UNIVERSITAT DE BARCELONA

Mecanismos de protección frente al déficit hídrico reiterado en plantas

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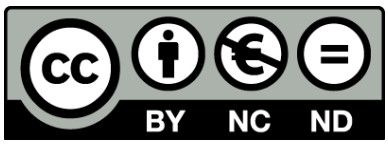

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Eva Fleta Soriano

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Memoria presentada por Eva Fleta Soriano para optar al título de Doctora por la Universidad de Barcelona.

Programa de Doctorado en Ecología, Ciencias Ambientales y Fisiología Vegetal de la Universidad de Barcelona.

Este trabajo se ha realizado en el Departamento de Biología Evolutiva, Ecología y Ciencias Ambientales de la Facultad de Biología de la Universidad de Barcelona bajo la dirección del Dr. Sergi Munné Bosch.

Doctoranda — Eva Fleta Soriano

Director de Tesis — Dr. Sergi Munné Bosch

El trabajador obligado a luchar penosamente por la vida nunca llega a conocer los altos goces de la ciencia y de la creación artística.

Para que todo el mundo llegue a estos placeres, que hoy se reservan al menor número, para que tenga tiempo y posibilidades de desarrollar sus capacidades intelectuales, la renovación debe garantizar a cada uno el pan cotidiano. Y luego, tiempo libre.

Este es nuestro propósito supremo.

Priot Kroptkin

Al buscar lo imposible el hombre
siempre ha realizado y reconocido lo posible.

Y aquellos que sabiamente se han limitado a lo que creían
posible, jamás han dado un solo paso adelante.

Mijaíl Bakunin

ÍNDICE

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Podría escribir muchos agradecimientos concretos a la gente que durante estos años (o durante toda la vida como mi familia) me ha ayudado/ apoyado que no ha sido poca (compañeros, amigos, Mikel, mi gatita Katu…) pero también creo que toda esa ayuda no hubiera servido de nada si antes mucha otra gente (algunos coinciden) no hubiera luchado por defender mi derecho a la educación y la cultura. Aun así me gustaría hacer un agradecimiento concreto a Sergi por su comprensión y libertad a la hora de trabajar y por supuesto por su forma de organizar el grupo, que aunque pueda derivar en muchos problemas por los individualismos a los que la sociedad nos tiene acostumbrados, en este grupo se trabaja en equipo y todos nos ayudamos y apoyamos mutuamente, como decía Kropotkin en su libro "Ayuda mutua: un factor en la evolución".

En resumen, muchas gracias a todas las personas que a lo largo de los tiempos han luchado, luchan y lucharán por hacer la educación accesible a todas las personas, independientemente de sexo o clase social. Sin esa lucha, yo, mujer de clase obrera, nunca podría haber llegado hasta aquí. Aunque aun queda mucho por avanzar gracias a su lucha, esfuerzo y vida en muchos casos, puedo yo ahora estar aquí sin que esto sea una heroicidad.

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Eva, Abril 2017

ABREVIATURAS

$^1O_2$	Oxígeno singlete
^{3}**Chl***	Clorofila triplete
ABA	Ácido abscísico
ABRE	Elementos de respuesta al acido abscísico
CO_2	Dióxido de carbono
DPS	Estado de de-epoxidación del ciclo de las xantofilas
F_m	Fluorescencia máxima
F_0	Fluorescencia mínima
F_v	Fluorescencia variable
F_v/F_m	Eficiencia máxima del fotosistema II
F_v'/F_m'	Rendimiento efectivo del fotosistema II
H_2O_2	Peróxido de hidrogeno
O_2^-	Radical superóxido
LMA	Masa foliar por unidad de superficie
PC-8	Plastocromanol 8
PS	Peso seco
PSII	Fotosistema II
ROS	Especies reactivas de oxígeno
RWC	Contenido hídrico relativo

INTRODUCCIÓN

1.- Déficit hídrico en plantas

El déficit hídrico en plantas ocurre cuando no hay suficiente agua en la zona radicular de las mismas para poder satisfacer sus necesidades en un momento y lugar determinados, es decir, cuando el agua transpirada excede el agua absorbida (Lawlor & Cornic 2002). Este tipo de estrés por sequía se conoce como sequía hidroedáfica o agrícola y no hay que confundirla con aridez, algo intrínseco a una región y por lo tanto dependiente del clima, a diferencia del estrés por sequía que se considera un episodio transitorio para las plantas (Maliva & Missimer 2012).

Los modelos climáticos actuales predicen que debido al cambio climático la aridez se va a continuar incrementando y por tanto, la disponibilidad de agua disminuirá (Dai 2012), especialmente en las zonas de clima mediterráneo (Bussotti et al. 2014). De hecho, en las últimas décadas en España, las sequías han aumentado debido a la disminución de las precipitaciones y al aumento en la evapotranspiración (Vicente-Serrano et al. 2014). Estas condiciones conllevan una menor disponibilidad hídrica para las plantas y por tanto, más episodios de déficit hídrico, lo cual provoca un estrés más severo del que las plantas están adaptadas en las diferentes regiones (Allen et al. 2010).

El déficit hídrico en plantas afecta a la mayor parte de sus funciones vitales, de hecho, entre un 80-90% del peso fresco en especies herbáceas es agua, y su déficit afecta tanto a la morfología o estructura, como a su fisiología y metabolismo (Sánchez-Díaz &

Aguirreolea 2008). A nivel estructural el estrés por sequía puede causar varias alteraciones a nivel de planta entera, de órganos y células, como cambios en la distribución de las raíces en el suelo (Kuster et al. 2012), disminuciones en el número de hojas y/o su área foliar (Zhang et al. 2014), aumentos en la masa foliar por unidad de área (LMA, Peña-Rojas et al. 2005), y/o reducciones en la expansión celular debido a reducciones en la turgencia (Davies et al. 1986). Estos cambios estructurales permiten a la planta perder menos agua mediante la transpiración al mismo tiempo que el cambio de distribución de su sistema radicular le puede permitir mantener la absorción de agua (Farooq et al. 2009).

Si el déficit hídrico se da en condiciones que conllevan un exceso de luz, como ocurre habitualmente en clima mediterráneo cuando el estrés hidroedáfico ocurre simultáneamente a aumentos en la temperatura ambiental y la radiación solar durante los meses de verano (Chaves et al. 2002), las plantas se aclimatan mediante cambios estructurales adicionales incluso más sofisticados, como cambios en la estructura y distribución de los cloroplastos a nivel celular (Zhang et al. 2014), y/o la reducción del tamaño de las antenas o complejos captadores de luz del aparato fotosintético, lo cual puede venir indicado por cambios en la relación clorofila a/b (Kurasová et al. 2000, 2002).

En cuanto a la secuencia temporal de eventos en la respuesta de las plantas al estrés por sequía, uno de los acontecimientos más rápidos es la pérdida parcial de volumen celular debida a la pérdida de agua, lo cual reduce la presión de turgencia en las célu-

las (Lawlor & Cornic 2002). Esta presión es indispensable para la expansión celular y por tanto, para el crecimiento de la planta, lo cual conlleva una rápida reducción del mismo en condiciones de sequía (Taiz et al. 2006). Al mismo tiempo, puede producirse un cierre estomático debido a la pérdida de volumen de las células oclusivas (mecanismo regulado por la hormona ácido abscísico –ABA –) que son las encargadas de la apertura estomática (Pirasteh -Anosheh et al. 2016). En los procesos fisiológicos y metabólicos que ocurren en situaciones de déficit hídrico, cabe destacar también la importancia del agua en otros aspectos, pues es el medio de transporte y distribución de metabolitos, en el cual se producen reacciones bioquímicas, y es además el disolvente de sales y azúcares, por lo que su déficit afecta prácticamente a todos los procesos metabólicos de la planta (Sánchez-Díaz & Aguirreolea 2008). Es por ello que la sequía es considerada uno de los estreses ambientales que más afectan al crecimiento y desarrollo de las plantas (Ciais et al. 2005); de hecho es el estrés abiótico que más limita la producción de los cultivos (Reddy et al. 2004).

Uno de los procesos más afectados por la sequía es la fotosíntesis (Chaves 1991). La reducción en la incorporación de dióxido de carbono (CO_2) causada por el cierre estomático (para evitar la pérdida de agua), junto a alteraciones en el transporte de electrones y metabolismo fotosintético (desde reducciones en la actividad de Rubisco a alteraciones en la biosíntesis de sacarosa o almidón) conllevan una compleja aclimatación del aparato fotosintético al estrés (Flexas et al. 2004; Gupta & Kaur 2005; Aranjuelo et al. 2011). Entre otros aspectos, estos cambios, básicamente debido al

desequilibrio producido entre la producción de ATP y poder reductor por parte del transporte fotosintético de electrones y su consumo por parte del ciclo de Calvin (encargado de la fijación del CO_2), pueden desencadenar un exceso de energía en los cloroplastos (Asada 1999, 2006). Este exceso de energía provoca que las clorofilas que en principio transferían la energía para ser utilizada en el transporte de electrones, cedan su energía al oxígeno molecular para formar oxigeno singlete ($^1O_2$), una de las especies reactivas del oxígeno (ROS) potencialmente más dañinas, ya que puede provocar procesos de oxidación de lípidos, proteínas y/o ácidos nucleicos (Triantaphylides & Havaux 2009; Pintó-Marijuan & Munné-Bosch 2014). Este $^1O_2$ se produce básicamente en el PSII, pero se ha sugerido que se podría formar también a nivel de fotosistema I (Takagi et al. 2016). En este último se producen mayoritariamente radicales superóxido (O_2^-) cuando hay un exceso de reducción de la ferredoxina en el transporte fotosintético de electrones y no se puede formar NADPH en el transporte lineal de electrones (Asada 2006). Mediante la enzima superóxido dismutasa (SOD), este O_2^- se transforma rápidamente en peróxido de hidrógeno (H_2O_2), el cual se puede convertir en agua por el ciclo ascorbato-glutatión cerrándose el conocido ciclo del agua-agua, o bien a radicales hidroxilo (OH^-) debido a un mal funcionamiento o un funcionamiento insuficiente del mencionado ciclo junto a la acumulación de cobre o hierro en los cloroplastos (Asada 2006). La formación de estas moléculas ($^1O_2$, O_2^-, H_2O_2, OH^-) en cloroplastos debido al exceso de energía se muestra en la **Figura 1**.

Estas moléculas ($^1O_2$, O_2^-, H_2O_2, OH^-) son todas ellas conocidas como ROS y pueden tener tanto efectos beneficiosos actuando como señalizadores celulares para regular las repuestas al estrés, pero también pueden ser muy nocivas para las plantas causando daños irreversibles e incluso la muerte celular (Mittler 2002). Las ROS se producen constantemente como resultado de las diferentes vías metabólicas que ocurren en las plantas, no sólo en cloroplastos sino también en otros compartimentos subcelulares (Munné-Bosch et al. 2013), y aumentan en situaciones de estrés, como el déficit hídrico (Miller et al. 2010), de tal forma que el desequilibrio entre la producción de ROS y su eliminación por antioxidantes desencadena un estrés oxidativo en la planta (Apel & Hirt 2004). Un ejemplo de estrés oxidativo causado por déficit hí-

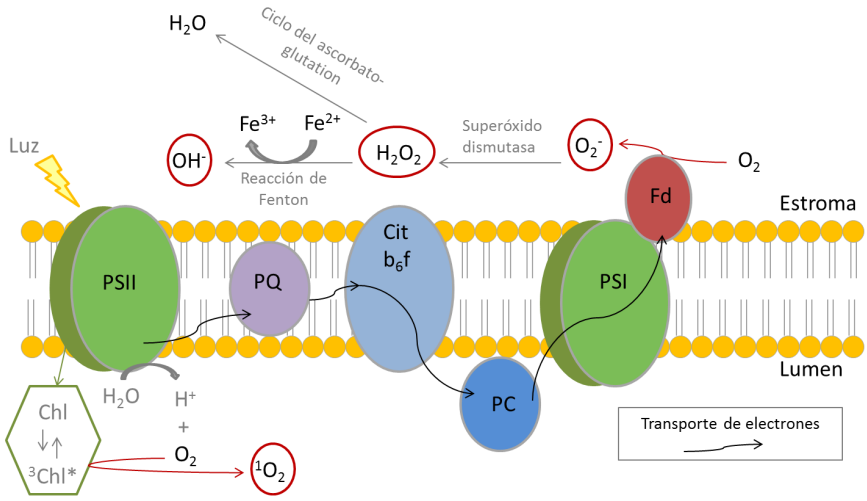


Figura 1. Esquema de la formación de las especies reactivas de oxigeno (ROS) en cloroplastos, incluyendo la formación de oxígeno singlete ($^1O_2$), radical superóxido (O_2^-), peróxido de hidrógeno (H_2O_2) y radical hidroxilo (OH^-) debido a un exceso de energía en la cadena de transporte de electrones durante la fotosíntesis.

drico puede observarse en la oxidación por parte del $^1O_2$ de los ácidos grasos poliinsaturados de la membrana tilacoidal (Triantaphylidès et al. 2008). Esta oxidación produce una reacción en cadena conocida como la peroxidación lipídica que aumenta la permeabilidad de la membrana, disminuye su fluidez y causa daños en las proteínas de la membrana tilacoidal (Møller et al. 2007).

El exceso de luz en los cloroplastos puede conllevar pues un aumento de las ROS en cloroplastos, básicamente de $^1O_2$, O_2^-, H_2O_2, los cuales pueden desencadenar procesos de señalización redox o bien daño oxidativo, según si estas ROS se producen de forma transitoria y a bajas concentraciones, o bien de forma sostenida en altas concentraciones, respectivamente (Munné-Bosch et al. 2013). La temporalidad en la acumulación y la concentración de ROS en cloroplastos depende de hecho de la capacidad fotoprotectora y de la protección antioxidante de los mismos, aspecto que se tratará más adelante en el apartado 3.2.

2.- Déficit hídrico reiterado y memoria en plantas

La respuesta de las plantas al estrés hídrico es constante y repetida en el tiempo. Aunque todavía poco estudiado, el estrés hídrico reiterado, es decir, los ciclos repetidos de estrés, son mucho más frecuentes en la naturaleza que la exposición de las plantas a un solo período de estrés por sequía, de tal forma que es indispensable comprender mejor la respuesta de las plantas al estrés hídrico reiterado para comprender mejor la respuesta de las plantas en relación

a su medio ambiente. Aunque el estrés hídrico reiterado es un fenómeno bien conocido desde los estudios clásicos de estrés por sequía en plantas, los mecanismos por los cuales las plantas se adaptan a él están todavía lejos de ser totalmente comprendidos (Li & Liu 2016).

La respuesta diferencial por parte de la planta frente a un solo episodio de estrés o frente a repetidos episodios es lo que se conoce como memoria. La memoria al estrés se puede definir como la capacidad de los organismos de responder mejor frente a un estrés concreto cuando la planta ya ha estado previamente expuesta a dicho estrés en comparación con las plantas que se enfrentan a él por primera vez (Trewavas 2003, 2005). En otras palabras, es la habilidad de acceder a la experiencia pasada y de incorporar la información relevante del pasado en las nuevas respuestas (**Figura 2**). Sin embargo, esa mejor respuesta para resistir frente a un estrés en ocasiones puede comprometer la productividad de la planta a corto plazo. Por ejemplo, la disminución de la fotosíntesis puede incrementar la resistencia a un estrés y por tanto la productividad a largo plazo, aunque compromete al mismo tiempo la productividad a corto plazo (Bruce et al. 2007).

Actualmente existe un gran interés en conocer mejor este fenómeno ya que el conocimiento sobre los mecanismos implicados en la memoria y su regulación es aun limitado, y una profundización en este tema nos permitiría entender mejor los sistemas que las plantas tienen para hacer frente a los diferentes estreses (Li & Liu 2016). Además, este fenómeno tiene un gran interés, no sólo

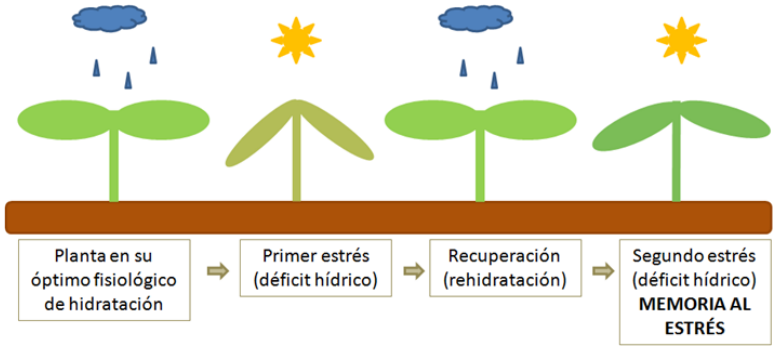


Figura 2. Esquema del proceso de memoria al estrés por déficit hídrico en plantas.

agronómico de cara a "entrenar" a las plantas para futuros estreses e incrementar la resistencia y por tanto la producción, sino también en el ámbito medioambiental para una mejora de la gestión de los recursos hídricos en ecosistemas naturales.

La memoria al estrés en plantas se puede dar a diferentes niveles de organización, los cuales incluyen tres fenómenos bien diferenciados pero a la vez relacionados entre ellos conceptualmente:

- Memoria en semillas, también conocida como *seed priming* en inglés, consiste en mejorar el porcentaje y tiempo medio de germinación de las semillas por una exposición previa a un agente osmótico (*osmopriming*) o una imbibición parcial de las semillas a través de diferentes métodos (*matrix*- o *hydropriming*, Sharma et al. 2014).
- Memoria en plantas, también conocida como *stress imprint* en inglés, hace referencia a una mejora de las respuestas al

estrés como consecuencia de una exposición previa al mismo. Si es un estrés previo diferente se conoce este fenómeno como tolerancia cruzada al estrés (*cross-stress tolerance* en inglés) y si la tolerancia al estrés se consigue a través del pre-tratamiento con un agente químico se conoce como *chemical priming* en inglés (Saavides et al. 2016).

- Memoria transgeneracional, también conocida como *transgenerational stress memory* en inglés, hace referencia al hecho que esta memoria se puede trasmitir de generación en generación, es decir que el estrés que experimenta un individuo se pueda trasmitir a la siguiente generación (Molinier et al. 2006).

3.- Mecanismos de respuesta frente al déficit hídrico reiterado

El proceso de respuesta de la planta frente a cualquier estrés como puede ser el estrés hídrico reiterado comienza con la percepción de éste. Una vez detectado el estrés, las plantas deben ser capaces de procesar esta información y transmitirla para regular una respuesta integrada a nivel de planta entera, desde cambios estructurales a cambios en la expresión génica, en favor de la supervivencia bajo las nuevas condiciones (Tadeo & Gómez-Cadenas 2008). Los mecanismos a través de los cuales las plantas hacen frente al déficit hídrico pueden agruparse en dos grandes estrategias: evitar el es-

trés o tolerarlo. Los mecanismos para evitar el estrés pueden incluir: (a) escapar de él, como los geófitos que tienen órganos subterráneos llenos de agua que les permiten evitar la sequía (como los tubérculos); (b) conservar el agua, lo que le permite a la planta mantener la turgencia celular, y para lograrlo puede servirse de diferentes mecanismos como la presencia de una cutícula gruesa e impermeable, o una gran capacidad de almacenar agua, como sucede en plantas crasas o plantas CAM; y por último (c) ser capaces de absorber más agua, para lo cual disponen de un mayor sistema radicular o algunas especies incluso pelos en la superficie de la hoja, capaces de absorber agua del vapor de agua presente en el aire por ejemplo en casos de niebla o rocío (Chaves et al. 2002; Touchette et al. 2006; Limm et al. 2009). Por otro lado, la estrategia para tolerar el estrés engloba el mantenimiento de la turgencia y la tolerancia a la desecación. El mantenimiento de la turgencia se lleva a cabo mediante un ajuste osmótico, basado en la acumulación de iones en la vacuola y osmolitos como la prolina en el citoplasma pero también ajustando la elasticidad de la pared (Sanders & Arndt 2012; Tenhaken 2014). Por otro lado, los mecanismos para tolerar la desecación o pérdida de agua en la célula incluyen el incremento en los niveles de ácido abscísico (ABA) y la activación de mecanismos de fotoprotección y protección antioxidante, como se describe a continuación.

3.1.- Ácido abscísico

Las hormonas vegetales, también conocidas como fitohormonas,

son moléculas encargadas de regular la fisiología de la planta en la respuesta a estreses ambientales reiterados, desde la regulación de procesos de crecimiento, básicamente regulados por hormonas como las auxinas, las citoquininas y las giberelinas, al cierre de los estomas, el cual está regulado por una de las hormonas del estrés más importantes, el ácido abscísico o ABA.

El ácido abscísico ($C_{15}H_{20}O_4$) se encuentra especialmente implicado en procesos fisiológicos como son el desarrollo embrionario en semillas, la inhibición del crecimiento en procesos de dormición y la respuesta a estreses ambientales como el déficit hídrico (Davies 2010). Su síntesis se produce en los plastidios, aunque las últimas etapas se produzcan en el citosol como puede verse en la **Figura 3**, como consecuencia de la formación de xantoxina (un sesquiterpeno), a partir de los carotenoides, neoxantina y violaxantina (tetraterpenos) como consecuencia de la activación de la 9-*cis*-epoxicarotenoide dioxigenasa, un enzima clave en la biosíntesis de ABA (Zhang et al. 2009).

Frente al estrés por déficit hídrico los niveles de ABA se incrementan tanto en hojas como en raíces con el objetivo de reducir la transpiración e inducir la síntesis de moléculas osmoprotectoras y proteínas que aumentan la resistencia a la desecación (Tseng et al. 2013). El cierre estomático se produce porque el ABA inhibe una bomba H^+-ATPasa en la membrana plasmática de las células de guarda. Esta enzima transfiere protones fuera de la célula, lo que facilita la entrada de K^+ y esto a su vez la entrada de agua. Por lo tanto, su inhibición reduciría la turgencia celular lo

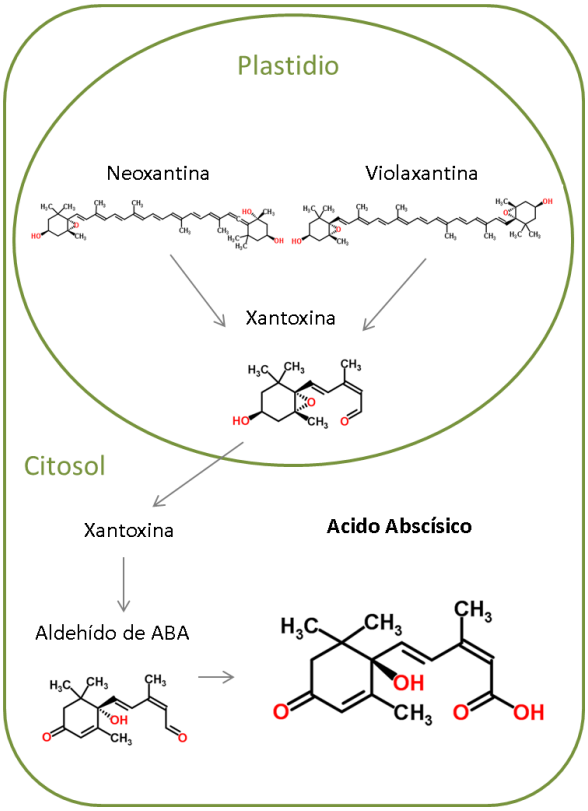


Figura 3. Esquema de biosíntesis de ABA a partir de la neoxantina y la violaxantina. Las estructuras están disponibles de manera gratuita en www.chemspider.com. Chemspider IDs: neoxantina 4444659, violaxantina 395237, xantoxina 4445403, aldehído de ABA 4445405, ABA 558841.

que causaría el cierre estomático (Davies 2004). De hecho, aunque inicialmente se creyó que sin una reducción en el estado hídrico de las hojas podía haber un incremento de ABA que permitiría cerrar los estomas, ya que puede sintetizarse en las raíces y transportarse hasta las hojas por el xilema (Zacarías and Lafuente 2008), se ha descrito recientemente que bajo condiciones de estrés hídrico reiterado la acumulación de ABA en las raíces es fuertemente dependiente de la biosíntesis de ABA en hojas y su transporte desde la parte aérea hacía las raíces (Mazini et al. 2015).

A pesar de la gran importancia que tiene el ABA en la respuesta de las plantas al déficit hídrico, cabe destacar que se han

realizado muy pocos estudios de forma sistemática con el fin de evaluar el papel de esta hormona en la memoria de las plantas al estrés. Goh et al. (2003) mostraron no obstante que el ABA puede tener un papel fundamental en la respuesta de las plantas al estrés reiterado. La exposición repetida al ABA provocó alteraciones en la apertura de los estomas inducida por luz en plantas de *Arabidopsis thaliana*, de tal forma que la exposición repetida a esta hormona parece que provoca una regulación negativa de la respuesta de los estomas, sugiriendo un proceso de memoria.

3.2.- Fotoprotección y protección antioxidante

Como se ha descrito anteriormente en el apartado 1 el estrés por sequía puede muchas veces ir acompañado de un exceso de luz. Este estrés lumínico se da cuando se produce un desequilibrio entre la actividad fotosintética que la planta puede realizar y la energía recibida mediante la luz. Cuando esto sucede y la planta absorbe más energía de la que puede utilizar por limitaciones como puede ser la baja disponibilidad de CO_2, el aparato fotosintético se sobrexcita y la planta debe disipar esa energía para no incrementar la producción de ROS en la cadena de transporte de electrones (Asada 2006).

Las clorofilas de las antenas son capaces de captar la energía de la luz y transmitirla mediante su excitación que va pasando de una molécula a otra. Este aumento en la energía de excitación (excitón) se dirige al centro de reacción de los fotosistemas donde es transformada en energía química (fotoquímica). Sin embargo,

aparte de la fracción de energía que se dirige al centro de reacción del fotosistema (*quenching* fotoquímico), en la antena del fotosistema la energía puede ser disipada mediante la disipación térmica a través del ciclo de las xantofilas (*quenching* no fotoquímico) y la fluorescencia de las clorofilas (**Figura 4**).

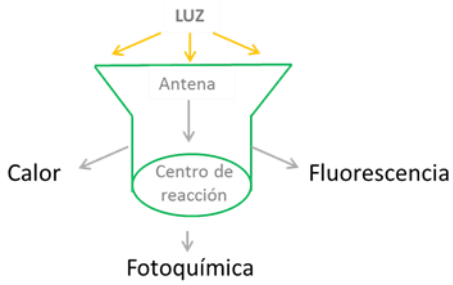


Figura 4. Esquema de distribución de la energía luminosa recibida por los fotosistemas del aparato fotosintético de las plantas.

Las clorofilas del complejo captador de luz o antena son capaces de emitir un 3-4% de la energía absorbida en forma de fluorescencia. La medida de la fluorescencia de las clorofilas permite conocer el funcionamiento del aparato fotosintético y el uso de la energía, ya que cuanta más energía es usada fotoquímicamente o disipada térmicamente menor será la fluorescencia emitida. La diferencia entre la florescencia mínima (F_0), cuando todos los centros de reacción están abiertos (oxidados) y la florescencia máxima (F_m), cuando todos los centros están cerrados (reducidos), es la fluorescencia variable (F_v). El parámetro F_v/F_m se usa como una estimación del máximo rendimiento cuántico o la máxima eficiencia del PSII en plantas adaptadas a la oscuridad y su valor es de 0.75-0.85. La disminución de este parámetro nos indica fotoinhibición (Murata et al. 2007).

En las antenas de los fotosistemas las plantas también son capaces de disipar la energía emitiendo calor. Mientras que a través de la fluorescencia se disipa un 3-4%, mediante la disipación térmica la planta puede disipar más de un 95% de la energía absorbida (Artetxe 2005). Este proceso de disipación térmica está mediado por las xantofilas. Cuando hay un exceso de luz se produce la conversión de violaxantina a anteraxantina, que a su vez se transforma en zeaxantina, ambas reacciones por medio de la enzima violaxantina de-epoxidasa (**Figura 5**). Esta enzima permite la acumulación de zeaxantina, pigmento directamente implicado en la disipación térmica (Jahns & Holzwarth 2012). Para conocer la capacidad que poseen las xantofilas de disipar energía mediante este ciclo, es decir, la cantidad de xantofilas que están disipando calor respecto al total de xantofilas se usa el siguiente cociente, conocido como estado de deepoxidación de las xantofilas (DPS): (Z+0.5A)/

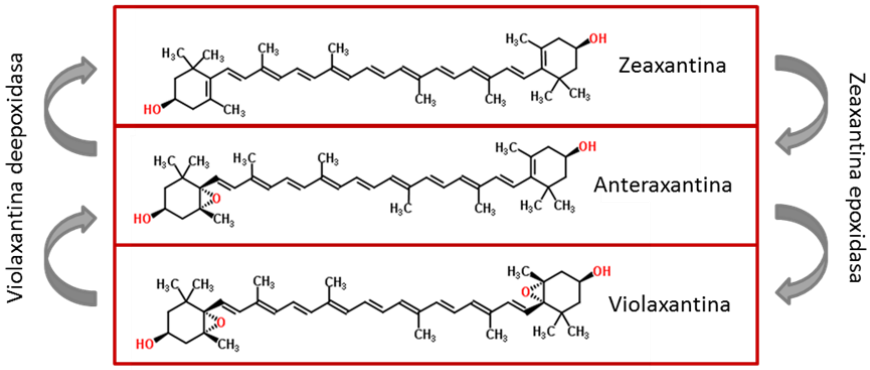


Figura 5. Esquema del ciclo de las xantofilas (zeaxantina, anteraxantina y violaxantina) con las enzimas catalizadoras de las conversiones (violaxantina deepoxidasa y zeaxantin epoxidasa). Las estructuras están disponibles de manera gratuita en www.chemspider.com. Chemspider IDs: zeaxantina 4444421, anteraxantina 4444635, violaxantina 395237.

V+Z+A, donde A es anteraxantina, Z es zeaxantina y V es violaxantina (Demmig-Adams & Adams 1996).

