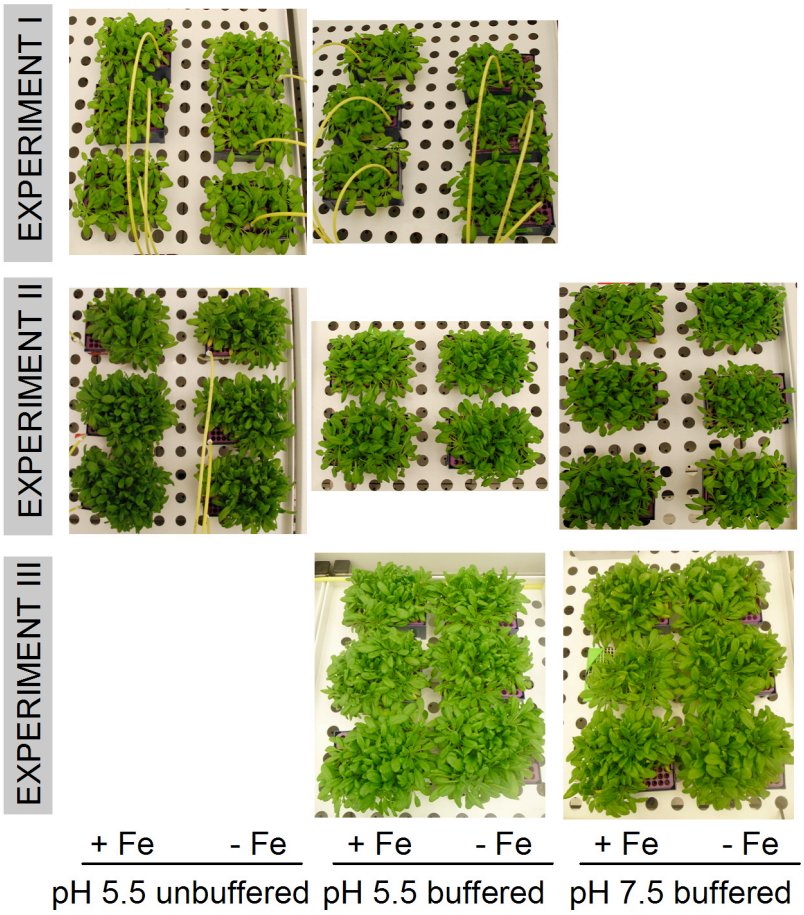


Fig. SII.4 Leaf total chlorophyll concentrations (in µmol m^{-2}) in young leaves of control (+Fe) and Fe-deficient (-Fe) *Arabidopsis thaliana* plants of experiments I, II and III. Plants were sampled at the end of the treatment period. The plant growth conditions are described in Figure SII.1. Data are means ± SE (n=2-3). Significant differences among treatments (at $p < 0.05$) are marked with different letters above the columns.

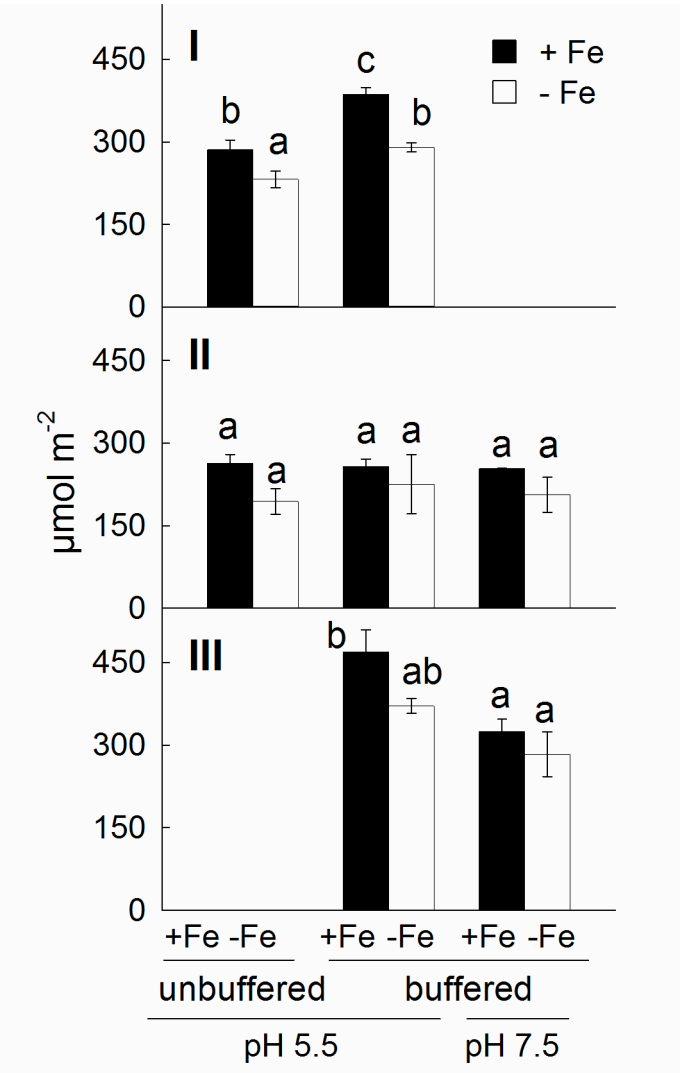


Fig. SII.5 Concentrations (in nmol g^{-1} root FW) of coumarins, coumarin precursors, and coumarinolignans in roots of control (+Fe) and Fe-deficient (-Fe) *Arabidopsis thaliana* plants of experiment II. Plants were sampled at the end of the treatment period. The plant growth conditions are described in Figure SII.1. Coumarins and their precursors were: scopolin (a), fraxetin hexoside (b), fraxinol and isofraxidin hexoside (c), coniferyl aldehyde hexoside (d), ferulic acid hexoside (e), sinapyl aldehyde hexoside (f), scopoletin (g), fraxetin (h), fraxinol and isofraxidin (i). The coumarinolignans were: cleomiscosin A (j), cleomiscosin B (k) and cleomiscosin C (l). The concentrations of ferulic acid hexoside are expressed as fraxin. The cleomiscosin levels are expressed in peak area ratio, relative to the lignan matairesinol used as internal standard. Data are means ± SE (n=3-5).

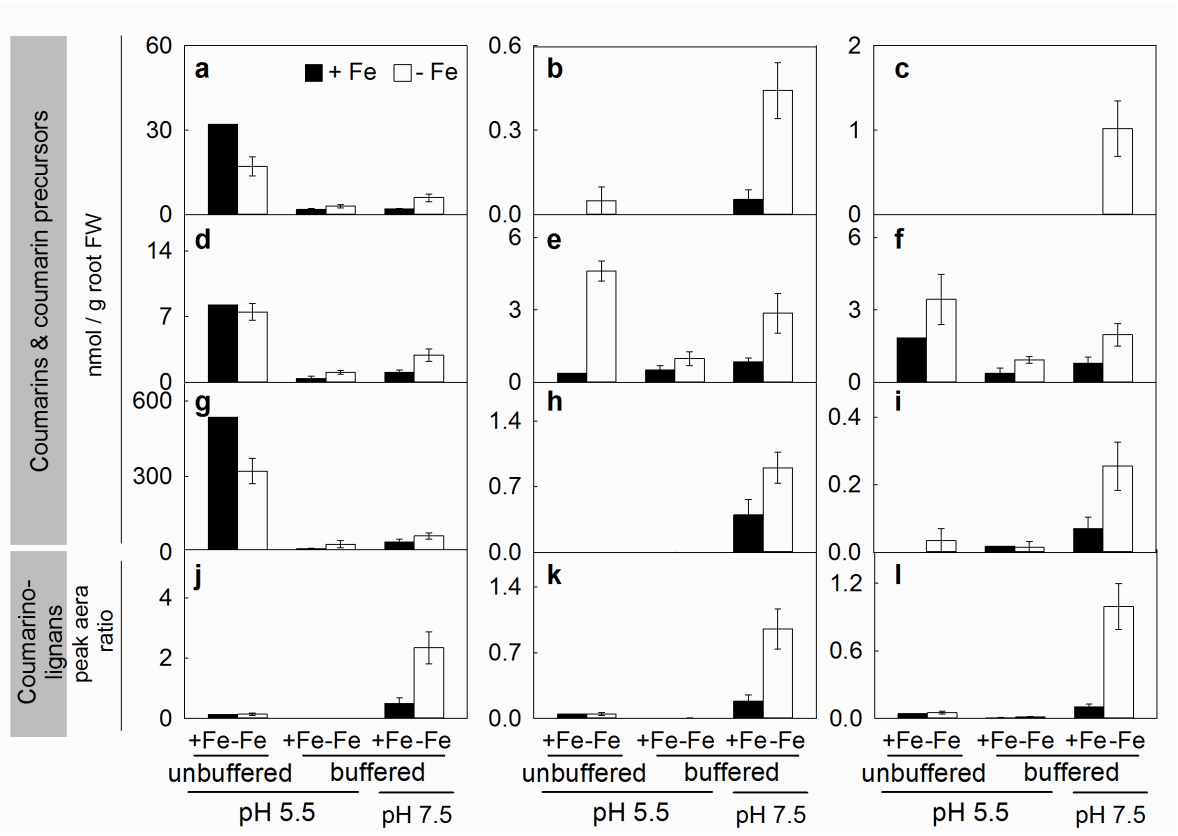
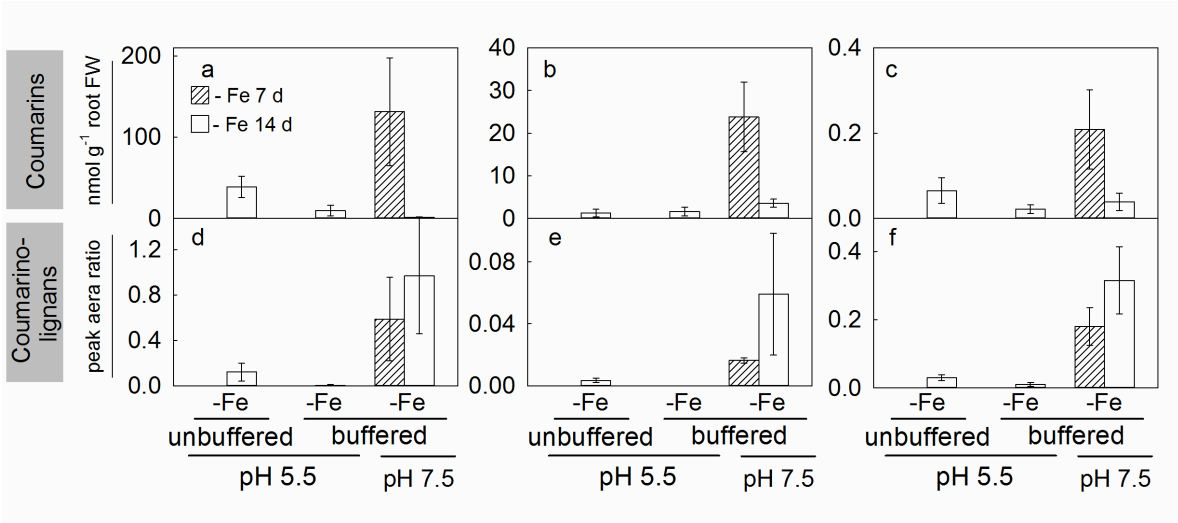


Fig. SII.6 Concentrations (in nmol g^{-1} root FW) of coumarins and coumarinolignans in the nutrient solutions of control (+Fe) and Fe-deficient (-Fe) *Arabidopsis thaliana* plants of experiment II. Nutrient solutions were sampled at 7 and 14 d. The plant growth conditions are described in Figure SII.1. Coumarins were: scopoletin (a), fraxetin (b), fraxinol and isofraxidin (c). The coumarinolignans were: cleomiscosin A (d), cleomiscosin B (e) and cleomiscosin C (f). The cleomiscosin levels are expressed in peak area ratio, relative to the lignan matairesinol used as internal standard. Data are means ± SE (n=3-5).



3.3. Coumarin accumulation in roots of iron-deficient tomato (*Solanum lycopersicum*)

Coumarin Accumulation in Roots of Iron-Deficient Tomato (*Solanum lycopersicum*)

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Summary

The Solanaceae species tomato (*Solanum lycopersicum*) is widely used as a model crop species for the investigation of Strategy I mechanisms. Prompted by the similarities between the Fe acquisition mechanisms of *A. thaliana* and *S. lycopersicum*, and as a follow-up of our previous studies on *A. thaliana*, we present in this study the characteristics of the root synthesis, accumulation and secretion of phenolics in *S. lycopersicum*. The root extracts and exudates from Fe-sufficient and Fe-deficient *S. lycopersicum* plants of three different genotypes, "Tres Cantos", FER and the mutant *fer*, were analyzed for phenolics and flavins, using advanced HPLC-MS-based methods specifically designed to detect, identify and quantify such compounds. Results show that *S. lycopersicum* behaves as *A. thaliana* in that accumulates in the roots coumarin-type phenolics in response to Fe deficiency, with the transcription factor FER being required. Genotype and Fe deficiency time affected quantitatively, but not qualitatively, the coumarin profile. In the conditions used in the present study, *S. lycopersicum* did not secrete coumarins to the growth media, but instead accumulated hexosylated forms of highly oxygenated coumarins in roots, as it occurs in the *abcg37 A. thaliana* mutant that lacks the plasma membrane transporter protein necessary for coumarin secretion. This may be related to the *S. lycopersicum* low tolerance to Fe deficiency. Also, *S. lycopersicum* did not produce flavins in response to Fe deficiency, thus differing from several other Solanaceae species, supporting the view that the production of phenolics and flavins in response to Fe deficiency is mutually exclusive.

Introduction

Iron (Fe) is essential for plant growth and development. Soils are usually rich in Fe, but in well-aerated and alkaline ones the availability of Fe for plant uptake is very limited. All plants except members of the monocotyledonous family *Poaceae* use a Fe reduction-based mechanism (also termed Strategy I) for acquisition of Fe from the soil. This mechanism includes the release of protons by a H^+-ATPase into the rhizosphere to increase the soil solubility of Fe(III), the reduction of soluble Fe(III) to Fe(II) on the root surface by an Fe(III) chelate reductase (FCR) and the subsequent plant uptake of Fe(II) by Fe(II) transporters (Marschner, 2012). Other elements of this Strategy are the secretion by roots of compounds called Fe facilitators (Sisó-Terraza y col., 2016a), including phenolics, carboxylates and flavins (Cesco *y col.*, 2010; Mimmo *y col.*, 2014), as well as morphological root changes such as the formation of transfer cells and an increased root hair formation (Li *y col*, 2016).

In the two past decades, many of the key components for increased Fe uptake have been identified at the molecular level, mainly in the model plant *Arabidopsis thaliana* and also in some crop species such as tomato (*Solanum lycopersicum*). In *A. thaliana*, transcripts of the H^+-ATPase gene *AtAHA2*, the Fe(III) reductase gene *AtFRO2* and the Fe(II) transporter gene *AtRT1* accumulate upon Fe deficiency in roots (Eide *y col.* 1996; Robinson *y col.* 1999; Vert *y col.* 2002, 2003; Santi and Schmidt, 2009), and the mutants *fro2* and *irt1* showed strong leaf chlorosis and growth impairment. The *AtAHA2*, *AtFRO2* and *AtIRT1* genes are subject to complex transcriptional and post-translational regulation, and the basic helix-loop-helix (bHLH) transcription factor FIT (FE-DEFICIENCY INDUCED TRANSCRIPTION FACTOR1, also known as FRU and bHLH29) controls them (along with other genes in a subset of

Fe-regulated ones) in *A. thaliana* (Ivanov *y col.*, 2012). Also in *A. thaliana*, recent studies have unveiled that Fe deficiency induces the root synthesis and secretion into the rhizosphere of coumarin-type phenolics (Fourcroy *y col.*, 2014; Schmid *y col.*, 2014; Schmidt *y col.*, 2014; Sisó-Terraza y col., 2016a,b) and that this is critical for Fe uptake from scarcely bioavailable Fe sources (Rodríguez-Celma *y col.*, 2013). The 2-oxoglutarate-dependent dioxygenase enzyme feruloyl-CoA6'-hydroxylase1 gene (*AtF6'H1*) which encodes a key enzyme in the synthesis of the coumarin scopoletin (Kai *y col.*, 2008), increases with Fe deficiency in *A. thaliana* roots (Schmid *y col.*, 2014; Schmidt *y col.*, 2014) and is also regulated by FIT (Schmid *y col.*, 2014). The plasma membrane-bound ABC (ATP-binding cassette) transporter, ABCG37/PDR9, expressed predominantly in the lateral root cap and in epidermal cells of the root tip (Ito and Gray, 2006), is involved in the root-secretion of coumarins in Fe-deficient *A. thaliana* and its transcript accumulates in roots upon Fe deficiency (Fourcroy *y col.*, 2014). In Fe-deficient *Atpdr9* plants roots accumulate glycosylated forms of an array of coumarins, whereas the growth media is almost coumarin-free (Fourcroy *y col.*, 2014). The mutants *Atf6'h1-1* and *Atpdr9-2* show severe reductions in growth and Fe contents as well as leaf chlorosis when grown on media containing insoluble Fe compounds, but not when grown on media containing soluble Fe (Rodríguez-Celma *y col.*, 2013).

In *S. lycopersicum*, the transcripts of the Fe reductase gene *SlFRO1*, the metal transporter genes *SlIRT1* and *SlNRAMP1* (Eckhardt *y col.* 2001; Bereczky *y col.* 2003; Li *y col.* 2004) and the bHLH-type transcriptional factor FER (a functional FIT ortholog) are also increased in roots in response to Fe deficiency (Ling *y col.*, 2002; Bauer *y col.*, 2004; Brumbarova and Bauer, 2005). FER is essential for the up-regulation of Fe mobilization responses in *S. lycopersicum* roots, with *fer* mutant plants being inefficient in taking up Fe from the soil into the root and unable to induce the root expression of *SlFRO1* and *SlIRT1* (Brown *y col.*, 1971; Ling *y col.*, 2002; Bauer *y col.*, 2004). Also, it was reported in the 1980's that Fe-deficient *S. lycopersicum* plants secrete into the growth media compounds capable of reducing Fe(III) to Fe(II) (Brown *y col.*, 1971; Brown y Ambler, 1974; Olsen *y col.*, 1981; Hether *y col.*, 1984), whereas the *fer* mutant was defective in the production/exudation of Fe reductants (Brown *y col.*, 1971; Brown and Ambler, 1974; Olsen *y col.*, 1981). The intermediate phenylpropanoid pathway metabolite *para*-coumaric acid, but not *meta*- or *ortho*-coumaric acid, was a precursor for the production of these Fe reductants by Fe-deficient *S. lycopersicum* roots (Olsen *y col.*, 1981). At that time, the major components of the Fe reductant fraction of Fe-deficient *S. lycopersicum* growth media were putatively identified, using high performance liquid chromatography (HPLC) separation coupled to UV-visible detection, as two phenolic compounds, caffeic acid (Olsen *y col.*, 1981) and chlorogenic acid (Hether *y col.*, 1984); from a current point of view, this analytical approach could be considered as poorly selective, since many of the *ca*. 10,000 individual phenolic compounds already known to exist in plants share similar UV/VIS properties (Croteau *y col.*, 2000).

Prompted by the similarities between the Fe acquisition mechanisms of *A. thaliana* and *S. lycopersicum*, and in continuation of our previous studies on *A. thaliana*, we present here the characteristics of the root synthesis, accumulation and secretion of phenolics in the economically significant crop species *S. lycopersicum*. Root extracts and exudates from Fe-sufficient and Fe-deficient *S. lycopersicum* plants of three different genotypes ("Tres Cantos", FER and the mutant *fer*), have been analyzed by HPLC coupled to photodiode array and mass spectrometry (MS) detectors. Coumarins were found to accumulate in Fe-deficient *S. lycopersicum* roots, and this accumulation was dependent of *FER* gene. The coumarin profile of Fe-deficient *S. lycopersicum* roots differ from that reported for *A. thaliana*, since in *S. lycopersicum* the hexosides of highly oxygenated coumarins such as dihydroxydimethoxycoumarin and trihydroxymethoxycoumarin were prominent. In addition, in contrast with what occurred in the growth media of Fe-deficient *A. thaliana* plants, in the conditions used in the present study the growth media of Fe-deficient *S. lycopersicum* plants were coumarin-free, as it occurs in the *abcg37 A. thaliana* mutant. On the other hand, *S. lycopersicum* was different

from other several Solanaceae species in that it did not respond to Fe deficiency by enhancing the root accumulation and/or secretion of flavins.

Materials and methods

Plant culture and experimental design

Tomato (*S. lycopersicum* Mill) seeds from the cv. "Tres Cantos", the Fe uptake-inefficient mutant [T3238*fer* (*fer*)] and its wild type [T3238 (FER)] were germinated, pre-grown and grown in a controlled environment chamber (Fitoclima 10000 EHHF, Aralab, Albarraque, Portugal), at 25ºC, 70% relative humidity and a photosynthetic photon flux density of 400 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR) with a photoperiod of 16 h light/8 h dark. The cv. "Tres Cantos" was purchased from Semillas Fito S. A. (Barcelona, Spain) and the genotypes FER and *fer* were multiplied from seeds generously provided by Dr. Frits Bienfait. Seeds were germinated and grown in vermiculite for 10 days.

For pre-growth, seedlings were transferred into 10-L plastic buckets containing aerated half-strength Hoagland nutrient solution, pH 5.5, with 45 µM Fe(III)-ethylenediaminetetraacetate [Fe(III)-EDTA], and grown for 10 d. During this period, the Fe-inefficient *fer* plants were supplied with Fe by foliar-sprays 2-3 days with 2-5 mM of $FeSO_4$ and 0.2% of a non-ionic, organo-silicon surfactant (Break-Thru S 233, Evonik Industries AG, Essen, Germany). In absence of this treatment, plants developed strong leaf chlorosis.

Then, plants were grown as follows: i) "Tres Cantos" plants were cultivated for 17 d in 10 L plastic buckets (8 plants per bucket) in unbuffered nutrient solution (initial pH 5.5), with (45 µM Fe(III)-EDTA; Fe-sufficient) or without Fe (Fe-deficient), with nutrient solutions being renewed daily. Both nutrient solutions and plants (four plants grown in the same bucket) were sampled at days 6, 11, 14 and 17 after the onset of Fe deficiency treatment; ii) FER and *fer* plants were grown for 10 d in 4-L plastic buckets (4 plants per bucket) in nutrient solution with (45 µM Fe(III)-EDDHA; Fe-sufficient) or without Fe (Fe-deficient), buffered at pH 7.5 with 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid)-NaOH. Nutrient solutions were not renewed during the experiment. Iron-sufficient *fer* plants were also foliar-treated every 2-3 days with Fe as indicated above. Nutrient solutions and plants were sampled at days 5 and 10 after the onset of Fe deficiency treatment. Two batches of plants were grown and analyzed.

In all cases, the development of Fe deficiency was assessed in young leaves using a SPAD 502 device (Minolta, Osaka, Japan). At each sampling time, nutrient solutions (aliquots of 0.5 L) were immediately stored at -20ºC until extraction for flavins and phenolics analyses, shoot and root mass were determined, and then roots were washed with tap water and after with type I water, dried with filter paper, frozen immediately (in aliquots of approximately 800 mg) in liquid N_2 and stored at -80ºC until extraction for flavins and phenolics analyses. Pots without plants, containing only aerated nutrient solution (with and without Fe) were also placed in the growth chamber and the nutrient solutions sampled as in pots containing plants; these samples were later used as blanks for root exudate analyses.

Root microscopy

FER and *fer* root pieces were observed at x7.3 magnification with a digital stereo microscope (DFC 420C, Leica Microsystems GmbH, Wetzlar, Germany, with a 5 megapixel CCD). Fluorescence was detected with a filter set GFP Leica MZ FLIII (including a 440/70 nm filter for excitation and a 500/50 nm filter for emission).

Extraction of flavins and phenolic compounds from roots and nutrient solutions

Flavins and phenolic compounds were extracted from roots and nutrient solutions as described in Chapter 3.2, with some modifications. First, an extraction was carried out without adding internal standards (IS) to identify relevant compounds, including those increasing (or appearing) with Fe deficiency. This extract was also used to check for the possible presence of the compounds used as IS and of other endogenous isobaric compounds

that may coelute with them, since analytical interferences in the quantification process would exist in both cases. Then, the extraction was carried out adding three IS compounds: i) artemicapin C (Chapter 3.2 Fig. 1d), a methylenedioxycoumarin, for quantification of the coumarins fraxetin, dihydroxydimethoxycoumarin, scopoletin and fraxinol (compounds *12*, *13*, *14* and *17* in Table 1, respectively); ii) esculin (Chapter 3.2 Fig. 1a), the glucoside form of the coumarin esculetin, for quantification of coumarin glycosides, and iii) the hydroxycinnamic acid isoferulic acid, for quantification of ferulic acid.

Frozen roots (*ca*. 200 mg) were ground in liquid N_2 for 3 min using a Retsch M301 ball mill (Restch, Dusseldorf, Germany) and then phenolic compounds were extracted with 1 ml of 100% LC-MS grade methanol, either alone or supplemented with 20 µl of a solution containing the three IS (50 µM artemicapin C, 50 µM esculin and 250 µM isoferulic acid) by homogenization in the same mill for 5 min. The supernatant was recovered by centrifugation (12,000 x*g* at 4°C and 5 min), and stored at -20°C. The pellet was re-suspended in 1 ml of 100% methanol, homogenized again for 5 min and the supernatant recovered. After that, the pellet was re-suspended again in 1 ml of 100% methanol, homogenized again for 5 min and the supernatant recovered. The three supernatant fractions were pooled, vacuum dried in a SpeedVac (SPD111V, Thermo-Savant, Thermo Fisher Scientific, Waltham, Massachusetts, MA, USA) and dissolved with 250 µl of a solution containing 15% methanol and 0.1% formic acid. Extracts were filtered through polyvinylidene fluoride (PVDF) 0.45 µm ultrafree-MC centrifugal filter devices (Millipore) and stored at -80°C until analysis.

In the case of nutrient solutions, flavins and phenolics were extracted from 500 ml of solution as described in Chapter 3.1.

Analysis of flavins and phenolics by HPLC-UV/VIS/ESI-MS(TOF)

Flavins and phenolics were analyzed by HPLC-UV/VIS/ESI-MS(TOF) using an Alliance 2795 HPLC system (Waters) coupled to a UV/VIS (Waters PDA 2996) detector and a time-of-flight mass spectrometer [MS(TOF); MicrOTOF, Bruker Daltonics, Bremen, Germany] equipped with an electrospray (ESI) source. Separations were performed using an analytical HPLC column (Symmetry® C_{18}, 15 cm x 2.1 mm i.d., 5 µm spherical particle size, Waters) protected by a guard column (Symmetry® C_{18}, 10 mm x 2.1 mm i.d., 3.5 µm spherical particle size, Waters) and a gradient mobile phase built with 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in methanol. Chromatographic conditions including the elution program are described in Chapter 3.2 (Elution program 2; Supplementary Table S2). ESI-MS (TOF) operating conditions and software used were as described in Chapters 3.1 and 3.2. Mass spectra were acquired in positive and negative ion mode in the range of 50-1000 mass-to-charge ratio (*m/z*) units. Concentrations of phenolics were quantified using external calibration with internal standardization. For many compounds there was no commercially available standard, with only a few exceptions: the coumarins fraxetin, scopoletin and fraxinol (compounds *12*, *14* and *17*, respectively in Table 1), the coumarin glucoside scopolin (compound *5* in Table 1) and the scopoletin precursor ferulic acid (compound *16* in Table 1). Some of the remaining compounds were not quantified because they had low signals, either below or close to the limit of quanti- fication (compounds *2**, *3**, *9**, *10** and *15*). Other compounds were quantified tentatively using the following structurally related molecules (see structures in Chapter 3.2 Fig. 1a,b): i) fraxin (7,8-dihydroxy-6-methoxycoumarin 8-O-glucoside) for trihydroxymethoxycoumarin dihexoside (compound *1**), dihydroxymethoxycoumarin hexoside (compound *4**) and trihydroxymethoxycoumarin hexoside (compound *8**); ii) scopolin (7-hydroxy-6-methoxycoumarin 7-glucoside) for dihydroxydimethoxyscoumarin hexoside (compound *7**) and hydroxyldimethoxycoumarin hexoside (compound *11**); iii) scopoletin (7-hydroxy-6-methoxycoumarin) for dihydroxydimethoxycoumarin (compound *13*), and iv) ferulic acid for ferulic acid hexoside (compound *6**). Quantification was done using the peak areas of analytes and ISs obtained from chromatograms extracted at the *m/z* (± 0.05) ratios corresponding to the $[M+H]^+$ ions, with the exception of hexosides and dihexosides, for which the *m/z* ratios corresponding to $[M\text{-hexose}+H]^+$ ions were used.

Analysis of phenolics by HPLC/ESI-MS(Ion trap)

HPLC/ESI-MS(Ion trap) analysis was carried out as described in Chapter 3.2.

Results

Changes in leaf chlorophyll with Fe deficiency

Leaf chlorophyll concentration was assessed using SPAD values in young leaves of the three *S. lycopersicum* genotypes ("Tres Cantos", FER and *fer*) during the treatment period (Fig. 1), 17 days for "Tres Cantos" and 10 days for FER and *fer*. In "Tres Cantos" plants the SPAD values of Fe-sufficient leaves increased slightly with time, and were in the range 35-43, whereas in Fe-deficient plants the leaf SPAD values decreased from 36 at day 0 to 18 at day 17 (Fig. 1a).

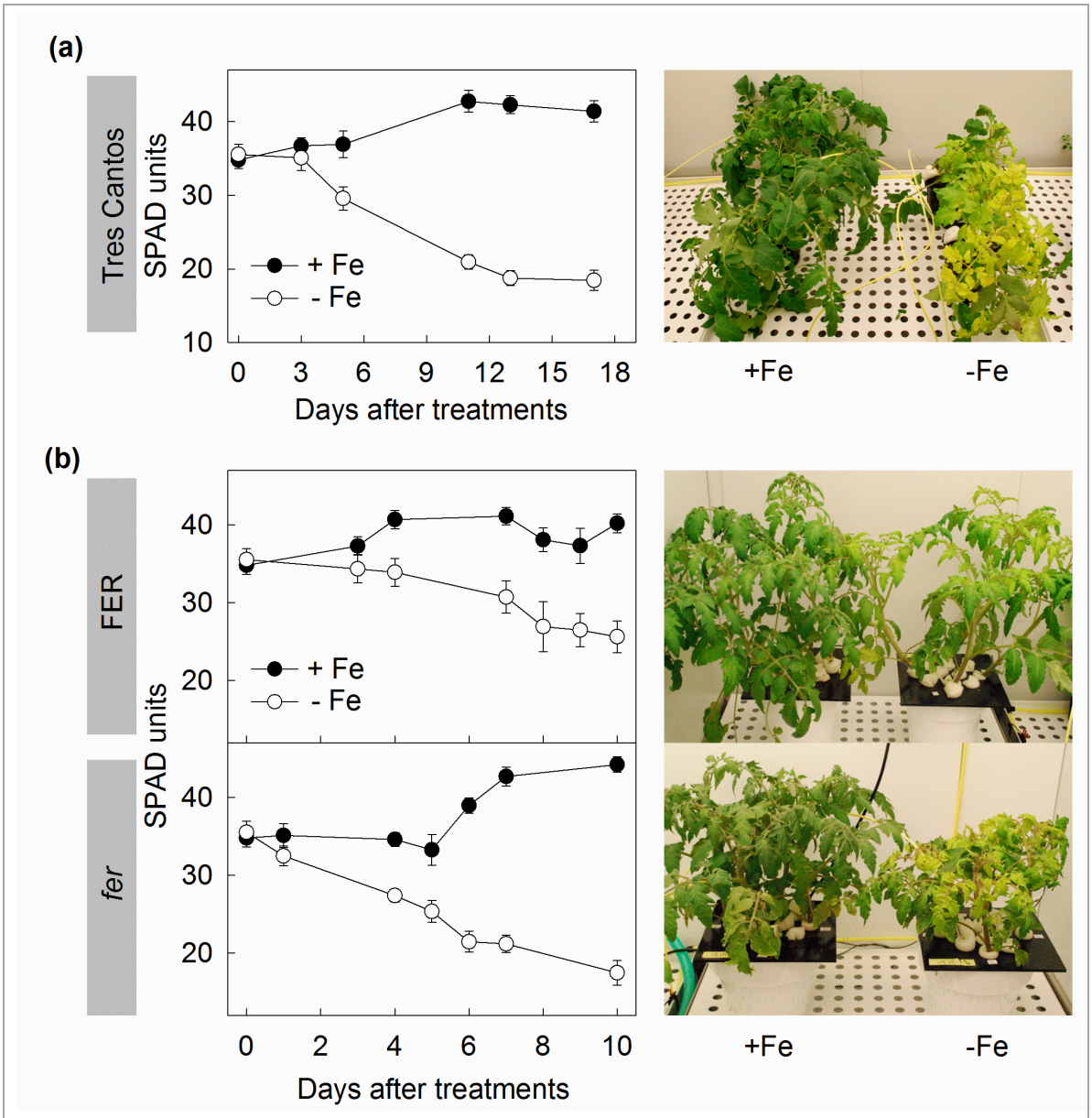

Figure 1 Time-course of the SPAD values of young leaves of tomato plants as affected by Fe deficiency. (a) Tres Cantos plants grown for 17 days in nutrient solution with 0 (-Fe) or 45 µM Fe (+Fe) Fe and without Fe (-Fe) and adjusted to pH 5.5. Data are means ± SE (n = 8 plants). Pictures of plants taken at day 14 after imposing treatments. (b) FER and *fer* plants grown for 10 days in nutrient solution with 0 (-Fe) or 45 µM Fe (+Fe) buffered at pH 7.5 with 5 mM HEPES-NaOH. Data are means ± SE (n = 8 plants). Pictures of plants taken at day 10 after imposing treatments.

Therefore, from day 5 on the SPAD reading in Fe-deficient leaves was 7 to 24 units lower than in the leaves of the corresponding Fe-sufficient plants, with the highest relative decrease occurring at day 13. Pictures of "Tres Cantos" plants at day 14 are shown in Fig. 1a.

In FER plants the SPAD values of Fe-sufficient leaves varied only slightly with time, and were in the range 35-41, whereas in Fe-deficient plants the leaf SPAD values decreased from 36 at day 0 to 26 at day 10 (Fig. 1b). Therefore, from day 4 on the SPAD in Fe-deficient leaves was 7 to 15 units lower than in the corresponding Fe-sufficient leaves, with the highest relative decrease occurring at day 10. Pictures of FER plants at day 10 are shown in Fig. 1b.

In *fer* plants the SPAD values in Fe-sufficient leaves were quite constant in the first 5 days of the experiment, in the range 33-35, and then increased to 44 at day 10 after an Fe foliar treatment. Iron foliar treatments (see above) are necessary for Fe-sufficient *fer* plants, since the roots of this mutant are completely inefficient in taking up Fe. In Fe-deficient plants the leaf SPAD values decreased gradually over time from 36 at day 0 to 17 at day 10. Therefore, the SPAD reading of Fe-deficient leaves at days 4-10 were 7-27 units lower than those of the corresponding Fe-sufficient leaves. Pictures of *fer* plants at Day 10 are shown in Fig. 1b.

Changes in biomass with Fe deficiency

The shoot and root biomass of the "Tres Cantos", FER, and *fer* plants were also assessed at different times after the onset of the Fe deficiency treatment (Fig. 2). In "Tres Cantos" plants the shoot biomass increased gradually over time, regardless of the Fe treatment, with the increases being much larger in Fe-sufficient than in Fe-deficient plants (Fig. 2a). The shoot biomass of Fe-sufficient plants increased from 29 (at day 6) to 193 FW plant^{-1} (at day 17), whereas in Fe-deficient plants it increased from 23 to 82 g FW plant^{-1} in the same time period. Therefore, Fe deficiency decreased the shoot biomass by 21 and 57% at days 6 and 17. The root biomass of Fe-sufficient and Fe-deficient "Tres Cantos" plants also increased over time, from 6 (at day 6) to 28-41 g FW plant^{-1} (at day 17) (Fig. 2a). The only decreases in root biomass with Fe deficiency were 24 and 26% at days 14 and 17, respectively, although these changes were not statistically significant.

In FER plants, the shoot biomass of Fe-sufficient plants increased with time from 24 (at day 5) to 56 g FW plant^{-1} (at day 10) (Fig. 2b). In Fe-deficient plants, shoot biomass also increased with time, from 13 (at day 5) to 46 g FW plant^{-1} (at day 10). Therefore, Fe deficiency decreased the shoot biomass by 45 and 18% at days 5 and 10, respectively. The root biomass of FER plants increased with time but was unaffected by Fe deficiency, with values of *ca.* 2 (at day 5) and *ca.* 12 g FW plant^{-1} (at day 10) (Fig. 2b).

In *fer* plants the shoot biomass of Fe-sufficient plants increased with time from 21 (at day 5) to 42 g FW plant^{-1} (at day 10) (Fig. 2b). In Fe-deficient plants the shoot biomass also increased with time from 19 (at day 5) to 27 g FW plant^{-1} (at day 10). The time-dependent increases in shoot biomass of Fe-sufficient plants were much larger than those in the corresponding Fe-deficient plants, and Fe deficiency decreased shoot biomass at day 10 (by 37%) but not at day 5. As in the case of the shoot biomass, the root biomass of *fer* plants increased with time for both treatments, from 4 g FW plant^{-1} (at day 5 for both Fe-sufficient and Fe-deficient plants) to 10 and 6 g FW plant^{-1} (at day 10 for Fe-sufficient and Fe-deficient plants, respectively) (Fig. 2b). Therefore, Fe deficiency did not decrease root biomass at day 5 but it did at day 10 (by 37%).

Changes in root morphology with Fe deficiency

Changes in root morphology were looked at only in the genotypes FER and *fer*. In the FER genotype, the secondary roots of Fe-deficient plants at day 10 were markedly shorter than those in Fe-sufficient plants and they showed also a more intense green fluorescence in the apical and basal areas (Fig. 3). In the *fer* mutant, the secondary roots of Fe-deficient plants at Day 10 were shorter and thicker than those of Fe-deficient FER plants. The green fluorescence was much more intense in secondary roots of Fe-deficient *fer* plants than in those of Fe-sufficient *fer* plants.

