Universitat de Lleida

Caracterización de la respuesta a sequía e identificación de genes asociados al uso eficiente del agua (UEA) en portainjertos Prunus spp.

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TESI DOCTORAL

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Dña. Maria Pilar Muñoz Odina, Dra. en Biología, Profesora titular en la Universidat de Lleida, Investigadora en el Departamento de Producción Vegetal y Ciencia Forestal de dicha Universidad,

CERTIFICAN

Que la tesis doctoral titulada "**Caracterización de la respuesta a sequía e identificación de genes asociados al uso eficiente del agua (UEA) en portainjertos *Prunus* spp.**" ha sido realizada íntegramente por la Ingeniera Agrónoma Dña. Beatriz Bielsa Pérez bajo nuestra dirección en dicha Unidad de Hortofruticultura, habiéndose cumplido todos los objetivos planteados, reúne las condiciones requeridas para optar al título de Doctor.

Zaragoza, Septiembre de 2017

María José Rubio Cabetas Maria Pilar Muñoz Odina

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una sabiduría interior las acompaña,
porque cada semilla sabe
cómo llegar a ser árbol…

"Sueños de semilla" – Jorge Bucay

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RESUMEN

El reto en los programas de mejora de portainjertos es la combinación de tolerancias de estreses abióticos en nuevos híbridos interespecíficos para obtener portainjertos adaptados a un amplio rango de condiciones edafoclimáticas. La sequía es uno de los principales estreses abióticos con un gran impacto ecológico y socioeconómico en España para los Frutales de Hueso. Existe una necesidad urgente de identificar portainjertos tolerantes a la sequía que puedan responder a la escasez de agua.

En este trabajo se estudió la respuesta adaptativa de tolerancia a la sequía en posibles portainjertos *Prunus* mediante tres enfoques: (i) Comprender los mecanismos fisiológicos implicados en el estrés hídrico considerando el uso eficiente del agua (UEA) en distintos genotipos; (ii) determinar la inducción bioquímica provocada por el ácido abscísico (ABA) para entender las relaciones entre las respuestas fisiológicas y bioquímicas; y (iii) elucidar la respuesta genética a niveles transcriptómicos (a corto y largo plazo) y proteómico (a corto plazo) para identificar genes candidatos involucrados en la respuesta a la sequía.

Se han identificado seis especies silvestres de *Prunus* con mejor UEA de un total de 48 genotipos de *Prunus* fenotipando el contenido foliar en cenizas y la discriminación del isótopo 13C ($\Delta^{13}C$), los cuales están relacionados negativamente con el UEA.

Se determinó la respuesta de 'Garnem' y de otros genotipos híbridos evidenciándose los diferentes mecanismos de evitación a la sequía presentes en ellos que permiten mantener el contenido hídrico, a pesar de la disminución del potencial hídrico foliar (LWP). Además, la respuesta bioquímica producida a las 24h mediante la acumulación foliar de ABA, confirmó el papel clave de dicha fitohormona en la respuesta a la sequía, a través de la regulación estomática en 'Garnem'. Por otro lado, se han podido comprobar las diferencias de comportamiento existentes entre la respuesta a estrés hídrico en condiciones controladas y en campo. Donde, a pesar de producirse una respuesta fisiológica similar, los árboles no experimentaron el nivel de estrés necesario para desencadenar una respuesta bioquímica de producción de ABA.

El estudio molecular se realizó mediante cuatro enfoques diferentes. En primer lugar, el análisis de las regiones promotoras de dos genes relacionados con la sequía, *PpDhn2* y *DREB2B*, reveló el importante papel de los elementos *cis* identificados no sólo en el estrés hídrico, sino también su posible implicación en el estrés por bajas temperaturas. Además se realizó el estudio filogenético de estos genes en una colección de *Prunus*. En segundo lugar, el análisis transcriptómico en raíz de 'Garnem'

sometido a estrés hídrico, ha permitido identificar genes involucrados en las cascadas de señalización y control transcripcional, genes osmoprotectores y los genes implicados en el transporte de agua e iones. En tercer lugar, el análisis proteómico reveló cambios significativos en los niveles de abundancia de una serie de proteínas en raíz de 'Garnem' a las 24h de estrés. De las cuales, 15 fueron identificadas en diferentes procesos biológicos, descritos también en el análisis transcriptómico. Finalmente, mediante un análisis con microsatélites (SSRs) en cuatro progenies hibridas, se identificó una región genómica específica de almendro diferente de melocotón y ciruelo. Está región podrá ser útil a nivel de genómica comparativa para identificar genes de interés codificados por el almendro.

Este estudio molecular ha permitido elegir ocho genes candidatos para la selección de portainjertos *Prunus* tolerantes a la sequía. Estos fueron el gen de la proteína LEA, los genes *PpDhn1*, *PpDhn2* y *DREB2B*, además de los DEGs: *ERF023*; *LRR receptor-like serine/threonine-kinase ERECTA* y *NF-YB3,* por su relación con una mejor UEA; y finalmente el DEG *Myb44*, represor de la fosfatasa PPC2, validado mediante qRT-PCR.

En general, estos resultados pueden contribuir a mejorar el conocimiento existente sobre los cambios fisiológicos, bioquímicos y moleculares en respuesta a la sequía. La comprensión de las estrategias de evitación y tolerancia ayudarán a proponer nuevos retos en la mejora de la tolerancia a la sequía en portainjertos *Prunus*.

ABSTRACT

The challenge in the rootstock breeding programs is the combination of abiotic stress tolerances in the new interspecific hybrids in order to obtain rootstocks adapted to a wide range of edaphoclimatic conditions. Drought is one of the main abiotic stresses with far-reaching ecological and socioeconomic impact in Spain, for stone fruit crops. Thus, there is an urgent need to identify drought resilient rootstocks that can respond to the water scarcity.

In this work the adaptive response leading to tolerance to drought was studied in possible rootstock in *Prunus* through three approaches: (i) to understand the physiological mechanisms involved in water stress controlling water use efficiency (WUE) in different genotypes; (ii) to determine the biochemical induction related to abscisic acid (ABA) to understand the link between physiological and biochemical responses; and (iii) to elucidate the genetic response at transcriptomic (long- and short-term) and proteomic levels (short-term) to identify useful candidate drought-related genes.

Six wild-relative *Prunus* species were identified with the best WUE from a total of 48 genotypes phenotyping the foliar ash content and Carbon isotope discrimination ($\Delta^{13}C$), which are negatively related to WUE.

The response to drought was determined in 'Garnem' and other genotypes, which evidenced the different drought avoidance mechanisms allowing them to maintain the water content in spite of a in the leaf water potential (LWP) decrease. In addition, the biochemical response by foliar ABA accumulation at 24h, confirmed the key role of this phytohormone in the drought response, through the stomatal regulation in 'Garnem'. Moreover, a different behavior between controlled and field-grown conditions was also confirmed. Although a similar physiological response is produced, the studied trees did not experience enough stress level that triggers into a biochemical response based on the ABA production.

The molecular study was carried out through four different approaches. Firstly, the analysis of the promoter region of two drought-related genes, *PpDhn2* and *DREB2B* revealed the important role of the *cis*-elements identified not only in drought, but also their possible involvement in low temperature stress. In addition, a phylogenic study with the two genes was made in a *Prunus* collection. Secondly, the time-course transcriptome analysis in 'Garnem' roots allowed us to identify genes involved in signaling cascades and transcriptional control, genes in acting as osmoprotectants, and genes implicated in water and ion transport. Thirdly, the proteomic analysis revealed significant changes in the abundance levels of a number of proteins in 'Garnem' roots

at 24h of stress. Out of these, 15 proteins were identified in different biological processes, described also in the transcriptomic study. Finally, a specific almond genomic region different from peach and plum background was identified by a microsatellite (SSR) approach in four hybrid progenies. This region could be useful at comparative genomic level in order to identify interesting traits from almond.

From the molecular study, eight candidate genes were chosen for drought tolerant *Prunus* rootstocks screening. Namely the gene codifying a LEA protein, the genes *PpDhn1*, *PpDHn2* and *DRE2B*, as well as the DEGs *ERF023* TF, LRR receptor-like serine/threonine-kinase ERECTA, and *NF-YB3* TF, responsible of WUE improvement were selected; and finally the *Myb44* transcription factor (FT), a repressor of PP2C phosphatase, validated by qRT-PCR.

Overall, these results may contribute to improve the existing knowledge on the physiological, biochemical and molecular changes in response to drought. The understanding of the avoidance and tolerance strategies will be helpful to suggest new drought-tolerance breeding approaches in *Prunus*.

RESUM

Un repte bàsic dels programes de millora de patrons és la combinació de toleràncies als estressos abiòtics en els nous híbrids interespecífics per tal d'obtenir patrons adaptats a un ampli rang de condicions edafoclimàtiques. La sequera és un dels principals estressos abiòtics amb un gran impacte ecològic i socioeconòmic a Espanya, en conreus com els *Prunus*. Hi ha una necessitat urgent d'identificar patrons tolerants a la sequera que puguin respondre a la manca d'aigua.

En aquest treball es va estudiar la resposta adaptativa de tolerància a la sequera en diferents portaempelts de *Prunus* a través de tres enfocaments: (i) comprendre els mecanismes fisiològics implicats en l'estrès hídric controlant l'ús eficient de l'aigua (UEA) en diferents genotips; (ii) determinar la inducció bioquímica relacionada amb l'àcid abscísic (ABA) per a establir relacions entre les respostes fisiològiques i bioquímiques; i (iii) elucidar la resposta genètica a nivells transcriptòmics (a curt i llarg termini) i proteòmic (a curt termini) per a identificar gens candidats relacionats amb la sequera.

S'ha aconseguit d'identificar sis espècies silvestres de *Prunus* amb la millor UEA d'un total de 48 genotips d'aquest gènere mitjançant l'estudi fenotípic del contingut foliar en cendres i la discriminació de l'isòtop 13C ($\Delta^{13}C$), relacionats negativament amb la UEA.

Es va determinar la resposta de 'Garnem' i d'altres genotips híbrids evidenciant els diferents mecanismes d'evitació de la sequera presents que permeten de mantenir el contingut hídric, tot i la disminució del potencial hídric foliar (LWP). A més, es va confirmar la resposta bioquímica produïda a les 24h mitjançant l'acumulació foliar d'àcid abscísic (ABA), ratificant el paper clau d'aquesta fitohormona en la resposta a la sequera, i específicament, en la regulació estomàtica a 'Garnem'. D'altra banda, s'han pogut comprovar les diferències de comportament existents entre la resposta a l'estrès hídric en condicions controlades i al camp. Encara que es produeix una resposta fisiològica similar, els arbres no van experimentar un nivell d'estrès sever suficient per a desencadenar una resposta bioquímica basada en la producció d'ABA.

L'estudi molecular es va realitzar mitjançant quatre enfocaments diferents. En primer lloc, l'anàlisi de les regions promotores de dos gens relacionats amb la sequera, *PpDhn2* i *DREB2B* revelà l'important paper dels elements *cis* identificats no només a la sequera, sinó també la seva possible implicació en l'estrès a les baixes temperatures. A més es va realitzar l'estudi filogenètic amb els dos gens en una col·lecció de *Prunus*. En segon lloc, amb l'anàlisi transcriptòmica a l'arrel de 'Garnem' sotmès a estrès hídric, s'han identificat gens involucrats en les cascades de senyalització i control transcripcional, gens osmoprotectors i gens implicats en el transport d'aigua i ions. En

tercer lloc, l'anàlisi proteòmica va revelar canvis significatius en els nivells d'abundància d'una sèrie de proteïnes a l'arrel de 'Garnem' a les 24h d'estrès. Quinze d'aquestes proteïnes van ser identificades en diferents processos biològics, descrits també en l'anàlisi transcriptòmica. Finalment, mitjançant una anàlisi amb microsatèl·lits (SSRs) en quatre progènies híbrides, es va identificar una regió genòmica específica d'ametller diferent de préssec i prunera. Aquesta regió podrà ser útil a nivell de genòmica comparativa per a identificar gens d'interès codificats per l'ametller.

Aquest estudi molecular ha permès de triar vuit gens candidats per a la selecció de patrons de *Prunus* tolerants a la sequera. Aquests van ser el gen de la proteïna LEA, els gens *PpDhn1*, *PpDhn2* i *DREB2B*, a més dels DEGs: *ERF023*; *LRR receptor-like serine / threonine-kinase* ERECTA i *NF-Yb3*, per la seva relació amb el millor UEA; i finalment el DEG *Myb44*, repressor de la fosfatasa PPC2, validat mitjançant qRT-PCR.

En general, aquests resultats poden contribuir a millorar el coneixement existent sobre els canvis fisiològics, bioquímics i moleculars en resposta a la sequera. La comprensió de les estratègies d'evitació i tolerància serà d'ajuda per a la proposta de nous reptes en la millora de la tolerància a la sequera a patrons de *Prunus*.

1. INTRODUCCIÓN GENERAL

1.1. ORIGEN Y TAXONOMÍA DEL GENERO *PRUNUS* L.

El género *Prunus* L. está formado por árboles y arbustos caducifolios. Pertenece a la subfamilia Amygdaloideae, la cual está dentro de la familia Rosaceae. Este género es considerado como uno de los principales dentro de las Angiospermas ya que está constituido por casi 200 especies con gran variedad de cultivares distintos entre sí (Bortiri et al., 2006). El número cromosómico de las especies que forman este género es de x = 8, variando su ploidía dentro del género de 2x a 22x (Rehder, 1940). Varias de estas especies tienen gran importancia económica ya que se cultivan por sus frutos como son el melocotonero (*P. persica* (L.) Batsch.), el albaricoquero (*P. armeniaca* L.), el cerezo (*P. avium* L.), el ciruelo japonés (*P. salicina* L.) y europeo (*P. domestica* L.), y el almendro [*P. amygdalus* Batsch, syn *P. dulcis* (Mill.).], el fruto seco con mayor importancia a nivel mundial (Hummer y Janick, 2009). Existen otras especies dentro de este género utilizadas por su valor ornamental como son especies de la sección *Pseudocerasus*, en la industria maderera y también con fines medicinales (Potter, 2011). La clasificación más extendida es la descrita con 5 subgéneros: *Amygdalus* (L.) Focke (almendros y melocotoneros), *Cerasus* Pers (cerezos), P*runus* [=*Prunuophora* Focke] (ciruelos y albaricoqueros), *Laurocerasus* Koehne y *Padus* (Moench) Koehne. Además de estos, hay que considerar el subgénero *Emplectocladus* (con 6 especies de las zonas áridas de Norteamérica) y el subgénero *Maddenia* del cual se ha comprobado su monofilia a partir de estudios filogenéticos llevados a cabo a nivel molecular con secuencias ITS ribosomales tanto nucleares como de plásmidos (Chin et al., 2010). Así mismo, también hay que tener en cuenta otras especies silvestres del melocotonero como *P. davidiana* (Carr.), Franch, *P. ferganiensis* (Kost y Rjab) Kov. & Kost, *P. kasuensis* Rehd y *P. mira* Koehne kov et. kpst. También las silvestres relacionadas con el almendro como *P. bucharica* (Korsh.) Fetdsch., *P. kuramica* (Korsh.), *P. webbii* (Spach) Vieh. y *P. kotschii* (*A. kotschii* Boiss.). Estas especies son utilizadas como ornamentales, o como portainjertos tanto directamente, como parentales en cruzamientos para el desarrollo de híbridos interespecíficos, p. ej. *P. webbii* × almendro. Estas especies son consideradas fuente natural de genes de interés debido a que presentan una gran adaptación bajo condiciones de estrés abiótico y biótico (Alimohammadi et al., 2013; Byrne et al., 2012; Gradziel et al., 2001).

Existen diferentes centros de origen dependiendo de la especie. Así, el ciruelo europeo se originó en el Cáucaso y en la región del Mar Caspio como un híbrido entre *P. cerasifera* y *P. spinosa* (Hummer y Janick, 2009). Mientras que el ciruelo japonés, tiene su origen en China, al igual que el melocotonero, de donde fueron llevados al Oeste Asiático, y de allí a Europa y América. El almendro es originario de las regiones montañosas y áridas del Centro de Asia, aunque se halla espontáneo en el Oeste de China, Cáucaso, Grecia y países del Norte de África. El albaricoquero tiene su origen en la parte más oriental del Oeste de China y algunas variedades rústicas proceden de Siberia. Por último, el cerezo apareció en el Centro y Sur de Europa, y en Asia Menor. Gracias a las conquistas romanas y las rutas comerciales, estos frutales se extendieron por Europa, especialmente por la Zona Mediterránea. Ya en los siglos XVI y XVII, su cultivo fue extendido al continente americano por los colonos españoles y portugueses (Byrne et al., 2012; Gradziel et al., 2001; Grasselly, 1976; Hummer y Janick, 2009).

El cultivo de frutales del género *Prunus* se distribuye principalmente por las zonas templadas del Hemisferio Norte: Norteamérica, Europa y Norte de Asia. Su cultivo también se ha extendido a las zonas subtropicales y tropicales de Sur y Sureste de Asia, África, Centroamérica y América del Sur, así como en Australia (Kalkman, 1965; Rehder, 1940).

1.2. IMPORTANCIA ECONÓMICA DE LOS FRUTALES DE HUESO

La producción de los frutales de hueso es muy relevante a nivel mundial. Según datos de la Organización Mundial para la Alimentación (FAO), la producción mundial de frutas de hueso ha aumentado en los últimos años, llegando a las 42.39 mill. t en 2014 (FAOSTAT, 2017). España es uno de los principales productores de frutas de hueso con un total de 2.26 mill. t producidas y una superficie total dedicada a su cultivo de 674.224 ha en 2014 (FAOSTAT, 2017). Su situación en la zona mediterránea le proporciona una climatología propicia para la producción de este tipo de frutas, pero la variabilidad climática y edáfica de su geografía, hacen que no todas las regiones existan situaciones favorables para el cultivo de todas ellas. Así, nuestro país se encuentra entre los 10 primeros productores a nivel mundial de frutales de hueso y del

almendro. En España destacan el cultivo del melocotón, sólo superada por China tanto en superficie dedicada (86.118 ha) como en producción (1.6 mill. t); y el cultivo del almendro, con una mayor superficie cultivada (527.058 ha), siendo la tercera productora por detrás de los Estados Unidos y Australia con un total de 195.704 t en 2014 (FAOSTAT, 2017).

En 2015, a nivel nacional, la producción de frutales de hueso representó un 20% del total de frutales más representativos en nuestra Agricultura (Fig. 1.1A). Destaca el cultivo del almendro, el cual teniendo una producción del 2%, es el cultivo con mayor superficie dedicada, un 52% correspondiente a 527.029 ha (Fig. 1.1B).

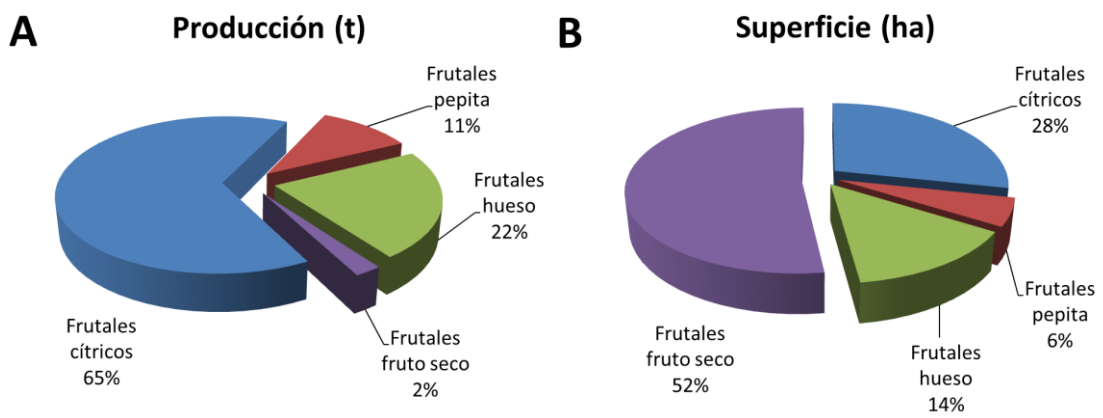

Figura 1.1. Producción (A) y superficie dedicada al cultivo (B) de los principales frutales cultivados en España en el año 2014. Fuente: (MAPAMA, 2017).

En relación a los frutales de hueso, la evolución del melocotonero ha experimentado un gran incremento en la última década (Fig. 1.2) como consecuencia tanto de la entrada en el mercado de nuevas variedades y portainjertos que permiten un cultivo más adaptado, como del aumento en la utilización de riego por goteo, mejorando el rendimiento del cultivo (Llácer et al., 2009). Mientras que en el resto de cultivos, esta producción es mucho menor, estabilizándose a lo largo de los años. Solamente, la producción del almendro desde 2013 también ha experimentado un importante ascenso (Fig. 1.2) y se espera un gran aumento en los próximos años, cuando las nuevas plantaciones entren en producción. En nuestro país, el cultivo del melocotonero se lleva a cabo principalmente en el Valle del Ebro (Cataluña y Aragón), Murcia, Extremadura y Andalucía. Mientras que el del almendro se extiende por las

regiones de Andalucía, Valle del Ebro, Comunidad Valenciana, Castilla la Mancha y Murcia (MAPAMA, 2017).

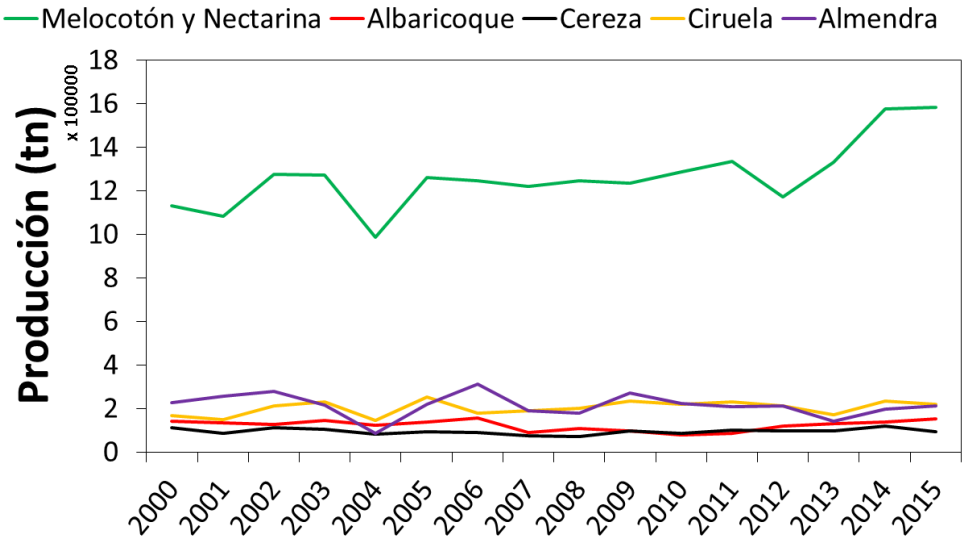

Figura 1.2. Evolución de la producción en frutales del género *Prunus* L. entre los años 2000 y 2015 en España. Fuente: FAOSTAT, (2017); MAPAMA, (2017).

1.3. EL ESTRÉS EN LAS PLANTAS

El estrés en términos biológicos es una desviación en la fisiología, desarrollo y funciones normales de las plantas que puede ser perjudicial e infligir un daño irreversible en el sistema de la planta (Nagarajan, 2010) afectando negativamente al crecimiento y productividad del cultivo. Cuando los agentes causantes de estas alteraciones son las temperaturas extremas, la salinidad, el encharcamiento, la sequía, la deficiencia natural de minerales o la irradiación extrema, el estrés se denomina como estrés abiótico.