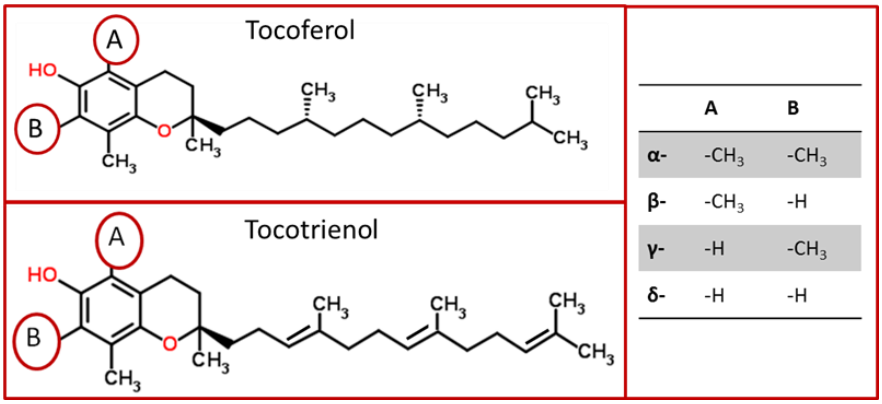
Pero a pesar de los mecanismos que la planta dispone para disipar el exceso de energía y evitar la producción de ROS, es inevitable que en momentos de estrés las ROS produzcan daños en el aparato fotosintético y por tanto se reduzca la actividad fotosintética. A esta reducción en la tasa fotosintética se le llama fotoinhibición, aunque se ha demostrado que se da por la inhibición de la reparación del PSII más que por el daño directo causado por las ROS (Nishiyama et al. 2006). Por ello, la fotoinhibición se produce cuando la tasa de reparación del PSII es menor a la tasa de daño (Takahashi & Badger 2011), concretamente cuando la proteína D_1 del PSII se recupera más lentamente de lo que se daña (Goh et al. 2012).

Aunque las ROS producidas en plantas debido a uno o varios estreses pueden causar daños en las plantas, estas disponen de antioxidantes como tococromanoles o carotenoides que son los antioxidantes lipofílicos más abundantes en cloroplastos (Munné-Bosch & Alegre 2002; DellaPenna & Pogson 2006; Kruk et al. 2014) para hacerles frente. De esta manera, las plantas tratan de mantener el equilibrio entre la producción de ROS y su eliminación por medio de antioxidantes para evitar el daño oxidativo.

Los tococromanoles son un grupo de moléculas anfipáticas sintetizadas solo por organismos fotosintéticos. Tienen una cadena poliprenil y un anillo cromanol que es el que le otorga su gran capacidad antioxidante al ser capaz de donar sus hidrógenos del gru-

po hidroxilo a las ROS. Tocoferoles, tocotrienoles y el plastocromanol-8 (PC-8) contienen esta estructura. Tocoferoles y tocotrienoles son conocidos también con el nombre de vitamina E debido al rol esencial que tienen en la nutrición y salud animal (Falk & Munné-Bosch 2010). Los tocoferoles, al igual que los tocotrienoles, están formados por 4 homólogos (α, β, γ y δ) que difieren en el número y posición de los grupos metilos en el anillo, y a diferencia de los tocotrienoles donde la cadena está 3 veces insaturada, los tocoferoles tienen la cadena completamente saturada (**Figura 6**). El PC-8 en cambio tiene una cadena prenil más larga e insaturada que tocoferoles y tocotrienoles (**Figura 7**), lo que aumenta su capacidad como *quencher* (atenuador o disipador) frente al $^1O_2$ en ambientes hidrofóbicos (Gruszka et al. 2008).

La principal función de los tococromanoles es la de antioxidante pero también se ha descrito que podrían desempeñar otras



	A	B
α-	-CH_3	-CH_3
β-	-CH_3	-H
γ-	-H	-CH_3
δ-	-H	-H

Figura 6. Estructura de tocoferoles y tocotrienoles con la posición de los grupos metilo en el anillo cromanol de α-, β-, γ- y δ-tococromanol. Modificadas a partir de las estructuras obtenidas en www.chemspider.com. Chemspider IDs: tocoferol 14265, tocotrienol 8105532.

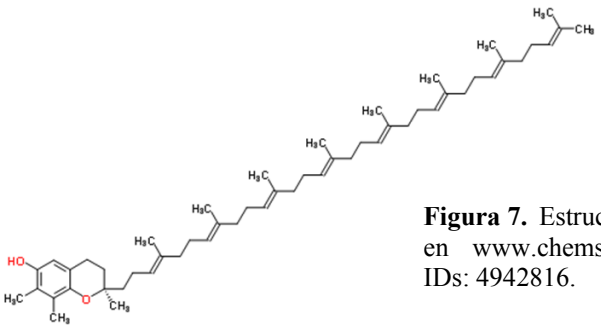


Figura 7. Estructura del PC-8, Obtenida en www.chemspider.com. Chemspider IDs: 4942816.

funciones en diferentes procesos biológicos ya que en plantas deficientes en tocoferol se han observado alteraciones en la germinación, crecimiento, senescencia foliar y transporte de fotoasimilados (Falk & Munné-Bosch 2010). El α-tocoferol es el más abundante en órganos fotosintéticos como las hojas y en la membrana tilacoidal del cloroplasto ayuda a prevenir la propagación de la peroxidación lipídica desactivando el $^1O_2$ (Munné-Bosch 2005).

En estudios previos se han descrito correlaciones entre ABA y la biosíntesis de vitamina E bajo condiciones de déficit hídrico en plantas como *Aptenia cordifolia* (Cela et al. 2009) o *Cistus creticus* (Munné-Bosch et al. 2009). Además en arroz se ha demostrado que genes relacionados con la biosíntesis de tocoferol tienen elementos de respuesta al ABA (ABREs) en su región promotora (Chaudhary & Khurana 2009).

Los carotenoides son pigmentos fotosintéticos que forman parte del aparato fotosintético y que ejercen un papel protector frente al exceso de luz recibida por el fotosistema I y II (fotoprotección), no solo por su función en la disipación del exceso de energía en forma de calor (ciclo de las xantofilas), sino también

por su función antioxidante. Los carotenoides son lípidos isoprenoides formados por 40 átomos de carbono y pueden dividirse en carotenos y xantofilas (**Figura 8**) (De las Rivas 2000). Se han descrito más de 600 carotenoides estructuralmente distintos (Ladygin 2000). Los carotenos no tienen ningún grupo oxigenado. Dentro de este grupo se encuentra el β-caroteno que es capaz de eliminar el $^1O_2$ (Telfer 2005). Las xantofilas sin embargo poseen algún grupo oxigenado y dentro de este grupo se encuentran: luteína, violaxantina, anteraxantina, zeaxantina y neoxantina.

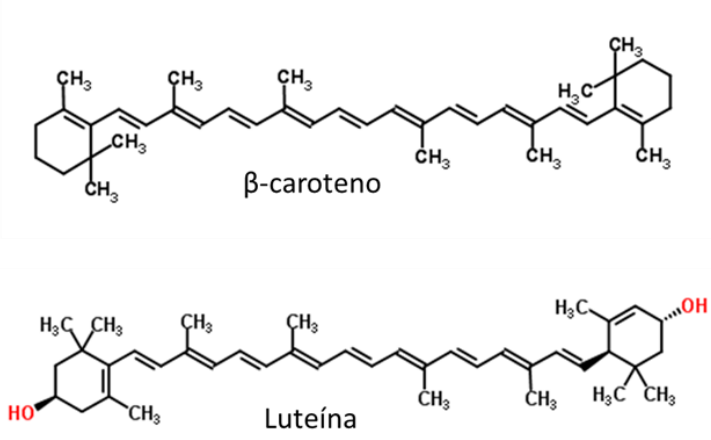


Figura 8. Estructura de ejemplo de carotenos (β-caroteno) y xantofilas (luteína). Obtenidas en www.chemspider.com. Chemspider IDs: β-caroteno 4444129, luteína 4444655.

La luteína por su parte, es capaz de desactivar clorofilas triplete (clorofilas excitadas, ^{3}Chl*) (Jahns & Holzwarth 2012) y la neoxantina es capaz de proteger el PSII eliminando sobretodo el O_2^- (Dall'Osto et al. 2007). La zeaxantina además de estar implica-

da en la disipación térmica también es capaz de eliminar el $^1O_2$ (Havaux et al. 2007).

3.3.- Melatonina

La melatonina (N-acetil-5-metoxitriptamina) es una molécula anfipática de bajo peso molecular presente en organismos evolutivamente distantes, desde bacterias hasta mamíferos. Las plantas son capaces de sintetizar melatonina a partir del aminoácido L-triptófano. La función de la melatonina es actualmente un tema abierto a discusión puesto que no está claro si actúa como regulador del crecimiento y/o como antioxidante. Recientemente, se ha descrito que podría regular ciertos procesos implicados en el desarrollo de raíces, brotes, frutos y el retraso de la senescencia, pero también se ha visto que tiene una gran capacidad antioxidante (Arnao & Hernández 2014; Gao et al. 2016).

Como regulador del crecimiento la melatonina parece tener un comportamiento similar al de las auxinas, ya que regula el desarrollo de raíces y brotes independientemente de la señalización de las auxinas. De hecho, la melatonina está relacionada estructuralmente con el ácido índol-3-acético (**Figura 9)**, la auxina más abundante en plantas, con la que comparte el triptófano como precursor en su ruta biosintética y enzimas implicadas también en su biosíntesis (Pelagio-Flores et al. 2012). También parece estar involucrada en el control del desarrollo de frutos, pues en tratamientos con melatonina en frutas como el melocotón se ha dado un retraso en la senescencia del fruto (Gao et al. 2016). Además produce un retraso

en la senescencia foliar tras tratar a las plantas con melatonina, como se ha descrito en hojas de manzano (*Malus domestica*) a través del incremento en la actividad enzimática para eliminar las ROS y regulando el ciclo del ascorbato-glutatión que se encarga como ya se ha dicho en el apartado 1 de eliminar el peróxido de hidrógeno (Wang et al. 2011).

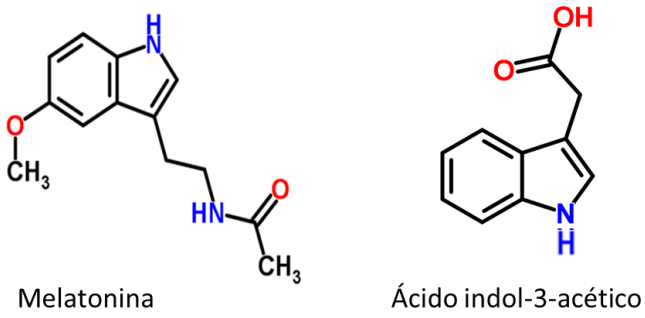


Figura 9. Estructura de la melatonina y el ácido índol-3-acético. Obtenidas en www.chemspider.com. Chemspider IDs: melatonina 872, ácido índole-3-acético 780.

4.- Modelos de estudio

4.1.- *Silene dioica*

La borbonesa, también conocida como carapitera, doble campeón o por su nombre científico *Silene dioica* (de la familia de las Cariofiláceas) es una planta ornamental con metabolismo C3. Es una especie dioica perenne de vida corta que crece frecuentemente en bosques húmedos y en vegetaciones densas (Karrenberg & Favre 2008), y es por tanto sensible al estrés por sequía (**Figura 10**).

Figura 10. Fotografías de plantas de *Silene dioica.* A: plantas usadas en los experimentos en condiciones de estrés por sequía a la izquierda y control bien hidratada a la derecha. B: planta con flor.

4.2.- *Aptenia cordifolia*

El rocío, escarcha o también conocida por su nombre científico como *Aptenia cordifolia* (de la familia de las Aizoáceas) es una planta ornamental muy popular debido, el menos en parte, a su gran resistencia a condiciones ambientales adversas (**Figura 11**). Es una planta con metabolismo CAM (Herppich & Peckmann 1997), considerado como un mecanismo adaptativo que permite a muchas plantas sobrevivir en hábitats secos. En estas plantas el carbono se asimila durante la noche evitando una pérdida excesiva de agua (en comparación con el metabolismo C3) ya que los estomas se abren cuando hay menos evapotranspiración (Herrera 2009). Procede de los desiertos costeros de Sudáfrica y se ha extendido ampliamente debido en parte a su gran popularidad como planta ornamental. Además, es muy competitiva con las especies

autóctonas y una vez establecida puede recuperarse fácilmente de heladas o sequías ya que necesita muy poca agua (Cela et al. 2009; Cela & Munné-Bosch 2012).

Figura 11. Fotografía de una *Aptenia cordifolia* usada en los experimentos.

4.3.- *Zea mays*

El maíz o *Zea mays* es una gramínea anual originaria de América con metabolismo C4 (a diferencia de *Silene dioica* y *Aptenia cordifolia*). Este metabolismo lleva a cabo la fotosíntesis con una separación espacial entre la captación del CO_2 y el uso de este por la Rubisco. Esta separación le permite una mayor concentración de CO_2 en torno a la Rubisco reduciendo así la fotorespiración y au

mentando su eficacia ya que esta enzima cataliza la fijación de CO_2 pero también la fijación del O_2. Actualmente es el segundo cultivo con mayor volumen de producción a nivel mundial (tras el trigo) y el cereal con mayor rendimiento de grano por hectárea (FAO 2017). Dada la importancia económica de este cultivo, y el hecho que la sequía está en aumento disminuyendo la productividad de los cultivos (Lawlor 2002) es muy importante estudiar el déficit hídrico reiterado en esta especie (**Figura 12**), así como los mecanismos implicados en la tolerancia de las plantas al estrés y los mecanismos existentes para contrarrestar los efectos negativos del estrés por sequía a través del uso del *chemical priming*.

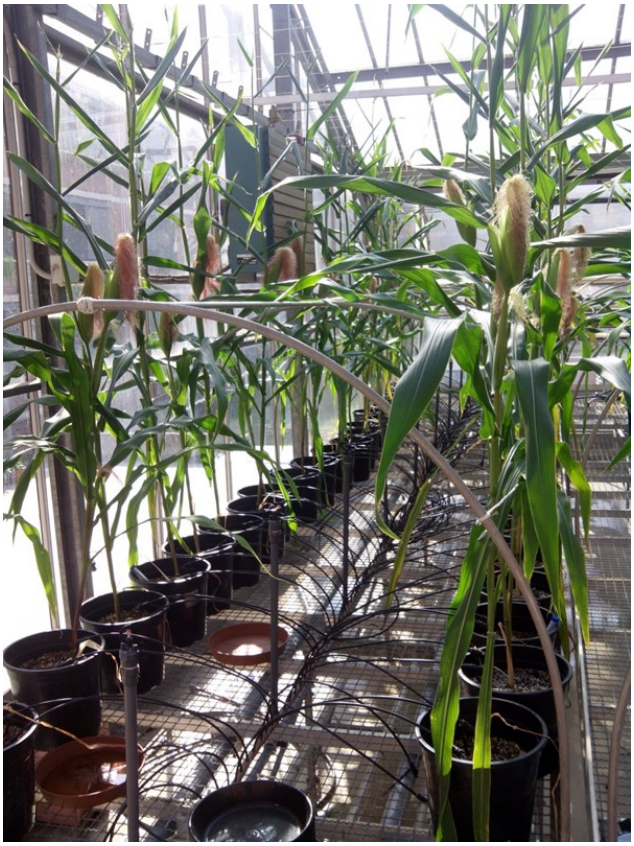


Figura 12. Fotografía de plantas de *Zea mays* usadas en los experimentos.

OBJETIVOS

Comprender mejor los mecanismos de respuesta de las plantas frente al déficit hídrico reiterado, con un énfasis especial en mecanismos que puedan conferir memoria al estrés. Para cumplir este objetivo general se marcaron los siguientes objetivos específicos:

- Estudiar la posible existencia del fenómeno de memoria al estrés por sequía desde una perspectiva fisiológica en plantas de hábitats y tipos de metabolismo muy diferentes

- Determinar si el ABA podría estar implicado en procesos de memoria al estrés en plantas

- Estudiar el impacto del déficit hídrico reiterado en la fotoprotección y acumulación de antioxidantes en plantas

- Examinar el papel de la melatonina en la respuesta de las plantas al déficit hídrico reiterado y su posible aplicación en *chemical priming*

INFORME DEL DIRECTOR DE TESIS

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Barcelona, 3 de abril de 2017

El Dr. Sergi Munné Bosch, como director de la Tesis Doctoral titulada "**Mecanismos de protección frente al déficit hídrico reiterado en plantas**" presentada por la doctoranda Eva Fleta Soriano,

INFORMA sobre el factor de impacto y la participación de la doctoranda en cada uno de los artículos incluidos en la memoria de esta Tesis Doctoral

Capítulo 1. Artículo "**Stress memory and the inevitable effects of drought: A physiological perspective**", publicado en la revista *Frontiers in Plant Science*, índice de impacto (2015) de 4.495. En este trabajo se da una nueva perspectiva a los trabajos actuales de memoria al estrés en plantas recientemente publicados en el campo de la epigenómica. Se destaca la importancia de los cambios estructurales/ morfológicos que experimentan las plantas en respuesta al estrés hídrico reiterado y la importancia de su evaluación como complemento a aproximaciones "ómicas" utilizadas recientemente en otros estudios de memoria al estrés. Cabe destacar el buen manejo de esta tipología de artículos en los que se discuten temas actuales de la especialidad combinando nuevas aproximaciones teóricas con algunos datos experimentales, en este caso obtenidos en la planta ornamental *Silene dioica.* La doctoranda muestra un buen manejo de los conceptos básicos teóricos de la especialidad y ha realizado todo el muestreo, el análisis de las muestras, el tratamiento estadístico y la elaboración de los resultados, además de participar en la idea conceptual del artículo

y su discusión, constando por tanto como primera autora del trabajo. La doctoranda ha demostrado una notable capacidad de trabajo, así como un buen manejo de las herramientas básicas de muestreo y análisis espectrofotométricos en estudios de bioquímica vegetal. La doctoranda demuestra también una notable capacidad de análisis e interpretación de los resultados, y una excelente motivación para el aprendizaje y su formación.

Capítulo 2. Artículo "**Evidence of drought stress memory in the facultative CAM, *Aptenia cordifolia*: Possible role of phytohormones**", publicado en la revista *PLoS ONE*, índice de impacto (2015) de 3.057. En este trabajo se evalúa de forma minuciosa el posible papel de las fitohormonas en el mecanismo de memoria al estrés hídrico en una planta de gran interés ecofisiológico, *Aptenia cordifolia*. El estudio demuestra por primera vez que esta especie, utilizada como modelo de tolerancia al estrés, modula de forma específica los contenidos endógenos de ABA en la respuesta de las plantas al estrés hídrico reiterado. Además, el estudio muestra una estrecha correlación entre los niveles de ABA y tocoferoles (vitamina E) en la respuesta de las plantas al estrés, resultados que apoyan la idea que esta hormona está implicada en la regulación de los contenidos endógenos de vitamina E en hojas. La doctoranda ha realizado todo el muestreo, los análisis de las muestras, el tratamiento estadístico y la elaboración de los resultados, y además ha participado en el diseño experimental y la discusión de los resultados, constando por tanto como primera autora del trabajo. La doctoranda ha demostrado una gran capacidad de trabajo, así como una excelente predisposición a la mejora de los resultados realizados. Además, la doctoranda se introduce en los análisis de fitohormonas por cromatografía líquida acoplada a espectrometría de masas

en tándem (LC-MS/MS), técnica en la que muestra una alta motivación y capacidad de aprendizaje.

Capítulo 3. Artículo "**Drought stress memory in the photosynthetic mechanisms of an invasive CAM species, *Aptenia cordifolia***", publicado en la revista *Photosynthesis Research*, índice de impacto (2015) de 4.122. En este trabajo se describe, entre otros aspectos, la importancia de la modulación de la composición y contenidos de pigmentos fotosintéticos en hojas de *A. cordifolia*. Cabe destacar la aproximación experimental, con un diseño experimental muy ambicioso y la obtención de resultados muy robustos, originales y de un gran valor científico, ya que se demuestra de forma concluyente que las plantas modulan de forma muy específica la composición y los contenidos de los pigmentos antena en los cloroplastos, aspecto ya tratado, aunque no con tanta profundidad, en el Capítulo 1 de esta Tesis Doctoral. La doctoranda ha realizado parte de los muestreos y análisis de las muestras, ha colaborado en el tratamiento estadístico y elaboración de los resultados, y además ha participado en el diseño experimental, constando por tanto como tercera autora del trabajo. La doctoranda demuestra una gran capacidad de trabajo, así como un excelente madurez en la realización de muestreos en equipo. La doctoranda adquiere además mayor experiencia en los análisis de cromatografía líquida de alta resolución (HPLC), específicamente de pigmentos fotosintéticos, así como de moléculas antioxidantes y fotoprotectoras en plantas.

Capítulo 4. Artículo "**Enhanced plastochromanol-8 accumulation during reiterated drought in maize (*Zea mays* L.)**", publicado en la revista *Plant Physiology and Biochemistry*, índice de impacto (2015) de

2.928. En este trabajo se describe la importancia del plastocromanol-8, un compuesto derivado del plastoquinol, como antioxidante en plantas, así como su posible implicación como mecanismo de tolerancia al estrés reiterado en plantas de maíz. En este trabajo, la doctoranda profundiza y adquiere todavía mayor especialización en los análisis por HPLC de antioxidantes de fase lipídica en plantas. La doctoranda ha realizado todo el muestreo, los análisis de las muestras, el tratamiento estadístico y la elaboración de los resultados, y además ha participado en el diseño experimental y la discusión de los resultados, constando por tanto como primera autora del trabajo. La doctoranda demuestra una gran capacidad de trabajo y ha participado también en la redacción del artículo, mostrando una notable madurez científica.

Capítulo 5. Artículo "**Melatonin may exert a protective role against drought stress in maize**", publicado en la revista *Journal of Agronomy and Crop Science*, índice de impacto (2015) de 2.565. En este último trabajo se realiza un análisis del posible efecto protector de la melatonina en la respuesta de las plantas al déficit hídrico reiterado. Este estudio tiene una gran relevancia debido al posible uso de la melatonina en el "chemical priming", y por tanto, en la mejora de la respuesta de las plantas al estrés, en este caso de gran interés agronómico. Como muestra de este interés aplicado cabe destacar que este estudio se ha realizado en el marco de un proyecto de transferencia con la empresa Biovert S.L., en el cual la doctoranda ha tenido un papel fundamental y de gran valor añadido no solo para su Tesis Doctoral sino también para todo el grupo de investigación. Este estudio demuestra el gran potencial de la melatonina como agente químico para su aplicación en la mejora de la respuesta de las plantas al estrés. La doctoranda ha realizado todo los muestreos, los análisis de las muestras, el tratamiento estadístico y la

elaboración de los resultados, y además ha participado en el diseño experimental y en la discusión de los resultados, constando por tanto como primera autora del trabajo. La doctoranda ha demostrado una gran capacidad de trabajo y ha participado también activamente en la redacción del artículo. La doctoranda muestra un excelente grado de madurez.

Y, para que así conste a los efectos oportunos,

Dr. Sergi Munné Bosch

RESULTADOS

CAPÍTULO 1

Memoria al estrés y los efectos inevitables de la sequía: una perspectiva fisiológica

CHAPTER 1

Stress memory and the inevitable effects of drought: A physiological perspective

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RESUMEN DEL CAPÍTULO 1

Las plantas crecen y se desarrollan ajustando su fisiología a los cambios en el ambiente. Los cambios abióticos en el ambiente ocurren a lo largo de los años, las estaciones, los días, pero también a lo largo de minutos o incluso segundos. En este ambiente siempre cambiante, las plantas deben ajustar su estructura y función para optimizar su crecimiento y reproducción. Las respuestas de las plantas frente a déficits hídricos reiterados (por ej. ciclos repetidos de déficit hídrico) difieren respecto a las expuestas a un solo estrés; de hecho, en la naturaleza las plantas están normalmente expuestas a diferentes ciclos de déficit hídrico que varían en intensidad y duración. Actualmente ha incrementado el interés en comprender mejor los mecanismos de las plantas para responder a déficits hídricos reiterados, debido al menos en parte, al descubrimiento de los cambios epigenéticos que desencadenan la memoria al déficit hídrico en plantas. Sin embargo, más allá de los cambios epigenéticos, hay otros aspectos que también deberían ser considerados en el estudio de las respuestas de las plantas a déficits hídricos reiterados: desde los cambios en otros enfoques "ómicos" (trasncriptómica, proteómica y metabolómica), hasta cambios en la estructura de la planta, los cuales pueden ayudarnos a entender mejor la memoria al estrés en plantas y sus mecanismos subyacentes. Aquí presentamos un ejemplo donde el déficit hídrico reiterado afecta a la composición de pigmentos en las hojas de la planta ornamental *Silene dioica* y discutimos la importancia de los cambios estructurales (en este caso del aparato fotosintético) en la

respuesta de la planta a déficits hídricos reiterados; estos cambios representan una memoria al estrés que puede afectar a la respuesta por parte de la planta. Se hace un énfasis especial sobre la importancia de considerar los cambios estructurales, además de los ajustes fisiológicos al nivel de “ómicas”, para entender mejor la memoria al estrés en plantas.

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Stress Memory and the Inevitable Effects of Drought: A Physiological Perspective

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Plants grow and develop by adjusting their physiology to changes in their environment. Changes in the abiotic environment occur over years, seasons, and days, but also over minutes and even seconds. In this ever-changing environment, plants may adjust their structure and function rapidly to optimize growth and reproduction. Plant responses to reiterated drought (i.e., repeated cycles of drought) differ from those to single incidences of drought; in fact, in nature, plants are usually exposed to repeated cycles of drought that differ in duration and intensity. Nowadays, there is increased interest in better understanding mechanisms of plant response to reiterated drought due, at least in part, to the discovery of epigenomic changes that trigger drought stress memory in plants. Beyond epigenomic changes, there are, however, other aspects that should be considered in the study of plant responses to reiterated drought: from changes in other "omics" approaches (transcriptomics, proteomics, and metabolomics), to changes in plant structure; all of which may help us to better understand plant stress memory and its underlying mechanisms. Here, we present an example in which reiterated drought affects the pigment composition of leaves in the ornamental plant *Silene dioica* and discuss the importance of structural changes (in this case in the photosynthetic apparatus) for the plant response to reiterated drought; they represent a stress imprint that can affect plant response to subsequent stress episodes. Emphasis is placed on the importance of considering structural changes, in addition to physiological adjustments at the "omics" level, to understand stress memory in plants better.

Keywords: drought stress, drought tolerance, long-term memory, photosynthesis and the environment, chloroplasts

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INTRODUCTION

The environment is constantly changing, not only over seasons and years, as we currently experience with global warming effects, but also daily and even over a few minutes and sometimes seconds, as occurs with the variations in light intensity at dawn or dusk. Therefore, plants may adjust their metabolism, structure, and function rapidly to optimize growth and reproductive capacity at any given moment. At the same time, the capacity of plants to adjust the mechanisms at work in them to an ever-changing environment determines their capability to respond to future environmental conditions. Diurnal and seasonal cycles in climate conditions force plants to adjust their metabolism; and stress memory allows them to select, at least to some extent, the most appropriate response to certain changes in the environment. Thus, the capacity of plants to adjust

the mechanisms that function within them to an ever-changing environment shapes their future fitness and ultimately makes it possible for plants to live in the great diversity of habitats they have colonized. Plant stress responses can be characterized by an initial alarm phase, in which mechanisms for coping with the stress are activated and growth-related processes slow down. This is generally followed by a resistance phase, in which the plant modulates its structure and function in ways that allow it to withstand the stress and repair any damage already caused. If the stress persists or if it is too severe, the plant dies; if the stress subsides, however, the plant may recover and can reach a new optimal physiological status in the recovery phase. Whatever the future holds for a plant, the first stress episode will leave an imprint on it that will affect its response to subsequent stresses.

It is possible to categorize plant responses to drought stress in accordance with the organizational level of study: from a more molecular perspective, where one can find the "omics" approach, to a more "classical" approach that includes structural changes. However, structural changes also occur at different levels of organization, from the whole plant (e.g., changes in the number of leaves, leaf area, or leaf thickness) to the genetic level (e.g., histone modification); so the two approaches overlap. Within the "omics" approach, we find changes in epigenomics, which affect DNA activity without modifying the gene sequence; transcriptomics, which are changes in gene expression; proteomics, referring to changes in proteins; and finally metabolomics, which are changes in metabolites (Singh et al., 2015). At the structural level, changes in the root/shoot biomass ratio, number of leaves, leaf area, leaf mass per area ratio (LMA), leaf size, and/or structure of the photosynthetic apparatus coupled to chloroplast organization and shape (Pallardy and Kozlowski, 2008; Aroca, 2012) may all also affect plant response to subsequent stresses.

Here, we discuss the importance of both "omics" and structural changes, and present an example in which reiterated drought affects the pigment composition of leaves in the ornamental plant *Silene dioica*. Structural changes that result from the plant response to reiterated drought may be considered important stress imprints that can affect plant response to subsequent stresses and should therefore be carefully considered, in addition to "omics" approaches, in the study of plant responses to reiterated drought or other abiotic stress factors.

DROUGHT STRESS MEMORY IN PLANTS

Of all the environmental stresses, drought is one that has the most negative effects on plant growth and development, and can lead to important losses of productivity capacity (Ciais et al., 2005). The effects of drought stress vary depending of many factors, such as the intensity and duration of the stress, the plant genotype or growth phase, and also the imprint previous stress episodes have left on the plant. This imprint, or stress memory, can be defined as the structural, genetic, and biochemical modifications that have occurred as a consequence of stress exposure and which make the plant more resistant (although it might also be more sensitive in some cases) to future exposure to the same stress factor (if the later stress is different, the term "cross-stress tolerance" is more appropriate). Although the increase in resistance may compromise plant productivity in the short term, for example through a reduction of photosynthesis, it represents increased tolerance to subsequent stress and therefore favors productivity in the long term (Bruce et al., 2007). If the stress is too severe, however, productivity may be negatively affected in both the short and long term.