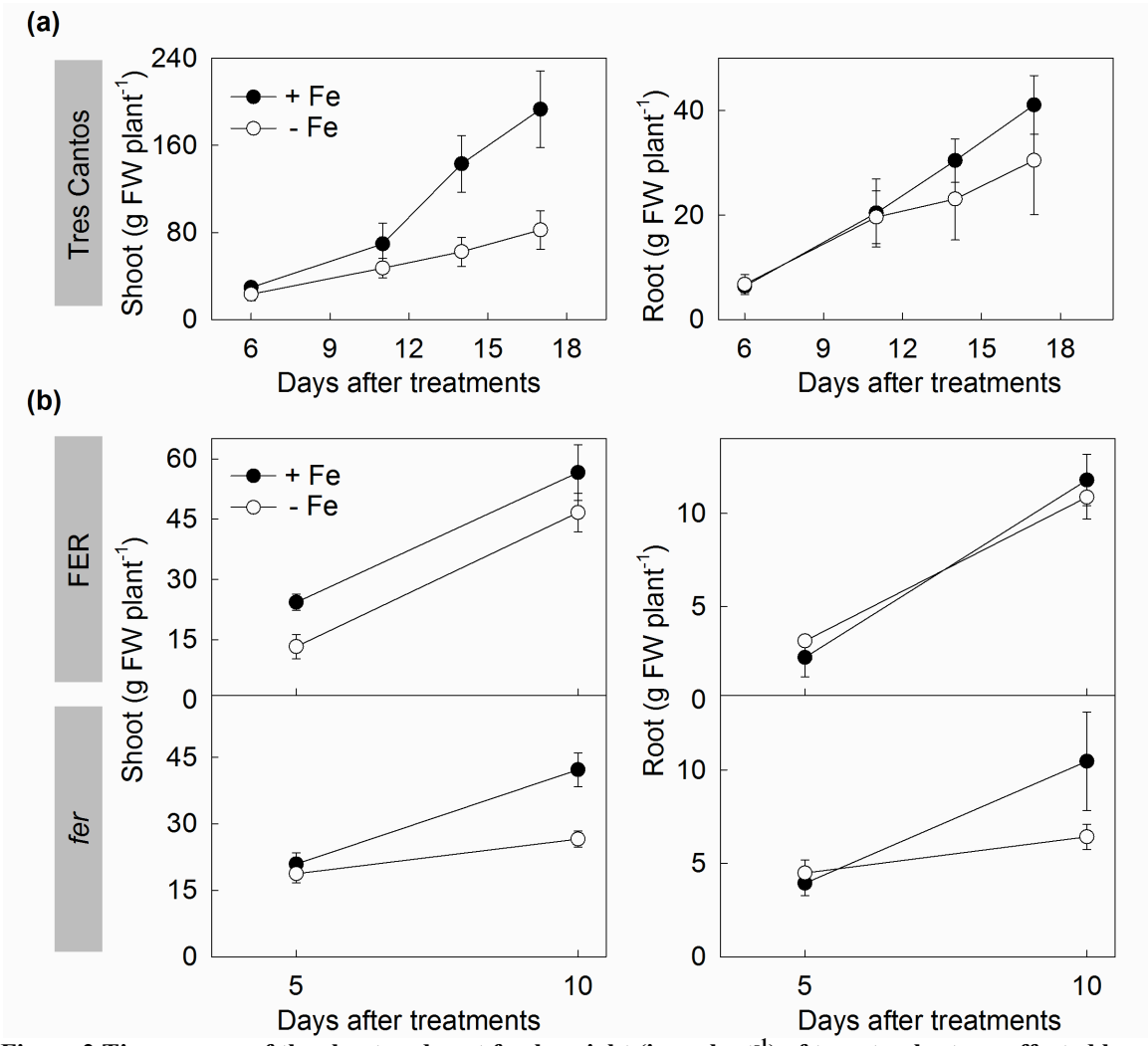

Figure 2 Time-course of the shoot and root fresh weight (in g plant^{-1}) of tomato plants as affected by Fe deficiency. Plants were grown as indicated in Fig. 1. (a) Fe-sufficient (+Fe) and Fe-deficient (-Fe) Tres Cantos plants. Data are means ± SE (n = 4 plants). (b) Fe-sufficient (+Fe) and Fe-deficient (-Fe) FER and *fer* plants. Data are means ± SE (n = 8 plants).

Tomato roots accumulate an array of phenolic-type compounds with Fe deficiency

The methanolic extracts of "Tres Cantos", FER and *fer* roots and those of their corresponding nutrient solutions were analyzed using the reverse phase C_{18} HPLC-based method described in Chapter 3.2 (Elution program 2 of Supplementary Table S2) coupled to UV/VIS detection in the range 200-600 nm. The elution program 2 separates phenolic-type compounds of different classes (Chapter 3.2 Supplementary Figs. S1 and S2) and flavin-type compounds (Fig. 4 shows the chromatogram obtained with a mixture of flavin standards). Both flavin-type and phenolic-type compounds absorb light in the UV region, with flavin-type compounds having absorption maxima in the ranges 310-400 and 400-490 nm and phenolic-type compounds (coumarins, their derivatives and precursors; *e.g.*, ferulic and other cinnamic acids) having absorption maxima in the range 290-330 nm. Therefore, both types of compounds can be detected at 320 nm, whereas at 445 nm only flavin-type compounds can be detected.

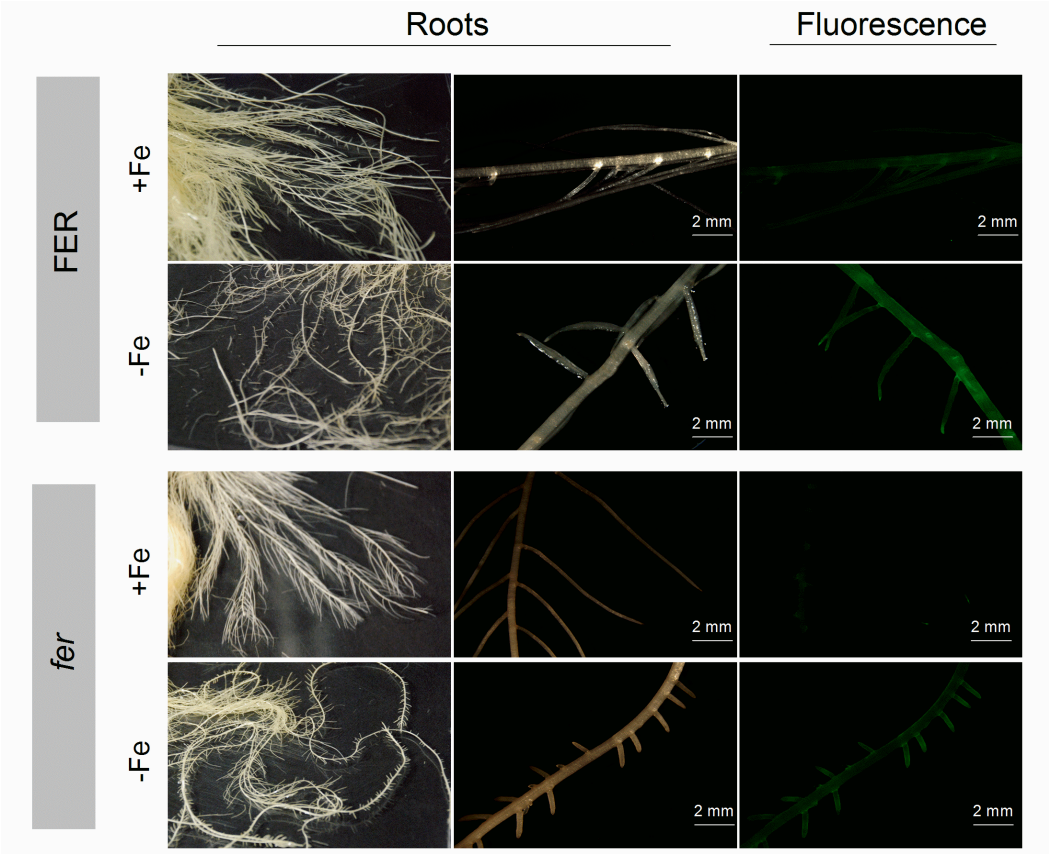

Figure 3 Appearance of roots (part of the root systems, excised root sections of *ca.* 15 mm) excised from tomato plants as affected by Fe deficiency. Iron-sufficient (+Fe) and Fe-deficient (-Fe) FER and *fer* plants were grown for 10 days as indicated in Fig. 1. Light microscopy and fluorescence microscopy views of the 15 mm-root sections are shown.

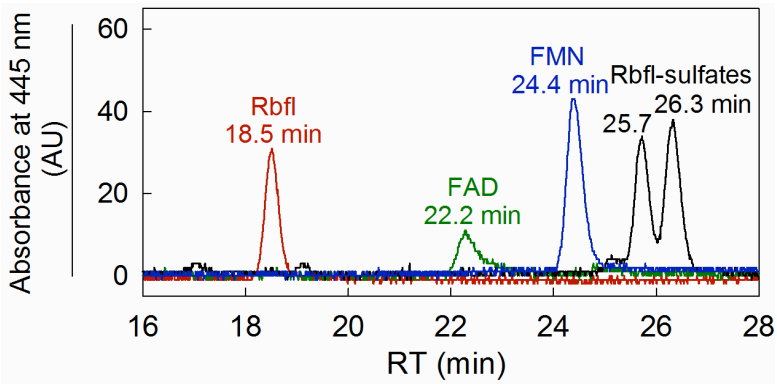

Figure 4 Chromatographic separation of riboflavin (Rbfl), Rbfl-5′-phosphate (FMN), flavin adenine dinucleotide (FAD), Rbfl-3′-sulfate and Rbfl-5′-sulfate (Rbfl-sulfates) using the Elution program 2 indicated in Chapter 3.2 (Supplementary Table S2). Absorbance chromatograms were extracted at a wavelength of 445 nm. AU, absorbance units and RT, retention time.

Root extracts of "Tres Cantos" and FER Fe-sufficient plants had absorbance chromatograms at 320 nm with either no peaks or only small peaks at 6 and 18.5 min, whereas those of Fe-deficient plants had peaks at approximately 6, 10, 13, 14, 16.5, 18.5, 22 and 24 min (Fig. 5). All these peaks are identified in sections below. In contrast, in the case of Fe-sufficient and Fe-deficient *fer* the root extract chromatograms at 320 nm were quite similar and had only small peaks in the range 3-25 min. The absorbance chromatograms at 445 nm of the same root samples are also shown in Fig. 5, and in all cases there was only a very minor peak at 18.5 min corresponding to riboflavin.

The chromatograms at 320 nm and 445 nm of the methanolic concentrated extracts of nutrient solutions did not show any peak, regardless of the genotype, Fe supply and time of Fe deficiency treatment (data not shown).

Therefore, Fe deficiency induced the synthesis and root accumulation of phenolic-type compounds in *S. lycopersicum* plants, without any apparently secretion of these compounds to the nutrient solution. On the other hand, the lack of phenolic compounds in the extracts of *fer* roots indicates that this synthesis was dependent on the Fe deficiency-induced transcription factor FER.

Identification of the phenolic-type compounds induced by Fe deficiency as coumarins and coumarin precursors

To identify the compounds found in the Fe-deficient "Tres Cantos" and FER root extracts, samples were analyzed using two HPLC-UV/VIS/ESI-MS(TOF) protocols described in Chapter 3.2, including Elution program 2 (Chapter 3.2 Supplementary Table S2) and two electrospray (ESI) ionization modes (positive and negative). These HPLC/ESI-MS(TOF) analyses provided highly accurate (error ≤5 ppm) measurements of the mass-to-charge (*m/z*) ratio of the detected ions, therefore allowing for accurate elemental formulae assignments (Bristow 2006). Raw datasets (time, *m/z* and ion intensity) were analyzed as described in Chapter 3.2.

From a total of approximately 125 possible mass spectral features analyzed per run and sample, only 17 complied with the following two requirements: i) occurring at chormatographic RTs where absorbance at 320 nm was observed, and ii) showing peak area increases (or appearing) with Fe-deficiency.

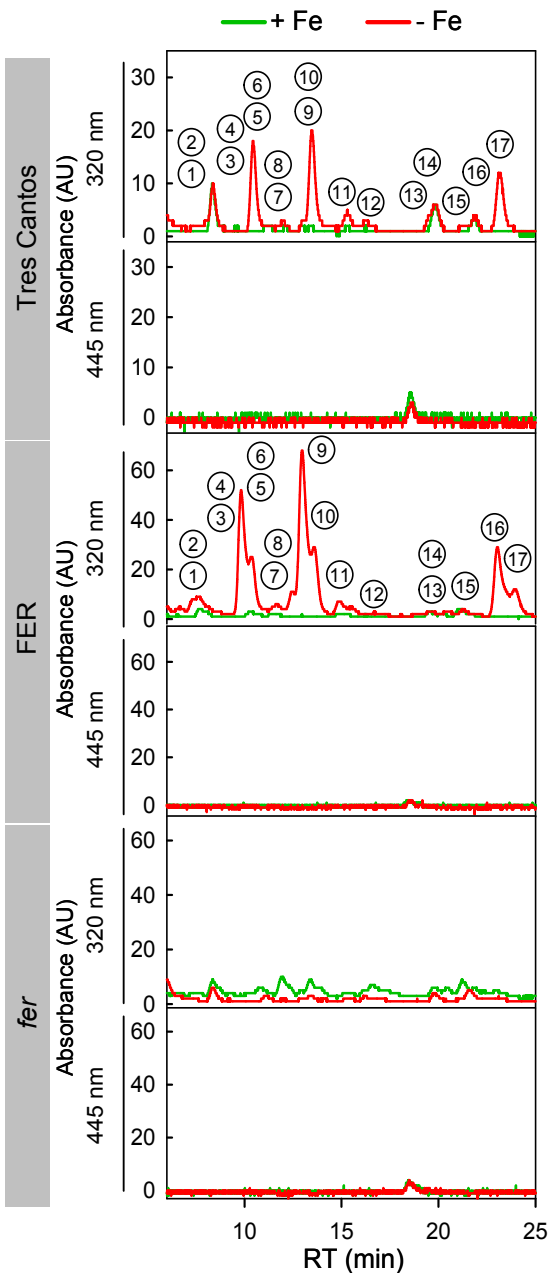

Figure 5 Chromatographic separation of a range of phenolic-type compounds produced by tomato roots in response to Fe deficiency. Typical absorbance chromatograms, extracted at 320 and 445 nm for root extracts from Fe-sufficient (+Fe) and Fe-deficient (-Fe) Tres Cantos, FER and *fer* plants grown for 17 (Tres Cantos) and 10 (FER and *fer*) days as indicated Fig. 1. The encircled numbers above each peak correspond to the phenolic compounds listed in Table 1. AU absorbance units, and RT, retention time.

Then, associated ions coming from adducts (with salts or solvents), dimers and trimers were discarded (with some exceptions, see below), and the ion chromatograms of all major remaining ions (including non-fragmented ones as well as fragment ions produced in the ESI source) were extracted with a precision of ±0.02 *m/z*. From these, we selected major ions showing large changes in peak areas in response to Fe deficiency, without considering fragments and minor ions. The localization in the chromatograms of the 17 selected compounds is depicted in Fig. 5, and the RT, exact *m/z* and assigned elemental formulae are shown in Table 1.

These 17 compounds were subjected to HPLC/ESI-MS(ion trap) analyses, including MS^2 and/or MS^3 and/or MS^4 experiments with the $[M+H]^+$ or $[M-H]^-$ ions (Table 1). None of the 17 compounds was detected in the extracts of nutrient solutions. These 17 compounds include some phenolic compounds already reported in roots and/or nutrient solutions of Fe-deficient *A. thaliana* plants [Chapters 3.1 and 3.2 and/or in Schmid *y col*. (2014) and/or in Schmidt *y col*. (2014)], as explained in detail below. To ease description, those found to be hexosides are marked with an asterisk.

Coumarins and coumarin precursors present in roots of tomato plants grown in Fe-deficiency conditions and previously reported in *A. thaliana* Some compounds (nine out of 17; compounds *3**, *5**, *6**, *7**, *8**, *12*, *14*, *16* and *17* in Table 1) have retention times (RTs) and exact *m/z* values matching with those of coumarins and that of a coumarin precursor (compound *16*; ferulic acid) previously reported in roots and/or exudates of Fe-deficient Col0 and/or *abcg37 A. thaliana* plants (Chapter 3.1 Table 1 and Chapter 3.2 Table 1). Five of them, compounds *5**, *12*, *14*, *16* and *17*, were assigned to scopolin, fraxetin, scopoletin, ferulic acid and fraxinol, respectively, with these annotations being confirmed using the RT and exact *m/z* values of authenticated standards as well as their MS^2 and MS^3 fragmentation spectra (Table 1 *vs*. Chapter 3.2 Table 2). The comparisons of fragmentation spectra showed a perfect match between analytes and the corresponding standards both in terms of *m/z* values and relative intensities of the major fragment ions.

Compound *6** was assigned to ferulic acid hexoside basing on i) the presence of a major ion (positive/negative) at *m/z* 195.0646/ 193.0505 in the MS(TOF) spectrum, which is consistent with the elemental formula of ferulic acid $[M+H]^+$/ $[M-H]^-$ ion (Chapter 3.2 Table 2) and with a neutral loss of an hexosyl moiety ($C_6H_{10}O_5$, 162.05 Da) from the $[M+H]^+$/ $[M-H]^-$ ions (with absolute errors of 3.1 and 4.9 ppm), and ii) the good fit between the MS^3 spectra of the $[M-H]^-$ ion of *6** (355→193) (Table 1) and the MS^2 spectra of the standard of ferulic acid (Chapter 3.2 Table 2).

The compounds *3**, *8** and *7** have *m/z* values, but not RT values, matching with those of some coumarin hexosides that accumulate in roots of Fe-deficient *abcg37 A. thaliana* plants (compounds *1*, *5* and *4* in Chapter 3.1 Table 1). These previously reported coumarin hexosides were annotated as two different glucosides of dihydroxyscopoletin (*1** and 5*; thereafter called trihydroxymethoxycoumarin hexosides) and one glucoside of hydroxymethoxyscopoletin (*4**; there after called dihydroxydimethoxycoumarin hexoside), respectively. The discrepancy in RT values are due the different elution programs used to analyze the roots of *abcg37 A. thaliana* and *S. lycopersicum* (Elution program 1 and 2 of Chapter 3.2 Supplementary Table S2, respectively). When the Fe-deficient *abcg37* root extracts were analyzed using the Elution program 2, the two trihydroxymethoxycoumarin hexosides and the dihydroxydimethoxycoumarin hexoside eluted at 7.9, 12.9 and 12.7 min, respectively, matching the RTs of compounds *3**, *8** and *7** occurring in Fe-deficient *S. lycopersicum* roots (Table 1). Therefore, *3** and *8** were preliminary annotated as trihydroxymethoxycoumarin hexosides as well as *7** as dihydroxydimethoxycoumarin hexoside.

The annotations for *3*, 8** and *7** were confirmed using the MS(TOF), MS^2 and MS^3 spectra. In the MS(TOF) spectra, ions (positive/negative) at *m/z* 225.0393/223.0227, 225.0395/223.0226, and 239.0546/237.0398 for *3**, *8**, and *7**, respectively, were consistent with the loss of a hexosyl moiety (162.05 Da) from their corresponding$[M+H]^+$/$[M-H]^-$ ions (see *m/z* values in Table 1).

Table 1. **Phenolic compounds accumulated in roots in response to Fe deficiency in tomato plants (cvs. Tres Cantos and FER):** retention times (RT), exact mass-to-charge ratios (*m/z*), molecular formulae, error *m/z* (in ppm) and MS^n fragmentation data. The *m/z* ratios for $[M+H]^+$ and $[M-H]^-$ were determined from the HPLC-ESI-MS(TOF) data obtained in positive and/or negative mode, respectively. For compound *11*, the *m/z* shown is that measured for the Na ($[M+Na]^+$) adduct, because it was more intense than that for $[M+H]^+$. Common names for coumarins are also indicated in parentheses. The ion having 100% relative intensity of the MS^2, MS^3 and MS^4 spectra is indicated in bold. All the compounds referred with a number (#) followed by an asterisk are hexosides. Double asterisk denotes spectra obtained from a fragment of the compound produced in source.

#	RT (min)	Measured *m/z*	Molecular formula	Calculated *m/z*	Error *m/z* (ppm)	MS^n *m/z* (Relative intensity %)	Annotation
*1**	5.8	547.1306	$C_{22}H_{27}O_{16}^-$	547.1294	2.2	MS^2 [547]: 532 (15), **385 (100)**, 370 (12), 369 (18), 353 (5), 223 (5), 222 (32), 221 (13), 207 (29), 206 (8) MS^3 [547→385]: 370 (58), 239 (5), 233 (5), 223 (85), **222 (100)**, 208 (37), 207 (65), 205 (18) *MS^2 [387]: **225 (100)**, 210 (5) *MS^3 [387→225]: **210 (100)**, 165 (11)	trihydroxymethoxycoumarin dihexoside
*2**	6.3	547.1310	$C_{22}H_{27}O_{16}^-$	547.1294	2.9	MS^2 [547]: **385 (100)**, 370 (3), 223 (7), 207 (4) MS^3 [547→385]: 370 (44), 223 (80), **222 (100)**, 208 (59), 207 (46), 206 (9) *MS^2 [387]: **225 (100)**, 210 (5) *MS^3 [387→225]: **210 (100)**, 165 (11)	trihydroxymethoxycoumarin dihexoside
*3**	8.0	387.0912 385.0758	$C_{16}H_{19}O_{11}^+$ $C_{16}H_{17}O_{11}^-$	387.0922 385.0765	-2.6 -1.8	MS^2 [385] : 370 (51), **223 (100)**, 208 (44), 206 (2) MS^3 [385→223]: **208 (100)**	trihydroxymethoxycoumarin hexoside
*4**	9.2	371.0971 369.0826	$C_{16}H_{19}O_{10}^+$ $C_{16}H_{17}O_{10}^-$	371.0973 369.0816	-0.5 2.7	MS^2 [369]: 337 (5), **207 (100)**, 192 (45) MS^3 [369→207]: **192 (100)**	dihydroxymethoxycoumarin hexoside
*5**	10.3	355.1021 353.0877	$C_{16}H_{19}O_9^+$ $C_{16}H_{17}O_9^-$	355.1024 353.0867	-0.8 2.8	MS^2 [355]: 337 (6), 313 (19), 201 (4), **193 (100)** MS^3 [355→193]: 178 (9), 165 (27), 137 (2), **133 (100)**	hydroxymethoxycoumarin hexoside (scopolin)
*6**	10.6	357.1180 355.1025	$C_{16}H_{21}O_9^+$ $C_{16}H_{19}O_9^-$	357.1181 355.1024	0.3 0.3	MS^2 [355]: **193 (100)**, 178 (4), 149 (5), 134 (7) MS^3 [355→193]: 178 (9), **149 (100)**, 134 (47)	ferulic acid hexoside
*7**	12.7	401.1066 399.0920	$C_{17}H_{21}O_{11}^+$ $C_{17}H_{19}O_{11}^-$	401.1078 399.0922	-3.0 -0.5	MS^2 [401]: **239 (100)**, 224 (4) MS^3 [401→239]: **224 (100)**, 206 (86), 179 (7)	dihydroxydimethoxycoumarin hexoside
8*	12.9	387.0920 385.0775	$C_{16}H_{19}O_{11}^+$ $C_{16}H_{17}O_{11}^-$	387.0922 385.0765	-0.5 2.6	MS^2 [385]: **370 (100)**, 223 (88), 208 (65), 207 (29) MS^3 [385→223]: **208 (100)**, 179 (1) MS^4 [385→223→208]: **180 (100)**	trihydroxymethoxycoumarin hexoside
9*	13.0	401.1080 399.0937	$C_{17}H_{21}O_{11}^+$ $C_{17}H_{19}O_{11}^-$	401.1078 399.0923	0.5 3.5	MS^2 [401]: **239 (100)**, 224 (4) MS^3 [401→239]: **224 (100)**, 206 (59), 179 (4)	dihydroxydimethoxycoumarin hexoside
10*	13.7	387.0921 385.0778	$C_{16}H_{19}O_{11}^+$ $C_{16}H_{17}O_{11}^-$	387.0922 385.0765	-0.3 3.4	MS^2 [385]: 370 (30), 325 (6), 295 (3), 265 (8), 247 (48), **223 (100)**, 208 (27), 205 (53), 190 (10), 164 (8), 149 (4) MS^3 [385→223]: **208 (100)**	trihydroxymethoxycoumarin hexoside
11*	14.2	407.0943	$C_{17}H_{20}O_{10}Na^+$	407.0949	-1.5	MS^2 [407]: **245 (100)**, 185 (4) MS^3 [407→245]: **245 (100)** 217 (7)	hydroxydimethoxycoumarin hexoside
12	16.4	209.0438	$C_{10}H_9O_5^+$	209.0445	-3.3	MS^2 [209]: 194 (24), 191 (29), 181 (51), 177 (14), 173 (6), 167 (17), 163 (62), 153 (9), **149 (100)**, 135 (19), 123 (6), 107 (12)	dihydroxymethoxycoumarin (fraxetin)
13	19.8	239.0551	$C_{11}H_{11}O_6^+$	239.0550	0.4	MS^2 [239] : **224 (100)**, 206 (74), 193 (3), 179 (9), 131 (12)	dihydroxydimethoxycoumarin
14	20.0	193.0492	$C_{10}H_9O_4^+$	193.0495	-1.6	MS^2 [193]: 178 (10), 165 (30), 151 (21), 149 (15), 137 (11), **133 (100)**, 128 (5), 119 (4), 105 (4)	7-hydroxy-6-methoxycoumarin (scopoletin)
15	21.6	239.0552	$C_{11}H_{11}O_6^+$	239.0550	0.8	MS^2 [239]: **224 (100)**, 206 (59), 193 (4), 179 (5), 131 (19)	dihydroxydimethoxycoumarin
16	23.0	195.0653 193.0490	$C_{10}H_{11}O_4^+$ $C_{10}H_9O_4^-$	195.0652 193.0495	0.5 -2.6	MS^2 [193]: 178 (57), **149 (100)**, 134 (87)	ferulic acid
17	23.8	223.0597	$C_{11}H_{11}O_5^+$	223.0601	-1.8	MS^2 [223]: **208 (100)**, 195 (13), 190 (50), 179 (5), 163 (38), 135 (8), 107 (3)	6-hydroxy 5,7dimethoxycoumarin (fraxinol)

The hexoside nature of *3**, *8** and *7** was further confirmed from the MS^2 spectra: major fragment ions (100% relative intensity at *m/z* 223, 223 and 239 in the MS^2 spectra of *3**, *8** and *7**, respectively; Table 1) corresponded to the $[M-H]^-$ or $[M+H]^+$ ions (at *m/z* 385 for *3** and *8** and at *m/z* 401 for compound *7**) after a mass loss of 162 Da. This mass loss is typical in the MS^2 fragmentation of coumarin hexosides and it is also reported in previous Chapters for the standards of two coumarin hexosides: scopoletin 7-O-glucoside (scopolin) and fraxetin 8-O-glucoside (fraxin) (Chapter 3.1 Table 2 and Chapter 3.2 Table 2). The aglycone moieties of *3**, *8** and *7** were identified from ion trap MS^3 and also taking advantage of having the dehexosylated ions in the MS(TOF) spectra. First, their MS^3 spectra (385→223 for *3** and *8**, and 401→239 for *7**; Table 1) have a major ion (100% relative intensity) consistent with a mass loss of 15 Da, which is also common in the MS^2 fragmentation of coumarins (Table 2); these MS^3 spectra also match those of the two trihy-

Table 2 Mass losses (ML) of the MS^2 and/or MS^3 product ions (P. ion), from their corresponding parent ion, for the compounds *3*, *7-10*, *13* and *15* produced by tomato roots in response to Fe deficiency and for coumarin standards. The numbers in italics (#) refer to the labels used for each compound in Fig. 5 and Table 1. Common names for coumarins are indicated in parentheses. The major product ion and its corresponding mass loss are indicated in bold. All the compounds referred with a number (#) followed by an asterisk are hexosides.

#	*3#*		*7#*		*8#*		*9#*		*10#*		*13*		*15*	
Anno-tation	trihydroxy-methoxy-coumarin hexoside		dihydroxydi-methoxy-coumarin hexoside		trihydroxy-methoxy-coumarin hexoside		dihydroxydi-methoxy-coumarin hexoside		trihydroxy-methoxy-coumarin hexoside		dihydroxydi-methoxy-coumarin		dihydroxydi-methoxy-coumarin	
Parent ion	$[M-H-C_6H_{10}O_5]^-$		$[M+H-C_6H_{10}O_5]^+$		$[M-H-C_6H_{10}O_5]^-$		$[M+H-C_6H_{10}O_5]^+$		$[M-H-C_6H_{10}O_5]^-$		$[M+H]^+$		$[M+H]^+$	
Transi-tion	MS^3(385 →223)		MS^3(401 →239)		MS^3(385 →223)		MS^3(401 →239)		MS^3(385 →223)		MS^2(401)		MS^2(401)	
	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)
	208	**15**	**224**	**15**	**208**	**15**	**224**	**15**	**208**	**15**	**224**	**15**	**224**	**15**
			206	33			206	33			206	33	206	33
			179	60			179	60			193	46	193	46
											179	60	179	60
											131	108	131	108

Name	**7-hydroxy-6-methoxycoumarin (scopoletin)**				**7,8-dihydroxy-6-methoxycoumarin (fraxetin)**				**7-hydroxy-6,8-dimethoxycoumarin (isofraxidin)**				**6-hydroxy-5,7-dimethoxycoumarin (fraxinol)**			
Parent ion	$[M+H]^+$ MS^2 (193)		$[M-H]^-$ MS^2 (191)		$[M+H]^+$ MS^2 (209)		$[M-H]^-$ MS^2(207)		$[M+H]^+$ MS^2 (223)		$[M-H]^-$ MS^2 (221)		$[M+H]^+$ MS^2 (223)		$[M-H]^-$ MS^2 (221)	
	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)	P. ion *m/z*	ML (Da)
	178	**15**	**176**	**15**	194	15	**192**	**15**	**208**	**15**	**206**	**15**	**208**	**15**	**206**	**15**
	165	28			181	28			195	28			195	28		
	149	44			177	32			190	33			190	33		
	137	56			163	46			163	60			163	60		
	133	60			**149**	**60**			135	88			135	88		
					135	74			107	116			107	116		
					107	102										

droxymethoxycoumarin hexosides and the dihydroxydimethoxycoumarin hexoside found in Fe-deficient *abcg37* root extracts (Chapter 3.1 compounds *1*, *5* and *4* of Supplementary Fig. S3). This confirms that compounds *3**, *8** and *7** are coumarin hexosides with $[M+H]^+$/$[M-H]^-$ 225/223 *m/z* for *3** and *8**, and $[M+H]^+$/$[M-H]^-$ 239/237 *m/z* for *7**. From the positive and negative MS(TOF) spectra, the *m/z* values for dehexosylated ions (see above) of *3**, *8** and *7** were assigned to the elemental formulae $C_{10}H_8O_6$, $C_{10}H_8O_6$ and $C_{11}H_{10}O_6$, respectively (with errors ≤5 ppm). The elemental formula $C_{10}H_8O_6$ was consistent with the addition of two hydroxyl (-OH) groups to the coumarin 7-hydroxy-6-methoxycoumarin (scopoletin), resulting in a change of +31.99 Da, whereas the elemental formula $C_{11}H_{10}O_6$ was consistent with the addition of a hydroxyl (-OH) and a methoxy group ($-OCH_3$) to 7-hydroxy-6-methoxy-coumarin, resulting in a change of +46.00 Da. Therefore, *3** and *8** were confirmed as two different trihydroxymethoxycoumarin hexosides and *7** was confirmed as dihydroxy-dimethoxycoumarin hexoside.

Coumarins present in roots of tomato plants grown in Fe-deficiency conditions but not previously reported in *A. thaliana*

The eight remaining compounds (*1**, *2**, *4**, *9**, *10**, *11**, *13* and *15*) were absent in Fe-deficient (and Fe-sufficient) *A. thaliana* plants. Two of them (*9** and *10**) are isomers of some of the compounds identified above: *9** is an isomer of *7**, whereas *10** is an isomer of *3** and *8**. For the two sets of isomers we found a good fit among the corresponding MS^2 and MS^3 spectra (Tables 1 and 2) and also among the positive and negative MS(TOF) spectra (the dehesoxylated ions of *9** and *10** gave signals at 239.0553/237.0398 and 225.0395/223.0234 *m/z* in the positive/negative MS(TOF) spectra, respectively). Therefore, compound *9** was annotated as a dihydroxydimethoxycoumarin hexoside (such as compound *7**), and compound *10** was annotated as a trihydroxymethoxycoumarin hexoside (such as compounds *3** and *8**) (Table 1). The differences in RT among these isomers could be ascribed to differences both in the position of the hexose unit in their structure (more than to the identity of the hexose) and also to differences in the position of the -OH and $-OCH_3$ substituents in the coumarin backbone. The lack of commercially available authenticated compounds makes impossible to assign by a MS approach the position and identity of the hexose unit and the position of the -OH and $-OCH_3$ substituents in the isomers.

Four more compounds (*1**, *2**, *4** and *11** in Table 1) were confirmed as hexoside-type compounds from the RT values and the MS(TOF) and MS^2 spectra of the $[M-H]^-$ or $[M+H]^+$ ions. First, the polar nature of *1**, *2**, *4** and *11** was evidenced from their low RTs (5.8, 6.3, 10.3 and 13.0 min, respectively) compared to those of different coumarins, phenylpropanoids, flavonoids, stilbenes and lignans in both glycoside and aglycone forms (Chapter 3.2 Table 2 and Supplementary Figures S1 and S2). Their RTs were lower (in the case of *1** and *2**) or similar (in the case of *4** and *11**) than those of the coumarin glucosides scopolin (10.3 min) and fraxin (13.0 min). In terms of MW, *4** (370.31 g mol^{-1}) and *11** (384.33 g mol^{-1}) were comparable to those of scopolin (354.31 g mol^{-1}) and fraxin (370.31 g mol^{-1}), whereas the MW of *1** and *2** (584.45 g mol^{-1} for both compounds) were larger. Therefore, *1**, *2**, *4** and *11** behaved as polar (*i.e.*, glycosylated) forms of coumarins. In fact, *4** has exact *m/z* values for $[M+H]^+$ and $[M-H]^-$ matching with those of fraxin (Table 1 and Chapter 3.2 Table 2). The polar nature of *1**, *2**, *4** and *11** was also supported by the presence of a major ion at *m/z* 387.0920, 387.0918, 209.0443 and 223.0600, respectively, consistent with $[M-hexosyl+H]^+$ ion in their positive MS(TOF) spectra, as well as from similar findings observed in the negative MS(TOF) spectra. The hexoside character of these four compounds was finally confirmed using the MS^2 spectra: major fragments (100% relative intensity at *m/z* 385, 385, 207 and 245, respectively; Table 1) correspond to the $[M-H]^-$ (*m/z* 547, 547 and 385 for *1**, *2** and *4**, respectively) or $[M+H]^+$ (*m/z* 407 for *11*) ions after a mass loss of 162 Da. The rest of ions in the MS^2 spectra of *1**, *2**, *4** and *11** showed significantly lower relative intensities (<45%), indicating that the hexosyl loss is favored in all cases.

The aglycone moieties of compounds *1**, *2**, *4** and *11** were identified carrying out MS^2 and MS^3 experiments, taking advantage

of having the dehexosylated ions in the MS(TOF) and using their chromatographic mobility. Compounds *1** and *2** were annotated as two different trihydroxy-methoxycoumarin dihexoside isomers (Table 1) based on the following findings: i) the negative MS^3 spectra of *1** and *2** (547→385; Table 1) showed a common major ion (80-85% relative intensity) at *m/z* 223 that was consistent with the corresponding $[M-H]^-$ ions after two simultaneous mass losses of 162 Da, supporting that *1** and *2** were dihexosides of a coumarin with $[M-H]^-$ 223 *m/z*; ii) in the positive MS(TOF) spectrum of *1** and *2**, ions at *m/z* 225.0393 and 225.0392, respectively, were consistent with a neutral loss of two hexosyl moieties ($C_{12}H_{20}O_{10,}$ 324.10 Da) from their corresponding $[M+H]^+$ ion (see the experimental exact *m/z* in Table 1) and with the elemental formula ($C_{10}H_8O_6$) of a trihydroxymethoxycoumarin, the aglycone of the already identified hexosides *3**, *8** and *10** (see above); iii) the RT values of *1** (5.8 min) and *2** (6.3 min) were lower than those of *3**, *8** and *10** (8.0-13.7 min), which is consistent with dihexosides being more polar than hexosides; iv) to confirm that *1** and *2** harbored a trihydroxymethoxycoumarin, the MS^4 spectra (547→385→223) were acquired, but these spectra did not show any signal, probably due to the already low signal of the parent ions, and v) the MS^2 and MS^3 (387→225) fragmentation in the ESI source of the $[M\text{-hexose}+H]^+$ ion (*m/z* 387) match well with those of *3**, *8** and *10** acquired in negative mode (Table 1). The assignment of the positions and identity of the hexose units and the position of the -OH and $-OCH_3$ substituents in the isomers *1* and *2* was not possible due to the lack of authenticated standards.

Compounds *4** and *11** were annotated as hexosides of dihydroxymethoxycoumarin and hydroxydimethoxycoumarin, respectively (Table 1), based on the following data: i) the aglycones of *4** and *11** were assigned to the elemental formulae $C_{10}H_9O_5^+$ and $C_{11}H_{11}O_5^+$, respectively (with absolute errors < 1 ppm) from the exact *m/z* values (see above) of the dehexosylated ions observed in the corresponding MS(TOF) spectra, formulae consistent with those of the $[M+H]^+$ ions of two coumarins already identified in *S. lycopersicum* roots, dihydroxymethoxy-coumarin (*e.g.*, fraxetin, *12* in Table 1) for $C_{10}H_9O_5^+$ and hydroxydimethoxycoumarin (*e.g.*, fraxinol, *17* in Table 1) for $C_{11}H_{11}O_5^+$; ii) the MS^2 and MS^3 (369→207) spectra of compound *4** (Table 1) match with those of 7,8-dihydroxy-6-methoxycoumarin 8-O-glucoside (Chapter 3.2 Table 2); iii) the different RT values of compound *4** (9.2 min) and 7,8-dihydroxy-6-methoxycoumarin 8-O-glucoside (13.0 min) indicate the presence of structural differences such as the identity and/or position of the hexosyl unit and the position of the -OH and $-OCH_3$ substituents in the coumarin backbone, and iv) compound *11** (at 14.2 min RT) is an isomer of a hydroxydimethoxycoumarin hexoside previously reported in Fe deficient Col0 *A. thaliana* roots (at 14.7 min) (Chapter 3.2 compound *5* in Tables 1 and 3), with the difference in RT revealing structural differences. Further confirmation of the identity of the aglycone of *11** was not possible because of the low intensity of their $[M+H]^+$ and $[M-H]^-$ ions, which prevented the acquisition of the MS^2 and MS^3 spectra.