La sequía es uno de los mayores problemas ambientales en la agricultura y determina tanto la productividad como la distribución de las plantas (Bartels y Sunkar, 2005), especialmente en climas áridos y semi-áridos. El estrés hídrico se asocia a ambientes donde la pluviometría es escasa o la distribución de las precipitaciones es irregular. Esta menor disponibilidad de agua produce una reducción de la conductividad hidráulica en las raíces que prepara a la planta para las condiciones deficitarias, aumentando la resistencia al flujo de agua y así, disminuir las pérdidas por

evaporación (Chaves et al., 2009). Durante este periodo de estrés, la planta desencadena diferentes mecanismos de respuesta asociados a la regulación de diferentes procesos fisiológicos y bioquímicos, afectando a la morfología de la planta. Se produce la regulación estomática, un ajuste osmótico, la estabilidad de la membrana citoplasmática, una disminución de la expansión foliar y de la actividad fotosintética, y se regula el crecimiento con el fin de minimizar la pérdida de agua (Belin et al., 2010; Golldack et al., 2014; Lind et al., 2015; Verslues et al., 2006). Una de las principales hormonas que regulan estos procesos es el ácido abscísico (ABA), que está asociada particularmente a la regulación del cierre estomático (Kim et al., 2010), pero también está implicada en la inducción a la latencia, modificaciones de la arquitectura radicular, además de impulsar la comunicación cruzada entre varias rutas de señalización en condiciones de estrés (Basu y Rabara, 2017).

1.3.1. Respuestas de las plantas al estrés hídrico

El agua es un compuesto esencial en la fisiología de las plantas. Es la forma en la que se absorbe el átomo de Hidrógeno en la fotosíntesis, por lo que puede considerarse un nutriente; es un disolvente de gran cantidad de sustancias y el medio en el que se realizan las reacciones bioquímicas; es esencial para el transporte de nutrientes y metabolitos; y permite mantener la turgencia de los órganos vegetales. En procesos de deshidratación, la falta de agua puede provocar daños irreversibles en células y tejidos.

Al igual que otros organismos vivos, las plantas han desarrollado mecanismos de señalización sofisticados para adaptar su metabolismo celular al medioambiente cambiante. De hecho, debido a su ciclo de vida sésil, las plantas pueden responder o protegerse a sí mismas de todas las formas de estrés abiótico a niveles molecular, celular, fisiológico y bioquímico para poder ser capaces de sobrevivir (Nakashima et al., 2009). Estos mecanismos se pueden clasificar en mecanismos de escape, evasión y tolerancia (Fig. 1.3) (Varela, 2010).

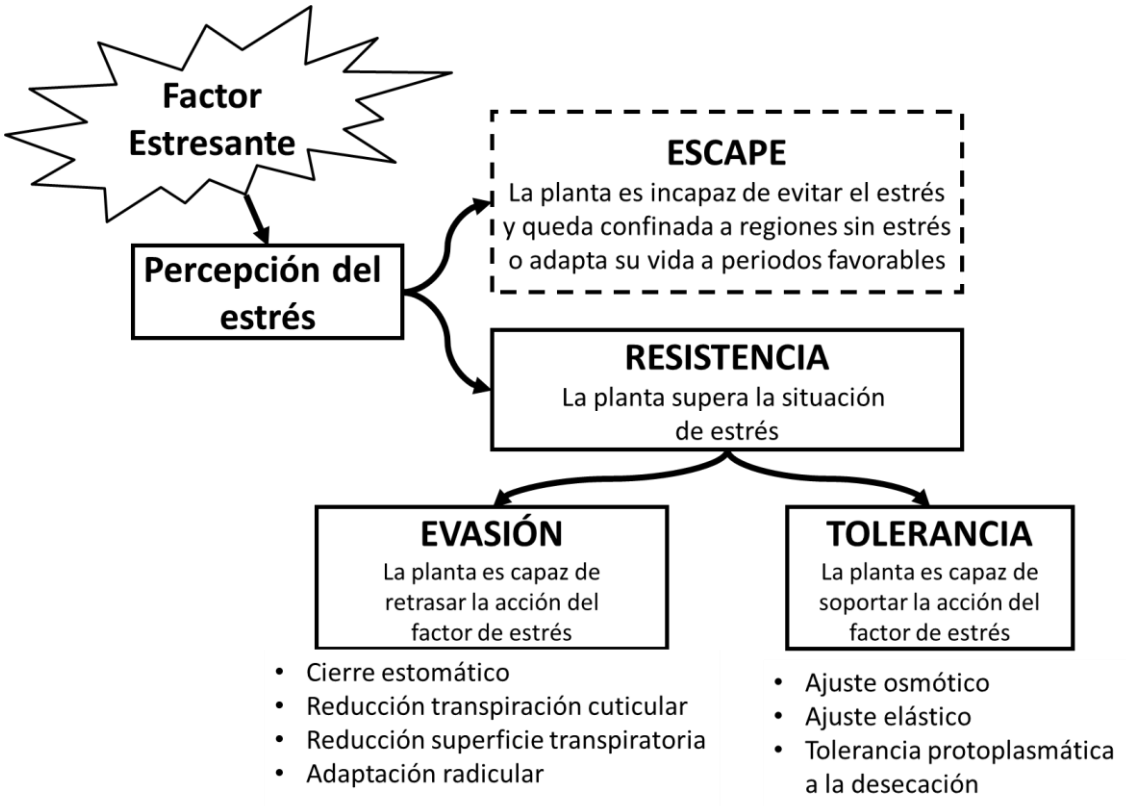

Figura 1.3. Tipos de mecanismos que pueden desencadenar las plantas frente al estrés. Fuente: Varela, (2010).

Determinar la manera en que las plantas perciben el estrés hídrico es complejo. Las señales pueden ser diferentes y provenir de distintos procesos. Primeramente, habría que identificar los lugares en donde la planta percibe dicho estrés y produce la señal, y los procesos de traducción de esta señal en una respuesta por parte de la célula o el tejido. Finalmente, los genes serían activados o desactivados teniendo lugar los consecuentes cambios fisiológicos, haciendo que la planta reprograme su desarrollo (Chaves et al., 2003).

Respuesta molecular

Ante una situación de estrés hídrico, la planta desarrolla su respuesta en tres pasos: (i) percepción del estrés; (ii) traducción de la señal de estrés; e (iii) inducción de los genes de respuesta al estrés. Así, la percepción del estrés se produce en receptores específicos presentes en la membrana celular como el complejo histidina-kinasa que actúa como osmosensor ante el déficit de agua (Ye et al., 2017). Cuando estos receptores se activan, se inicia la traducción de la señal en la que se generan segundos mensajeros como el calcio (Ca^{2+}), especies reactivas del oxígeno (EROs) e inositol

fosfatos (Mahajan y Tuteja, 2005). Estos segundos mensajeros activan, a su vez, una cascada de fosforilación, la cual termina con la inducción de los genes implicados en la respuesta adaptativa a la sequía (Fig. 1.4) (Mahajan y Tuteja, 2005; Roychoudhury et al., 2013; Yamaguchi-Shinozaki y Shinozaki, 2006). Estos genes de respuesta a la sequía se pueden clasificar en dos grupos en relación a su función: (i) genes reguladores como son factores de transcripción (FTs), kinasas, fosfatasas, y enzimas encargadas de la biosíntesis de fitohormonas; y (ii) genes efectores donde se incluyen chaperonas, proteínas LEA (*late embryogenesis abundant*), enzimas encargadas de la biosíntesis de osmolitos y proteínas relacionadas con los canales de agua, etc. Mientras que la función de los genes reguladores se centra en la síntesis de las proteínas que modulan la expresión génica, los genes efectores se encargan de la acumulación de osmolitos, del transporte pasivo a través de las membranas, de los sistemas de transporte de agua y energía, y de la protección, así como de la estabilización de las estructuras celulares ante la deshidratación y daño por EROs, además de sintetizar enzimas para la protección de macromoléculas (Roychoudhury et al., 2013; Shinozaki y Yamaguchi-Shinozaki, 2007). Son estos compuestos los que conducen a la adaptación de la planta y su supervivencia bajo condiciones de sequía.

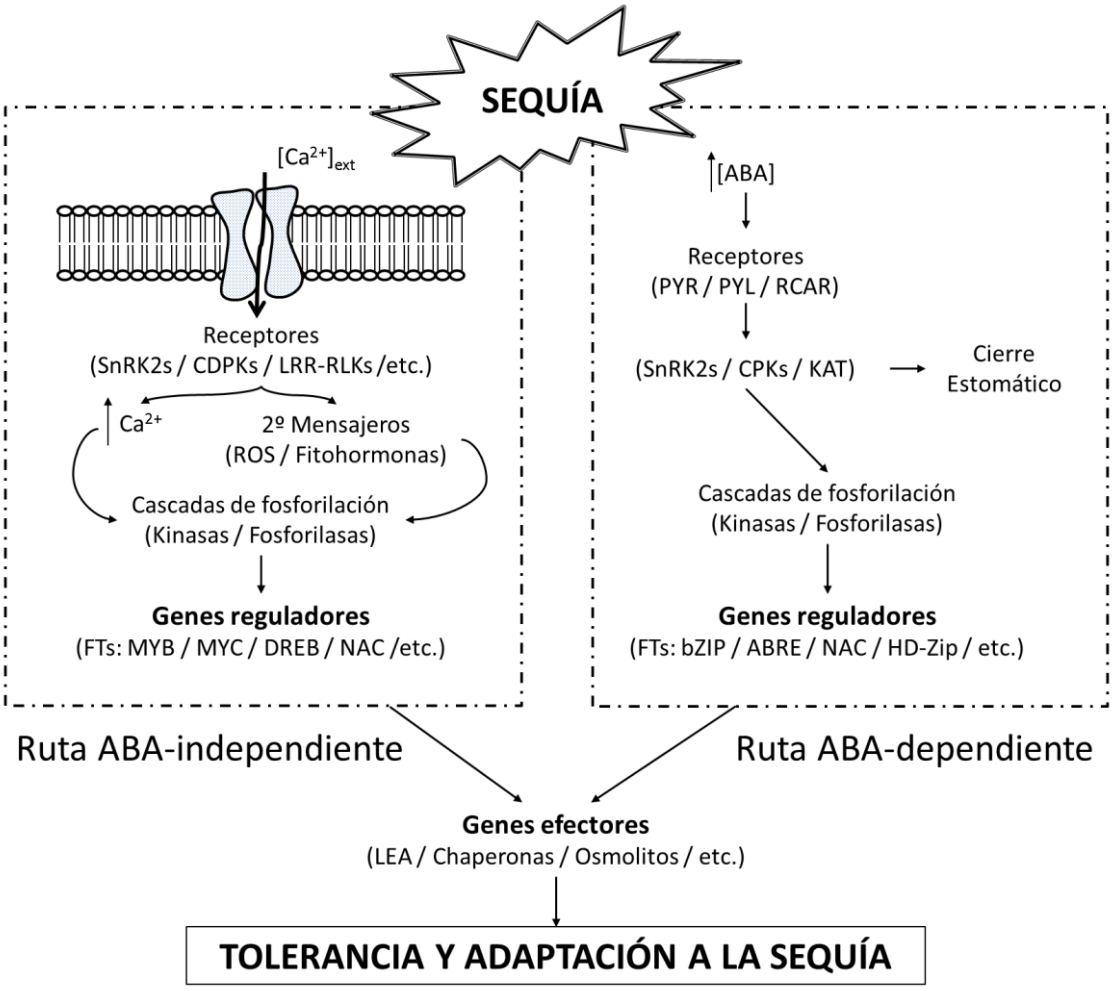

Figura 1.4. Ruta de señalización y traducción de la señal bajo estrés hídrico.

Esta respuesta al estrés puede seguir dos posibles rutas en función de la implicación o no del ABA en ella (Fig. 1.4). En la ruta ABA-dependiente la acumulación de esta fitohormona activa diferentes genes asociados al estrés. Estos genes contienen en sus regiones promotoras elementos *cis* característicos como los elementos de respuesta al ABA (ABRE, *ABA Responsive Elements*). La activación de estos elementos por varios FTs, p. ej. proteínas bZIP, hace que se inicie la expresión de los genes efectores de tolerancia a sequía, como las dehidrinas o enzimas que catalizan osmolitos de gran peso molecular. En la ruta ABA-independiente están involucrados entre otros, los elementos reguladores denominados proteínas de unión a elementos que responden a la deshidratación (DREB, *Dehydration-Responsive Element Binding protein*) que actúan sobre el promotor de los genes, los cuales finalmente, inducen la respuesta al estrés (Roychoudhury et al., 2013; Yoshida et al., 2014). Aunque

normalmente se ha considerado que estas dos rutas son independientes la una de la otra, existen interacciones entre ellas (Roychoudhury et al., 2013).

Respuesta bioquímica

La aclimatación de las plantas sometidas a sequía se produce por la acumulación de metabolitos asociados con la capacidad estructural que mejoran las funciones de la planta bajo las condiciones de estrés. Tras la activación de la respuesta multigénica al estrés, entran en juego los llamados solutos compatibles, también llamadas osmoprotectores. Estos solutos actúan regulando el ajuste osmótico de la célula, disminuyendo el riesgo de daños causados por la síntesis de EROs, previniendo el daño de la membrana y estabilizando las proteínas y enzimas (Singh et al., 2015), que permiten su adaptación a la sequía. Existen diferentes grupos de osmoprotectores en función de su naturaleza química (revisado por Singh et al., 2015): (i) Las poliaminas, compuestos alifáticos que contienen nitrógeno de bajo peso molecular, están involucradas en la eliminación de radicales libres, así como en procesos de crecimiento y división celular; (ii) las betaínas, compuestos de amonio cuaternario en las que se incluyen la glicina betaína, que se acumula en los cloroplastos, favoreciendo el flujo de agua para mantener el equilibrio osmótico y regulando la cascada de señalización de la transducción; (iii) los carbohidratos como fructano y trehalosa que actúan estabilizando la membrana y evitando la desnaturalización de las proteínas; en el caso de la trehalosa, protegiendo el PSII contra la fotooxidación manteniendo la capacidad fotosintética (Lyu et al., 2012); (iv) los polialcoholes como manitol, sorbitol y D-Ononitol, que funcionan como eliminadores de radicales libres y previenen la pérdida de agua; y finalmente (v) los aminoácidos, como prolina, el soluto compatible más estudiado (Kiran y Abdin, 2012; Roosens et al., 2002; Sofo et al., 2004), que está relacionado con las cascadas de señalización ABA-dependiente e independiente (Yoshiba et al., 1997).

Respuesta fisiológica

La respuesta fisiológica de las plantas al estrés hídrico varía dependiendo tanto de la severidad como de la duración del mismo (Shao et al., 2008). Para que la planta

pueda ser funcional a bajos potenciales hídricos manteniendo su estado hídrico alto, y que esta pueda recuperarse tras un periodo de sequía se hace necesaria la contribución de diferentes procesos fisiológicos (Verslues et al., 2006). Los principales mecanismos fisiológicos en respuesta a la sequía incluyen estrategias de evasión y tolerancia al estrés (Fig.1.3).

Entre los mecanismos de evasión o evitación se encontraría una de las respuestas más rápidas en producirse durante el estrés, el cierre estomático, controlando el intercambio gaseoso y por tanto la pérdida de agua por transpiración. Por un lado, se ve influenciada la entrada de CO_2 en las hojas a través de los estomas afectando a la eficiencia fotosintética, y por otro, el intercambio de vapor de agua es crucial para el mantenimiento del balance hídrico en la planta (Belin et al., 2010). El ABA juega un papel importante en la regulación del cierre estomático (Kim et al., 2010). Sin embargo, existen otros muchos parámetros que interactúan junto con la sequía regulando este proceso como son la luz y la concentración de CO_2, entre otros (Basile et al., 2003; Belin et al., 2010). Además de la transpiración estomática, también se ve reducida la transpiración cuticular, la cual puede representar hasta el 50% del total (Murata y Mori, 2014). Otro mecanismo de evasión es la reducción de superficie transpiratoria. La planta puede reducir su superficie mediante el enrollamiento foliar o la abscisión foliar y así mantener los potenciales hídricos en los meristemos y las raíces (Engelbrecht y Kursar, 2003; Kozlowski y Pallardy, 2002). Incluso también se puede reducir por una reorientación de las hojas y los brotes, disminuyendo así la captación de energía (Torrecillas et al., 1996). Las plantas también experimentan una reducción de su superficie radicular y una disminución de la permeabilidad para evitar pérdidas en suelos muy secos. Aumentan la resistencia hidráulica para disminuir el flujo hídrico hasta la copa. O por el contrario, la absorción se puede mantener incrementando la superficie radicular pero no la aérea. Así, cada unidad de superficie foliar está abastecida por más unidades de superficie radicular, que exploran un mayor volumen de suelo y extraen más agua. Esto conlleva un agotamiento de recursos más rápido, que en ausencia de una disminución de la transpiración lleva a la marchitez (Shao et al., 2008).

Los principales mecanismos de tolerancia son el ajuste osmótico y el ajuste elástico. Mediante el ajuste osmótico se produce una acumulación de solutos como se ha mencionado anteriormente, haciendo que disminuya el potencial osmótico y manteniendo la turgencia celular a bajos potenciales hídricos. Con ello, la planta mantiene su crecimiento celular, la apertura estomática y la fotosíntesis, favoreciendo la supervivencia a la deshidratación y explorando un mayor volumen de agua del suelo (Shao et al., 2008). El ajuste elástico hace que sea posible la extensión permanente de las células en crecimiento. Además de estos dos ajustes existe otro mecanismo denominado de tolerancia protoplasmática a la desecación, el cual permite mantener las células vivas a potenciales hídricos muy bajos (Botella y Campos, 2005).

1.4. LA MEJORA GÉNETICA EN PORTAINJERTOS

Los árboles frutales están formados por dos partes independientes unidas mediante injerto: la parte aérea, denominada variedad y la parte subterránea que aporta el sistema radicular, el patrón o portainjerto. Cada una de las partes procede de individuos diferentes, que pueden pertenecer o no a la misma especie.

Los portainjertos se pueden clasificar en función de su origen. Estos pueden ser francos, clonales e híbridos interespecíficos (Felipe, 1989; Rubio-Cabetas, 2010). Los portainjertos francos son aquellos que se obtienen a partir de semillas de la especie que son consideradas. La semilla puede proceder de una sola variedad o bien de una mezcla de estas dentro de una misma especie (Felipe, 1989). Estos patrones son menos uniformes que los clonales. La ventaja que tienen es que al propagarse por semilla están libres de virus (Wertheim, 1998) y son los más baratos de producir. Los portainjertos clonales son los obtenidos mediante propagación vegetativa a partir de una planta seleccionada por sus características específicas. Los individuos obtenidos son genéticamente idénticos, por lo que expresan un comportamiento homogéneo en condiciones externas iguales (Felipe, 1989). Finalmente, debido a la facilidad de hibridación entre especies cercanas dentro del género *Prunus*, se han obtenido diferentes híbridos interespecíficos aumentando la variabilidad disponible y

permitiendo una mayor adaptación en condiciones edáficas limitantes (Gradziel, 2003; Rubio-Cabetas et al., 2005).

Mientras que la variedad es seleccionada por su productividad y calidad de fruto, el portainjerto se selecciona en función de otras características. Los patrones son un factor primordial en la producción ya que en ellos recae la responsabilidad de la absorción de agua y nutrientes, además de la capacidad de adaptación a las diferentes condiciones ambientales y prácticas culturales (Gainza et al., 2015). Por lo tanto, los portainjertos proporcionan caracteres de interés ausentes en la variedad tales como control del vigor, buen anclaje al terreno, resistencia a nematodos, bacterias y hongos, y tolerancias a estreses provocados por la sequía, la salinidad o el encharcamiento, por nombrar algunos de los caracteres más importantes seleccionados en los programas de mejora (Gainza et al., 2015; Layne, 1987; Rubio-Cabetas et al., 2017).

Debido la escasez de agua en la Cuenca Mediterránea cada vez más acentuada, la selección de portainjertos adaptados a condiciones de estrés hídrico se hace imprescindible. Los portainjertos de almendro francos, los cuales están adaptados a un amplio rango de disponibilidades hídricas (Isaakidis et al., 2004; Rubio-Cabetas et al., 2017), han sido utilizados durante siglos debido a las condiciones de secano en la mayoría de los cultivos del Mediterráneo. Sin embargo, estos portainjertos son susceptibles a nematodos y a asfixia radicular (Rubio-Cabetas et al., 2017). La selección de híbridos interespecíficos entre especies relacionadas en los que es posible combinar varios caracteres de interés en un mismo individuo, ofrece un amplio abanico de oportunidades en los programas de mejora de *Prunus*, principalmente en programas de mejora en patrones de almendro y melocotonero llevados a cabo en Francia, EEUU, España o ex-Yugoslavia (Rubio-Cabetas et al., 2017). Los cruzamientos interespecíficos más empleados han sido, principalmente, almendro × melocotonero, pero también existen otros entre melocotonero × *P. davidiana*, o *P. webbii* × almendro, en los que las especies silvestres sirven de fuente natural de genes relacionados con resistencias a estreses bióticos y abióticos para su introgresión en portainjertos de *Prunus* ya cultivados (Alimohammadi et al., 2013; Bielsa et al., 2014; Byrne et al., 2012; Felipe, 2009; Gradziel et al., 2001; Lecouls et al., 2004).

Desde los años 70, el patrón más utilizado en la zona mediterránea fue el híbrido melocotonero × almendro 'GF-677', seleccionado en la estación francesa de La Grand Ferrade (INRA, Burdeos), por su tolerancia a clorosis y su buena compatibilidad de injerto. Sin embargo, es sensible a asfixia, *Agrobacterium* y nematodos, e induce un excesivo vigor (Cinelli y Loreti, 2004) para el melocotonero. Se introdujeron también otros híbridos interespecíficos como 'Barrier' y 'Cadaman', procedentes del cruzamiento entre *P. davidiana* × melocotonero por su resistencia a las cuatro principales especies de *Meloidogyne* spp. También los ciruelos 'Puebla de Soto' y 'Montizo' por la mejor adaptación en suelos calizos y pesados (Rubio-Cabetas et al., 2005), Sin embargo, en los últimos años, se ha producido un sustitución del híbrido 'GF-677' por los híbridos de 'Garfi' × 'Nemared' (G×N) (Felipe, 2009) (Fig. 1.5). Los parentales de estas series G×N fueron seleccionados por su buena propagación mediante estaquillado leñoso procedente del almendro 'Garfi' y por la resistencia a nematodos procedente del melocotonero 'Nemared' (Socias i Company et al., 2009). De esta serie, fueron seleccionados los patrones 'Garnem', 'Felinem' y 'Monegro', que presentan resistencia a nematodos agalladores de la especie *Meloidogyne* spp., tolerancia a clorosis y a sequía, además de un buen comportamiento en condiciones de replantación y en suelos calizos, proporcionando también un buen vigor (Felipe, 2009). Estos portainjertos fueron seleccionados inicialmente para el cultivo de almendro, pero debido a la buena compatibilidad de injerto con melocotonero, también se está extendiendo su uso al cultivo de este frutal. La limitación de estos híbridos es la mala adaptación a la asfixia radicular y a los suelos pesados (Amador et al., 2012). Existen otras líneas de mejora con nuevos cruzamientos entre ciruelos mirobolanes (*P. cerasifera* Ehrh) y los híbridos G×N con el fin de obtener nuevos portainjertos con tolerancia a la asfixia radicular provocada por el encharcamiento (Xiloyannis et al., 2007).