Despite the fact that the mechanisms underlying the stress imprint or memory are still not fully understood, it has been shown that an accumulation of signaling compounds and transcription factors together with epigenomic modifications may play a major role in them (Bruce et al., 2007; Conrath et al., 2009). For example, it has been reported that abscisic acid (ABA) may be involved in drought stress memory in the short term, such as over days or weeks (Ding et al., 2012; Fleta-Soriano et al., 2015) and also that epigenomic changes play a role in aspects related to meristem functioning (Kaya et al., 2001) and seed development (Wu et al., 2000), which will in turn affect plant development and productivity in the long term.

Among the plethora of responses that plants have evolved to withstand drought stress, photoinhibition of photosynthesis occurs in several plant species and directly affects productivity in the short term. A reduction in the function of the photosynthetic electron transport chain causes an excess of energy in chloroplasts that may, among other consequences, lead to increased production of reactive oxygen species (ROS; Ghosh and Xu, 2014). Photoinhibition of photosynthesis in drought-stressed plants is preceded by an increase in ABA levels that leads to stomatal closure, which prevents dehydration. At the same time, however, ABA promotes the production of protective substances (e.g., osmolytes) and helps maintain membrane structure (Verslues et al., 2006), thereby regulating genes with ABA-response elements (ABREs) in their promoter region (Evers et al., 2010). This raises the following important questions. If the plant recovers from the water deficit, could such responses persist over time and benefit the plant if it is challenged again by a new period of drought? Will double-stressed plants respond differently from single-stressed plants? What methodological approaches can we use to understand the mechanisms underlying drought stress memory?

"OMICS": NEW CHALLENGES

Nowadays, there is increased interest in better understanding the mechanisms involved in plant responses to reiterated drought, in part due to the discovery of epigenomic changes. This discovery, in parallel with the ongoing development of massive gene expression analysis, such as that conducted using microarrays and deep sequencing, has revolutionized the field. Furthermore, proteomics and metabolomics have helped to solve the puzzle by providing important new insights into our understanding of plant responses to drought stress (Ruan and Silva, 2011).

Global warming has led to forecasts of an increase in drought in some areas of the world and, even more importantly, an

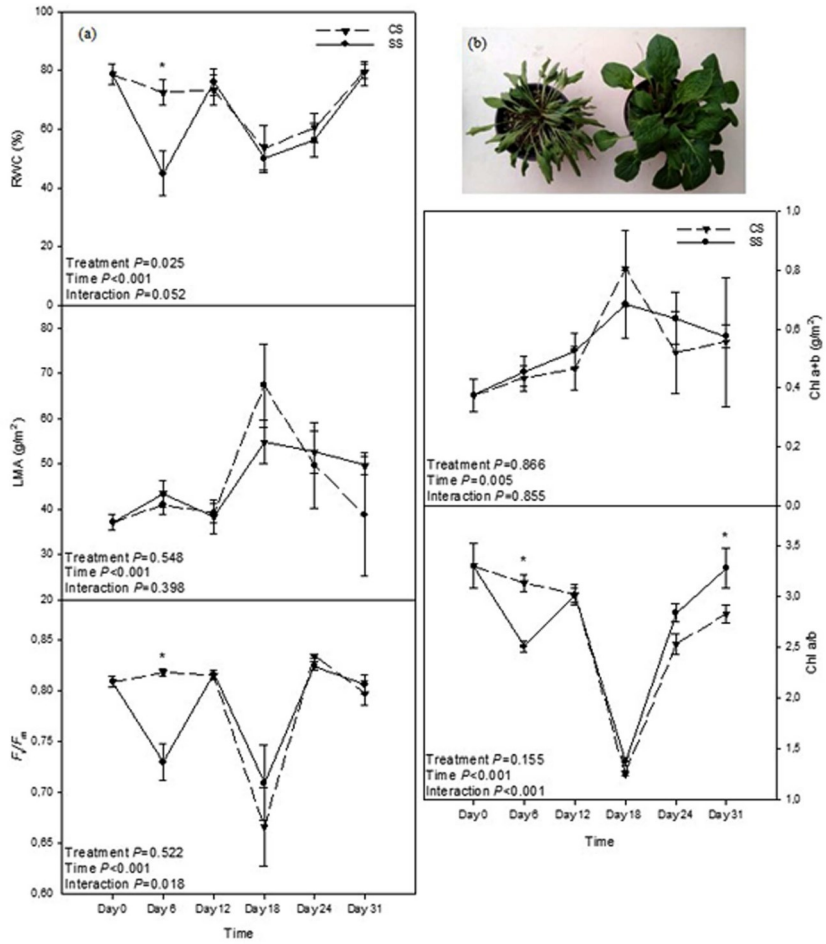

FIGURE 1 | (a) Relative water content (RWC), leaf mass per area ratio (LMA), F_v/F_m ratio, chlorophyll (Chl) a + b and the ratio Chl a/b in leaves of *S. dioica* exposed to reiterated drought (double-stressed – SS – plants) or exposed to water deficit only once (single-stressed – CS – plants). Data represent the mean ± SE of $n = 6$–8 individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA) and Tukey *post hoc* tests ($P < 0.05$). An asterisk indicates significant differences between SS and CS plants. **(b)** Note that water deficit dramatically alters plant structure, showing the inevitable effects of drought. Aside from changes in the composition of the photosynthetic apparatus, severely stressed plants, as those shown here *(left)*, will respond in a different way to controls *(right)* when challenged with a subsequent stress.

increase in the areas potentially exposed to severe drought over the next few decades, due to an increase in temperatures of between 1.4 and 5.8°C by the end of the 21st century (Salinger, 2005). Global warming effects are, however, occurring at the same time as important advances in "omics" technology (epigenomics, transcriptomics, proteomics, and metabolomics). This technology provides us with very useful information that allows us to understand drought stress responses and the mechanisms underlying plant stress memory better. This in turn may lead to improved plant productivity under changing climatic conditions and could balance the possible losses due to the effects of global warming.

Abscisic acid is known to be involved in plant responses to reiterated drought. In some plant species, ABA levels are higher under drought conditions if the plants have previously been challenged by water deficit, that is in double-stressed plants than in single-stressed plants; thus indicating drought stress memory (Fleta-Soriano et al., 2015). Since ABA plays an essential role in plant responses to drought stress, important transcriptional effects can be assumed, since several genes contain ABREs in their promoter regions (Evers et al., 2010). Virlouvet and Fromm (2015) showed that previously stressed plants have stomatal apertures that remain partially closed during a recovery period, which reduces transpiration during subsequent dehydration stress. Interestingly, this response was associated with increased expression of *9-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) and *ALDEHYDE OXIDASE 3* (*AAO3*), which are key modulators of ABA biosynthesis. This is in agreement with a drought memory effect, in which ABA plays a key regulatory role.

Histone modifications and DNA methylation can trigger important changes in gene transcription (Chinnusamy and Zhu, 2009). Alterations in the chromatin structure, such as modifications of the histone H3K4me3 in rice, have been associated with changes in the expression of some genes related to drought stress (Chen et al., 2013). Meanwhile, it has been reported that DNA hypermethylation occurs in salt-stressed *Mesembryanthemum crystallinum* when metabolism shifts from C3 to CAM (Dyachenko et al., 2006) and also in the root tips of pea plants under drought stress (Labra et al., 2002). Therefore, changes in chromatin structure and DNA methylation are currently considered a general response not only to drought stress, but also to other abiotic stresses, conferring both stress memory and cross-stress tolerance (reviewed by Urano et al., 2010; Kim et al., 2015). However, whether or not changes in chromatin structure and DNA methylation are modulated by ABA in drought stress memory is still to be determined. Furthermore, more research is needed to determine the effects these epigenomic changes trigger at the proteomic and metabolomic levels.

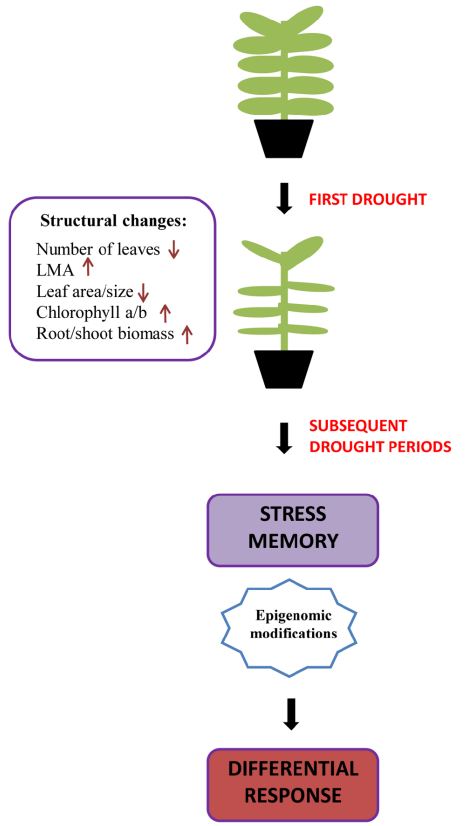

FIGURE 2 | Diagram to illustrate the biological significance of structural changes in plant responses to reiterated drought. Stress memory effects that lead to a differential response to repeated drought periods is not only determined by epigenomic changes, but also by the structural changes caused as a result of the first drought.

STRUCTURAL CHANGES: A MORE "CLASSICAL" PERSPECTIVE?

Beyond the "omics" approaches, there are, however, other aspects that should be considered in the study of plant responses to reiterated drought. These include structural changes, which in turn are the result of changes in "omics" during previous stress exposure and will severely affect the "omics" and overall physiological response during subsequent stress episodes. By exposing *Silene dioica* plants to reiterated drought in a greenhouse (including two cycles of 6 days of water deficit by withholding water, followed by subsequent periods of six days of recovery), it was found that, despite the relative water content (RWC), LMA, maximum efficiency of the photosystem II (F_v/F_m ratio) and the total amount of chlorophylls (Chl a + b) not differing between double-stressed and single-stressed plants (SS and CS, respectively), the Chl a/b ratio was higher in SS plants than in CS plants at the end of the experiment (**Figure 1**). It is interesting to note that changes in the Chl a/b ratio were only observed after recovery; this suggests a change in the structure of the photosynthetic apparatus, since it has been reported that there is a reduction in the size of the light harvesting complex of the photosystem II (LHCII) under excess light (Čajánek et al., 1999; Kurasová et al., 2000, 2002). This change in the pigment composition of leaves is therefore indicative of a reduction of the pigment antenna size in double-stressed plants, which might help plants to reduce ROS production and photo-oxidative stress in chloroplasts, if they are challenged by a new stress in the future.

These results are just an example of structural changes, in this case in the photosynthetic apparatus, as a stress imprint that prepares the plant to respond better to a subsequent period of drought. There are, however, other structural changes that have been reported to occur in plant responses to drought stress in various species. These include a decrease in the leaf area and size, and reductions in the shoot/root ratio in *Quercus ilex* (Peña-Rojas et al., 2004, 2005); a decrease in the number of leaves in *Saccharum* sp. (Zhang et al., 2014); a change in the distribution of roots moving toward the lower layers of the soil in search for water in oaks (Kuster et al., 2012); and even changes in the chloroplast structure and position within the cell in sugarcane (Zhang et al., 2014). Although it has still to be determined to what extent these structural changes contribute to drought stress memory, we propose a model in which structural changes may constitute a stress imprint with significant effects on the plant response to reiterated drought (**Figure 2**). To what extent some changes occur or not will undoubtedly depend not only on the species, but also on the duration and severity of the stress to which the plants are exposed. For instance, Walter et al. (2010) showed that severe drought in grasses not only resulted in biomass loss, but also in reductions in photosynthesis and photoinhibition of the photosynthetic apparatus when plants were challenged by a second drought. Therefore, severe stress in double-stressed plants may result in negative effects; but it is well known that acclimation to small periods of water deficit and/or some water shortage can help improve water use efficiency in ornamental plants and this constitutes a general practice in horticulture (Davies et al., 1992). A first exposure to drought will have inevitable effects on plant structure and function. However, if the stress is not too severe and the plant can recover, it may then respond better to subsequent stresses by showing not only epigenomic changes but also by deploying a different physiological response related to the new adapted structure. This may involve overall reduced transpiration at the whole-plant level, due to reduced size, or changes in photosynthesis and photoprotection, due to an altered pigment composition of the leaves, among a plethora of other possible effects resulting from the first drought. Therefore, the inevitable effects of the first drought can serve to improve the physiological response to reiterated drought.

CONCLUSION

Drought is one of the abiotic stresses that most severely affects plant growth and development; consequently, plants rapidly adjust their structure, metabolism and function to withstand it. Nowadays, "omics" approaches, such as epigenomics, transcriptomics, metabolomics, and transcriptomics, provide us with a unique opportunity to solve the complex but at the same time fascinating puzzle of plant responses to drought stress. Combining such approaches with the study of structural changes at various levels of organization (from histone modifications to changes at the whole-plant level) will undoubtedly contribute to our understanding of the mechanisms underlying drought stress memory. An integrated approach is therefore encouraged in future studies of plant responses to reiterated drought to help us understand general water management practices in plant production.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: EF-S and SM-B. Performed the experiments: EF-S. Analyzed the data: EF-S and SM-B. Contributed reagents/materials/analysis tools: SM-B. Wrote the paper: EF-S and SM-B.

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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.

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CAPÍTULO 2

Evidencia de memoria al déficit hídrico en la CAM facultativa, *Aptenia cordifolia*: posible papel de las fitohormonas

CHAPTER 2

Evidence of drought stress memory in the facultative CAM, *Aptenia cordifolia*: Possible role of phytohormones

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RESUMEN DEL CAPÍTULO 2

Aunque las respuestas de las plantas al estrés por sequía han sido estudiadas con detalle en algunas especies, incluidas las plantas CAM, la memoria al estrés y sus posibles mecanismos de regulación aún están poco comprendidos. En un intento de entender mejor este hecho y sus posibles mecanismos de regulación en plantas, medimos la concentración de fitohormonas en *Aptenia cordifolia* expuesta a reiteradas sequías, junto con varios indicadores de estrés, incluidos el contenido hídrico foliar, fotosíntesis y mecanismos de fotoprotección y protección antioxidante. Los resultados muestran que las plantas expuestas al déficit hídrico responden de manera diferente si se han enfrentado previamente a un primer estrés. Tras la primera exposición al estrés los niveles de giberelinas descienden y se mantienen más bajos en las plantas doblemente estresadas que en las expuestas al estrés por primera vez. Por el contrario, los niveles de ácido abscísico fueron mayores en las doblemente estresadas que las expuestas a un solo estrés. Paralelamente se dan alteraciones en los niveles de hidroperóxidos pero no en los de malondialdehido, lo que sugiere un incremento en el estado de oxidación que no acaba en daño oxidativo en las plantas doblemente estresadas. Se concluye que (i) en las plantas de *A. cordifolia* se da memoria al estrés, (ii) tanto giberelinas como ácido abscísico pueden jugar un papel en la respuesta a déficits hídricos reiterados, y (iii) los cambios en los niveles de ácido abscísico en las plantas doblemente estresadas pueden tener un efecto positivo ajustando el estado redox celular con un papel en la señaliza-

ción, más que causando daño oxidativo a la célula.

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RESEARCH ARTICLE

Evidence of Drought Stress Memory in the Facultative CAM, *Aptenia cordifolia*: Possible Role of Phytohormones

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Abstract

Although plant responses to drought stress have been studied in detail in several plant species, including CAM plants, the occurrence of stress memory and possible mechanisms for its regulation are still very poorly understood. In an attempt to better understand the occurrence and possible mechanisms of regulation of stress memory in plants, we measured the concentrations of phytohormones in *Aptenia cordifolia* exposed to reiterated drought, together with various stress indicators, including leaf water contents, photosynthesis and mechanisms of photo- and antioxidant protection. Results showed that plants exposed to drought stress responded differently if previously challenged with a first drought. Gibberellin levels decreased upon exposure to the first drought and remained lower in double-stressed plants compared with those exposed to stress for the first time. In contrast, abscisic acid levels were higher in double- than single-stressed plants. This occurred in parallel with alterations in hydroperoxide levels, but not with malondialdehyde levels, thus suggesting an increased oxidation state that did not result in oxidative damage in double-stressed plants. It is concluded that (i) drought stress memory occurs in double-stressed *A. cordifolia* plants, (ii) both gibberellins and abscisic acid may play a role in plant response to repeated periods of drought, and (iii) changes in abscisic acid levels in double-stressed plants may have a positive effect by modulating changes in the cellular redox state with a role in signalling, rather than cause oxidative damage to the cell.

Introduction

Stress memory in plants, also known as stress imprint or priming, is considered to be an important component of the behavioral ecology of plants and it is becoming an increasingly important part of plant stress physiology textbooks nowadays. Stress memory is defined as the capacity of organisms to respond better to a given stress factor when individuals have already been challenged previously with the same stimulus relative to those that have not been exposed to the stress before [1]. Indeed, there is an increasing interest in stress memory effects, since

this feature has important implications in plant stress physiology [2]. Unfortunately, the occurrence of stress memory, either leading to positive or negative effects in plant stress responses, and the mechanisms underlying stress memory are still very poorly understood [2]. Therefore, a better knowledge on stress memory effects is urgently needed not only to better understand the physiology and ecology of plants, but also to improve crop production and environmental management practices.

Mechanisms of stress memory may largely vary depending on the organizational level to what the studies are carried out, from changes in leaf anatomy to epigenomics, including phenological, biochemical and physiological mechanisms that may operate in an integrated way to fulfill a role in plant stress tolerance. Among these mechanisms, it appears that phytohormones may have a prominent role. Gibberellins (GAs) have long been known to be involved in vernalization, which implies epigenetic changes and long-term memory effects [3]. On the other hand, recent studies suggest that abscisic acid (ABA) may be involved in short-term drought stress acclimation in the model plant, *Arabidopsis thaliana*. It has been shown that *A. thaliana* increase the transcription of several ABA-induced genes in response to reiterated dehydration, while maintaining leaf water contents [4,5]. Guard cells appear to have a dehydration stress memory so that plants produce ABA to keep partially closed stomata in order to reduce water loss under reiterated water deficit conditions [6].

Crassulacean acid metabolism (CAM) is an adaptive mechanism to survive in extreme habitats characterized by severe drought in which the carbon dioxide is assimilated during the night avoiding an excessive water loss [7]. In fact, CAM plants are able to keep a minimal metabolically active state for a long time during severe droughts, while they are able to recover quickly during re-watering [8]. Therefore, drought stress memory in CAM plants, which are specialized in drought stress tolerance, may have a tremendous biological significance. While drought stress responses have been extensively studied in CAM plants [9–11], the occurrence of stress memory in CAM plants and the possible mechanisms for its regulation are still very poorly understood.

Here, we hypothesized that the CAM plant, *Aptenia cordifolia* may show a drought stress memory in plant response to drought stress, in which phytohormones could play a role. Since *A. cordifolia* is an invasive species, this capacity could help displace other species less resistant to drought stress and colonize new habitats. Specifically, in the present study, we examined whether or not *A. cordifolia* show any stress memory to reiterated drought. With this aim, we measured the endogenous levels of phytohormones, together with various markers of physiological stress, in double- compared with single-stressed plants.

Materials and Methods

Plant material, treatments and sampling

Sixty plants of baby sun rose (*Aptenia cordifolia* (L.f.) Schwantes) were purchased in a local garden (Ca L'Agustí, Barcelona, Spain) and were transferred to 0.5 L-pots with peat:perlite:vermiculite (2:1:1, v/v). Plants were grown in a greenhouse at the Faculty of Biology of the University of Barcelona (Barcelona, Spain). Prior to experiments, plants were watered 3 times per week with half-diluted Hoagland nutrient solution. Experiments, which started on 11th June 2014, consisted in developing two water regimes on plants: CS plants were watered for 13 days, exposed to drought by withholding water for 10 days, and then re-watered for 4 days; while SS plants were stressed by withholding water for 9 days, recovered for 4 days and then exposed again to drought for 10 days, followed by a final recovery of 4 days. Therefore, SS were double-stressed, while CS plants were stressed during a single period. Samplings were performed at the beginning of the experiment (day 0) and after 9, 13, 23 and 27 days of treatments, that is at the

points of maximum stress and during recovery. All measurements were performed at midday (between 11 and 13h solar time). At each sampling point, fully-expanded young leaves of 7 individuals were used to estimate the endogenous contents of phytohormones, together with various physiological indicators, including leaf water contents and gas exchange, chlorophyll fluorescence, levels of photosynthetic pigments and antioxidants, and the extent of lipid peroxidation in leaves. Samples for phytohormone and other biochemical analyses were collected, immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Hormonal profiling

The extraction and analysis of GAs, including the bioactive GA_1 and GA_4, and their precursors GA_9, GA_{19}, GA_{20} and GA_{24}, the bioactive auxin, indole-3-acetic acid (IAA), the cytokinins, zeatin (Z), zeatin riboside (ZR), 2-isopentenyl adenine (2iP) and isopentenyl adenosine (iPA), and the stress-related phytohormones, ABA, jasmonic acid (JA) and salicylic acid (SA) were carried out by UPLC-MS/MS as described [12]. Deuterium-labelled phytohormones were used as internal standards.

Leaf water contents, gas exchange and chlorophyll fluorescence

Samples were weighed to estimate the fresh matter (FW), immersed in distilled water at 4°C for 24h to estimate the turgid matter (TW) and then oven-dried at 80°C to constant weight to estimate the dry matter (DW). Relative water content (RWC) was then calculated as 100x (FW-DW)/(TW-DW). Net photosynthesis (A), stomatal conductance (g_s) and the maximum efficiency of photosystem II phptochemistry (F_v/F_m) were estimated by using a portable infrared gas analyzer with a leaf chamber fluorometer (LI-COR 6400 system, LI-COR, Lincoln, NE, USA). Light intensity was set at 700 μmol quanta m^{-2} s^{-1} with a 10% of blue light; CO_2 concentration at 400 ppm; leaf temperature at 20–25°C, and relative humidity ranging 50–60% with a flow of 500 μmol s^{-1}. The F_v/F_m ratio, which was measured after adapting the leaves to darkness for 2 h, was calculated as $(F_m\text{-}F_0)/F_m$, where F_m and F_0 are the maximum and basal fluorescence yields, respectively, of dark-adapted leaves [13].

Photosynthetic pigments and antioxidants

For pigment and tocopherol analysis, leaf samples (50 mg) were ground in liquid nitrogen and extracted with cold methanol (v/v) using ultrasonication. After centrifuging at 8000 rpm for 10 min and 4°C, the supernatant was collected and the pellet was re-extracted with the same solvent until it was colourless. Then, supernatants were pooled and filtered through a 0.5 μm syringe filter. Total chlorophylls and carotenoids were estimated spectrophotometrically as described [14]. Levels of neoxanthin and violaxanthin, ABA precursors, were measured by high performance liquid chromatography (HPLC) as described [15]. Tocopherols were measured by HPLC as described [16].

Estimation of lipid peroxidation

The extent of lipid peroxidation was estimated by measuring the levels of lipid hydroperoxides (primary stable products of lipid peroxidation) and malondialdehyde (MDA) equivalents (secondary products of lipid peroxidation) in leaves. Lipid hydroperoxides levels were estimated spectrophotometrically following the ferrous oxidation-xylenol orange assay as described [17]. MDA levels were estimated spectrophotometrically following the thiobarbituric acid-reactive assay considering the effect of potential interfering compounds, as described [18].

Statistical analysis

In the first set of results, which included a time-course evolution of water contents, leaf gas exchange and phytohormone levels, data was analyzed by using two-way factorial analysis of variance (ANOVA) with treatment and time (sampling day) as factors, and by additionally using Duncan posthoc tests. In the second set of results, in which differences in photosynthetic pigments, antioxidants and the extent of lipid peroxidation in a single time point were analyzed, Student´s t-tests were used. In all cases, differences were considered significant at a probability level of $P<0.05$. All statistical tests were carried out using the SPSS 15.0 statistical package (SPSS, Inc., Chicago, IL, USA).

Results and Discussion

Drought-stressed *A. cordifolia* plants induce CAM metabolism

A number of CAM plants are extremely resistant to drought, as they are commonly adapted to deserts and other arid or semi-arid environments [19]. Aside from stomatal closure during the day, which largely increases water use efficiency, CAM plants adopt a series of strategies to resist water deprivation during long periods, including, among others, specific hormonal responses and activation of mechanisms of photo- and antioxidant protection [9]. *A. cordifolia* was first described as a facultative CAM species [20], and later thought to be an obligate CAM [21]. In the present study, however, it is shown that it is a facultative CAM plant that opens stomata at midday when water is available and closing them completely when water is withheld (Fig 1). After 9 days of withholding water, the RWC decreased from 68% to 40% at midday. This was associated with a sharp reduction in CO_2 assimilation and stomatal conductance rates, which reached values of and close to zero, respectively (Fig 1). The stress caused to plants, as indicated by RWC values, was quite severe. When challenged with a new drought, plants reduced again the RWC and gas exchange values to a similar extent. Double-stressed plants did not respond differently as those stressed for the first time in terms of leaf water contents and gas exchange (Fig 1), thus indicating that a second stress did impact neither positively nor negatively on these parameters.

GAs and ABA levels reveal stress memory

Among the analysed phytohormones, the bioactive GA_4 was the one showing the most relevant results (Figs 2 and 3, Table 1). GA_4 levels decreased during drought and did not recover, so that double-stressed plants showed slightly, but consistently lower GA_4 levels throughout the experiment compared with plants challenged by drought for the first time (Fig 2). While GA_9 levels were not affected by drought stress, the endogenous concentrations of GA_{24} increased during the first drought, to decrease later during the second drought in double-stressed plants (Fig 2). Since GA_{24} is a precursor of GA_4 [22], it is likely that conversion to bioactive GAs is reduced during the first drought, thus leading to an accumulation of GA precursors. When challenged again with a second stress, however, it seems that this effect disappears, so that GA precursors do not accumulate (despite bioactive GA levels were kept at low levels). Although still to be confirmed using enzymatic assays and molecular tools, these results suggest a memory effect on GA metabolism in plant response to reiterated drought, in analogy to the regulation of GA metabolism by vernalization [23]. It is noteworthy that levels of GAs from the GA_4 pathway were affected by reiterated stress (Figs 2 and 3), and that neither auxin, cytokinins, salicylic acid nor jasmonic acid levels were affected by repeated periods of drought (Table 1).

ABA showed differences in double-stressed plants compared with plants challenged with drought for the first time (Fig 4). ABA levels increased 40-fold in response to the first drought

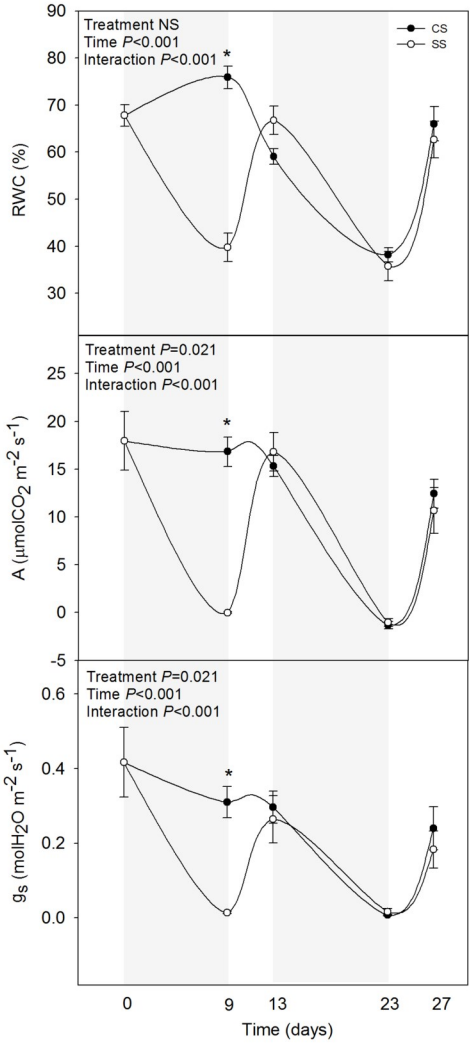

Fig 1. Relative water content (RWC), CO_2 assimilation (A) and stomatal conductance (g_s) in leaves of *A. cordifolia*. Data represent the mean ± SE of $n = 7$ individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA) and Duncan posthoc tests.

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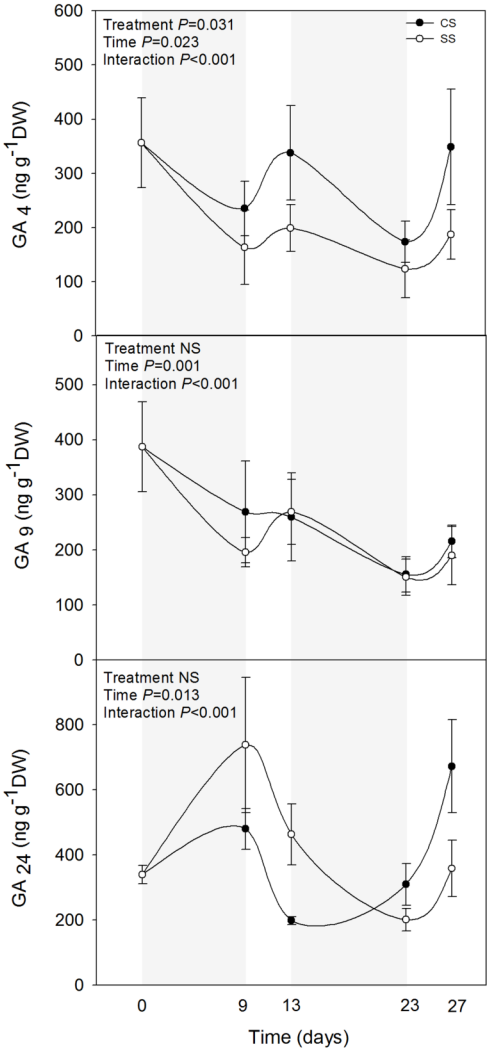

Fig 2. Endogenous concentrations of gibberellin 4 (GA_4), and its precursors, gibberellin 9 (GA_9) and gibberellin 24 (GA_{24}) in leaves of *A. cordifolia*. Data represent the mean ± SE of $n = 7$ individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA) and Duncan posthoc tests.