The last two compounds (*13* and *15*) were annotated as two dihydroxydimethoxy-coumarins isomers, based on the following data: i) the exact *m/z* values were consistent with the elemental formula $C_{11}H_{10}O_6$; ii) both compounds had a coumarin nature since their MS^2 fragmentation produced ions consistent with the mass losses of 15, 33, 46 and 60 Da, common in the MS^2 fragmentation of the coumarins scopoletin, fraxetin, isofraxidin and fraxinol (Table 2); iii) the formula $C_{11}H_{10}O_6$ corresponded to the addition of a hydroxyl (-OH) and a methoxy group ($-OCH_3$) to 7-hydroxy-6-methoxycoumarin (scopoletin); iv) their RT (19.8 and 21.6 min, respectively) were close to those of simple coumarins such as fraxetin, scopoletin, isofraxidin and fraxinol (16.4-23.8 min), and longer than those of the two dihydroxydimethoxycoumarin hexosides also found in *S. lycopersicum* roots (*7* and *9*, with RTs 12.7-13.0 min), and v) the differences in RT between *13* and *15* could be ascribed to differences the position of the -OH and $-OCH_3$ substituents in the coumarin backbone, although the assignment of such positions was not possible due to the lack of authenticated standards.

Changes in root coumarin concentrations with Fe deficiency

The concentrations of the 10 most abundant compounds from the total 15 coumarins identified were determined using the hexoside and/or aglycone signals obtained by HPLC-UV/VIS/ESI-MS(TOF). These were plotted in five different groups, considering compounds sharing the same coumarin backbone as follows: i) dihydroxydimethoxycoumarin compounds *7** and *13* (Fig. 6a,f); ii) trihydroxymethoxycoumarins *1** and *8** (Fig. 6b,g); iii) hydroxydimethoxycoumarins *11** and *17* (Fig. 6c,h); iv) dihydroxymethoxycoumarins *4** and *12* (Fig. 6d,i), and v) scopolin (*5**) and scopoletin (*14*) (Fig. 6e,j). All compounds were determined in Fe-sufficient and Fe-deficient roots from *S. lycopersicum* plants of three genotypes ("Tres Cantos", FER and *fer*) at different days of treatment (Fig. 6). The rest of coumarins in Table 1 (five out of 15 coumarins; compounds *2**, *3**, *9**, *10** and *15*) were always at levels below the quantification limit, and therefore are not included in Fig. 6.

In the "Tres Cantos" genotype, roots of Fe-sufficient plants contained only one coumarin, the dihydroxydimethoxycoumarin hexoside (compound *7**) at very low concentrations (≤0.2 nmol g^{-1} root FW). Six days after the onset of the Fe deficiency treatment, "Tres Cantos" roots contained low concentrations (*ca.* 0.8 nmol g^{-1} root FW) of dihydroxydimethoxycoumarin hexoside, along with even lower concentrations of its aglycone, as well as very low concentrations two further coumarins, trihydroxymethoxycoumarin dihexoside and the aglycone scopoletin; dihydroxydimethoxycoumarin hexoside accounted for 76% of the total coumarins (1.0 nmol g^{-1} root FW). Eleven days after the onset of the Fe deficiency treatment, the concentration of these three coumarins increased markedly (32- to 108-fold) when compared to the values at 6 days (Fig. 6a,b,e). At this time, more coumarins appeared, including the hexosides of trihydroxymethoxycoumarin and scopoletin (Fig. 6b,e), as well as two further coumarins (hydroxydimethoxycoumarin and dihydroxymethoxycoumarin) in hexoside and aglycone forms (Fig. 6c,d). Dihydroxydimethoxycoumarin, trihydroxymethoxycoumarin and hydroxyldimethoxycoumarin (including in the mass balance the hexoside and aglycone forms) were the three most abundant coumarins, accounting for 53, 31 and 14% of the total (116.8 nmol g^{-1} root FW), respectively (Fig. 7). At longer times (14 and 17 days), the concentrations of all coumarins present at day 11 decreased progressively, reaching in some cases values below the quantification limit (Fig. 6a-e). Comparing concentrations at day 14 with those at day 11, scopoletin hexoside disappeared (Fig. 6e) and decreases were very large (75-78%) for dihydroxymethoxycoumarin hexoside and its aglycone (Fig. 6d) and large (28-56%) for the rest of coumarins (Fig. 6a,b,c,e). Dihydroxydimethoxycoumarin, trihydroxymethoxycoumarin and hydroxydimethoxycoumarin remained as the three most abundant coumarins, accounting for 58, 27 and 13% of the total coumarins (70.3 nmol g^{-1} root FW), respectively (Fig. 7). Comparing coumarin concentrations at day 17 with those at day 14, dihydroxymethoxycoumarin hexoside disappeared (Fig. 6d) and decreases were very large (87%) for hydroxydimethoxycoumarin hexoside (Fig. 6c), large (36-51%) for trihydroxymethoxycoumarin dihexoside and its hexoside (Fig. 6b) and the aglycone scopoletin (Fig. 6e) and either moderate (11-12%) or non significant for the rest of coumarins (Fig. 6a,c,d). Therefore, at day 17 the most abundant coumarin was by far dihydroxydimethoxycoumarin, accounting for 77% of the total coumarins (48.5 nmol g^{-1} root FW), with trihydroxymethoxycoumarin being the second most abundant, accounting for 19% of the total (Fig. 7).

In the FER and *fer* genotypes, no coumarins were found in the roots of Fe-sufficient plants (Fig. 6f-j; no data are shown for *fer*). No coumarins were still found 5 days after the onset of the Fe deficiency treatment in roots of FER and *fer*. At 10 days after the onset of the Fe deficiency treatment, *fer* roots remained free of coumarins, whereas FER roots contained relevant concentrations of dihydroxydimethoxycoumarin hexoside (15.0 nmol g^{-1} root FW) and minor concentrations of its aglycone (Fig. 6f) and three other coumarins, including trihydroxymethoxycoumarin dihexoside (Fig. 6g) and the hexosides of hydroxydimethoxycoumarin (Fig. 6h) and dihydroxymethoxycoumarin (Fig. 6k).

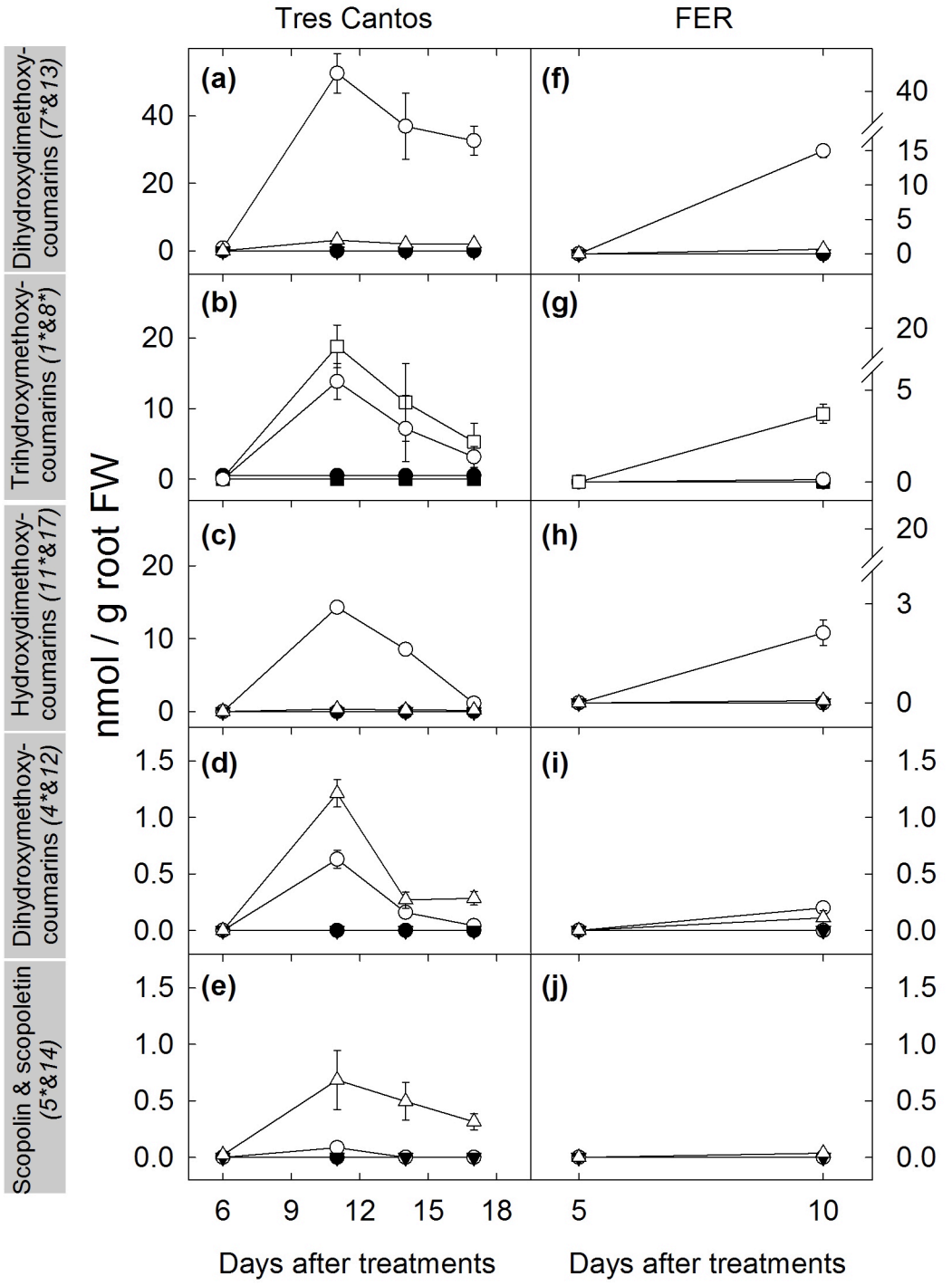

Figure 6 Time-course of the root concentration of coumarins of tomato plants as affected by Fe deficiency. Iron-sufficient (+Fe) and Fe-deficient (-Fe) Tres Cantos, FER and *fer* plants were grown as indicated in Fig. 1. The coumarins are dihydroxydimethoxycoumarins (compounds *7** and *13*), trihydroxymethoxycoumarins (compounds *1** and *8**), hydroxydimethoxycoumarins (compounds *11** and *17*), dihydroxymethoxycoumarins (compounds *4** and *12*) and scopolin and scopoletin (compounds *5** and *14*). Data are means of n=3-4. Squares, circles and triangles represent the diglycoside, glycoside and aglycone forms, respectively. Filled symbols and empty symbols are used for Fe-sufficient plants and Fe-deficient plants, respectively.

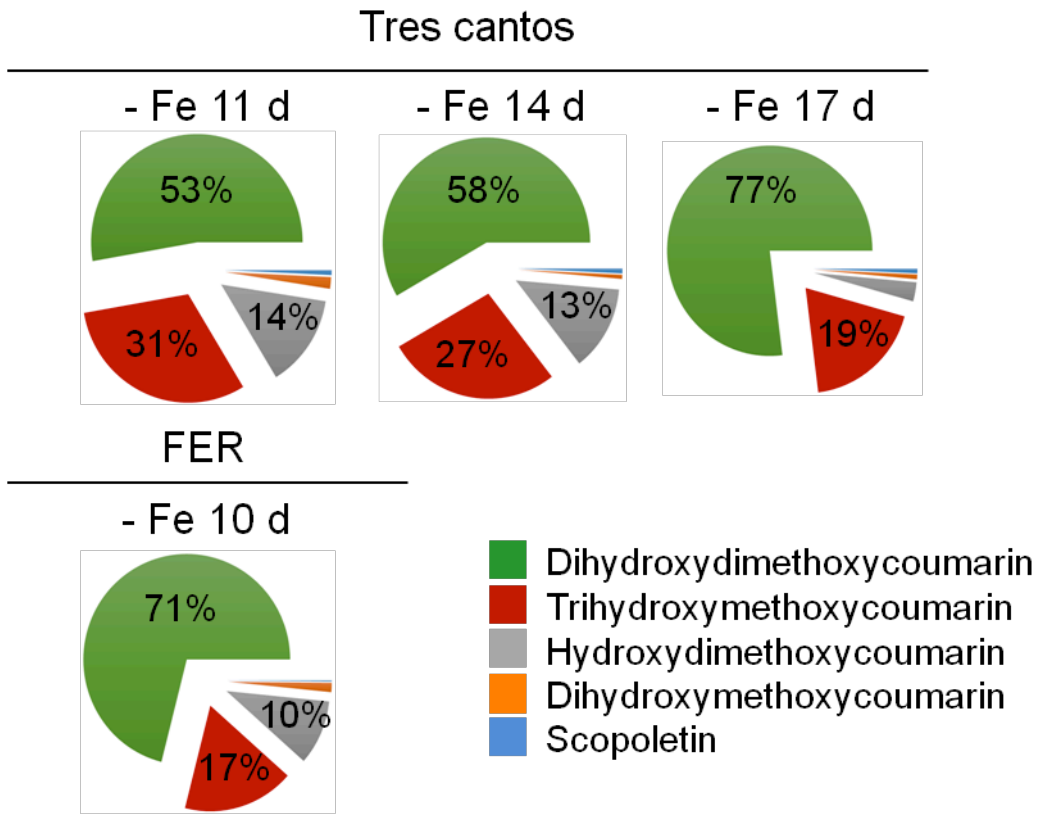

Figure 7 Time-course of the relative root concentrations of coumarins and ferulic acid of Fe-deficient tomato plants. Tres Cantos, FER and *fer* plants were grown as indicated in Fig. 1. The coumarins are dihydroxydimethoxycoumarin, trihydroxymethoxycoumarin, hydroxydimethoxycoumarin, dihydroxymethoxycoumarin and scopoletin.

Therefore, the most abundant coumarin in Fe-deficient FER roots after 10 days of Fe deficiency was by far dihydroxydimethoxy-coumarin, accounting for 71% of the total (21.0 nmol g^{-1} root FW), followed by trihydroxymethoxycoumarin and hydroxy-dimethoxycoumarin, accounting for 17 and 10% of the total, respectively (Fig. 7).

Since most coumarins were detected as hexoside and aglycone forms (with the exception of trihydroxymethoxycoumarin), the predominance of each form was considered individually for every coumarin (Fig. 8). For the first and the third most abundant coumarins, dihydroxydimethoxycoumarin and hydroxydimethoxycoumarin, respectively, the hexoside form was predominant, accounting for 86-96% of the total (depending on the coumarin, genotype and time of Fe deficiency). In the case of the second most abundant coumarin, trihydroxymethoxy-coumarin, the dihexoside form was found alone or in combination with similar amounts of the hexoside form (Fig. 8). For the rest of coumarins, dihydroxymethoxycoumarin and scopoletin, the aglycone form was predominant, with the exception of dihydroxy-methoxycoumarin in Fe-deficient FER roots 10 days after the onset of Fe deficiency treatment (Fig. 8).

Changes in root ferulic acid concentrations with Fe deficiency

The root concentrations of the hexoside and aglycone forms of ferulic acid (compounds *6* and *16*) were also determined by HPLC UV/VIS/ESI-MS(TOF) in Fe-sufficient and Fe-deficient plants from the three genotypes

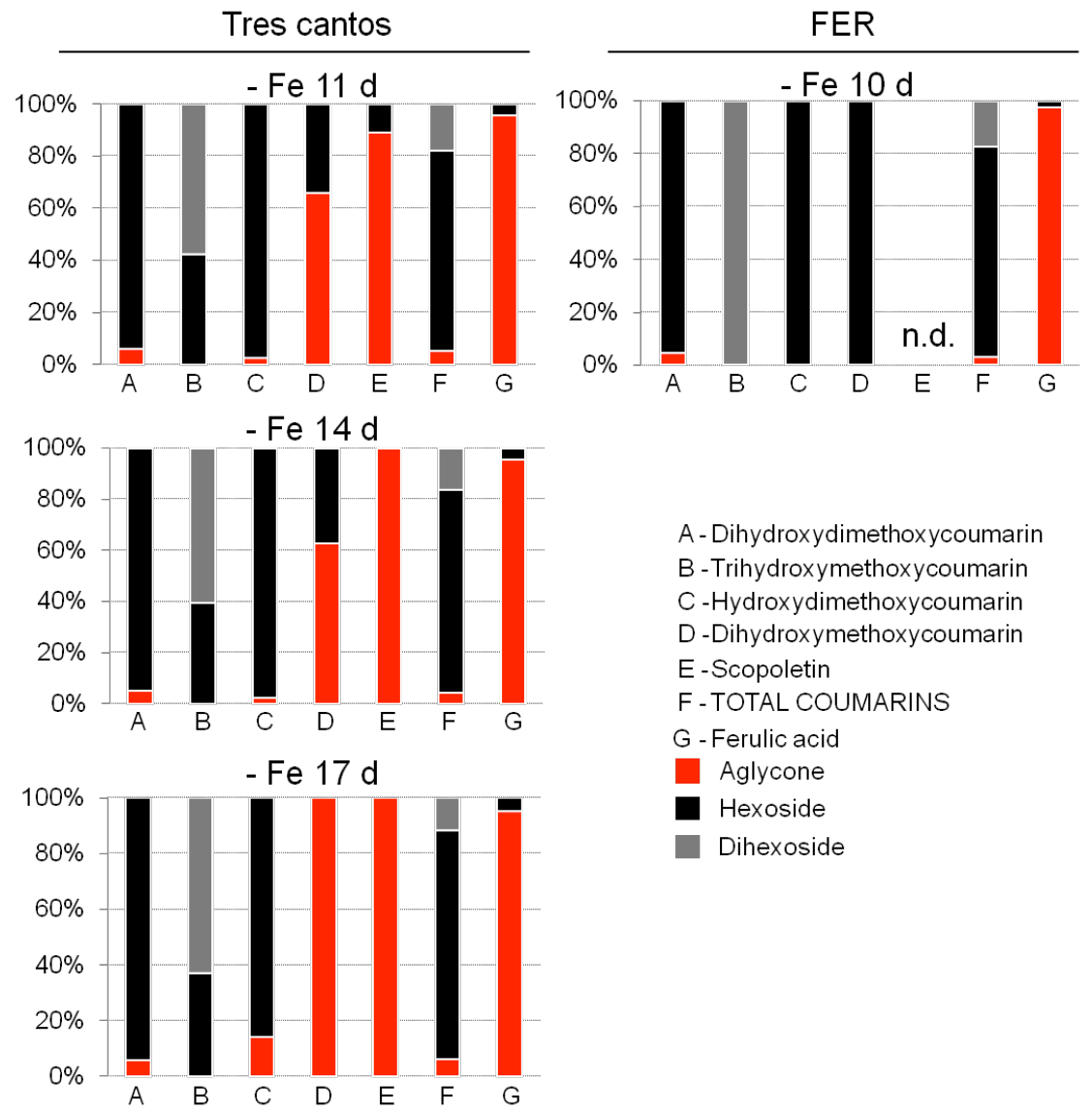

Figure 8 Time-course of the relative root concentration of the aglycone and hexoside forms of coumarins and ferulic acid of Fe-deficient tomato plants. Tres Cantos, FER and *fer* plants were grown as indicated in Fig. 1. The coumarins are dihydroxydimethoxycoumarin, trihydroxymethoxycoumarin, hydroxydimethoxycoumarin, dihydroxymethoxycoumarin and scopoletin.

("Tres Cantos", FER and *fer*) at different days of treatment (Fig. 9). In "Tres Cantos", ferulic acid was not present in Fe-sufficient plants, whereas Fe-deficient plants contained both ferulic acid forms, the hexoside and the aglycone. The concentration of ferulic acid hexoside did not change with time (0.3 nmol g^{-1} root FW), whereas the concentration of ferulic acid increased from approximately 4.7 nmol g^{-1} root FW at days 6-11 to approximately 8.7 nmol g^{-1} root FW at days 14-17.

In the FER and *fer* genotypes, ferulic acid was not present in roots of Fe-sufficient plants, and the same occurred 5 days after the onset of the Fe deficiency treatment (Fig. 9). Ten days after the onset of the Fe deficiency treatment, *fer* roots remained free of ferulic acid, whereas FER roots contained significant concentrations of the aglycone (29.9 nmol g^{-1} root FW) and hexoside ferulic acid forms (0.7 nmol g^{-1} root FW).

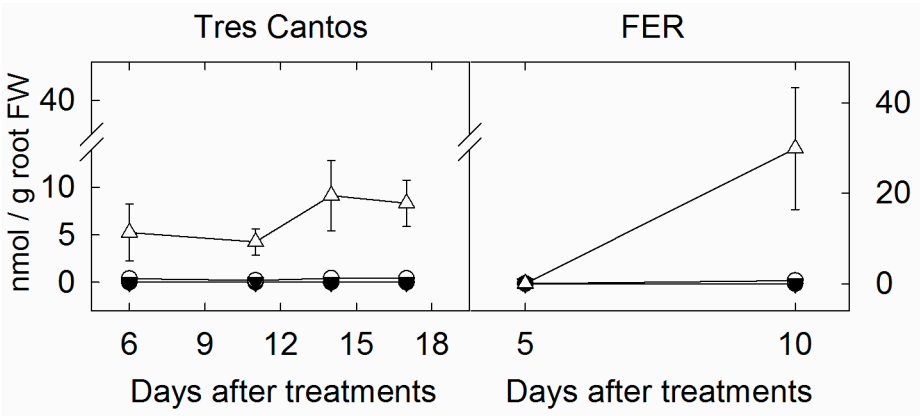

Figure 9 Time-course of the root concentration of ferulic acid of tomato plants as affected by Fe deficiency. Iron-sufficient (+Fe) and Fe-deficient (-Fe) Tres Cantos, FER and *fer* plants were grown as indicated in Fig. 1. Data are means of n=3-4. Circles and triangles represent the glycoside and aglycone forms, respectively. Filled symbols and empty symbols are used for Fe-sufficient plants and Fe-deficient plants, respectively.

When Fe the deficient roots of "Tres Cantos" and FER plants contained ferulic acid the aglycone form was predominant, being 93-98% of the total (depending on genotype and time of Fe deficiency) (Fig. 8).

Discussion

Solanum lycopersicum plants accumulate an array of phenolics in their roots in response to Fe deficiency. In contrast, flavin-type compounds were in low concentrations and did not accumulate with Fe deficiency. In this study we report for the first time the identification of 17 phenolic compounds in Fe-deficient *S. lycopersicum* roots, including 15 oxygenated coumarins (with 11 of them being O-hexosylated and the rest aglycones) and the coumarin precursor ferulic acid and its hexoside. Also, we show that the root-accumulation of coumarins was controlled by the transcription factor FER. Furthermore, we show that the coumarin profile was unaffected qualitatively by the genotype and the duration of Fe deficiency. Some of the phenolics found in Fe-deficient *S. lycopersicum* roots were previously reported in roots of Fe-deficient *A. thaliana* plants, but the coumarin profiles in both species was different, since the most numerous and predominant coumarin group in *S. lycopersicum* (the tetra-oxygenated coumarins) was not present in *A. thaliana* WT. Interestingly, tetra-oxygenated coumarins were previously reported in roots of *A. thaliana* mutant (*abcg37*) and both *S. lycopersicum* and *Atabcg37* plants did not export coumarins to the nutrient solution in response to Fe deficiency.

The production and accumulation of phenolics in *S. lycopersicum* roots was observed with two different genotypes, "Tres Cantos" and FER (Fig. 5), and this is, to the best of our knowledge, the first report of this kind for members of the Solanaceae. Phenolics accumulation was previously reported in different species, including *Parietaria diffusa* (Dell'Orto y col., 2003; Donnini y col., 2012), *Medicago ciliaris* (M'Sehli y col., 2008), *Pisum sativum* (Jelali y col., 2010), *Parietaria judaica* (Tato y col., 2013) and *A. thaliana* (Fourcroy y col., 2014; Schmid y col., 2014; Schmidt y col., 2014; Sisó-Terraza y col. 2016b). On the other hand, it has been known for a long time that *S. lycopersicum* roots respond to Fe deficiency by secreting phenolics to the growth medium (Olsen y col., 1981, 1982; Hether y col, 1984). However, in the conditions used in the present study phenolics were not detected in the nutrient solutions of Fe-deficient plants of both genotypes (data not shown). Since most of the former studies also used the genotype FER, this inconsistency seems related not to the genotype, but rather to differences in plant growth conditions and/or in protocols for exudate collection and isolation of organic compounds from the growth/exudation medium. The root secretion of phenolics from Fe-deficient plants has been shown not only in

S. lycopersicum but also in the dicotyledoneous species cited above as well as in others, including *Arachis hypogeae* (Römheld and Marschner, 1983), *Medicago sativa* (Masaoka y col., 1993), *Trifolium pratense* (Jin y col., 2007), *Glycine max* (Brown and Ambler, 1973; Zocchi y col., 2007) and *Cucumis sativus* (Pii y col., 2015). It should be taken into account that in these previous studies total phenolics in root extracts and/or exudates were usually estimated by Folin-Ciocalteau method, with the only exception of *S. lycopersicum* (Olsen *y col*., 1981; Hether *y col*., 1984) and *A. thaliana* (Fourcroy y col., 2014; Schmid y col., 2014; Schmidt y col., 2014; Sisó-Terraza y col., 2016b) for which analysis of individual phenolic compounds or in-depth phenolics profiling analyses have been made. The Folin-Ciocalteau phenolics determination method is known to have major interferences, for instance with ascorbic acid and reducing sugars among others (Sánchez-Rangel y col., 2013).

The phenolics accumulated in Fe-deficient *S. lycopersicum* roots consisted in an array of simple coumarins and the coumarin precursor ferulic acid and its hexoside. The 15 individual coumarin-type phenolics identified in *S. lycopersicum* include di-, tri- and tetra-oxygenated coumarins having -OH, -OCH_3 and -O-hexoside substituents and are described in Table 1. As far as we know, none of such coumarins has been previously reported in *S. lycopersicum*. However, it has been known since the 1970's that this species is capable of producing the simple coumarins hernianin (a mono-oxygenated coumarin with a -OCH_3 substituent) and esculetin (a di-oxygenated coumarin with two -OH substituents), as well the furanocoumarins marmesin and bergapten (Méndez and Brown, 1971). No flavonoids were found in *S. lycopersicum* roots, although a microarray analysis study showed that Fe deficiency induces the up-regulation of transcripts involved in the biosynthesis and glycosylation of flavonoids (Zamboni y col., 2012). On the other hand, no traces of other two phenolics, caffeic and chlorogenic acids, previously reported to exist in root exudates of Fe-deficient *S. lycopersicum* plants (Olsen y col., 1981; Hether y col., 1984), were detected in roots and nutrient solutions, regardless of the genotype and the Fe supply.

The apparent inconsistency among the nature of phenolics identified in *S. lycopersicum* roots (this study) and root exudates (older studies) could be due to the low selectivity of the analytical techniques (HPLC-UV/VIS) used to analyze individual phenolics in the older studies, which could have led to incorrect compound identifications. Also, other factors such as the different protocols for establishing Fe deficiency should be considered. For instance, in the older Fe deficiency *S. lycopersicum* studies roots had a light-brown coating, likely due to Fe oxide, at the time plants were transferred to the zero Fe nutrient solution, and this did not occur in our experiments. The old studies reported the acidification of the medium from pH 7.0 up to pH 4.0 within a few hours, concomitantly with the release of Fe-reducing compounds identified as caffeic and/or chlorogenic acids. Both events, acidification and release of Fe reducing compounds, were elicited along with the first symptoms of Fe-chlorosis in the apical leaves, and phenolics disappeared from the medium when the pH rose toward the initial value and apical leaves re-greened. When the pH of the medium was decreased artificially, the secretion of Fe reducing compounds also occurred. In contrast to those old studies, in the present one the nutrient solution analyzed for phenolics was sampled when plants were chlorotic, and the acidification of the nutrient solutions was prevented either by preparing them with a buffer (experiments with FER and fer plants) or by renewing them daily (experiments with "Tres Cantos").

Differences between the root coumarin profiles of the two *S. lycopersicum* genotypes used were mainly quantitative (Fig. 7). "Tres Cantos" plants accumulated in the roots higher concentrations of coumarins than those of FER in a similar time-period (10-11 days) of Fe deficiency. This could not be attributed to differences in plant size or to differences in Fe status, since the shoot biomass and leaf SPAD levels were similar in both genotypes (Figs. 1, 2). Indeed, the root biomass of "Tres Cantos" plants was 2-fold higher than that of FER (Fig. 1), thus stressing even more the higher ability of the "Tres Cantos" genotype to produce

coumarins in comparison with FER. In line with that, at the same time of Fe deficiency (10-11 days) the root concentration of the coumarin precursor ferulic acid was much lower in "Tres Cantos" than in FER (Fig. 9); this suggests that in *S. lycopersicum*, as it occurs in *A. thaliana* (Kai y col., 2008; Rodríguez-Celma y col., 2013), there is an activation of the phenylpropanoid/coumarin pathway upstream of the coumarate:CoA ligases (4CLs) in both genotypes, but downstream of the 4CLs this pathway is more active in "Tres Cantos" than in FER. Genotypic differences in the ability to accumulate/release phenolics in response to Fe deficiency, related to Fe deficiency tolerance, have been previously reported in *M. ciliaris* and *P. sativum* (M'Sehli y col., 2008; Jelali y col., 2010). Quantitative differences in the coumarin profile were also found with time (Fig. 6). This is in agreement with the fact that genotype and time are well-known factors modulating other root responses typical of dicotyledoneous species such as acidification, FCR activity and Fe(II) uptake.

These findings show that *S. lycopersicum* shares with the Brassicaceae species *A. thaliana* the production of coumarin-type phenolics in response to Fe deficiency. Also, this study revealed that *fer S. lycopersicum* plants, as it occurs in the *fit A. thaliana* mutant, did not produce coumarins (Fig. 5 and Schmid y col., 2014) proving its coordination with other Fe deficiency responses in roots (*e.g.*, IRT and FRO) (Rodríguez-Celma y col., 2013). However, marked qualitative changes between the coumarin profile of roots of *S. lycopersicum* and *A. thaliana* were observed. In fact, the presence of at least one -OH and one $-OCH_3$ substituent in the coumarin backbone, as in the di-oxygenated coumarin, scopoletin and in the scopoletin precursor ferulic acid (Sisó-Terraza y col., 2016a), is the only common structure feature of all the coumarins accumulated in Fe-deficient roots of both species. *S. lycopersicum*, unlike *A. thaliana*, accumulated in roots many (13 out of 15) coumarins having three or four oxygen-containing substituents in the coumarin backbone (Table 3). Tetra-oxygenated coumarins were the most numerous (nine out of 15) and abundant (accounting for at least 84% of the total coumarins accumulated) in *S. lycopersicum*, whereas they were not reported in *A. thaliana* (Fourcroy y col., 2014; Schmid y col., 2014; Schmidt y col., 2014; Sisó-Terraza y col. 2016a). The hexosylation status of the major coumarins accumulated in roots of *S. lycopersicum* and *A. thaliana* under Fe deficiency was also different: hexoxides were the major forms (>86%) in the three most abundant coumarins (accounting for 98% of the total) in *S. lycopersicum* (Figs. 7, 8) whereas aglycones were the major forms (>75%) for the two most abundant coumarins (accounting for 95% of the total) in *A. thaliana* (Sisó-Terraza y col., 2016b).

Table 3 Classification of coumarins accumulated in tomato roots in response to Fe deficiency according to the type and number of oxygen substituents in coumarin backbone.

	Di-oxygenated	**Tri-oxygenated**		**Tetra-oxygenated**		
	Number of substituents					
Hydroxy substituents	1	2	1	2	3	
Methoxy substituents	1	1	2	2	1	
	Number of coumarins					**Total**
Simple coumarins	1	1	1	2	0	**5**
Coumarin hexosides	1	1	1	2	3	**8**
Coumarin dihexosides	0	0	0	0	2	**2**
Total coumarin hexosides	1	1	1	2	5	**10**
Total	**2**	**4**		**9**		**15**

It is well-known that the glycosylation status, regulated by glycosyltransferases and glycoside hydrolases, can change both the compartmentalization and biological activity of phenolics, as well as their solubility, stability and toxic potential (Le Roy y col., 2016). In fact, a β-glucosidase, bglu42, is induced by Fe deficiency in roots of *A. thaliana* (García y col., 2010, Yang y col., 2010, Lan y col., 2011, Rodríguez-Celma y col., 2013), and the Fe-deficient *bglu42* plants apparently fail to secrete coumarins (Zamioudis y col., 2014). Remarkably, coumarins were not secreted to the growth media by *S. lycopersicum* and the *abcg37 A. thaliana* mutant, and in both cases the coumarin profile in roots were quite similar, with hexosides of tetra- and tri-oxygenated coumarins being predominant (Figs. 7, 8 and Fourcroy y col., 2014). In both cases, the accumulation of coumarins in root cells could activate detoxification mechanisms involving oxidation and hexosylation modifications of toxic compounds as well as internal-sequestration into vacuoles.

Solanum lycopersicum plants did not respond to Fe deficiency by enhancing the root accumulation and/or secretion of flavins (Fig. 5). These results, obtained using highly sensitive and selective analytical techniques to analyze flavins, confirms previous results obtained with much less advanced techniques and two different *S. lycopersicum* genotypes (Welkie y col., 2000). Therefore, *S. lycopersicum* plants produce phenolics, but not flavins, in response to Fe deficiency, as it occurs in *A. thaliana* (Fourcroy y col., 2014; Schmid y col., 2014; Schmidt y col., 2014; Sisó-Terraza y col., 2016a) and *T. pratense* (Jin y col., 2007). This extends the view that the root production of phenolics and flavins elicited by Fe deficiency could be mutually exclusive, as recently proposed in a study on the root transcriptome changes induced by Fe deficiency, using the phenolics producer *A. thaliana* and the flavin producer *Medicago truncatula* (Rodríguez-Celma y col., 2013). In line with this, this Thesis also revealed that *Beta vulgaris* is flavin producer but not phenolic producer (Sisó-Terraza y col., 2016a). The Solanaceae family appears to be mainly a flavin producer under Fe deficiency (eight out of 14 Solanaceae species; Welkie y col., 2000; Rodríguez-Celma y col., 2011), although the only Solanaceae species analyzed for phenolics has been *S. lycopersicum*. In contrast, the Fabaceae family appears to be mainly a phenolics producer under Fe deficiency: six members of the family, *G. max* (Brown and Ambler, 1973; Zocchi y col., 2007), *A. hypogeae* (Römheld and Marschner, 1983), *M. sativa* (Masaoka y col., 1993), *T. pratense* (Jin y col., 2007), *M. ciliaris* (M'Sehli y col., 2008) and *P. sativum* (Jelali y col., 2010) are phenolic producers, whereas only one (*M. truncatula*) out of 10 species tested was found to produce flavins (Welkie y col., 2000; Jin y col., 2007; Rodríguez-Celma y col., 2011).

In summary, the roots and nutrient solutions from Fe-deficient and Fe-sufficient plants of three different genotypes of the Solanaceae species *S. lycopersicum* have analyzed for phenolics and flavins, using advanced HPLC-MS-based methods specifically designed to detect, identify and quantify such compounds. Results show that *S. lycopersicum* behaves as *A. thaliana*, accumulating coumarin-type phenolics in roots in response to Fe deficiency, with the transcription factor FER being required. Furthermore, in the conditions used in the present study *S. lycopersicum* accumulated hexosylated forms of highly oxygenated coumarins in roots and did not secrete coumarins to the growth media, as it occurs in the *abcg37 A. thaliana* mutant. This might be related to the low tolerance to Fe deficiency of *S. lycopersicum*. Also, *S. lycopersicum* did not produce flavins in response to Fe deficiency, differing from several other Solanaceae species, supporting the view that the production of phenolics and flavins in response to Fe deficiency seems to be mutually exclusive.

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Author contributions

A.A-F. planned and designed the research. P.S-T. conducted the experiments, identified phenolics, made root microscopy, analyzed the results, made the figures and tables and drafted the manuscript. A.L-V. quantified phenolics. J.A., A.A. and A.A-F. analyzed critically the results. J.A., A.A. and A.A-F. wrote the manuscript.

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3.4. Flavins secreted by roots of iron-deficient *Beta vulgaris* enable mining of ferric oxide via reductive mechanisms

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Research

Flavins secreted by roots of iron-deficient *Beta vulgaris* enable mining of ferric oxide via reductive mechanisms

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Summary

• Iron (Fe) is abundant in soils but generally poorly soluble. Plants, with the exception of *Graminaceae*, take up Fe using an Fe(III)-chelate reductase coupled to an Fe(II) transporter. Whether or not nongraminaceous species can convert scarcely soluble Fe(III) forms into soluble Fe forms has deserved little attention so far. We have used *Beta vulgaris*, one among the many species whose roots secrete flavins upon Fe deficiency, to study whether or not flavins are involved in Fe acquisition.

• Flavins secreted by Fe-deficient plants were removed from the nutrient solution, and plants were compared with Fe-sufficient plants and Fe-deficient plants without flavin removal. Solubilization of a scarcely soluble Fe(III)-oxide was assessed in the presence or absence of flavins, NADH (nicotinamide adenine dinucleotide, reduced form) or plant roots, and an Fe(II) trapping agent.

• The removal of flavins from the nutrient solution aggravated the Fe deficiency-induced leaf chlorosis. Flavins were able to dissolve an Fe(III)-oxide in the presence of NADH. The addition of extracellular flavins enabled roots of Fe-deficient plants to reductively dissolve an Fe(III)-oxide.

• We concluded that root-secretion of flavins improves Fe nutrition in *B. vulgaris*. Flavins allow *B. vulgaris* roots to mine Fe from Fe(III)-oxides *via* reductive mechanisms.

Introduction

Iron (Fe) is an essential micronutrient for plants and the fourth most abundant element in the lithosphere (Marschner, 1995). However, the solubility of soil Fe is generally controlled by those of scarcely soluble Fe(III)-oxides and oxyhydroxides, which are minimal at pH values typical of calcareous soils (7.4–8.5); the Fe concentration in these soil solutions is *c.* 10^{-10} M (Lindsay, 1995), considerably lower than those required by plants (10^{-8} M; Römheld & Marschner, 1986). Because calcareous soils constitute one third of the world's agricultural land, Fe deficiency is one of the most important factors limiting crop production worldwide (Briat *et al.*, 2015).