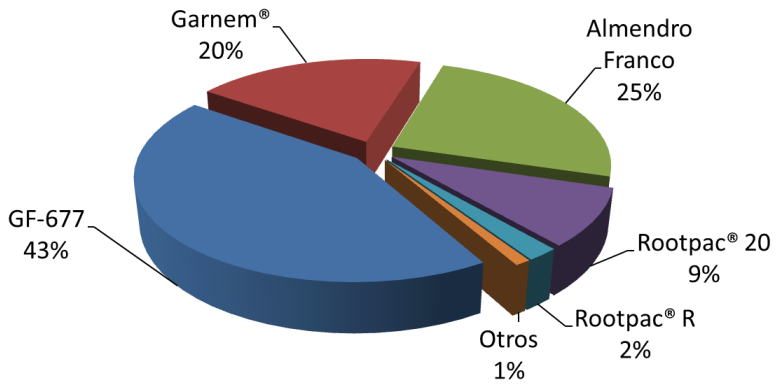

Figura 1.5. Patrones para almendro más utilizados en España durante 2014 - 2105. Fuente: Rubio-Cabetas et al., (2017).

1.5. ESTRATEGIAS EN MEJORA GENÉTICA PARA LA TOLERANCIA A ESTRÉS HÍDRICO EN LEÑOSOS

La complejidad de los mecanismos de tolerancia a sequía ha hecho que el progreso en la mejora de este carácter haya sido lento (Tuberosa y Salvi, 2006). La obtención de variedades tolerantes a la sequía se ha llevado a cabo sobre todo con estrategias de mejora clásica. La introducción de genes a partir especies silvestres a través de la hibridación interespecífica, como se ha mencionado en el apartado 1.4., ha sido ampliamente utilizada, permitiendo la selección de patrones tolerantes a sequía mediante esa introgresión de genes.

Para tratar de comprender la respuesta de las plantas al estrés hídrico, se hace necesario entender las bases fisiológicas y genéticas de dicha respuesta (Mir et al., 2012). Por un lado, los estudios fisiológicos ayudan a comprender el complejo entramado de mecanismos relacionados con la tolerancia y así mejorar la eficiencia en la selección de plantas tolerantes a la sequía (Mir et al., 2012). En esta línea, durante los últimos años se han realizado diversos estudios en olivo (Boussadia et al., 2008), pistacho (Gijón et al., 2010; Memmi et al., 2016), vid (Rodriguez-Dominguez et al., 2016; Tombesi et al., 2015), manzano (Liu et al., 2012), almendro (Espadafor et al., 2017; Karimi y Yadollahi, 2012; Yadollahi et al., 2011), melocotonero (Basile et al., 2003; Solari et al., 2006), así como en híbridos interespecíficos del género *Prunus* (García Brunton et al., 2004; Jiménez et al., 2013; Martinazzo et al., 2011; Rickes et al., 2017; Sofo et al., 2005; Xiloyannis et al., 2007). Por otro, diferentes estrategias basadas

en la genética han permitido en los últimos años desarrollar otras técnicas como el uso de líneas casi isogénicas (NILs - *Near Isogenic Lines*) (Sánchez-Pérez et al., 2004), o el desarrollo nuevas estrategias de mejora mediante Introgresión Asistida por Marcadores (MAI - *Marker Assistant Introgression*) (Serra et al., 2016), las cuales presentan una gran utilidad en estudios genéticos para la identificación de genes involucrados en la tolerancia a la sequía. Así mismo, la identificación de genes candidatos y QTLs (*Quantitative Trait Loci*) asociados a esos mecanismos (Tuberosa y Salvi, 2006), o el empleo de técnicas para el análisis de expresión génica como qRT-PCR han permitido identificar y estudiar genes relacionados con la respuesta a sequía. De esta manera, distintas dehidrinas han sido identificadas en melocotonero (Artlip et al., 1997; Bassett et al., 2009; Wisniewski et al., 2006), otros genes relacionados con la síntesis de prolina como el *P5SC* han sido analizados en el híbrido 'GF-677' sometido a estrés hídrico (Jiménez et al., 2013), o genes que sintetizan proteínas entre las que se incluyen la zeaxanthina epoxidasa, implicada en la síntesis de ABA se han estudiado mediante cDNA-AFLPs en *P. scoparia* (Alimohammadi et al., 2013). También se hacen imprescindibles los estudios de las regiones promotoras, que ayudan a comprender la regulación de esos genes de respuesta (Alimohammadi et al., 2013; Bassett et al., 2009). Más recientemente, el avance en las técnicas de secuenciación y genotipado han permitido disponer de genomas de referencia como el del melocotonero (Verde et al., 2013) ofreciendo nuevas oportunidades en la mejora genética. Así, con marcadores SNPs (*Single Nucleotide Polymorphism*) se hace posible la saturación de mapas de ligamiento existentes y, junto al genoma de referencia, localizar genes candidatos en las regiones en estudio. En los últimos años, el desarrollo de las tecnologías de nueva generación (-*omicas*) han facilitado la identificación de nuevos genes candidatos, permitiendo una mejor comprensión de los mecanismos moleculares de respuesta a la sequía. En esta línea, los estudios transcriptómicos, tanto por *microarray*, como por secuenciación de ARN (RNAseq), han revolucionado la capacidad de discernir sobre estos mecanismos de regulación en diferentes especies vegetales (Barghini et al., 2015; Cossu et al., 2014; Ksouri et al., 2016; K.-Q. Li et al., 2016; Tang et al., 2013; J. Wang et al., 2015). Sin embargo, ya que los niveles de ARNm pueden no estar correlacionados con la cantidad de proteínas y sus funciones debido a posibles alteraciones en la traducción, o modificaciones post-transcripcionales como la

fosforilación, la glucosilación, ubiquitinación y sumoilación (Alam et al., 2010), los estudios proteómicos son una herramienta esencial para la identificación de proteínas relacionadas con la respuesta a sequía (Bonhomme et al., 2009; Macarisin et al., 2009; Valdés et al., 2013; Valero-Galván et al., 2013; Wisniewski et al., 2009; Zhou et al., 2015). En *Prunus*, estos estudios proteómicos han sido más limitados y se han centrado más en la calidad de fruto (D'Ambrosio et al., 2013; S. Li et al., 2016), en estudios de auto-(in)compatibilidad (Martínez-García et al., 2015) o en estreses abióticos relacionados con estrés por frío y fotoperiodo (Nilo et al., 2010; Renaut et al., 2008), y el calor (Lara et al., 2009).

1.6. ANTECEDENTES

La variabilidad del clima durante los últimos años ya ha comenzado a ser una amenaza para el sector agrario. Debido al cambio climático, se están produciendo cambios en la frecuencia de eventos meteorológicos extremos y en las estaciones del clima que provocan la reducción de la producción, proliferación de malas hierbas, plagas y enfermedades (FAO, 2014), y afectan a la rentabilidad de las explotaciones agrícolas. En España, según el informe Evaluación del Grupo Intergubernamental de Expertos de Naciones Unidas sobre el Cambio Climático (IPPCC), el cambio climático afectará directamente a los principales cultivos con un impacto negativo en la producción debido a aumentos de la temperatura de 2 °C. Para el periodo de 2030-2049 se esperan pérdidas superiores al 25%, aumentando en los años siguientes. Este impacto está relacionado con la disponibilidad de agua y su suministro entre otros factores (Intergovernmental Panel on Climate Change, 2014). Además de los recursos hídricos, los problemas edáficos, ya presentes en los suelos españoles como desertificación, pérdida de fertilidad o incendios forestales, se verán agravados provocando un aumento de la aridez del suelo (Piqueras, 2007). Un claro ejemplo de este cambio en la climatología española es la extrema sequía sufrida durante este año, 2017, pudiendo superar a la de 2012, la cual es la sequía más grave descrita por la Agencia de Meteorología Española (AEMT) en España (Tejedor et al., 2016).

A corto plazo, los agricultores pueden adoptar estrategias sencillas como los cambios de fecha o replantaciones en zonas más altas. Sin embargo, a largo plazo, son necesarias soluciones que permitan la adaptación a las nuevas condiciones climáticas (Medina, 2014). Entre estas soluciones destacaría la reconversión varietal con variedades adaptadas a estas nuevas condiciones limitantes. Por tanto, un reto fundamental de la agricultura española es su adaptación al cambio climático, en particular, a los estreses ambientales como la sequía y la salinidad. En este marco, desde la Unidad de Hortofruticultura del Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), se están llevando a cabo programas de mejora centrados en la respuesta a los estreses abióticos en patrones de frutales, dentro de los cuales se incluye el presente trabajo. La obtención de portainjertos tolerantes a la sequía se ha llevado a cabo, sobre todo, con estrategias de mejora clásica. Sin embargo, debido a que la tolerancia al estrés hídrico, es un carácter cuantitativo, en el que están implicados una gran serie de procesos adaptativos a nivel fisiológico, bioquímico y molecular, hace que su estudio sea complejo.

Por todo ello, son necesarios los estudios centrados en el análisis del comportamiento y respuesta de nuevos portainjertos bajo condiciones de sequía, con el fin de comprender los mecanismos e identificar genes candidatos implicados en la respuesta al estrés hídrico, y aplicar *a posteriori* métodos de selección genómica como complemento a la mejora genética vegetal clásica.

1.7. OBJETIVO DE LA TESIS

El objetivo general es establecer las bases fisiológicas y moleculares del estrés hídrico en patrones del género *Prunus* L. Este objetivo se puede dividir en los siguientes sub-objetivos:

1. Estudio de mecanismos fisiológicos involucrados en la respuesta a la sequía provocada por el estrés hídrico a corto y largo plazo mediante diferentes parámetros.

2. Determinación del contenido de ácido abscísico (ABA) para dilucidar su implicación en la respuesta al estrés hídrico y su participación en el desencadenamiento de los mecanismos fisiológicos y moleculares.
3. Estudios moleculares a corto y largo plazo (i) la expresión diferencial de factores de transcripción (FTs) y genes diana involucrados en la respuesta a la sequía y su implicación en la ruta de síntesis del ABA; (ii) la búsqueda de otras fuentes de tolerancia a sequía en especies silvestres de *Prunus* mediante el análisis de regiones promotoras y fenotipado; y (iii) la búsqueda de genes candidatos mediante la secuenciación de ARN (RNAseq) y el análisis proteómico en tejido de 'Garnem'.

2. PHYSIOLOGICAL CHARACTERIZATION OF DROUGHT STRESS RESPONSE AND EXPRESSION OF TWO TRANSCRIPTION FACTORS AND TWO LEA GENES IN THREE *PRUNUS* GENOTYPES

ABSTRACT

Global warming has led to a progressive decrease in rainfall, which is reflected by a reduction of water resources in the soil and a negative effect on crop production in Mediterranean areas. Under drought stress, many plants react by inducing a different series of responses at both physiological and molecular levels, allowing them to survive for a variable period of time. Therefore, in order to understand the response of roots to drought conditions, the genotypes peach × almond 'Garnem' [*P. amygdalus* Batsch × *P. persica* (L.) Batsch] and their progeny, the hybrid 'P.2175' × 'Garnem'-3 and OP-'P.2175' (*P. cerasifera* Ehrh.) were subjected to a period of water deficit. Drought conditions with a subsequent re-watering period were tested for potted plants for one month. Stomatal conductance (gs) and leaf water potential (LWP) were measured to monitor the plant physiological responses. Significant differences among the drought stress and drought stress recovery treatments and among the genotypes were observed. In addition, four genes related to the abscisic acid (ABA) biosynthesis pathway were studied for their expression by qRT-PCR: an AN20/AN1 zinc finger protein (*ppa012373m*); a bZIP transcription factor (*ppa013046m*); a dehydrin (*ppa005514m*) and a LEA protein (*ppa008651m*). Their expression profiles correlated with our physiological results of drought response, being higher in roots than in phloem tissue. In general, the expression of the four studied genes was higher after 15 days under drought conditions. Under drought and recovery conditions, the zinc finger and bZIP transcription factors showed significant differences in their relative expression levels from LEA and dehydrin. These results suggest the role of LEA and dehydrin in the regulatory response to drought stress in *Prunus* genotypes. Therefore, the dehydrin and the protein LEA might be potential biomarkers to select rootstocks for tolerance to drought conditions.

Keywords: ABA, LEA protein, qPCR, Transcription Factor, Water deficit.

2.1. INTRODUCTION

Stress can be defined as a physiological deviation from normal plant functions that can damage or cause irreversible damage to the plant (Nagarajan, 2010), negatively affecting crop growth and yield. Drought stress is one of the biggest problems in agriculture, especially in arid and semi-arid climates (Bartels and Sunkar, 2005) in the Mediterranean region where water availability is the most important factor for plant survival. Since Mediterranean countries are the main stone fruit producers (FAOSTAT, 2017), the use of adapted rootstocks is necessary for such limited edaphoclimatic conditions. Currently, the challenge in rootstock breeding programs is the combination of abiotic tolerances in a new generation of interspecific hybrids resulting from the cross of almond × peach hybrids by plum genotypes. Peach × almond hybrids such as 'Garnem', 'Felinem' and 'Monegro' (which come from the cross 'Garfi' almond × 'Nemared' peach) show good vigor, nematode resistance, and adaptation to calcareous soils (Felipe, 2009). Myrobalan plums such as 'P.2175' provide a wide spectrum of root-knot nematode resistance (Rubio-Cabetas et al., 2000) and tolerance to waterlogging (Amador et al., 2012).

During the stress period, plants undergo some morphological and physiological changes due to hormones such as abscisic acid (ABA) and ethylene (Bruce et al., 2002; Munns, 2002). ABA accumulation under water deficit conditions activates different genes linked to stress (Narusaka et al., 2003). The ABA-inducible genes have *cis*-elements in their promoter regions including *ABA-responsive elements* (ABRE) (Yamaguchi-Shinozaki and Shinozaki, 2005). The activation of these elements through different transcription factors (TFs) ABA-responsive element binding proteins, such as ABI / ABF / AREB / bZIP families (Hossain et al., 2010; Sakuma et al., 2006; Uno et al., 2000), induces the expression of many downstream genes involved in drought tolerance or enzymes involved in the catalysis of low molecular weight osmolytes (Beck et al., 2007). Jakoby et al., (2002) identified 75 different bZIP TFs divided in ten groups. One of them is the Group S, whose TFs are transcriptionally activated after stress treatment, such as drought (Jakoby et al., 2002). *AtbZIP53* TF, found inside this group S, functions as transcriptional activator of the *ProDH* gene in *Arabidopsis* (Satoh

et al., 2004) with leads to the decomposition of proline accumulated during dehydration period (Satoh et al., 2004; Yoshiba et al., 1997). In addition to these TFs, among others, there are genes belonging to the *Stress Associated Protein* (SAP) genes family which encodes proteins containing A20/AN1 zinc-finger domains (Saad et al., 2010). Proteins with zinc-fingers A20/AN1 type are described in numerous species such as *Oryza sativa* (Vij and Tyagi, 2006), *Populus trichocarpa* (Jin et al., 2007), and *Aeluropus littoralis* (Saad et al., 2010) among others, suggesting an important role in abiotic stress responses in plants, such as cold, salt, dehydration, heavy metals, submergence, wounding as well as stress hormone abscisic acid (Vij and Tyagi, 2006).

After the early response to stress of TFs, the expression of different target genes coding proteins, such us chaperones, late embryogenesis abundant (LEA) proteins, osmotin, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases take place (Shinozaki and Yamaguchi-Shinozaki, 2007). In particular, protecting function of LEA proteins has been widely demonstrated in literature. For example, overexpression of *HVA1* confers drought tolerance in transgenic rice (Babu et al., 2004; Chen et al., 2015). LEA-type proteins play a main role in storage of seeds as well as acclimation and adaptive response to stress processes conferring molecular protection of cellular components during abiotic stress (Battaglia et al., 2008; Xiao et al., 2007) by the influence of ABA concentration changes (Hong-Bo et al., 2005). ABA accumulation produced by drought stress induces the activation of ABRE *cis*-elements regulating the transcription of most *LEA* genes (Hundertmark and Hincha, 2008), which are organized in several groups depending on sequence similarity, and therefore, on functionality (Battaglia et al., 2008). One of them is group II, known as D-11 family whose proteins are called dehydrins (Allagulova et al., 2003). Dehydrins have been studied in several species (Lopez et al., 2001; Yamasaki et al., 2013), and more particularly in woody plants (Artlip and Wisniewski, 1997; Bassett et al., 2009; Velasco-Conde et al., 2012; Vornam et al., 2011; Wisniewski et al., 2009, 2006). Up to date, three dehydrin genes (*Ppdhn1*, *Ppdhn2* and *Ppdhn3*) have been described in peach confirming its induction by drought and its implication in cold acclimation (Artlip and Wisniewski, 1997; Bassett et al., 2009; Wisniewski et al., 2006).

Due to the complexity of drought tolerance mechanisms, improvements in the breeding of this trait have been slow (Tuberosa and Salvi, 2006). New cultivars obtained, showing drought tolerance, have been mostly released in classical breeding programs. Gene introgression from other species through interspecific hybridization has been used in many breeding programs: crossing almond × apricot, but also peach with wild species such as *P. webbii*. This gene introgression led to the production of drought-tolerant rootstocks (Felipe, 2009; Martínez-Gómez et al., 2003b). A variety of studies have been undertaken in order to understand the physiological and genetic basis of the hydric stress response on fruit trees (Basile et al., 2003; Karimi et al., 2012; Liu et al., 2012), and also, on interspecific hybrids from *Prunus* genus (Jiménez et al., 2013; Sofo et al., 2005; Xiloyannis et al., 2007). Furthermore, molecular biology as well as genomics led to the identification of candidate genes. In peach, different genes that encode for dehydrins have been identified (Artlip et al., 1997; Bassett et al., 2009; Wisniewski et al., 2006). Alimohammadi et al., (2013) categorized five candidate genes responsive to water-deficit stress and emphasized the importance of starch synthesis, sugar and ABA in *P. scoparia*. More recently, improvements in sequencing and genotyping techniques provide reference genomes in *Prunus* genus, such as peach (Verde et al., 2013) and Japanese apricot (Zhang et al., 2012), representing a new tool for breeding. Molecular studies mainly focused on transcriptomics, have led to rapid generation of information about all the genes expressed under drought conditions in a particular genotype. RNAseq analysis studies in Mongolian almond identified genes involved in drought response (J. Wang et al., 2015). In the same way, Eldem et al., (2012) identified miRNAs responsive to drought in peach by Illumina deep sequencing technology.

The objective of this study was the evaluation of the response to drought stress of three *Prunus* rootstocks by measuring genotype differences in different physiological parameters and studying the expression profiles of two TFs as well as two key genes involved in drought tolerance. The development of drought-tolerant biological markers involved in drought stress is useful in breeding programs for the selection of more drought tolerant rootstocks.

2.2. MATERIALS AND METHODS

2.2.1. Plant material and experimental conditions

The material presenting different levels of resistance against nematodes of *Meloidogyne* spp included two hybrid genotypes from a breeding program (EU funded project FAIR-6-CT-98-4139) and the commercial rootstock 'Garnem'. A total of 30 two-year-old plants were considered for the experiment: six plants from the almond × peach hybrid 'Garnem'; 12 plants from the 'P.2175' x 'Garnem'-3 hybrid, formerly named 'Tri-hybrid-3'; and 12 plants from the OP-'P.2175' (*P. cerasifera* Ehrh.). This plant material was propagated by hardwood cuttings at the CITA (Centro de Investigación y Tecnología Agrolaimentaria de Aragón) facilities in Zaragoza, Spain.

These plants were placed in 20 cm diameter pots with a mix of turf, 30% coconut fiber and 20% sand. The experimental design was a two randomized block: Control and Treatment (3 plants from 'Garnem', 6 plants from 'Tri-hybrid-3' and 6 plants from OP-'P.2175' for each group). The pots were covered with black plastic in order to minimize evapotranspiration from the soil surface and to avoid the entrance of precipitation into the soil. The experiment was carried out in a shaded greenhouse located in the CITA facilities in Zaragoza (41°43'N, 0°48'W). Plants underwent a drought period beginning from July 5 to 19, 2011, followed by a re-watering period of 15 days. Before beginning the water-stress period, the water content was maintained in optimal conditions for all plants. During the treatment period, stressed plants had no water supply, whereas control plants were watered three times weekly until field capacity to maintain optimal soil water content by drip irrigation (flow dripper of 2 l/h – 15 min). After 15 days of water stress, treatment plants were re-watered supplying the same irrigation level and frequency as the control plants during 15 days more to restore the water soil conditions. The average climatic conditions during the experimental period were the following: temperature of 22.3 °C; relative humidity of 54.8%; solar radiation of 26.9 MJ m^{-2} day^{-1}; rainfall of 0.14 mm day^{-1}; and ETo of 6.5 mm day^{-1}. (Extended environmental data are shown in Supplementary Material S2.1; Annex 1). Samples of root and phloem tissues from each plant were collected, considering two biological replicates, from the control and treated plants on days 0, 10

and 15 during the drought stress period and on days 10 and 15 during the re-watering period. For root sampling, each plant was de-potted, sampled, and re-potted again until next sampling. Phloem sampling was done in each plant. Stems were cut, the bark removed and the phloem tissue isolated using a scalpel. These samples were immediately frozen at -80 ºC for subsequent RNA extraction and gene expression analysis.

2.2.2. Physiological characterization

Physiological measurements

Plant water status was determined by measuring the leaf water potential (LWP) twice a week at 11 am, using a Scholander-type pressure chamber (Soil Moisture Equipment Corp. Santa Barbara, CA, USA) (Scholander et al., 1964). The values of LWP were obtained from healthy old leaves from each plant of the median segment of the shoot. The selected leaves were covered with aluminum foil in order to stop transpiration before picking up them for measuring LWP. The resultant LWP data was the average of three measurements as technical replicates. Stomatal conductance (gs) was also measured twice a week at 11 am from a leaf of each plant of the median segment of the shoot with a Leaf Porometer (Decagon Devices Inc., Pullman, WA, USA). Finally, the percentage of leaf epinasty was determined in stressed plants by counting leaves without visible drought stress symptoms like leaf curling, yellowing, loss of turgidity and leaf falling, twice a week before sampling for LWP and gs according to the following equation:

$$\text{Epinasty } \% = \frac{\text{total leaves - leaves without stress symptoms}}{\text{total leaves}} \times 100$$

Ash content

Three shoots with a length of approximately 35 cm were picked up, as technical replicates, from each plant during the experiment, cut into small pieces and dried at 60 °C for 48h in an oven. Once the wood was dried, it was ground up. Approximately 0.5 g of powder from each sample was placed in a preheated ceramic vessel and incubated

at 70 °C overnight. Finally, samples were burnt in a muffle at 550 °C for 24h. The results of the ash content were expressed as a percentage of dry mass (Glenn and Bassett, 2011).

2.2.3. Molecular analysis

RNA isolation and cDNA synthesis

Total RNA was extracted from 0.5 g of root and phloem samples as described by Meisel et al., (2005) with some modifications (Chang et al., 1993; Salzman et al., 1999; Zeng and Yang, 2002) (Supplementary Material S2.2; Annex 1). RNA integrity was verified by 1% agarose gel electrophoresis and ethidium bromide staining. Genomic DNA from RNA samples was removed by DNase I (TURBO DNA-free™, Ambion, Life Technologies, Austin, TX, USA) according to manufacturer's instructions. RNA (2500 ng) was reverse transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies, Carlsbad, CA, USA) in a total volume of 21 µl according manufacturer's instructions.