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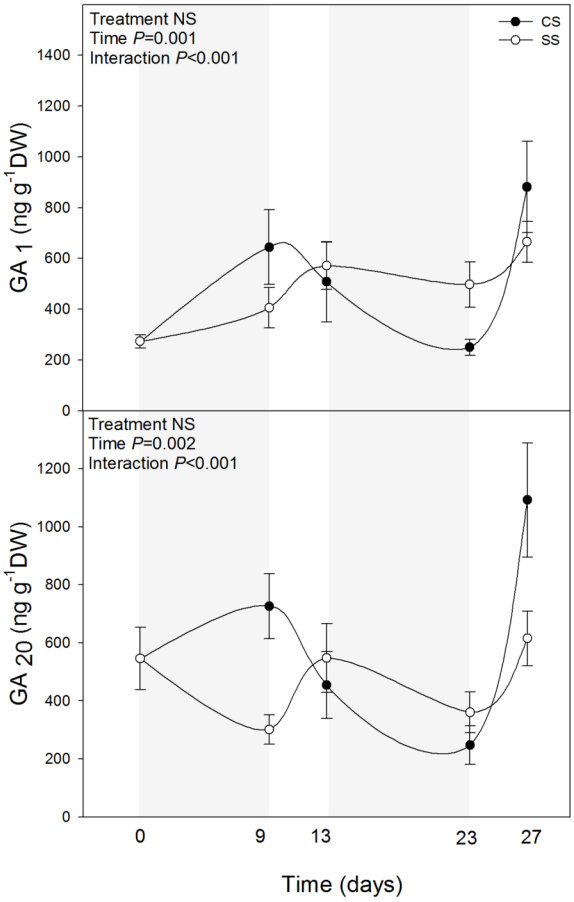

Fig 3. Endogenous concentrations of gibberellin 1 (GA_1), and its precursor, gibberellin 20 (GA_{20}) in leaves of *A. cordifolia*. Data represent the mean ± SE of $n = 7$ individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA) and Duncan posthoc tests.

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to recover later to initial pre-drought values. During the second period of stress, however, ABA levels increased more in double-stressed plants compared with plants challenged by drought for the first time (ABA values reaching 4.2 *vs*. 3.4 μg/g DW, respectively, Fig 4). Despite double-stressed plants did not respond differently in terms of leaf water contents and gas exchange (Fig 1), they did so in terms of ABA accumulation, enhancing the endogenous levels of this phytohormone during the second stress compared with plants challenged by drought for the first time. Increases of ABA levels under reiterated stress may have an effect of growth regulation [24], osmotic adjustment [25] and antioxidant responses [26–28]. Furthermore, previous

Table 1. *P* values of the analysis of variance (ANOVA) to test the effect of treatment, sampling time and its interaction on the levels of phytohormones in leaves of *A. cordifolia*.

Hormone	Treatment	Time	Interaction
IAA	NS	0.001	0.003
iPA	NS	0.001	NS
2iP	NS	0.001	0.008
Z	NS	0.041	NS
ZR	NS	0.001	0.001
SA	NS	NS	0.001
JA	NS	0.042	0.001

IAA, indole-3-acetic acid; iPA, isopentenyl adenosine; 2iP, isopentenyl adenine; Z, zeatin; ZR, zeatin riboside; SA, salicylic acid; JA, jasmonic acid. NS, not significant (*P*>0.050).

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studies have shown that ABA exerts a protective role under reiterated drought by reprogramming gene expression [4–6]. In the present study, plants did not suffer photo-inhibitory damage to the photosynthetic apparatus, as indicated by constant F_v/F_m values (Table 2), and plants were able to fully recover after 4 days of re-watering (Fig 1), thus indicating that the observed changes in the endogenous levels of phytohormones may indeed have a positive effect on the physiology of double-stressed plants.

ABA could exert a drought memory effect by modulating antioxidant responses. By comparing various photo-oxidative stress and lipid peroxidation markers in double- *vs*. single-stressed plants, it was found that reiterated stress had a significant impact on the chlorophyll a/

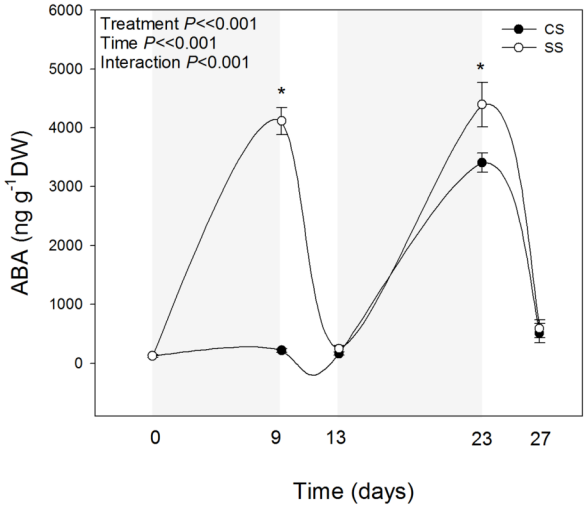

Fig 4. Endogenous concentrations of abscisic acid (ABA) in leaves of *A. cordifolia*. Data represent the mean ± SE of *n* = 7 individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA) and Duncan posthoc tests.

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Table 2. F_v/F_m ratio, photosynthetic pigment and antioxidant levels, and extent of lipid peroxidation in double-stressed plants (SS) compared with plants exposed to drought for the first time (CS). Data represent the mean ± SE of $n = 7$.

Parameter	CS	SS
F_v/F_m	0.68±0.02	0.68±0.01
Chlorophyll *a*+*b* (mg/gDW)	11.4±1.0	10.6±0.3
Chlorophyll *a*/*b* (g/g)	1.98±0.01	2.04±0.01*
Carotenoids/ Chlorophyll *a*+*b* (mg/g)	206±2	207±2
Neoxanthin/Chlorophyll *a*+*b* (mg/g)	12.5±1.4	12.0±1.6
Violaxanthin/Chlorophyll *a*+*b* (mg/g)	27.3±0.2	26.3±0.4*
α-Tocopherol/Chlorophyll *a*+*b* (mg/g)	21.9±3.5	23.8±2.0
γ-Tocopherol/ Chlorophyll *a*+*b* (mg/g)	7.3±1.1	6.5±0.5
Ascorbate (μmol/gDW)	11.41±0.67	10.3±0.58
Dehydroascorbate/Total ascorbate (%)	12.43±1.95	11.3±1.95
Lipid hydroperoxides (μmol equiv. H_2O_2/gDW)	3.9±0.4	7.4±0.8*
MDA (nmol equiv./gDW)	9.6±2.7	13.6±2.9

* Significant differences between treatments (Student's *t*-test, P<0.050)

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b ratio and the extent of lipid peroxidation in leaves (Table 2). The chlorophyll a/b ratio increased, however, by 3% only in double- compared with single-stressed plants. In contrast, the extent of lipid peroxidation increased by 90% in double- *vs*. single-stressed plants, as indicated by lipid hydroperoxide levels, respectively. In contrast, malondialdehyde levels were not affected by reiterated drought (Table 2). Lipid hydroperoxide and malondialdehyde levels are the primary and secondary stable products of lipid peroxidation, respectively [29]. Therefore, results obtained suggest that double-stressed plants experienced an increased oxidative stress compared with plants challenged by drought for the first time. However, neither

Table 3. Correlation coefficients and *P* values (in parentheses) of the Spearman rank's correlations between the endogenous concentrations of all phytohormones analyzed and the levels of α- and γ-tocopherol in *A. cordifolia* leaves. Significant correlations are indicated in bold (Bonferroni corrected, *P*<0.004).

Hormone	α-Tocopherol	γ-Tocopherol
GA_1	-0.010 (0.470)	-0.177 (0.107)
GA_4	-0.286 (0.012)	-0.316 (0.011)
GA_9	0.001 (0.497)	-0.160 (0.111)
GA_{20}	0.141 (0.138)	-0.066 (0.32)
GA_{24}	0.157 (0.112)	-0.128 (0.191)
IAA	0.057 (0.331)	-0.217 (0.045)
Z	0.048 (0.357)	-0.262 (0.020)
ZR	0.046 (0.362)	0.072 (0.290)
2iP	0.279 (0.015)	0.285 (0.012)
IPA	-0.223 (0.042)	-0.272 (0.018)
ABA	0.331 (0.005)	**0.552 (<0.001)**
JA	-0.125 (0.167)	-0.218 (0.044)
SA	-0.224 (0.040)	**-0.374 (0.001)**

GAs, gibberellins; IAA, indole-3-acetic acid; Z, zeatin; ZR, zeatin riboside; 2iP, isopentenyl adenine; iPA, isopentenyl adenosine; SA, salicylic acid; JA, jasmonic acid.

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malondialdehyde levels nor the F_v/F_m ratio were affected by reiterated drought, thus indicating absence of increased photo-oxidative damage in double- *vs.* single-stressed plants [30]. Results obtained appear therefore to be consistent with a hormonal and redox-related gene reprogramming, an aspect that warrants further research. In this respect, it has been previously shown that ABA may trigger activation of antioxidant defences, including vitamin E biosynthesis [31,32]. Despite neither α- nor γ-tocopherol increased in double- *vs.* single-stressed plants (Table 2), a correlative analysis revealed that ABA concentrations positively correlated with γ-tocopherol levels (Table 3). A relationship between ABA and vitamin E biosynthesis is also supported by previous correlative studies in the same species [9] and by studies on model plants, showing that tocopherol-biosynthesis genes have ABA-response elements in their promoter region [33]. Furthermore, analysis of ABA precursors, the carotenoids neoxanthin and violaxanthin, revealed a reduction by 3.7% in violaxanthin levels in double- compared with single-stressed plants (Table 2). Although this reduction appears to be small, it should be considered that it is in the order of 1 mg/g chlorophyll (equivalent to 1.5 mmol/mol chlorophyll), which corresponds to 350 nmol/g DW, while the observed enhanced ABA levels in double- compared with single-stressed plants were in the order of 0.8 μg/g DW (Fig 4), which corresponds to 3 nmol/g DW. Therefore, a 3.7% reduction in violaxanthin levels was more than sufficiently enough to account for the 24% increase of ABA levels in double- compared with single-stressed plants. Further research is however needed to confirm a memory effect on ABA metabolism by directly measuring gene expression, amounts and/or activity of 9-*cis*-epoxycarotenoid dioxygenases, the committed step in ABA biosynthesis from carotenoids [34]. Moreover, it is still to be demonstrated whether or not ABA catabolism is additionally affected in double- compared with single-stressed plants.

Conclusions

Phytohormones may be involved in plant response to reiterated drought stress in the invasive CAM plant, *A. cordifolia*. GA levels decreased upon exposure to the first drought and remained lower in double- compared with single-stressed plants. In contrast, ABA levels were higher in double- than in single-stressed plants. This occurred in parallel with alterations in primary products of lipid peroxidation, but not with changes in malondialdehyde levels and the F_v/F_m ratio, thus suggesting an increased oxidative stress that did not result in photo-oxidative damage in double-stressed plants. It is concluded that (i) drought stress memory occurs in double-stressed *A. cordifolia* plants, (ii) both GAs and ABA may play a role in plant response to repeated periods of drought, and (iii) changes in ABA levels in double-stressed plants may have a positive effect by modulating changes in the cellular redox state with a role in signalling, rather than cause oxidative damage to the cell. In other words, changes in ABA levels in double-stressed plants may be associated with an increased oxidative stress that did not result in photo-oxidative damage. Further research is needed to better understand the GA-, ABA- and redox-mediated effects in plant stress acclimation to reiterated drought in plants.

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Author Contributions

Conceived and designed the experiments: EFS MPM SMB. Performed the experiments: EFS MPM. Analyzed the data: EFS MPM SMB. Contributed reagents/materials/analysis tools: SMB. Wrote the paper: EFS SMB.

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CAPÍTULO 3

Memoria al estrés por sequía en los mecanismos fotosintéticos de una especie CAM invasiva, *Aptenia cordifolia*

CHAPTER 3

Drought stress memory in the photosynthetic mechanisms of an invasive CAM species, *Aptenia cordifolia*

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RESUMEN DEL CAPÍTULO 3

Las plantas son conocidas por su alta capacidad para aclimatarse a las fluctuantes condiciones ambientales. Un amplio rango de condiciones ambientales pueden permitir una subóptima eficiencia fisiológica. Sin embargo, estudios recientes han mostrado que las plantas pueden resistir repetidos periodos de estrés. Para encontrar como lo hacen, en este trabajo estudiamos los ajustes fotosintéticos a repetidos ciclos de estrés hídrico en *Aptenia cordifolia*: una especie CAM facultativa invasora. Las plantas fueron expuestas a tres ciclos de déficit hídrico y se cuantificaron los parámetros fotosintéticos y los antioxidantes de los cloroplastos para obtener una compresión de los mecanismos por los cuales hacen frente a repetidos periodos de estrés. En plantas expuestas previamente a un estrés hídrico se observó una modificación significativa de la composición en pigmentos de la antena, lo que permite una mayor eficiencia del fotosistema II comparando con plantas que se enfrentan por primera vez a la sequía. Estos hallazgos subrayan la importancia biológica de la memoria al estrés y muestran como las plantas pueden ajustar su composición del aparato fotosintético a las fluctuantes condiciones ambientales y por tanto optimizar la fotosíntesis y la fotoprotección bajo condiciones de sequía.

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ORIGINAL ARTICLE

Drought stress memory in the photosynthetic mechanisms of an invasive CAM species, *Aptenia cordifolia*

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Abstract Plants are known for their high capacity to acclimatise to fluctuating environmental conditions. A wide range of environmental conditions can lead to sub-optimal physiological efficiency. However, recent studies have shown that plants can withstand repeated periods of stress. To find out how they do it, we studied photosynthetic adjustments to repeated water stress in *Aptenia cordifolia*: a facultative, invasive CAM species. Plants were subjected to three cycles of water deficit, and photosynthetic parameters and chloroplast antioxidants were quantified to gain an understanding of the mechanisms by which they cope with repeated stress periods. Significant modification of the photosystems' antenna and reaction centres was observed in plants subjected to previous water stress cycles, and this led to higher PSII efficiency than in plants challenged with drought for the first time. These findings underline the biological significance of stress memory and show how plants can adjust their photosynthetic apparatus to fluctuating environmental conditions and thus optimise photosynthesis and photoprotection under drought conditions.

Keywords Abiotic stress · Chloroplast pigments · Crassulacean acid metabolism · Photoprotection · Photosynthesis · Vitamin E

Abbreviations

Chl	Chlorophyll
HPLC	High-performance liquid chromatography
PS	Photosystem
ROS	Reactive oxygen species
Toc	Tocopherol

Introduction

Under natural conditions, plants experience a wide range of climatological and environmental difficulties known as stress factors, including water scarcity or flooding, extreme temperatures, excessive incident light, salinity and nutrient deficiencies. These unfavourable conditions limit the physiological efficiency of plants and activate several regulation mechanisms that allow them to overcome stress factors and acclimatise to the new situation (Chaves et al. 2009). The scientific community is concerned with understanding how plants can be better prepared for future stress once a stress period has passed. Trewavas (2003) described 'plant memory' as 'an ability to access past experience so that new responses incorporate relevant information from the past'. It has been suggested that several mechanisms contribute to 'stressful memories', including the accumulation of signalling proteins or transcription factors (Bruce et al. 2007). Moreover, plants have the capacity not only to acclimatise, but also to adapt to adverse growth conditions through epigenetic regulation (Chinnusamy and Zhu 2009). The ways in which plants 'remember' previous stress periods so that they are better equipped to deal with future stress is an increasingly hot topic, as it will help shed light on functioning systems in the acclimatisation and adaptation mechanisms of plants. Few studies have been conducted on abiotic stress memory

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in plant species other than *Arabidopsis thaliana* (Sahu et al. 2013; Fleta-Soriano et al. 2015).

One of the most problematic abiotic stress factors is drought. According to global climate change projections, drought will be an increasing problem, especially in the world's Mediterranean climate zones (Bussotti et al. 2013). The capacity of plants to reach water has been studied extensively, to address the significant reduction in the area available for crop production (Deikman et al. 2012; Wasson et al. 2012). The lack of available water is currently a core issue for international research funding agencies (e.g. the European Commission H2020 and the National Water Research Institute in the USA), since drought has become the most common environmental disaster related to climate change in recent decades (IPCC 2012; Thornton et al. 2014). Abiotic stress factors slow down CO_2 incorporation in the Calvin-Benson cycle, which leads to an overreduction in the electron transport chain. The excited triplet chlorophyll (^{3}Chl*) in photosystem II (PSII) can pass excitation energy to molecular oxygen and form singlet oxygen ($^1O_2$), while superoxide radicals (O_2^-) formed in photosystem I (PSI) (due to an excess of reduced ferredoxin in thylakoids) are easily converted to hydrogen peroxide (H_2O_2) (Asada 2006). Therefore, the production of reactive oxygen species (ROS) signals the stress situation and may lead to oxidative cell damage if plants do not have an adequate capacity to reduce ROS. Both carotenoids and antioxidant systems are responsible for quenching or scavenging reactive molecules. Some ecophysiological traits, such as photosynthetic pigments and antioxidant compound composition, provide clues about phenotypic plasticity advantages against drought stress tolerance (Pintó-Marijuan and Munné-Bosch 2014).

In the plant kingdom, there is a special photosynthetic pathway of CO_2 fixation that optimises water-use efficiency (WUE), known as the crassulacean acid metabolism (CAM) (Cushman and Borland 2002). Species that use this metabolic strategy are much more resistant to arid conditions than C_3 and C_4 crops (Davis et al. 2014). CAM species only open their stomata at night. Consequently, CO_2 uptake occurs nocturnally, when the water gradient between the plant and the atmosphere is the lowest, which minimises water loss (Neales et al. 1968). CO_2 is fixed into C_4 molecules (usually malate) by phospho*enol*pyruvate carboxylase (PEPC) and stored in the vacuoles (Black and Osmond 2003). When light is available to activate the electron transport chain in the photosystems and obtain NADPH and ATP to regenerate ribulose-1,5-bisphosphate, the C_4 molecules are decarboxylated and a high concentration of carbons is released around RuBisCO, which allows the Calvin–Benson cycle to start (Borland et al. 2014). Moreover, high photosynthetic plasticity is found in the majority of CAM plants (Dodd et al. 2002). Among a wide range of metabolic peculiarities, there is a special group of plants called 'facultative CAM' that fine-tune their metabolism to adapt to the environment conditions. When there are no limitations, they use C3 metabolism, but they can switch to CAM when either drought or salinity problems are detected (Winter and Holtum 2014). Under these conditions, the photosynthetic machinery unquestionably participates in the plant's responses to abiotic stresses. However, Esteban et al. (2015a) reviewed the photosynthetic pigment composition in 525 studies and found that CAM species were underrepresented.

Under the new, changing conditions, the most successful species will be those that can cope with future environmental stress factors, those that present adaptive phenotypic plasticity and those that are fittest (Nicotra et al. 2010). These are the main characteristics of invasive species that enable them to survive the colonisation period and spread within a broad range of environments (Davidson et al. 2011). Invasive species threaten biodiversity, especially in areas that are more susceptible to changes in climatological conditions (Chown et al. 2012). Therefore, studying the ecophysiological traits of invasive plants could be key to understanding plant physiological adaptation and evolution in future scenarios (Pintó-Marijuan and Munné-Bosch 2013). In this study, we selected a succulent species from the Aizoaceae family, *Aptenia cordifolia* (L.f.) Schwantes, a facultative CAM species (Treichel 1975). *A. cordifolia* is native to southern Africa, but has been widely used as an ornamental plant, which has led to it becoming invasive in many areas of the world, including some parts of southern USA (California, Oregon and Florida), Australia, Tasmania, New Zealand, Hawaii and several habitats of southern and northern Europe, particularly Mediterranean climate zones. *A. cordifolia* is characterised by very high water-use efficiency that has been studied under salt stress (Cela and Munné-Bosch 2012) and drought stress (Cela et al. 2009). It has already demonstrated an extraordinary capacity to rehydrate after long drought periods (10 days) (Herppich and Peckmann 1997; Peckmann and Herppich 1998). Moreover, in a recent study to evaluate the cooling capacity of several roof systems (Schweitzer and Erell 2014), this species was found to have special hydraulic traits: it presented the highest water-use efficiency and the highest cooling benefit per unit of irrigation. The present study aimed to gain an in-depth understanding of how *Aptenia cordifolia* plants remember previous stressing conditions and are highly resistant to water stress, withstanding several repeated drought periods. We focused on how protection of photosynthetic function (photosynthetic pigments and antioxidant composition) is trained to deal with future stress periods.

Materials and methods

Plant materials and experimental design

Sixty *Aptenia cordifolia* plants were purchased from Ca L'Agustí (Barcelona, Spain) on 16 May 2014. The stems of each plant were about 20 cm long, and the leaf size was homogeneous. Each plant was grown in a 500-mL container with a soil mixture of peat/perlite/vermiculite (2:1:1, by vol.). On 29 May, all pots were placed inside the greenhouse in the experimental fields (Faculty of Biology, University of Barcelona), where the 60 plants were acclimatised for 13 days before the experiment began on 11 June. They were watered with half Hoagland nutrient solution. Typical Mediterranean spring weather occurred throughout the experiment. All water treatments and sampling points are outlined in Fig. S1 (see Supplementary Fig. S1), and the treatments selected to summarise the most important results are shown in Fig. 1. The weather conditions during the experiment were recorded every 30 min by the weather station *Barcelona—Zona Universitària*, located 200 m from the experimental fields (Fig. 2).

Sampling point days were full sunny days, as summarised in Fig. 1 (marked with dashed lines in Fig. 2), and the precise weather conditions during the sampling process are summarised in Table 1. T_0 (time zero) was the first sampling to characterise *A. cordifolia* plants, just before the experiment started. Plants underwent three cycles of water stress (S) lasting from 9 to 12 days, followed by the respective water stress recovery (R) for 4–5 days. At the start of the water treatments (indicated in grey in the figures), the 60 *A. cordifolia* plants were divided into two groups: 30 (W) that were well-watered every other day and 30 (S) that had no water added (T_1). Both W and S plants were subjected to a recovery period (WR and SR, respectively) (R_1). In the second cycle, treatment and recovery was again applied to the same plants: WW/WWR and SS/SSR (T_2/R_2, respectively). In the third cycle (T_3 and R_3), each of the two groups was divided into two subgroups of 15 plants each: half of the WWR and SSR groups were kept under well-watered conditions (WWW and SSW, respectively), and the other half were submitted to a water stress period (WWS and SSS, respectively). At each sampling point, 6–7 plants from each treatment were selected randomly to be measured, and pools of 15–20 sun-exposed, fully expanded young leaves per plant were collected at midday (11:00 to 13:00 solar time), frozen in liquid nitrogen and stored at −80 °C until analysis.

Relative water content and leaf morphology

Relative water content (RWC) and leaf mass per area (LMA) of one leaf per plant were measured in seven plants per treatment at each sampling point. The RWC was obtained using the formula (FW − DW)/(FSW − DW) × 100, where FW is the fresh weight, FSW is the fresh saturated weight after rehydrating samples for 24 h in the dark at 4 °C, and DW is the dry weight after oven-drying samples at 65 °C until constant weight. Leaf area (LA) was measured with a

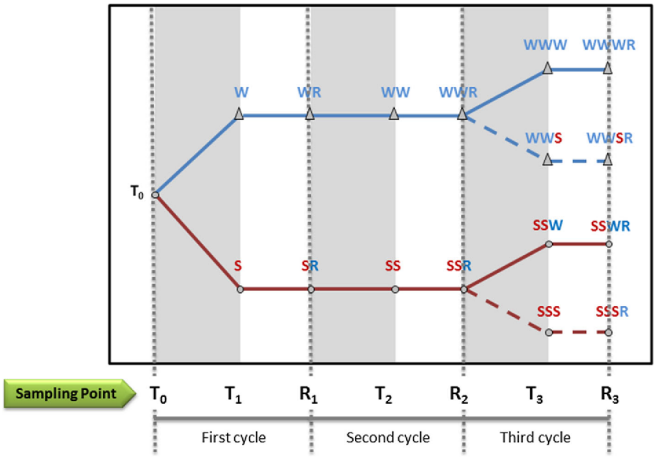

Fig. 1 Experimental design scheme. Abbreviations, sampling points and history of the *A. cordifolia* plants under the different water treatments during the three cycles. W, well-watered plants; S, water-stressed plants; T, treatment sampling point; R, recovery sampling point. *Grey stripes* indicate the moment at which a treatment (W or S) was applied. Symbols indicate initial water treatment (first and second cycles): well-watered plants represented in triangles, and water-stressed plants in circles. *Continuous* and *dashed lines* in the third cycle represent well-watered and water-stressed plants, respectively

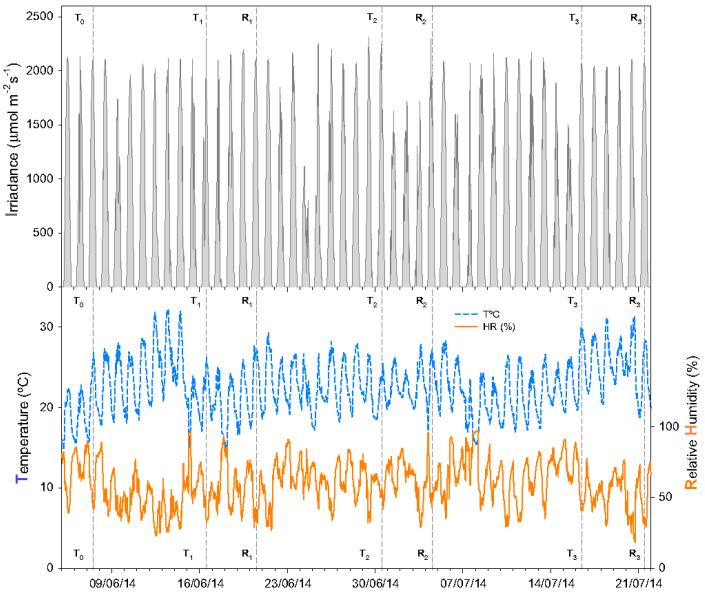

Fig. 2 Experimental weather conditions. Irradiance (µmol m ^{2} s ^{1}), temperature (°C) and relative humidity (%) throughout the experiment. The three cycles of treatment and recovery are indicated in the *time axis*. T, treatment sampling point; R, recovery sampling point

Table 1 Climatological conditions during the sampling points

	Day	PPFD (µ mol m ^{2} s ^{1})	*T* (°C)	RH (%)	Accum. PPFD (mol m ^{2} TP ^{1})
T_0	07/06/2014	1970.12 ± 103.77	26.30 ± 0.44	42.33 ± 0.58	
T_1	16/06/2014	2011.68 ± 224.43	25.83 ± 0.35	34.00 ± 2.00	498.06
R_1	20/06/2014	1972.27 ± 99.28	26.67 ± 0.06	34.33 ± 2.31	216.16
T_2	30/06/2014	1902.03 ± 316.45	23.57 ± 0.23	55.00 ± 1.00	523.09
R_2	04/07/2014	1666.25 ± 236.49	26.63 ± 0.55	45.67 ± 2.52	179.75
T_3	16/07/2014	1970.12 ± 84.76	29.27 ± 0.45	41.67 ± 1.15	642.83
R_3	21/07/2014	2033.90 ± 27.45	28.30 ± 0.10	33.00 ± 1.73	280.97

Photosynthetic photon flux density (PPFD; µmol m ^{2} s ^{1}), temperature (°C) and relative humidity (%), all averages of three points measured during the midday sampling points; accumulated irradiance during the period of treatment (TP) or recovery (accum. PPFD; mol m ^{2} TP ^{1}) before the sampling point

flat-bed scanner (Epson Perfection V30) and processed using the LA Measurement (version 1.3, University of Sheffield 2003) image analyser software. The LMA was calculated as DW/LA (gDW m^{-2}).

Gas exchange and chlorophyll fluorescence

Leaf gas exchange measurements coupled to chlorophyll fluorescence were taken between 10:00 and 17:00 on one representative attached leaf from each sampled plant at each sampling point, with a portable infrared gas analyser (LI-6400 system, LI-COR, Inc., Lincoln, NE, USA) using a leaf chamber fluorometer. Measurement settings were maintained as follows: photosynthetic photon flux density (PPFD): 700 ± 0.72 µmol m^{-2} s^{-1} (with 10 % blue light); leaf temperature: 26.3 ± 0.21 °C; supplied CO_2 concentration inside the cuvette (*Ca*): 400.00 ± 0.10 µmol mol^{-1} CO_2; and relative humidity of the incoming air (%RH): 47 ± 0.64 %; flow 500 µmol s^{-1}. Parameters related to leaf water content status were also recorded: leaf temperature (T_{leaf} in °C) and leaf vapour pressure deficit (VpdL in KPa). The maximum operating efficiency in the light-

adapted state (F_v'/F_m') (Murchie and Lawson 2013) was determined concomitantly with each gas exchange measurement in the light-adapted leaves. The maximum photochemical efficiency of PSII (F_v/F_m) was estimated in dark-adapted (for a minimum of 45 min) leaves.

Photosynthetic pigments quantification

Photosynthetic pigments were extracted under ice atmosphere and low-light conditions. Frozen leaves (100 mg) were ground to powder in liquid nitrogen and extracted with 1 mL of cold 85 % (w/w) acetone using ultrasonication (Bransonic Ultrasonic Bath, Emerson Industrial Automation, Danbury, CT, USA). The extract was centrifuged for 10 min at 4 °C and 13,000 rpm, and the supernatant was transferred to a 15-mL Falcon tube. The pellet was re-extracted once more with 85 % acetone and twice more with 100 % acetone, and the four supernatants were combined. The final acetone concentration of the supernatants was 92.5 %. A precise volume of H_2O was added to the supernatants to obtain a final acetone concentration of 82.5 %, and the extracts were filtered through filters with a 0.22-μm pore size. Pigments were separated by high-performance liquid chromatography (HPLC) on a Dupont non-endcapped Zorbax ODS-5-μm column (250 mm long, 4.6 mm i.d.; 20 % carbon, Teknokroma, Sant Cugat, Spain) at 30 °C at a flow rate of 1 mL min^{-1} for 38 min. The solvent mixture for the gradient and detection at 445 nm (diode array detector, HP1100 Series HPLC System, Agilent Technologies, Santa Clara, CA, USA) was performed as described by Munné-Bosch and Alegre (2000). The conversion factors from peak area units to pmol per injection were: chlorophyll a (167), chlorophyll b (163), lutein (359), zeaxanthin (359), β-carotene (283), neoxanthin (344), violaxanthin (400) and antheraxanthin (372), which were consistent ($R^2 = 0.9996$) with those used by Thayer and Björkman (1990).