Plants deploy different strategies for mining soil Fe. Members of the *Graminaceae* use the chelation-based Strategy II, which involves the synthesis of polyaminocarboxilic chelators (the so-called phytosiderophores, PS; Kobayashi *et al.*, 2006) that are secreted into the rhizosphere by the TOM (transporter of mugineic acid) transporter (Nozoye *et al.*, 2011). Iron is mined from the soil via the formation of Fe(III)-PS complexes that are taken up intact by YS1 transporters (Curie *et al.*, 2001). Nongraminaceous species use the reduction-based Strategy I that involves the reduction of soluble Fe(III) to Fe(II) by an Fe(III) chelate reductase (FCR) (FRO2; Robinson *et al.*, 1999), and the subsequent uptake of Fe(II) by Fe(II) transporters (IRT1; Eide *et al.*, 1996) at the root cell plasma membrane (PM). However, whether or not Strategy I plants are capable of mining Fe from the soil and supply soluble Fe forms to the PM uptake sites is still not fully known.

It has been known for a long time that many Strategy I plant species/cultivars secrete what can be described as a toolbox of root-borne Fe facilitators, including phenolics, carboxylates and flavins in response to low Fe availability (Cesco *et al.*, 2010; Mimmo *et al.*, 2014). Removal from the nutrient solution of phenolics secreted by roots of Fe-deficient *Trifolium pratense* plants resulted in a more intense Fe deficiency (Jin *et al.*, 2007). Recently, the root synthesis and secretion of coumarin-type phenolic compounds has been identified as a key component of the Fe acquisition machinery in *Arabidopsis thaliana* growing under Fe deficiency (Rodríguez-Celma *et al.*, 2013; Fourcroy *et al.*, 2014; Schmid *et al.*, 2014). These phenolics are released into the rhizosphere by the ABCG37 ATP binding cassette (ABC) transporter (Rodríguez-Celma *et al.*, 2013; Fourcroy *et al.*, 2014), and some of them are capable of solubilizing Fe(III)-oxide at high pH, likely by forming Fe(III)-catechol complexes (Schmid *et al.*, 2014; Schmidt *et al.*, 2014).

Flavin accumulation and/or export from roots upon Fe shortage is a phenomenon widely reported in dicotyledenous species, with flavins being present in at least 29 species from eight families and absent in 37 species from 13 families (Welkie, 2000;

Rodríguez-Celma *et al.*, 2011b). Flavin root secretion does not follow obvious taxonomical categories: it does occur in all tested *Amaranthaceae* species (including *Beta vulgaris*), whereas in the *Fabaceae* family it occurs in *Medicago truncatula* but not in *Lupinus albus*, and it has not been reported so far in any *Brassicaceae* species (which includes *A. thaliana*). The most common flavin found in exudates and roots of Fe-deficient plants is riboflavin (Rbfl), but other derivatives have also been reported, including Rbfl 3′- and 5′-sulfates in *B. vulgaris* and 7-carboxy- and 7-hydroxy-Rbfl in *M. truncatula* (Rodríguez-Celma *et al.*, 2011b). In *B. vulgaris* and *M. truncatula* flavins are secreted to the nutrient solution when external pH is low, whereas at high external pH flavins accumulate in the distal root parts (0–5 mm from the apex) (Susín *et al.*, 1993, 1994; López-Millán *et al.*, 2000; Rodríguez-Celma *et al.*, 2011b). In both species, transcriptomic and/or proteomic studies using Fe-deficient roots have revealed a strong upregulation of enzymes involved in Rbfl biosynthesis, as well as a link between the Rbfl biosynthesis pathway and the core genes of the Fe acquisition machinery (Rellán-Álvarez *et al.*, 2010; Rodríguez-Celma *et al.*, 2011a, 2013).

Flavins share an isoalloxacine ring capable of undergoing oxidation–reduction reactions. Riboflavin and their derivatives flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are well-known prosthetic groups used as redox centers in many proteins (Senda *et al.*, 2009). Less known are the metal (e.g. Fe) ion-affinities of the half-reduced flavins (Hemmerich & Spence, 1966; Müller *et al.*, 1968) and the capability of reduced flavin forms to dissolve Fe(III)-oxides (von Canstein *et al.*, 2008). It has been hypothesized that flavins in Fe-deficient roots may act as electron donors or cofactors for the FCR, whereas flavins secreted to the rhizosphere may mediate extracellular Fe solubilization and/or act as antimicrobial agents (Jordan *et al.*, 1992; González-Vallejo *et al.*, 1998; Higa *et al.*, 2010).

In this study, we have tested the hypothesis that the root-secretion of flavins by Fe-deficient plants is an additional key component for Strategy I Fe acquisition that allows for soil Fe mining. We have used *B. vulgaris*, an Fe-deficiency tolerant species where a wide characterization of the biochemical, proteomic, and metabolomic changes induced by Fe deficiency is available (Rellán-Álvarez *et al.*, 2010, and references therein). Results found contribute to the understanding of the mechanism(s) by which flavins may improve Fe acquisition.

Materials and Methods

Plant culture

Sugar beet (*Beta vulgaris* L. cv Orbis) seeds were germinated and grown in half-strength Hoagland nutrient solution, pH 5.5, with 45 μM Fe(III)-ethylenediaminetetraacetate [Fe(III)-EDTA] for 13 d, as indicated in Sagardoy *et al.* (2009). Then, plants (with six leaves at this stage) were treated as follows.

Set-up 1 A first set-up was designed to investigate the effect of flavin removal from the nutrient solution on the plant Fe status. Plants were grown for 13 d in 10-l buckets (four plants per bucket) in nutrient solution pH 5.5, with Fe (45 μM Fe(III)-EDTA), without Fe or without Fe and using a flavin removal system. To remove flavins, the nutrient solution was recirculated continuously through a C_{18} resin column using a pump (see Supporting Information Fig. S1). The pH was readjusted daily to 5.5 in all buckets using 1 M NaOH. The development of Fe deficiency was assessed in leaves using a soil and plant analyzer development (SPAD) device (Minolta, Osaka, Japan). Ten-microliter aliquots of the nutrient solutions were sampled every 2–3 d and stored at −20°C for flavin analysis. At the end of the treatment period, plants had 10 leaves, eight of them fully developed, and all organs were individually processed as in Sagardoy *et al.* (2009).

Set-up 2 A second set-up was used to test if extracellular flavins allow plants to solubilize poorly soluble Fe(III) forms. Plants were grown for 10 d in 20-l buckets (four plants per bucket) in nutrient solution with 0 or 45 μM Fe, buffered at pH 5.5 (with 20 mM MES (2-(N-morpholino)ethanesulfonic acid)-NaOH) or 7.5 (with 1 $g\,l^{-1}$ of $CaCO_3$ and 1 mM NaOH; Susín *et al.*, 1993) to counteract the root acidification of nutrient solution and maintain a stable pH during the whole treatment period. Iron(III)-EDTA and Fe(III)-ethylendiaminedi(*o*-hydroxyphenylacetate) (Fe(III)-EDDHA; Sequestrene, Syngenta, Madrid, Spain) were used as Fe source for pH 5.5 and pH 7.5, respectively. At the end of the treatment period, root Fe(III) reduction activity assays were carried out.

Set-up 3 Plants were grown for 17 d in zero-Fe, unbuffered nutrient solution (initial pH 5.5) in 20-l buckets (Susín *et al.*, 1994). Nutrient solutions were sampled at the end of the treatment period, and Rbfl-sulfates were isolated to be used for solubilization of an Fe(III)-oxide.

Riboflavin sulfates isolation from nutrient solutions

Nutrient solutions of plants grown under Set-up 3 were passed (1 l per cartridge) through Sep-Pak C_{18} cartridges (Vac 35 cc/10 g; Waters, Milford, MA, USA), using a vacuum manifold system. Flavins were retained in the cartridges as a broad yellow band and eluted using 30 ml of methanol. Eluates were dried in a SpeedVac. The flavin isolate was analyzed by high-performance liquid chromatography (HPLC) coupled to ultraviolet/visible (UV/VIS) spectroscopy and time-of-flight (TOF) mass spectrometry (MS) (see later), and stored at -80°C until further use in FCR measurements and *in vitro* solubilization of Fe(III)-oxide.

Extraction and analyses of flavins and phenolics in roots and nutrient solutions

Total flavins in the nutrient solutions were analyzed directly by spectrofluorimetry using a multimode microplate reader (Synergy™ HT; Biotek Instruments Inc., Winooski, VT, USA) equiped with fluorescence excitation (360/40 nm) and emission filters (528/20 nm). The concentration of total flavins was estimated using Rbfl (purity ≥98%, Sigma-Aldrich).

Phenolics and flavins were extracted from 500 ml of solution and *c.* 200 mg of frozen roots as described by Fourcroy *et al.* (2014) with some modifications (see Notes S1 for details).

Individual flavins and phenolics were analysed by HPLC (Waters Alliance 2795 HPLC) coupled to UV/VIS (Waters PDA 2996) and MS time-of-flight (MicrOTOF; Bruker Daltonics, Bremen, Germany) detectors. Phenolics and flavins were analyzed according to Fourcroy *et al.* (2014) and the method described in Notes S2, respectively. The flavin isolate obtained was found to consist of 46% Rbfl 3′-sulfate and 54% Rbfl 5′-sulfate (Fig. S2).

Root Fe(III) reductase activity

Root Fe(III) reductase activity was measured by the Fe(II) bathophenanthrolinedisulphonate (BPDS) method, using whole roots of intact plants and excised root sections, and either Fe(III)-EDTA (Bienfait *et al.*, 1983) or a poorly crystalline Fe(III)-oxide as substrate. The Fe(III)-oxide was obtained by precipitation from $Fe(III)Cl_3$ with NaOH as described by Pérez-Sanz & Lucena (1995).

After 2–3 h of light onset, the whole root of intact, illuminated plants (kept in aluminum foil-wrapped beakers in the growth chamber) was placed in 250 ml of the assay medium, containing 300 μM BPDS, 500 μM Fe(III)-EDTA or 50 mg of Fe(III)-oxide, supplemented with flavins at concentrations from 0 to 100 μM. The assay media was buffered at pH 5.5 (with 5 mM MES-KOH) or 7.5 (with 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-KOH). Flavins used were Rbfl and the Rbfl-sulfate isolate. Reactions were run for 20 (soluble Fe) or 60 min (Fe(III)-oxide). Blanks without roots were always used to correct for any reduction of Fe(III) not associated to plants. The possible contribution of soluble reductants released from roots to overall root Fe(III) reduction was assessed by using an assay medium without Fe source and BPDS; after roots were removed, the Fe source and BPDS were added to the medium and left for the times indicated above. Aliquots were taken from the assay medium, filtered through polyvinylidene fluoride (PVDF) 0.45 μm ultrafree-MC centrifugal filter devices (Millipore) at 10 000 ***g*** for 3 min, and absorbance was measured at 535 nm. Iron(II)-$BPDS_3$ concentration was calculated using an extinction coefficient of 22.14 mM^{-1} cm^{-1}. In the absence of roots, 100 μM Rbfl produced a measurable FCR activity (not shown); the absorbances of blanks without and with 100 μM Rbfl were equivalent to *c.* 2 and 18 nmol Fe(II) reduced g^{-1} root FW min^{-1} (all root FCR values shown in the corresponding figures were corrected for these basal values).

When using excised roots, root material (60 mg; sections taken 0–5 and 5–10 mm from the root tip) was placed with 10 mg of Fe(III)-oxide and 1.5 ml of the assay medium in 2-ml tubes, and the assay was run (at room temperature and in the dark) for 30 min. Assays were carried out as indicated previously.

In vitro solubilization of Fe(III)-oxide mediated by flavins

Ten micrograms of poorly crystalline Fe(III)-oxide was incubated (at room temperature and in the dark) for 6 h with 1.5 ml of an assay solution containing: 0 or 500 μM NADH as an electron donor, 0 or 10 μM oxidized flavins (Rbfl or the Rbfl-sulfates isolate) as electron mediators, and 0 or 600 μM of BPDS as Fe(II) trapping agent. The assay medium was buffered at pH 5.5 (with 5 mM MES-KOH) or pH 7.5 (with 5 mM HEPES-KOH). After 6 h, the assay medium was filtered through PVDF 0.45 μm centrifugal filters (Millipore) at 10 000 ***g*** for 3 min. Iron reduction was monitored using BPDS as indicated above. Iron solubilization from Fe(III)-oxide was determined by diluting 50 μl of the filtered assay solutions with 100 μl of 65% ultrapure H_3NO (TraceSELECT Ultra, Sigma-Aldrich), and then measuring Fe by graphite furnace atomic absorption spectrometry (GFAAS; Varian 3000, Palo Alto, CA, USA).

Plant tissue mineral analyses

Plant tissues (*c.* 0.2 g of ground dry tissues) were digested as in Fourcroy *et al.* (2014). Digests were analyzed for Fe, manganese (Mn), copper (Cu) and zinc (Zn) by AAS (SOLAAR 969; Thermo, Cambridge, UK). The content of apoplastic Fe in fine roots was estimated with the EDTA-based Bienfait's method (Fodor *et al.*, 2005) with some modifications (see Notes S3 for details).

Statistical analyses

Statistical analysis was carried out with SPSS for PC (v.23.0, IBM, Armonk, NY, USA), using ANOVA or nonparametric tests ($P \leq 0.05$), and a Levene test for checking homogeneity of variances. *Post hoc* multiple comparisons of means were carried out ($P \leq 0.05$) using Duncan test when variances were equal and Games–Howell's test when variances were unequal.

Results

Flavin removal from the nutrient solution affects Fe plant status

Beta vulgaris plants grown in zero-Fe nutrient solution adjusted daily to pH 5.5 secreted flavins to the nutrient solution (Fig. 1). The flavin concentration increased in the nutrient solution from Day 5 after imposing Fe deficiency, and at Day 11 the estimated flavin concentration was *c.* 0.65 μM, a value similar to that found with a different *B. vulgaris* cv (Susín *et al.*, 1994).

Flavin concentration in the nutrient solution is depleted by recirculation through a C_{18} resin column Flavins were depleted in the nutrient solution by recirculation through a C_{18} resin column. This system was similar to that used by Jin *et al.* (2007) to remove phenolics from the nutrient solution of Fe-deficient *T. pratense*. In the recirculated solution, flavins were barely detectable during the first 6 d of treatment, and at Day 11 the concentration of flavins was still *c.* 0.1 μM (Fig. 1). This indicates that the system was able to remove up to 86% of the flavins secreted to the growth medium by *B. vulgaris* roots.

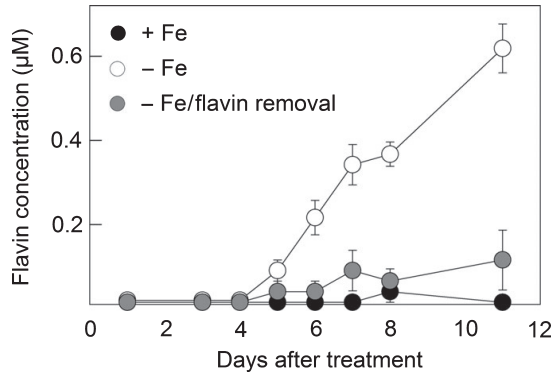

Fig. 1 Flavins in the nutrient solution of iron (Fe)-deficient plants and flavin removal using a recirculation system. Data show the time-course of the total concentration of flavins in nutrient solutions of *Beta vulgaris* plants grown with Fe (+Fe), without Fe (−Fe), and without Fe using the flavin removal system (−Fe/flavin removal). Plants were grown with experimental Set-up 1: plants were pre-grown for 13 d in the presence of 45 μM Fe (III)-EDTA at pH 5.5, and then grown for 13 d in a medium with 0 or 45 μM Fe (III)-EDTA (pH was readjusted daily to 5.5 in all pots). Flavin concentration was measured using fluorescence (excitation 360 nm/ emission 528 nm). Data are means ± SE ($n = 4$; solutions were from three different plant batches).

Flavin depletion intensifies leaf chlorosis and decreases the biomass of Fe-deficient *B. vulgaris* plants Leaf chlorophyll concentration was assessed using SPAD values in developed (5^{th}–6^{th}) and young (7^{th}–8^{th}) leaves during the 13-d treatment (Fig. 2). Iron deficiency caused a significant decrease in the SPAD value of developed leaves from Day 3 to the end of the experiment (Fig. 2a). The young leaves of Fe-deficient plants were always very chlorotic, with the SPAD readings being at least 10 units lower than those of Fe-sufficient plants (Fig. 2b).

Flavin depletion intensified significantly the chlorosis of the developed leaves from Day 3 until the end of the experiment (Fig. 2a). In the young leaves, the chlorosis was also aggravated by flavin removal, and these leaves always had the lowest SPAD values (Fig. 2b). Pictures of plants and nutrient solutions at the end of the 13-d treatment period are shown in Fig. 2(c) and (d), respectively.

At the end of the 13-d treatment, plant biomass was only slightly affected by Fe deficiency: the DW of young and old leaves and stems did not change significantly, whereas the DW of developed leaves and storage and fine roots decreased (Fig. 3). Upon removal of flavins, the DW of young and developed leaves and storage and fine roots decreased significantly when compared with those of Fe-deficient plants, whereas those of old leaves and stems did not change (Fig. 3). Changes in FW were similar to those in DW (Fig. S3).

Flavin depletion alters uptake and distribution of Fe and other metals in Fe-deficient *B. vulgaris* plants Iron deficiency affected the micronutrient contents of different tissues of *B. vulgaris* plants (Fig. 4). Iron deficiency caused a decrease in Fe content in most plant parts, with the only exceptions being stems and storage roots. Also, the content of apoplastic Fe in fine roots

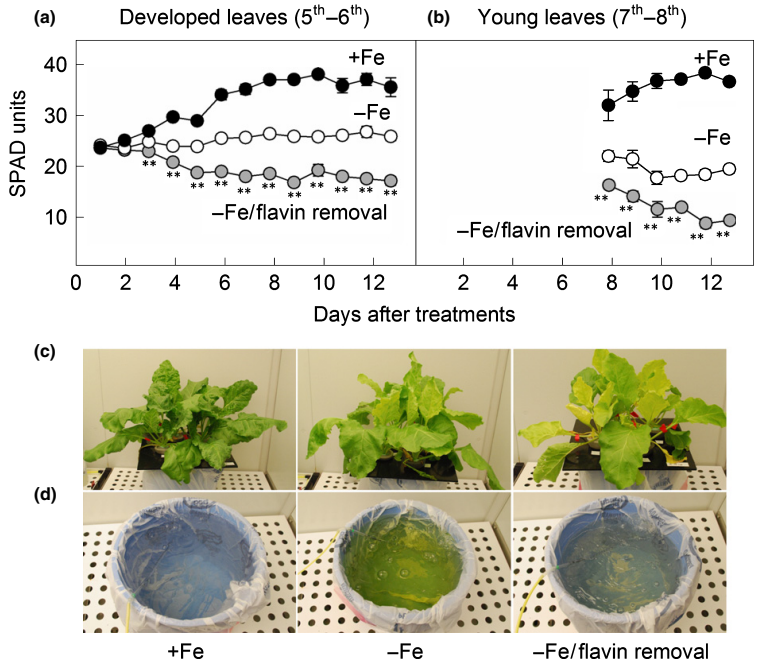

Fig. 2 Leaf chlorophyll changes upon iron (Fe) deficiency and flavin removal from the nutrient solution. Time-course of the soil and plant analyzer development (SPAD) values of (a) developed (leaves 5^{th}–6^{th}) and (b) young (leaves 7^{th}–8^{th}) leaves of *Beta vulgaris* plants grown with Fe (+Fe), without Fe (−Fe), and without Fe using the flavin removal system (−Fe/flavin removal). Plants were grown with experimental Set-up 1 as indicated in Fig. 1, and leaves 7^{th}–8^{th} were the youngest ones at the end of the treatment period. Data are means ± SE ($n = 30$–60; plants were from six different batches). Significant differences (at $P < 0.05$) between means for the −Fe and −Fe/flavin removal treatments at each time point are marked (**). Pictures of (c) plants and (d) nutrient solutions taken at day 13 after imposing treatments.

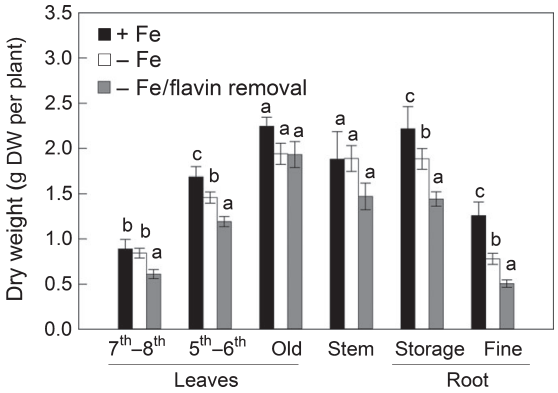

Fig. 3 Plant biomass changes upon iron (Fe) deficiency and flavin removal from the nutrient solution. Data show the DW of the different parts of *Beta vulgaris* plants grown for 13 d with Fe (+Fe), without Fe (−Fe), and without Fe using the flavin removal system (−Fe/flavin removal). Plants were grown with experimental Set-up 1 as indicated in Fig. 1. Data are means ± SE ($n = 12$ for stems and old leaves, and 28–55 for the rest of plant parts). For a given plant part, significant differences among treatments (at $P < 0.05$) are marked with different letters above the columns.

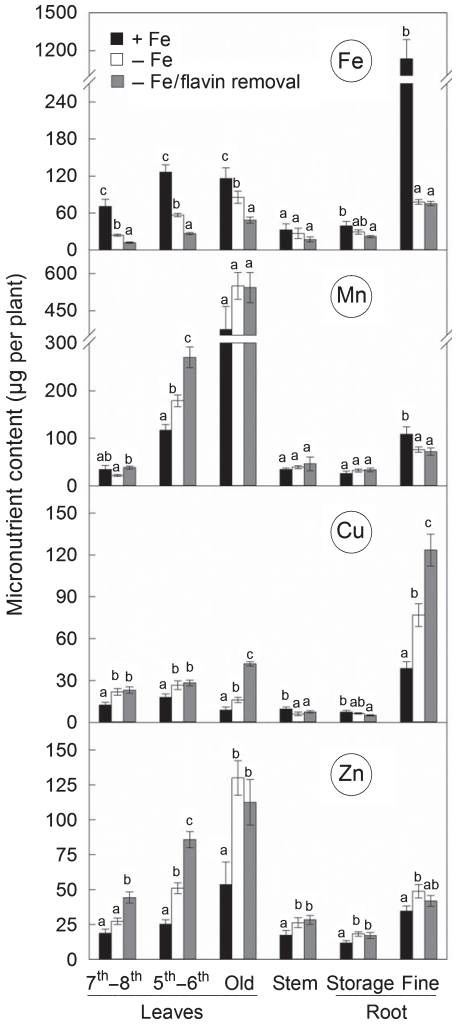

Fig. 4 Plant micronutrient content changes upon iron (Fe) deficiency and flavin removal from the nutrient solution. Fe, manganese (Mn), copper (Cu) and zinc (Zn) contents (µg metal per plant) in different parts of *Beta vulgaris* plants grown for 13 d with Fe (+Fe), without Fe (−Fe), and without Fe using the flavin removal system (−Fe/flavin removal). Plants were grown with experimental Set-up 1 as indicated in Fig. 1. Data are means ± SE ($n = 6$ for stem and oldest leaves, and 24–47 for the rest of plant parts). For a given plant part, significant differences among treatments (at $P < 0.05$) are marked with different letters above the columns.

decreased markedly with Fe deficiency (Fig. S4); apoplastic Fe was 68% and 44% of the total root Fe in control and Fe-deficient plants, respectively. On the one hand, Mn contents increased with Fe deficiency in developed leaves and decreased in fine roots; Cu contents increased with Fe deficiency in all leaves and fine roots, whereas decreases were found in stems. On the other hand, Zn contents increased significantly with Fe deficiency in all tissues except in young leaves.

Flavin depletion led to additional changes in micronutrient contents when compared with those found in Fe-deficient plants (Fig. 4). Flavin depletion further decreased the Fe contents in all leaves, whereas those of stems and roots were unaltered. The content of apoplastic Fe in fine roots was 46% of the total, a value similar to that observed when flavins were present (Fig. S4). Flavin removal led to increases in Mn and Zn contents only in the upper (young and developed) leaves, and to increases in Cu contents in old leaves and fine roots. Similar trends were generally found when considering the effects of flavin removal on metal concentrations (µg g DW^{-1}) instead of total contents (µg per plant) (Fig. S5).

Flavin depletion does not affect the root proton extrusion and Fe(III)-chelate reductase activities of Fe-deficient *B. vulgaris* plants Plants respond to Fe deficiency by lowering the pH of the growth media and increasing the root Fe(III) reductase activity (Susín *et al.*, 1994, 1996). When *B. vulgaris* plants were grown under zero-Fe in unbuffered nutrient solution (four plants per 10 l of nutrient solution), the pH had to be readjusted daily to 5.5, because Fe-deficient plants had decreased the pH of the nutrient solution from 5.5 to 4.0 in 24 h; the only exception was the first day, when the pH did not change (Fig. 5a). This rate of pH decrease was equivalent to *c.* 170 nmol H^+ per plant min^{-1}. Flavin removal did not have any effect on proton extrusion activity (Fig. 5a).

The root FCR activity was measured with whole roots of intact plants (at pH 6.0) 12 d after imposing Fe deficiency, following the classical method of Bienfait *et al.* (1983). The root FCR activity increased two-fold with Fe deficiency, and flavin removal did not have any effect on the root FCR activity (Fig. 5b).

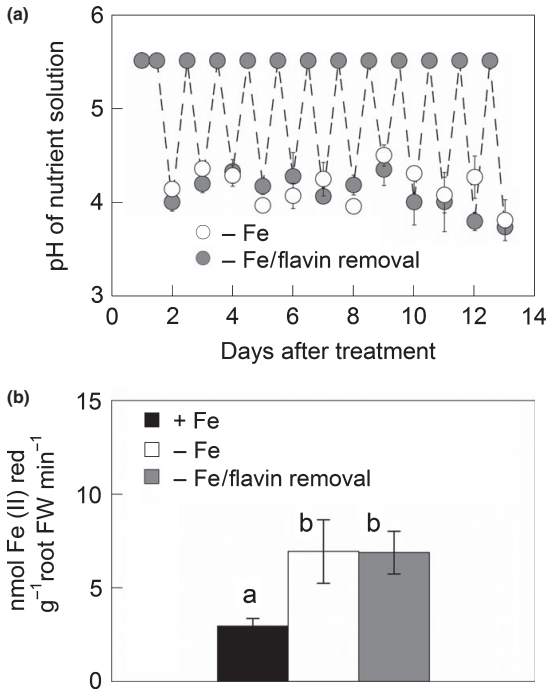

Fig. 5 Strategy I root responses with iron (Fe) deficiency and flavin removal from the nutrient solution. Plants were grown with the experimental Set-up 1 as indicated in Fig. 1. (a) Time-course of the changes in nutrient solution pH of *Beta vulgaris* plants grown with Fe (+Fe), without Fe (−Fe), and without Fe using the flavin removal system (−Fe/flavin removal). After each pH measurement, the pH was readjusted to 5.5. Data are means ± SE ($n = 12$). (b) Whole root Fe(III) chelate reductase activity of *B. vulgaris* plants after 12 d of treatment. Data are means ± SE ($n = 7$–11). Significant differences among treatments (at $P < 0.05$) are marked with different letters above the columns.

Flavins allow *B. vulgaris* plants to mine Fe from Fe(III)-oxides

In a different experimental set-up (Set-up 2), plants were grown with 0 or 45 μM Fe at pH 5.5 or 7.5 (Susín *et al.*, 1993, 1994, 1996). This set-up uses a two-fold larger solution volume per plant ratio and nutrient solutions with suitable buffer capacity to counteract acidification caused by roots during the whole treatment period. Unlike Set-up 1, Set-up 2 generally leads to a stronger chlorosis (Fig. S6) and higher root FCR activities (see later). These Fe-deficient plants have roots with particularly high concentrations of flavins when grown at pH 7.5, especially in the 0–5-mm section at the root tip, whereas they also have flavins, although in lower amounts, when grown at pH 5.5 (Figs S7, S8). Root flavins consist of Rbfl-sulfates (Fig. S8), which are released by Fe-deficient plants to the nutrient solution at pH 5.5 but not at pH 7.5 (Fig. S9). All of these data confirm results obtained previously with a different *B. vulgaris* genotype (Susín *et al.*, 1993, 1994; López-Millán *et al.*, 2000). Riboflavin sulfate concentrations in the soil solution surrounding the root (apex) can be in the range of 100–300 μM (calculated as detailed in Römheld, 1991, for phytosiderophores).

Flavin supplementation does not affect the root Fe(III)-chelate reductase activities of Fe-deficient *B. vulgaris* plants In order to investigate the possible effect of flavins on the FCR activities of whole roots from intact *B. vulgaris* plants, assays were carried out with (soluble) Fe(III)-EDTA by supplementing the assay medium with 0 or 100 μM Rbfl – in the oxidized form – at two different assay pH values, 5.5 and 7.5. The FCR activity was affected by Fe deficiency, the pH of the growth nutrient solution and the pH of the assay medium, but never by the addition of 100 μM Rbfl (Fig. 6a). When the assay was carried out without Rbfl and at pH 5.5, the FCR activity of Fe-deficient plants increased *c.* 11- to 19-fold (depending on the plant growth pH)

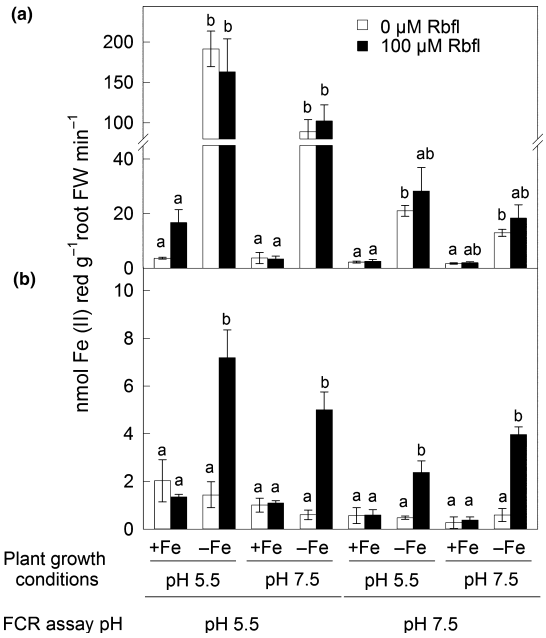

Fig. 6 Effects of flavin supplementation on the whole root Fe(III) reduction rates. Iron (Fe)-sufficient (+Fe) and Fe-deficient (−Fe) roots of *Beta vulgaris* plants were assayed with: (a) a soluble Fe(III)-chelate (500 μM Fe (III)-EDTA) or (b) a scarcely soluble solid ferric form (50 mg of poorly crystalline Fe(III)-oxide). All assays were carried out with 300 μM bathophenanthrolinedisulphonate (BPDS) in the presence or absence of 100 μM Rbfl for 20 and 60 min using Fe(III)-EDTA and Fe(III)-oxide, respectively. Plants were grown with experimental Set-up 2: plants pregrown as indicated earlier were grown for 10 d in 20-l buckets in nutrient solution with 0 or 45 μM Fe (Fe forms are indicated later), buffered at pH 5.5 (with 20 mM MES-NaOH) or 7.5 (with 1 mM NaOH and 1 g l^{-1} $CaCO_3$). Iron(III)-EDTA and Fe(III)-ethylendiaminedi(*o*-hydroxyphenylacetic) (Fe(III)-EDDHA) were used as Fe source in the nutrient solutions with pH 5.5 and 7.5, respectively. Data are means ± SE ($n = 3$–6). For a given pH of plant growth and Fe(III) chelate reductase (FCR) assay, significant differences among treatments (at $P < 0.05$) are marked with different letters above the columns.

when compared with the Fe-sufficient controls. Using an assay pH of 7.5 in the absence of Rbfl all FCR activities were markedly lower than at pH 5.5, but the activities of Fe-deficient plants were still four- to eight-fold higher than those of Fe-sufficient plants (depending on the plant growth pH). The addition of 100 μM Rbfl to the FCR assay medium did not cause changes in the root Fe(III)-EDTA reduction activities.

Flavin supplementation facilitates the reduction of a scarcely soluble Fe(III) source by roots of Fe-deficient *B. vulgaris* plants Root reduction assays were also carried out with a poorly crystaline Fe(III)-oxide as a substrate (Fig. 6b), instead of (soluble) Fe(III)-EDTA chelate (Fig. 6a). Iron reduction values were much lower with Fe(III)-oxide than with Fe(III)-EDTA (maximum values *c.* 8 and 200 nmol Fe(II) reduced g^{-1} root FW min^{-1}, respectively). In the absence of Rbfl, the reduction of Fe(III)-oxide by roots of intact plants was never affected by Fe deficiency, irrespective of the plant growth and assay pHs. However, when the assay medium was supplemented with 100 μM Rbfl, the root reduction of Fe(III)-oxide was markedly affected by Fe deficiency: it was unaffected in Fe-sufficient plants but increased markedly in Fe-deficient ones (four- to seven-fold, depending on the plant growth and assay pHs). This increase indicates that a flavin-dependent process accounts for up to 80% of the extracellular electron transport in Fe-deficient *B. vulgaris* plant roots. It is remarkable that in Fe-deficient plants assayed at pH 7.5, typical of calcareous soils, the addition of 100 μM Rbfl increased the rate of Fe(III)-oxide reduction three- to five-fold. The enhancement in the reduction of Fe(III)-oxide by Fe-deficient plants with 100 μM Rbfl occurred consistently using assay times from 20 min to 2 h (not shown; values in Fig. 6b are for 60 min).

The possible differences between the Rbfl-sulfates secreted by Fe-deficient *B. vulgaris* plants and Rbfl on the root reduction of Fe(III)-oxide were studied using excised root sections (0–5 and 5–10 mm from the apex; Figs. 7a,b). Root sections were used instead of whole roots from intact plants because of the limited amount of Rbfl-sulfates isolate. The supplementation of the assay medium with 1, 10 and 100 μM Rbfl caused four-, nine- and 16-fold increases, respectively, in the reduction of Fe(III)-oxide by excised Fe-deficient roots (Fig. S10); 10 μM flavin was used in the rest of the assays due to the low amount of Rbfl-sulfates available. In the absence of Rbfl-sulfates, the reduction of Fe(III)-oxide by 0–5 mm root sections was not affected significantly by Fe deficiency, irrespective of the plant growth and assay pHs (Fig. 7a). However, when the assay medium was supplemented with 10 μM Rbfl or Rbfl-sulfates, the reduction of Fe(III)-oxide was markedly increased, irrespective of the plant growth and assay pHs, with the effects of Rbfl and Rbfl-sulfates being similar.

When the assay was run with root sections of Fe-deficient plants taken 5–10 mm from the apex and flavins were not included in the assay, the reduction of Fe(III)-oxide was somewhat higher than that observed with the 0–5 mm sections (Fig. 7b). When the assay medium was supplemented with 10 μM Rbfl or Rbfl-sulfates, an increase (< two-fold) in the reduction rate of Fe(III)-oxide by 5–10 mm root sections was found only for plants grown and assayed at pH 5.5 (Fig. 7b).

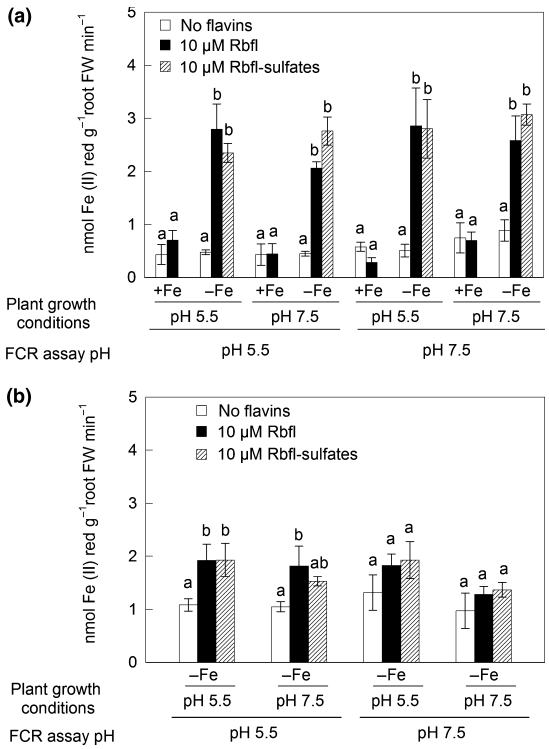

Fig. 7 Effects of flavin supplementation on the Fe(III) reduction rates of excised roots using a scarcely soluble Fe(III)-oxide as substrate. Root sections were taken (a) 0–5 mm or (b) 5–10 mm from the apex from iron (Fe)-sufficient (+Fe) and/or Fe-deficient (−Fe) *Beta vulgaris* plants and assayed with 0 or 10 μM of Rbfl or Rbfl sulfates. Plants were grown with experimental Set-up 2 as indicated in Fig. 6, and Rbfl-sulfates were obtained using experimental Set-up 3. Data are means ± SE ($n = 3–6$). For a given pH of plant growth and Fe(III) chelate reductase (FCR) assay, significant differences among treatments (at $P < 0.05$) are marked with different letters above the columns.

Flavins facilitate the *in vitro* solubilization of a scarcely soluble Fe(III) source In order to investigate the effect of flavins on the solubilization of poorly crystaline Fe(III)-oxide, *in vitro* incubation assays were carried out with 0 and 10 μM Rbfl or Rbfl-sulfates (in the oxidized forms) in the presence or absence of the electron donor NADH and the Fe(II) trapping agent BPDS. Assay media were buffered at pH 5.5 or 7.5. Iron solubilization was assessed by measuring total Fe in the assay solution with GFAAS (Fig. 8). In the absence of NADH and BPDS, the solubilization of the Fe(III)-oxide was *c.* 1-2 nmol Fe g^{-1} Fe(III)-oxide min^{-1}, irrespective of the presence or absence of flavins and the assay pH (Fig. 8a). When the medium was supplemented with 500 μM NADH no changes were found when flavins were absent. However, when 10 μM Rbfl was present statistically significant increases in the solubilization of Fe(III)-oxide were found (Fig. 8b), with the increases being larger at pH 7.5 than at pH 5.5 (six- vs four-fold).