Gene expression analysis

Two microliters of a 40X diluted synthesized cDNA was used for each amplification reaction in a final volume of 20 µl. For each of two biological replicates, quantitative real-time PCR (qRT-PCR) reactions were triplicated. qRT-PCR was performed on an Applied Biosystems 7900HT Fast PCR System using PerfeCTa SYBR Green SuperMix, ROX Master Mix (Quanta Biosciences Gaithersburg, MD, USA). Specific primers corresponding to dehydrin (*ppa005514m*), the LEA protein (*ppa008651m*), the A20/AN1 zinc finger TF (*ppa012373m*) (Leida et al., 2012) and the bZIP TF were designed based on the nucleotide sequence of the *ppa013046m* gene present in the assembled and annotated peach genome (*Prunus persica* genome v1.0; http://www.rosaceae.org/) (Table 1). The amplification conditions consisted of an initial denaturation at 95 ºC for 10 min, followed by 40 cycles of 15 s at 95 ºC for denaturation, and 1 min at 60 ºC for annealing and extension. Amplification was followed by a melting curve analysis. The control reaction for qRT-PCR was performed

using actin primers designed from the available *P. persica* actin DNA sequence (Gene Bank accession number AB046952). Relative expression was measured by the standard curve procedure.

Table 2.1. Primer sequences used in the qRT-PCR analysis.

Primer Name	Gene	5' to 3' Sequence	Prmier Reference
Dehydrin F	*ppa005514m*	GTACTCTCATGACACCCACAAAACTAC	Leida et al. 2012
Dehydrin R		CCCGGCCCCACCGTAAGCTCCAGTT	
LEA protein F	*ppa008651m*	GCAAAAGGTAGGGCAAACAG	Leida et al. 2012
LEA protein R		TGGCTTTGCTTCTTTGGTCT	
Zn-Finger F	*ppa012373m*	ACACAGGCTTCCTCTACTCCATCTTT	Leida et al. 2012
Zn-Finger R		GAACCCTCATTCCGAGACATTTATCAG	
ppn070g03 F	*ppa013046m*	GGGTTGAAACACCCAAAAGA	
ppn070g03 R		GCGATTCGACAACATCCTCT	
Actin F	*ppa007242m*	CAGATCATGTTTGAGACCTTCAATGT	
Actin R		CATCACCAGAGTCCAGCACAAT	

Physiological parameters

For each genotype, the differences among days and within each treatment were determined using analysis of one-way variance (ANOVA) for gs, LWP, epinasty and ash content. The significant difference was assessed with Tukey's test ($p \leq 0.05$).

Gene expression profiles

The statistical differences in the relative gene expression values were determined by the Student's t-test ($p \leq 0.05$) between the control (day 0) and treatment values for each gene. Furthermore, statistical differences among genotypes for each day of treatment in both phloem and root tissue were evaluated by ANOVA. The significant difference was assessed with Tukey's test ($p \leq 0.05$).

All the statistical analyses were performed with GenStat Discovery Version 4 (VSN International, 2013).

2.3. RESULTS AND DISCUSSION

2.3.1. Physiological characterization of the drought stress response

Effects of drought stress on water status, stomatal conductance and leaf epinasty

During the experiment, the control plants presented constant LWP values, most of them higher than -1MPa, indicating an optimal and stable water status (Fig. 2.1A). These values were similar to found by Jiménez et al., (2013) in control plants of a drought experiment with four *Prunus* rootstocks. In contrast, the LWP progressively decreased in the stressed plants, confirming that this parameter depends on the soil water conditions (Davies et al., 1994; Gollan et al., 1992). Therefore, the water absorption by the roots and its movement along the plant is reduced when the water content falls (Nagarajan, 2010). In our work, this reduction was different in 'Garnem' with respect to the 'Tri-hybrid-3' and OP-'P.2175' (Fig. 2.1A). 'Garnem' dramatically reduced its LWP at 10 days of treatment, reaching -3.80 MPa, whereas in 'Tri-hybrid-3' and OP-'P.2175' this reduction was slower, showing less reduced LWP values (-1.65 MPa and -2.57 MPa, respectively). The lowest values were obtained in all genotypes after two weeks of drought, which represented the period of maximum stress (Fig. 2.1A), when the LWP value in OP-'P.2175' was significantly higher than the values in 'Tri-hybrid-3' and 'Garnem' (Supplementary Material S2.3; Annex 1). After 10 days of re-watering, the LWP values recovered their original status, reaching a water potential similar to those of the control plants (Fig. 2.1A) and revealing a rapid recovery, as it is reflected in their leaf water potential. Similar results were obtained for *Prunus* interspecific hybrids, which also reached comparable LWP values to those of the control plants after 15 days of water status recovery (Sofo et al., 2005).

Furthermore, other significant differences between the two experimental hybrids and 'Garnem' were observed. In adequate water conditions as in day 0 and the recovery period, the LWP in the two hybrids was lower than in 'Garnem', while the LWP was lower for the latter with respect to the hybrids in drought stress conditions (Fig. 2.1A). Similar results were documented by characterization of the drought and chlorosis tolerances in several *Prunus* tri-hybrids (Xiloyannis et al., 2007). The

performance of these rootstocks could be explained by the vigor influence in the plant water balance (Basile et al., 2003; Hajagos and Végvári, 2013; Weibel, 1999). 'Garnem' is a vigorous rootstock (Bielsa et al., 2015; Felipe, 2009), although its vigor was not reflected in the cuttings studied. Therefore, this genotype could have a greater transport and water consumption under good water conditions. This corresponds to a higher LWP value due to the amount of water present in the plant. In contrast, the stored water in 'Tri-hybrid-3' and OP-'P.2175' plants was lower, probably due to their less vigor, and hence their LWP values were correspondingly low.

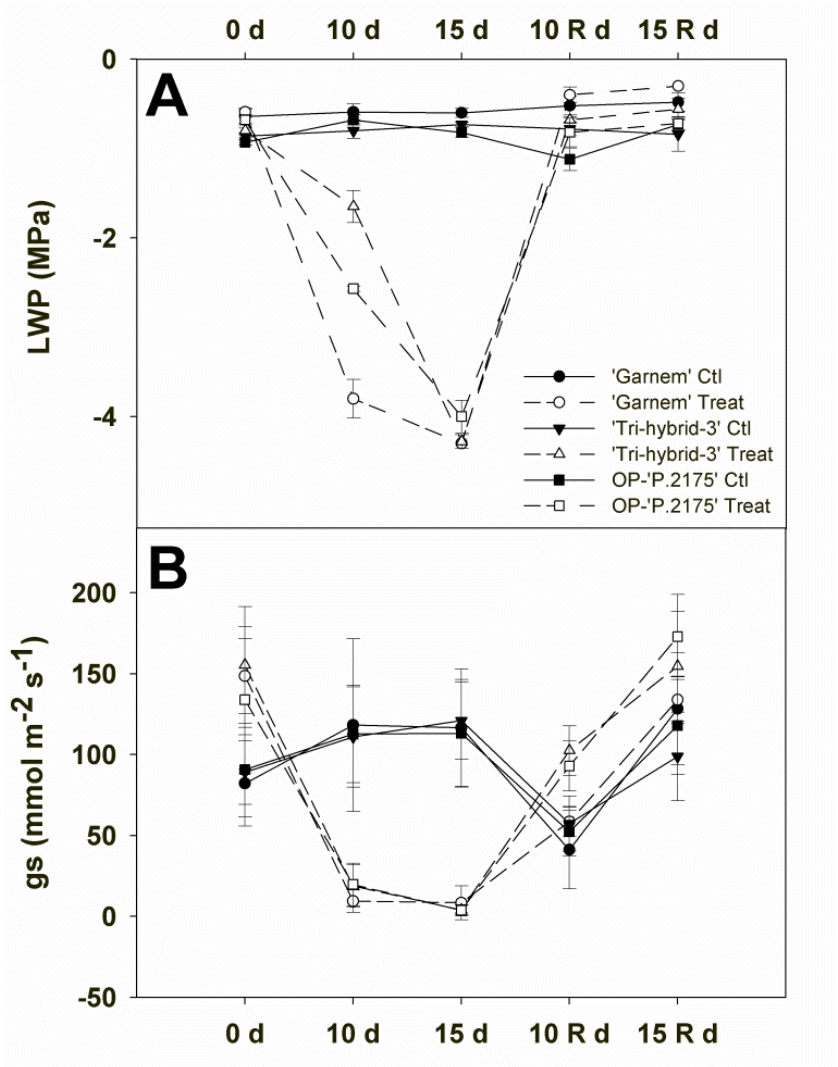

Figure 2.1. Leaf water potential (LWP) (A) and stomatal conductance (gs) (B) during the drought experiment for the studied genotypes. Continuous lines indicate water supplied plants while dot lines indicate hydric conditions in plants under drought treatment. Error bars represent the standard error of the mean. (d = days, R= Recovery; Ctl: Control; Treat: Treatment).

Although stomatal closure is not yet a fully understood phenomenon, LWP is one of the major factors in its regulation because the stomatal aperture responds directly to maintain cellular turgor (Franks and Cowan, 1995). Rahmati et al., (2015) also observed this response. They confirmed in peach that a low stomatal conductance was because of the low LWP for the three water deficit levels studied in their work. The stomatal conductance showed a similar tendency to LWP (Fig. 2.1A and B). The control plants presented high gs values, although there were no significant differences among the genotypes for each day. In contrast, gs average levels decreased from 147.68 mmol m^{-2} s^{-1} on day 0 to 5.39 mmol m^{-2} s^{-1} on day 15 of treatment in the stressed plants (Fig. 2.1B). By 10 days of recovery, gs levels in stressed plants reached similar values as in the control plants, the hybrid genotypes showing even higher values (Fig. 2.1B). However, the gs value was significantly lower in 'Garnem' than in the two hybrids (Supplementary Material S2.3; Annex 1). After two weeks of recovery, 'Garnem' showed a lower gs value than the two hybrids again, but the differences in this case were not significant (Fig. 2.1B, Supplementary Material S2.3; Annex 1).

One possible reason can explain these observations during the drought stress period; 'Garnem' quickly consumed its water reserves, which led to a fast drop of LWP, behaving like a water spender plant (Jones and Sutherland, 1991) that absorbs all the available water in order to maintain its growth rate. In contrast, 'Tri-hybrid-3' and OP-'P.2175' would use a water saver plant strategy (Jones and Sutherland, 1991). These plants would carry on a strict stomatal control of the LWP in order to avoid the hydraulic conductivity loss. They can avoid high water deficits in the stem and maintain a minimum water level, but as a counterpart they employ a relatively risky strategy to maintain a high gs value (Vilagrosa et al., 2003; Zhang et al., 2013). This hypothesis would explain why 'Tri-hybrid-3' and OP-'P.2175' maintained a higher water level than 'Garnem' by 10 days of treatment, also showing a slightly higher gs levels, although without significant differences among them (Fig. 2.1A). By day 15 of treatment, the performance of 'Garnem' was similar to that of the 'Tri-hybrid-3' and OP-'P.2175'. This suggests that 'Garnem' may transform its water spender strategy into a water saver strategy once its water reserve was depleted (Jones and Sutherland, 1991; Varela, 2010). During the recovery period, 'Garnem' reached less negative LWP values than

the 'Tri-hybrid-3' and OP-'P.2175' (Fig. 2.1A). 'Garnem' being a vigorous rootstock (Bielsa et al., 2015; Xiloyannis et al., 2007) could have a greater water transport capacity, thus this genotype would be faster in restoring the water loss in order to hold a high LWP (Zhang and Cao, 2009; Zhang et al., 2013). However, their lower gs values indicated that the gas exchange was lower, and therefore their stomata were more sealed than the stomata of their progeny. This contradiction could be due to other factors involved in the regulation of the stomatal mechanisms in the plants (Basile et al., 2003).

In addition to the decrease of LWP and gs levels as avoidance mechanisms against drought stress, a reduction in exposed leaf area was shown by leaf curling (epinasty) until reaching loss of foliar biomass during the most severe stress time. This reduction of leaf area by epinasty and loss of biomass by leaf shedding is a typical avoidance mechanism that lowers water demand and helps to maintain the water potential in the meristems and the roots (Engelbrecht and Kursar, 2003; Kozlowski and Pallardy, 2002). A rate of 100% of epinastic leaves was reached on day 15 of treatment for all genotypes (Fig. 2.2). The leaf area reduction process was slower in 'Garnem' (66.7% of leaf epinasty) than in 'Tri-hybrid-3' (92.2% of leaf epinasty) and OP-'P.2175' (80.9% of leaf epinasty) on day 10 of treatment (Fig. 2.2). After 10 days of the recovery period, the percentage of leaf epinasty in 'Garnem' was 18.52% compared to 83.01% in OP-'P.2175' and 67.02% in 'Tri-hybrid-3', indicating a faster recovery in this genotype than in the two hybrids. In contrast, after 15 days of recovery period, the 'Tri-hybrid-3' and OP-'P.2175' showed slightly lower leaf epinasty values than those of 'Garnem' (Fig. 2.2), which could be related to lower gs levels presented by this rootstock (Fig. 2.1B). A possible explanation is that a higher new healthy leaves in 'Tri-hybrid-3' and OP-'P.2175', a higher gas exchanging capacity in these genotypes in comparison to 'Garnem'.

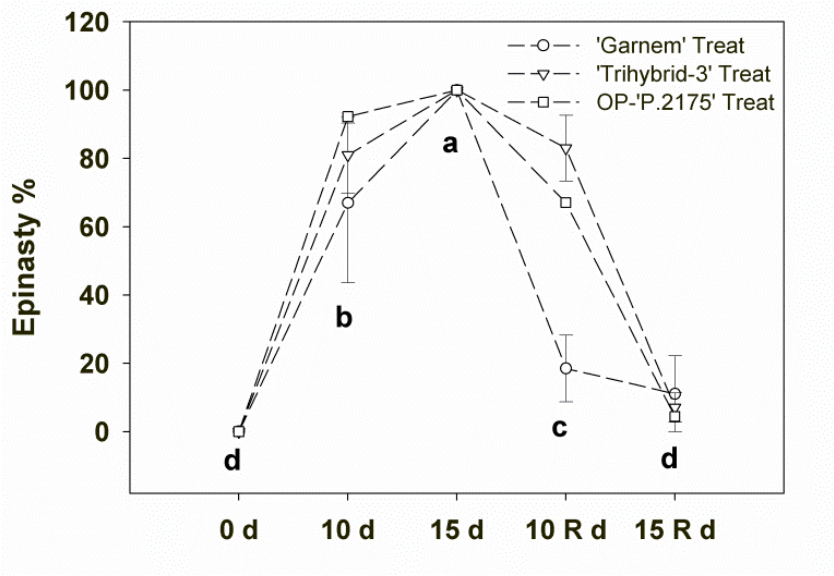

Figure 2.2. Leaf epinasty percentage during the experiment for the genotypes under drought conditions. Similar letter values indicate no significant difference ($p \leq 0.05$) following Tukey's post-hoc test. Error bars represent the standard error of the mean. (d = days, R = Recovery; Treat: Treatment).

Ash content

Ash content increased with the stress level until 10 days of drought ,with 'Garnem' showing 3.8%, significantly higher than the percentage obtained by OP-'P.2175' and higher (but not significantly) than by the 'Tri-hybrid-3' (Fig. 2.3). Mineral accumulation in growing and transpiring tissues occurs by passive transport in the xylem (Masle et al., 1992). Thus, a higher transpiration rate correlates with a higher mineral transport to the transpiring tissues where transpiration occurs, leading to increased ash content (Araus et al., 1998; Glenn and Bassett, 2011; Zhu et al., 2008).

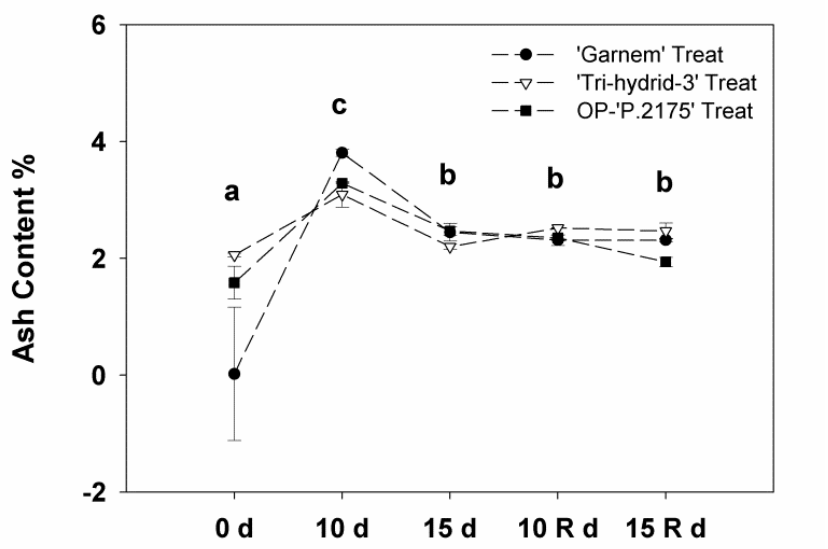

Figure 2.3. Ash content percentage in wood tissue during the experiment for the genotypes under drought conditions. Similar letter values indicate no significant difference ($p \leq 0.05$) following Tukey's post-hoc test. Error bars represent the standard error of the mean. (d = days, R = Recovery; Treat: Treatment).

The higher mineral content by 10 days of treatment in 'Garnem' could be explained by the water spender hypothesis. As a water spender plant, 'Garnem' consumes its water reserves quickly requiring a high transpiration flow along the xylem and causing a drop in the LWP (Fig. 2.1A). The amount of stored water would be greater in 'Garnem' than in the 'Tri-hybrid-3' and OP-'P.2175', so when the water was consumed, the mineral concentration in the tissues would also be higher. It is also true that the gs value in 'Garnem' was the lowest (Fig. 2.1B), which suggests a lower transpiration in this genotype. However as previously mentioned, the lack of correlation between both LWP and mineral content values in relation to the stomatal conductance could be due to other factors implicated in the stomatal closure mechanisms (Basile et al., 2003). From day 15 of treatment, the ash content significantly decreased in all genotypes, remaining stable throughout the recovery period with values that did not exceed 2.4% (Fig. 2.3), below the values obtained by the control plants (Fig. 2.1). Although 'Tri-hybrid-3' had a higher ash percentage after two weeks with an optimum water supply, this value did not differ significantly from those in the other genotypes (Fig. 2.3). Several previous studies have been conducted on the ash content by different authors, considering its relationship to the rate of transpiration (Masle et al., 1992), the carbon isotope discrimination ($\Delta^{13}C$) and the water use efficiency (WUE) in cereals (Araus et al., 2002, 1998; Blum, 2005; Cabrera-

Bosquet et al., 2009; Merah et al., 2001), and in fruit trees (Glenn, 2014; Glenn and Bassett, 2011). In these studies, the plant material showed seasonal or annual differences with a clear response in the mineral content from the plants under drought conditions in different environments (Cabrera-Bosquet et al., 2009) and in different years (Glenn, 2014; Glenn and Bassett, 2011; Merah et al., 2001). In our study, the lack of variation observed after 15 days of treatment and held throughout the recovery period could be due to the short considered period of two weeks that did not allow for any significant change in the percentage of ash. We are aware that also a longer period of study would be required, perhaps annual or seasonal, in order to measure new stem growth and thus, find differences.

2.3.2. Molecular analysis of the drought stress response

The response to drought stress of two supposed target genes, the dehydrin *ppa005514m* and the gene encoding the LEA protein *ppa008651m*, was analyzed throughout the drought and recovery periods. Both genes are related to one of the ABA synthesis pathways (Allagulova et al., 2003; Battaglia et al., 2008; Leida et al., 2012). In addition, two TFs were analyzed including the bZIP TF *ppa013046m* belonging to the S group of the bZIP family (Jakoby et al., 2002) and related to proline synthesis (Kiran and Abdin, 2012; Lee et al., 2006), and *ppa012373m* which encodes an A20/AN1 zinc-finger protein involved in responses to different abiotic stresses as cold, salt, dehydration and bud dormancy entrance (Giri et al., 2011; Leida et al., 2012; Mukhopadhyay et al., 2004). The gene expression patterns were studied in young tissue from the phloem and roots by qRT-PCR in 'Garnem', 'Tri-hybrid-3' and OP-'P.2175' plants. A higher response at the root level was observed in comparison to the phloem for the TFs and dehydrin genes, but not the LEA gene, whose expression in OP-'P.2175' at 15 day of treatment was similar both phloem and root tissue (Fig. 2.4). These observations demonstrate that the primary response to drought stress occurs in the root by a lack of water in the soil (Aguado et al., 2014; Wisniewski et al., 2004). This trend was observed in all four of the studied genes in both tissues and in all genotypes. The gene expression levels were the highest in OP-'P.2175' and the lowest in 'Garnem' (Fig. 2.4).

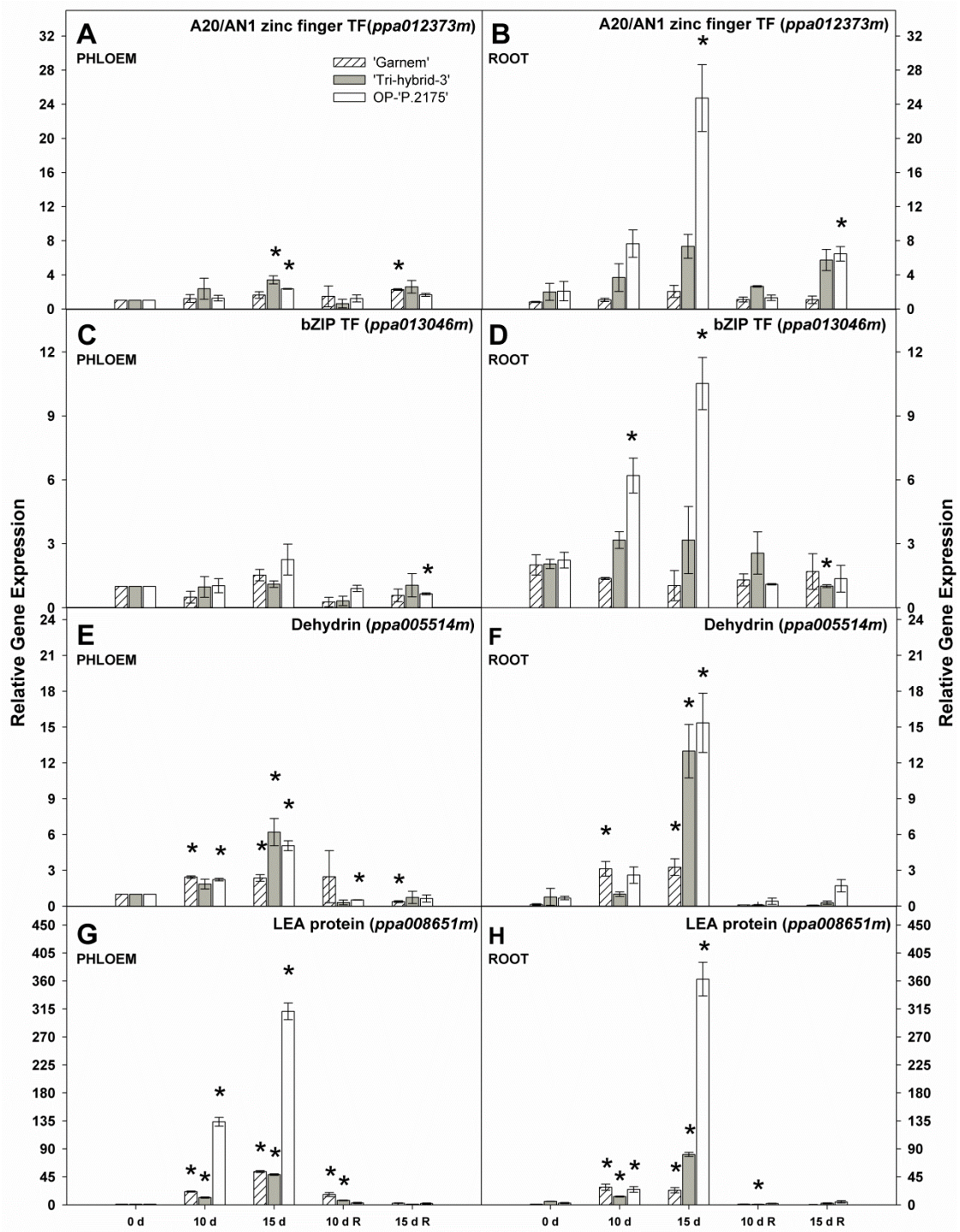

Figure 2.4. Relative expression of the A20/AN1 zinc finger TF (*ppa012373m*) (A and B); the bZIP TF (*ppa013046m*) (C and D); the dehydrin (*ppa005514m*) (E and F); and the LEA protein (*ppa008651m*) (G and H). Expression levels were compared to the actin gene. The relative value of 1 was assigned to the phloem sample on day 0 (control day value). Data show the average relative expression of two biological samples with three technical replicates each one. Asterisks indicate significantly different expression values ($p \leq 0.05$) for each genotype with respect to day 0 following the Student's t-test. (d = days, R = Recovery). Error bars represent the standard error of the mean.