Vitamin E quantification

Tocopherols were extracted from 50 mg of ground fresh leaf material with 500 μL of cold 100 % methanol (v/v). After vortexing the extract, it was ultrasonicated for 20 min and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected, and the pellet was re-extracted with the same solvent three times. The supernatants were polled and filtered through a syringe filter with a pore size of 0.45 μm. The HPLC analysis was performed as described (Amaral et al. 2005). In short, the HPLC equipment consisted of an integrated system with a PU-2089 Plus quaternary gradient pump, an AS-2055 Plus intelligent sampler and a FP-1520 fluorescence detector (Jasco). Tocopherols were separated on an Inertsil 100A (30 × 205 mm, 5 μm, GL Sciences Inc.) normal-phase column, operating at room temperature. The mobile phase used was a mixture of *n*-hexane and 1,4-dioxane (95.5:4.5, v/v) at a flow rate of 0.7 mL/min. Detection was carried out for excitation at 295 nm and emission at 330 nm. Quantification was based on the fluorescence signal response compared with authentic standards of each compound (Sigma-Aldrich).

Data analysis

Statistical analysis was performed using the SPSS 15.0 statistical package (SPSS, Inc., Chicago, IL, USA). The significance level was set to $P < 0.05$. To evaluate the effect of water treatments on the traits, the values obtained for each parameter in each plant were analysed using a one-way ANOVA, in which the only factor was treatment. When a significant difference was found at sampling points T_3 and R_3, the post hoc Duncan test was applied to determine individual differences between means. Linear regression analyses were used to relate RWC with T_{leaf} and VpdL, and bivariate Pearson correlations were run. The number of replicates is indicated in each figure legend. Graphs were prepared using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA).

Results

Aptenia cordifolia plants prepared the efficiency of their photosystems for the third water stress cycle

Aptenia cordifolia plants were subjected to severe drought stress by reducing the RWC to half for three cycles of 9–12 days under water stress (Fig. 3a). RWC decreased from 65.6 ± 1.0 % under well-watered conditions (ranging from 58.2 to 75.9 %) to 34.2 ± 1.6 % at the end of the water stress period (ranging from 28.9 to 39.8 %). After each water stress treatment, full recovery of the leaf water content to the same values as the well-watered treatment plants was observed (with no significant differences among treatments at any *R* sampling points). In the third cycle, there were no significant differences due to the previously experienced stress periods; only the differences between present water treatments were significant.

Leaf morphology was also affected by water stress, especially from T_2 sampling point onwards, when stressed plants (SS, SSS and WWS) presented significantly lower LA and higher LMA (see Supplementary Fig. S3) than the respective well-watered plants (WW, WWW and SSW). Regarding morphological leaf characteristics, there were two clear groups of leaves: pre-T_2 and post-T_2. The first group was formed of leaves that became unsuitable for

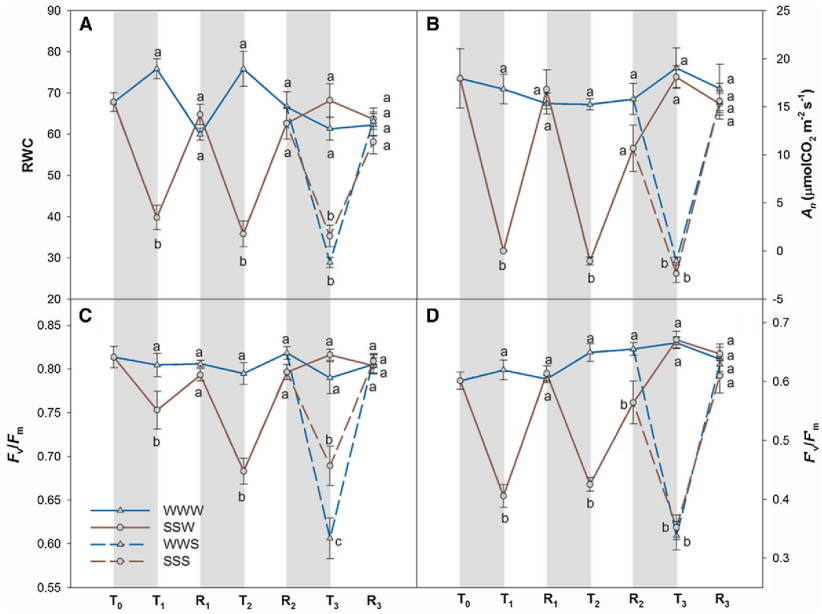

Fig. 3 Water content and stress memory of photosystems. **a** Relative water content (RWC). **b** Net CO_2 assimilation (A_n in μmol CO_2 m^{-2} s^{-1}). **c** Maximum PSII efficiency (F_v/F_m). **d** Intrinsic efficiency of open PSII centres (F_v'/F_m'). Water stress treatments are represented by *grey stripes*. *Symbols* indicate initial water treatment (first and second cycles): well-watered plants represented in *triangles*, and water-stressed plants in *circles*. *Dashed lines* indicate water stress treatment in the third cycle. Values are the mean ± SE of 6–7 measurements per treatment and sampling point. *Different letters* indicate significant differences between treatments ($P < 0.05$)

collection in both water treatments after the R_1 sampling point: (a) in WW and WWW plants, they were almost senescent (violet circles in Fig. S4B); (b) in SS and SSS plants, they were excessively desiccated, and little plant material was available for the gas exchange measurements (see violet circles in Supplementary Fig. S4). The second group of leaves were sampled from T_2 sampling point onwards (see orange squares in Supplementary Fig. S4). They were too young and immature to be sampled at the beginning of the experiment, but fully expanded during the second and third cycles (as shown by orange squares in Fig. S4). When plants were watered, recovery to full turgidity was very fast and leaf area (LA) rapidly increased as a result. Two photographs were taken in an attempt to understand such a rapid change in LA (see Supplementary Fig. S2) during the rehydration process: an SSS plant (Fig. S2A) and the same plant after 10 h with available soil water (Fig. S2B).

Daytime photosynthetic performance was significantly influenced by the previous history of the plants. The average value of net daytime CO_2 uptake in well-watered *A. cordifolia* plants (A_n, Fig. 3b; Supplementary Table S1) was 15.71 ± 0.53 μmol CO_2 m^{-2} s^{-1}, while at the end of the stress periods, CO_2 assimilation rates were always negative during the midday measurements. During the repeated stresses, stomatal conductance (g_s) and the ratio of internal to atmospheric CO_2 concentration (C_i/C_a) (Supplementary Fig S5) showed the same pattern of responses as in A_n, with no evident sign of memory of the preceding drought periods. Photosynthetic machinery efficiency was also measured by chlorophyll fluorescence parameters. The observed PSII efficiency measured by both F_v/F_m in the dark (Fig. 3c) and F_v'/F_m' (at 700 μmol PPFD m^{-2} s^{-1}, Fig. 3d) at the available water sampling points of the first and third treatment cycles was maintained above 0.79 in F_v/F_m and above 0.56 in F_v'/F_m'. However, the recovery of the second cycle (R_2) indicated a different response between WWR and the previously stressed SSR plants, especially since F_v'/F_m' values were significantly below those of the WWR. Moreover, at this sampling point, the A_n values of the SSR plants were 32 % lower than those of the WWR. The limitation of both parameters indicated that

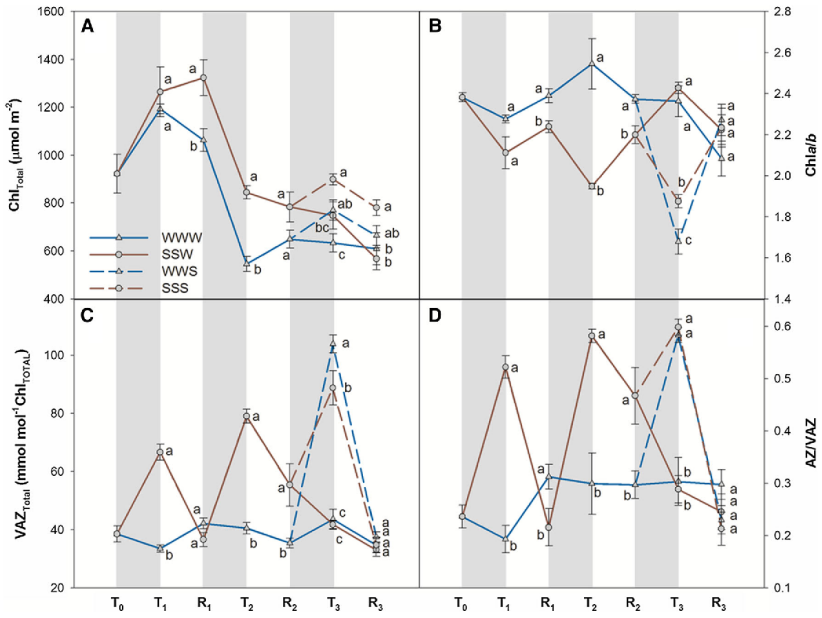

Fig. 4 Chloroplast pigment stress memory. **a** Total content of chlorophylls (Chl_{Total}). **b** Chl*a*/*b* ratio. **c** Total content of the VAZ cycle xanthophylls (VAZ_{Total}). **d** De-epoxidation state (AZ/VAZ). Water stress treatments are represented by *grey stripes*. *Symbols* indicate initial water treatment (first and second cycles): well-watered plants represented in *triangles*, and water-stressed plants in *circles*. *Dashed lines* indicate water stress treatment in the third cycle. Values are the mean ± SE of 6–7 measurements per treatment and sampling point. *Different letters* indicate significant differences between treatments ($P < 0.05$)

the photosynthetic machinery did not experience full recovery at the R_2 sampling point. Although *A. cordifolia* plants showed negative CO_2 assimilation rates when water was a limiting factor, PSII was still working, as demonstrated by F_v'/F_m' values of around 0.37 (ranging from 0.34 to 0.43). The maximum efficiency of the PSII also decreased after each S treatment. Interestingly, in the third stress cycle, the F_v/F_m values of plants subjected to three stress periods (SSS) indicated that their photosystems were significantly less damaged than those of plants that were stressed for the first time (WWS). In fact, with respect to the four water stress treatments (see Supplementary Table S1B) at this sampling point (T_3), the F_v/F_m values exhibited a gradient of plants with different backgrounds between SSS (0.69 ± 0.03) and WWS (0.61 ± 0.02). This information indicates that the photosystem structure alters in preparation for future stress periods, since plants that were stressed twice before the last stress cycle were less damaged at the same level of stress intensity than plants that were only stressed once, and these latter were less damaged than plants that had never been stressed before (WWS).

Photosynthetic pigment content depends on previous plant history

In the photosystems, chlorophyll content per area of sampled leaf changed during the experiment (Fig. 4; Supplementary Table S2), while the plants grew in the greenhouse for 6 weeks. These results are in line with the data on morphological leaf characteristics: (1) in the initial samplings (from T_0 to R_1), the leaves had 1153.9 ± 72.2 μmol m^{-2} of total chlorophyll (Chl_{TOTAL}), (2) in the last two cycles, the fully expanded leaves that were sampled had a slightly lower Chl_{TOTAL} concentration at 708.7 ± 22.5 μmol m^{-2}. A comparison between water treatments during the experiment showed that there was a higher Chl_{TOTAL} concentration in stressed leaves (893.7 ± 63.8 μmol m^{-2}) than in well-watered ones (751.0 ± 44.8 μmol m^{-2}), especially when the LA was significantly lower than that of leaves from well-

watered plants (see Supplementary Table S2A and Fig. S3). Therefore, all the chloroplast component results (carotenoids and lipophilic antioxidants) are expressed relative to Chl_{TOTAL} units. The ratio Chl*a*/*b* was about 7 % lower in stressed plants than in well-watered plants at T_1 and had not recovered at the R_1 sampling point. At T_2, the differences between stressed and non-stressed plants increased (23 % lower in stressed plants) and, again, had not recovered at R_2. In the third cycle, there were differences between the two stressed groups at the T_3 sampling point. The *A. cordifolia* plants that had previously been stressed (SSS) showed a lower reduction in the Chl*a*/*b* ratio (from 2.31 ± 0.05 to 1.97 ± 0.03), while the previously well-watered plants (WWS) experienced a higher reduction in the ratio (from 2.49 ± 0.02 to 1.76 ± 0.06). When the T_3 sampling point was examined in detail and the two chlorophyll molecules were analysed separately (see Supplementary Fig. S6 and Table S2), a different increase was observed in the chlorophyll content in each treatment from R_2 to T_3. From SSR to SSS, plants that had previously been stressed twice increased their Chl*a* content by 21.21 % and their Chl*b* content by 42.05 %. However, the WWS plants, which had never been stressed before, increased their Chl*a* content by 13.79 % and their Chl*b* content by 61.94 %. As a consequence, Chl*a*/*b* values in WWS were significantly lower than those of SSS, which indicates that the Chl*a*/*b* ratio had a different response to the same level of stress intensity depending on the previous stress history, as WWS did not synthesise enough Chl*a* to maintain the ratio.

Water stress cycles also affected the carotenoid content of the chloroplasts; xanthophyll concentration in particular increased with every water stress period affected by previous stress periods. For example, VAZ_{Total} (violaxanthin [V] + antheraxanthin [A] + zeaxanthin [Z]) increased more than twofold compared to the content at the beginning of each stress period (Fig. 3c). Moreover, throughout the whole experiment, stressed plants were always richer in de-epoxidised xanthophylls than well-watered plants, so the de-epoxidation state (AZ/VAZ: A + Z/VAZ_{Total}; Fig. 3d) of the stressed plants increased during each stress period, albeit with different intensities. Interestingly, significant differences were found in two sampling points, depending on the previous history of the plants: during the recovery of the second water stress cycle (R_2) and the third water stress cycle (T_3). First, at R_2, neither VAZ_{Total} nor AZ/VAZ regained R_1 values, which indicates that the *A. cordifolia* plants slowed their recovery down to the rewatered status. Second, at T_3, WWS plants experienced a significantly higher increase in VAZ_{Total} concentration (198.46 %) than SSS plants (76.24 %), but there were no significant differences in the AZ/VAZ ratio between the same treatment groups of plants. Other xanthophylls such as neoxanthin (Nx; Fig. 5a) and lutein (Lut; Fig. 5b)

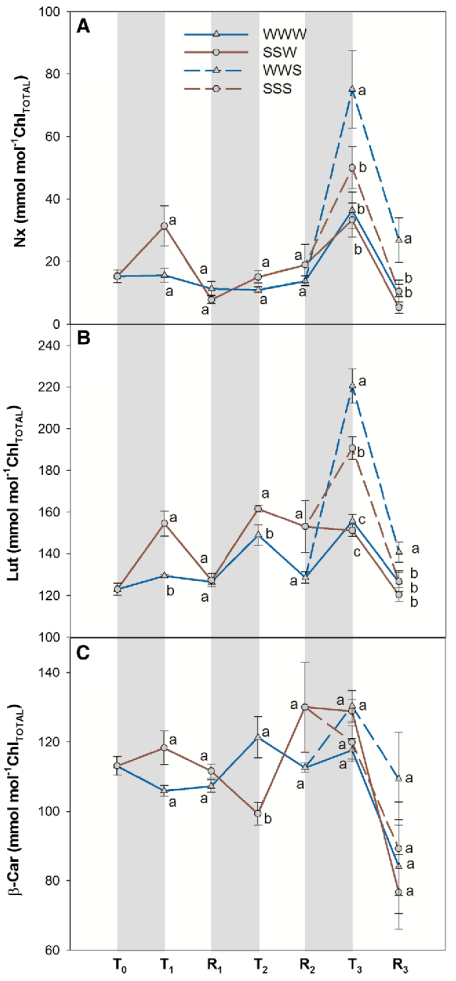

Fig. 5 Chloroplast pigment stress memory. **a** Neoxanthin (Nx). **b** Lutein content (Lut). **c** β-Carotene content (β-Car). Water stress treatments are represented by *grey stripes*. *Symbols* indicate initial water treatment (first and second cycles): well-watered plants represented in *triangles*, and water-stressed plants in *circles*. *Dashed lines* indicate water stress treatment in the third cycle. Values are the mean ± SE of 6–7 measurements per treatment and sampling point. *Different letters* indicate significant differences between treatments ($P < 0.05$)

showed a similar outline to the VAZ_{Total} content throughout the three water stress cycles. However, not all carotenoids followed the same pattern: β-carotene (β-Car;

Fig. 5c; Supplementary Table S3) did not show a particular trend throughout the different water stress cycles, with values of 108.3 ± 3.1 mmol mol^{-1}Chl$_{Total}$ (ranging from 76.7 to 95.9) in well-watered plants and 114.3 ± 4.6 mmol mol^{-1}Chl$_{Total}$ (ranging from 95.9 to 130.3) in water-stressed plants. The content of Lut and VAZ$_{Total}$ remained in parallel throughout the whole experiment, and Nx remained constant during the two first cycles, while at the T_3 sampling point, Nx also confirmed the general pattern, i.e. a significantly higher concentration of xanthophylls in the WWS plants than in the SSS plants. Therefore, the carotenoid dynamics presented different patterns depending on the previous history of the plants.

Tocopherols: ready to scavenge in every water stress period

During the first two drought stress cycles, *A. cordifolia* plants responded to the lack of water (S and SS plants) with a significantly higher content of both tocopherols (α-Toc and γ-Toc; Fig. 6) than in the well-watered plants (W and WW, respectively). During water recovery, the tocopherol content of previously stressed plants decreased to control values (α-Toc decreased by 24.78 and 27.09 %, while γ-Toc decreased by 53.94 and 40.89 % during R_1 and R_2, respectively). In T_3, γ-Toc differences between water treatments were more significant than the differences observed in α-Toc. Regarding all T_3 sampling situations (see Supplementary Table S3), both tocopherols had a higher content in stressed plants: α-Toc averaged 54.8 ± 2.6 mmol mol^{-1}Chl$_{Total}$ compared to 39.8 ± 2.7 mmol mol^{-1}Chl$_{Total}$ in well-watered plants, and γ-Toc averaged 16.8 ± 0.7 mmol mol^{-1}Chl$_{Total}$ under drought conditions compared to 7.7 ± 0.5 mmol mol^{-1}Chl$_{Total}$ in well-watered plants. When plants that were stressed with different water treatment histories were compared, SSS showed a slightly lower concentration of both α-Toc and γ-Toc than WWS plants. However, these differences were not significant. At R_3, the tocopherol content of all stressed plants decreased (about 28 % in α-Toc and over 60 % in γ-Toc), which meant that there were no significant differences between plants with different treatment histories. Therefore, under water stress conditions, no significant differences in lipophilic antioxidant system functioning were observed between the two plant groups with different stress histories.

Discussion

In this study, drought stress memory was studied to analyse two key questions that have been asked in previous studies: When is stress memory used? How does stress memory affect chloroplast mechanisms? Previous studies have found that most transcriptional responses modified in *A. thaliana* due to stress memory processes were related to chloroplasts (24 %) and the thylakoid membrane (17 %) (Ding et al. 2013). However, in *Zea mays*, which has a C_4 photosynthetic pathway, the number of genes regulated by memory processes that were associated with the thylakoid membrane or chloroplast was much lower (1–6 %) (Ding et al. 2014). Here, we investigated what makes a plant so resistant to repeated drought stress periods at chloroplast level in the invasive species *Aptenia cordifolia*, with a facultative CAM photosynthetic pathway.

The studied species switched rapidly from CAM to C_3 metabolism during the recovery periods when water was available, as demonstrated by the recovery of diurnal CO_2 uptake to WW values (Cushman and Borland 2002). The

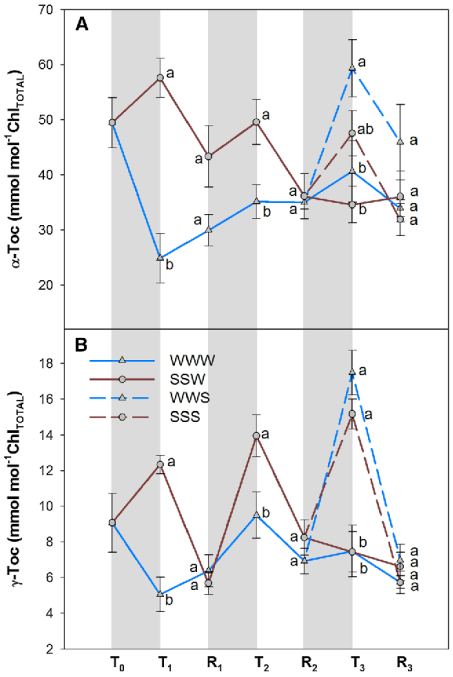

Fig. 6 Vitamin E stress memory. **a** α-Tocopherol (α-Toc); **b** γ-tocopherol (γ-Toc). Water stress treatments are represented by *grey stripes*. T, treatment sampling point; R, recovery sampling point; W, well-watered plants; S, water-stressed plants. *Symbols* indicate initial water treatment (first and second cycles): well-watered plants represented in *triangles* and water-stressed plants in *circles*. *Dashed lines* indicate water stress treatment in the third cycle. Values are the mean ± SE of 6–7 measurements per treatment and sampling point. *Different letters* indicate significant differences between treatments ($P < 0.05$)

first water stress memory signal was detected after two stress cycles. At R_2, the SSR *A. cordifolia* plants had not fully recovered from water deprivation; not only did the reaction centres present lower photosynthetic performance, with significantly lower Chl*a*/*b* and photosynthetic PSII efficiency in the light-adapted state (F_v'/F_m') (together with a 32 % reduction in the net CO_2 assimilation rate) than WWR, but the VAZ cycle did not recover the well-watered plant values either in terms of VAZ_{Total} or the AZ/VAZ ratio. Z in particular remained significantly high in SSR (24.59 ± 7.01 mmol $mol^{-1}Chl_{TOTAL}$) compared to WWR (7.20 ± 1.37 mmol $mol^{-1}Chl_{TOTAL}$, in Supplementary Table S3H) at R_2. This indicates slow recovery of the carotenoids used for energy dissipation, which provides better photoprotection through xanthophyll cycle activation (Garcia-Plazaola et al. 2012). Moreover, a high pool of Z was found to be related to stress memory under severe stress conditions and contributed to sustaining non-photochemical quenching (NPQ) after the stress was over (Jahns and Holzwarth 2012) and to decreasing the NPQ relaxation rate (Ruban and Johnson 2010) for several days to allow complete recovery (Esteban et al. 2015b). The increased need for higher photoprotection during sudden rehydration, due to the resumption of metabolic activity and the resulting cellular oxidative stress, has already been described in desiccation-tolerant plants (Fernández-Marín et al. 2010). When photoprotective mechanisms were studied in mosses during water recovery, Beckett et al. (2005) described the priorities that plants assumed during 'hardening': firstly photoprotection and secondly the efficiency of photosystems. In concordance with a previous study on drought stress memory in *A. cordifolia* (Fleta-Soriano et al. 2015), we confirmed that this species remembers previous stress periods and also prioritises photoprotection before growth during rehydration.

The second signal was detected at the final water stress sampling point (T_3). The weather conditions in our experiment meant that the accumulated irradiance (Table 1) increased at each sampling point ($T_1 < T_2 < T_3$), which is correlated with higher water evapotranspiration (Vialet-Chabrand et al. 2013; Bond and Bumbaco 2015). This led to an increasing level of stress in every water deprivation cycle. Moreover, the T_3 sampling point was much warmer than the previous two sampling points, which gave rise to a particularly stressful environment. Such unusual weather conditions were exemplified through the clear lineal correlation between RWC and both VpdL and T_{leaf} (see Supplementary Fig. S8), which showed how stressed T_3 plants experienced higher T_{leaf} and greater atmospheric dryness.

This weather scenario meant that the effect of drought stress memory on SSS plants showed significantly different photoprotective mechanisms and photosynthetic pigment composition during the maximum water stress of the third cycle. The functions of carotenoids are not only to bind to the photosynthetic complexes for more efficient light harvesting (as accessory pigments in the light-harvesting complex, LHC) and to influence membrane structure and fluidity (Gruszecki and Strzałka 2005), but also to increase chloroplast photoprotection (Cazzonelli 2011) by quenching ^{3}Chl*, scavenging $^1O_2$ and other ROS, and dissipating excess energy through NPQ (Havaux and García-Plazaola 2014). Xanthophylls are a group of oxygenated carotenoids composed mainly of VAZ, Nx and Lut. At the maximum stress sampling point, SSS presented significantly lower xanthophyll content (VAZ_{Total}, Nx and Lut) than WWS. Moreover, as previously described by Niinemets et al. (2003), differences in photoprotection-related parameters between sampling points showed a linear correlation with increased accumulated irradiance (with the following R^2 values: VAZ_{Total}, $R^2 = 0.825$; AZ/VAZ, $R^2 = 0.609$; Lut, $R^2 = 0.999$; Z, $R^2 = 0.738$). It has already been suggested that Lut content presents a photoprotective memory by maintaining energy dissipation for more than 1 month in shade-acclimated avocado leaves (Förster et al. 2009). Both Z and Nx have also been described as necessary for protein stabilisation in the PSII by promoting more efficient photoprotection (Ballottari et al. 2013). Our data therefore indicate that plants subjected to two previous stress periods needed less xanthophylls/Chl than plants stressed for the first time at T_3, which means that WWS had a higher LHC with a higher carotenoid content (and LHC was lower in SSS). However, the Chl*a*/*b* ratio, which indicates the proportion of Chl that is part of the LHC (Walters 2005), was lower in WWS than in SSS. Low Chl*a*/*b* ratio values have been widely described in low-light-adapted plants, where higher levels of LHCII were accompanied by a compensating reduction in levels of PSII reaction centres (Walters et al. 1999). The Demmig-Adams research group also confirmed the relationship between higher de-epoxidation of xanthophylls (Z + A) and the reorganisation of PSII components (D1 protein, pheophytin and oxygen-evolving complex) (Zarter et al. 2006). Therefore, the suggested differences implied an increase in photosystems, and especially an increase in reaction centres, in the plants that were subjected to two previous stress periods (SSS) (compared to the plants that were well-watered before the stress, WWS). As observed in the results, SSS plants did not need more antenna components for efficient light capture, as F_v'/F_m' showed no significant differences at T_3 and demonstrated even better photoprotection (higher F_v/F_m). Previous studies performed with grasses also showed that recurrent water stress led to a faster, more protective response to future water scarcity (Walter et al. 2011). In our study, higher carotenoid content in the WWS plants did not correlate with better photoprotection. It has been suggested that damaged reaction

centres in WWS, as revealed by a reduction in F_v/F_m values, are related to higher production of reactive oxygen species that would disrupt the PSII structure (Walters 2005; Takahashi and Badger 2011). Many studies have reported that higher xanthophyll content in large PSII antennae correlates with decreased intrinsic PSII efficiency and lower F_v/F_m values (Walters 2005; Camarero et al. 2012).

Although both β-Car and tocopherols played a crucial role in the redox regulation of drought stress in our study, neither compound was specifically regulated by the drought stress memory processes. β-Car was described as bound to core complexes in PSI and PSII, with antioxidant and protective properties as a quencher of both ^{3}Chl* and $^1O_2$ (Cazzaniga et al. 2012). Its functional concentration in *A. cordifolia* plants did not differ significantly depending on water availability, and it has been described as a stable parameter in most abiotic stress studies (Esteban et al. 2015a). The vitamin E components analysed are also responsible for lipophilic antioxidant properties and regulate membrane fluidity in plant chloroplasts. Two main functions are attributed to tocopherol molecules: lipid peroxy radical scavenging and physically quenching or chemically scavenging $^1O_2$ (Munné-Bosch and Alegre 2002). However, no effect was observed depending on the plant stress history in this experimental design. In fact, when the involvement of both photoprotective isoprenoids (xanthophylls and tocopherols) was compared in the stress memory process, Esteban et al. (2015b) found that the xanthophylls group played a more significant role.

In this study, we emphasised the importance of chloroplast composition and structure on the oxidative stress memory processes. In view of the fact that redox signals modify the expression of nuclear and chloroplast genes (Fey et al. 2005; Barajas-López et al. 2013), we suggest that communication between chloroplast and nucleus and vice versa (Pfalz et al. 2012) could be the key mechanism by which this species remembers previous stress periods. The largest physiological changes measured included chloroplast and thylakoid function, in line with previous studies (Ding et al. 2014), which reported that most of the genes modified by repeated dehydration stress encoded chloroplast and thylakoid membrane proteins.

In conclusion, this is the first time that a stress memory experimental design has proved that photosynthetic performance adapts to improve the photoprotective mechanisms of double-stressed plants so that they can cope with a third stress period more effectively. The molecular mechanisms of these adaptations at chloroplast level to better prepare for subsequent stress events, and the possible role of epigenetic modifications, are a motivating field of study for future investigations. The outcomes published in this paper expand our limited knowledge of stress memory and will help to focus future research on the mechanisms by which plants 'remember' previous stressing conditions. Moreover, *A. cordifolia* plants were found to be an excellent model for studying stress memory responses associated with chloroplast functions, and unravelling the mechanisms by which they prepare for future stress periods. The fact that this species is a facultative CAM may have been crucial for these findings. Future studies to examine the nocturnal responses of facultative CAM species during repeated stress periods would increase knowledge of the discovered mechanisms.

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CAPÍTULO 4

Incremento en la acumulación de plastocromanol-8 durante déficits hídricos reiterados en maíz (*Zea mays*)

CHAPTER 4

Enhanced plastochromanol-8 accumulation during reiterated drought in maize (*Zea mays* L.)