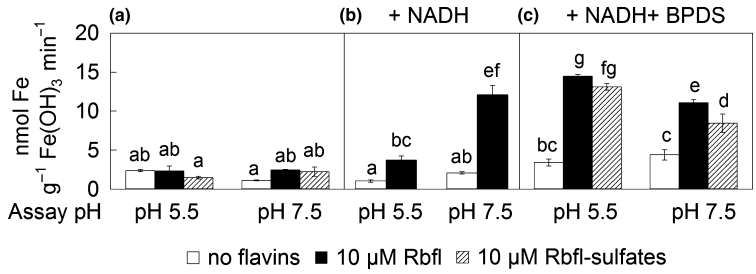

Fig. 8 *In vitro* solubilization of scarcely soluble Fe(III)-oxide as affected by flavins, NADH and bathophenanthrolinedisulphonate (BPDS). Incubation assays were run at two different pH values, 5.5 and 7.5, in the presence of 0 or 10 μM of Rbfl or Rbfl-sulfates, (a) 0 or (b, c) 500 μM NADH as an electron donor, and (a, b) 0 or (c) 600 μM BPDS as Fe(II) trapping agent. Riboflavin-sulfates were obtained using experimental Set-up 3. Data are means ± SE ($n = 3$). Significant differences among treatments (at $P < 0.05$) are marked with different letters above the columns.

When incubation assays were run in the presence of 500 μM NADH and 600 μM BPDS, the solubilization of Fe(III)-oxide by the buffer solutions was *c.* 3–4 nmol Fe g^{-1} Fe(III)-oxide min^{-1}, irrespective of the assay pH (Fig. 8c). However, when 10 μM Rbfl or Rbfl-sulfates were added to the assay medium, the solubilization of Fe(III)-oxide increased markedly (two- to four-fold, depending on the assay pH and the flavin used). The solubilization of Fe(III)-oxide was higher with Rbfl than with Rbfl-sulfates at pH 7.5. For both flavins, the solubilization of Fe(III)-oxide was somewhat higher at pH 5.5 than that at pH 7.5. Therefore, the addition of BPDS to the assay medium containing NADH enhanced the Rbfl-induced solubilization of Fe(III)-oxide only at pH 5.5 (Fig. 8b,c).

The formation of the Fe(II)(BPDS)$_3$ complex was also measured in the final assay solutions, to investigate if the solubilization of Fe(III)-oxide involved the reduction of Fe(III) to Fe(II). The amount of Fe reduced from Fe(III)-oxide using solutions containing 500 μM NADH and 600 μM BPDS was *c.* 1 nmol Fe (II) reduced g^{-1} Fe(III)-oxide min^{-1}, irrespectively of the pH (Fig. 9). When 10 μM Rbfl or Rbfl-sulfates were added to the medium containing NADH and BPDS, the reduction of Fe(III)-oxide increased markedly (five- to eight-fold, depending on the pH and the flavin used). The reduction of Fe(III)-oxide was higher with Rbfl than with Rbfl-sulfates, both at pH 5.5 and 7.5 (relative increases were 21% and 31%, respectively), and for both flavins the reduction rate of Fe(III)-oxide was higher at pH 5.5 than at pH 7.5. In the presence of flavins, the amount of Fe(II) trapped by BPDS accounted for 70–77% of the total Fe solubilized, depending on the pH and the flavin used, whereas in the absence of flavins it accounted for 28–40% (Figs 8c, 9).

The reduction of Fe(III)-oxide increased progressively when the concentration of Rbfl increased from 1 to 25 μM, to remain constant from 25 up to 100 μM (Fig. S11). The reduction of Fe (III)-oxide was generally lower at pH 7.5 than at pH 5.5, irrespective of the Rbfl concentration, with the exception of the assay with 1 μM Rbfl. Iron(III) oxide reduction also occurred in the 0.01–1.00 μM Rbfl concentration range, and even at incubation times of 1 h, the addition of Rbfl concentrations as low as 1 μM led to a significant reduction of Fe(III)-oxide (Fig. S12).

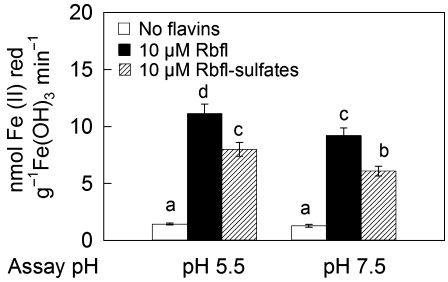

Fig. 9 *In vitro* reduction of scarcely soluble Fe(III)-oxide as affected by flavins in the presence of NADH and bathophenanthrolinedisulphonate (BPDS). Incubation assays were run at two different pH values, 5.5 and 7.5, in the presence of 0 or 10 μM of Rbfl or Rbfl-sulfates, 500 μM NADH as an electron donor and 600 μM BPDS as Fe(II) trapping agent. Riboflavin-sulfates were obtained using experimental Set-up 3. Data are means ± SE ($n = 6$). Significant differences among treatments (at $P < 0.05$) are marked with different letters above the columns.

Discussion

The roots of many Strategy I plants secrete flavins to the growth medium in response to low Fe availability. However, whether or not flavins are involved in Fe acquisition is still an unresolved question. In this study we show that the depletion of flavins in the nutrient solution of Fe-deficient *Beta vulgaris* plants has a negative impact on the plant Fe nutritional status. Also, we show that flavins promote the reductive dissolution of a scarcely soluble Fe(III)-oxide both in presence of NADH and Fe-deficient plant roots, and that this process occurs preferentially in the first 5 mm from the root apex, the same zone where the Fe(III)-reducing activity, flavin concentrations and O_2 consumption are localized in Fe-deficient *B. vulgaris* (Susín *et al.*, 1993; López-Millán *et al.*, 2000).

Beta vulgaris plants respond to Fe deficiency by enhancing the root secretion (Figs 1, 2d, S9) and accumulation of flavins (Figs S7, S8), which consist in two different Rbfl-sulfates (Figs S2, S8). This response is elicited in combination with an early and strong acidification of the nutrient solution (Fig. 5a). This can be relevant for flavin secretion in natural conditions, which is absent at

pH 7.5 (Fig. S9), typical of the bulk calcareous soils, but it is present at high rates at the lower pH values that can be expected near the roots when ATPase activity is enhanced. *Beta vulgaris* plants also respond with an enhancement of the root FCR activity (Figs 5b, 6a), which is common to other Strategy I species tolerant to Fe deficiency. All of this is in agreement with previous studies with *B. vulgaris* (Susín *et al.*, 1993, 1994, 1996; see Notes S4 for details) and other flavin-producing species (Welkie, 2000; Andaluz *et al.*, 2009; Rodríguez-Celma *et al.*, 2011a,b). We did not detect coumarin-type phenolics, previously associated to Fe-deficiency in *Arabidopsis*, in the root extracts and nutrient solutions of Fe-deficient *B. vulgaris* plants (Figs S13, S14). Conversely, Fe-deficient *T. pratense* plants produce and secrete phenolic compounds, whereas they do not accumulate or secrete flavins (Jin *et al.*, 2007). This extends the view that the root production of phenolics and flavins induced by Fe shortage could be mutually exclusive, as recently shown for *M. truncatula* (a flavin producer) and *Arabidopsis* (a phenolics producer) (Rodríguez-Celma *et al.*, 2013).

The depletion of flavins from the nutrient solution aggravated the Fe deficiency-induced chlorosis in *B. vulgaris*. A much more developed Fe chlorosis was observed when the nutrient solution of Fe-deficient plants was recirculated through a C_{18} resin column (Fig. 2); using this system, flavins were retained in the columns (Fig. S1) and the flavin concentrations in the nutrient solution decreased up to 86% (Figs 1, 2d). When plants were grown in the low-flavin medium, they showed significant decreases in total biomass (21%; Fig. 3) and leaf Fe content (47%; Fig. 4), whereas the plant uptake of other metals was not impaired (the Cu, Mn and Zn plant contents were actually higher, with increases being 49%, 11% and 10%, respectively, when compared with those of Fe-deficient plants; Fig. 4). However, flavin removal did not affect other Strategy I root responses, because root FCR and ATPase activities were similar to those found in Fe-deficient plants in the presence of flavins in the medium (Fig. 5a,b). Similar findings were observed when phenolics were removed from the nutrient solution of Fe-deficient *T. pratense* (Jin *et al.*, 2007) and Arabidopsis plants (Fourcroy *et al.*, 2014) using similar C_{18}-based recirculation removal systems.

Recent studies with *Arabidopsis* support the hypothesis that the function of phenolics in Fe nutrition is an intrinsic component of the chelation-based Strategy I in some plant species. Coumarins, some of which have catechol groups, were identified in exudates of Fe-deficient *Arabidopsis* roots (Fourcroy *et al.*, 2014; Schmid *et al.*, 2014; Schmidt *et al.*, 2014). These exudates were more capable of mobilizing Fe(III) from Fe(III)-oxides than those from Fe-sufficient plants or low coumarin-producing *f6′h1* mutant plants, and this was ascribed to the formation of Fe(III) catechol complexes (Schmid *et al.*, 2014). Also, *Arabidopsis* mutants defective in the production (*f6′h1*) or secretion of coumarins (*abcg37*) were inefficient in taking up Fe from a poorly available Fe(III) source (Rodríguez-Celma *et al.*, 2013). Based on this information, it has been suggested that phenolic-producing Strategy I plant species, such as *Arabidopsis*, use a chelation-based Fe acquisition strategy when grown with scarcely soluble Fe(III)-oxide forms instead of soluble Fe(III) forms (Rodríguez-Celma *et al.*, 2013; Schmid *et al.*, 2014).

Flavins, including Rbfl-sulfates secreted by *B. vulgaris*, were able to dissolve scarcely soluble Fe(III)-oxide in the presence of the electron donor NADH and at physiologically relevant pH values (5.5–7.5; Fig. 8). Because most of the dissolved Fe (70–77%) was trapped by BPDS, the dissolution process appears to involve a reduction of Fe(III) (Figs 8, 9). This is in line with the recent findings that reduced flavin forms ($RbflH_2$ and $FMNH_2$) – in the absence of O_2 – reduce and dissolve not only a freshly prepared Fe(III)-oxide (von Canstein *et al.*, 2008), but also more crystalline Fe(III) forms such as ferrihydrite, lepidocrocite, goethite and hematite, and this occurs over a wide pH range (Shi *et al.*, 2012, 2013). The reductive dissolution of Fe(III)-oxide occurs at low flavin concentrations (0.01–1 μM), with rates increasing when the concentration of flavins increased (Figs S11, S12). Whereas the flavin-induced reductive dissolution of Fe (III)-oxide occurs both at pH 5.5 and 7.5, the rate of reduction at pH 5.5 was enhanced by the Fe(II) trapping agent BPDS (Fig. 8). The two latter conditions (lower pH, Fe(II) sequestering) are likely to exist near to the root cell plasma membrane (PM) in Fe-deficient plants, where the ATPase is highly active and Fe(II) is actively transported into the cell by IRT1.

The addition of extracellular flavins enabled roots of Fe-deficient *B. vulgaris* plants (but not those of Fe-sufficient plants) to increase markedly (five-fold) the mobilization of Fe from a scarcely soluble Fe source. This was demonstrated by spiking flavins (Rbfl or Rbfl-sulfates) in a medium containing whole or excised roots from Fe-deficient plants and Fe(III)-oxide, both at pH 5.5 and 7.5 (Figs 6b, 7a). Conversely, flavins did not increase the already high reduction rate of a soluble Fe form, (Fe(III)-EDTA), by Fe-deficient plant roots (Fig. 6a). In fact, acquisition from Fe(III) soluble forms is likely to be little relevant in natural conditions in calcareous soils, where very low Fe concentrations in the soil solution surrounding the root tips are expected, because of the active incorporation of Fe into the cells and the limited replenishment of soluble Fe (because the bulk of the soil solution has pH 7.4–8.5 and soluble Fe is *c.* 10^{-10} M; Lindsay, 1995).

Flavin concentration appears to be critical to control the rate of Fe(III) reduction from Fe(III)-oxide. Whereas Rbfl and Rbfl-sulfates led to similar Fe(III)-oxide reduction rates when used at the same concentration (both in the presence of roots and NADH; Figs 7a, 8, respectively), increases in flavin concentrations led to a marked enhancement in reduction rates (Fig. S10). The solubility of flavins differ greatly, because the maximum solubility of Rbfl in solution is 130–150 μM, whereas the solubility of the Rbfl-sulfates found in *B. vulgaris* is one to two orders of magnitude higher (Susín *et al.*, 1993), and the same is likely to occur with the hydroxylated Rbfl derivatives found in *M. truncatula* (Rodríguez-Celma *et al.*, 2011b). Therefore, the production of highly soluble Rbfl derivatives can provide a competitive advantage in low Fe environments.

On the one hand, the flavin-mediated mobilization of Fe from Fe(III)-oxide occurred mainly in the first 5 mm from the root apex (Fig. 7a), where the Fe(III) chelate reductase (FCR) activity

and flavin accumulation are localized in Fe-deficient *B. vulgaris* plants (López-Millán *et al.*, 2000, 2001). On the other, the flavin-mediated mobilization in the 5–10 mm root zone, known to have lower FCR activity (López-Millán *et al.*, 2000, 2001), was much lower (Fig. 7b). These findings support the idea that flavins could mediate extracellular electron transfer between the PM FCR enzyme and the Fe(III)-oxide. A number of microorganisms living in anaerobic environments use a PM reductase in connection with secreted extracytoplasmic flavins (Rbfl and FMN) to mediate both assimilatory Fe reduction (in *Campylobacter jejuni*, *Helicobacter pylori* and three species of methanotrophic bacteria; Fassbinder *et al.*, 2000; Crossley *et al.*, 2007; Balasubramanian *et al.*, 2010) and respiration from insoluble minerals, including Fe(III)-oxide (in *Shewanella oneidensis*, *Geothrix fermentans*, *Bacillus* sp. *WS-XY1* and *Pichia stipitis*; von Canstein *et al.*, 2008; Marsili *et al.*, 2008; Mehta-Kolte & Bond, 2012; Wu *et al.*, 2014). For instance, flavins secreted by *S. oneidensis* (a well-studied facultative dissimilatory metal-reducing, anaerobic, Gram-negative gamma-proteobacterium), account for at least 70% of the extracellular electron transport capacity (Marsili *et al.*, 2008; Kotloski & Gralnick, 2013), a value similar to the 80% value found in this study with *B. vulgaris* plants (Figs 6b, 7a). The use of endogenous extracellular small molecules – such as flavins – as an electron shuttle is a powerful strategy for many bacteria, enabling long-distance electron transfer between the organism and insoluble electron acceptors such as Fe(III)-oxides (Marsili *et al.*, 2008; Brutinel & Gralnick, 2012; Fuller *et al.*, 2014). During anaerobic respiration, bacteria transfer electrons from organic carbon to electron acceptors outside the cell, and use the energy obtained from these coupled reactions to translocate protons from the cytoplasm to the periplasm (Madigan *et al.*, 2003).

When plants are grown under Fe deficiency, roots have to cope with a low O_2 environment both at the symplastic and apoplastic level. Numerous proteomic and/or biochemical studies support the idea that Fe-deficient roots revert to a low O_2 (anaerobic-like) metabolism in the symplast associated to the high energy demand caused by the enhancement of the FCR and ATPase activities (both in *B. vulgaris* and other Strategy I species; López-Millán *et al.*, 2000, 2001, 2009, 2013; Zocchi, 2006; Andaluz *et al.*, 2009; Donnini *et al.*, 2010; Rellán-Álvarez *et al.*, 2010; Rodríguez-Celma *et al.*, 2011a; Vigani, 2012). These activities consume NADPH and ATP, and the recharge of these substrates consumes O_2 at high rates, leading to a functional hypoxia similar to that found in burst-type muscular activity in animals. The recharging of ATP has been associated to an upregulation of glycolysis – increasing the cytosolic production of ATP – and a highly enhanced activity of phospho*enol*pyruvate carboxylase (PEPC), an enzyme that is the downstream driving force for glycolysis and supplies oxalacetate to the TCA cycle. For instance, the first 5 mm in the yellow root tips of Fe-deficient *B. vulgaris* had large increases in the activities of PEPC (60-fold) and the two enzymes typical of anaerobic metabolism, pyruvate decarboxylase (six-fold) and lactate dehydrogenase (13-fold), as well as a four-fold increase in O_2 consumption (López-Millán *et al.*, 2000). Furthermore, O_2 is also depleted at the apoplastic level, because 20% of the total O_2 consumption by roots of Fe-deficient plants is still present when mitochondrial respiration is inhibited by KCN and salicylhydroxamic acid (SHAM) (López-Millán *et al.*, 2000; Vigani & Zocchi, 2010). Because this residual O_2 consumption ceased with the addition of Fe(III)-EDTA and anaerobiosis increased Fe(III)-EDTA reduction activity, it is likely that the FCR can donate electrons directly to O_2 (López-Millán *et al.*, 2000).

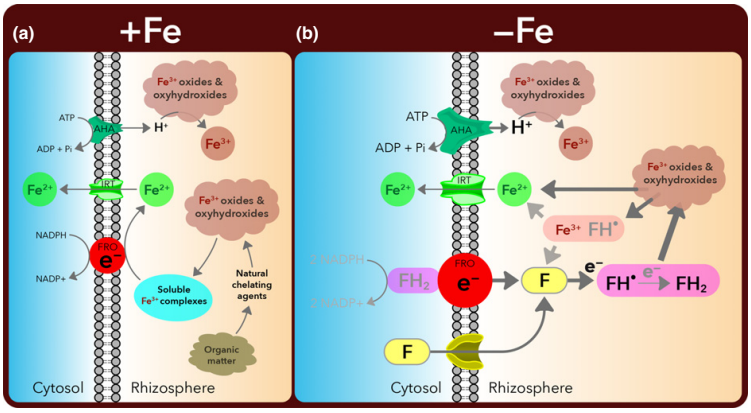

Fig. 10 Schematic representation of iron (Fe) mining from the soil by flavin-producing Strategy I plant species. (a) Mechanisms of Fe uptake by plants when sufficient soluble Fe^{3+} is available. Naturally occurring chelating agents in the rhizosphere solubilize Fe^{3+}-oxides/oxyhydroxides by forming soluble Fe^{3+} organic complexes, which are then reduced and taken up by the Fe^{3+} chelate reductase (FRO) and the Fe^{2+} IRT transporter, respectively, at the root cell plasma membrane (PM). (b) Mechanisms of Fe uptake by flavin-producing plants when soluble Fe is in low concentrations. Secreted flavins act as a soluble redox shuttle, carrying electrons from the enhanced FRO at the PM towards soil Fe^{3+} oxides/oxyhydroxides, leading to a reductive dissolution and therefore increasing plant Fe availability. Thin dark gray arrows indicate processes already known. Thick dark gray arrows indicate processes related to flavins shown in this study. Light gray arrows and letters indicate still hypothetical processes. The changes in activity of FRO, IRT and H^+-ATPase (AHA) upon Fe-deficiency are indicated by the size of the corresponding symbols. F, $FH^•$, FH_2, oxidized, semi-reduced radical and fully reduced flavin forms.

In summary, the results of this study provide support for the view that flavins secreted by Fe-deficient plant roots may act as a soluble redox shuttle, carrying electrons from the PM FCR to Fe (III)-oxide, leading to a reductive dissolution and therefore increasing plant Fe availability (Fig. 10). The use by the root FCR in Fe-deficient plants of alternative electron acceptors such as Fe(III)-oxides would allow a continued energy generation as well as the simultaneous production of soluble Fe that can be used by the Strategy I uptake system. Accumulation of flavins in the cell and/or at the cell surface, in combination with a flavin reductase to generate reduced flavins, would constitute a potent Fe-reduction system. A flavin-based Fe uptake system would be particularly useful in environments with a low Fe availability (e.g. in the absence of soluble Fe(III) forms), because the FCR enzyme may donate electrons not only to flavins but also to O_2, which would become depleted in the close vicinity of the PM uptake sites, in turn preventing the oxidation of reduced flavin forms and Fe(II). Among eukaryotes, the use of environmentally available electron acceptors other than O_2 (such as Fe(III)-oxide) is still considered to be rare, but it might be more widespread than currently thought, because it would allow for a facilitation of organism survival in adverse conditions (Müller *et al.*, 2012).

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Author contributions

A.A-F. planned and designed the research. P.S-T. carried out flavin removal study, flavin supplementation study, and Fe(III)-oxide solubilization assays. J.J.R. carried out mineral analyses, and root microscopy. P.S-T. prepared and analyzed the results. J.A., A.A. and A.A-F. analyzed critically the results. P.S-T., J.A., A.A. and A.A-F. wrote the manuscript. All authors read and approved the final manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 System used to remove flavins from the nutrient solution.

Fig. S2 Characterization of the flavin isolate from the nutrient solution of Fe-deficient plants.

Fig. S3 Plant biomass changes upon Fe deficiency and flavin removal from the nutrient solution.

Fig. S4 Changes in apoplastic Fe contents in plants upon Fe deficiency and flavin removal from the nutrient solution.

Fig. S5 Plant micronutrient concentration changes upon Fe deficiency and flavin removal from the nutrient solution.

Fig. S6 Time-course of SPAD values of leaves of Fe-sufficient and Fe-deficient plants grown at pH values 5.5 or 7.5.

Fig. S7 Optical view and flavin fluorescence of excised root sections of Fe-sufficient and Fe-deficient plants grown at pH values 5.5 or 7.5.

Fig. S8 Flavin concentrations in excised root sections of Fe-sufficient and Fe-deficient plants grown at pH values 5.5 or 7.5.

Fig. S9 Time-course of flavin secretion to the nutrient solution by Fe-sufficient and Fe-deficient plants grown at pH values 5.5 or 7.5.

Fig. S10 Effect of Rbfl concentration on Fe(III) reduction rates using excised root tips and Fe(III)-oxide.

Fig. S11 Effects of Rbfl concentration and pH on the *in vitro* reduction of Fe(III)-oxide in the presence of NADH and BPDS.

Fig. S12 Effects of Rbfl concentration and reaction time on the *in vitro* reduction of Fe(III)-oxide in the presence of NADH and BPDS.

Fig. S13 HPLC-MS(TOF) analysis of phenolics in root extracts of Fe-deficient plants.

Fig. S14 HPLC-MS(TOF) analysis of phenolics in nutrient solutions of Fe-deficient plants.

Notes S1 Detailed information on the extraction of flavins and phenolics from roots.

Notes S2 Detailed information on the analyses of flavins and phenolics.

Notes S3 Detailed information on the root apoplastic Fe extraction.

Notes S4 Detailed information on the differences between *Beta vulgaris* cultivars in the intensity of the root responses to Fe deficiency.

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Supplementary Material

Flavins secreted by roots of iron-deficient *Beta vulgaris* enable mining of ferric oxide via reductive mechanisms

Patricia Sisó-Terraza, Juan J. Rios, Javier Abadía, Anunciación Abadía, and Ana Álvarez-Fernández

The following Supplementary Data is available for this article:

Notes S1 Detailed information on the extraction of flavins and phenolics from roots.

Notes S2 Detailed information on the analyses of flavins and phenolics.

Notes S3 Detailed information on the root apoplastic Fe extraction

Notes S4 Detailed information on the differences between *Beta vulgaris* cultivars in the intensity of the root responses to Fe deficiency.

Fig. S1 System used to remove flavins from the nutrient solution.

Fig. S2 Characterization of the flavin isolate from the nutrient solution of Fe-deficient solution.

Fig. S3 Plant biomass changes upon Fe deficiency and flavin removal from the nutrient solution.

Fig. S4 Changes in apoplastic Fe contents in plants upon Fe deficiency and flavin removal from the nutrient solution.

Fig. S5 Plant micronutrient concentration changes upon Fe deficiency and flavin removal from the nutrient solution.

Fig. S6 Time-course of SPAD values of leaves of Fe-sufficient and Fe-defficient plants grown at pH values 5.5 or 7.5.

Fig. S7 Optical view and flavin fluorescence of excised root sections of Fe-sufficient and Fe-defficient plants grown at pH values 5.5 or 7.5.

Fig. S8 Flavin concentrations in excised root sections of Fe-sufficient and Fe-defficient plants grown at pH values 5.5 or 7.5.

Fig. S9 Time-course of flavin secretion to the nutrient solution by Fe-sufficient and Fe-defficient plants grown at pH values 5.5 or 7.5.

Fig. S10 Effect of Rbfl concentration on Fe(III) reduction rates using excised root tips and Fe(III)-oxide.

Fig. S11 Effects of Rbfl concentration and pH on the *in vitro* reduction of Fe(III)-oxide in the presence of NADH and BPDS.

Fig. S12 Effects of Rbfl concentration and reaction time on the *in vitro* reduction of Fe(III)-oxide in the presence of NADH and BPDS.

Fig. S13 HPLC-MS(TOF) analysis of phenolics in root extracts of Fe-deficient plants.

Fig. S14 HPLC-MS(TOF) analysis of phenolics in nutrient solutions of Fe-deficient plants.

Note S1 Detailed information on the extraction of flavins and phenolics from roots.

Frozen roots (ca. 100 mg) were ground with 1 ml of 100% LC-MS grade methanol in a Retsch M301 ball mill (Restch, Düsseldorf, Germany) for 5 min. The procedure was repeated with 100 mg of frozen sample. The supernatants were recovered by centrifugation (12,000 *g*, 4 ºC and 5 min) and stored at -20 °C, and the pellets were resuspended in 1 ml of 100% methanol, homogenised for 5 min and the supernatants recovered again. All four supernatants per sample were pooled, vacuum concentrated and diluted with a solution of 15% methanol and 0.1% formic acid to a final volume of 250 µl. Extracts were filtered through polyvinylidene fluoride (PVDF) 0.45 µm ultrafree-MC centrifugal filter devices (Millipore, Billerica, MA, USA) and stored at -80°C until analysis.

Note S2 Detailed information on the analyses of flavins and phenolics.

Flavin analysis

The HPLC system was equipped with a Waters Symmetry® C_{18} column (3.5 µm particle size, 2.1 x 150 mm) and a Waters Symmetry® C_{18} guard column (3.5 µm particle size, 2.1 x 10 mm), and a gradient built with two solutions: A (1 mM ammonium acetate in methanol at pH 6.0) and B (1 mM ammonium acetate in Milli-Q water at pH 6.0). Injection volume and flow rate were 20 µL and 250 µL min^{-1}, respectively. Autosampler and column temperatures were 6 and 30 ºC, respectively. The gradient started at 10% B, the solvent composition was maintained for 1 min and then increased linearly to 17.5% B for 4 min. Afterwards, the solvent composition was maintained for 50 min and then decreased linearly to 10% B for 2 min. Finally, to regenerate the column, the solvent was maintained linearly at 10% B until 70 min, when a new sample could be injected. The HPLC was coupled to a photodiode array detector, with the exit flow connected *via* an electrospray (ESI) source to a MS(TOF) detector. UV-VIS absorption spectra were recorded from 200 to 600 nm and MS spectra were acquired in the 100-800 mass/charge ratio range. The MicrOTOF was operated in positive ion mode with endplate and spray tip potentials of -0.5 and 4.5 kV, respectively. Nebulizer gas (N_2) pressure and drying gas (N_2) flow rate were kept at 2.9 bar and 9.3 L min^{-1}, respectively, orifice voltage was 70 V and drying gas temperature was 200 ºC. The mass axis was calibrated using Li-formate adducts (10 mM LiOH, 0.2% [v/v] formic acid and 50% [v/v] 2-propanol). The system was controlled with MicrOTOF Control v.2.2 and HyStar v.3.2, and data were processed with Data Analysis v.4.0 (all software items from Bruker Daltonik, Bremen, Germany). Standards used include Rbfl, Rbfl-5'-phosphate (FMN; purity ≥95%, Sigma) and flavin adenine dinucleotide (FAD; purity ≥95%, Sigma). Rbfl-sulfates were identified using retention time, UV-VIS spectra and exact molecular mass (with errors <5 ppm). All flavins were quantified using peak areas at 445 nm and calibration curves of their corresponding standards, with the exception of Rbfl-sulfates, where concentrations were estimated using FMN as standard.

Phenolics analysis

The LC-UV/VIS-MS(TOF) method used for analysis of phenolics (Fourcroy y col., 2014) is highly selective and has a very good sensitivity for the detection of different phenolic compounds (in the range of 2-500 nM). This method has been used to identify the phenolics accumulated/secreted in response to Fe deficiency by roots in Arabidopsis (Fourcroy y col., 2014) and in four more plant species (unpublished data).

The limits of detection for standard solutions are as follows. Coumarins: 2 nM for isofraxidin and unckalin, 3 nM for fraxinol, 4 nM for scopoletin, fraxetin and esculetin, 5 nM for esculin, 10 nM for scopolin, 50 nM for fraxin; Flavonoids: 40 nM for myricetin and myricitrin; Stilbenes: 70 nM for resveratrol; Lignan: 2.5 for matairesinol; Cinnamic acid derivatives: 50 nM for ferulic acid and coniferyl aldehyde, 500 nM for caffeic acid, sinapic acid, sinapyl aldehyde and sinapyl alcohol.

Note S3 Detailed information on the root apoplastic Fe extraction.

Roots were centrifuged between filter paper sheets at 200 g to remove traces of the nutrient solutions, and then transferred into 200 ml of 10 mM Na_2EDTA and 0.5 mM $CaSO_4$ for 1.5 h to remove apoplastic Fe. After washing, roots were centrifuged again, dried and analyzed for non-apoplastic Fe. Apoplastic Fe contents were calculated as the difference between total and non-apoplastic contents.

Note S4 Detailed information on the differences between *Beta vulgaris* cultivars in the intensity of the root responses to Fe deficiency.

Differences in the intensity of Strategy I responses to Fe stress between species and within cultivars in a given species have long been recognized. In *B. vulgaris*, the root flavin accumulation and the time course of flavin secretion to the nutrient solution were similar in two different cultivars, the cv. “Orbis” used in the present study (Figs. S7 and S8 and Figs. 1 and S9, respectively) and the cv. “Monohil” (Susín *y col.* 1993, 1994, López-Millán *y col.*, 2000 and Rellán-Álvarez *y col.*, 2010). Iron-deficient *B. vulgaris* plants exhibited comparable increases in root FCR activity using Fe(III)-EDTA [20- and 40-fold over control values for “Monohil” (Susín *y col.*, 1996) and “Orbis” (Fig. 6a), respectively; these differences are probably associated to a difference of 0.5 pH units in the FCR assay pH]. It should be noted that the plants/volume ratio and/or the nutrient solution buffering had a strong effect on the Fe deficiency-induced enhancement in root-FCR activity: the increase was 2- and 40-fold in plants grown at plant/volume (L) ratios of 0.4 (Fig. 5b) and 0.2 (Fig. 6a) with and without pH buffering at 5.5, respectively.

Fig. S1 System used to remove flavins from the nutrient solution. **(a)** Fe-deficient *B. vulgaris* plants growing with the Set-up 1 in a 10-liter nutrient solution bucket; **(b)** peristaltic pump (flow of 480 mL h^{-1}; Minipuls Evolution cartridge pump, Gilson, Middleton, WI, USA) used for continuous recirculation of the nutrient solution; and **(c)** 10 cm-column (Sigma, St. Louis, MO, USA) filled with 10 g of spherical C_{18} bonded flash silica (45-75 µm particle size). Columns were changed every 1-3 days to avoid saturation. Flavins were accumulated (see yellow zone) in the column.

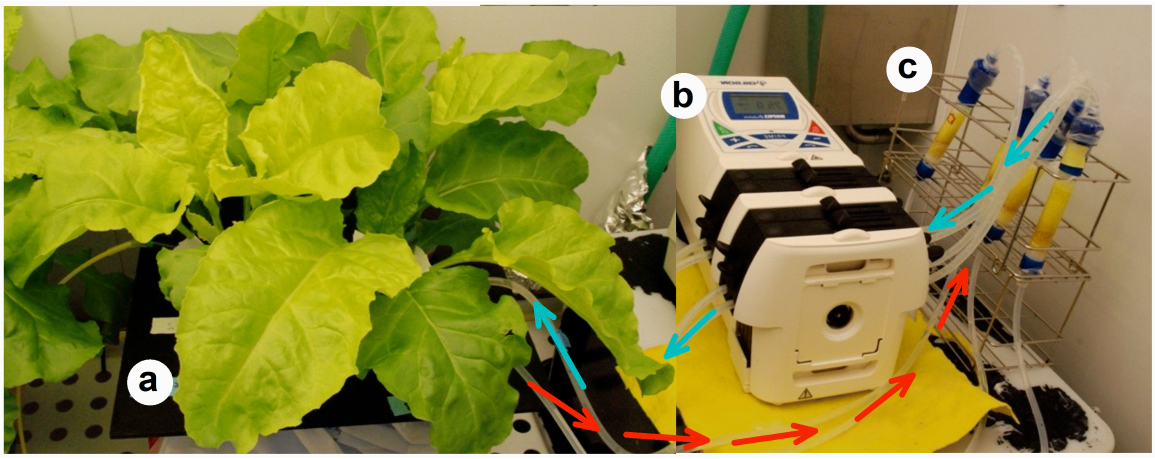
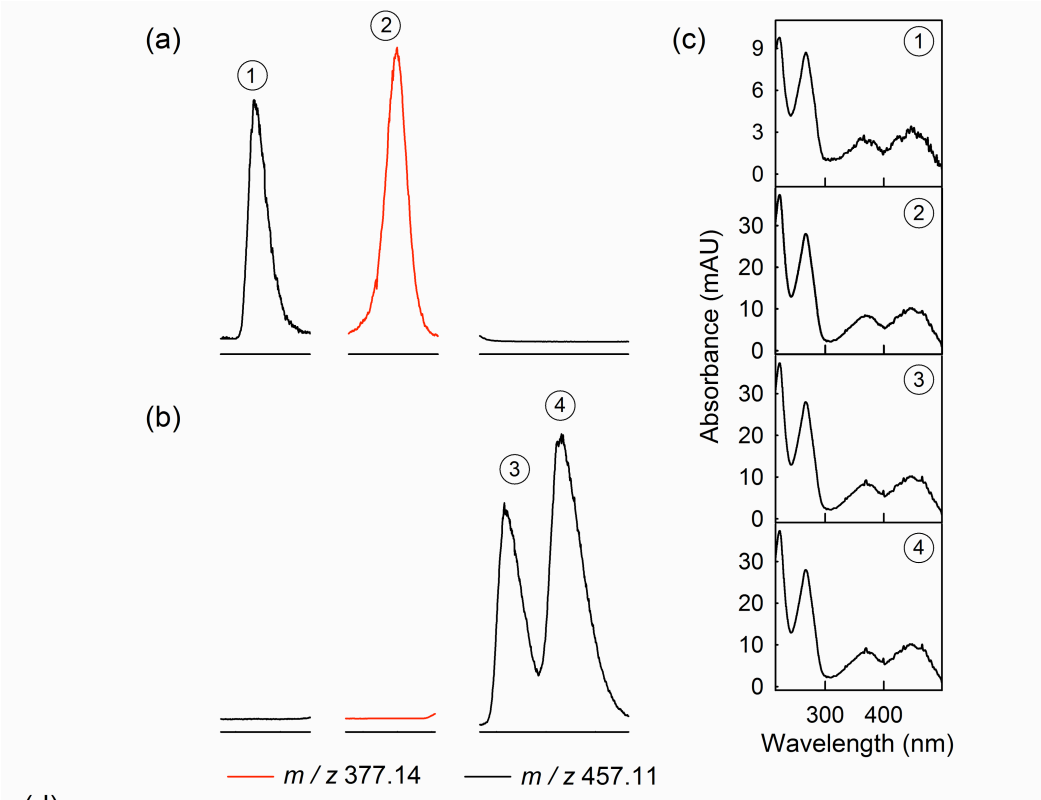


Fig. S2 Characterization of the flavin isolate obtained from the nutrient solution of *B. vulgaris* plants grown without Fe (-Fe) at pH 5.5 using the Experimental Set-up 3. **(a)** Chromatogram of FMN and Rbfl standards. **(b)** Chromatogram of the flavin isolate. **(c)** UV-VIS spectrum of flavins in (a) and (b). **(d)** Name, retention time (in min), measured *m/z*, molecular formulae, calculated *m/z* and error *m/z* for each flavin.



(d)

#	Name	R. T. (min)	Measured *m/z* [M+H]+	Molecular formula	Calculated *m/z* $[M+H]^+$	Error *m/z* (ppm)
1	FMN	32.5	457.1187	$C_{17}H_{22}N_4O_9P^+$	457.1189	-2.4
2	Rbfl	33.8	377.1459	$C_{17}H_{21}N_4O_6^+$	377.1455	1.0
3	Rbfl 3'-sulfate	36.5	457.1010	$C_{17}H_{21}N_4O_9S^+$	457.1023	-2.8
4	Rbfl 5'-sulfate	40.0	457.1003	$C_{17}H_{21}N_4O_9S^+$	457.1023	-4.3

Fig. S3 Plant biomass changes upon Fe deficiency and flavin removal from the nutrient solution. Data show the fresh weight (FW) of the different parts of *B. vulgaris* plants grown for 13 days with Fe (+Fe), without Fe (-Fe), and without Fe using the flavin removal system (-Fe / flavin removal). Plants were grown with the Experimental Set-up 1 as indicated in Fig. 1. Data are means ± SE (n = 12 for stems and old leaves, and 28-55 for the rest of plant parts). For a given plant part, significant differences among treatments ($p < 0.05$) are marked with different letters above the columns.