Expression profiles of the TFs

The expression levels of the *ppa012373m* gene, encoding the A20/AN1 zinc-finger protein, changed slightly throughout the stress period in phloem tissue in all genotypes. Comparing the expression levels between each day of treatment to day 0 (control expression level) in phloem, significant differences were found in 'Tri-hybrid-3' (3-fold higher) and in OP-'P.2175' (2-fold higher) on 15 days of treatment and in 'Garnem' genotype (1.6-fold higher) on 15 days after recovery (Fig. 2.4A). Only significantly differences were observed among genotypes on 15 days of treatment in phloem tissue, being 'Tri-hybrid-3' expression significantly different from 'Garnem' expression (2-fold higher) (Supplementary Material S2.4; Annex 1). In root tissue, both 'Garnem' and 'Tri-hybrid-3' did not show significant differences in *ppa012373m* expression throughout the experiment compared to the control level (day 0), although an increase of expression was observed on day 15 of the stress period and on day 15 of the recovery period (Fig. 2.4B). Expression peaks were observed in OP-'P.2175' roots on day 15 of the treatment (12-fold increase) and 15 days after recovery (3-fold increase) compared to day 0 levels, showing significant differences in both cases (Fig. 2.4B). Among genotypes, significant differences were found along the days of treatment (Supplementary Material S2.4; Annex 1). So, the gene expression rate in 'OP-P.2175' was significantly different to the rates in 'Garnem' at 10 days of treatment. At 15 days of treatment, gene expression values in OP-'P.2175' were significant different to rates reached in 'Garnem' and 'Tri-hybrid-3'. During the recovery period, 'Tri-hybrid-3' was the genotype with a significant higher gene expression rate compared to the other genotypes at 10 days of recovery. Finally, after 15 days of recovery, the gene expression values in hybrids were significant higher than the gene expression rate in 'Garnem' (Supplementary Material S2.4; Annex 1). The gene encoding the A20/AN1 zinc-finger protein, *ppa012373m*, is homologous to the *SAP-8* gene of *Vitis vinifera, P. mume* and *Malus domestica*. In these species, this gene belongs to *Stress Associated Protein (SAP)-like* (SAP) family, which is characterized by the presence of A20/AN1 zinc-finger domains. SAP-like proteins have also been described in other species such as *Populus trichocarpa* (Jin et al., 2007), *Oryza sativa* (Vij and Tyagi, 2006) and *Aeluropus littoralis* (Saad et al., 2010), suggesting that they

are involved in the response to different stresses such as low temperatures, drought and salinity. The overexpression of different genes belonging to this family in rice (Giri et al., 2011; Huang et al., 2008; Kanneganti and Gupta, 2008; Mukhopadhyay et al., 2004) confirmed its regulatory role in these stresses, showing a higher expression during the early phase of the stress response. In our experiment, the higher expression at 10 and 15 days of treatment in this TF would suggest its role in acclimatization phase. In addition, Ben Saad et al., (2010) observed that the upregulation of several *LEA* genes in *AlSAP* transgenic lines suggesting that *SAP* gene would active the expression of these target genes. (Mukhopadhyay et al., 2004) suggested a role of the *OSISAP1* gene in preventing damages caused by stress and also promote a better recovery after the stress period. This hypothesis could also be valid for this experiment and would explain the trend followed by 'Tri-hybrid-3' and OP-'P.2175' in both tissues (Fig. 2.4).

The *bZIP* gene, *ppa013046m*, is orthologue to the *bZIP3 cis-element-binding factor 1* gene from *M. domestica* and *AtbZIP53* from *A. thaliana*. These TFs belong to the S group described by (Jakoby et al., 2002), and they function as transcriptional activators of the *ProDH* gene. Signals deriving from H_2O_2 and the ABA-dependent synthesis pathway during drought and salinity stress activate the *P5CS* gene, which induces the accumulation of proline (Saradhi et al., 1995; Strizhov et al., 1997; Yoshiba et al., 1997). During the first hours of rehydration, the metabolism of proline (which accumulated during stress) to glutamate is regulated by the *ProDH* gene (Satoh et al., 2004; Yoshiba et al., 1997). In our study, the *ppa013046m* gene did not show significant differences in 'Garnem' both phloem and root tissues (Fig. 2.4C and D), as well as 'Tri-hybrid-3' (Fig. 2.4C and D). Nevertheless, the *bZIP* gene was significant under-expressed in 'Tri-hybrid-3'at 15 day of recovery compared to control expression level in root tissue (Fig. 2.4D). During the stress period, *ppa013046m* expression was significantly higher in the roots from OP-'P.2175' (Fig. 2.4D), reaching levels 3-fold higher at 10 days and 4-fold higher at 15 days compared to day 0, but not in phloem tissue (Fig. 2.4C). However, the level expression of the TF was significantly lower in phloem from OP-'P.2175' after 15 days of the recovery period (Fig. 2.4C). Among genotypes for each day of treatment, no significant differences were found in phloem

(Supplementary Material S2.4; Annex 1). While, in the roots, the level expression of *ppa013046m* was significant higher in OP-'P.2175' than in 'Garnem' at 10 days of treatment and significant higher than 'Garnem' and 'Tri-hybrid-3' at 15 days of drought stress (Supplementary Material S2.4; Annex 1). Since *ProDH* gene is active during the first hour of rehydration, we would expect that its transcriptional activator would also be expressed under these conditions. On the contrary, our results were not consistent with the assumptions discussed above. A possible reason could be due to other metabolic factors involved in the induction of the *ppa013046m* gene during the stress period that require consideration in the future. Even if it seems not to be involved in rehydration process, the higher expression in OP-'P.2175' makes it useful as a marker of drought stress; even if the reasons and the mechanism that stand below are still to be unraveled.

In spite of the most of reports studying TFs expression had been done at short-term stages of the drought response (Giri et al., 2011; Huang et al., 2008; Kanneganti and Gupta, 2008; Mukhopadhyay et al., 2004), Su et al., (2013) observed the overexpression of different TFs at long-term experiment, demonstrating the important role of TFs, not only as transcriptional activators of target genes at early response to drought, but during the acclimatization phase.

Expression profiles of the target genes

The expression levels increased both in the dehydrin gene (*ppa005514m*) and in the gene encoding the LEA protein (*ppa008651m*) throughout the stress period, reaching an expression peak by 15 days of treatment, and their levels dropped significantly during the recovery period (Fig. 2.4E - H). The same trend was observed in all genotypes, both in phloem and root tissues. These two genes belong to the LEA protein family (Allagulova et al., 2003; Battaglia et al., 2008), which plays a main role in acclimatization and the adaptive response to stress processes by conferring tolerance under drought conditions, low temperatures and osmotic stress (Battaglia et al., 2008; Xiao et al., 2007). The expression of *LEA* genes is not specific for a particular tissue. These genes can be expressed in both leaves and roots or stems and even in the cotyledons (Hong-Bo et al., 2005).

The dehydrin expression levels (*ppa005514m*) showed statistically significant increases in phloem tissue at all stages of the experiment in comparison to day 0 (control), while in root tissue the expression levels increased significantly only during the stress period decreased dramatically during recovery (Fig. 2.4E and F). In 'Garnem', the expression level of *ppa005514m* was significantly 2.4-fold higher at 10 and 15 days of treatment in comparison to day 0 in phloem (Fig. 2.4E). In root tissue, 'Garnem' increased significantly the expression of the dehydrin gen being 24-fold higher on day 10 and 25-fold higher at 15 days of treatment in comparison to control (Fig. 2.4F). The *ppa005514m* expression in 'Trihybrid-3' was significantly higher (6-fold) at 15 days of treatment in phloem (Fig. 2.4E). In the root tissue, the expression level was significantly 17-fold higher at 15 days (Fig. 2.4F). Meanwhile, OP-'P.2175' showed a 2-fold higher expression in phloem by 10 days and 5-fold higher by 15 days of drought period (Fig. 2.4E). After 15 days, *ppa005514m* expression was 23-fold higher in roots (Fig. 2.4F). During the recovery period, there were only significant differences in *ppa005514m* expression levels in phloem. The dehydrin expression was less than that on day 0 in OP-'P.2175' by 10 days and in 'Garnem' at two weeks (Fig. 2.4E). Among genotypes, significant differences were found at 15 days of treatment, when the dehydrin expression in 'Tri-hybrid-3' was significantly different to the expression in 'Garnem' in the phloem (Supplementary Material S2.4; Annex 1), as well as in root tissue at 15 days, when 'Tri-hybrid-3' and 'OP-'P.2175' genotypes presented a significant higher expression levels than 'Garnem' (Supplementary Material S2.4; Annex 1). In the same tissue, *ppa005514m* expression was significantly higher in 'OP-'P.2175' than the others genotypes at 15 days of recovery (Supplementary Material S2.4; Annex 1). The *ppa005514m* gene encodes a dehydrin belonging to group 2, also known as D-11 group (Battaglia et al., 2008). Dehydrins have been studied in woody plants (Artlip and Wisniewski, 1997; Bassett et al., 2009; Velasco-Conde et al., 2012; Vornam et al., 2011; Wisniewski et al., 2009, 2006), confirming the existence of a direct relationship between the accumulation of dehydrins in tissues and tolerance to abiotic stresses. Artlip et al., (1997) identified the *Ppdhn1* gene and they demonstrated its protective role during dehydration caused by low temperatures and drought stress in *P. persica* and showed its induction by ABA. Wisniewski et al., (2006) observed that the accumulation of *Ppdhn1* in peach bark was higher than in leaves under drought

stress. Moreover, as in our work, Wisniewski et al., (2006) found that after a week of severe drought stress, the accumulation of *Ppdhn1* transcripts decreased in bark when the plants recovered their water status (Wisniewski et al., 2006). On the contrary, under low-temperature conditions, *Ppdhn1* transcripts did not accumulate in root tissues due to the minimum temperature changes that the roots might suffer throughout the seasons as compared to the damages suffered in buds where *Ppdhn1* accumulation was higher (Wisniewski et al., 2004). So this gene is supposed to be involved in drought and low temperature tolerance mechanisms. These observations are consistent with the results describing the dehydrin tendency in the tissues studied in our work. Roots would be more sensitive to the lack of water in the substrate, resulting in higher gene expression levels in root tissue than in phloem. This condition is also true for the TFs analyzed above. It was observed that the expression of 24-kd dehydrin was stronger in drought-tolerant plants than in sensitive plants at a higher water potential (Lopez et al., 2003, 2001), as it is consistent with our findings. 'Tri-hybrid-3' and OP-'P.2175'registered higher LWP and dehydrin expression levels than 'Garnem' (Figs. 2.1A and 2.4 E and F), suggesting that the accumulation of dehydrin would be related to the better drought tolerance showed by the 'Garnem' progeny.

The gene encoding the LEA protein (*ppa008651m*) was identified in a transcriptomic study of genes subjected to low temperatures in peaches (Ogundiwin et al., 2008). This gene is homologous to the gene encoding a D-29 LEA protein belonging to the 3B group described by Battaglia et al., (2008). When the relative expression of the *ppa008651m* gene was analyzed, significant differences were found in comparison to day 0 levels both in phloem and root tissues throughout the stress period, and on 10 days after recovery (Fig. 2.4G and H). For the 'Garnem' genotype, the expression showed a peak at 15 days of stress in phloem with a value 53-fold higher than control levels (Fig. 2.4G), whereas the expression values were 31- and 26-fold higher in root tissue on 10 and 15 days of the stress period, respectively (Fig. 2.4H). For the two hybrids, the highest expression level was reached on day 15 of the stress period, highlighting OP-'P.2175' on the other genotypes with a value 311-fold higher in phloem (Fig. 2.4G) and 130-fold higher in roots with respect to the reference status at day 0 (Fig. 2.4H). During the recovery period, *ppa008651m* gene expression dropped to

similar levels as those on day 0, showing statistical differences at 10 days for phloem in 'Garnem' (Fig. 2.4G) and in 'Tri-hybrid-3' genotype in both phloem (Fig. 2.4G) and root tissues (Fig. 2.4H). Significant differences were found when the *LEA* gene expression levels were compared among genotypes. So, this gene expression was significantly higher at 10 and 15 days of treatment in 'OP-'P.2175' than in 'Garnem' and 'Tri-hybrid-3', as well as significantly higher at 10 days of recovery in 'Garnem' than in the other genotypes in the phloem (Supplementary Material S2.4; Annex 1). Furthermore, its expression level was significantly higher at 15 days of drought stress in OP-'P.2175' than in 'Garnem' and 'Tri-hybrid-3' in root tissue. It is noteworthy that the control level expression in 'Tri-hybrid-3' was significantly higher than in the others genotypes in this same (Supplementary Material S2.4; Annex 1). Various studies showed the relationship of group 3 LEA proteins in the response to abiotic stress. For example, the *Hva1* gene, identified in barley, confers drought tolerance in transgenic rice, due to its protective role of the cellular membrane (Babu et al., 2004). In rice, the *OsLEA3*-1 gene was also identified and overexpressed showing that the transgenic plants improved their drought tolerance and maintaining the yield (Xiao et al., 2007). In addition, (Leida et al., 2010) found that the *ppa008651m* gene was associated with dormancy in peaches under low-temperature conditions. In our experience, we verified that *ppa008651m* expression is activated not only under low temperatures, but that it is also induced by dehydration caused by drought.

2.4. CONCLUSIONS

From the physiological and molecular data under our specific experimental conditions, the two hybrid genotypes showed a better adaptive response to drought than the 'Garnem' genotype, this is especially true for OP-'P.2175'. All genes studied had the maximum expression level in root tissue (Fig. 2.4), while LWP and gs reached the minimum value at 15d of treatment (Fig. 2.1), confirming a drought stress response. The genes encoding the LEA and dehydrin proteins can be proposed as biomarkers in the selection of more tolerant plants within a drought tolerance breeding program. In this work, we demonstrated their correlation by showing higher expression in the best adaptive response plants. It would be interesting to confirm our

results also in other species and hybrids. On the other side, the gene expression of the TFs tested was confirmed at long-term stage. Nevertheless, additional experiments are required in order to test their involvement during the early hours of exposure to drought stress.

3. IDENTIFICATION OF *CIS*-REGULATORY ELEMENTS INVOLVED IN DEHYDRATION IN TWO WATER USE EFICENCY-RELATED GENES IN A *PRUNUS* COLLECTION

ABSTRACT

Leaf ash content and Carbon isotope discrimination ($\Delta^{13}C$), which are strongly correlated to water use efficiency (WUE), are useful as selection criteria for drought stress. On the other hand, abscisic acid (ABA) accumulation under drought stress triggers multiple survival mechanisms in plants. Excess water losses are reduced by stomata closure via ABA, thereby improving WUE in plants. Thus, ABA is also involved in activation of both transcription factor (TF) genes, such as dehydration-responsive element-binding (DREB) TFs, and genes encoding osmoprotectant proteins, such as late embryogenesis abundant (LEA) and dehydrin genes. The presence of *cis*-regulatory elements (CREs) in the promoter regions of these genes provides differential expression. In this work, a population of *Prunus* species, including seven almond wild-relative species, nine cultivated hybrid rootstocks and their parentals, were subjected to leaf ash content and $\Delta^{13}C$ analysis. *P. mira* Koehne kov et. kpst, *P. davidiana* (Carr.) Franch, 'Garfi' (*P. amygdalus* (L.) Batsch) × *P.persica* (L.) Batsch) individuals and six of the almond wild-relative species showed the best WUE. Drought-related CREs were identified in the promoter regions of *PpDhn2* and *DREB2B*, and the phylogenetic analysis revealed seven clusters for *PpDhn2* and four clusters for *DREB2B*. Our results showed that *PpDhn2* and *DREB2B* are involved in WUE, and they could shed light on future studies for selection as biomarkers for WUE in a rootstock breeding program.

Keywords: Almond wild-relative species, Ash content, Carbon isotope discrimination, Rootstock, Drought, Transcription regulation.

3.1. INTRODUCTION

Drought stress is a key problem in agriculture, especially in arid and semi-arid climates (Bartels and Sunkar, 2005) in the Mediterranean region where water availability is the most important factor for plant survival. Plants respond to drought stress by activating several processes at the molecular, cellular, biochemical and physiological levels (Agarwal et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2005). Abscisic acid (ABA) is one of the main hormones involved in these processes. Under drought conditions, ABA accumulation is triggered, inducing expression of stress-related genes (Lata and Prasad, 2011; Shinozaki and Yamaguchi-Shinozaki, 2007). Genes regulated by ABA are classified in two groups. The first group is formed by genes encoding genes involved in the synthesis of osmoprotectants, such as late embryogenesis abundant (LEA) proteins. Dehydrins belong to group II LEA proteins (Allagulova et al., 2003; Hundertmark and Hincha, 2008) and their expression is associated with response to ABA, cold, salinity and drought tolerances in several species (Allagulova et al., 2003; Hundertmark and Hincha, 2008; Lopez et al., 2003; Melišová et al., 2015; Sivamani et al., 2000; Velasco-Conde et al., 2012). Particularly, three dehydrin genes (*Ppdhn1*, *Ppdhn2* and *Ppdhn3*) have been described in peach confirming its induction by cold and drought and the presence of specific *cis*-regulatory elements (CREs) in their promoter regions. Those are ABA responsive elements (ABREs), or MYB and MYC binding domains, as well as a dehydration/C-repeat responsive element (DRE/CRT) (Artlip and Wisniewski, 1997; Bassett et al., 2009; Wisniewski et al., 2006). The second group are genes encoding transcription factors (TFs) like dehydration-responsive element-binding factor (DREB), which are involved in expression of stress-inducible genes by interaction with their CREs in promoter regions (Shinozaki and Yamaguchi-Shinozaki, 2007; Tavakol et al., 2014). *DREB* TFs are members of the AP2/ERF family (Liu et al., 1998), one of which is DREB2B TF induced by dehydration and salinity, but not by cold stress (Liu et al., 1998; Nakashima et al., 2000). These proteins bind to DRE/CRT *cis*-elements in the promoter region of target genes, for example genes encoding LEA proteins (Kobayashi et al., 2008). Many stress-inducible genes are controlled by this ABA-dependent pathway, but some may also be regulated by other ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki,

2006). Furthermore, there is evidence of coordinated interaction between these two responsive pathways (Roychoudhury et al., 2013).

As a physiological consequence of ABA accumulation, water loss is reduced by leaf stomata closure, enhancing water use efficiency (WUE) (Lata and Prasad, 2011). This may be the most important fact associated with plant drought adaptation (Blum, 2009; de Almeida Silva et al., 2012). WUE can be defined as a function of CO_2 assimilation in photosynthesis and water loss by transpiration (Bassett et al., 2014; Condon et al., 2004; Tomás et al., 2012), leading to the productivity of plants under drought conditions (Melišová et al., 2015). Carbon isotope discrimination ($\Delta^{13}C$) is a time-average technique used to estimate long-term WUE at leaf level (Farquhar and Richards, 1984; Melišová et al., 2015; Moghaddam et al., 2013). The basis of this indirect method has been extensively studied (Farquhar and Richards, 1984; Melišová et al., 2015; Moghaddam et al., 2013), and suggests a negative correlation between WUE and $\Delta^{13}C$. Furthermore, the relationship between $\Delta^{13}C$ and ash content has been studied in cereals (Araus et al., 1998; Zhu et al., 2008), in apple (Glenn, 2014) peach (Glenn and Gasic, 2015) in order to improve phenotyping and breeding for WUE. The association among these three parameters (WUE, $\Delta^{13}C$ and leaf ash content) is grounded on the passive transport of minerals via xylem and their accumulation in growing and transpiring tissues. Therefore, the higher transpiration, the higher mineral transport to those tissues leading to an increase in ash content (Glenn and Bassett, 2011). The correlation of high WUE with low leaf ash content and low $\Delta^{13}C$ has been well demonstrated (Blum, 2011; Glenn, 2014; Masle et al., 1992).

Prunus L. is a diverse genus including approximately 200 species with most of them growing in the temperate zone and some in the tropical and subtropical regions. This genus is economically important due to its diverse uses as fruit, oil, timber, and ornamentals (Lee and Wen, 2001). Rootstocks are responsible for water and nutrient uptake, resistance to soil-borne pathogens, and tolerance to environmental stresses, to name a few of the more important traits developed in breeding programs (Layne, 1987). Several *Prunus* species such as *P. amygdalus* Batsch, *P. persica* (L.) Batsch, *P. cerasifera* Ehrh., *P. davidiana* (Carr.) Franch, *P. mira* Koehne kov et. Kpst, *P. domestica*

L., *P. insititia* L. are used as rootstocks. Also interspecific hybrids rootstock have also been developed from almond × peach and peach × *P. davidiana* (Bielsa et al., 2014; Byrne et al., 2012; Felipe, 2009; Lecouls et al., 2004).

Currently, the aim of several stone fruit rootstock breeding programs is to create more interspecific hybrids to desirable and useful traits from different *Prunus* species. Wild relative species have also been utilized both for direct rootstock such us, *P. bucharica* (Korsh.) Fetdsch., *P. kuramica* (Korsh.), *P. webbii* (Spach) Vieh. or *P. kotschii* (*A. kotschii* Boiss.), and to create interspecific hybrids. e.g., *P. webbii* × almond, due to their natural abiotic and biotic resistances to introgress genes in cultivated *Prunus* rootstocks (Alimohammadi et al., 2013; Gradziel et al., 2001). Recently, Bielsa et al., (2016) evaluated the response to drought stress in three interspecific hybrids of *Prunus* identifying two genes a LEA protein and a dehydrin with different expression.

The aim of this study was to estimate WUE from leaf ash content and $\Delta^{13}C$ in a number of almond wild-relative species and in cultivated rootstocks in order to evaluate their use as a new source of drought-tolerance genes. Also to identify drought-related CREs found in the promoter regions of *PpDhn2* and *DREB2B* of several genotypes for a better understanding of gene regulation under drought stress.

3.2. MATERIALS AND METHODS

3.2.1. Plant material and growth conditions

A total of 48 individuals, listed in Table 3.1, were used in this study. The genotypes were located at the CITA (Centro de Investigación y Tecnología Agroalimentaria de Aragón) facilities in Zaragoza, Spain (41°43'N, 0°48'W) belonging to a rootstock and wild relatives collections, respectively. Conventional orchard practices were used in tree training and weed control. Water requirements were supplied by surfaced irrigation for the hybrids and their parentals, and drip irrigation for the almond wild-relative species.

Table 3.1. List of the 48 individuals used in this study.