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RESUMEN DEL CAPÍTULO 4

El plastocromanol-8 (PC-8) pertenece al grupo de los tococromanoles, y junto con tocoferoles y carotenoides, podrían ayudar a proteger el fotosistema II de la fotoinhibición durante los estreses ambientales. Aquí tratamos de desentrañar la evolución temporal de los contenidos de PC-8, junto con los de los compuestos del grupo de la vitamina E, en plantas de maíz (*Zea mays*) expuestas a déficits hídricos reiterados. Las medidas se realizaron en plantas (crecidas en un invernadero) sujetas a dos ciclos de déficit hídrico-recuperación consecutivos. Los niveles de PC-8, que representan más del 25% de los tococromanoles en las hojas de maíz, incrementaron significativamente en respuesta a repetidos periodos de déficit hídrico. Los niveles de PC-8 variaron en paralelo con los de vitamina E, particularmente con los de α-tocoferol. El perfil de las hormonas relacionadas con el estrés (ácido abscísico, ácido jasmónico y ácido salicílico) fue consistente con el papel del ácido abscísico en la regulación del PC-8 y la síntesis de vitamina E durante el déficit hídrico. Los resultados sugieren que el PC-8 puede ayudar a los tocoferoles a frenar el daño al aparato fotosintético. Un mejor conocimiento de la regulación del PC-8 dependiente del ácido abscísico nos puede ayudar a manipular el contenido de este importante antioxidante en los cultivos.

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Short communication

Enhanced plastochromanol-8 accumulation during reiterated drought in maize (*Zea mays* L.)

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ABSTRACT

Plastochromanol-8 (PC-8) belongs to the group of tocochromanols, and together with tocopherols and carotenoids, might help protect photosystem II from photoinhibition during environmental stresses. Here, we aimed to unravel the time course evolution of PC-8 together with that of vitamin E compounds, in maize (*Zea mays* L.) plants exposed to reiterated drought. Measurements were performed in plants grown in a greenhouse subjected to two consecutive cycles of drought-recovery. PC-8 contents, which accounted for more than 25% of tocochromanols in maize leaves, increased progressively in response to reiterated drought stress. PC-8 contents paralleled with those of vitamin E, particularly α-tocopherol. Profiling of the stress-related phytohormones (ABA, jasmonic acid and salicylic acid) was consistent with a role of ABA in the regulation of PC-8 and vitamin E biosynthesis during drought stress. Results also suggest that PC-8 may help tocopherols prevent damage to the photosynthetic apparatus. A better knowledge of the ABA-dependent regulation of PC-8 may help us manipulate the contents of this important antioxidant in crops.

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RESULTADOS

1. Introduction

Together with tocopherols and tocotrienols (vitamin E compounds), plastochromanol-8 (PC-8) belongs to the group of tocochromanols, all of which contain a chromanol ring that is responsible for their strong antioxidant properties (Kruk et al., 2014). The prenyl chain of PC-8 differs, however, from that of vitamin E compounds; PC-8, tocopherols and tocotrienols being formed from condensation of homogentisate with solanesyl-diphosphate, phytyl-diphosphate and geranylgeranyl-diphosphate, respectively. Therefore, they share the same chromanol structure, but differ in the nature of the prenyl chain, being much longer and unsaturated in PC-8 compared to vitamin E compounds (Falk and Munné-Bosch, 2010; Kruk et al., 2014). This structural difference is indeed very important because a longer, unsaturated prenyl chain provides a much higher quenching activity towards singlet oxygen in PC-8 compared to vitamin E compounds in hydrophobic environments (Gruszka et al., 2008).

Although present in several species, PC-8 and tocotrienols are not ubiquitous in the plant kingdom (Falk and Munné-Bosch, 2010; Kruk et al., 2014), so they may be considered secondary metabolites. In contrast, tocopherols are present in most photosynthetic organisms examined thus far, including all land plants and most cyanobacteria (Esteban et al., 2009; Falk and Munné-Bosch, 2010; Dłużewska et al., 2016). PC-8 has generally been found to be less abundant than tocopherols in leaves, with the exception of *Cecropia* sp., *Pseudobombax munguba* and *Hevea brasiliensis* (Kruk et al., 2014). PC-8 contents in leaves are species-specific, but may also be influenced by leaf age, plant developmental stage and growth conditions. In *Arabidopsis thaliana*, PC-8 contents increase in young leaves under strong light conditions (Szymanska and Kruk, 2010). Unfortunately, however, no information is still available on the effects of other abiotic stresses, such as water deficit and salinity, on PC-8 accumulation in plants.

PC-8, a derivative from plastoquinol (Szymanska and Kruk, 2010), is located in chloroplasts (Dunphy et al., 1966). Tocopherol cyclase (VTE1), which is located in plastoglobules (Vidi et al., 2006), is a key enzyme for the biosynthesis of both PC-8 and vitamin E compounds (Sattler et al., 2003; Szymanska and Kruk, 2010; Dłużewska et al., 2016). As the content of plastoglobules is in equilibrium with thylakoid membranes (Austin et al., 2006), both PC-8 and vitamin E compounds are found in thylakoids where they fulfill an antioxidant function protecting lipids from the

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propagation of lipid peroxidation and preventing photosystem II damage as a result of singlet oxygen attack, the latter together with carotenoids (Munné-Bosch and Alegre, 2002; Falk and Munné-Bosch, 2010; Zbierzak et al., 2010; Kruk et al., 2014).

Photosynthesis is a major source of reactive oxygen species, which are generated through processes associated with energy transfer and electron transport. When water deficit limits CO_2 assimilation, saturation of the photosynthetic electron transport may occur due to a reduced consumption of NAPDH and ATP in the Calvin cycle (Miret and Munné-Bosch, 2015). There exist a number of mechanisms to prevent photo-oxidative damage in chloroplasts (e.g., xanthophyll cycle-dependent excess energy dissipation, photorespiration, water-water cycle, chloroplast movements), among which, formation and scavenging of singlet oxygen may represent an important safety valve for excess energy dissipation in drought-stressed plants (Asada, 2006; Noctor et al., 2014; Pintó-Marijuan and Munné-Bosch, 2014). Therefore, PC-8 may potentially serve an important function together with vitamin E and carotenoid compounds in chloroplast membranes of drought-stressed plants. Unfortunately, however, it is still unknown whether or not PC-8 biosynthesis increases under drought stress.

With the aim of getting new insights into the possible function of PC-8 in chloroplasts, we investigated whether or not the contents of this tocochromanol increase in response to water deficit in maize (*Zea mays* L.). We examined the response of tocochromanols to two consecutive drought-recovery cycles, and evaluated to what extent the drought-induced response in the accumulation of these antioxidants might be modulated by the endogenous contents of the stress-related phytohormones, ABA, jasmonic acid and salicylic acid.

2. Materials and methods

2.1. Plant material, treatments and sampling

Seeds of maize (*Zea mays*) were sown in 2L-pots on peat:perlite:vermiculite (2:1:1, v/v). After 2 months of growth, plants were transferred to 9L-pots with the same substrate. During all experiments, plants were grown in a greenhouse at the Faculty of Biology at the University of Barcelona (Barcelona, NE Spain). Prior to experiments, plants were watered daily with half-diluted Hoagland nutrient solution. Experiments started on 16th February 2014 by exposing plants to two treatments: half of the plants were subjected to drought (water-stressed plants) by withholding water for 9 days, recovered for 5 days and then exposed again to drought for 8 days, followed by a final recovery of 5 days; while the other half (irrigated plants) were watered every other day during the whole experiment. Samplings were performed at the beginning of the experiment and after 9, 14, 22 and 27 days of treatments, that is at the points of maximum stress and during recovery. All samplings were performed 1 h before dawn with temperatures around 20 °C throughout the experiment. At each sampling point, the mid region of the blade of the fifth leaf of 5 individuals were used to measure leaf water contents, chlorophyll contents, the F_v/F_m ratio, and the contents of PC-8, vitamin E and the stress-related phytohormones, ABA, jasmonic acid and salicylic acid. Samples for biochemical analyses were collected, immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Leaf water contents, chlorophyll contents and the F_v/F_m ratio

Samples were weighed to estimate the fresh matter (FW), immersed in distilled water at 4°Cfor 24h to estimate the turgid matter (TW) and then oven-dried at 80 °C to constant weight to estimate the dry matter (DW). Relative water content (RWC) was then calculated as 100 x (FW-DW)/(TW-DW).

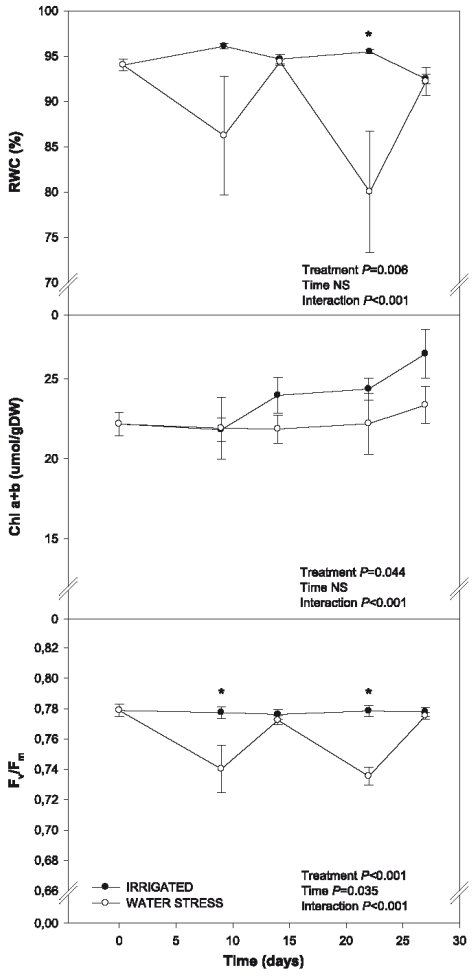

Fig. 1. Relative water content (RWC), chlorophyll a+b (Chl a+b) contents and F_v/F_m ratio in leaves of *Zea mays*. Data represent the mean ± SE of n = 5 individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA, $P < 0.05$). An asterisk indicates significant differences between water-stressed and irrigated plants (Duncan posthoc tests, $P < 0.05$).

For pigment analysis, leaf samples (50 mg) were ground in liquid nitrogen and extracted with cold methanol (v/v) using ultrasonication. After centrifuging at 8000 rpm for 10 min and 4 °C, the supernatant was collected and the pellet re-extracted with the same solvent until it was colorless; then, supernatants were pooled and total chlorophylls and carotenoids estimated spectrophotometrically as described by Lichtenthaler (1987).

The maximum efficiency of the photosystem II (F_v/F_m ratio) was

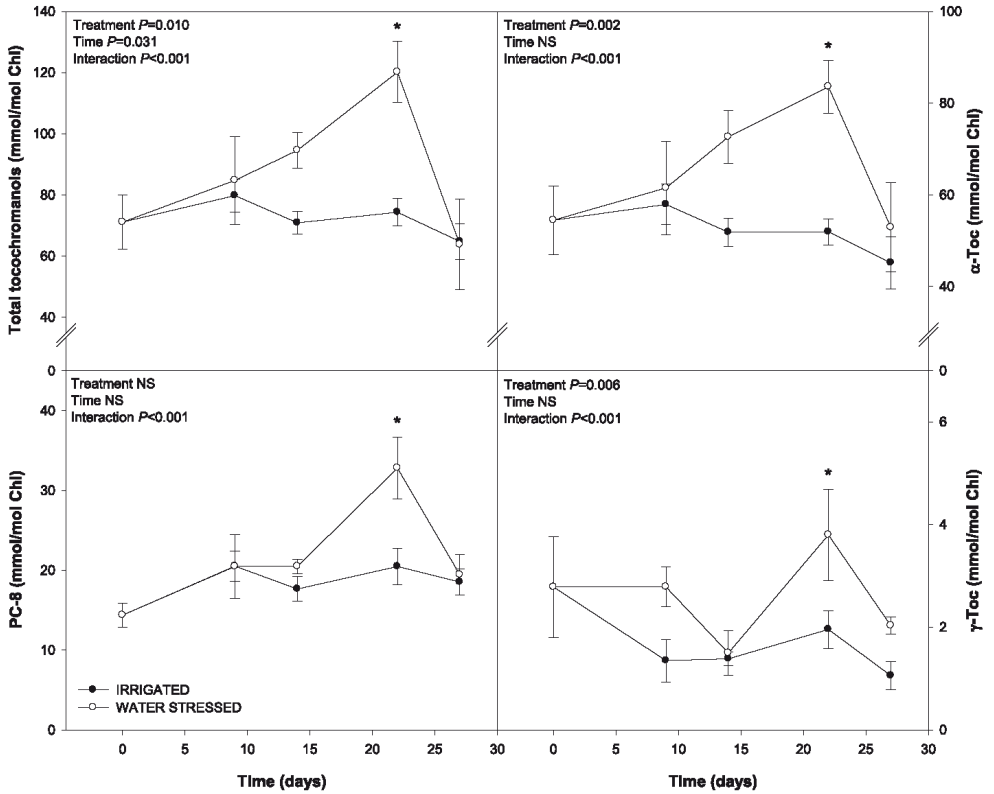

Fig. 2. Contents of total tocochromanols, plastochromanol-8 (PC-8), γ-tocopherol (γ-Toc) and α-tocopherol (α-Toc) in leaves of *Zea mays*. Data represent the mean ± SE of n = 5 individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA, $P < 0.05$). An asterisk indicates significant differences between water-stressed and irrigated plants (Duncan posthoc tests, $P < 0.05$).

determined measuring the chlorophyll fluorescence of leaves at predawn by using a pulse-modulated fluorimeter (Mini PAM; Walz, Effeltrich, Germany).

2.3. PC-8 and vitamin E contents

For analyses of PC-8 and vitamin E contents, leaf samples (50 mg) were ground in liquid nitrogen and extracted with cold methanol (v/v) using ultrasonication. After centrifuging at 8000 rpm for 10 min and 4 °C, the supernatant was collected and the pellet re-extracted with the same solvent until it was colorless; then, supernatants were pooled, filtered and injected into the HPLC. PC-8 and vitamin E contents were separated isocratically on a normal-phase HPLC system using a fluorescent detector as described (Cela et al., 2011). Compounds were identified by co-elution with authentic standards and quantified by using a calibration curve.

2.4. Profiling of stress-related phytohormones

For analyses of ABA, jasmonic acid and salicylic acid, leaf samples (50 mg) were ground in liquid nitrogen and extracted with cold methanol (v/v) using ultrasonication. After centrifuging at 8000 rpm for 10 min and 4 °C, the supernatant was collected and the pellet re-extracted with the same solvent until it was colorless; then, supernatants were pooled, filtered and injected into the UHPLC-MS/MS. Stress-related phytohormones were separated using an elution gradient on a reverse-phase UHPLC system and quantified using tandem mass spectrometry in multiple reaction monitoring mode as described (Müller and Munné-Bosch, 2011).

2.5. Statistical analysis

Data was analyzed by using two-way factorial analysis of variance (ANOVA) with treatment and time (sampling day) as factors, and by additionally using Duncan posthoc tests. In all cases, differences were considered significant at a probability level of $P < 0.05$. All statistical tests were carried out using the SPSS 20.0

statistical package. Furthermore, linear regressions between ABA and tocochromanol contents were performed by using SigmaPlot (version 10.0, Systat, USA).

3. Results and discussion

3.1. Plant response to reiterated drought

A first exposure to drought stress for 9 days did not significantly decrease the relative water content in maize leaves, while a second drought stress exposure of a similar intensity and duration significantly reduced the relative leaf water content from 95% to 80% (Fig. 1). In contrast, the maximum efficiency of photosystem II photochemistry (F_v/F_m ratio) decreased significantly and to a similar extent during both the first and the second drought, attaining values of 0.74, which are indicative of photoinhibition to the photosynthetic apparatus (Long et al., 1994; Werner et al., 2002; Takahashi and Murata, 2008). However, the F_v/F_m ratio fully recovered during both the first and second re-watering periods to attain initial values (Fig. 1). Chlorophyll a+b contents differed between irrigated and drought-stressed plants, differences being more clearly evidenced after the first drought (Fig. 1). Therefore, although reductions in the leaf water contents and chlorophyll contents seemed to accumulate under reiterated drought, the integrity of photosystem II seemed to respond similarly during both stresses, being equally well recovered during drought periods. Although several studies have focused on understanding mechanisms underlying photosystem II integrity during water deficit and other abiotic stresses (reviewed by Takahashi and Murata, 2008), fewer studies report on changes in photosystem II integrity in drought-recovery cycles or, in other words, the study of photoinhibition and mechanisms to prevent it under reiterated drought (Munné-Bosch and Alegre, 2000; Munné-Bosch and Peñuelas, 2003; Gomes et al., 2008). It is however highly relevant to study such responses because reiterated drought is more common than single periods of drought stress in both agro- and natural ecosystems.

3.2. PC-8 contents increase in response to reiterated drought

PC-8 accounted for more than 25% of tocochromanols in maize leaves both in irrigated and drought-stressed plants (Fig. 2). Among the tocochromanols examined (PC-8 and the four tocopherol and tocotrienol homologues: α, β, γ and δ), α-tocopherol was the major form, followed by PC-8 and then γ-tocopherol (Fig. 2). Absolute values shown here for PC-8 and tocopherols (Fig. 2) were similar to those reported for maize plants previously (Dłużewska et al., 2016). The time course evolution of the three tocochromanols detected and quantified in maize leaves (PC-8, and α- and γ-tocopherol) was similar, with significant increases during the second period of drought only (Fig. 2). Total tocochromanol contents in drought-stressed plants increased by 70%, those of α-tocopherol and PC-8 by 65%, and those of γ-tocopherol doubled relative to irrigated plants (Fig. 2). Tocopherol contents have been reported to increase in response to drought in several species (reviewed in Munné-Bosch, 2005), but the response of PC-8 to drought has not been investigated thus far, being the present one the first study showing significant increases in the contents of this antioxidant in drought-stressed plants. It is worthy to note that the dynamics of drought-induced changes in PC-8 and α-tocopherol were very similar, with significant increases during the second drought only, and following a very similar trend during the first drought and during both recovery phases (Fig. 2). In contrast, γ-tocopherol contents, which represented less than 5% of total tocochromanols, seemed to be more responsive to the recovery phases, particularly after the first drought (Fig. 2).

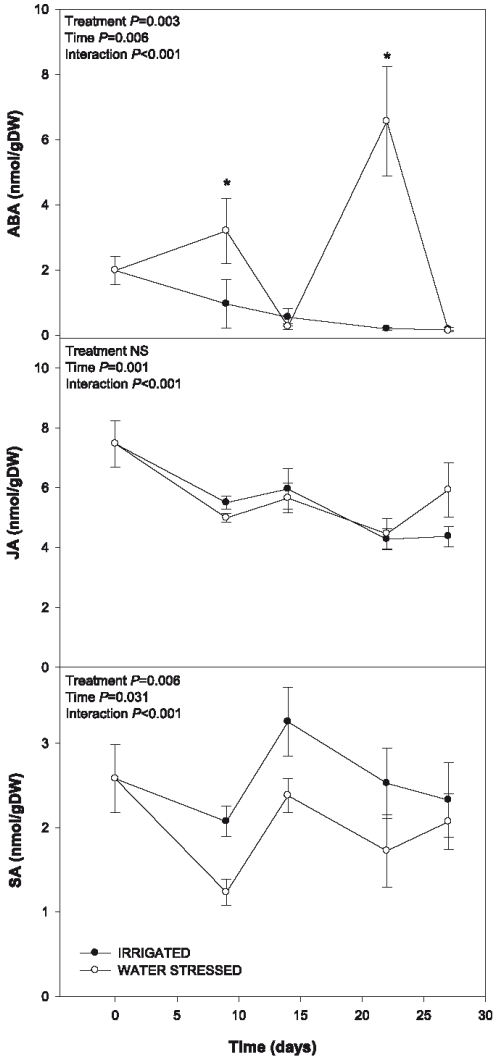

Fig. 3. Contents of ABA, jasmonic acid (JA) and salicylic acid (SA) in leaves of *Zea mays*. Data represent the mean ± SE of n = 5 individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA, $P < 0.05$). An asterisk indicates significant differences between water-stressed and irrigated plants (Duncan posthoc tests, $P < 0.05$).

Drought training seemed to enhance tocochromanol accumulation in leaves. PC-8 and both α- and γ-tocopherol significantly differed between drought-stressed and irrigated plants during the second period of drought only. The first period of drought had a significant effect on the F_v/F_m ratio (Fig. 1), but not on the contents of tocochromanols (Fig. 2). Interestingly, however, tocochromanol contents increased significantly during the second drought, while

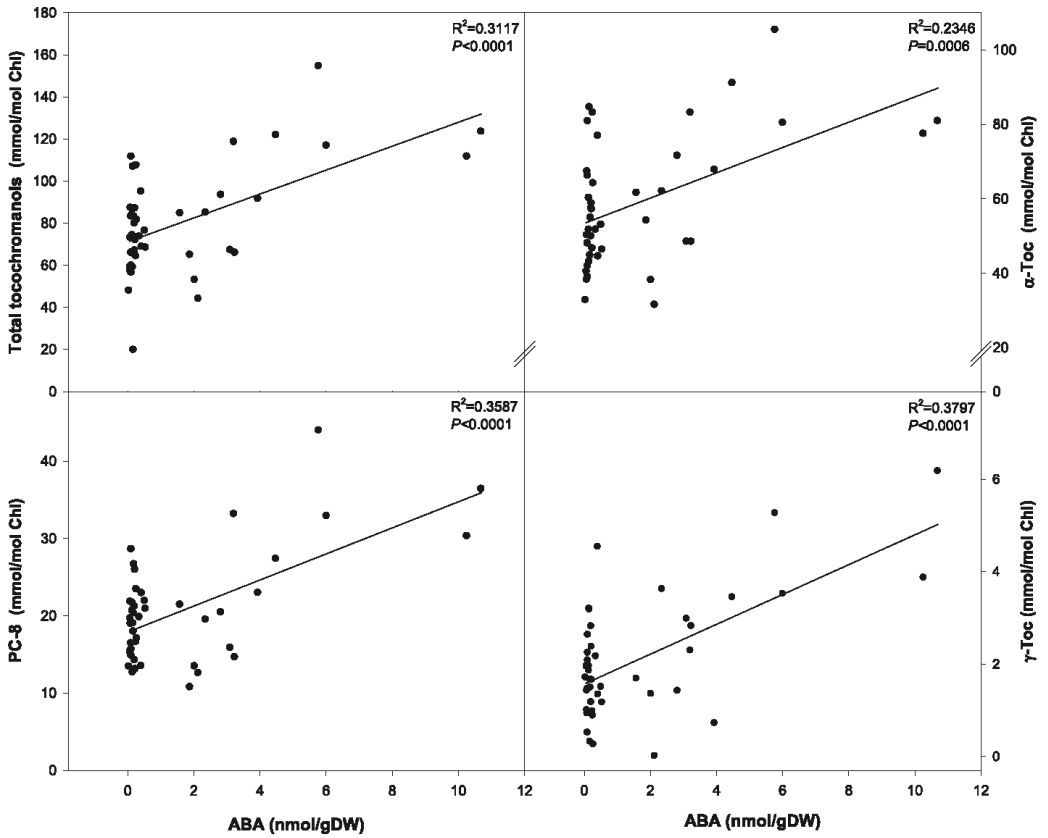

Fig. 4. Linear regression between contents of ABA and total tocochromanols, plastochromanol-8 (PC-8), γ-tocopherol (γ-Toc) and α-tocopherol (α-Toc) in leaves of *Zea mays*.

F_v/F_m ratio did not decrease further, despite the significant reductions in the relative leaf water content and chlorophyll contents (Fig. 2). Since it is known that α-tocopherol protects thylakoid membranes from lipid peroxidation and helps maintain, together with carotenoids, the integrity of photosystem II in plants subjected to water deficit and other abiotic stresses (Havaux et al., 2005; Espinoza et al., 2013), results suggest that accumulation of α-tocopherol during the second drought may help prevent damage to the photsynthetic apparatus, allowing plants to recover photosystem II integrity after drought. Furthermore, results suggest that the first drought was very mild but probably helped plants to better respond to a second drought preventing damage to photosystem II by increasing tocopherol contents. Since (i) PC-8 is an efficient quencher of singlet oxygen (Rastogi et al., 2014), even more than α-tocopherol in hydrophobic environments due to its longer and more unsaturated prenyl chain (Gruszka et al., 2008), and (ii) singlet oxygen can significantly reduce photosystem II efficiency by damaging and altering the turnover of the D1 protein (Nishiyama et al., 2004), results support a protective role for PC-8 in thylakoid membranes of maize leaves. However, it is still to be determined if both α-tocopherol and PC-8 play a synergistic protective role, or if they differentially accumulate within the PSII reaction center, the thylakoid membrane and plastoglobules, which should be investigated in future studies.

3.3. PC-8 and α-tocopherol contents may be modulated by ABA under drought, but not during recovery

PC-8 and vitamin E accumulation may be modulated by ABA contents. Previous studies have shown the presence of an ABA-responsive element (ABRE) in the promoter region of *hydroxyphenylpyruvate dioxygenase* (*hppd*), which encodes for the enzyme responsible of the formation of homogentisate, needed for the biosynthesis of both PC-8 and all vitamin E compounds (Falk and Munné-Bosch, 2004; Chaudhary and Khurana, 2009). Therefore, it is likely that ABA regulates the biosynthesis of vitamin E compounds, as it has been shown in previous studies (Chaudhary and Khurana, 2009; Munné-Bosch et al., 2009), and that of PC-8. Furthermore, jasmonates have been shown to be involved in the regulation of *tyrosine aminotransferase* (*tat*), which is needed for hydroxyphenylpyruvate biosynthesis (Sandorf and Holländer-Czykto, 2002). Other studies have also shown a strong positive correlation between salicylic acid and tocopherols (Munné-Bosch and Peñuelas, 2003), although no molecular evidence of such

relationship has been provided yet. Here, we found that drought stress caused a significant increase of ABA contents in maize leaves, showing the highest increases during the second drought (Fig. 3), which is consistent with stronger decreases in the relative leaf water content compared to the first drought (Fig. 1). Interestingly, however, jasmonic acid contents did not differ between irrigated and drought-stressed plants, and those of salicylic acid decreased under drought (Fig. 3). Therefore, from the three classes of stress-related phytohormones examined, ABA was the one showing parallel changes with PC-8 and vitamin E compounds, particularly with γ-tocopherol (Figs. 2 and 3). Regression analyses revealed a positive relation between contents of ABA and those of tocochromanols, with the strongest one being observed for γ-tocopherol, followed by PC-8, and finally α-tocopherol (Fig. 4). The latter was the one showing a more slow response during recovery phases (Fig. 2), while ABA contents recovered very fast during re-watering (Fig. 3), which might explain the smaller regression values obtained between ABA and α-tocopherol, compared to those obtained for ABA and PC-8, or ABA and γ-tocopherol (Fig. 4). Another reason was the absence of significant increases of PC-8 and vitamin E contents during the first drought (Fig. 2), while ABA contents increased during this period (Fig. 3). In any case, however, our results support the contention that tocochromanol contents may be modulated, at least in part, by drought-induced increases in ABA contents. This was not only observed for vitamin E compounds, but also for PC-8, which may have important implications for our understanding of the regulation and manipulation of the biosynthesis of this antioxidant in crops.

4. Conclusion

It is concluded that (i) PC-8 accounts for more than 25% of tocochromanols in maize leaves, (ii) PC-8 contents increase in response to repeated periods of drought stress, (iii) drought training may increase PC-8 and vitamin E accumulation in leaves, and (iv) PC-8 and vitamin E accumulation may be modulated by ABA contents. Further research is needed to better understand the differential regulatory mechanisms governing the biosynthesis of PC-8 and vitamin E during recovery phases, as well as the possible synergistic effects exerted by PC-8 and α-tocopherol in the protection of photosystem II.

Contribution

Eva Fleta-Soriano carried out experiments, analyzed and discussed the results. Sergi Munné-Bosch planned and wrote the paper.

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CAPÍTULO 5

La melatonina puede ejercer un papel protector frente al estrés por sequía en maíz

CHAPTER 5

Melatonin may exert a protective role against drought stress in maize

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RESUMEN DEL CAPÍTULO 5

La melatonina (N-acetil-5-metoxitryptamina) es un compuesto anfifilico de bajo peso molecular encontrado en organismos vivos evolutivamente distantes, desde bacterias a mamíferos. Puede ser sintetizada por plantas y actuar en ellas como un potente antioxidante y/o como regulador del crecimiento y desarrollo. Aquí investigamos el papel de la melatonina en la respuesta al déficit hídrico y la recuperación en plantas de maíz (*Zea mays*), con un énfasis en su posible papel como fotoprotector y antioxidante y/o su función en la señalización relacionada con las fitohormonas vinculadas con el estrés, ácido abscísico, ácido salicílico y ácido jásmonico. Los resultados muestran una correlación positiva entre concentraciones endógenas de melatonina y fotoprotección, como indica la máxima eficiencia del fotosistema II (relación F_v/F_m), lo cual fue confirmado adicionalmente mediante aplicaciones exógenas de melatonina durante la recuperación del déficit hídrico. Las aplicaciones de melatonina durante la recuperación del déficit hídrico mejoraron la relación F_v/F_m en las plantas de maíz. Además, las concentraciones endógenas de melatonina se correlacionaron positivamente con las de las fitohormonas vinculadas al estrés, especialmente con la del ácido salicílico, aunque las aplicaciones exógenas de melatonina no alteraron los niveles de estos compuestos de defensa. Se concluye que en las plantas de maíz expuestas al déficit hídrico la melatonina puede ejercer un papel defensivo, en particular mejorando la eficiencia fotoquímica del fotosistema II.