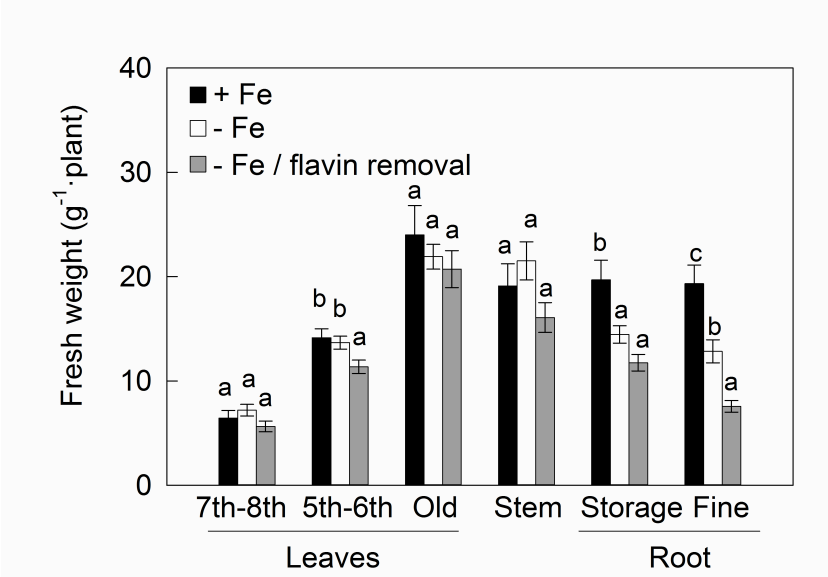


Fig. S4 Changes in apoplastic Fe contents in *B. vulgaris* plants upon Fe deficiency and flavin removal from the nutrient solution. Data show Fe contents (in µg plant^{-1}) in the apoplast of plants grown for 13 days with Fe (+Fe), without Fe (-Fe), and without Fe using the flavin removal system (-Fe / flavin removal). Plants were grown with the Experimental Set-up 1 as indicated in Fig. 1. Data are means ± SE (n = 6 - 12). Significant differences among treatments (at $p < 0.05$) are marked with different letters above the columns.

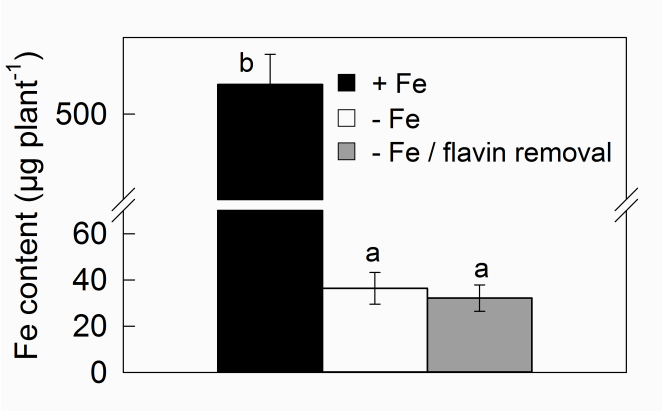


Fig. S5 Plant micronutrient concentration changes upon Fe deficiency and flavin removal from the nutrient solution. Iron, Mn, Cu, and Zn concentrations (in µg g^{-1} DW) in different parts of *B. vulgaris* plants grown for 13 days with Fe (+Fe), without Fe (-Fe), and without Fe using the flavin removal system (-Fe / flavin removal). Plants were grown with the Experimental Set-up 1 as indicated in Fig. 1. Data are means ± SE (n = 6 for stem and oldest leaves, and 24 - 47 for the rest of plant parts). For a given plant part, significant differences among treatments (at $p < 0.05$) are marked with different letters above the columns.

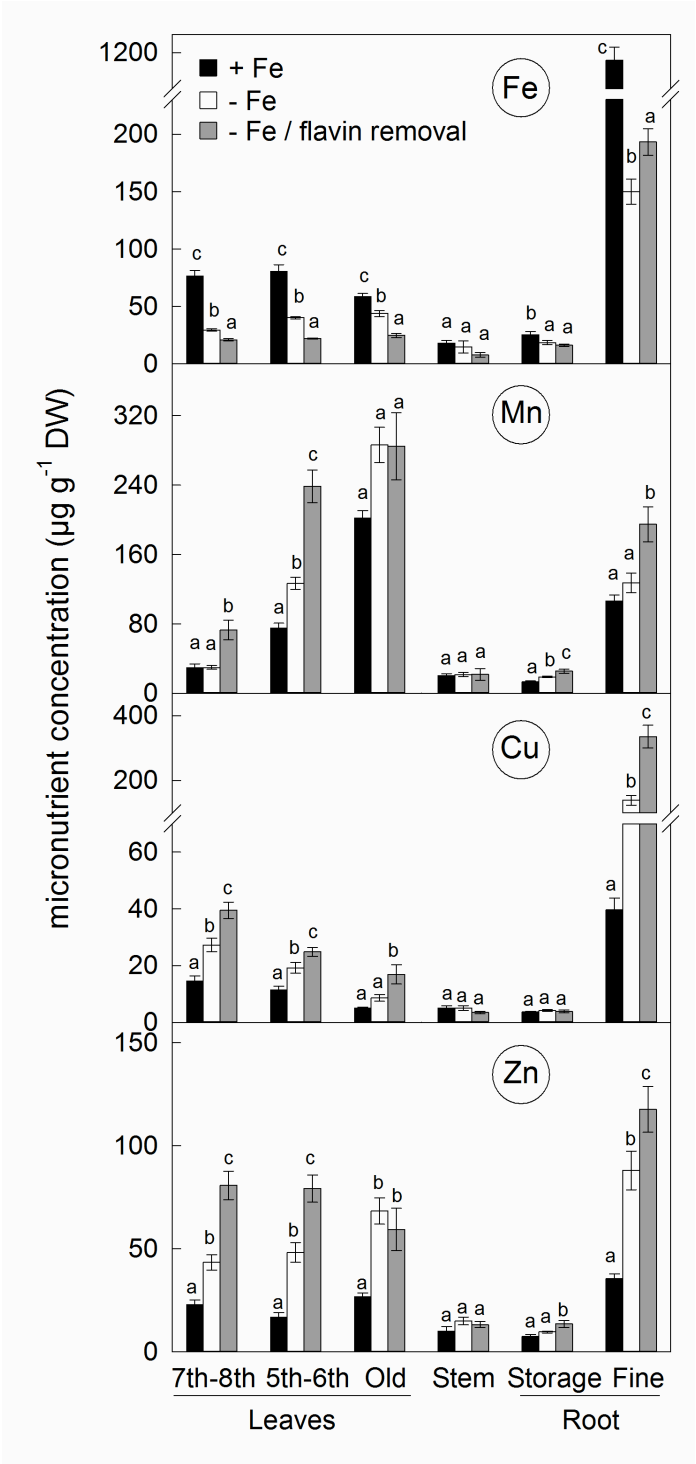


Fig. S6 Time-course of SPAD values of **(a)** developed (leaves 5th-6th) and **(b)** young (leaves 7th-8th) leaves of *B. vulgaris* Fe-sufficient (+Fe; black symbols) and Fe-deficient plants (-Fe; white symbols) plants grown with the Experimental Set-up 2 for 10 days at pH values 5.5 (circles) or 7.5 (squares) (as indicated in Fig. 7). Data are means ± SE (n = 4; plants).

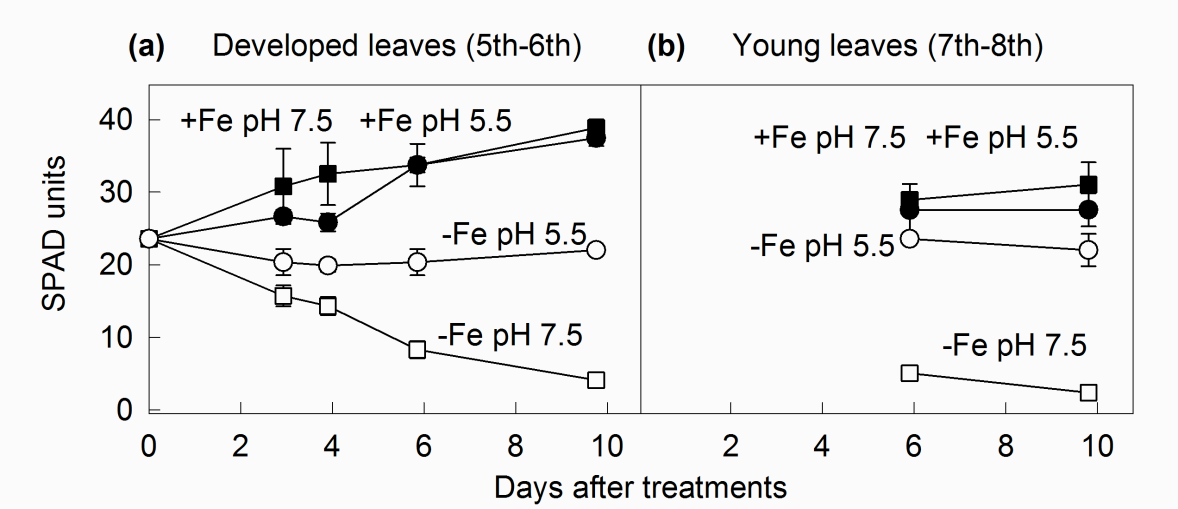


Fig. S7 Optical view **(a)** and flavin fluorescence **(b)** of excised root sections (*ca.* 15 mm), depicting the areas 0-5 mm or 5-10 mm from the apex, from *B. vulgaris* Fe-sufficient (+Fe) and Fe-deficient plants (-Fe) plants grown with the Experimental Set-up 2 for 10 days at pH values 5.5 or 7.5. Root pieces were observed at x7.3 magnification with a digital stereo microscope (DFC 420C, Leica Microsystems GmbH, Wetzlar, Germany, with a 5 megapixel CCD). Fluorescence was detected with a filter set GFP Leica MZ FLIII (440/70 excitation wavelength and 500/50 nm cut-off filter).

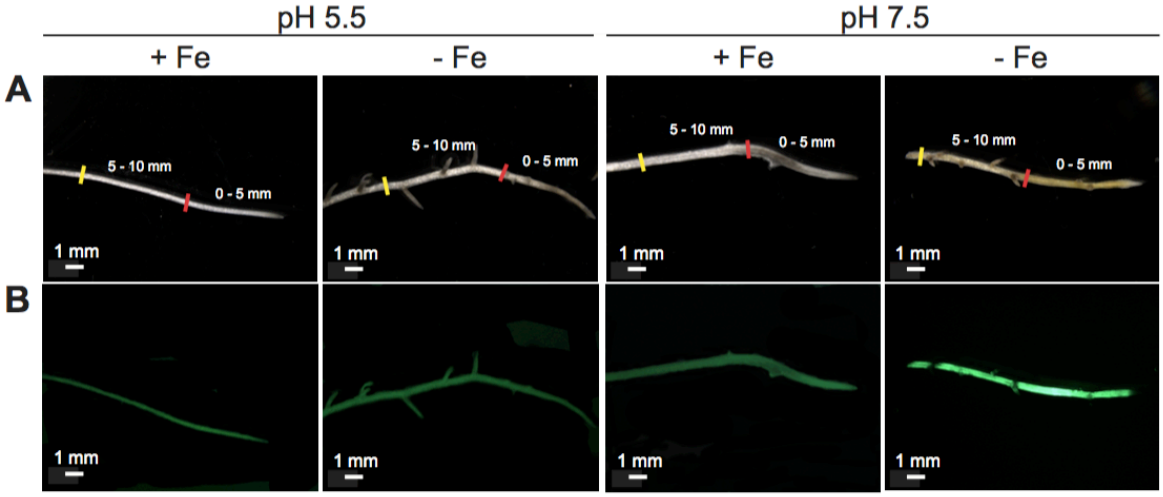


Fig. S8 Flavin concentrations in excised root sections, taken 0-5 mm or 5-10 mm from the apex, from *B. vulgaris* Fe-sufficient (+Fe) and Fe-deficient plants (-Fe) plants grown with the Experimental Set-up 2 for 10 days at pH values 5.5 or 7.5. Data are means ± SE (n = 2; plants).

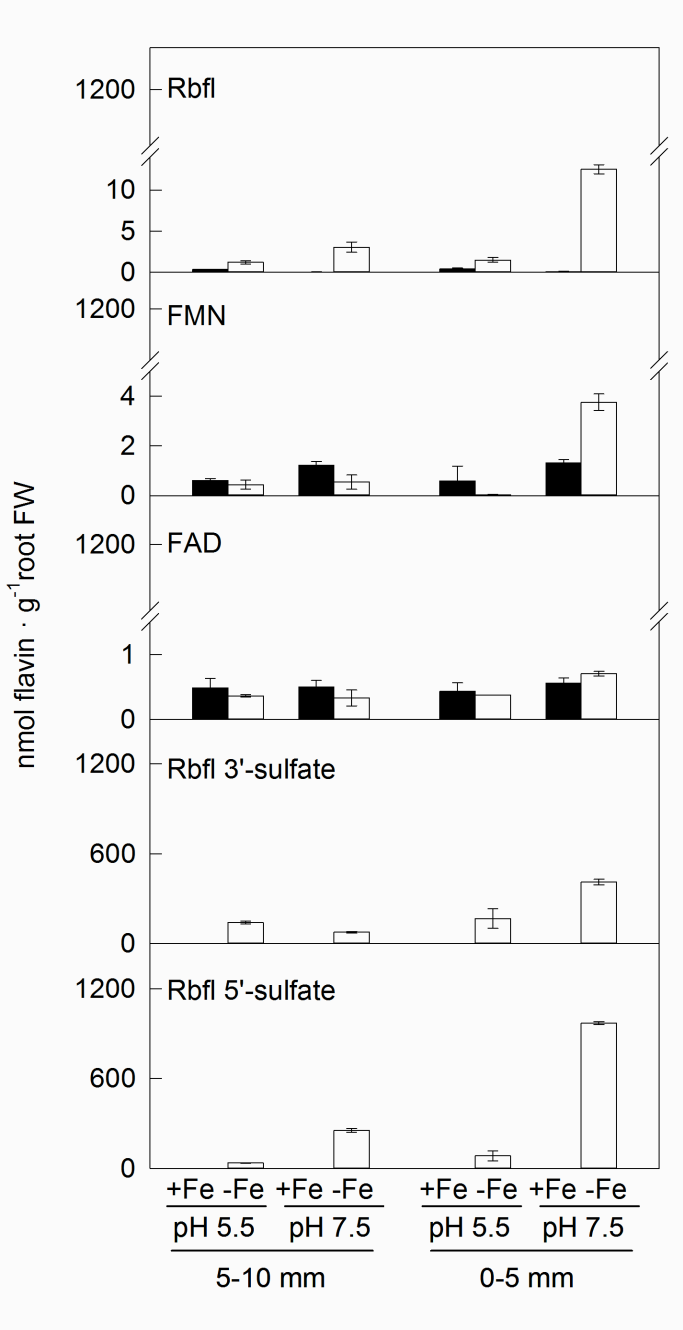


Fig. S9 Time course of flavin secretion to the nutrient solution by *B. vulgaris* Fe-sufficient (+Fe) and Fe-deficient plants (-Fe) plants grown with the Experimental Set-up 2 for 10 days at pH values 5.5 or 7.5. All data corresponding to treatments +Fe pH 5.5, +Fe pH 7.5 and –Fe pH 7.5, were below the detection limit and depicted as 0 µM. The white square symbols are under the black symbols (squares and circles) and all of them are 0 µM.

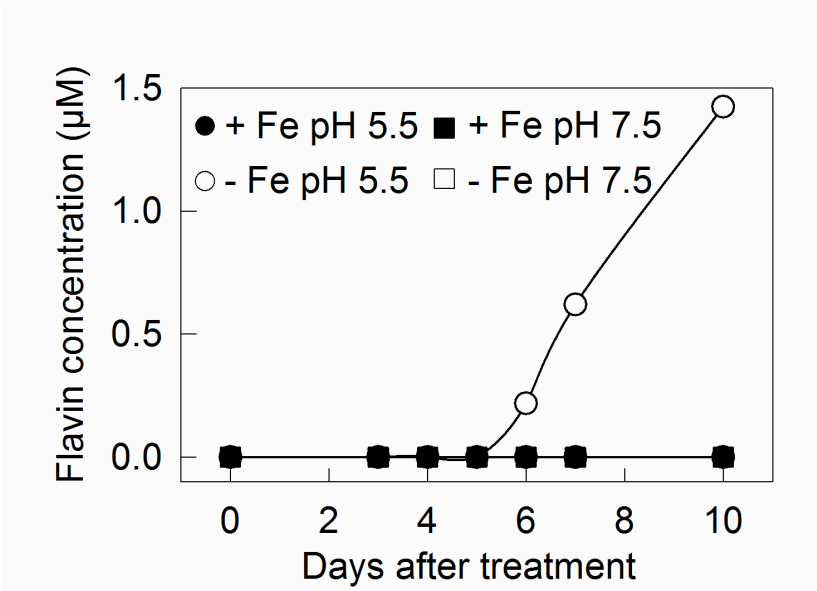


Fig. S10 Effect of Rbfl concentration on Fe(III) reduction rates using excised root tips and scarcely soluble Fe(III) oxide as substrate. Root tip sections were taken 0-5 mm from the apex from Fe-deficient (-Fe) *B. vulgaris* plants grown for 10 d at pH 7.5 (with 1 mM NaOH and 1 g L^{-1} $CaCO_3$), and assayed with 0, 1, 10 or 100 µM of Rbfl. Data are means ± SE (n = 2).

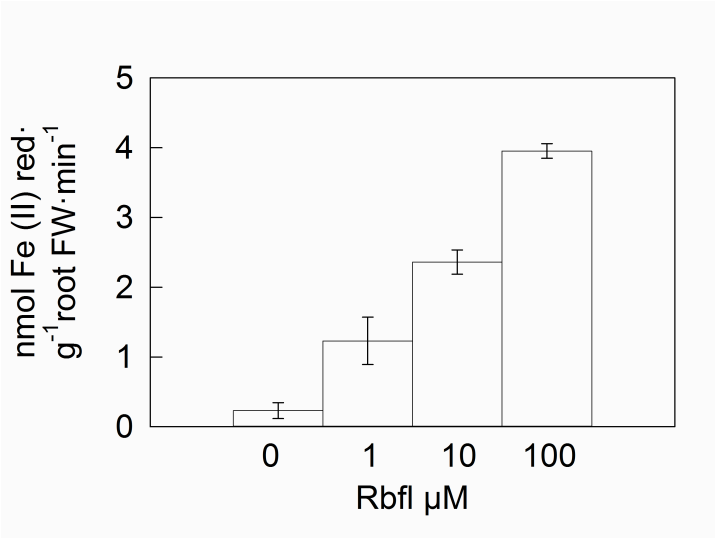


Fig. S11 Effects of Rbfl concentration and pH on the *in vitro* reduction rates of scarcely soluble Fe(III) oxide in the presence of NADH and BPDS. Incubation assays were run at two different pH values, 5.5 and 7.5 for 1 h, in the presence of 0, 3, 6, 12, 25, 50 and 100 µM of Rbfl, 500 µM NADH as an electron donor and 600 µM BPDS as Fe(II) trapping agent. Data are means (n = 2-1).

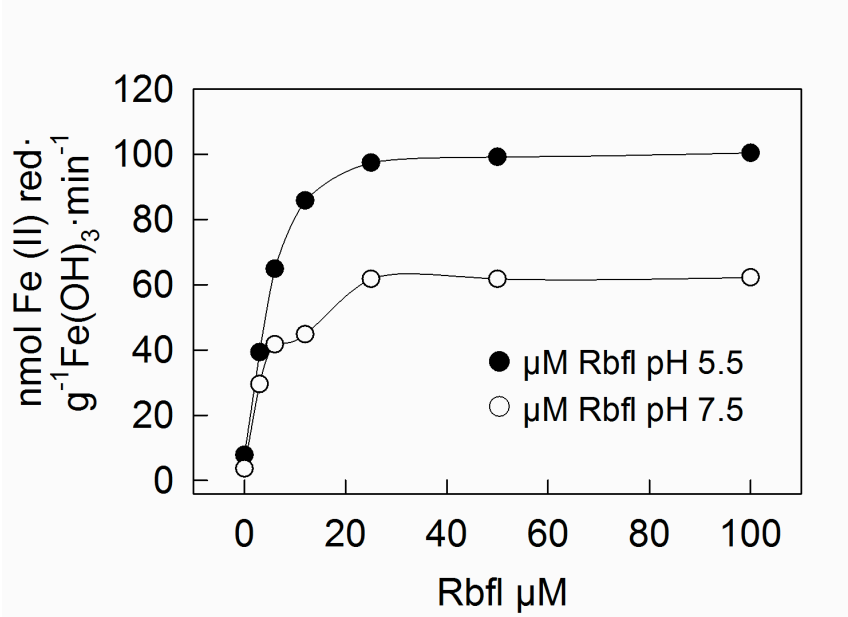


Fig. S12 Effects of Rbfl concentration and reaction time on the *in vitro* reduction of scarcely soluble Fe(III) oxide in the presence of NADH and BPDS. Incubation assays were run at pH 5.5 for 1, 3, or 6 h, in the presence of 0, 0.01, 0.1, 1 and 10 µM of Rbfl, 500 µM NADH as an electron donor and 600 µM BPDS as Fe(II) trapping agent. Data are means (n = 2-1).

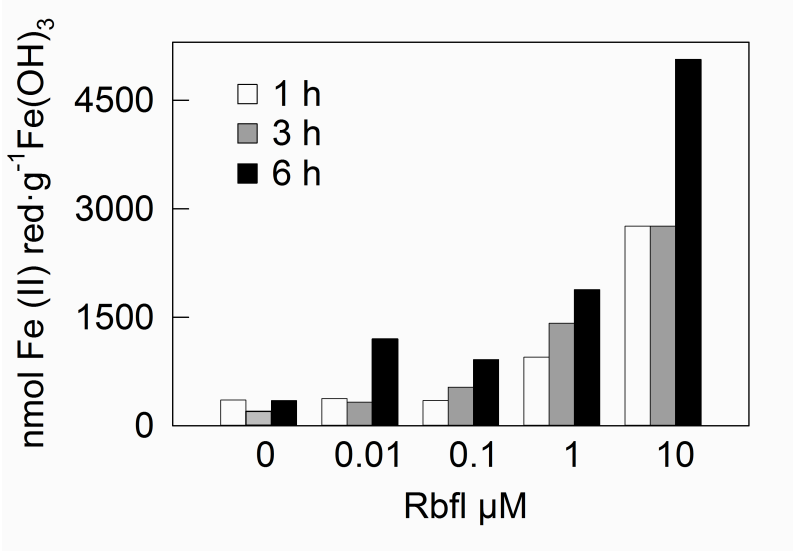


Fig. S13. HPLC-MS(TOF) analyes of phenolics in root extracts of *B. vulgaris* Fe-deficient plants. Each graph shows chromatograms corresponding to the root extract, the root extract spiked with known phenolics, and water spiked with known phenolics. The chromatograms were zoomed to show the peaks corresponding to phenolics used for spiking both samples and water. Scopolin (a), fraxin (b), caffeic acid (c), fraxetin (d), coniferyl alcohol (e), sinapyl alcohol (f), scopoletin (g), ferulic acid (h), sinapic acid (i), coniferyl acid (j), sinapyl acid (k), myricitrin (l), resveratrol (m), myricetin (n) and matairesinol (o). The chromatograms were extracted at *m/z* (± 0.05) ratios corresponding to $[M+H]^+$ ions, with the exception of alcohols and acids that were extracted at the *m/z* ratios of both $[M+H]^+$ and $[M-H_2O+H]^+$ ions.

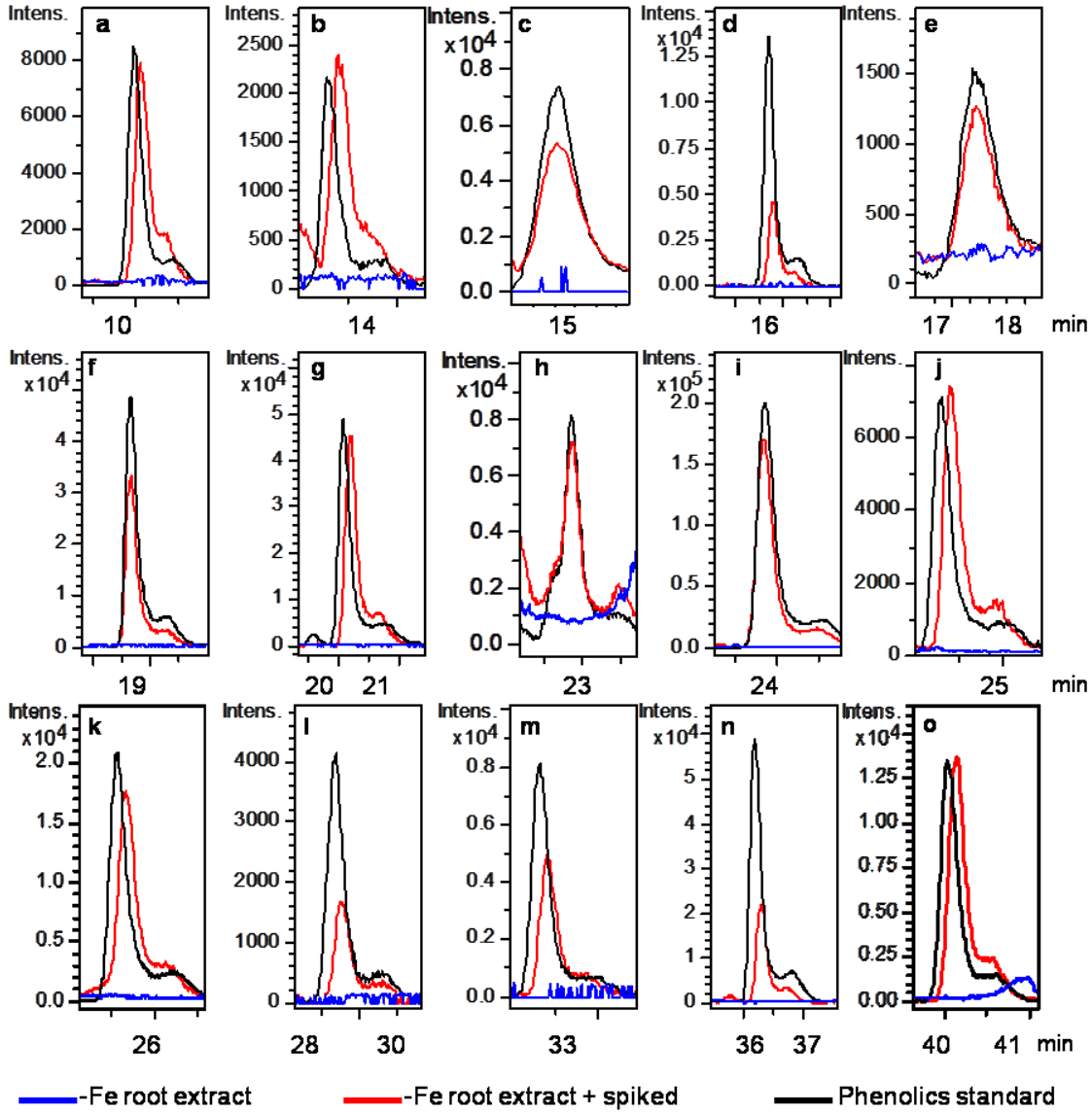
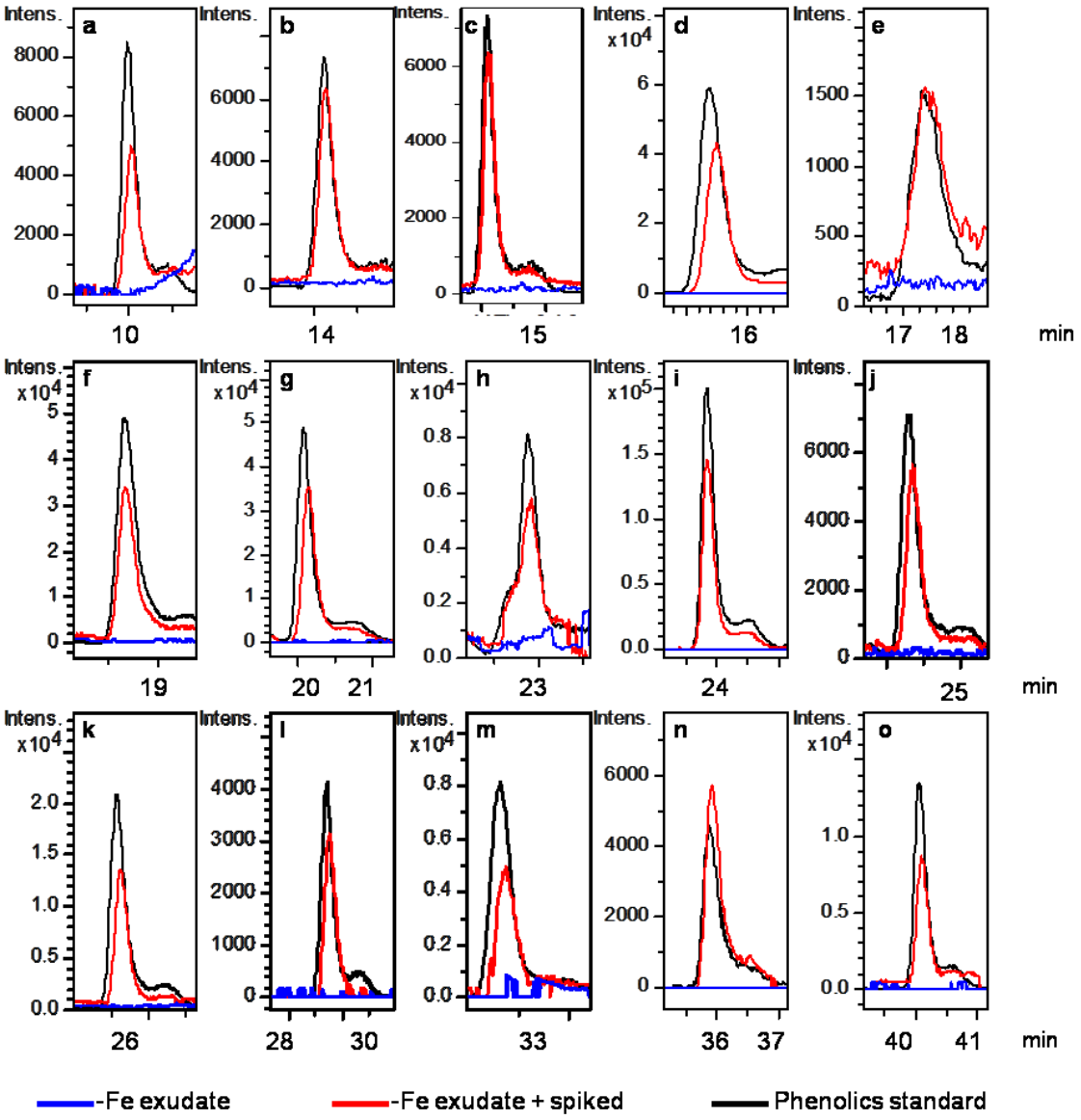


Fig. S14 HPLC-MS(TOF) analyes of phenolics in extracts of nutrient solutions of *B. vulgaris* Fe-deficient plants. Each graph shows chromatograms corresponding to the root extract, the root extract spiked with known phenolics, and water spiked with known phenolics. The chromatograms were zoomed to show the peaks corresponding to phenolics used for spiking both samples and water. Scopolin (a), fraxin (b), caffeic acid (c), fraxetin (d), coniferyl alcohol (e), sinapyl alcohol (f), scopoletin (g), ferulic acid (h), sinapic acid (i), coniferyl acid (j), sinapyl acid (k), myricitrin (l), resveratrol (m), myricetin (n) and matairesinol (o). The chromatograms were extracted at *m/z* (± 0.05) ratios corresponding to $[M+H]^+$ ions, with the exception of alcohols and acids that were extracted at the *m/z* ratios of both $[M+H]^+$ and $[M-H_2O+H]^+$ ions.



CAPÍTULO 4. DISCUSIÓN GENERAL

En esta Tesis se ha estudiado la exudación radicular de algunos metabolitos secundarios, compuestos fenólicos y flavinas, inducida por la deficiencia de hierro (Fe), en plantas pertenecientes a la Estrategia I, utilizando la especie modelo por excelencia para la investigación fitobiológica y 'mala hierba' cuando nace en terrenos cultivados, *Arabidopsis thaliana*, y dos especies de interés agronómico como son tomate (*Solanum lycopersicum*) y remolacha azucarera (*Beta vulgaris* var. *saccharifera*). Este estudio ha abordado los siguientes aspectos: la caracterización de los compuestos acumulados en raíz y exudados al medio de cultivo, el transporte de dichos compuestos a la rizosfera, y la función que estos compuestos desempeñan allí en relación a la nutrición férrica de las plantas. El conjunto de los resultados obtenidos permite ampliar el conocimiento de la toma de Fe de las plantas de Estrategia I con el estudio de un mecanismo, especialmente necesario en condiciones de baja disponibilidad de Fe, basado en la extracción de Fe del suelo por procesos de quelación y/o redución mediados por los compuestos fenólicos o flavinas exudados. Al final de esta Discusión General se muestran de forma esquemática los mecanismos propuestos para la toma de Fe facilitado por compuestos fenólicos y flavinas, respectivamente, señalando las partes derivadas de los resultados de esta Tesis.

Caracterización

El presente trabajo constituye el primer análisis exhaustivo y simultáneo de los compuestos fenólicos y las flavinas acumulados en raíces y exudados al medio de cultivo, como respuesta de las plantas a la deficiencia de Fe. Para este estudio se ha utilizado una aproximación analítica basada en la separación de los compuestos por HPLC y su detección por la combinación de técnicas de espectroscopía de fluorescencia y de UV-VIS con técnicas de espectrometría de masas de diferentes modalidades (TOF, Q-TOF y trampa de iones). Las tres especies estudiadas pertenecen a familias diferentes (*A. thaliana* a Brassicaceae, *S. lycopersicum* a Solanaceae, y *B. vulgaris* a Amaranthaceae) y han sido cultivadas en hidroponía a pHs de interés agronómico (5,5 y/o 7,5) en condiciones controladas.

Esta Tesis muestra por primera vez que la deficiencia de Fe causa la síntesis, acumulación y/o exudación radicular de compuestos fenólicos de tipo cumarina en *A. thaliana* y tomate. Los resultados obtenidos en esta parte de la Tesis a partir de *A. thaliana*, han sido publicados en Fourcroy y col. (2014) y Sisó-Terraza y col. (2016b), y simultáneamente confirmados por los resultados de otros autores (Schmid y col., 2014; Schmidt y col., 2014). El presente trabajo también muestra que la deficiencia de Fe no afecta a la concentración radicular de flavinas ni a su exudación en las especies *A. thaliana* (Fourcroy y col., (2014); Sisó-Terraza y col., 2016b) y tomate (Sisó-Terraza y col., 2017). Sin embargo, la acumulación de flavinas y su exudación es una característica conocida de la respuesta radicular de remolacha ante la deficiencia de Fe (Susín y col., 1993, 1994), confirmada tanto en trabajos posteriores (López-Millán y col., 2000, 2001; Rellán-Álvarez y col., 2010) como en esta Tesis, en la que se ha utilizado un cultivar diferente de dicha especie. Además, los resultados del presente trabajo muestran que las plantas de remolacha no parecen responder a la deficiencia de Fe incrementando la concentración radicular de compuestos fenólicos ni su exudación al medio de cultivo. Por consiguiente, considerando en conjunto todos estos resultados parece confirmarse la idea de que la producción de compuestos fenólicos y flavinas causada por la deficiencia de Fe podría ser mutuamente excluyente. Esta respuesta fue encontrada por primera vez en *A. thaliana* y *Medicago truncatula* (especie Fabaceae que produce flavinas; ver Tabla 1) en base a los resultados de un estudio que utilizó técnicas de secuenciación masiva de ARN (RNA-seq) para comparar los cambios inducidos por deficiencia de Fe en el transcriptoma de la raíz (Rodríguez-Celma y col., 2013). Dicho estudio demostró que la síntesis de cumarinas aumenta en *A. thaliana* con la deficiencia de Fe pero no en *M. truncatula* y que lo contrario ocurre con la síntesis de flavinas. Además, esta idea está en consonancia con los resultados obtenidos en el único estudio realizado con anterioridad a esta Tesis en el que se analizaron flavinas y fenólicos totales en exudados radiculares y que desveló que la especie Fabaceae *Trifolium pratense* produce fenólicos pero no flavinas en respuesta a la deficiencia de Fe (Jin y col.,

Tabla 1. Especies de plantas estudiadas en relación a la acumulación y/o exudación radicular de compuestos fenólicos y flavinas como respuesta a la deficiencia de Fe. El símbolo ✔ indica que la deficiencia de Fe causa un aumento de fenólicos o flavinas en raíces y/o exudados, mientras que el símbolo ✗ indica que no hubo cambio. N. A.: no analizada.

Famila y especie	Fenólicos	Flavinas	Famila y especie	Fenólicos	Flavinas
Aizoceae			Fabaceae		
Cryophytum crystallinum	N. A.	✗[1]	*Arachis hypogeae*	✔[12]	N. A.
Tetragonia tetragonioides	N. A.	✗[1]	*Glycine max*	✔[13,14]	✗[1]
Amaranthaceae			*Lupinus albus*	N. A.	✗[1]
Amaranthus caudatus	N. A.	✔[2]	*Medicago ciliaris*	✔[15]	N. A.
Beta vulgaris	✗[3]	✔[1,2]	*Medicago truncatula*	✗[7]	✔[2]
Spinacea oleracea	N. A.	✔[1,2]	*Medicago sativa*	✔[16]	N. A.
Chenopodium quinoa	N. A.	✔[1]	*Phaseolus coccineus*	N. A.	✗[1]
Chenopodium murale	N. A.	✔[1]	*Phaseolus lunatus*	N. A.	✗[1]
Gomphrena globosa	N. A.	✔[1]	*Phaseolus vulgaris*	N. A.	✗[1]
Beta maritima	N. A.	✔[1]	*Pisum sativum*	✔[17]	N. A.
Amaryllidaceae			*Trifolium pratense*	✔[18]	✗[18]
Allium cepa	N. A.	✔[2]	*Vicia faba*	N. A.	✗[1]
Apiaceae			*Vigna sinensis*	N. A.	✗[1]
Anthriscus crefolium	N. A.	✗[1]	Lamiaceae		
Petroselinum crispum	N. A.	✔[2]	*Mentha x piperita*	N. A.	✗[2]
Asteraceae			*Ocium basilicium*	N. A.	✗[1]
Anthemis arvensis	N. A.	✗[2]	*Satureja hortensis*	N. A.	✗[1]
Calendula officinalis	N. A.	✗[2]	Malvaceae		
Carthamus tinctorius	N. A.	✗[1]	*Gossypium hirsutium*	N. A.	✗[1]
Cichorium envidia	N. A.	✗[1]	*Hybiscus esculentus*	N. A.	✗[1]
Franseria dumosa	N. A.	✔[1]	Papaveraceae		
Hellianthus annuus	N. A.	✔[1]	*Eschscholzia californica*	N. A.	✗[2]
Lactuca sativa	N. A.	✔[1]	Poaceae		
Tagates erecta	N. A.	✗[1]	*Triticum aestivum*	N. A.	✗[9]
Zinnia elegans	N. A.	✗[1]	*Zea mays*	N. A.	✗[9]
Brassicaceae			Portulaceceae		
Arabidopsis thaliana	✔[4,5,6,7,8]	✗[4,7,8]	*Portulaca grandiflora*	N. A.	✗[2]
Brassica campestris	N. A.	✗[1]	*Portulaca oleracea*	N. A.	✗[2]
Brassica rapa	N. A.	✗[9]	Solanaceae		
Brassica oleraceae	N. A.	✗[1]	*Capsicum annuum*	N. A.	✔[1]✗
Eruca sativa	N. A.	✗[1]	*Datura meteloides*	N. A.	✔[1]
Nasturtium officinale	N. A.	✗[2]	*Datura stramonium*	N. A.	✔[1]
Raphanus sativa	N. A.	✗[1]	*Hyoscyamus albus*	N. A.	✔[19]
Boraginaceae			*Nicotiana alata*	N. A.	✔[1]
Borago officinalis	N. A.	✗[2]	*Nicotiana rustica*	N. A.	✔[1]
Caryophylllaceae			*Nicotiana tabacum*	N. A.	✔[1]
Silene armeria	N. A.	✗[1]	*Nicotiana physaloides*	N. A.	✗[1]
Curcubitaceae			*Petunia x hybrida*	N. A.	✗[2]
Citrullus lanatus	N. A.	✔[1]	*Physalis alkekengi*	N. A.	✔[1]
Curcubita moschata	N. A.	✔[1]	*Physalis ixocarpa*	N. A.	✔[1]
Cucumis melo	N. A.	✔[2]	*Solanum lycopersicum*	✔[20,21,22]	✗[1,22]
Curcubita pepo	N. A.	✔[1]	*Solanum melongena*	N. A.	✗[1]
Cucumis sativus	✔[10]	✔[1,11]	*Solanum tuberosum*	N. A.	✗[1]
Lagenaria siceraria	N. A.	✔[1]	Urticaceae		
Luffa aegypticra	N. A.	✔[1]	*Parietaria judaica*	✔[23,24,25]	N. A.