Parental Genotypes			No. ind.
P. davidiana (Carr.) Franch			3
P. mira Koehne kov et. kpst			2
P. persica (L.) Batsch			1
'Garfi' (*P. amygdalus* (L.) Batsch)			1
'Nemared' (*P. persica* (L.) Batsch)			1
Wild Species			
P. bucharica (Korsh.) Fetdsch.			5
P. zabulica Seraf.			5
P. webbii (Spach) Vieh.			5
P. vavilovi (Spach)			4
P. orientalis (Mill.) [syn. *P. argentia* (Lam)]			4
P. gorki (Fristch)			4
P. kotschii (*A. kotschii* Boiss.)			4
Hybrid Genotypes	♀	♂	
'Mira×Pecher'	*P. mira* Koehne kov et. kpst	*P. persica* (L.) Batsch	1
'Barrier'	*P. davidiana* (Carr.) Franch	*P. persica* (L.) Batsch	1
'Cadaman'	*P. persica* (L.) Batsch	*P. davidiana* (Carr.) Franch	1
'GF-677'	'Garfi'	*P. persica* (L.) Batsch	1
'Garnem'	'Garfi'	'Nemared'	1
'Felinem'	'Garfi'	'Nemared'	1
'Monegro'	'Garfi'	'Nemared'	1
'GN-8'	'Garfi'	'Nemared'	1
'GN10'	'Garfi'	'Nemared'	1

3.2.2. Leaf ash content and carbon isotope discrimination ($\Delta^{13}C$) analyses

To evaluate the water use efficiency (WUE) in our individuals, we determined ash content and carbon isotope discrimination in leaves. Approximately 15 leaves per tree were collected, washed with deionized water, air dried at 60 °C for 48 h. The tissue was re-dried at 70 °C for 72h, ground to pass a 40 mesh screen, and analyzed for ^{13}C content (University of California, Davis Stable Isotope Facility, Department of Plant Sciences, Davis, CA, USA). Carbon isotope discrimination ($\Delta^{13}C$) was calculated according to (Farquhar et al., 1989). The carbon dioxide isotope composition in air was assumed to be -7.8 parts per thousand (Francey et al., 1995). The same sample leaf tissue weight (0.5 g approximately) was placed in a preheated porcelain crucible and

burnt in a muffle at 550 °C for 24 h to determine ash content using a thermogravimetric analyzer (Leco, Inc., St. Joseph, MO, model TGA701). Correlation analysis was performed to relate leaf ash content with $\Delta^{13}C$ using IBM SPSS Statistics v21.0 (SPSS Inc./IBM Corp., Chicago, IL, USA).

3.2.3. DNA isolation

Leaves were collected and stored at -20 °C. Total DNA was extracted from 50 mg of frozen leaves as described by Doyle and Doyle, (1987). The sample was ground in a mortar with liquid N_2. The powder was homogenized with 700 µl of CTAB (100 mM Tris-HCl $C_4H_{11}NO_3$, 20 mM EDTA, 2% CTAB, 1.4 M NaCl, pH 8, 1% PVP-40, 0.1% $NaHSO_3$) and 0.4 µl of 2-mercaptoethanol, and transferred to a 1.5 ml Eppendorf tube. Then, the sample was incubated at 65 °C for 25 min. After incubation, 700 µl of chloroform-isoamyl alcohol (24:1, v/v) were added. Once the sample was homogenized, it was centrifuged at 5,590 × g and room temperature for 15 min. After centrifugation, 450 µl from the upper phase were transferred to a new 1.5 ml Eppendorf tube and an equal volume (450 µl) of cold isopropanol was added and homogenized. The precipitated nucleic acid was recovered by centrifugation at 10,956 × g at room temperature for 5 min, washed in 800 µl of 10 mM ammonium acetate in 76% ethanol during 45 min. After the washing step, the sample was centrifuged again at 10,956 × g at room temperature for 5 min. Finally, the supernatant was removed and the pellet dried at room temperature. DNA was re-suspended in 100 µl of TE solution (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at 4 °C overnight. The following day, the samples were quantified using a NanoDrop® ND-1000 UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

3.2.4. PCR amplification

In order to clone the approximately 1,000 bp upstream sequence of the translation start codon primers were designed based on the nucleotide sequences of the *PpDhn2* gene (*ppa011637m*) and *DREB2B* gene (*ppa022996m*) present in the assembled and annotated peach genome (*P. persica* genome v1.0; http://www.rosaceae.org/). Approximately 150 ng of genomic DNA were amplified

using a Platinum Taq DNA Polymerase High Fidelity kit according to the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA) and the *PpDhn2*-specific primers, forward 5'-TTGAGCAGCAGTATCACAAGC-3', reverse: 5'-GGTGGTTCCGGTCGTAGTAG-3'; and the *DREB2B*-specific primers, forward 5'-ACGTGGGACAAAACAGGGTA-3', reverse: 5'-TACCAAGCCAAAGACGACTG-3'. The PCR conditions consisted in an initial denaturation during 1 min at 94 °C, 35 cycles of 30 s at 94 °C, 1 min for the annealing temperature of 60 °C and 2 min at 68 °C, followed by a final extension of 10 min at 72 °C. After agarose electrophoresis, the PCR products were purified using a DNA Clean & concentrator™-5 kit (Zymo Research, Orange, CA, USA) following the manufacturer's recommendations.

3.2.5. Cloning and sequencing

The gDNA fragments of 1,074 bp and 1,003 pb obtained from our genotypes for *PpDhn2* and *DREB2B* genes, respectively, were cloned into the pCR™2.1-TOPO® vector (Invitrogen, Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The plasmid DNA of the positive transformants was isolated using GeneJET™ Plasmid Miniprep kit (Thermo Fisher Scientific, Waltham, MA, USA). After digestion with *Eco*R1 using EcoR1-HF™ RE-Mix® (New England, BioLabs Inc., Ipswich, MA, USA) for checking the quality and the integrity of the gDNA insert within the vector, positive clones were sent to Beckman Coulter Genomics (Danvers, MA, USA) and Secugen S.L. (Madrid, Spain) for sequencing using the universal M13 forward and reverse primers.

3.2.6. *In silico* analysis of *PpDhn2* and *DREB2B* promoter regions

Chromatograms from the sequencing of the studied fragments were edited by BioEdit software version 7.2.5 (Hall, 1999), vector sequences were removed using VecScreen software from NCBI (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). Then, resulting sequences were aligned using MUSCLE software from EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/muscle/) (Edgar, 2004) and assembled by the Contig Assembly Program CAP3 (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::cap3) (Huang and Madan, 1999).

The phylogenetic trees for each promoter region of *PpDhn2* and *DREB2B* genes were constructed to classify our individuals using MEGA 6.0 (Tamura et al., 2013) with the Neighbour-Joining (NJ) method (Saitou and Nei, 1987), and a bootstrap analysis was conducted using 1,000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980).

Two databases of *cis*-acting regulatory elements (CREs) motifs: PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002) and PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/promoter.php) (Chang et al., 2008) were used to identify CREs involved in drought response along the 5' regulatory regions of each gene.

3.3. RESULTS

3.3.1. Relationship between leaf ash content and carbon isotope discrimination ($\Delta^{13}C$)

Mean $\Delta^{13}C$ ratios varied among genotypes and ranged from 17.71‰ to 23.17‰ and mean ash content varied from 5.89 to 17.97 % (Table 3.2). There was a significant ($P < 0.05$) positive relationship between $\Delta^{13}C$ and leaf ash content (Figure 3.1) *P. davidiana* individuals had the lowest value both $\Delta^{13}C$ ratio and leaf ash content (Table 3.2 and Figure 3.1). The $\Delta^{13}C$ values of almond-related wild species were close to the average (20.99‰) with ratios between 19.96‰ to 20.87‰ (Table 3.2). Genotypes with highest $\Delta^{13}C$ ratios were 'Nemared' (23.17‰), 'Monegro' (23.10‰) and 'Mira×Pecher' (22.94‰). $\Delta^{13}C$ ratios of the individuals belonging to G×N series, except to 'GN-8', were upper the average (Table 3.2). GF-677 had the highest leaf ash content and the fourth highest $\Delta^{13}C$ values (Table 3.2). Variability of $\Delta^{13}C$ values was low with an overall standard deviation value of 1.30 and coefficient of variation (CV%) of 6.20, while the overall standard deviation of ash content values was 3.61 with a CV% of 34.19 (Table 3.2).

Table 3.2. Carbon isotope discrimination [$\Delta^{13}C$ (‰)] and leaf ash content (%) of 21 *Prunus* genotypes. (SD: Standard Deviation; nd: no data; CV: Coefficient of variation).

Genotypes	$\Delta^{13}C$ (‰)	Ash (%)	SD $\Delta^{13}C$ (‰)	SD Ash (%)
P. davidiana	17.714	5.957	0.491	0.202
P. mira	19.273	7.640	0.465	0.608
P. persica	20.627	11.570	nd	nd
'Garfi' almond	20.994	7.380	nd	nd
'Nemared' peach	23.169	14.600	nd	nd
P. bucharica	20.518	8.076	1.289	0.771
P. zabulica	20.408	7.076	0.842	1.101
P. webbii	20.188	11.646	0.705	2.092
P. vavilovi	19.964	6.993	0.893	0.211
P. orientalis	20.661	8.150	0.902	0.719
P. gorki	20.285	7.690	1.264	0.991
P. kotschii	20.868	6.563	0.662	0.409
'Mira×Pecher'	22.948	17.600	nd	nd
'Barrier'	21.292	10.860	nd	nd
'Cadaman'	20.880	9.660	nd	nd
'GF-677'	22.235	17.970	nd	nd
'Garnem'	21.979	14.470	nd	nd
'Felinem'	22.146	14.450	nd	nd
'Monegro'	23.105	13.240	nd	nd
'GN-8'	20.548	9.890	nd	nd
'GN-10'	21.154	11.540	nd	nd
Mean	20.998	10.620	1.303	3.631
CV (%)	6.203	34.192		

Comparing, both $\Delta^{13}C$ and ash content values, *P. davidiana* individuals had the lowest values (Fig. 3.1), indicating higher WUE than the other genotypes. Conversely, WUE in 'GF-677' and 'Mira×Pecher' hybrids had the highest values for ash content and $\Delta^{13}C$ indicating the lowest WUE (Table 3.2 and Fig. 3.1). Almond wild-relative species had similar low ash content values to *P. davidiana* except for *P. webbii* F3 and F17 and low $\Delta^{13}C$ values compared to peach and peach hybrid values. Overall, peach and peach hybrids had higher $\Delta^{13}C$ and ash content values than Almond wild-relative species. Among the G×N series, 'GN-8' and 'GN-10' had lower ash and $\Delta^{13}C$ values than 'Felinem', 'Garnem', 'Monegro' and 'Nemared' (Table 3.2).

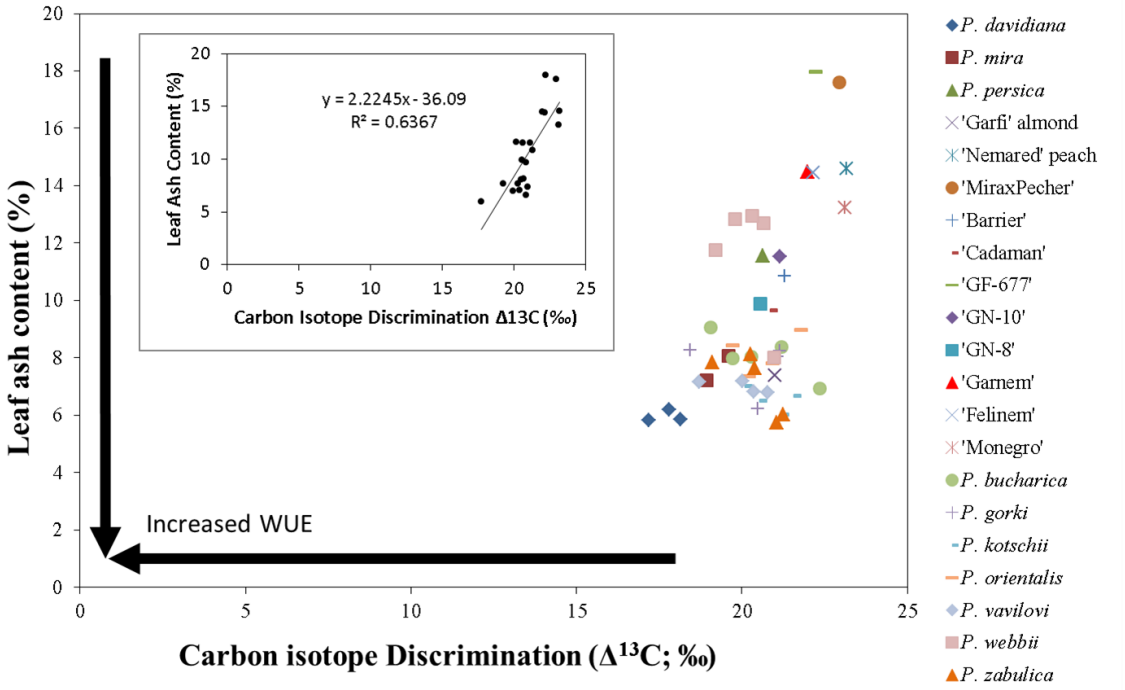

Figure 3.1. Relationship between carbon isotope discrimination [$\Delta^{13}C$ (‰)] and leaf ash content (%) for *Prunus* genotypes. Arrows indicate the negative relation between these two parameters and WUE.

3.3.2. Phylogenetic analysis

The 5' regulatory region of *PpDhn2* gene from 47 nucleic acid sequences were classified in six clusters (Fig. 3.2) based on the dendrogram tree obtained by NJ method. Cluster I contained 28 individuals, including all the hybrids and their parents, except one individual belonging to *P. mira* genotype, which was found in cluster II, as well as 12 individuals belonging to 6 different wild relative species (Fig. 3.2). In cluster II are included one *P. mira* individual, the *P. mira* T1, as above-mentioned, and other 6 individuals from 4 different wild-relative species (Fig. 3.2). Clusters III, IV and V were the only clusters containing just one individual from 2 wild-relative species, *P. gorkii* for Cluster III and *P. webbii* for clusters IV and V (Fig. 3.2). Finally, cluster VI was formed by 9 wild-relative almond species (Fig. 3.2). These results revealed the diversity in the promoter region of *PpDhn2* gene.

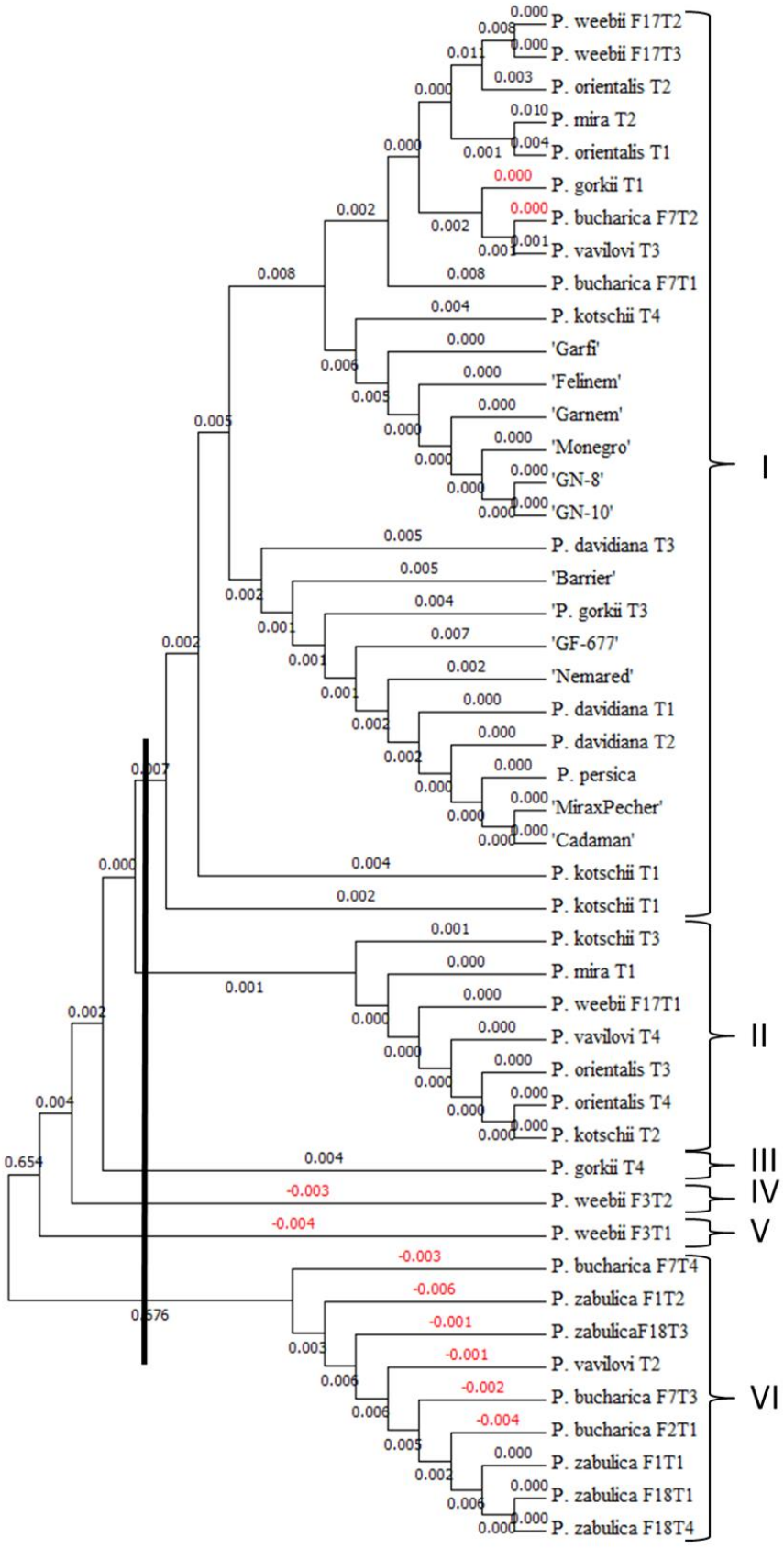

Figure 3.2. Dendrogram representing the phylogenetic differences in *PpDhn2* promoter gene regions. The tree was constructed using the Neighbor-Joining method with 1,000 bootstrap replicates.

The 48 promoter regions of *DREB2B* TF were grouped in five clusters (Fig. 3.3). The largest cluster I contained 29 individuals including the 'Garfi' almond and one hybrid the 'GF-667'with most wild-relative almond species (Fig. 3.3). Cluster II were formed by 4 wild-relative almond species belonging to *P. zabulica* and *P. kotchii* species (Fig. 3.3). The smallest group was cluster III with only one individual *P. mira* T2 (Fig. 3.3). All hybrid individuals and most of the parents were found in cluster IV (Fig. 3). This dendrogram showed evolutionary distances close to 0, indicated a high level of conservation in the 5' regulatory region of *DREB2B* TF.

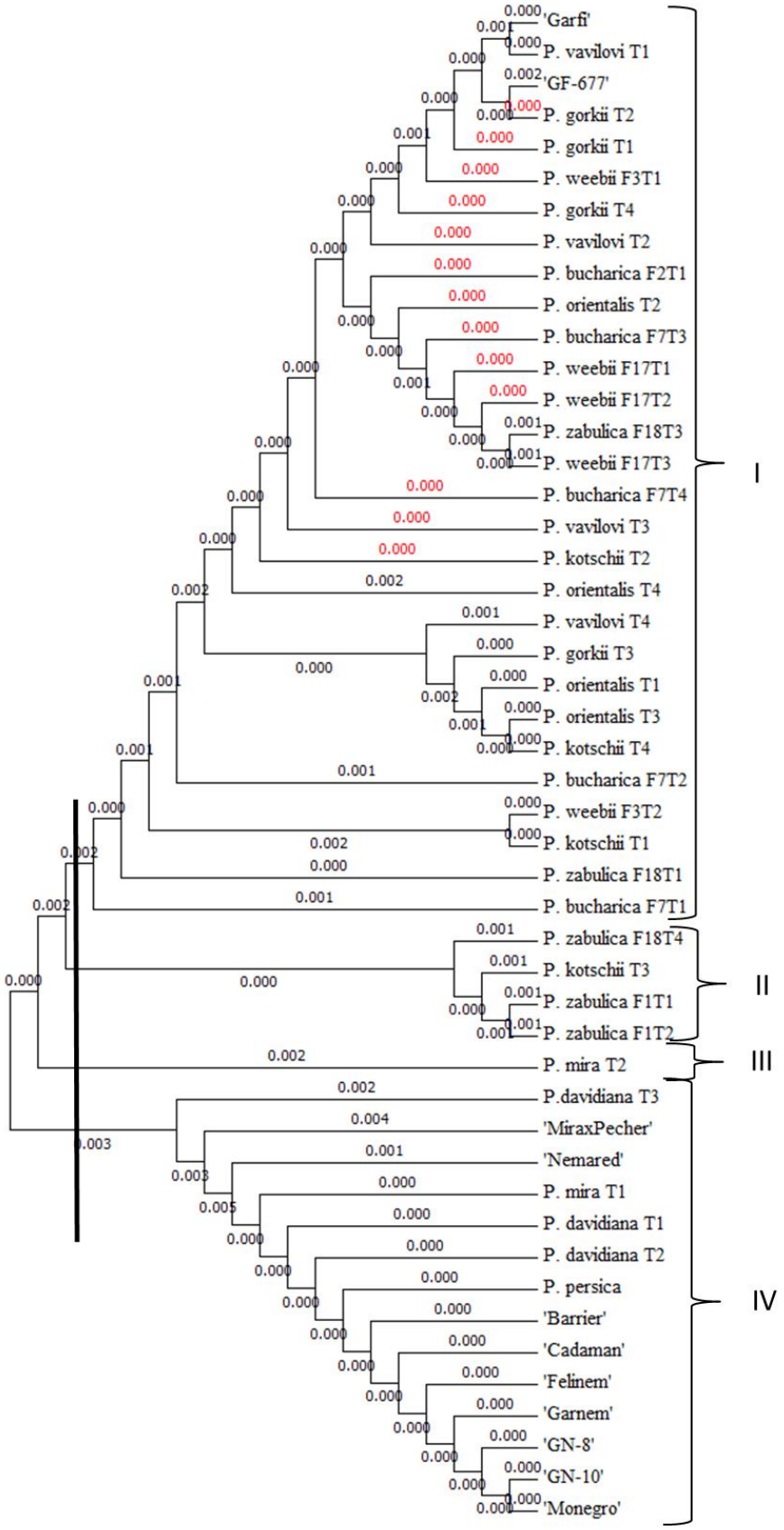

Figure 3.3. Dendrogram representing the phylogenetic differences in *DREB2B* promoter gene regions. The tree was constructed using the Neighbor-Joining method with 1,000 bootstrap replicates.

3.3.3. *PpDhn2* gene and *DREB2B* TF promoter region analysis

To understand the regulation of *PpDhn2* gene and *DREB2B* TF in response to drought, we identified CREs in their genomic sequences approximately 1,000 bp upstream of the translation start codon. Based on the dendrograms resulting from the phylogenetic analysis, the nucleic acid sequences of selected individuals from each cluster were aligned. Individuals of each group of the alignment were selected again depending on the nucleic acid differences found in the alignment analysis. Finally, CREs were found not only responsive to drought stress, but also to others processes and stresses such us light, development, hormone, biotic and abiotic stress responses in both promoter regions of the individuals which represented the alignment results after a deep search done by PlantCARE and PlantPAN 2.0 databases.