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Melatonin may exert a protective role against drought stress in maize

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Abstract

Melatonin (*N*-acetyl-5methoxytryptamine) is an amphiphilic low-molecular-weight compound found in evolutionary distant living organisms, from bacteria to mammals. It can be synthesized by plants and acts as a potent antioxidant and/or a regulator of plant growth and development. Here, we investigated the role of melatonin in plant response to drought stress and recovery in maize (*Zea mays* L.) plants, with an emphasis on its possible photoprotective and antioxidant role and/or signalling function in relation to the stress-related phytohormones, abscisic acid, salicylic acid and jasmonic acid. Results show a positive correlation between endogenous contents of melatonin and photoprotection, as indicated by the maximum efficiency of photosystem II photochemistry (F_v/F_m ratio), which was confirmed further by exogenous application of melatonin during recovery from drought stress. Melatonin applications during drought recovery improved the F_v/F_m ratio in maize plants exposed to a subsequent drought stress. Furthermore, endogenous contents of melatonin positively correlated with those of stress-related phytohormones, particularly with those of salicylic acid, although exogenous application of melatonin did not alter the contents of these defence compounds. It is concluded that melatonin can exert a defensive role in maize plants exposed to drought stress, particularly improving the efficiency of photosystem II photochemistry.

KEYWORDS
abscisic acid, drought stress, jasmonic acid, melatonin, salicylic acid, vitamin E, *Zea mays*

1 | INTRODUCTION

Melatonin (*N*-acetyl-5methoxytryptamine) is an amphiphilic low-molecular-weight compound found in seeds, roots, stems, shoots, leaves, flowers, bulbs and fruits, and its content ranges, depending on the organ, species and growth conditions, from picograms to micrograms per gram of tissue (Arnao & Hernández-Ruiz, 2014). Plants can take it from the soil but also synthesize it from L-tryptophan (Nawaz et al., 2016). The role of melatonin in plants is still not fully understood, and there is currently an active discussion whether this compound acts as an antioxidant and/or as a growth regulator (Arnao & Hernández-Ruiz, 2014).

As a growth regulator, melatonin seems to have auxin activity regulating both root and shoot development but acting independently of auxin signalling (Pelagio-Flores et al., 2012). Also, it may be involved in the control of fruit development and in delaying senescence (Gao et al., 2016; Liang et al., 2015). Besides it has been described that seed priming with melatonin increases crop yield (Janas, Malgorzata, & Posmyk, 2013) and that exogenous melatonin can improve drought tolerance in tomato (Liu, Wang, Wang, & Sun, 2015). However, melatonin crosstalk with stress-related phytohormones has not been studied thus far, except for two studies linking melatonin with ABA (Li, Tan, Liang, et al., 2015; Li, Tan, Jiang, & Liu, 2016). In the first study, it was shown that melatonin pre-treatment represses the expression of *MdNCED3* (involved in ABA biosynthesis) and promotes the upregulation of *MdCYP707 A1* and *MdCYP707 A2* (involved in ABA catabolism), reducing this way ABA contents under drought stress in

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two *Malus* species (Li, Tan, Liang, et al., 2015). In the second one, it was shown that exogenously applied melatonin in drought-primed barley plants results in higher ABA contents when exposed to cold stress (Li et al., 2016).

As an antioxidant, melatonin has been shown to have higher antioxidant capacity than vitamins E, C and K in some systems (Tan et al., 2007). Such roles make this molecule very useful in agronomic applications aimed at increasing crop production. Melatonin has been shown to increase crop tolerance against cold, drought, heat, chemical pollutants and other abiotic stressors, but also against biotic stressors such as pathogens (Tan et al., 2012; Yin et al., 2013). Furthermore, it has been shown to increase seed germination under stress (Zhang et al., 2014), stimulate root development (Pelagio-Flores et al. 2012) and protect the photosynthetic apparatus in green algae (Lazar et al., 2013).

Vitamin E, a potent group of lipophilic antioxidants that include both tocopherols and tocotrienols with four homologues each (α-, β-, γ-, δ-), is exclusively synthesized in photosynthetic organisms. Vitamin E is preferentially found in the form of α-tocopherol, and to a lesser extent γ-tocopherol, in leaves of higher plants (Miret & Munné-Bosch, 2015). Together with carotenoids, tocopherols play a key role preserving photosystem II structure and function, as well as the functionality of thylakoid membranes by quenching and scavenging reactive oxygen species. Furthermore, tocopherols specifically inhibit the propagation of lipid peroxidation. As a result, variations in vitamin E contents have been used as a stress marker (Munné-Bosch, 2005). It has been previously shown that vitamin E biosynthesis may be regulated by ABA (Chaudhary & Khurana, 2009; Munné-Bosch et al., 2009), so that drought-induced increases in this phytohormone may lead to enhanced vitamin E-mediated photoprotection and antioxidant protection (Fleta-Soriano, Pintó-Marijuan, & Munné-Bosch, 2015).

Here, we hypothesized that melatonin may exert a role in maize plants against drought stress. We investigated the interplay between melatonin, stress-related phytohormones and vitamin E under drought stress by (i) measuring the endogenous contents of melatonin, and those of stress-related phytohormones and vitamin E together with some physiological stress markers during water stress and recovery, and (ii) evaluating the effects of exogenous applications of melatonin in the irrigation water in the response of maize plants to reiterated drought stress.

2 | MATERIALS AND METHODS

2.1 | Plant material and samplings

Seeds of maize (*Zea mays* L.) plants were sown in 2-L pots on peat: perlite:vermiculite (2:1:1, v/v) in a greenhouse at the Faculty of Biology at the University of Barcelona (Barcelona, NE Spain). After 2 months of growth, plants were transferred to 9-L pots with the same substrate and were watered daily with half-diluted Hoagland nutrient solution. Then, two independent experiments were performed on juvenile (non-flowering plants), as follows.

For the first experiment, plants were exposed on 15th March 2014 to one treatment with two levels: no irrigation and full irrigation. Half the plants were subjected to drought stress (water-stressed plants) by withholding water for 8 days, followed by a recovery period of 5 days, while the other half (irrigated plants) were watered every other day with half-diluted Hoagland nutrient solution during the whole experiment. Samplings were performed at the beginning of the experiment (day 0) and after 8 and 13 days of treatment, that is at the points of maximum stress and during recovery.

For the second experiment, another set of plants were exposed to water deficit stress as described for experiment 1, but then, during recovery, 1 mM melatonin was added to the irrigation water (half-diluted Hoagland nutrient solution) to half of the plants (melatonin-treated plants), while no melatonin was added to the other half (control plants). After an additional period of 8 days of drought stress by withholding water, samples were taken on melatonin-treated and non-treated (control) plants. This experimental design allowed us to examine the effects of melatonin treatment in the irrigation water during recovery on the physiology of plants during a subsequent period of drought stress, that is whether or not melatonin applied with irrigation water can protect plants from reiterated periods of water deficit stress.

For both experiments, samplings were performed as follows. All samplings were performed at predawn (1 hr before dawn). At each sampling point, five plants for each treatment were randomly selected. The mid region of the blade of the fifth leaf from each individual was used to estimate the endogenous contents of melatonin, leaf water contents, photosynthetic pigment and lipid hydroperoxide contents, the F_v/F_m ratio, vitamin E contents, and the endogenous contents of the stress-related phytohormones, abscisic acid, jasmonic acid and salicylic acid. Samples for biochemical analyses were collected, immediately frozen in liquid nitrogen and stored at −80°C until analyses.

2.2 | Leaf water and pigments contents, F_v/F_m ratio and lipid peroxidation

The samples were weighed to estimate the fresh matter (FW), immersed in distilled water at 4°C for 24 hr to estimate the turgid matter (TW) and then oven-dried at 80°C to constant mass to estimate the dry matter (DW). Relative water content (RWC) was then calculated as 100 × (FW − DW)/(TW − DW).

For pigment analyses, the leaf samples (50 mg) were ground in liquid nitrogen and extracted with cold methanol using ultrasonication. After centrifuging at 3,000 g for 10 min and 4°C, the supernatant was collected and the pellet re-extracted with the same solvent until it was colourless; then, supernatants were pooled and chlorophylls and carotenoids estimated spectrophotometrically as described (Lichtenthaler, 1987).

The maximum efficiency of the photosystem II (F_v/F_m ratio) was determined at predawn (1 hr before dawn) by measuring the chlorophyll fluorescence of leaves in darkness with a pulse-modulated

fluorimeter (Mini PAM; Walz, Effeltrich, Germany) as described (Bilger, Schreiber, & Bock, 1995).

The extent of lipid peroxidation was estimated by measuring the contents of lipid hydroperoxides (primary stable products of lipid peroxidation) in leaves. Lipid hydroperoxides contents were estimated spectrophotometrically in methanolic extracts following the ferrous oxidation–xylenol orange assay as described (DeLong et al., 2002).

2.3 | Vitamin E contents

For analysis of vitamin E, 50 mg of frozen leaf tissue was ground in liquid nitrogen and extracted with cold methanol using ultrasonication. After centrifuging at 3,000 g for 10 min and 4°C, the supernatant was collected and the pellet re-extracted with the same solvent until it was colourless; then, supernatants were pooled, filtered and injected into the HPLC. Vitamin E contents were separated isocratically on a normal-phase HPLC system using a fluorescent detector as described (Cela, Chang, & Munné-Bosch, 2011). Compounds were identified by co-elution with authentic standards and quantified using a calibration curve.

2.4 | Melatonin and stress-related phytohormones

For analysis of melatonin, abscisic acid, jasmonic acid and salicylic acid, 50 mg of frozen leaf tissue was ground in liquid nitrogen and extracted with cold methanol using ultrasonication. After centrifuging at 3,000 g for 10 min and 4°C, the supernatant was collected and the pellet re-extracted with the same solvent until it was colourless; then, supernatants were pooled, filtered and injected into the UHPLC-MS/MS. Stress-related phytohormones were separated using an elution gradient on a reverse-phase UHPLC system and quantified using tandem mass spectrometry in multiple reaction monitoring mode as described (Müller & Munné-Bosch, 2011) Recovery rates (>80% in all cases) were calculated for each hormone on every sample using deuterated compounds.

2.5 | Statistical analysis

Data were analysed using two-way factorial analysis of variance (ANOVA) with treatment and time (sampling day) as factors for the first experiment, and one-way ANOVA for the second experiment, and by additionally using Duncan post hoc tests. In all cases, differences were considered significant at a probability level of $p < .05$. All statistical tests were carried out using the SPSS 20.0 statistical package (IBM Analytics, New York, NY, USA). Furthermore, Spearman correlations between melatonin and all other measured parameters, and stress-related phytohormones and vitamin E contents were performed using SigmaPlot (version 10.0, Systat Software Inc., Chicago, IL, USA).

3 | RESULTS

3.1 | Physiological effects of drought stress and recovery

We used various stress indicators, including the RWC, the F_v/F_m ratio, the extent of lipid peroxidation, chlorophyll (Chl) contents and the Chl a/b ratio, to evaluate the physiological effects of drought and recovery in maize plants (Figure 1). After 8 days of drought stress, the F_v/F_m ratio and the RWC decreased significantly in drought-stressed plants relative to controls, with reductions by 6% and 23%, respectively, while hydroperoxide contents, an indicator of lipid peroxidation, and Chl contents were not altered (Figure 1). However, the amount of Chls and the Chl a/b ratio were altered during recovery, so that drought-stressed plants showed lower Chl contents and higher Chl a/b ratios compared to irrigated plants during recovery (Figure 1). It is noteworthy that Chl contents were affected during recovery, but not during stress, while the contrary occurred with the RWC and the F_v/F_m ratio (Figure 1).

While endogenous contents of melatonin were not affected either by drought stress or recovery in maize leaves, ABA contents increased 20-fold during drought stress to return to initial contents after 5 days of recovery, and those of jasmonic acid decreased by 30% relative to controls after 8 days of water deficit and recovered later (Figure 2). Furthermore, salicylic acid contents remained unaltered during the experiment, as it occurred with melatonin contents (Figure 2).

Among the vitamin E compounds studied, leaves of maize plants contained both α-tocopherol and its precursor, γ-tocopherol, with the former being more abundant (Figure 3). Water deficit stress doubled α-tocopherol contents, while those of γ-tocopherol were not affected. When expressed on a Chl basis, however, it was clear that both α- and γ-tocopherol contents were affected by drought stress, as indicated by enhanced contents of both homologues during recovery. Contents of both α- and γ-tocopherol per unit of Chl were threefold higher in drought-stressed plants than in irrigated plants during recovery (Figure 3), which was partly due to the 50% reductions in Chl contents in drought-stressed plants compared to irrigated plants during recovery (Figure 1). Total carotenoid contents changed in parallel with Chls, showing a reduction by 35% in water-stressed plants compared to controls during recovery only (Figure 4).

In summary, while drought stress transiently affected leaf water contents, PSII efficiency, ABA, α-tocopherol and jasmonic acid contents; changes in photosynthetic pigment contents occurred during recovery only.

3.2 | Physiological effects of exogenous melatonin application

To elucidate to what extent melatonin applications in the irrigation water may influence subsequent drought stress responses in maize plants, 1 mM melatonin was applied with irrigation water during recovery and the effect evaluated after 8 additional days of drought

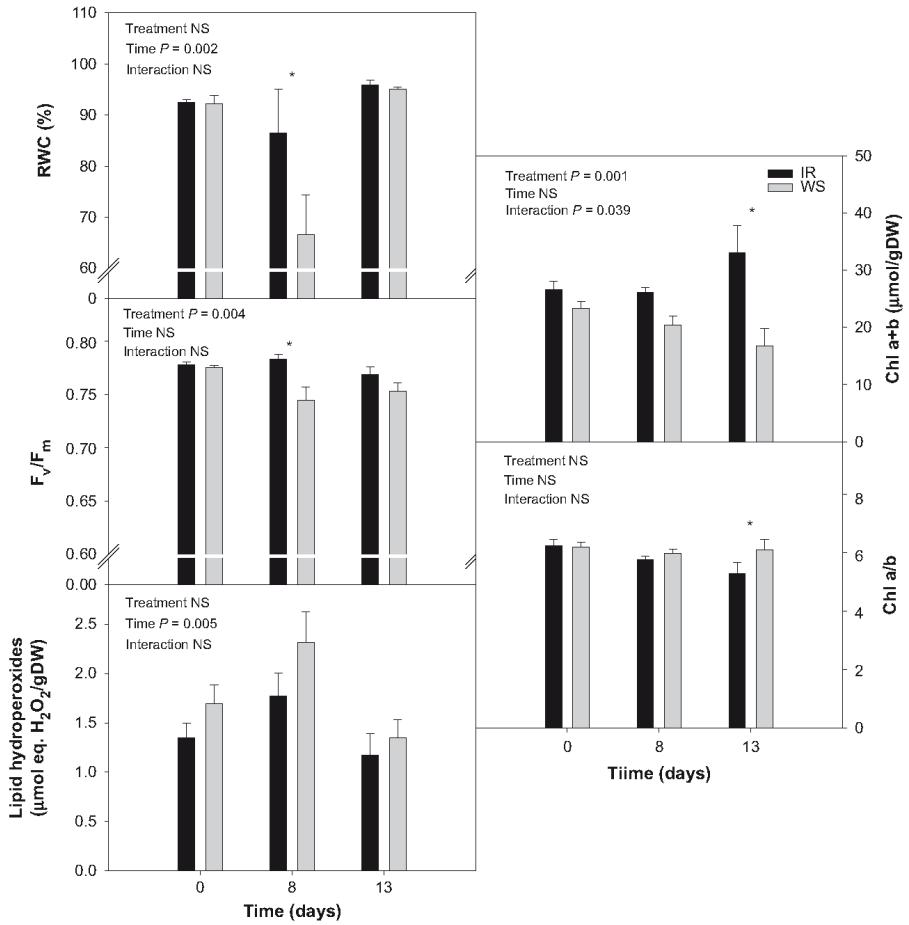

FIGURE 1 Relative water content (RWC), F_v/F_m ratio, extent of lipid peroxidation (estimated as lipid hydroperoxide contents), chlorophyll (Chl) a+b contents and Chl a/b ratio in leaves of maize plants exposed to water stress and recovery. Data represent the mean ± SE of $n = 5$ individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA, $p < .05$). An asterisk indicates significant differences between drought-stressed and irrigated plants (Duncan post hoc tests, $p < .05$). DW, dry matter

stress. After 13 days of melatonin application with irrigation water (5 days of recovery plus 8 days of additional water deficit stress), endogenous contents of melatonin were 3.5-fold higher in treated plants compared to non-treated plants (controls, Figure 5). Melatonin contents just after application (24 hr after the application in the irrigation water during recovery) attained contents by up to 4.2 µmol/gDW (data not shown) to decrease to 7 nmol/gDW at the time of samplings, so endogenous melatonin contents were reduced by 99.8% during the time elapsed between drought recovery and the subsequent period of drought stress. Among all parameters analysed, melatonin treatment had a positive effect on the F_v/F_m ratio only, but this was significant. The F_v/F_m ratio was maintained at 0.787, that is 7% higher than non-treated, control plants (with values of 0.735, Figure 6). This improvement was not observed after 24 hr of application during recovery (data not shown), but during the subsequent period of water deficit stress. Despite differences were small quantitatively, it is noteworthy that melatonin applications kept the F_v/F_m ratio above 0.75 in drought-stressed plants, a value below which photodamage occurs. Leaf water, pigment, hydroxyperoxide, vitamin E and stress-related phytohormone contents were, however,

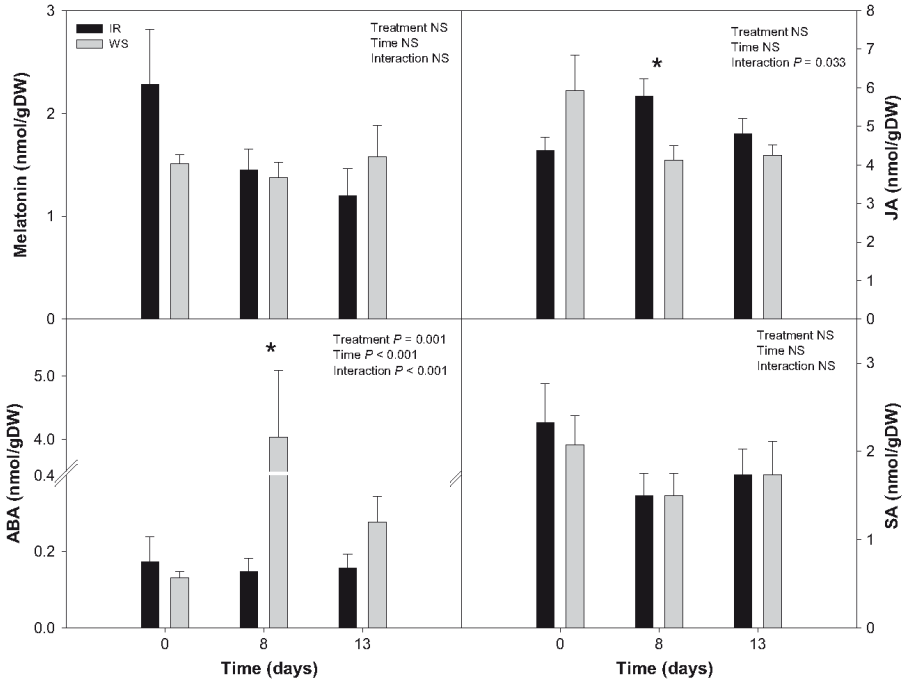

FIGURE 2 Endogenous contents of melatonin, abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) in leaves of maize plants exposed to water stress and recovery. Data represent the mean ± SE of $n = 5$ individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA, $p < .05$). An asterisk indicates significant differences between drought-stressed and irrigated plants (Duncan post hoc tests, $p < .05$). DW, dry matter

not affected by melatonin application neither just after application (24 hr later, data not shown) nor during a subsequent period of water stress (Figs S1-S4).

In summary, exogenous application of melatonin with irrigation water during the recovery phase improved the efficiency of the photosynthetic apparatus, as indicated by increases in the F_v/F_m ratio, during a subsequent period of water deficit stress in maize plants, while other parameters measured in this study were not altered.

4 | DISCUSSION

Melatonin may exert a protective effect against both abiotic and biotic stresses in plants, but mechanisms are still relatively unknown, with some studies suggesting it acts as an antioxidant and others as a growth regulator (Arnao & Hernández-Ruiz, 2014). In the present study, endogenous melatonin was found at relatively low contents in leaves, in the nmol/gDW range, which is comparable to the contents found for stress-related phytohormones. Indeed, melatonin contents were in the range of those found for salicylic acid, jasmonic acid and ABA, thus suggesting melatonin may exert an endogenous hormonal effect in maize leaves. While the water deficit stress experienced by maize plants during 8 days of withholding water significantly decreased RWC and F_v/F_m, increased ABA contents and decreased those of jasmonic acid, melatonin contents remained unaltered. On the other hand, melatonin applications in the irrigation water improved the maximum efficiency of PSII efficiency during a subsequent period of water deficit stress, an effect that seemed to occur independently of changes in stress-related phytohormones, or variations in photosynthetic pigment and antioxidant (vitamin E and carotenoid) contents. The high concentrations of melatonin applied to maize plants in the irrigation water resulted in very high contents of endogenous melatonin in leaves 24 hr after the application, but endogenous contents decreased rapidly, thus suggesting a rapid consumption of this compound during recovery and the subsequent drought stress. Considering the positive effect on the F_v/F_m ratio, it is likely that exogenous melatonin could exert, at least in part, a direct antioxidant role, as this compound has been found to display high antioxidant properties (Gao et al., 2016). This is the first study that shows such a protective antioxidant role of melatonin in a crop

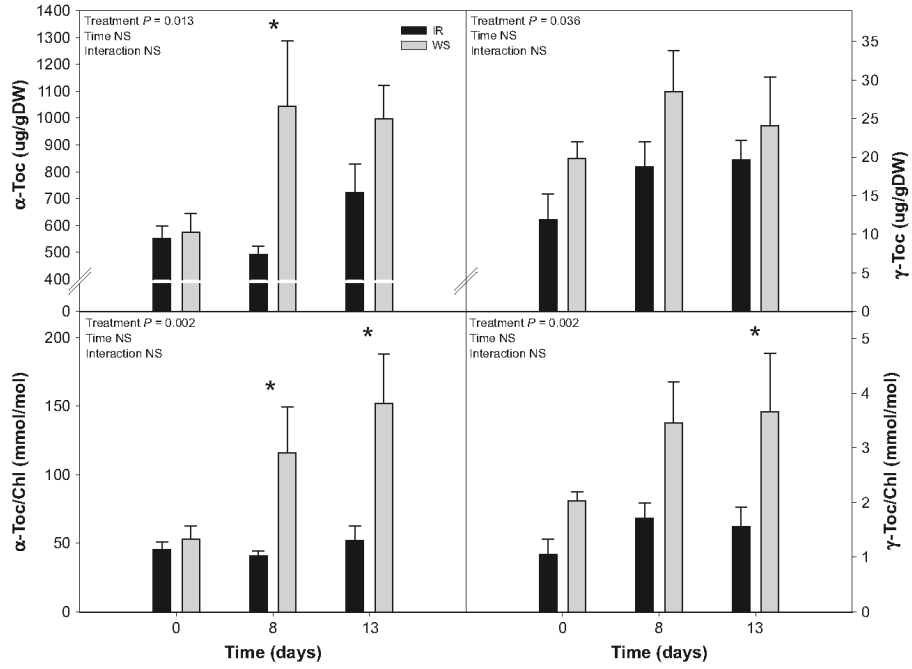

FIGURE 3 Endogenous contents of α-tocopherol (α-Toc) and γ-tocopherol (γ-Toc) in leaves of maize plants exposed to water stress and recovery. Data represent the mean ± SE of $n = 5$ individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA, $p < .05$). An asterisk indicates significant differences between drought-stressed and irrigated plants (Duncan post hoc tests, $p < .05$). DW, dry matter. Chl, chlorophyll a+b

plant, which further enlightens the strong potential of this compound in agriculture. However, the effect of exogenous melatonin was rather limited possibly because it was rapidly metabolized.

It has been suggested that melatonin evolved at the time organisms began their transition from an anaerobic to aerobic metabolism, so that the original and primary function of melatonin in organisms might has been to serve as an antioxidant to detoxify the free radicals generated during the process of aerobic metabolism (Gao et al., 2016). Although still relatively poorly studied to date, it has been suggested both chloroplasts and mitochondria synthesize melatonin in plants (Tan et al., 2013). The capacity of chloroplasts to synthesize melatonin is based on studies showing that rice serotonin *N*-acetyltransferase (SNAT, the penultimate step in melatonin biosynthesis) presents a transit sequence that targets the enzyme into chloroplasts (Byeon et al., 2014; Tan et al., 2013). Indeed, confocal microscopy studies have shown that SNAT is localized in chloroplasts, whereas *N*-acetylserotonin methyltransferase (ASMT), which catalyses the last step in melatonin biosynthesis, is found in the cytoplasm in rice (Byeon et al., 2014). Although it is still unknown whether or not melatonin can be transported into chloroplasts, it is very likely exogenous applications of melatonin resulted not only in an enhanced accumulation of melatonin in leaves, as shown in our study, but also in increased contents of this compound in chloroplasts, whose antioxidant properties might help in photoprotection, as indicated by improved F_v/F_m ratios in melatonin-treated plants.

Is melatonin synthesized by plants, however, exerting a protective role in vivo? In other words, could also endogenous melatonin play a protective role? To get some insight into this question, we applied Spearman's rank correlation analyses to our data. As indicated in Table 1, melatonin contents not only positively correlated with the F_v/F_m ratio, but also with Chl contents and the endogenous contents of stress-related phytohormones. Correlation coefficients obtained were, however, generally low (always below 0.5, and 0.129 for the F_v/F_m ratio), thus suggesting melatonin was not the main driver of protection to the PSII in maize plants against drought. Indeed, melatonin contents did not increase during drought stress, thus suggesting that (i) other antioxidants may endogenously play such a protective role, and (ii) endogenous melatonin plays only a minor role in the protection of the photosynthetic apparatus during drought stress in vivo.

Tocopherols have been shown to increase against drought and exert a photoprotective and antioxidant role in several species

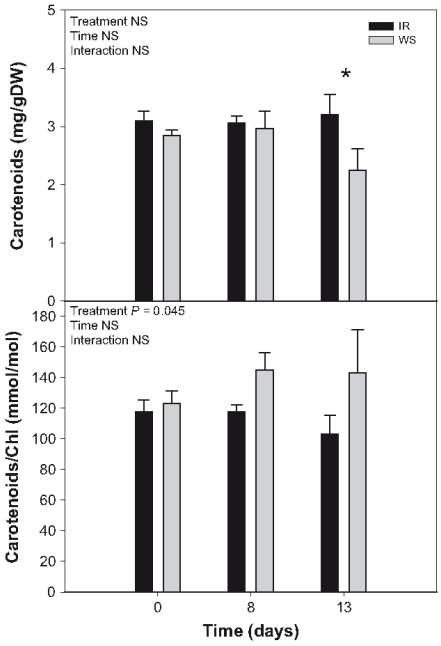

FIGURE 4 Endogenous contents of total carotenoids in leaves of maize plants exposed to water stress and recovery. Data represent the mean ± SE of $n = 5$ individuals. Significant differences between groups were tested by two-way analysis of variance (ANOVA, $p < .05$). An asterisk indicates significant differences between drought-stressed and irrigated plants (Duncan post hoc tests, $p < .05$). DW, dry matter. Chl, chlorophyll a+b

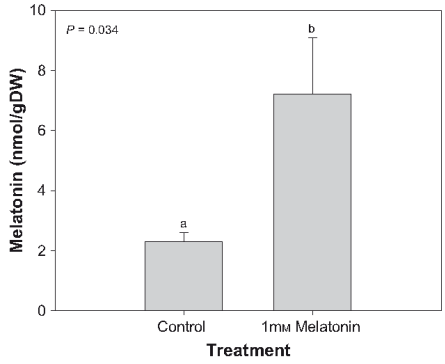

FIGURE 5 Endogenous contents of melatonin in leaves of maize plants treated with 1 mM melatonin in the irrigation water and exposed to a subsequent drought stress relative to non-treated plants (controls). Data represent the mean ± SE of $n = 5$ individuals. Significant differences between groups were tested by one-way analysis of variance (ANOVA, $p < .05$). Different letters indicate significant differences according to Duncan's post hoc tests ($p < .05$). DW, dry matter

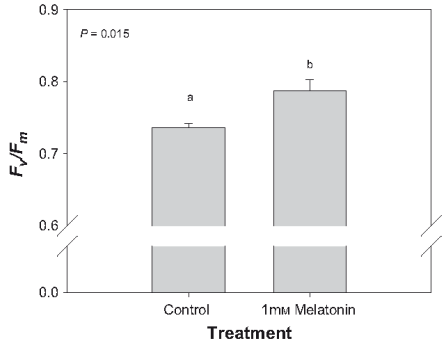

FIGURE 6 F_v/F_m ratio in leaves of maize plants treated with 1 mM melatonin in the irrigation water and exposed to a subsequent drought stress relative to non-treated plants (controls). Data represent the mean ± SE of $n = 5$ individuals. Significant differences between groups were tested by one-way analysis of variance (ANOVA, $p < .05$). Different letters indicate significant differences according to Duncan's post hoc tests ($p < .05$)

(Munné-Bosch, 2005). In the present study, α-tocopherol contents increased during water deficit stress, concomitantly with enhanced ABA contents, thus presumably helping protect the photosynthetic apparatus (Fleta-Soriano et al., 2015). Could this function be exerted in cooperation with melatonin? Variations in endogenous contents of tocopherols and melatonin in drought-stressed maize plants do not support this contention in vivo. Although α-tocopherol contents increased, those of melatonin remained unaltered. Furthermore, it should be noted that tocopherol contents were in the order of 1,000-fold higher than those of melatonin in maize leaves, which suggests tocopherols, rather than endogenous melatonin, exert a photoprotective role in chloroplasts of drought-stressed maize plants in vivo. The F_v/F_m ratio was improved by melatonin after exogenous application only, thus suggesting that high contents of melatonin may assist tocopherols in the protective role against photosystem II damage under drought stress.