[1]Welkie (2000) y sus referencias; [2]Rodríguez-Celma y col. (2011) y sus referencias; [3]Sisó-Terraza y col. (2016a); [4]Fourcroy y col. (2014); [5]Schmid y col. (2014); [6]Schmidt y col. (2014); [7]Sisó-Terraza y col. (2016b); [8]Rodríguez-Celma y col. (2013); [9]Stocks (1964); [10]Pii y col. (2015); [11]Satoh y col. (2016); [12]Römheld y Marschner (1983); [13]Brown y Ambler (1973); [14]Zocchi y col. (2007); [15]M'Sehli y col. (2008); [16]Masaoka y col. (1993); [17]Jelali y col. (2010); [18]Jin y col. (2007); [19]Higa y col. (2010); [20]Olsen y col. (1981); [21]Hether y col. (1984); [22]Esta Tesis (Chapter 3.3); [23]Dell'Orto y col. (2003); [24]Donnini y col. (2012); [25]Tato y col. (2013).

2007). El resto de estudios realizados hasta la fecha analizan bien fenólicos totales o bien flavinas, y hasta donde sabemos se han estudiado 71 especies pertenecientes a 16 familias diferentes (Tabla 1). A pesar de que el número de especies es ciertamente limitado, de que los estudios son incompletos, y de que las técnicas de análisis utilizadas podrían cuestionarse en la mayoría de los casos como ya se comentó en capítulos anteriores, los resultados obtenidos para las familias con un mayor número de especies estudiadas (al menos 6) sugieren que las especies que producen fenólicos o flavinas podrían distribuirse taxonómicamente. Así, la gran mayoría de las especies de las familias Amaranthaceae, Cucurbitaceae y Solanaceae producen preferentemente flavinas mientras que las pertenecientes a las familias Fabaceae y Brassicaceae producen preferentemente compuestos fenólicos (Tabla 1). No obstante, es evidente que se requieren estudios masivos y exhaustivos de los exudados de especies silvestres y domesticadas de éstas y otras familias para validar o rechazar esta hipótesis.

Este trabajo presenta el perfil cualitativo de los fenólicos de tipo cumarina producidos en respuesta a la deficiencia de Fe en tomate y *A. thaliana*, permitiendo por primera vez establecer diferencias comparativas entre los compuestos fenólicos producidos por especies diferentes. Aunque ambas especies producen cumarinas simples que presentan sustituciones oxigenadas (-OH, -OCH_3 y -O-hexosil), el grado de oxigenación de las cumarinas producidas es diferente. Así, en tomate predominan las cumarinas tetra-oxigenadas (p. ej. trihidroximetoxicumarina) frente a las tri- (p. ej. fraxetina, fraxinol) y di-oxigenadas (p. ej. escopoletina) mientras que en *A. thaliana* las cumarinas son di- y tri-oxigenadas y se producen junto con cumarinolignanos de tipo cleomiscosina. Otra diferencia relevante entre ambas especies es que, en las condiciones en las que se ha realizado este trabajo, sólo *A. thaliana* exuda las cumarinas al medio de cultivo. Sin embargo, trabajos anteriores realizados en tomate describieron que la exudación radicular de compuestos fenólicos en respuesta a la deficiencia de Fe estaba regulada por el pH de manera que ocurría concomitantemente con una acidificación, natural o artificial, del medio de cultivo (Olsen y col., 1981; Hether y col., 1984). De forma similar, la exudación radicular de flavinas en remolacha, *M. truncatula* y otras especies también está regulada por el pH del medio de cultivo (Susín y col., 1993, 1994; Rodríguez-Celma y col., 2011). Así, la exudación de flavinas se observa cuando se produce una acidificación del medio, mientras que no se produce cuando las plantas crecen en medios tamponados a pHs altos en cuyo caso las flavinas son acumuladas en la raíz. En el presente estudio, la acidificación del medio de cultivo se ha evitado en los experimentos con tomate y *A. thaliana*, y sin embargo en esta última especie se ha visto que la exudación radicular de cumarinas aumenta con el pH. Por consiguiente, o bien hay otros factores que podrían explicar la ausencia de cumarinas en las soluciones nutritivas de tomate (p. ej. degradación microbiana elevada de las cumarinas tetra-oxigenadas), o bien es necesaria la acidificación del medio de cultivo para la exudación de cumarinas en tomate pero no en *A. thaliana*. Otro aspecto que diferencia ambas especies, y que además podría estar relacionado con la falta de exudación de cumarinas en tomate, es el grado de glicosilación de las cumarinas ya que éste puede afectar a la disponibilidad y a la actividad fisiológica de los compuestos fenólicos (Le Roy y col., 2016). En este trabajo se muestra que el grado de glicosilación de las cumarinas acumuladas en raíz de *A. thaliana* (<25%) es mucho menor que el correspondiente para tomate (>86%). Por tanto, la incapacidad de tomate para activar algún proceso fisiológico que permitiese la liberación activa de las formas agliconas de las cumarinas podría conducir a su acumulación en raíz, y por consiguiente, dada su citotoxicidad, activaría los mecanismos de detoxificación como oxidación y glicosilación del tóxico y su secuestro en vacuola (Shoji, 2014). Este comportamiento a su vez explicaría las diferencias cualitativas entre los perfiles de cumarinas acumuladas en las raíces en las dos especies. Por otra parte, en los medios de cultivo de las plantas de *A. thaliana* deficientes en Fe, no se detectaron los hexósidos de cumarinas (resultados de esta Tesis; publicado en Fourcroy y col., 2014; Sisó-Terraza y col., 2016b) o fueron minoritarias (Schmid y col., 2014), lo que podría sugerir un transporte activo de agliconas hacia la rizosfera. En línea con esta idea otros autores encuentran que se necesita

la acción de una ß-glucosidasa (BGLU42) para la exudación de cumarinas en *A. thaliana* en respuesta a una baja disponibilidad de Fe (Zamioudis y col., 2014). Las pequeñas cantidades de hexósidos ocasionalmente encontradas en el medio de cultivo podrían provenir de la lisis de las células epidérmicas desprendidas de la raíz. De hecho, las células epidérmicas y las corticales de la parte basal de la raíz principal es donde se acumulan las cumarinas y donde se expresa la enzima clave para que se produzca su síntesis en *A. thaliana*, la feruloil-CoA 6'-hydroxylasa (F6'H1), en condiciones de deficiencia de Fe (Schmid y col., 2014).

El perfil cualitativo de los fenólicos de tipo cumarina producidos en respuesta a la deficiencia de Fe está también afectado por el pH del medio de cultivo y el tiempo de exposición de las plantas a la deficiencia de este elemento. Los cambios cualitativos del perfil de cumarinas parecen asociados a la intensidad de la exudación, ya que ocurren en *A. thaliana*, son menores en el perfil de cumarinas de la raíz que en el del medio de cultivo (Sisó-Terraza y col. 2016b), y no se observan en el perfil de cumarinas de las raíces de tomate (Sisó-Terraza y col., 2017). De hecho, en tomate ni siquiera se observan cambios cualitativos del perfil de cumarinas entre los dos genotipos estudiados ("Tres Cantos" y FER). En *A. thaliana*, se observa que cuando aumenta el tiempo de exposición a la deficiencia de Fe de las plantas y cuando éstas se cultivan a pH 7,5 *versus* 5,5, se produce una disminución de la cantidad relativa de la cumarina di-oxigenada no catecol escopoletina junto con un aumento proporcional de la cantidad de las cumarinas tri-oxigenadas, principalmente de la cumarina de tipo catecol fraxetina. Independientemente del pH del medio de cultivo, la cumarina que disminuye, escopoletina, está mayoritariamente (>80%) en raíz mientras que la cumarina que aumenta, fraxetina, está mayoritariamente (>80%) en el medio de cultivo. Por tanto, los cambios cualitativos del perfil de cumarinas del medio de cultivo son mayores que los de las raíces.

El genotipo y el tiempo de exposición a la deficiencia de Fe también afectan a la producción de cumarinas desde un punto de vista cuantitativo. El genotipo de tomate "Tres Cantos" acumula en raíz una concentración de cumarinas 6 veces mayor que el genotipo FER en plantas expuestas al mismo tiempo de deficiencia de Fe (10-11 días) (Sisó-Terraza y col., 2017). Diferencias genotípicas en la síntesis, acumulación y exudación radicular de compuestos fenólicos han sido descritas en *Medicago ciliaris* y *Pisum sativum* (M'Sehli y col., 2008; Jelali y col., 2010). Estos autores muestran que los genotipos más tolerantes a la deficiencia de Fe producen y exudan más cantidad de compuestos fenólicos aunque no identifican el tipo de compuestos fenólicos que se producen. Está ampliamente demostrado, tanto en estos trabajos citados como en otros, que la exudación de fenólicos y flavinas ocurre concomitantemente con respuestas radiculares claves para la adquisición de Fe como acidificación y aumento de la actividad reductasa férrica y de la toma de Fe(II), que son también moduladas tanto por el genotipo como por el tiempo de exposición a la deficiencia y, por lo tanto, se infiere una regulación común. Así, la producción de cumarinas inducida por deficiencia de Fe no se observa en los mutantes *Slfer* (resultados de esta Tesis) y *Atfit* (Schmid y col., 2014) que carecen del factor de transcripción FER/FIT responsable de la regulación de la expresión de los genes clave para la adquisición de Fe (FRO, IRT y HA). Asimismo, más recientemente se mostró que en *Cucumis melo* (una especie productora de flavinas; Tabla 1) el aumento de la expresión de los genes de la síntesis de flavinas en respuesta a la deficiencia de Fe ocurre en raíces de WT pero no en las del mutante *fefe* (Hsieh y Waters, 2016) que carece del gen que codifica la proteína tipo bHLH que interacciona con FIT regulando su nivel de expresión (Waters y col., 2014). En línea con estos resultados, la expresión ectópica de los genes *AtbHLH38* y *AtbHLH39* (cuyas proteínas interaccionan con AtFIT) en tabaco (*Nicotiana tabacum*) y girasol (*Hellianthus annuus*) (ambas especies productoras de flavinas; Tabla 1) causó un aumento de la síntesis, acumulación y exudación radicular de flavinas que, además, fue independiente de la deficiencia de Fe y que no se observó en plantas de *A. thaliana* y tomate que sobre-expresaban *AtbHLH38* y *AtbHLH39* (Vorwieger y col., 2007). Otros autores, utilizando RNA-seq, observaron una fuerte asociación entre la expresión de los

transcritos de los genes de la síntesis de cumarinas (PAL, 4CL, CCoOAMT, COMT y F6'H1) y los de la toma de Fe (FIT, FRO2, IRT1, AHA2) en raíces de plantas de *A. thaliana* crecidas en deficiencia de Fe (Rodríguez-Celma y col., 2013). Estos mismos autores también obtuvieron resultados similares en relación a la expresión de los genes involucrados en la síntesis de flavinas (GCH, PD, PR y DMRL) inducida por deficiencia de Fe en raíces de *M. truncatula* (Fig. 2).

Transporte

Con respecto al transporte hacia la rizosfera de los compuestos identificados, una aportación relevante del presente trabajo es que se demuestra que un transportador de tipo ABC de la subfamilia G, AtABCG37 (también llamado AtPDR9 y AtPIS1), está involucrado en la exudación radicular de cumarinas inducida por deficiencia de Fe en *A. thaliana* (Fourcroy y col., 2014). La deficiencia de Fe aumenta la expresión del gen *PDR9* (resultados de esta Tesis; Rodríguez-Celma y col., 2013) en *A. thaliana* así como de su ortólogo (*NtPDR3*) en tabaco (Ducos y col., 2005). Otros autores habían descrito que AtPDR9 se expresa en la membrana plasmática de las células epidérmicas de las puntas de las raíces permitiendo la salida del precursor de auxinas, ácido indol-3-butírico, y del análogo auxínico sintético, ácido 2,4-diclorofenoxiacético y que podría transportar compuestos fenólicos (Růžička y col., 2010). En esta Tesis se muestra que las plantas *Atpdr9* producen cumarinas en respuesta a la deficiencia de Fe pero no las exudan al medio de cultivo. La imposibilidad para liberar estas cumarinas también se refleja en cambios cualitativos y cuantitativos en el perfil de cumarinas de las raíces de las plantas *Atpdr9* deficientes en Fe. Estas raíces acumulan más cantidad de cumarinas y de su precursor, el ácido ferúlico, que las de las plantas WT, y además tienen hexósidos de cumarinas tetra-oxigenadas que no están presentes en las plantas WT. Por tanto, parece que existe un mecanismo que involucra modificaciones de las cumarinas (oxidación y glicosilación), y probablemente su secuestro en la vacuola, junto con una posible inhibición parcial de la síntesis y que permite a las raíces de *Atpdr9* acumular niveles mucho mayores de cumarinas que la planta WT. Cabe destacar que estos hexósidos de cumarinas tetra-oxigenadas acumuladas en *Atpdr9* son también acumulados en las raíces de tomate deficientes en Fe. Esto sugiere de nuevo que, en nuestras condiciones de crecimiento, las raíces de tomate deficientes en Fe activan mecanismos de detoxificación de cumarinas.

Por otra parte, un análisis filogenético de las secuencias de las proteínas transportadoras ABC de la subfamilia G de tamaño completo (tipo PDR) de *A. thaliana*, tomate y remolacha junto con NtPDR3 revela que AtABGG37/AtPDR9 y NtPDR3 (cuya expresión aumenta con la deficiencia de Fe en raíz) están en el mismo clado que SlPDR3 (llamado Solyc01g101070.*2* en la Fig. 1) y Bv4_079970_zacs.tl, y por consiguiente podrían tener patrones de expresión y función biológica comunes. La secuencia de aminoácidos de AtPDR9 comparte un 67, 67 y 60% de los aminoácidos con las secuencias de NtPDR3, SlPDR3 y Bv4_079970_zacs.tl, respectivamente, mientras que el porcentaje de identidad de aminoácidos es mayor (91%) cuando se comparan las secuencias de NtPDR3 y SlPDR3 que corresponden a especies de la misma familia (Solanaceae). Aunque hasta la fecha se desconoce si SlPDR3 y Bv4_079970_zacs.tl se inducen con deficiencia de Fe, estudios con otras especies revelan que esto podría ocurrir. Así, un estudio con soja (*Glycine max*) utilizando técnicas de ARN-seq mostró que los dos ortólogos (*Glyma17g03863* y *Glyma07g36166*) de *AtPDR9* existentes en esta especie aumentan su expresión en raíces en respuesta a la deficiencia de Fe en un cultivar tolerante y esto ocurre concomitantemente con aumentos en la expresión de los ortólogos de los genes *AtF6'H1* (*Glyma03g23770* y *Glyma07g12210*) y *AtCCoAOMT* (*Glyma04g40591* y *Glyma06g14210*) (Moran Lauter y col., 2014) que son críticos en la síntesis de la cumarina escopoletina. Como este tipo de transportadores son poliespecíficos (transportan un amplia gama de sustratos estructuralmente no relacionados) (Crouzet y col., 2006; Hwang y col., 2016), cabe esperar que los ortólogos de AtPDR9 en otras especies pudiesen transportar otros sustratos. Por ejemplo, NtPDR3 y Bv4_079970_zacs.tl transportarían las flavinas

que son secretadas a la rizosfera en respuesta a la deficiencia de Fe en tabaco y remolacha. Hasta la fecha, no se han publicado estudios de actividad de AtPDR9 o de sus ortólogos en relación al transporte de cumarinas o flavinas (Shitan, 2016). Aunque no se han identificado transportadores de flavinas en plantas, en ciertos animales (ratones, vacas y humanos) la secreción de riboflavina hacia la leche está mediada por una proteína tipo ABCG (ABCG2 en humanos) (van Herwaarden y col., 2007; Horsey y col., 2016). Esta proteína se expresa en las glándulas mamarias durante la lactancia así como en otros tejidos que actúan como barreras (p. ej., colon e intestino delgado) y tiene actividad reconocida para

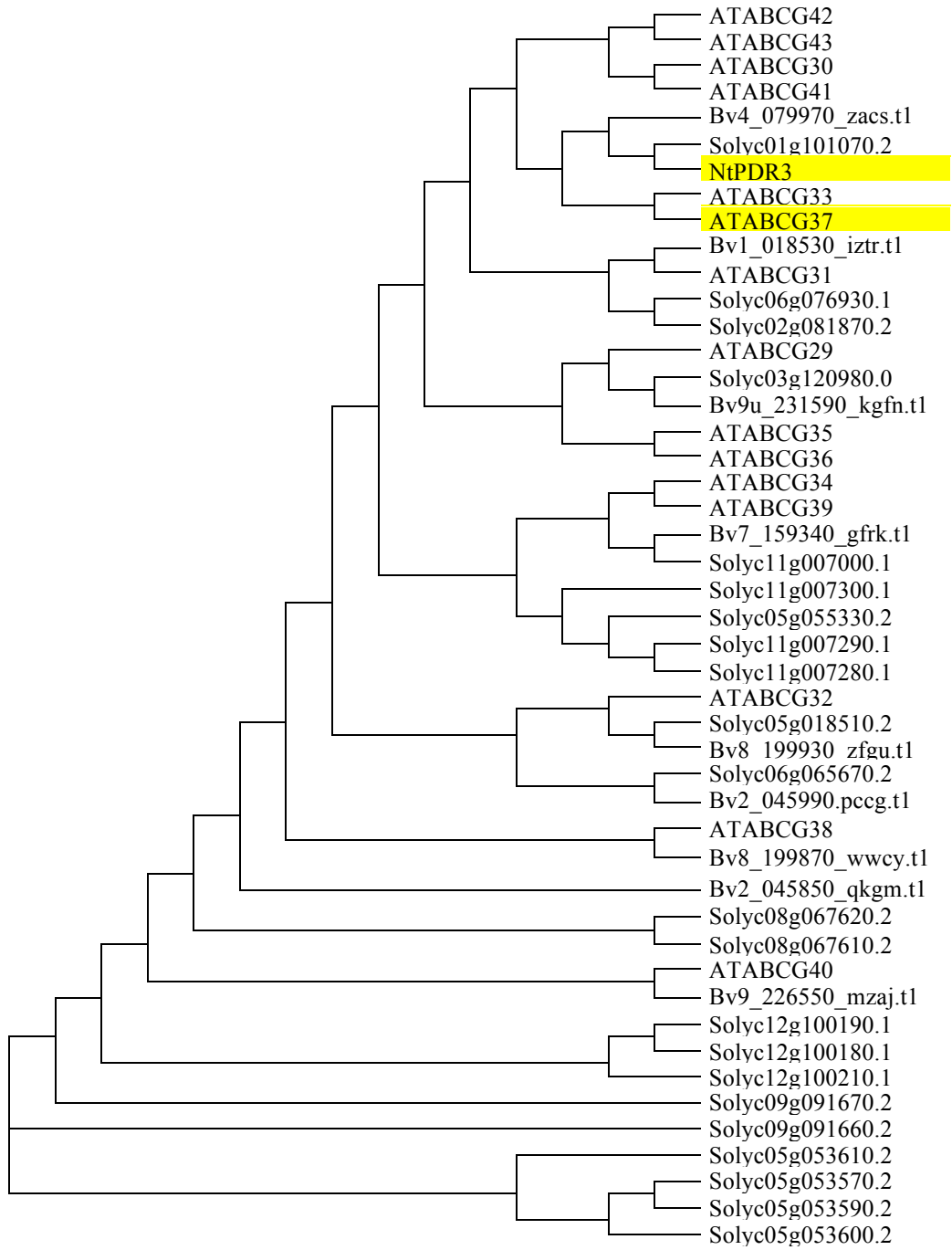


Figura 1 Análisis filogenético de los transportadores ABC de la subfamilia G de tamaño completo (tipo PDR). El análisis se realizó con el programa Clustal W utilizando las secuencias de aminoácidos de los PDRs de *A. thaliana*, tomate, remolacha y NtPDR3. El color amarillo destaca aquellos PDR que aumentan con la deficiencia de Fe en raíz.

más de 200 sustratos diferentes tanto de carácter endógeno (p. ej., riboflavina, FMN, ácido úrico) como xenobiótico (p. ej. fármacos, pesticidas, etc.) (Horsey y col., 2016). ABCG2 ha sido extensamente estudiado debido a que una elevada expresión confiere resistencia clínica a la quimioterapia (disminución de la eficacia) contra el cáncer, especialmente contra el cáncer de mama, contamina la leche con xenobióticos (p. ej. antibióticos) y reduce enfermedades relacionadas con la hiperuricemia (niveles altos de ácido úrico en sangre). Curiosamente, la administración de las cumarinas fraxetina y esculetina (así como de sus glicósidos, fraxina y esculina) aumenta la expresión de ABCG2 en ratones reduciendo los niveles de ácido úrico en sangre (Li y col., 2011). Variaciones en la actividad transportadora, la especificidad por determinados sustratos, su patrón de expresión tisular y funcionalidad de la proteína ABCG2 han sido descritas en relación tanto a diferencias inter-específicas como polimorfismos en la secuencia nucleotídica del gen (Horsey y col., 2016). Por tanto, sería interesante estudiar si las variaciones entre la secuencias de *SlPDR3* y *AtPDR9* afectan al transporte de cumarinas, lo que podría explicar las diferencias en la capacidad de exudar estos compuestos de *A. thaliana* y tomate en respuesta a la deficiencia de Fe. Esta diferencia también podría estar relacionada con la ß-glucosidasa de tomate, ya que, como se señaló anteriormente, en *A. thaliana* la ß-glucosidasa, BGLU42, es necesaria para la exudación radicular de cumarinas (Zamioudis y col., 2014).

El transporte de flavinas hacia la rizosfera (y puede que también el transporte de cumarinas en algunas especies como el tomate) podría estar mediado por proteínas transportadoras de membrana tipo MATE o MFS. A diferencia de los transportadores ABC, que consumen energía química derivada de la hidrólisis del ATP, los transportadores MATE y MFS son dependientes de un gradiente electroquímico siendo la fuente de energía en muchos ocasiones un gradiente de H^+ (Remy y Duque, 2014). Estos transportadores pueden mover dos o más moléculas (en la misma dirección o en direcciones contrarias), unas a favor del gradiente o potencial electroquímico y otras en contra. La exudación radicular de flavinas inducida por deficiencia de Fe es muy dependiente de la existencia de un gradiente de pH ya que no sucede cuando las plantas crecen en medios tamponados a pHs próximos a la neutralidad (el pH del citoplasma es 7,2) (Susín y col., 1994; Rodríguez-Celma y col., 2013). Sin embargo, la exudación radicular de flavinas sucede de forma simultanea a la acidificación del medio en las raíces de plantas deficientes cultivadas en medios con pH ligeramente ácidos (pH 5,5-6,5). Por consiguiente, cabría pensar en la acción de un transportador que mueva simultáneamente flavinas y H^+. Un caso similar podría ocurrir con la exudación de fenólicos en algunas especies como el tomate en el que se observa la acumulación radicular de fenólicos que pueden ser exudados sólo cuando se acidifica el medio. Dos miembros de la familia MATE, OsPEZ1 y OsPEZ2 transportan un compuesto fenólico, el ácido protocatecuico, al xilema en la especie arroz (*Oryza sativa*), modelo de la Estrategia II (Ishimaru y col., 2011; Bashir y col., 2011). En ese estudio se observó que las plantas *pez1* y *pez2* tenían en el xilema concentraciones de los ácidos protocatecuico y cafeico y de Fe inferiores a las de las plantas WT, y se atribuyó a PEZ1 y PEZ2 un papel en la solubilización del Fe apoplástico. Además, se propuso que PEZ2 está involucrado en la secreción radicular de compuestos fenólicos ya que se localizó en las células de la región de elongación de la raíz y los exudados de las plantas *pez2* no contenían los ácidos cafeico y protocatecuico, que sin embargo fueron detectados en los exudados de las plantas WT. Otros miembros de la familia MATE transportan flavinas en bacterias, dos transportadores de tipo MATE, Bfe y YeeO permiten la secreción de FMN al exterior en *Shewanella oneidensis* (Kotloski y Gralnick, 2013) y en *Escherichia coli* (McAnulty y Wood, 2014), respectivamente. Con respecto a los transportadores MFS, dos miembros de dicha familia, Medtr1g092870 y Medtr1g092880, han sido propuestos como los transportadores que median la exudación de flavinas hacia la rizosfera en *M. truncatula* en respuesta a la deficiencia de Fe. Esta propuesta se realizó en base a los resultados de un estudio de RNA-seq en raíces (Rodríguez-Celma y col., 2013) en el que las plantas fueron crecidas en un medio tamponado a pH 7,5, donde no se observa exudación de flavinas (Rodríguez-Celma y col., 2011) pero

sí su acumulación en raíz, por lo que Medtr1g092870 y Medtr1g092880 podrían estar en el tonoplasto, posibilitando la acumulación de flavinas en la vacuola.

Función

Efecto en la nutrición férrica de las plantas

En esta Tesis también se ha demostrado que la exudación radicular de coumarinas en *A. thaliana* y flavinas en remolacha tiene un efecto en la nutrición férrica, reduciendo la clorosis de las hojas y aumentado la eficiencia en la toma de Fe. Así, para *A. thaliana* se demuestra que la retirada continua de las cumarinas del medio de cultivo (mediante la recirculación de la disolución nutritiva a través de columnas llenas de un adsorbente hidrofóbico del tipo C_{18}) causa un agravamiento de la clorosis de plantas expuestas a un tratamiento con 0 µM de Fe soluble (Fourcroy y col., 2014). Este aumento de la clorosis, especialmente de las hojas jóvenes, está acompañado de un descenso de las concentraciones de Fe en hoja, mientras que la toma de otros micronutrientes no está limitada e incluso aumenta. Resultados similares habían sido obtenidos previamente con la especie leguminosa trébol rojo (*Trifolium pratense*) retirando del medio de cultivo los fenólicos (de carácter desconocido) exudados por las raíces en respuesta a la deficiencia de Fe (Jin y col., 2007). Otro resultado de esta Tesis en relación a la función de los fenólicos de tipo cumarina en la rizosfera es que su relevancia en la nutrición férrica se ciñe a condiciones en que la disponibilidad de Fe soluble es baja. Así, plantas de *Atpdr9* (ineficientes en la exudación de cumarinas) son más pequeñas y cloróticas que las plantas WT cuando crecen en condiciones de muy baja disponibilidad de Fe soluble (pH del medio de cultivo 6,5, próximo a la neutralidad) y esto no ocurre cuando las condiciones de pH (pH 5,5) permiten la solubilización de Fe insoluble disponible bien sea en el apoplasto de la raíz o en el agar utilizado como medio de crecimiento (Fourcroy y col., 2014). La disponibilidad del Fe es 1.000 veces menor a pH 6,5 que a pH 5,5 y alcanza su mínimo a valores de pH próximos a la neutralidad (Lindsay, 1995). Simultáneamente a este trabajo, otros autores obtienen resultados mostrando un papel de las cumarinas en la rizosfera en relación con Fe insoluble. Por una parte, Schmid y col. (2014) y Schmidt y col. (2014) observaron que las plantas *Atf6'h1* (ineficientes en la síntesis de cumarinas) presentaban menor crecimiento y menores contenidos de Fe en la parte aérea y mayor clorosis que las plantas WT cuando crecen en un substrato de turba tamponado a pH 7,2 (para reducir la disponibilidad de Fe soluble). Por otra parte, Rodríguez-Celma y col. (2013) mostraron en *A. thaliana* que tanto la síntesis de cumarinas como la exudación de las mismas aumentan la adquisición de Fe cuando se utilizan formas de Fe insoluble mientras que no existen diferencias entre las plantas mutantes y las WT cuando se usan formas de Fe solubles. Estos autores suplementaron con Fe insoluble un medio de agar tamponado a pH 7,0 y en esas condiciones observaron que las plantas *Atf6'h1* y *Atpdr9* eran más pequeñas, cloróticas y tenían contenidos de Fe menores que las plantas WT. Por el contrario, cuando suplementaron con Fe soluble (Fe-EDTA) un medio de agar a pH 5,5 las plantas WT, *Atf6'h1* y *Atpdr9* mostraron el mismo fenotipo. En otro experimento de este mismo estudio se recuperó parcialmente el fenotipo WT en plantas *Atf6'h1* y *Atpdr9* cuando éstas se co-cultivaron con plantas WT en agar suplementado con Fe insoluble a pH 7,0. Otro resultado que validó la hipótesis de que la exudación de cumarinas de las plantas WT facilita la toma de Fe de formas insolubles, se obtuvo utilizando agar enriquecido con Fe insoluble (50 µM $FeCl_3$ a pH 7,2) y suplementado con diferentes cumarinas (esculina, esculetina, escopoletina), ya que en estas condiciones las plantas *Atf6'h1* recuperaron el fenotipo WT (Schmid y col., 2014). Esta recuperación del fenotipo de las plantas *Atf6'h1* fue similar a la obtenida cuando el mismo agar se trata con el agente quelante sintético EDTA. Aún más recientemente, se ha demostrado que la toma de Fe insoluble facilitada por cumarinas precisa del sistema IRT/FRO integrado en la membrana plasmática, ya que plantas *Atirt1* y *Atfro2* muestran clorosis cuando se cultivan en agar enriquecido con Fe insoluble incluso cuando está suplementado con los fenólicos exudados por plantas WT (Fourcroy y col., 2016).

En cuanto al efecto de la exudación de flavinas en la nutrición férrica de plantas de remolacha esta Tesis muestra que también se produce un agravamiento de la clorosis cuando se retiran las flavinas de la solución nutritiva de las plantas (Sisó-Terraza y col., 2016a). Este agravamiento de la clorosis (especialmente intenso en hojas jóvenes) está asociado al Fe, ya que ocurre junto con un descenso de las concentraciones de Fe en hoja, mientras que la toma de otros micronutrientes no está limitada e incluso aumenta. Además, según otros autores las flavinas exudadas en el medio por una especie pueden mejorar la nutrición férrica de otra cuando crecen juntas (Rodríguez-Celma y col., 2013). Así, la co-cultivación de *M. truncatula* (especie productora de flavinas) con los mutantes de *A. thaliana f6'h1* o *pdr9* en agar suplementado con Fe insoluble (a pH 7,0) logró la recuperación parcial del fenotipo WT en las plantas mutantes en lo que se refiere a concentración de clorofila y concentración de Fe en hoja, así como al peso fresco de las hojas.

Mecanismo de acción

Una vez demostrado el efecto positivo de la exudación de cumarinas y flavinas en la nutrición férrica de las plantas, esta Tesis aborda el estudio del mecanismo de acción en la rizosfera. Ensayos *in vitro* de interacción con un óxido férrico permiten demostrar que cumarinas y flavinas pueden actuar en la rizosfera facilitando la disolución de óxidos férricos y, por consiguiente, aumentado la cantidad de Fe soluble a disposición de la planta (Figs. 2 y 3). Los óxidos e hidróxidos férricos son formas mayoritarias de Fe en el suelo y su solubilidad es escasa alcanzando un mínimo a pHs próximos a la neutralidad (pH 7,5-8,5) característicos de los suelos calizos (Lindsay, 1995). En cuanto a la disolución facilitada por cumarinas, esta Tesis y otros autores muestran que la estructura de la cumarina es determinante en su eficiencia para disolver óxido férrico. Se ha visto que la presencia de un grupo catecol en la estructura de la cumarina permite que pueda disolver el óxido (Schmid y col., 2014; Sisó-Terraza y col., 2016b). Así, de entre las dos cumarinas más abundantes en los exudados de plantas de *A. thaliana* cultivadas a pH 7,5, la cumarina catecol, fraxetina, y la cumarina no catecol, escopoletina, sólo la primera es realmente efectiva en la disolución de óxido férrico (Sisó-Terraza y col., 2016b). Tampoco resulta eficiente la forma glicosilada de la cumarina fraxetina (fraxina), ya que en fraxina el grupo glucosil está enlazado a uno de los 2 sustituyentes –OH de la parte catecol de fraxetina, y, por consiguiente, impide la acción de la misma (Sisó-Terraza y col., 2016b). En cuanto a la forma de acción tanto la reducción de Fe como la formación de complejos parecen mediar la disolución de óxido férrico facilitada por cumarinas. Así, en esta Tesis, se observa que aproximadamente el 42% del Fe total disuelto cuando fraxetina interacciona con óxido férrico es atrapado por un agente quelante específico para Fe(II) (Sisó-Terraza y col., 2016b). Otros autores han mostrado la formación *in vitro* de complejos de Fe con cumarinas utilizando espectrofotometría visible (Schmid y col., 2014) y ESI-MS (Schmidt y col., 2014).

Otra parte de los resultados de esta Tesis evidencia que la disolución de óxido férrico facilitada por cumarinas podría ocurrir en condiciones reales. Esta hipótesis se basa en la estimación de las concentraciones de cumarinas que podrían existir en las proximidades de la raíz, y en estudios tanto del efecto de la concentración de cumarina como del pH del medio sobre la disolución del óxido. Una concentración de 10 μM de fraxetina resulta suficiente para disolver cantidades significativas de óxido férrico y concentraciones en el intervalo 10-100 μM aumentan linealmente la cantidad de Fe disuelto a pH 7,5 (Sisó-Terraza y col., 2016b). Este intervalo de concentración está en el mismo orden de magnitud que la concentración de fraxetina (42 μM) estimada (utilizando un procedimiento descrito por Römheld en 1991 para fitosideróforos) en la proximidad de las raíces de *A. thaliana* a partir de los datos obtenidos en esta Tesis (Sisó-Terraza y col., 2016b). En cuanto al efecto del pH del medio, se observa que la fraxetina es más eficaz para disolver óxido férrico a pHs próximos a la neutralidad (pH 7,5) que a pHs ligeramente ácidos (pH 5,5) (Sisó-Terraza y col., 2016b). Esto es relevante ya que el pH del medio tiene un efecto opuesto sobre la actividad reductasa férrica (FCR) de raíz que permite a la planta acceder al Fe(III) soluble del medio. Así, la FCR de raíz muestra su

máximo de actividad a valores de pH ligeramente ácidos en las especies estudiadas (pH 5,0-5,5) y disminuye de forma rápida a pH más altos (Susín y col., 1996; Moog y col., 1995). Por consiguiente, la exudación radicular de fraxetina parece suponer una ventaja respecto del sistema de membrana IRT/FRO (FCR) ya que permitiría acceder al Fe insoluble del suelo a valores de pH característicos del suelo calizo. Por una parte, la disolución del óxido férrico por cumarinas catecol produce Fe(II) que sería susceptible de ser tomado directamente por la raíz mediante el transportador IRT aunque también podría ser re-oxidado a Fe(III) (dependiendo del nivel de O_2). Tanto el Fe(II) como el Fe(III) así producidos son susceptibles de ser quelados en la rizosfera por otros ligandos con alta afinidad por Fe y conocidos por mostrar una baja velocidad en la disolución de óxido férrico (p. ej. sideróforos). De esta manera se formaría un tándem de ligandos que sería muy efectivo en la extracción de Fe de óxido férrico y su estabilización en disolución. En línea con este probable modo de acción, el trébol rojo exuda compuestos fenólicos (de carácter desconocido, podrían ser cumarinas u otros) en respuesta a la deficiencia de Fe y estos exudados promueven el crecimiento de microorganismos capaces de exudar sideróforos en detrimento de otros micro-organismos que no los exudan (Jin y col., 2006). Por otra parte, la disolución de óxido férrico con cumarinas también produce complejos de Fe(III) susceptibles de ser reducidos a Fe(II) por la FCR de la raíz y posteriormente el Fe(II) se podría incorporar directamente a la célula mediante el transportador IRT.

En cuanto al mecanismo de acción de cumarinas y cumarinolignanos de tipo no catecol exudados por *A. thaliana*, estos compuestos podrían transformarse en cumarinas tipo catecol (y así contribuir a la disolución de óxido férrico del suelo) ya que todas poseen un grupo –OH y al menos otro sustituyente de tipo -OCH_3 (p. ej. escopoletina) u -O-hexosil (p. ej. esculina) en posición adyacente. Por tanto, enzimas demetilasas podrían actuar sobre sustituyentes tipo -OCH_3 y convertirlos en –OH y, por tanto, producir la respectiva cumarina tipo catecol (p. ej. la demetilación de la escopoletina produce esculetina). Igualmente la acción de glucosidasas típicas de la rizosfera podrían actuar sobre los sustituyentes –O-hexosil y convertirlos en sustituyentes –OH, y, por consiguiente, en la forma aglicona de una cumarina catecol (p. ej. la deglucosilación de esculina produce esculetina). Esta conversión de unas cumarinas inactivas a efectos de promover la disolución de óxido férrico en cumarinas activas ha sido propuesta para explicar el hecho de que la aplicación de las cumarinas no catecol, escopoletina y esculina, de forma exógena a plantas *Atf6'h1* (crecidas en agar con Fe insoluble) consiga la recuperación del fenotipo WT de forma tan eficiente como la aplicación de la cumarina catecol, esculetina (Schmidt y col., 2014).