For *PpDhn2* gene, we analyzed the promoter regions of *P. mira* T2 and *P. webbii* F17T2 from cluster I, *P. gorki* T4 from cluster III; and *P. zabulica* F1T2 from cluster VI as selected individuals. For CRE analysis, clusters II, IV and V were represented by *P. gorki* T4 from cluster III because the promoter regions of all these individuals had the same CREs in their promoter regions. Different families of CREs associated with drought stress and ABA response were predicted in both sense and antisense positions. Four CREs classes were found in all genotypes: different ABA- and dehydration-responsive elements as ABRELATERD1 (5'-ACGTG-3') and ACGTATERD1 (5'-ACGT-3'); ABRERATCAL (5'-MACGYGB-3') and ACGTABREMOTIFA2OSEM (5'-ACGTGKC-3'); the ASF-1 binding site (5'-TGACG-3'); the bZIP TF DPBFCOREDCDC3 (5'-ACACNNG-3'); the calmodulin-binding motif CAMTA3; SR1 (5'-[ACG]CGCG[GTC]-3'); a MYC element as EBOXBNNAPA (5'-CANNTG-3'); several MYB motifs such as MYB2CONSENSUSAT (5'-YAACKG-3'), MYBCORE (5'-CNGTTR-3'), MYBCOREATCYCB1 (5'-AACGG-3') and MYBST1 (5'-GGATA-3'); and the SEF4 TF SEF4MOTIFGM7S (5'-RTTTTTR-3') (Fig. 3.4A and Supplementary Material S3.1; Annex 2). Among the CREs, EBOXBNNAPA was the most abundant element with a repetition range of 18 to 4 in the promoter region of each genotype, followed by ACGTATERD1 with a range of 8 to 6 repetitions (Supplementary Material S3.1; Annex 2). Clear differences between individuals from cluster VI and individuals from the rest of the clusters (I, II, III, IV and V) were identified (Fig. 3.4A and

Supplementary Material S3.1; Annex 2) Three different CREs families were only represented in the promoter region of genotypes from cluster VI: a heat shock promoter element (HSE) (5'-AGAAnnTTCT-3'); a low-temperature-responsive element (LTRE-1) LTRE1HVBLT49 (5'-CCGAAA-3'); three MYB elements such as MYB1AT (5'-WAACCA-3'), MYBGAHV (5'-TAACAAA-3') and MYBPLANT (5'-MACCWAMC-3'); and (Fig. 3.4A and Supplementary Material S3.1; Annex 2). Six CREs were found in individuals from clusters I, II, III, IV and V, but not in individuals from cluster VI: the ABRE motif ABREDISTBBNNAPA (5'-GCCACTTGTC-3'); a T-box ACGTTBOX (5'-AACGTT-3'); the DRE element DRE1COREZMRAB17 (5'-ACCGAC-3'); the MYC elements MYCATERD1 (5'-CATGTG-3') and MYCATRD22 (5'-CACATG-3'), and the MYC recognition site G-box (5'-CACNTG-3'). Furthermore, individuals from clusters I, II, III, IV and V contained a GT3 box (5'-GGTAAA-3') in their promoters (Fig. 3.4A and Supplementary Material S3.1; Annex 2). The ERE element, ERELEE4 (5'-AWTTCAAA-3'), was only identified in cluster I, but not in clusters II, III, IV and V. Finally, five CREs were found only in individuals from clusters II, III, IV and V, but not in clusters I and VI: the DRE elements CBFHV (5'-RYCGAC-3'), DDF1 (5'-[AG]CCGAC-3'), DRE1COREZMRAB17 (5'-ACCGAC-3') and DRECRTCOREAT (5'-RCCGAC-3'); and a LTRE element LTRECOREATCOR15 (5'-CCGAC-3') (Fig. 3.4A and Supplementary Material S3.1; Annex 2).

The study of the *DREB2B* TF promoter region was done in 'Garfi', 'GF-677', *P. orientalis* T4, *P. vavilovi* T4, *P. bucharica* F7T2, *P. kotschii* T1 and *P. bucharica* F7T1 from cluster I; *P. kotschii* T3 from cluster II; *P. mira* T2 from cluster III; and *P. davidiana* T3, 'MiraxPecher', *P. persica* and 'Garnem' from cluster IV. CREs elements were located in sense and antisense orientation, presenting a more conserved sequence than the *PpDhn2* gene promoter region. We identified in all individuals several ABA-, dehydration-, responsive elements, e.g., the ABARE-element HEXMOTIFTAH3H4 (5'-ACGTCA-3'), ABRELATERD1, ABREMOTIFAOSOSEM, ABRERATCAL, ACGTABREMOTIFA2OSEM and ACGTATERD1; the ASF-1 binding site; the ERELEE4 motif; the HSE element (5'-AGAAnnTTCT-3'); the motif LTRE1HVBLT49 (5'-CCGAAA-3'); several MYB elements such us MYB1AT, MYBCORE, MYBCOREATCYCB1 (5'-AACGG-3'), the MYBGAHV (5'-TAACAAA-3'), MYBPLANT and MYBST1 (5'-GGATA-3'); the

calmodulin-binding motif CAMTA3; SR1, and the MYC element EBOXBNNAPA (Fig. 3.4B and Supplementary Material S3.2; Annex 2). The motif most repeated was ACGTATERD1 with 8 repetitions. The *cis*-element MYB2CONSENSUSAT was the only one located in individuals of cluster I, but not in individuals belonging to clusters II, III, IV and V. (Fig. 3.4B and Supplementary Material S3.2; Annex 2). The bZIP TF DPBFCOREDCDC3 was found in clusters I, II, III and IV, but not in cluster V. The motif ·SEF3MOTIFGM (5'-AACCCA-3') was presented in individuals from cluster II, III and IV (Fig. 3.4B and Supplementary Material S3.2; Annex 2). Finally, the SEF4 TF was only found in clusters I and V with the particularity that this TF was at different position in each cluster (Fig. 3.4B and Supplementary Material S3.2; Annex 2).

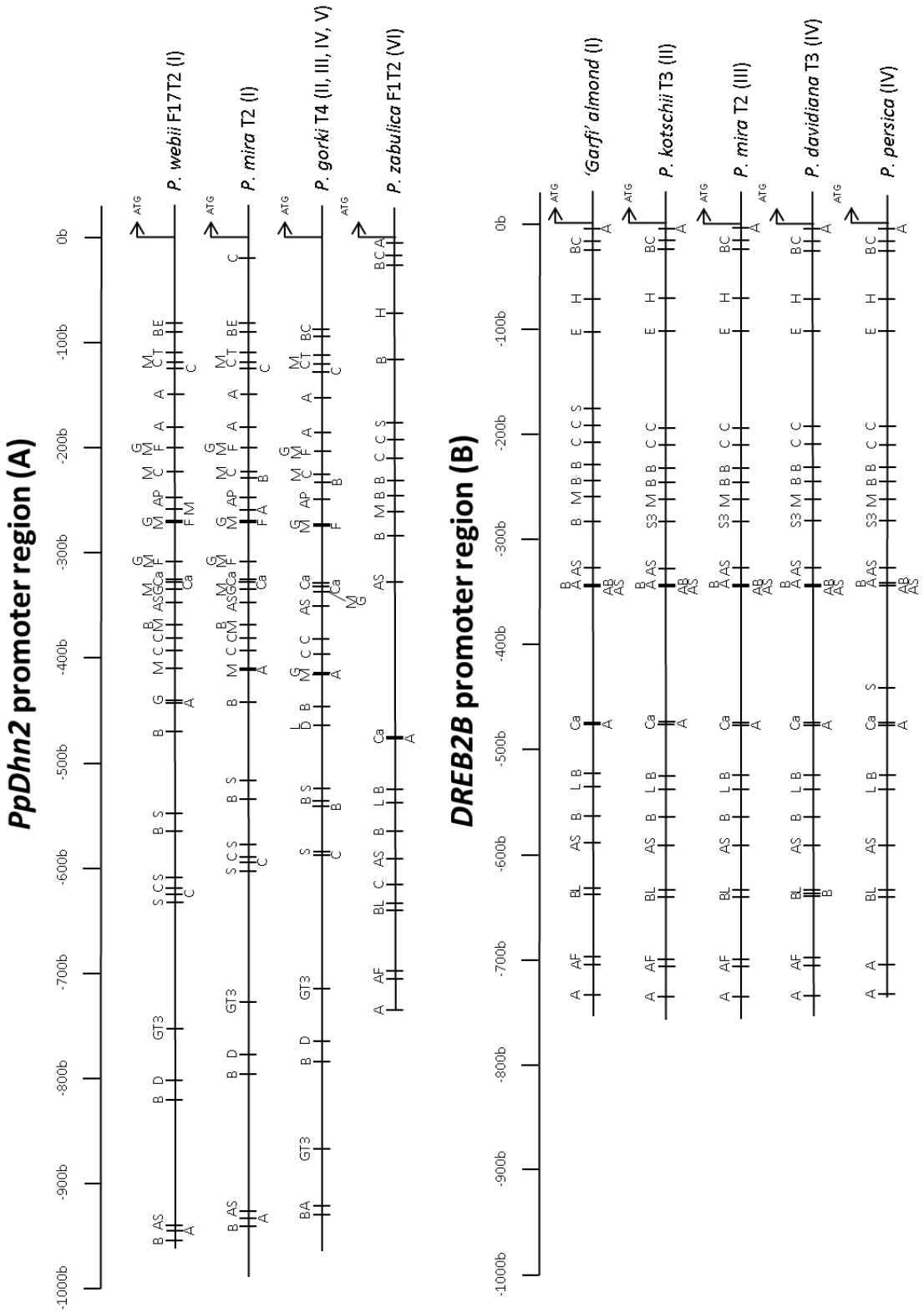

Figure 3.4. Schematic representation of the 1,000 pb region upstream of the *PpDhn2* (A) and *DREB2B* (B) promoters in each of the cluster-representing individuals. Promoter regions were defined as the first 1,000 pb 5' of the translation start site. A: ABRE-elements; AB: ABARE-element; AP: AP2; ERF TFs; AS: ASF-1 binding site; B: MYB-elements; C: CAAT-box; Ca: Calmodulin-binding motif; D: DRE-elements; E: ERE-element; F: bZIP TF; G: G-box; H: HSE-element; L: LTRE-element;; M: MYC-elements; S: SEF4 TF; S3: SEF3 TF; T: TATA-box; GT3: GT3-box.

3.4. DISCUSSION

Water scarcity must be a critical criterion when we choose a rootstock in areas in which water is the main constraint. Therefore, increasing WUE in rootstocks is important to ensure future economical fruit tree production. ABA accumulation reduces transpiration via stomata closure in response to drought stress in order to improve the WUE in plants (Lata and Prasad, 2011). Low leaf ash content and low carbon isotope discrimination are correlated with high WUE (Blum, 2011; Glenn, 2014; Masle et al., 1992). Furthermore, ABA biosynthesis induces the expression of a number stress-responsive genes during response to drought, including dehydrins, following both ABA-dependent and independent pathways (Shinozaki and Yamaguchi-Shinozaki, 2007). The important role played by dehydrins during drought response has widely been reported in herbaceous and woody plants (Allagulova et al., 2003; Lopez et al., 2003; Velasco-Conde et al., 2012; Wisniewski et al., 2006; Yang et al., 2012; Zolotarov and Strömvik, 2015). In particular, Wisniewski et al., (2006) confirmed induction of the *PpDhn2* gene by dehydration and ABA in peach. In order to activate the expression of dehydrins, different TFs have to bind to *cis*-elements contained in the dehydrin promoter region (Hanin et al., 2011). Likewise, the *DREB2B* TF is highly induced by drought stress, mainly through an ABA-independent pathway, but can also respond in an ABA-dependent manner (Lata and Prasad, 2011; Nakashima et al., 2000; Sazegari et al., 2015; Shinozaki and Yamaguchi-Shinozaki, 2007; Yamaguchi-Shinozaki and Shinozaki, 2005). *DREB2B* binds to CTR/DRE elements such as those found in the*PpDhn2* promoter region, thus contributing to its expression under drought stress signaling.

In this work, ash content and carbon isotopic composition were used to estimate plant WUE in several almond wild- relative species and in a number of interspecific *Prunus* hybrids. Since the expression of stress-responsive genes depends on the presence of CREs in their promoter regions, we analyzed promoters of the *PpDhn2* gene and *DREB2B* TF in a group of individuals showing genetic diversity in these regions to evaluate the hypothetical response to drought stress of these genotypes and enhance *Prunus* rootstock germplasm tolerance to drought.

Promoter analysis of *PpDhn2* and *DREB2B* revealed the presence of CREs associated with ABA- and dehydration-response. We found that all individuals shared ABREs in both gene promoter regions, although the number of ABREs varied depending on the genotype and the gene. ABRE is the most abundant CRE in ABA-responsive gene expression, and at least two copies of an ABRE are necessary for ABA-responsive induction of transcription (Yamaguchi-Shinozaki and Shinozaki, 2005). Different MYB motifs and a MYC element were also distributed throughout the promoter regions of both genes in all individuals. Both MYB and MYC recognition sequences confer drought responsiveness and are fundamental to ABA- and drought-responsive expression (Abe et al., 1997; Roychoudhury et al., 2013; Tran et al., 2004). We also located specific CREs for *PpDhn2* from each cluster, which indicate less evolution of that promoter region along time. The clearer differences were between the cluster VI in comparison with the rest of clusters: I, II, III, IV and V. The nine almond wild-relative species which formed that cluster also had in common a HSE, a LTRE, as well as three MYB motifs and more elements in the other individuals. However, in promoters of individuals from clusters I, II, III, IV and V were identified an ABRE motif, one DRE element, three MYC recognition sites, and an interesting element, the GT3 box, which is a Trihelix TF that is a negative regulator of WUE by the transcription repression of *SDD1* gene (Yoo et al., 2010). Other specific CREs were found in the almond wild-relative species and in a *P. mira* T1 belonging to clusters II, III, IV and V. Their *PpDnh2* promoter regions presented 3 DREs, one C-repeat binding factor and one LTRE. In previous reports, the promoters of *PpDhn2* gene in peach was studied, founding ABRE elements and MYC elements, but not MYB elements, DRE/CRT, LTRE elements in positive strand (Bassett et al., 2009; Wisniewski et al., 2006). In our work, the DRE motifs and LTRE were located in the negative strand, but not in sense position. The influence of the *cis*-element orientation in the promoter regions is a controversial issue. Although both dependent and independent orientation motifs have been reported (Guo et al., 1991; Lin et al., 2004). Recently a research did not find evidences of the influence of the motif orientation in regulatory gene expression in a number of *cis*-elements studied in *A. thaliana* (Lis and Walther, 2016). Similarly to our findings, Bassett et al., (2009) and Wisniewski et al., (2006) observed that no DRE elements were found in positive sense in the *PpDhn2* promoter and suggested that the absence

of this *cis*-element was related to the lack of expression in response to cold. However, other reports have confirmed the presence of one DRE/CRT element in the promoter region of the Y_nSK_n dehydrin class, which includes *PpDhn2* (Zolotarov and Strömvik, 2015). In spite of this observation, it is known that Y_nSK_n dehydrins are not expressed in response to cold. Garcia-Bañuelos et al., (2009) concluded that *MdDhn2* was accumulated after a period of acclimation in apple trees. Based on that, Zolotarov and Strömvik, (2015) afirmed that cold-induced expression of Y_nSK_n-type dehydrins would not be detected in some cases because of a limited time of exposure to low temperature. So that, the presence of the DRE and LTRE elements found in the anti-sense position in our individuals could have some effect in the expression of *PpDhn2* in a possible response to cold.

All species shared essentially the same CREs in their *DREB2B* promoter region. Furthermore, the elements described before, we identified in sense position a HSE element, which binds to heat shock factors responsible for heat stress tolerance (Larkindale and Vierling, 2008). Moreover, although several reports demonstrated that *DREB2B* is not induced by low temperatures (Bartels and Sunkar, 2005; Lata and Prasad, 2011; Liu et al., 1998) a LTRE element, which is an important motif for the induction of cold regulated genes (Dunn et al., 1998), was located upstream of the transcription start codon. The presence of ABREs motifs in the promoter region of *DREB2B* denoted the implication of this TF in ABA-dependent signal transduction pathway (Sazegari et al., 2015).

The promoter regions of both genes also contained multiple *cis*-elements related to other plant responses. For example, SORLIP or I-box motifs which are usually upstream elements are regulated by light and the circadian clock; other elements are relate to development responses, including the O2-site involved in zein metabolism regulation and a CAT-box linked to meristem expression. Some motifs are related to hormone responses including an ARR1AT motif (cytokinin response regulator), several CGTCA-motifs involved in methyljasmonate-responsiveness, and the GARE-motif associated with gibberellin-responsiveness, as well as others linked to additional stress. The presence of these CREs could reflect the role of *DREB2B* and *PpDhn2* in

other processes in addition to cold and drought (Ban et al., 2011; Li et al., 2003; Sazegari et al., 2015; Yang et al., 2012).

Leaf ash content and $\Delta^{13}C$ ratios were positively correlated with each other, and the ratios were similar to ratios obtained in previous reports in apple (Glenn, 2014) and peach (Glenn and Gasic, 2015). These two phenotypic parameters were used to identify individuals with a range of WUE. Using these criteria, all *P. davidiana* individuals, *P. bucharica*, *P. gorki*, *P. kotschii*, *P. orientalis*, *P. vavilovi* and *P. zabulica* individuals had higher WUE than *P. webbii* individuals, and the almond, peach and *P. mira* parentals, along with their hybrids. This improved WUE could be due to the natural adaptation of these species to severe conditions.

These species are original to arid steppes, deserts, and mountainous areas (Gradziel, 2009; Kester and Gradziel, 1996; Wang, 1985) in which the lack of water is a common factor. This classification did not correspond with the differences found in the approximate 1,000 bp promoter regions for each gene, in which *P. davidiana* (the highest WUE) were more related to the other parentals and their hybrids (the lowest WUE). Features acting as *trans*-elements much further upstream or located on other chromosomes could contribute to the regulation of these genes in drought response (Mizoi et al., 2012; Yamaguchi-Shinozaki and Shinozaki, 2006, 2005).

With the analysis of the different *cis*-elements presented in the promoter sequences of *PpDhn2* and *DREB2B*, we conclude that differential expression of these genes in various genotypes could be induced under drought conditions. In the literature the relation between dehydrin expression and an increase of WUE in cereals has been demonstrated. Sivamani et al., (2000) confirmed an improvement of biomass and WUE in transgenic barley plants expressing *HVA1* gene under drought conditions. Furthermore, Melišová et al., (2015) suggested that elevated expression of the *HvDhn4* gene, which is also a Y_nSK_n-type dehydrin and similar to *PpDhn2*, was associated with the high WUE observed in a drought-tolerant variety of barley at 12h after ABA treatment. Moreover, *DREB* TFs improved tolerance to abiotic stress in transgenic plants by regulation of genes involved in abiotic stress responses, so *DREB* TFs could increase WUE under water deficit conditions (Khan, 2011).

In conclusion, we found evidences to confirm the involvement of the *PpDhn2* and *DREB2B* TF genes in both ABA-dependent and ABA-independent signaling pathways, as well as a possible role in cold response in *Prunus*. Based on previous studies in other species, we chose these two genes as potential candidates for identifying individuals with better WUE. To expand our studies to test their potential in a drought-tolerant rootstock breeding program, the expression of the peach *PpDhn2* and *DREB2B* genes can be determined in clonally propagated *P. davidiana* and almond wild-relative species. Drought experiments where physiological parameters related to WUE would be monitored and their possible correlation with expression of *PpDhn2* and *DREB2B* would be determined. Positive results would suggest these genotypes as potential sources of drought tolerance.

4. IDENTIFICATION OF WATER USE EFFICIENCY-RELATED GENES IN 'GARNEM' ALMOND × PEACH ROOTSTOOK USING TIME-COURSE TRANSCRIPTOME ANALYSIS

ABSTRACT

Drought is one of the main abiotic stresses with far-reaching ecological and socioeconomic impact, especially in perennial food crops such as *Prunus*. There is an urgent need to identify drought resilient rootstocks that can adapt to changes in water availability. Currently, limited molecular information is available regarding responses to drought stress in *Prunus* rootstocks. We performed a time course transcriptome analysis of roots in an almond × peach hybrid [*P. amygdalus* Batsch, syn *P. dulcis* (Mill.) x *P. persica* (L.) Batsch]. Drought stress was induced in 'Garnem' genotype by exposure to a PEG-6000 solution. Root samples were harvested from control and stressed plants at 0, 2 and 24h time points and processed for RNAseq. Transcriptome analysis resulted in the identification of 83,110 differentially expressed contigs (DECs) with 12,693 unique DECs identified at the 2h time point and 7,705 unique DECs identified at 24h time point under drought treatment. Interestingly, three drought-induced genes, directly related to water use efficiency (WUE) namely, *ERF023* TF; LRR receptor-like serine/threonine-kinase ERECTA; and *NF-YB3* TF were found to be induced under stress. Furthermore, one differentially expressed gene (DEG) was identified as the *Myb 44* TF, a repressor of PP2C phosphatase, which was qRT-PCR validated along other seventeen DEGs. The present study provides valuable information regarding the transcriptomic events initiated during the first hours of stress-induced signaling in 'Garnem' roots. This information is expected to be useful in understanding the potential mechanisms underlying drought stress responses and drought adaptation strategies in *Prunus* species.

Keywords: Drought adaptation, *Prunus*, RNAseq, Water stress, Water use efficiency

4.1. INTRODUCTION

Prunus L. is a diverse and important genus belonging to the economically important Rosaceae family. It is comprised of approximately 200 species, most of them growing in the temperate zone with some also found to inhabit the tropical and subtropical regions. The economic importance of the genus is evident from the diverse uses of its members as a source of fruit, oil, and timber, as well as their use as ornamentals (Lee and Wen, 2001). Commercial production of *Prunus* species requires the use of rootstocks, which are derived from several members of the Amygdaloidae family, namely, *P. amygdalus* Batsch, *P. persica* (L.) Batsch, *P. cerasifera* Ehrh., *P. davidiana* (Carr.) Franch, *P. mira* Koehne kov et. Kpst, *P. domestica* L. and *P. insititia* L. Over the years, *Prunus* rootstock improvement via traditional breeding has been successful in incorporating various genetic traits such as, improved water and nutrient uptake, resistance to soil-borne pathogens, and tolerance to environmental stresses (Layne, 1987). Almond × peach hybrids such as 'Garnem', 'Felinem' and 'Monegro' (which have been derived from the cross between 'Garfi' almond × 'Nemared' peach) exhibit good vigor, nematode resistance, and adaptability to calcareous soils (Felipe, 2009). With hybrid rootstocks being increasingly derived from crosses between almond × peach and plum genotypes, the next challenge is to combine the tolerance to biotic and abiotic stresses in the new generation of rootstocks (Bielsa et al., 2014; Byrne et al., 2012; Felipe, 2009; Lecouls et al., 2004).

Drought is increasingly becoming one of the main abiotic stresses that threatens global agricultural production, particularly in the arid and semi-arid regions around the Mediterranean. Drought-tolerant plants utilize diverse approaches to survive under stress conditions, and it is critical to understand the molecular basis of the various survival mechanisms. Stress-inducing water limitation triggers the expression of a large number of drought-related genes, which in turn induce a set of molecular, cellular and biochemical processes including modifications in stomatal movement (Lind et al., 2015), accumulation of osmolytes (Singh et al., 2015), and antioxidant signaling (Baxter et al., 2013; Tognetti et al., 2012). The activation of these

processes allows for maintenance of cellular homeostasis through lipid and carbohydrate metabolism (Golldack et al., 2014). Based on current understanding, the drought-responsive genes can be classified into two groups depending on their function: (i) Regulatory genes (e.g. transcription factors (TFs), kinases and phosphatases, and enzymes for phytohormones biosynthesis) and, (ii) Effector genes (e.g. chaperones, late embryogenesis abundant (LEA) proteins, enzymes for osmolytes biosynthesis and water channel proteins) (Roychoudhury et al., 2013; Shinozaki and Yamaguchi-Shinozaki, 2007). Identification of these genes, and their functional, and mechanistic characterization will be critical for the improvement of drought tolerance in economically important crops (Valliyodan and Nguyen, 2006).