It is concluded that melatonin at high concentrations can exert a protective role to the photosynthetic apparatus when applied exogenously at high concentrations with irrigation water during recovery periods of reiterated water deficit stress in plants. This has indeed important agricultural implications, as this system could be used to improve the plant response to drought stress during periods of reiterated water deficit stress in maize plants, an aspect that warrants investigation in other crops. However, the fact that the effect of exogenous melatonin was rather limited, possibly because it was

TABLE 1 Spearman rank's correlation coefficients and *p* values (in parentheses) between the endogenous contents of melatonin and the F_v/F_m ratio, pigment and antioxidant contents, and endogenous contents of the stress-related phytohormones in maize leaves

Parameter	Melatonin (nmol/gDW)
F_v/F_m	**0.129 (0.034)**
Chl a+b (μg/gDW)	**0.273 (<0.001)**
Chl a+b (μmol/gDW)	0.060 (0.323)
Chl a/b	0.049 (0.428)
α-Toc (μg/gDW)	−0.142 (0.094)
γ-Toc (μg/gDW)	−0.065 (0.447)
Carotenoids (mg/gDW)	−0.055 (0.371)
α-Toc/Chl (mmol/mol)	−0.046 (0.592)
γ-Toc/Chl (mmol/mol)	−0.02 (0.82)
Carotenoids (mg/mmol)	−0.086 (0.162)
ABA (nmol/gDW)	**0.269 (<0.001)**
JA (nmol/gDW)	**0.340 (<0.001)**
SA (nmol/gDW)	**0.412 (<0.001)**

Significant correlations are indicated in bold.
Chl, chlorophyll; Toc, tocopherol; ABA, abscisic acid; JA, jasmonic acid; SA, salicylic acid.

rapidly metabolized, indicates that this compound should be applied more frequently and/or in combination with other compounds that prevent its rapid degradation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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DISCUSIÓN

1.- Memoria al deficit hídrico

La memoria al estrés se puede dar y por tanto estudiar a diferentes niveles organizativos, aunque estos estén relacionados entre sí. En este caso, el estudio de la memoria al estrés se ha llevado a cabo a nivel de planta. Esta memoria al estrés en plantas se conoce como la capacidad de responder mejor frente a un estrés concreto cuando la planta ya ha estado previamente expuesta a dicho estrés, en comparación con plantas que se enfrentan a él por primera vez (Trewavas 2003, 2005). En este caso concreto, dicho estrés es la sequía, por lo que la memoria al déficit hídrico es la capacidad de las plantas de responder mejor frente a un episodio de déficit hídrico cuando previamente ya ha estado expuesta a episodios de sequía. Este proceso de memoria tiene gran importancia pues el ambiente está en constante cambio y las plantas deben ajustar su metabolismo y estructura para optimizar su crecimiento, reproducción y asegurar su supervivencia. Y aunque ya se han descrito procesos de memoria al déficit hídrico (Kinoshita & Seki 2014), como se discute en el **Capítulo 1**, aún falta mucho por comprender del todo los mecanismos implicados en este proceso.

Es muy difícil categorizar las diferentes repuestas que las plantas pueden llevar a cabo frente a la sequía pues todo está interrelacionado, y pueden ir desde cambios a nivel de planta entera (como la reducción en el área foliar) hasta cambios genéticos (en el ADN). A pesar de esto he tratado de hacer una clasificación entre aspectos morfológicos o estructurales y bioquímicos. Dentro de

los aspectos morfológicos he querido incluir los cambios más "clásicos" como la reducción en el área foliar, en la relación raíces/brotes o incluso los cambios estructurales producidos en el aparato fotosintético y la organización y forma de cloroplastos, dejando fuera cambios también estructurales pero a un menor nivel (genético) como cambios en histonas y cromatina. Por otro lado en los aspectos bioquímicos incluyo los cambios epigenéticos que son aquellos que modifican la actividad del ADN sin modificar su secuencia como puede ser la metilación; transcriptómicos que son cambios a nivel de la expresión génica; proteómicos a nivel de proteína; y metabolómicos a nivel de metabolitos (Singh et al. 2015).

1.1.- Aspectos morfológicos/estructurales

Los aspectos morfológicos o estructurales modificados por la memoria son el resultado de cambios bioquímicos, los cuales junto con la fisiología de la planta también se verán afectados en la respuesta a nuevos episodios de sequía, debido a los cambios estructurales producidos durante previas exposiciones al déficit hídrico. Estos cambios producidos por la capacidad de las plantas de recordar (recuerdo fisiológico y estructural) pueden darse a diferentes niveles organizativos.

El incremento de la relación clorofila a/b puede ser un indicador de la reducción del tamaño de la antena del PSII (Čajánek et al., 1999; Kurasová et al., 2000, 2002), ya que la clorofila a está

localizada en los centros de reacción y en las antenas mientras que la clorofila b esta principalmente en las antenas (Croce & Van Amerongen 2011). Este incremento se observó en los modelos de estudio previamente comentados en la introducción. En *Silene dioica* fueron observados tras la recuperación del estrés al final del experimento al comparar plantas sujetas a dos episodios de déficit hídrico (SS) con plantas expuestas a solo uno (**Capítulo 1**). Sin embargo en *Aptenia cordifolia* este cambio se observó antes de la recuperación (tras el estrés), comparando plantas SS con CS (**Capítulo 2**) pero también en plantas tres veces estresadas (SSS) comparadas con plantas expuestas solo a un episodio de sequía (CCS) (**Capítulo 3**). Además tanto en *Silene dioica* como en *Aptenia cordifolia* estos cambios se dan mientras la cantidad de clorofilas total permanece sin diferencias significativas entre los dos tratamientos, y en el caso de las plantas SSS de *Aptenia cordifolia* con un incremento en F_v/F_m respecto a las plantas CCS y un descenso en la cantidad de xantofilas implicadas en el ciclo de las xantofilas (VAZ). Estos cambios sugieren que el incremento en F_v/F_m tendría más que ver con la reducción en el tamaño de la antena que con el aumento de las xantofilas implicadas en la disipación de calor o en diferencias en la cantidad de vitamina E (**Capítulo 3**). En maíz también se observó una reducción en la relación clorofila a/b en las plantas expuestas a la sequía respecto a aquellas bien irrigadas durante la recuperación del estrés, aunque en este caso no puede comprobarse si el proceso de memoria se da en este parámetro pues no hay un control específico (CS) para poder comparar y poder determinar si la memoria se da o no

(**Capítulo 5**). Estos resultados sugieren que el cambio en la composición de los pigmentos foliares indica una reducción del tamaño de la antena en el fotosistema, lo cual puede ayudar a las plantas que se enfrenten a un nuevo estrés en el fututo a reducir la producción de ROS y el estrés oxidativo en los cloroplastos.

Estos resultados son solo un ejemplo de los cambios estructurales, del aparto fotosintético en este caso, producidos por la memoria al estrés para preparar a la planta a nuevos periodos de sequía. Sin embargo, además de los cambios aquí descritos, hay otros cambios estructurales en respuesta a sequías reiteradas descritos en otras especies. Estos cambios incluyen la reducción del tamaño y área foliar y de la relación brotes/raíces (Peña-Rojas et al., 2004, 2005); la reducción en el número de hojas (Zhang et al., 2014); cambios en la distribución radicular moviéndose estas a través del suelo en busca de agua (Kuster et al., 2012); y hasta cambios en la posición y estructura de cloroplastos (Zhang et al., 2014). Todos estos cambios reducirían la transpiración (reducción área foliar), incrementarían la captación de agua (cambios radiculares) y reducirían la cantidad de luz que llega al centro de reacción de los fotosistemas (reducción área foliar, cambios en los cloroplastos y reducción de la antena de fotosistemas) ya que bajo condiciones de déficit hídrico un exceso de luz puede acabar en la formación de ROS. Aunque que estos cambios ocurran depende no solo de las especies sino también de la severidad y duración del estrés al que las plantas son expuestas.

1.2.- Aspectos bioquímicos

Actualmente hay un gran interés en entender los aspectos bioquímicos involucrados en las diferentes respuestas de las plantas al déficit hídrico. Esto se debe al menos en parte al descubrimiento de los cambios epigenómicos y al desarrollo de técnicas de análisis masivo de genes que junto a las ideas proporcionadas por la proteómica y metabolómica han ayudado a completar el puzzle de las respuestas de las plantas al déficit hídrico (Ruan & Silva 2011).

El ABA está involucrado en la respuesta a la sequía por parte de las plantas. En *Aptenia cordifolia* se ha observado que los niveles de ABA bajo condiciones de sequía son mayores en plantas previamente expuestas a la sequía (SS) en comparación con plantas que no han estado expuestas previamente (CS), mientras el contenido hídrico relativo (RWC), el intercambio gaseoso y F_v/F_m permanecen sin diferencias entre los dos tratamientos, lo que indica memoria al estrés por sequía. El incremento del ABA durante episodios de sequía reiterados puede tener efectos en la regulación del crecimiento (Sharp & LeNoble 2001), el ajuste osmótico (Verslues & Bray 2006) y en la respuesta antioxidante (Alonso et al. 2015; Bao et al. 2015; Morales et al. 2015) incluyendo la síntesis de vitamina E (El Kayal et al. 2006; Munné-Bosch et al 2009).

Además previamente se ha visto que el ABA puede ejercer un papel protector bajo reiterados episodios de sequía reprogramando la expresión génica (Ding et al. 2012; Virlouvet et al. 2014; Virlouvet & Fromm 2015). Aunque en este estudio los niveles de

vitamina E no incrementan en las plantas SS comparado con las CS sí que existe una correlación positiva entre los niveles de ABA y los de vitamina E. Esta correlación también se observa en plantas de maíz y no solo con la vitamina E sino también con el PC-8, siendo la correlación más fuerte con el γ-tocoferol seguida por el PC-8 (**Capítulo 4**). Además la correlación entre ABA y vitamina E es apoyada por estudios previos realizados en *Aptenia cordifolia* (Cela et al. 2009) y también por estudios realizados en *Arabidopsis thaliana* (planta modelo) donde se observó que hay elementos de respuesta al ABA (ABREs) en la región promotora de genes implicados en la síntesis de vitamina E (Chaudhary &, Khurana 2009).

Aunque al mismo tiempo que incrementó el ABA se observó una disminución en la cantidad de violaxantina, carotenoide precursor en la síntesis de ABA (**Capítulo 2**), haría falta medir la cantidad y/o actividad de la enzima 9-*cis*-epoxicarotenoide dioxigenasa que está implicada en la biosíntesis de ABA a partir de los carotenoides (Xiong & Zhu 2003) para confirmar el efecto de memoria en el metabolismo del ABA. El ABA tiene un papel esencial en la respuesta al estrés por sequía y estos cambios en sus niveles pueden tener importantes efectos en la transcripción ya que algunos genes contienen elementos de respuesta al ABA (ABREs) en su región promotora (Evers et al. 2010). De hecho, Virlouvet & Fromm (2015) mostraron que plantas estresadas previamente tienen estomas que permanecen parcialmente cerrados durante el periodo de recuperación, lo cual reduce la traspiración en los siguientes estreses. Esta respuesta se dio además junto a un incremento de la expresión de las enzima 9-*cis*-epoxicarotenoide dioxigenasa 3

(NCED3) y aldehído oxidasa 3 (AAO3), las cuales son moduladores clave en la biosíntesis del ABA. Todo esto concuerda con los efectos de la memoria al estrés por sequía donde el ABA juega un papel clave en la regulación.

Pero además de estos cambios, modificaciones en las histonas y metilaciones en el ADN pueden desencadenar cambios importantes en la transcripción génica (Chinnusamy & Zhu, 2009). Las alteraciones en la estructura de la cromatina, como la modificación de la histona H3K4me3 en arroz, han sido asociadas a cambios en la expresión de algunos genes involucrados en el estrés por sequía (Chen et al., 2013). Además, también se ha visto hipermitilación en el ADN en la puntas de las raíces de plantas de guisantes también bajo condiciones de estrés hídrico (Labra et al., 2002). Por lo que los cambios en la estructura de la cromatina y metilación del ADN son actualmente considerados una respuesta general no solo al estrés por sequía sino también frente a otros estreses abióticos, otorgándole a la planta mecanismos de protección como la memoria al estrés pero también como la tolerancia cruzada (Urano et al. 2010; Kim et al. 2015). Sin embargo se requiere más investigación para saber si los cambios en la estructura de la cromatina y la metilación del ADN bajo condiciones de sequía son regulados por el ABA y determinar qué efectos produce a nivel de proteínas y metabolitos.

2.- Interés ecofisiológico y agronómico de la memoria al estrés

Las plantas no van a responder igual frente a un estrés si es la primera vez que se enfrentan a él o si es un estrés frecuente en su hábitat. Estas son capaces de incorporar "conocimientos" y responder mejor cuando vuelven a toparse con ese estrés concreto. Un estrés severo puede tener efectos muy negativos para la planta, sin embargo si el estrés no es muy severo y la planta puede recuperarse podrá responder mejor a los siguientes episodios de sequía, no solo con cambios epigenéticos sino también desarrollando diferentes respuestas fisiológicas relacionadas con la nueva estructura adaptada. Esta capacidad es muy útil para la supervivencia de las plantas ya que el medio ambiente está en continuo cambio y las plantas tienen que ser capaces de ajustar su fisiología a las nuevas condiciones para sobrevivir.

A nivel ecofisiológico la memoria permite a las plantas adaptarse mejor al ambiente en el que se encuentran y sus cambios. El estudio de la memoria y sus mecanismos nos permiten entender mejor como las plantas son capaces de adaptarse a las nuevas condiciones y por tanto nos ofrece la posibilidad de predecir mejor como van a responder las plantas frente a posibles cambios. De esta manera podría realizarse una mejor gestión de los recursos hídricos pero también de las distintas poblaciones de plantas ya sean autóctonas o introducidas como en el caso de *Aptenia codifolia*. En este último caso concreto la comprensión de la

memoria y sus mecanismos podrían explicar el porqué de su gran éxito estableciéndose en otros hábitats y ayudar a gestionar mejor la dispersión de esta especie.

El interés agronómico se basa en el potencial uso de la memoria para mejorar la productividad de los cultivos. Como ya se ha comentado en la introducción, la sequía es un estrés cada vez más frecuente y puede causar por tanto reducciones importantes en la producción de los cultivos. Importantes pérdidas en la producción de cultivos por causa de la sequía se han descrito en diferentes cultivos como arroz, patata, cebada o maíz (Farooq et al. 2009). Concretamente en maíz, según modelos de cambio climático en Portugal, se prevé que para 2061-2080 se reducirá un 17% la producción, valores similares a los que fueron encontrados en España con un 16%, o en Italia con un 20% (Yang et al. 2017). La memoria es pues un mecanismo muy útil teniendo en cuenta el escenario de continuo cambio en el que nos encontramos, ya que permite a los cultivos mejorar su respuesta al estrés tras haber sido expuestos previamente a este, por tanto esta capacidad de las plantas les permitiría "entrenarse" de cara a responder mejor a un estrés concreto. Además conociendo qué mecanismos y como están implicados en la memoria se podría actuar de una forma mucho más efectiva y concreta que simplemente exponiéndolas al estrés de cara a entrenar sus respuestas. Es por este motivo que la memoria es actualmente un tema a la orden del día, dada la gran importancia que puede tener a nivel agronómico y de incremento de producción, mitigando así la perdida de producción que puede darse debido al cambio climático.

3.- Función del plastocromanol-8 en plantas

Aunque el PC-8 está presente en varias especies no es ubicuo en el reino Plantae, por lo que se puede considerar un metabolito secundario, a diferencia de los tocoferoles que están presentes en la mayoría de organismos fotosintéticos (Esteban et al. 2009; Kruk et al. 2014). A parte de estar menos extendido que tocoferoles su cantidad en hojas también suele ser menor (Kurk et al. 2014). Aunque su síntesis se da en los plastoglóbulos es en los tilacoides donde realizan su función de antioxidante protegiendo a los lípidos de la peroxidación lipídica y previniendo el daño del PSII producido por el $^1O_2$ junto con los carotenoides (Munné-Bosch & Alegre 2002; Falk & Munné-Bosch 2010; Zbierzak et al. 2010; Kruk et al. 2014).

La fotosíntesis es una fuente importante de ROS, las cuales son generadas a través del proceso de transferencia de la energía y el transporte de electrones. Por este motivo existen numerosos mecanismos para prevenir el daño fotooxidativo en los cloroplastos, como por ejemplo el ciclo de las xantofilas o los movimientos de los cloroplastos, pero también la formación y disipación del $^1O_2$, que puede ser una válvula de seguridad importante para disipar el exceso de energía en plantas bajo estrés por sequía (Asada 2006; Noctor et al. 2014; Pintó-Marijuan & Munné-Bosch 2014). En este escenario el PC-8 podría pues tener una importante función junto a la vitamina E y los carotenoides en las membranas de los cloroplastos de plantas bajo episodios de sequía. Aunque aún no se co-

nocía anteriormente si su biosíntesis incrementaba durante la sequía en el **Capítulo 4** se observó un incremento del 65% en las cantidades de PC-8 en el segundo periodo de estrés tras exponer las plantas de maíz a sequías reiteradas, siendo además el PC-8 más del 25% de los tococromanoles presentes en las hojas de maíz. Este incremento en los valores de PC-8 se da en paralelo con los de α-tocoferol y en contraste con los de γ-tocoferol que representan menos del 5% de los tococromanoles, y que parecen responder más durante la recuperación de la sequía que durante el estrés. El entrenamiento de las plantas producido por la exposición reiterada a la sequía parece incrementar los niveles de tococromanoles en las hojas de maíz, ya que en la primera exposición a la sequía pese al descenso en el RWC y de F_v/F_m los niveles de tococromanoles no aumentan de manera significativamente. Sin embargo es en el segundo estrés, donde aunque el RWC es menor aun que en el primer estrés F_v/F_m no decrece más y se da el incremento en los niveles de tococromanoles, especialmente del PC-8 y del α-tocoferol. La función del α-tocoferol protegiendo la membrana del tilacoides de la peroxidación y la integridad del PSII junto carotenoides (Havaux et al. 2005; Espinoza et al. 2013) concuerda con el incremento de α-tocoferol en el segundo estrés que podría ayudar a proteger la integridad del PSII tras la sequía. Estos resultados sugieren que el primer estrés probablemente ayudó a las plantas a responder mejor frente al segundo y por tanto ayudando a prevenir el daño en el PSII con el incremento de tococromanoles, ya que el PC-8 es un eficiente *quencher* del $^1O_2$ (Rastogi et al. 2014) y este, puede reducir la eficiencia del PSII

dañando y alterando la reparación de la proteína D1 del fotosistema (Nishiyama et al. 2004). De hecho, la actividad del PC-8 es similar a la del γ-tocoferol o γ-tocotrienol en un solvente polar pero es mayor que cualquier tococromanol en ambientes hidrofóbicos ya que su cadena prenil es más larga e insaturada (Gruszka et al. 2008). Todos estos resultados apoyan por tanto el papel protector del PC-8 en la membrana de los tilacoides de las hojas de maíz. Sin embargo aún habría que determinar si el PC-8 actúa sinérgicamente con el α-tocoferol o si su acumulación es diferencial en el centro de reacción del PSII, la membrana del tilacoides y/o los plastoglóbulos.

4.- Función de la melatonina en plantas

La melatonina podría ejercer un papel protector frente a estreses tanto bióticos como abióticos en plantas, aunque mediante qué mecanismos no está tan claro. Algunos estudios sugieren que actuando como antioxidante pero otros estudios indican que como regulador del crecimiento (Arnao and Herna 2014; Gao et al. 2016).

Los contenidos endógenos de melatonina en hojas de plantas de maíz (entre 2-3 nmol/g PS) fueron comparables a los niveles de las fitohormonas relacionadas con el estrés como son el ABA, el ácido jasmónico y el ácido salicílico (**Capítulo 5**). Estos resultados sugieren que la melatonina podría ejercer un papel como hormona endógena en las hojas de maíz. Además, la aplicacion exó-

gena de melatonina a una concentración de 1mM en el agua de riego durante la recuperación en plantas de maíz, provocó un aumento en los contenidos endógenos de melatonina en hojas con valores de 4.2 µmol/gPS al cabo de 24 h, valores que disminuyeron hasta los 7 nmol/g PS 13 días después de la aplicación. Sugiriendo por tanto estos resultados que la planta cataboliza muy rápidamente la melatonina en hojas para mantenerla a niveles endógenos comparables más a los de los reguladores de crecimiento que a los de los antioxidantes endógenos.

Mientras que los valores de melatonina endógena en plantas de maíz expuestas a sequía no se vieron alterados como los de ABA, RWC y el ácido jasmónico, la aplicación exógena de melatonina en el agua de riego durante la recuperación del estrés hídrico mejoró la eficiencia máxima del PSII (como muestran los valores de la relación F_v/F_m) durante el siguiente periodo de déficit hídrico. Esta mejora en la eficiencia máxima del PSII parece suceder de manera independiente a cambios en hormonas relacionadas con el estrés o a variaciones en los contenidos de pigmentos fotosintéticos y antioxidantes, como muestran los valores de vitamina E, carotenoides y hormonas que se mantienen sin variaciones (**Capítulo 5**). Aunque tras la aplicación de melatonina sus niveles en hojas de maíz aumentaran de forma notoria, como se ha comentado anteriormente estos se reducían rápidamente en un 99.8% entre la recuperación y el siguiente estrés, sugiriendo un rápido consumo de melatonina por parte de la planta durante la recuperación y la siguiente sequía. Aunque hasta ahora no se habían mostrado

propiedades antioxidantes de la melatonina en un cultivo, considerando la mejora en la relación F_v/F_m en hojas de maíz es posible que la melatonina pueda ejercer, al menos en parte, un papel directo como antioxidante cuando se aplica exógenamente a altas concentraciones, ya que la melatonina ha demostrado tener importantes propiedades antioxidantes (Gao et al. 2016). Además aunque hasta la fecha aún está relativamente poco estudiado, se ha sugerido que tanto los cloroplastos como las mitocondrias sintetizan melatonina en las plantas (Tan et al. 2013), lo cual está apoyado por la idea de que la melatonina evolucionó en el momento en que los organismos comenzaron su transición de un metabolismo anaeróbico a aeróbico, de modo que la función original y primaria de la melatonina en organismos pudo haber sido la de antioxidante para eliminar los radicales libres generados durante el proceso de metabolismo aeróbico (Gao et al.2016). Y aunque se desconoce si la melatonina puede ser transportada o no a los cloroplastos, es posible que las aplicaciones exógenas de melatonina no solo aumenten la cantidad de esta en hojas sino también en cloroplastos donde las propiedades antioxidantes de la melatonina podrían ayudar en la fotoprotección como indica el incremento en F_v/F_m (**Capítulo 5**).

Considerando el hecho de que la melatonina exógena parece desempañar un papel protector como antioxidante, cabe preguntarnos lo siguiente: ¿podría la melatonina endógena sintetizada por las plantas ejercer también este papel protector? Según muestran las correlaciones de Spearman de los datos del **Capítulo 5**, el contenido de melatonina no sólo correlacionó positivamente con la relación F_v/F_m, sino también con los contenidos de clorofilas y los

contenidos endógenos de las fitohormonas relacionadas con el estrés. Sin embargo, los coeficientes de correlación obtenidos fueron generalmente bajos lo que sugiere que la melatonina no fue el principal factor de protección de la PSII en las plantas de maíz frente a la sequía. De hecho, los contenidos de melatonina no aumentaron durante el estrés por sequía, lo que sugiere que (i) otros antioxidantes pueden jugar de forma endógena un papel protector, y (ii) la melatonina endógena desempeña un papel secundario en la protección del aparato fotosintético durante el estrés por sequía *in vivo*.

Así pues, en altas concentraciones la melatonina puede ejercer un papel protector en el aparato fotosintético cuando se aplica exógenamente a altas concertaciones en el agua de riego durante los periodos de recuperación del déficit hídrico reiterado en plantas. No obstante, su mecanismo de acción con o sin aplicaciones exógenas puede ser completamente diferente, actuando posiblemente como antioxidante cuando está presente en altas concentraciones y como regulador del crecimiento cunado está presente a bajas concentraciones

Se han descrito interacciones entre la melatonina y el ABA mostrado la supresión de genes implicados en la biosíntesis de ABA y promoviendo genes implicados en su catabolismo, reduciendo de esta manera la cantidad de ABA en *Malus domestica* bajo condiciones de déficit hídrico (Li et al. 2015) y también incrementado los contenidos de ABA en plantas expuestas a estrés por frío (Li et al. 2016). Y aunque los resultados del **Capítulo 4** sugieren que la melatonina puede actuar endógenamente como una hor-

mona, cuando no se aplican altas concentraciones exógenas de melatonina, esta podría actuar como tal pero no necesariamente mediante la regulación de los niveles de ABA en plantas de maíz.

5.- Interés agronómico de la melatonina

Diferentes estreses como la sequía pueden reducir la producción de los cultivos que crecen en condiciones de campo. Con el fin de preparar a las plantas para que respondan de una manera más exitosa frente al estrés, ya sea biótico o abiótico, se han desarrollado técnicas como el *chemical priming*, en la cual se añaden diferentes compuestos, ya sea a semillas o a plantas, para mejorar su respuesta frente a los diferentes estreses (Saavides et al. 2016).

El uso de estos compuestos ya ha demostrado ser efectivo en mejorar significativamente la tolerancia de las plantas contra diferentes estreses abióticos. Entre los diferentes compuestos que se ha demostrado tienen efecto en mejorar la respuesta de las plantas está el H_2O_2, una ROS que cuando se aplica a bajas concentraciones actúa de señalizador celular, lo que ha sido relacionado con la regulación transcripcional pero también con la post-trasncripcional. A parte de las ROS, también se han descrito de forma similar efectos de *chemical priming* en especies reactivas del nitrógeno como es el óxido nítrico, usando el nitroprusiato de sodio como donador de este compuesto. De forma análoga, las poliaminas y la melatonina son también usados en *chemical priming* para mejorar la tolerancia de las plantas bajo varias condiciones de estrés. Las poliaminas son capaces de unirse fuertemente a proteí-

nas cargadas negativamente, como numerosas enzimas de defensa y por tanto, modular la actividad de esas proteínas. Además, se ha demostrado que las poliaminas también tienen un papel en la producción de óxido nítrico y están relacionadas con las ROS a través de su catabolismo. Otro metabolito natural como las poliaminas que puede tener efecto de *priming* y actuar tanto a nivel homonal como antioxidante, al igual que las poliaminas, es la melatonina. La melatonina está involucrada en muchos procesos fisiológicos de las plantas y su potencial como compuesto usado en *chemical priming* se debe a su papel como antioxidante pero también a su papel como regulador desencadenante de respuestas antioxidantes (Saavides et al. 2016).

En aplicaciones exógenas de melatonina en el agua de riego a una concentración de 1mM en plantas de maíz durante la recuperación de periodos de sequía se observó que la melatonina aumentaba la fotoprotección como muestran los valores de F_v/F_m (**Capítulo 4**). Estos resultados concuerdan con los de estudios previos donde se ha descrito el papel de la melatonina en *chemical priming* en que puede ejercer una función como antioxidante, mejorando por tanto la respuesta de las plantas al estrés. En este caso es de especial importancia el hecho de que se ha demostrado el efecto beneficioso de la melatonina en la respuesta de las plantas al estrés hídrico reiterado, aplicándolo fácilmente en el agua de riego durante períodos de recuperación. Este hecho tiene por tanto importantes implicaciones agrícolas, ya que este sistema podría utilizarse para mejorar la respuesta de la planta al estrés por sequía durante períodos de déficit hídrico reiterado en cultivos de maíz,

aspecto que justifica la investigación también en otros cultivos. Sin embargo, el hecho de que el efecto de la melatonina exógena fuera bastante limitado, posiblemente porque se metabolizó rápidamente, indica que este compuesto debe aplicarse más frecuentemente y/o en combinación con otros compuestos que impida su rápida degradación.

Dada la importancia del *chemical priming* en la mejora de la resistencia de los cultivos al estrés, este tema requiere de más investigación ya que su uso en grandes cultivos por empresas del sector agronómico puede resultar en importantes mejoras en la resistencia de los cultivos, a pesar de los diferentes estreses a los que los cultivos pueden estar expuestos, y evitar así potenciales pérdidas de producción. Además también puede ser un tema de mucho interés no solo para empresas agronómicas sino también para el sector biotecnológico, en el cual se puede investigar para comprender mejor todos estos mecanismos y poder así usarlos en los cultivos cuya producción sea amenazada por algún estrés.

CONCLUSIONES

- Las tres especies de plantas estudiadas (*Silene dioica*, *Aptenia cordifolia* y *Zea mays*) presentan mecanismos de memoria al déficit hídrico reiterado. Estos mecanismos se han podido observar de forma muy evidente en la planta CAM, *A. cordifolia*.

- *Aptenia cordifolia* presenta memoria al estrés a varios niveles de organización, desde aspectos morfológicos/ estructurales (cambios en la estructura de las hojas y en la composición de las pantallas colectoras de luz del aparato fotosintético) hasta mecanismos bioquímicos (modulación de los contenidos endógenos de hormonas).

- La reducción de la antena del aparato fotosintético podría tener un papel importante en la fotoprotección (protección frente al exceso de luz) de plantas sujetas a déficits hídricos reiterados, permitiendo con ello captar menos energía luminosa y por tanto reducir un mayor potencial daño fotooxidativo causado por la sequía.

- Los contenidos endógenos de ABA aumentan en plantas doblemente estresadas, lo cual podría mejorar la respuesta de las plantas al estrés hídrico reiterado. Cambios en el metabolismo de gibberelinas podrían estar también implicados en el proceso de memoria al déficit hídrico reiterado.

- El PC-8 puede ayudar a la vitamina E a prevenir el daño fo-

tooxidativo del aparato fotosintético en situaciones de déficit hídrico reiterado. Tanto la síntesis de PC-8 como de vitamina E podría estar regulada por los niveles de ABA.

- La melatonina puede ejercer un papel protector como anti-oxidante en plantas de maíz sujetas a déficit hídrico reiterado cuando se aplica exógenamente en el agua de riego en altas concentraciones, lo cual confirma su importante papel como molécula de interés para ser usada en *chemical priming* para la mejora de cultivos.

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