Las cumarinas y los cumarinolignanos en la rizosfera podrían cumplir también otras funciones ya que son compuestos muy conocidos por su actividad biocida (fitocida, fungicida, bactericida, nematicida, insecticida, etc). Las concentraciones estimadas en la proximidad de la raíz para fraxetina (43 µM), escopoletina (21 µM), isofraxidina (14 µM) y fraxinol (0,5 µM) permitiría este tipo de función ya que estas concentraciones son del mismo orden que la concentración a la que cumarinas di- y tri-oxigenadas (1-12 µM) muestran actividad inhibitoria para el crecimiento de bacterias grampositivas y gramnegativas (Kayser y Kolodziej, 1999). Además, ciertas características estructurales de las cumarinas menos abundantes en los exudados de *A. thaliana* (p. ej. un alto número de sustituciones oxigenadas y un patrón de oxigenación consistente en dos grupos -OCH_3 y un grupo -OH) están asociadas con mayor actividad bactericida y/o fungicida. Esta función explicaría la exudación de un amplio conjunto de cumarinas muchas de las cuales no son efectivas en la disolución de Fe y otras que además son muy poco abundantes en los exudados. La alteración del microbioma de la rizosfera por la exudación de fenólicos podría perseguir que se reduzca la competición por el Fe de la rizosfera. Así, el estudio con trébol rojo mencionado anteriormente, mostró un efecto de los compuestos fenólicos exudados en la composición de microorganismos de manera que no sólo se favorece el crecimiento de microorganismos productores de sideróforos sino también de aquellos que producen compuestos de carácter auxínico (Jin

y col., 2006). Las auxinas regulan positivamente la acción de FIT y, por tanto, del sistema FRO/IRT de toma de Fe soluble por la raíz (Hindt y Guerinot, 2012; Romera y col., 2011). Esto implicaría que los fenólicos podrían indirectamente aumentar la capacidad de la raíz para tomar Fe soluble y también de forma directa (vía disolución de óxido férrico) o indirecta (vía aumento de concentración sideróforos) aumentar la concentración de Fe soluble en la proximidad de la raíz. Ambos aspectos junto con la eliminación de otros microorganismos no eficientes en la extracción de Fe del suelo (y competidores en la toma del Fe soluble) contribuirían conjuntamente a disminuir la competencia por el Fe soluble de la rizosfera.

Esta Tesis también presenta las primeras evidencias que sustentan un mecanismo de acción para las flavinas en la rizosfera. En el mecanismo propuesto, las flavinas actúan como transportadoras de electrones desde la raíz hacia los óxidos férricos del suelo lo que conduce a la disolución reductiva de los mismos y, por consiguiente, a un aumento de la concentración de Fe soluble a disposición de la planta. Por una parte, se muestra que los sulfatos de riboflavina y la riboflavina (Rbfl) (flavinas exudadas por las raíces de remolacha y otras especies en respuesta a la deficiencia de Fe) en forma reducida promueven la disolución de óxido férrico. La interacción de varias de dichas flavinas en forma oxidada con óxido férrico incrementa el Fe en disolución únicamente en presencia de un agente químico donador de electrones (NADH) (Sisó-Terraza y col., 2016a). Como en este trabajo el 70-77% del Fe en disolución es atrapado por BPDS, el proceso de disolución parece implicar la reducción de Fe(III), además ocurre tanto a pHs ligeramente ácidos (pH 5,5) como a pHs neutros (pH 7,5) característicos de los suelos calizos (Sisó-Terraza y col., 2016a). Esto confirma los resultados de otros autores que utilizando las flavinas Rbfl y FMN directamente en estado reducido y en ausencia de O_2, demostraron la disolución reductiva de diferentes óxidos férricos (óxido férrico recién preparado, ferrihidrita, lepidocrocita, goethita y hematita) (von Canstein y col., 2008; Shi y col., 2012, 2013). En esta Tesis se prueba que la disolución de óxido férrico facilitada por flavinas ocurre no sólo a las concentraciones estimadas en las proximidades de las raíces (aproximadamente de 100-300 μM) sino también a bajas concentraciones (0,1-1,0 μM) (Sisó-Terraza y col., 2016a). También se demuestra que la disolución del óxido está controlada por la concentración de flavinas, de manera que el grado de disolución aumenta con la concentración de flavinas en el medio. La riboflavina y los sulfatos de Rbfl utilizados a la misma concentración promueven la disolución de óxido en igual medida. La disolución de óxido férrico promovida por flavinas se encuentra afectada por el pH de una forma particular. Aunque el proceso ocurre tanto a pH ligeramente ácido (pH 5,5) como a pH neutro (pH 7,5) característico de los suelos calizos, la disolución del óxido es superior a pH 7,5 que a pH 5,5. Sin embargo, cuando el Fe(II) producido es atrapado por un agente quelante específico como el BPDS, la disolución de óxido a pH 5,5 aumenta y es mayor que la que ocurre a pH 7,5. A partir de este último resultado se infiere que la disolución de óxido férrico facilitada por flavinas puede ser relevante en suelos calizos tamponados a pH 7,5 así como en un área próxima a la raíz donde es más probable que existan pHs ligeramente ácidos y un secuestro de Fe(II) importante (debido a la alta actividad tanto de la H^+-ATPasa como del transportador IRT característicos de las raíces de muchas especies cuando crecen con baja disponibilidad de Fe soluble). Por otra parte, esta Tesis también demuestra que la presencia de flavinas en forma oxidada en el medio permite a las plantas de remolacha deficientes en Fe disolver óxido férrico (Sisó-Terraza y col., 2016a). Esto no se observa cuando se hace el mismo experimento con las plantas de remolacha suficientes en Fe (plantas control) que se caracterizan por tener menor actividad FCR en la raíz que las plantas deficientes en Fe. En línea con esto, el presente trabajo también demuestra que la presencia de flavinas en forma oxidada permite disolver óxido férrico a las puntas de las raíces (0-5 mm desde el ápice) zona donde se localiza principalmente la actividad FCR de la raíz de las plantas de remolacha deficientes en Fe (Landsberg y col., 1995; López-Millán y col., 2000 y 2001), y que la intensidad del proceso de disolución es mayor cuando se utiliza esta parte de la raíz que cuando se utilizan zonas adyacentes (5-10 mm desde el ápice). Estos hechos apoyan la hipótesis de que los sulfatos

de Rbfl exudados por las raíces de remolacha puedan actuar como compuestos solubles que medien la transferencia de electrones desde la FCR hasta el óxido férrico promoviendo su disolución y, por consiguiente, el aumento del Fe soluble disponible para la planta.

El uso de flavinas extracelulares como transportadores de electrones no es nuevo en la naturaleza y ya se ha descrito en otros organismos en condiciones de anaerobiosis. Así, algunos microorganismos anaeróbicos utilizan reductasas de la membrana plasmática junto con flavinas exudadas para reducir óxidos férricos del medio lo que les permite bien hacer la respiración anaeróbica (*Shewanella oneidensis*, *Geothrix fermentans* y otros; von Canstein y col., 2008; Marsili y col., 2008; Mehta-Kolte y Bond, 2012; Wu y col., 2014) o bien facilitar la asimilación de Fe (*Campylobacter jejuni*, *Helicobacter pylori* y otros; Fassbinder y col., 2000; Crossley y col., 2007; Balasubramanian y col., 2010). Las puntas de las raíces de las plantas deficientes en Fe de varias especies de Estrategia I que exudan flavinas (p. ej. remolacha) presentan cambios a nivel de actividades enzimáticas, cantidad de proteína y metabolitos que se pueden explicar por un paso hacia un metabolismo de tipo anaeróbico. Esta modificación sería consecuencia del gran gasto energético que suponen la activación de FCR y H^+-ATPasa que consumen NADH y ATP (Figs. 1 y 2) y generan una bajada del nivel de O_2 simplástico que va acompañado de disminuciones del O_2 apoplástico, éstas últimas producidas también en parte porque la propia FCR podría consumir O_2 (López-Millán y col., 2000; Vigani, 2012; Vigani y Zocchi, 2010).

Las flavinas podrían tener también un efecto en el microbioma de la rizosfera lo que podría afectar a la nutrición férrica de la planta. La exudación radicular de flavinas podría actuar como una barrera contra determinados organismos que compiten por el Fe soluble y también podrían promover asociaciones beneficiosas entre plantas y microorganismos. Así, se ha descrito que la Rbfl y los sulfatos de Rbfl inhiben el crecimiento de *Lactobacillus casei*, *Streptococcus faecialis*, *Phytophtora infestans* y otros (Susín y col., 1994; Ramsey y Wilson, 1957, Slotnick y Dougherty 1965; Jordan y col., 1992). La Rbfl contribuye claramente en la simbiosis entre leguminosas y miembros de la familia de bacterias del género *Rhizobium*. Por ejemplo, la presencia de flavina extracelular resultó importante para la colonización de las raíces de alfalfa (*Medicago sativa*) por *Sinorhizobium meliloti* (Yang y col., 2002).

Modelos de toma de Fe facilitada por la exudación de compuestos fenólicos y flavinas

Los mecanismos de acción de cumarinas y flavinas exudadas a la rizosfera que se desprenden de los resultados de esta Tesis y su discusión se presentan en los modelos de las Figuras 2 y 3. En ambos casos la deficiencia de Fe aumenta la síntesis, acumulación y/o exudación de cumarinas (Fig. 2) o flavinas (Fig. 3) en raíces. Las cumarinas diferentes de escopoletina (p. ej. fraxetina e isofraxidina) podrían producirse bien por hidroxilaciones y metilaciones de la escopoletina (Fig. 2) o bien partir de otros ácidos cinámicos (p. ej. síntesis de isofraxidina a partir del ácido sinápico). La hexosilación de las cumarinas podría regular su disponibilidad para el transportador de membrana que permite la salida de estos compuestos de la célula y, por consiguiente, sus posibles acciones en la rizosfera. En *A. thaliana* la acción coordinada de una glicosil transferasa desconocida -que formaría los hexósidos de cumarinas que se han encontrado acumulados en la raíz-, la glicosil hidrolasa BGLU42 que convertiría los hexósidos en las correspondientes formas agliconas de las cumarinas y el transportador de membrana PDR9 estarían involucrados en dichos procesos (Fig. 2). Otras especies aumentan la síntesis de Rbfl, flavina que se acumula en raíces, y que en algunas especies conduce a la formación de compuestos con la misma estructura pero con alguna sustitución bien en la cadena de ribosa (p. ej. sulfatación del hidróxilo sobre el carbono 5') o bien en la parte isoaloxacina (p. ej. hidroxilación del metilo en el carbono 7) (Fig. 3). Las flavinas pueden ser exudadas a la rizosfera, proceso realizado por un sistema de transporte todavía desconocido.

Una vez en la rizosfera, la acción de los compuestos fenólicos y las flavinas permite aumentar la disponibilidad de Fe soluble para el sistema de toma de Fe FRO/IRT. Las cumarinas de tipo catecol solubilizan óxidos férricos mediante mecanismos de quelación y/o reducción (Fig. 2). Las flavinas actúan como transportadores de electrones desde la reductasa férrica (FRO) hacia los óxidos férricos, produciendo formas reducidas de las flavinas que solubilizan óxidos férricos también mediante mecanismos de quelación y/o reducción (Fig. 3). La planta toma Fe soluble por el ya conocido mecanismo de absorción directa de iones Fe^{2+} mediado por la proteína de membrana IRT y/o por la reducción de quelatos férricos mediante la reductasa férrica FRO seguida de la absorción del Fe^{2+} resultante a través del IRT. Por otra parte, tanto las cumarinas (en especial aquellas altamente oxigenadas) como las flavinas podrían afectar al microbioma de la rizosfera limitando el crecimiento de microorganismos (tal vez de los menos eficientes en la toma de Fe) y/o promoviendo el crecimiento de otros (probablemente de los más eficientes en la solubilización de Fe del medio) y, de esta manera, contribuyendo indirectamente a man-

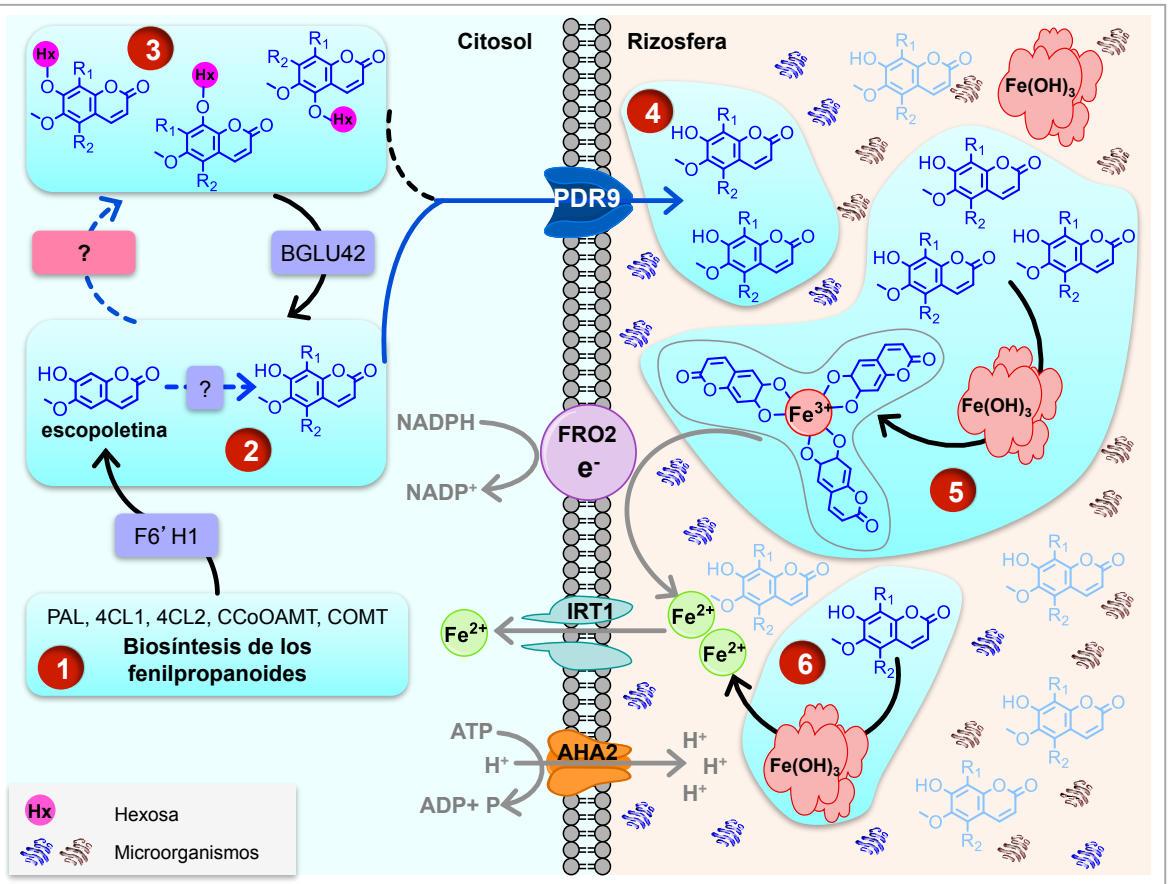


Figura 2 Modelo de extracción y toma del Fe del suelo por plantas de Estrategia I capaces de producir y exudar compuestos fenólicos del tipo cumarina en respuesta a una baja disponibilidad de Fe. **(1)** La síntesis de la coumarina escopoletina aumenta, siendo acumulada en raíz. **(2)** Compuestos de tipo cumarina derivados de escopoletina son sintetizados y acumulados en raíz. **(3)** Las cumarinas son glicosiladas y/o deglicosiladas en la raíz. **(4)** Las cumarinas y/o sus glicósidos son transportados a la rizosfera en un proceso mediado por la proteína de membrana PDR9/ABCG37 en *Arabidopsis thaliana*. **(5** y **6)** Las coumarinas de tipo catecol solubilizan óxidos férricos mediante mecanismos de quelación y/o reducción, aumentando la disponibilidad de Fe soluble para el sistema de toma de Fe FRO/IRT integrado en la membrana plasmática. La toma del Fe soluble de la rizosfera se produce por absorción directa de iones Fe^{2+} mediada por la proteína de membrana IRT1 y/o por la reducción de quelatos férricos mediante la reductasa férrica FRO2 seguida de la absorción del Fe^{2+} resultante a través del IRT1. Las flechas grises indican procesos ya conocidos relacionados con la toma de Fe. Las flechas **azules** y las negras representan procesos deducidos (flecha continua)/propuestos (flecha discontinua) a partir de los resultados de esta **Tesis** y de otros trabajos, respectivamente. AHA2, H+-ATPasa; BGLU42, glucosidasa; 4-CL, hidroxicinamoil CoA ligasa; CCoAOMT, cafeoil CoA-O-metiltransferasa; COMT, ácido cafeico O-metiltransferasa; F6'H1, feruloil-CoA 6'-hidroxilasa; PAL, fenilalanina amoníaco-liasa.

tener un reservorio mayor de Fe soluble en la proximidad de la raíz.

El conjunto de las acciones de cumarinas o flavinas conduciría a aumentar la cantidad de Fe soluble en la rizosfera y, por consiguiente, a mejorar la nutrición férrica de la planta. Finalmente, se puede afirmar que una mayor capacidad de síntesis y exudación de estos u otros metabolitos secundarios con funciones similares en la rizosfera, en combinación con un sistema de captación de Fe soluble muy activo en la membrana, supone una ventaja competitiva de unas especies y/o cultivares frente a otros cuando se cultivan en condiciones de baja disponibilidad de Fe soluble derivada tanto de las características del suelo/sustrato como de un cultivo muy intensivo del suelo.

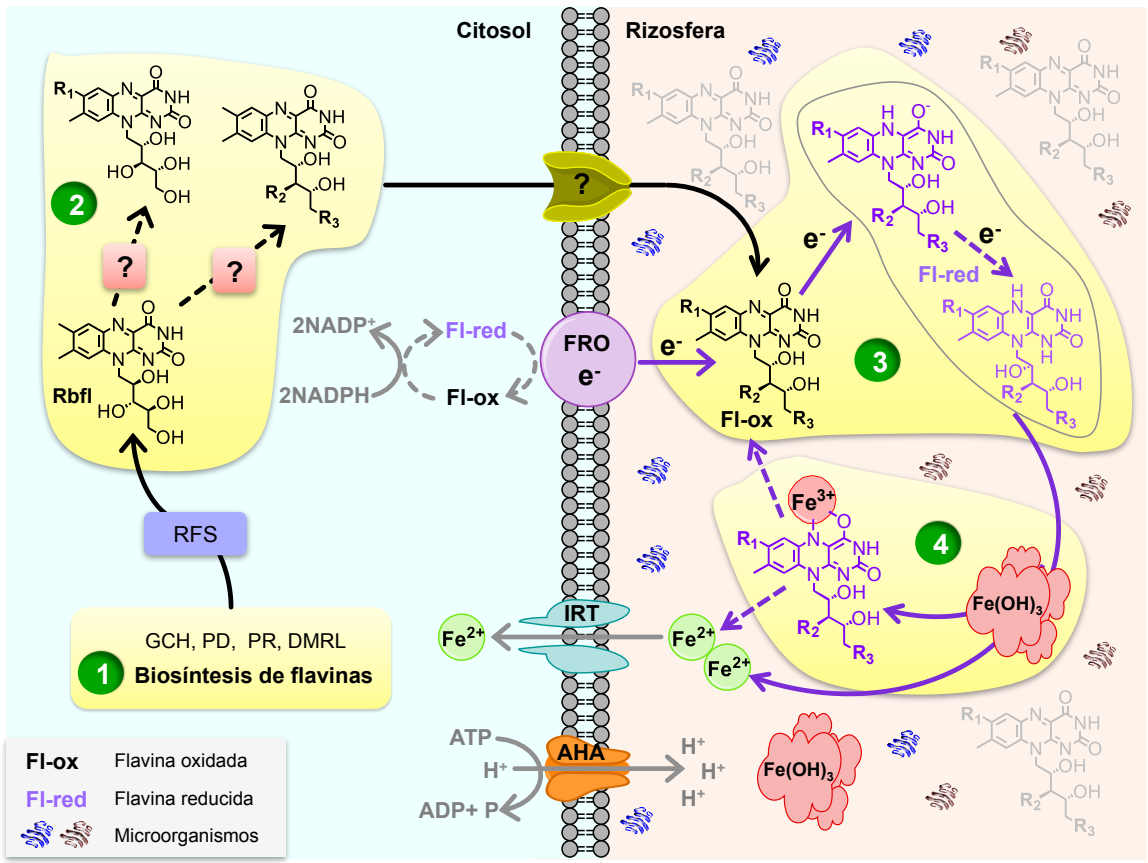


Figura 3 Modelo de extracción y toma del Fe del suelo por plantas de Estrategia I capaces de producir y exudar compuestos de tipo flavina en respuesta a una baja disponibilidad de Fe. **(1)** La síntesis de la riboflavina (Rbfl) aumenta , siendo acumulada en raíz. **(2)** Compuestos derivados de Rbfl son sintetizados y acumulados en raíz. **(3)** La Rbfl y/o sus derivados son transportados a la rizosfera por un proceso aún desconocido, allí actúan como transportadores de electrones (e-) desde la reductasa férrica (FRO) hacia los óxidos férricos. **(4)** Las formas reducidas de las flavinas solubilizan óxidos férricos mediante mecanismos de quelación y/o reducción,aumentando la disponibilidad de Fe soluble para el sistema de toma de Fe FRO/IRT integrado en la membrana plasmática. La toma del Fe soluble de la rizosfera se produce por absorción directa de iones Fe^{2+} mediado por la proteína de membrana IRT y/o por la reducción de quelatos férricos mediante la reductasa férrica FRO2 seguida de la absorción del Fe^{2+} resultante a través del IRT. Las flechas grises continuas/discontinuas indican procesos ya conocidos/propuestos relacionados con la toma de Fe. Las flechas **moradas** y las negras representan procesos deducidos (flecha continua)/propuestos (flecha discontinua) a partir de los resultados de esta **Tesis** y de otros trabajos, respectivamente. AHA2, H+-ATPasa; GCH, GTP ciclohidrolasa; PD, 2,5-diamino-6-ribosilamino-4(3H)-pirimidinona 5'-fosfato deaminasa; PR, 5-diamino-6-ribosilamino-2,4(1H,3H)-pirimidinediona 5'-fosfato reductasa; DMRL, 6,7-dimetil-8-ribitillumazina; RFS, riboflavina sintasa.

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Zamioudis C, Hanson J, Pieterse CMJ. 2014. β-glucosidase BGLU42 is a MYB72-dependent key regulator of rhizobacteria-induced systemic resistance and modulates iron deficiency responses in Arabidopsis roots. *New Phytologist* **204**: 368–379.

Zocchi G, De Nisi P, Dell'Orto M, Espen L, Marino Gallina P. 2007. Iron deficiency differently affects metabolic responses in soybean roots. *Journal of Experimental Botany* **58**: 993-1000.

CAPÍTULO 5. CONCLUSIONES

1- En raíces de *Arabidopsis thaliana* y tomate, la deficiencia de Fe induce la síntesis y acumulación de compuestos fenólicos de tipo cumarina y no afecta a la concentración de flavinas.

2- Los perfiles de compuestos de tipo cumarina producidos por *A. thaliana* y tomate son diferentes. Las raíces de *A. thaliana* deficientes en Fe acumulan las cumarinas simples escopoletina, fraxetina, isofraxidina y fraxinol, así como varios cumarinolignanos derivados de fraxetina, mientras que las raíces deficientes en Fe de tomate acumulan las cumarinas simples escopoletina, fraxetina, isofraxidina, trihidroximetoxicumarina y dihidroxidimetoxicumarina. Las coumarinas simples se acumulan tanto en formas hexósido como aglicona, con la forma predominante dependiendo de cada cumarina y/o especie vegetal.

3- La acumulación de compuestos de tipo cumarina en las raíces de *A. thaliana* y tomate es dependiente del tiempo de exposición a la deficiencia de Fe y del pH del medio de cultivo.

4- La deficiencia de Fe también induce la exudación radicular de compuestos de tipo cumarina en *A. thaliana*. El perfil de compuestos de tipo cumarina presentes en el medio de cultivo es diferente al observado en las raíces, presentándose en forma aglicona y siendo mayoritarias escopoletina y fraxetina.

5- La exudación radicular de compuestos de tipo cumarina por las plantas de *A. thaliana* depende del tiempo de exposición a la deficiencia de Fe y del pH del medio de cultivo.

6- El transportador de membrana ABCG37/PDR9 está involucrado en la exudación radicular de compuestos de tipo cumarina inducida por la deficiencia de Fe en *A. thaliana*.

7- Las raíces de las plantas de remolacha deficientes en Fe pueden utilizar flavinas endógenas extracelulares para promover la disolución reductiva de óxidos férricos. Este proceso forma parte de la Estrategia I que confiere tolerancia ante la deficiencia de Fe.

8- La exudación radicular de compuestos de tipo cumarina en plantas de *A. thaliana* y de flavinas en plantas de remolacha mejora la nutrición férrica.

ANEXO. CURRICULUM VITAE

MINISTERIO
DE ECONOMÍA
Y COMPETITIVIDAD

Currículum vitae

Impreso normalizado

Número de hojas que contiene: 5

Nombre: Patricia Sisó Terraza

Fecha: 11 de abril 2017

Apellidos: ***Sisó Terraza***	*N*ombre: ***Patricia***

FORMACIÓN ACADÉMICA

Titulación Superior	**Centro**	**Fecha**
Ingeniero Agrónomo	*Universitat de Lleida*	*2009*
Máster	**Centro**	**Fecha**
Investigación Química	*Universidad de Zaragoza*	*2011*

SITUACIÓN PROFESIONAL ACTUAL

Profesor Técnico de Formación Profesional. Especialidad en Operaciones y Procesos de Producción Agraria (Sep 15 – actualidad)

Organismo: Gobierno de Aragón

Departamento de Educación, Cultura y Deporte

ACTIVIDADES ANTERIORES DE CARACTER CIENTÍFICO O PROFESIONAL

Fechas	**Puesto**	**Institución**
Oct 10 – Oct 14	*Contrato predoctoral (Beca FPI)*	*Estación Experimental de Aula Dei. CSIC.*
Oct 09 – Sep 10	*Ayte. de investigación*	*C.I.T.A (GOBIERNO DE ARAGÓN)*
Abr 09 – Ago 09	*Aux. de laboratorio*	*AGROP. I SECC.CREDIT DE SOSES*
Ene 08 – Jul 08	*Técnico agrícola*	*FUNDACIÓ PRIVADA DEL MON RURAL*
Jul 06 – Oct 06	*Aux. de laboratorio*	*CATAFRUIT, S.A.*
Jul 05 – Oct 05	*Aux. de laboratorio*	*CODORNIU S.A.*
Jul 03 – Oct 03	*Aux. de laboratorio*	*FRUITS DE PONNENT, S.C.C.L.*

IDIOMAS DE INTERÉS CIENTÍFICO (R = regular, B = bien, C= correctamente)

Idioma	**Habla**	**Lee**	**Escribe**
Inglés	R	C	R
Francés	R	C	B
Catalán	C	C	C

PARTICIPACIÓN EN PROYECTOS DE I+D FINANCIADOS EN CONVOCATORIAS PÚBLICAS

TÍTULO DEL PROYECTO: Nuevos enfoques para el estudio de la disponibilidad, movimiento y localización del Fe en la fertilización de árboles frutales (AGL2009-09018)

ENTIDAD FINANCIADORA: MICINN-DGI (Programa Nacional de Recursos y Tecnologías Agroalimentarias)

DURACIÓN: *DESDE:* Enero 2010 *HASTA:* Diciembre 2012

INVESTIGADOR RESPONSABLE: Anunciación Abadía

TÍTULO DEL PROYECTO: Hacia el desarrollo de técnicas para un estudio fisiológico del olivo en cultivo hiper-intensivo (AP/040397/11)

ENTIDAD FINANCIADORA: AECID (España-Túnez)

DURACION: *DESDE:* Diciembre 2011 *HASTA*: Diciembre 2012

INVESTIGADOR PRINCIPAL: Anunciación Abadía

TÍTULO DEL PROYECTO: Estrategias innovadoras para mejorar la nutrición férrica en frutales (AGL2012-31988)

ENTIDAD FINANCIADORA: MINECO-DGI (Programa Nacional de Recursos y Tecnologías Agroalimentarias)

DURACION: *DESDE:* Enero 2013 *HASTA*: Diciembre 2015

INVESTIGADOR PRINCIPAL: Anunciación Abadía

PUBLICACIONES

Artículos en Revistas Internacionales SCI

1 Fourcroy P, **Sisó-Terraza P**, Sudre D, Savirón M, Reyt G, Gaymard F, Abadía A, Abadía J, Álvarez-Fernández A, Briat JF. (2014) Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by *Arabidopsis* roots in response to iron deficiency. **New Phytologist** 201:155-67. DOI: 10.1111/nph.12471. Nº de citas: 65.

Desde Julio/Agosto de 2016, este **artículo muy citado** recibió suficientes citas para incluirse en el 1% de los mejores artículos de su campo académico en función de un umbral de artículos muy citados para el campo y el año de publicación.

Datos de ***Essential Science Indicators***[SM]

2 **Sisó-Terraza P**, Ríos JJ, Abadía J, Abadía A, Álvarez-Fernández A. (2015) Flavins secreted by roots of iron-deficient *Beta vulgaris* enable mining of ferric oxide via reductive mechanisms. **New Phytologist** 209:733-745. DOI: 10.1111/nph.13633. Nº de citas: 4.

3 **Sisó-Terraza P**, Luis-Villarroya A, Fourcroy P, Briat JF, Abadía A, Gaymard F, Abadía J, Álvarez-Fernández A. (2016) Accumulation and secretion of coumarinolignans and other coumarins by *Arabidopsis thaliana* roots in response to iron deficiency at high pH. **Frontiers in Plant Science** 7:1711. DOI: 10.3389/fpls.2016.0171. Nº de citas: 0.

En preparación

4 **Sisó-Terraza P**, Luis-Villarroya A, Abadía J, Abadía A, Álvarez-Fernández A. Coumarin accumulation in roots of iron-deficient tomato (*Solanum lycopersicum*).

Artículos en Revistas no SCI

Sisó-Terraza P, Sanjuán López AI. (2010) Análisis de la situación interna del sector productor-comercializador de azafrán en la Comarca del Jiloca. Documento de Trabajo/ SIA. Unidad de Economía y Sociología Agrarias. Centro de Investigación y Tecnología de Agroalimentaria de Aragón (CITA). Zaragoza. pp: 34.

Capítulos en Libros

Sanjuán López AI, Zeballos MG**, Sisó-Terraza P,** Resano Ezcaray H**.** (2011) El uso actual y potencial del azafrán entre los consumidores aragoneses. En: Proyecto mejora integral del cultivo del azafrán del Jiloca. Centro de Investigación y Tecnología de Agroalimentaria de Aragón (CITA) (eds). Zaragoza. pp: 59-63. ISBN-978-84-694-0049-4

ESTANCIAS EN CENTROS EXTRANJEROS

CLAVE: D = doctorado, P = postdoctoral, I = invitado, C = contratado, O = otras (especificar).

CENTRO: Biochimie et Physiologie Moléculaire des Plantes (B&PMP/INRA). "Nutrition Minérale et Stress Oxydatif".

LOCALIDAD: Montpellier PAÍS: Francia CLAVE: D
AÑO: 2012 DURACIÓN: 3 meses

TEMA: Estudio del efecto de la deficiencia de Fe en la acumulación de compuestos fenólicos en raíz de la especie modelo *Arabidopsis thaliana* y la liberación de los mismos al medio de cultivo.

CENTRO: Biochimie et Physiologie Moléculaire des Plantes (B&PMP/INRA). "Nutrition Minérale et Stress Oxydatif".

LOCALIDAD: Montpellier PAÍS: Francia CLAVE: D
AÑO: 2013 DURACIÓN: 4 meses

TEMA: Estudio de la síntesis, acumulación y exudación radicular de compuestos fenólicos inducida por deficiencia de hierro en plantas de Estrategia I, *Arabidopsis thaliana* y *Solanum lycopersicum*.

ESTANCIAS EN CENTROS NACIONALES

CLAVE: D = doctorado, P = postdoctoral, I = invitado, C = contratado, O = otras (especificar).

CENTRO: Instituto de Ciencias de los Materiales de Aragón (CSIC-Universidad de Zaragoza). Servicio de Espectrometrías de Masas.

LOCALIDAD: Zaragoza PAÍS: España CLAVE: D
AÑO: 2012-2013 DURACIÓN: 3 semanas

TEMA: Identificación por espectrometría de masas en tándem de compuestos fenólicos exudados y acumulados por las raíces de *Arabidopsis thaliana* en respuesta a deficiencia de hierro.

CONTRIBUCIONES EN CONGRESOS NACIONALES E INTERNACIONALES

2012

***XIV Simposio Hispano-Luso de Nutrición Mineral de las Plantas**. Madrid, Spain*. (Asistencia).

(1) **Sisó-Terraza P**, Abadía J, Abadía A, Álvarez-Fernández A. The role of iron deficiency-induced release of flavins into the rizosphere – The case of *Beta vulgaris*. (Comunicación /Panel).

2013

***XVII International Plant Nutrition Colloquium. Plant Nutrition for Nutrient and Food Security**. Istanbul, Turkey*.

(2) **Sisó-Terraza P**, Abadía A, Abadía J, Álvarez-Fernández A. Secretion and accumulation of phenolic compounds by *Solanum lycopersicum* roots in response to iron deficiency. (Comunicación / Panel).

2014

***17th International Symposium on Iron Nutrition and Interactions in Plants**. Gatersleben, Germany*.

(3) Abadía J, **Sisó-Terraza P**, Pablo Díaz-Benito, Gutierrez-Carbonell E, Takahashi D, Abadía A, Uemura M, López-Millán AF, Álvarez-Fernández A. Advances in iron nutrition based on mass spectrometry approaches. (Ponencia invitada).

(4) **Sisó-Terraza P**, Abadía J, Abadía A, Gogorcena Y, Álvarez-Fernández A. Root secretion of phenolics plays a significant role for iron acquisition at high pH in *Prunus* rootstocks. (Comunicación / Panel).

2015

***II National Conference BIFI2015**. Zaragoza, España.*

(5) ***Sisó-Terraza P**, Gogorcena Y, Abadía J, Abadía A, Álvarez-Fernández A*. Plant root release of phenolics and flavins upon Fe deficiency. *(*Ponencia invitada*)*.

***IV Congreso Hispano-Luso de Fisiología Vegetal**. Toledo, España*.

(6) **Sisó-Terraza P**, Luis-Villarroya A, Abadía J, Abadía A, Álvarez-Fernández A. Arabidopsis root secretion of phenolics as affected by iron deficiency and external pH. (Comunicación / Panel).

2016

***18th International Symposium on Iron Nutrition and Interactions in Plants**. Madrid, España*.

(7) Izquierdo Alegre E, Fourcroy P, Boucherez J, Conéjéro G, Tissot N, Rothan C, Bres C, Causse M, **Sisó-Terraza P**, Álvarez-Fernández A, Abadía J, Briat J-F, Gaymard F, Dubos C. Impact of iron availability on tomato fruit quality. (Comunicación / Panel).

***XVI Simposio Hispano-Luso de Nutrición Mineral de las Plantas**. San Pedro del Pinatar, Murcia, España.*

(8) Álvarez-Fernández A, Luis-Villarroya A, **Sisó-Terraza P**, Fourcroy P, Lefèvre F, Venuti S, Gogorcena Y, Briat J-F, Tomasi N, Dubos C, Pinton R, Boutry M, Gaymard F, Abadía A, Abadía J. The role of coumarins, flavins and flavonoids in Fe acquisition by plants. (Ponencia invitada).

NOTAS DE PRENSA

- Interempresas, 18 Sep 2015. La vitamina B2 facilita la toma de hierro por plantas
- AZprensa, 16 Sep 2015. La vitamina B2 es una fuente de hierro para las plantas
- Página Web EEAD-CSIC, 15 Sept 2015. La vitamina B2 facilita la toma de hierro por las plantas
- Página Web CSIC, 15 Sep 2015. La vitamina B2 facilita la toma de hierro por las plantas
- Informaria Digital, 21 Sep 2015. Un estudio del CSIC indica que la vitamina B2 o riboflavina facilita la toma de hierro por las plantas
- Agencia Sinc, 7 Nov 2013. Identifican compuestos naturales que ayudan a las plantas a tomar hierro

GRANDES EQUIPOS QUE HA UTILIZADO

CLAVE : R= responsable, UA = usuario asiduo, UO = usuario ocasional

EQUIPO	*FECHA*	*CLAVE*
Cromatografía líquida de alta eficacia (HPLC)	2010-2014	UA
Espectroscopía ultra-violeta visible (HPLC-UV/VIS)	2010-2014	UA
Espectroscopía de fluorescencia	2010-2014	UO
Espectrometría de masas (ESI-MSTOF y HPLC-ESI/MSTOF)	2010-2014	UA
Espectrometría de masas tándem (HPLC-ESI/MS-MSQ-TOF y Ion-trap)	2010-2014	UA
Espectrometría de absorción y emisión atómica	2013-2014	UO

CURSOS Y SEMINARIOS RECIBIDOS

- Certificado de Aptitud Pedagógica (CAP). Universidad de Lleida. 2009.
- Curso de especialización: Especiación de elementos traza: metodología analítica y aplicaciones. Universidad de Barcelona. Julio 2012.
- Curso de botánica agrícola (63 h). Departamento de agricultura, ganadería, pesca y alimentación. Generalitat de Catalunya. Noviembre 2012.
- Curso de agua, suelo y fertilización. Ecosistemas (63 h). Departamento de agricultura, ganadería, pesca y alimentación. Generalitat de Catalunya. Junio 2013.
- Curso básico de producción integrada (50 h). Departamento de agricultura, ganadería, pesca y alimentación. Generalitat de Catalunya. Diciembre 2013.
- Curso básico de riego (50 h). Departamento de agricultura, ganadería, pesca y alimentación. Generalitat de Catalunya. Junio 2016.