Drought tolerance is a quantitative genetic trait. Due to the inherent complexity and the crosstalk between various molecular pathways involved, introgression of drought tolerance has been slow (Tuberosa and Salvi, 2006). However, over the last decade, different genomic and genetic tools have been used to identify the genes involved in drought response. The expression of three peach dehydrins was compared, which provided an insight into the role these genes may play during drought and cold-induced stress response (Bassett et al., 2009). In *P. scoparia* several water-deficit resistance genes were identified using the cDNA-AFLP technique (Alimohammadi et al., 2013). The advent of high-throughput approaches has revolutionized the capacity to elucidate drought responses in plants. Several studies based on microarray technique have been reported in tomato (Gong et al., 2010), rice (Rabbani et al., 2003) and other woody plants, such as *P. taeda* (Watkinson et al., 2003). The RNA sequencing (RNAseq) technology has made it possible to capture and compare entire transcriptomes of genotypes exposed to different stress conditions at various time points, while providing greater accuracy and sensitivity than other methods (Wang et al., 2009). RNAseq has been applied to characterize molecular responses under both biotic (Gusberti et al., 2013) and abiotic stresses, including low temperature in peach (Jiao et al., 2017), early freezing in maize and root hypoxia in *Prunus* rootstock (Arismendi et al., 2015). Particularly, transcriptomic approaches for drought response have been reported, mainly in herbaceous species, including barley (Bedada et al., 2014), wheat (Z. Liu et al., 2015), sorghum (Fracasso et al., 2016), *L.*

multiflorum (Pan et al., 2016) and soybean (Prince et al., 2015) among others. But also in woody plants, although more limited, RNAseq analysis have been reported, including poplar (Barghini et al., 2015; Cossu et al., 2014; Tang et al., 2013), *Q. suber* (Magalhães et al., 2016), *P. halepensis* (Pinosio et al., 2014) and *P. betulaefolia* (K.-Q. Li et al., 2016). Two recent studies identified drought-responsive genes under long-term drought exposure in Mongolian almond (J. Wang et al., 2015), and in leaf and root tissues of peach trees where 'Catherina' scion was grafted on to 'GF677' rootstock (Ksouri et al., 2016).

Drought or water limitation stress is expected to be initially perceived within the roots of a plant. The signal is then transmitted to the shoots, thereby activating different biochemical and morphological events to protect the plant against drought (Janiak et al., 2016). To gain a comprehensive understanding of the molecular and biochemical mechanisms underlying drought response, and identify drought responsive genes, we characterized the transcriptome of 'Garnem' roots under polyethylene glycol (PEG)-induced drought stress conditions (He et al., 2015; Meng et al., 2016; Michel and Kaufmann, 1973). The present study provides a global perspective of the genes involved in PEG-induced drought response, and putative biochemical and metabolic pathways related to drought response in 'Garnem'. Specifically, three drought-induced genes directly related to water use efficiency (WUE) were identified to be differentially expressed under drought conditions. These genes are expected to serve as important candidates for future investigations related to improvement of WUE and thus, drought tolerance in *Prunus*.

4.2. MATERIAL AND METHODS

4.2.1. Plant material and growth conditions

A total of 20 clonally propagated plants from the drought tolerant almond-peach hybrid [*P. amygdalus* Batsch, syn *P. dulcis* (Mill.) x *P. persica* (L.) Batsch] 'Garnem' were used for the experiment. The plants were acquired from Agromillora Iberia S.L. nursery (Barcelona, Spain). Prior to the drought experiment, the plants were placed in 5 cm diameter pot with a mix of turf, 30% coconut fiber and 20% sand and

maintained in a greenhouse at CITA facilities in Zaragoza, Spain (41°43'28.6''N, 0°48'31.1''W), where they were watered three times a week and fertilized monthly with 15:9:10 N:P:K + 0.2% MgO (Nitricol). Temperatures in the greenhouse during the growth period were in a range of 28 °C and 18 °C, respectively with 12h day / 8h night photoperiod.

4.2.2. Stress conditions and treatment

The plants were divided into control (n = 12 plants) and treatment groups (n = 8 plants). The control plants were maintained under optimal watering conditions, until field capacity, in their 5 cm diameter pots with peat moss substrate during the experiment. The root systems of the stressed plants were placed in a dialysis membrane containing a peat moss substrate and then were submerged in a Polyethylene glycol, PEG-6000, solution (Sigma-Aldrich, Co. St. Luis, MO, USA) (500 g l^{-1}) corresponding to an osmotic pressure of -2,68 Mpa as per the Michel and Kaufmann equation (Michel and Kaufmann, 1973) in order to simulate drought stress conditions. As the plants acclimatized to the PEG solution, the plant osmotic potential was controlled using a Scholander-type pressure chamber (Soil Moisture Equipment Corp. Santa Barbara, CA, USA) (Scholander et al., 1964) unitl day 7 (Supplementary material S4.1). The 0-hour time point for the experiment began at the termination of the 7-day acclimation period. Root samples were harvested at 0h (four control plants), 2h (four both control and treatment plants) and 24h (four both control and treatment plants) and flash frozen in liquid nitrogen prior to being transferred to storage at -80 °C for subsequent RNA extraction.

4.2.3. Plant water status

Leaf Water Potential (LWP) was measured in duplicate for each plant using a Scholander-type pressure chamber (Soil Moisture Equipment Corp. Santa Barbara, CA, USA) (Scholander et al., 1964). Stomatal conductance (gs) was measured for one leaf from each plant with a Leaf Porometer (Decagon Devices Inc. Pullman, WA, USA). Relative Water Content (RWC) was measured in duplicate as per previously published methods (Barrs and Weatherley, 1962). Briefly, three 1 cm diameter leaf discs were

weighed (W) and rehydrated to their turgid weight (TW) by floating them in *petri* plates containing deionized water for 4h at room temperature. The dry weight (DW) was obtained after 24h at 80 °C in an oven. RWC was calculated following the equation:

$$\mathrm{RWC}\ \% = \frac{\mathrm{W} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}} \times 100$$

Electrolyte Leakage (EL) was calculated from Cell Membrane Stability (CMS) rate. CMS was evaluated in duplicate following previously published protocols (Blum and Ebercon, 1981). Briefly, three 1 cm diameter leaf discs, previously cleaned twice with deionized water to remove surface-bound electrolytes, were submerged in a 50 ml vial containing 10 ml of deionized water and incubated in the dark for 24h at room temperature. Conductance was then measured with a conductivity meter (CRISON micro CM 2201, Barcelona, Spain). This measurement was taken as C1 (control samples) and as T1 (treated samples). After the measurement, the vials with the samples were autoclaved for 15 min at 121 °C. When the samples reached room temperature, a second reading was recorded (C2 for control samples; T2 for treated samples). CMS and EL were calculated according the following formulas:

$$\mathrm{CMS}\ \% = \frac{1 - \frac{\mathrm{T1}}{\mathrm{T2}}}{1 - \frac{\mathrm{C1}}{\mathrm{C2}}} \times 100 \qquad \mathrm{EL}\ \% = 100 - \mathrm{CMS}\ \%$$

Each of the parameters described above were measured and recorded at 0, 2 and 24h for both treatments.

4.2.4. RNA isolation, cDNA library construction and sequencing

Total RNA was extracted from 0.5 g of root tissue for each time point using the CTAB method described previously (Meisel et al., 2005) with minor modifications (Chang et al., 1993; Salzman et al., 1999; Zeng and Yang, 2002). Extracted RNA was quantified using a NanoDrop® ND-1000 UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was verified by electrophoresis on a 1% agarose gel. Contaminating genomic DNA was removed using DNase I (TURBO

DNA-free™, Ambion, Life Technologies) per the manufacturer's instructions. Samples were submitted to Lifesequencing S.L. (Paterna, Valencia, Spain) for sequencing library preparation and RNAseq. A total of 1 µg of cellular RNA (RIN > 7.6) was used for TruSeq RNA library construction (Illumina Inc. San Diego, CA, USA). The mRNA was purified using Oligo(dT) cellulose, and was subsequently fragmented into short pieces. First-strand cDNA and second-strand cDNA were synthesized using the fragmented RNA as template. Following purification with the QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), sequencing adapters with identification barcodes were ligated to the fragments in order to distinguish different samples. Fragments with lengths of 200-300 bp were purified by Ampure XP beads (Beckman Coulter, Brea, CA, USA), and selectively amplified via PCR in the final step of the library preparation. A total of 10 libraries were sequenced using an Illumina HiSeq™ 2000 configuration 100 PE (Illumina Inc. San Diego, CA, USA). The libraries represented the following samples: 0h Control (2 biological replicates), 2h control and 2h stress (2 biological replicates for each treatment), and 24h control and 24h stress (2 biological replicates for each treatment) (Table 4.1).

Table 4.1. Summary of reads from RNAseq in each generated library and number of genes mapping to the peach reference genome.

Library ID	Condition	Biological replicate	Number of raw reads	mean_Q	Number of reads after trimmed	Percentage trimmed (%)	Contig genes mapped	Contig genes mapped percentage (%)	Average lenght of contigs
131902	0h Control	1	42,379,234	36.28	40,841,953	96.37	117,356	79.43	534
131903		2	34,909,042	36.17	33,548,752	96.10			
131904	2h Control	1	39,490,574	36.27	38,061,652	96.38	140,041	94.79	527
131905		2	48,780,078	36.26	46,967,646	96.28			
131906	2h Stress	1	44,241,088	36.21	42,613,791	96.32	121,596	82.30	537
131907		2	44,777,684	36.14	43,044,853	96.13			
131908	24h Control	1	40,252,320	36.29	38,784,882	96.35	131,251	88.84	514
131909		2	38,424,802	36.25	37,020,672	96.35			
131910	24h Stress	1	43,514,954	36.29	41,948,499	96.40	138,682	93.87	500
131911		2	38,673,426	36.24	37,215,660	96.23			
Total			**415,443,202**		**400,048,360**				

4.2.5. RNAseq data processing

The Illumina HiSeq generated DNA sequence reads in the 2x100 paired format. The resulting fastq files were imported into the CLC Bio Genomics Workbench (ver 6.0.1) (Aarhus, Denmark) for quality assessment, pre-processing, and assembly. The quality of the paired reads was assessed using the CLC 'Create Sequencing QC report'. The CLC 'Trim Sequence' process was used to trim quality scores with a limit of 0.001 (Phred value of 30). In addition, all ambiguous nucleotides were trimmed, and 14 of the 5' terminal nucleotides were removed. Reads, less than 34 bp in length, were discarded. Overlapping pairs were then merged with the 'Merge Overlapping Pairs' tool. Once all of the preprocessing steps were finished, a *de novo* assembly was performed with the combined reads from all datasets using the following parameters: Map reads back to contigs = TRUE, Mismatch cost = 2, Insertion cost = 3, Deletion cost = 0.4, Similarity Fraction = 0.95, Global Alignment = TRUE, Minimum contig length = 200, Update contigs = true, Auto-detect paired distances = TRUE, Create list of un-mapped reads = TRUE, Perform scaffolding = TRUE. The *de novo* assembly generated 147,742 contiguous sequences (contigs). Contigs with less than 2x coverage and those less than 200 bp in length were filtered out. The original, non-trimmed reads from each individual dataset were then mapped back to the master transcriptome assembly. Default parameters were used for this process, with the exception of the 'length fraction' and 'similarity fraction' parameters, which were set to 0.5 and 0.9 respectively. Mapping allowed the number of individual sample reads per contig to be counted. The master transcriptome was then exported as a fasta file for downstream functional annotation, and the read counts for each dataset were exported and normalized via Reads Per Kilobase per Million reads (RPKM) method (Mortazavi et al., 2008). Finally, RPKM values were compared between drought and stressed treatments, using the 0-hour control as a baseline. Thereafter, the RPKM values used for differential expression analysis were derived from the total read count in a pairwise comparison of treatments (drought and control conditions, and 2h and 24h of treatment condition). Only genes with a log10 fold of change > 5 and p-value < 0.05 were selected for further analysis.

4.2.6. Functional annotation and GO enrichment analysis

Gene Ontology (GO) annotation was conducted using the Blast2GO v. 3.3 (Conesa et al., 2005). The sequence homology from other species was determined by conducting a Blastx search against the NCBI public BLAST database. The resulting top blast hits were then mapped with their respective Gene Ontology terms and annotated. The ontology annotations were refined using InterPro Scan and expanded using ANNEX. GoSlim was used as an additional annotation step to summarize the resulting information. Furthermore, the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was performed to map differentially expressed, annotated transcripts to respective metabolic pathways. Each of the previous steps was conducted using the Blast2GO default parameters. GO enrichment analysis was conducted using the two-tailed Fisher's exact test (FDR < 0.05) in order to reveal the over and underrepresented functions in control and treatment samples during PEG-induced drought stress.

4.2.7. Quantitative Real Time PCR validation of differentially expressed genes (DEGs)

RNA samples (2,500 ng) were reverse transcribed with SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies, Carlsbad, CA, USA) in a total volume of 21 µl according to the manufacturer's instructions. Primers were designed for 18 differentially expressed genes (DEGs) (Supplementary Material S.4.2; Annex3) using Primer3Plus software (Untergasser et al., 2007) and tested against genomic DNA from 'Garnem' genotype for quality assurance. Two microliters of 40X dilution of the synthesized cDNA was used for each amplification reaction in a final volume of 10 µl. qRT-PCR was performed in triplicate for each of the two biological replicates on an Applied Biosystems 7900HT Fast PCR System using iTAQ^{TM} Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The amplification conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C for denaturation, and 1 min at 60 °C for annealing and extension. Amplification was preceded by melting curve analysis. Primers for a translocation elongation factor gene (*TEF2*), designed from the available *P. persica TEF2* DNA sequence (Gene Bank

accession number TC3544), were used as an internal reference control reaction for the qRT-PCR experiments. Relative expression was measured by the -2ΔΔCt method (Pfaffl, 2001).

4.2.8. Statistical analysis

Physiological Parameters. Statistical analyses were performed with SPSS 21 software package (IBM SPSS Statistics, USA). Before carrying out any statistical analysis, the normality of all the data was studied using the Kolmogorov-Smirnov test. Data following a Normal distribution were subjected to ANOVA to test for significant differences between treatments and among hours. The significant difference was assessed with Tukey's test ($p \leq 0.05$). In case the hypothesis of normality was discarded at the 95 % confidence level, the data were subjected to non-parametric data Kruskal-Wallis' test ($p < 0.05$). Besides, the statistical differences between treatments for each time point were determined by the Student's *t*-test ($p \leq 0.05$).

4.3. RESULTS AND DISCUSSION

4.3.1. Physiological responses to PEG-induced drought

The induction of drought in plants treated with PEG-6000 (Michel and Kaufmann, 1973) was monitored by analysis of leaf water potential (LWP), stomatal conductance (gs), and relative water content (RWC), paramaters that have previously been used to indicate conditions of stress (Davies et al,. 1994; Gollan et al. 1992) (Fig.4.1). While the LWP values in the control plants were observed to be -0.77 and -0.48 MPa at the two time points (Fig. 4.1A), those of the PEG-treated 'Garnem' plants reached -1.30 MPa and -1.15 MPa in 2h and 24h, respectively. Moreover, significant differences between the control and stressed plants were observed at the 2h and 24h time points, and for stressed plants between 0h and 2h (Fig. 4.1A) (Table 4.2). Unlike LWP, gs did not change significantly between the two time points or the treatments (Table 4.2, Fig 4.1B); however, there was a notable decrease in the gs values from 60.57 mmol m^{-2} s^{-1} to 29.86 mmol m^{-2} s^{-1} between 2h and 24h in the PEG-treated plants (Fig. 4.1B). Induced stomatal regulation at 2h in the PEG treated plants is

consistent with published literature that reports stomatal closure in drought stress conditions (Negin and Moshelion, 2016; Verslues et al., 2006). RWC decreased in the PEG-treated plants throughout the course of the experiment. Significant differences were observed between the control and the treatment samples at 24h, at which the PEG-treated plants reached a minimun RWC value of 84.71% (Table 4.2, Fig 4.1C). Although RWC decreased at 24h of PEG treatment, EL rate was not significantly affected (Table 4.2, Fig. 4.1D). Furthermore, EL rates of the PEG-treated plants remained similar to control plants even at low LPW values, suggesting that PEG-treated plants may show an aviodance strategy as consequence of a solute accumulation. Such a strategy would allow an osmotic adjustment under stress conditions (Singh et al., 2015; Verslues et al., 2006).

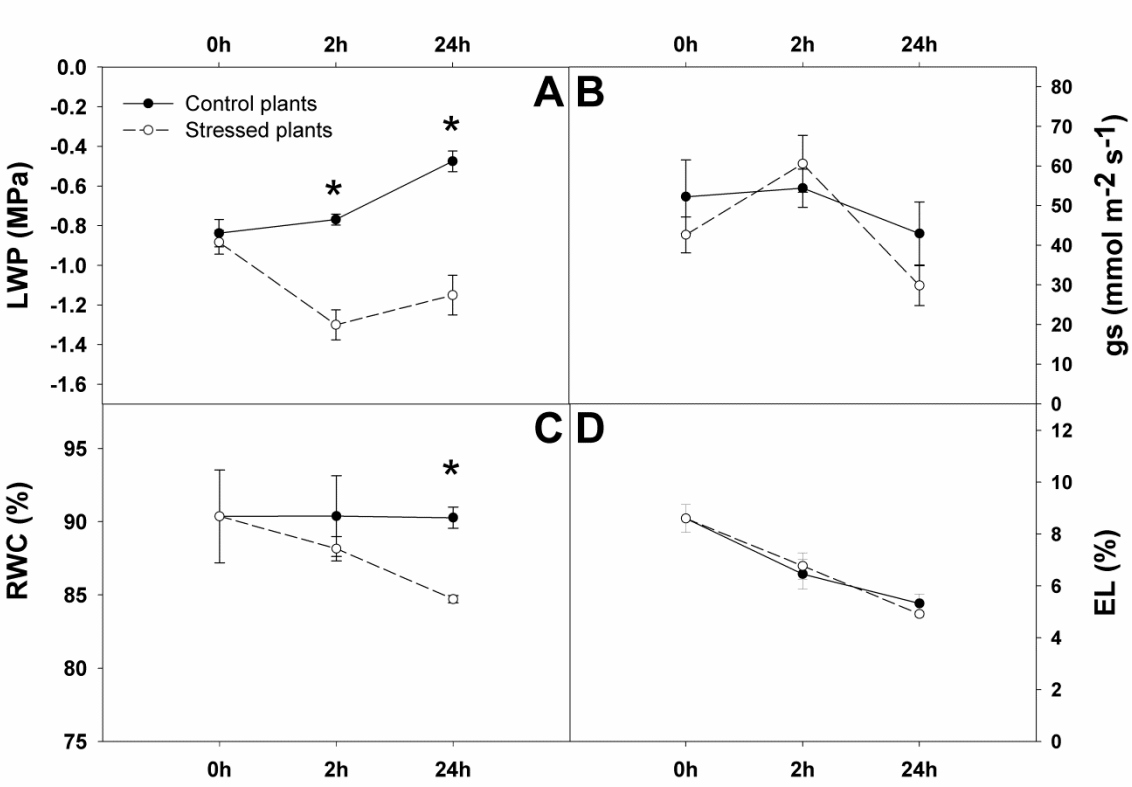

Figure 4.1. Leaf water potential (LWP) (A), stomatal conductance (gs) (B), relative water content (RWC) (C) and electrolyte leakage (EL) (D) during the drought experiment for control and stressed plants of 'Garnem'. Continuous lines indicate well-watered plants, while dash lines indicate stressed plants. Error bars represent the standard error of the mean. Asterisks represent significant differences ($p \leq 0.05$) beetween treatments (control and stressed) for each time point of the experiment. (h = hours).

Table 4.2. ANOVA results from Leaf water potential (LWP), stomatal conductance (gs), relative water content (RWC) and electrolyte leakage (EL) during the drought experiment. Same letter values indicate a no significant difference (p≤0.05) following Tukey's post hoc test (h: hours; SE: Standard Error).

	Control Plants			**Stressed Plants**		
	LWP (MPa)	**SE**		**LWP (MPa)**	**SE**	
0h	-0.838	0.069	a	-0.883	0.060	a
2h	-0.769	0.028	a	-1.300	0.076	b
24h	-0.475	0.052	b	-1.150	0.100	ab
	gs (mmol m^{-2} s^{-1})	**SE**		**gs (mmol m^{-2} s^{-1})**	**SE**	
0h	52.250	9.288	a	42.633	4.512	a
2h	54.400	4.814	a	60.567	7.154	a
24h	42.975	7.936	a	29.850	5.050	a
	RWC (%)	**SE**		**RWC (%)**	**SE**	
0h	90.367	3.169	a	90.367	3.169	a
2h	90.381	2.759	a	88.155	0.829	a
24h	90.276	0.718	a	84.713	0.247	b
	EL (%)	**SE**		**EL (%)**	**SE**	
0h	8.605	0.539	a	8.605	0.539	a
2h	6.456	0.582	a	6.767	0.513	a
24h	5.326	0.357	a	4.916	0.106	a

4.3.2. Processing and assembly of RNAseq data

RNAseq analysis was performed in duplicate biological samples from 'Garnem' roots, resulting in 10 sequenced libraries from 'Garnem' roots for representative of the 0h, 2h and 24 h time points in both in control and drought-stressed conditions. An approximate mean Q score of 36 for each library validated the quality of the assay (Table 4.1). In total, approximately 0.42 billion of reads, each 100 nucleotides long, were generated, of which. 96% (0.4 billion of reads) were obtained retained after trimming and filtering low quality reads stage. Mapping of the original, untrimmed reads from each individual condition and time point back to the master assembly generated 117,356 (79.4%); 140,041 (94.8%); 121,596 (82.3%); 131,251 (88.8%); 138,682 (93.9%) contigs for the 0h control, 2h control, 2h stress, 24h control and 24h stress time points, respectively (Table 4.1) with a mean contig size of 522 bp.

4.3.3. Identification of differentially expressed contigs (DECs) in response to PEG-induced drought

The RNAseq data was processed and assembled, and RPKM values calculated (see Supplementary Material S4.3; Annex 3). Thereafter, pairwise comparison of expression values from control and stressed samples at different time points was performed. Following comparison were made: 2h stressed vs. 24h stressed (2hS-24hS), which compares changes in transcript expression between PEG treatment time points, this allowed for identification of drought-responsive contigs; 2h control vs. 2h stressed normalized to 0h control (2hC-2hSN0C), which allowed for identification of contigs that were differentially expressed during the first 2 hours of drought; 24h control vs. 24h stressed normalized to 0h control (24hC-24hSN0C), which allowed for identification of genes that were differentially expressed after one day of stress; and 2h stressed vs. 24h stressed normalized to 0h control (2hS-24hSN0C), which enabled the identification of contigs that changed in expression as a result of PEG addition. RPKM values were used to identify the contigs that were differentially expressed by a Log fold change (logFC) > 5 in each comparison. In total, 83,110 DECs were found among the four comparison groups: 22,262 in the 2hS-24hS group; 44,883 in the 2hC-2hSN0C group; 29,524 in the in 24hC-24hSN0C group; and 46,005 in the 2hS-24hSN0C group (Fig. 4.2). Venn diagram analysis indicated that increased transcriptional activity occurred at the 2h stressed in comparison with that of the 24h stressed time point, considering the highest number of DECs were found exclusively in 2hC-2hSN0C pool (12,693 DECs) (Fig. 4.2). Notably, only 0.3% of DECs were expressed differentially across all four pools (Fig. 4.2). Interestingly, at 2h of PEG stress, more genes were upregulated (33,767 DECs) than downregulated (11,116 DECs). In contrast, at 24h of PEG stress, more genes were downregulated (19,424 DECs) than upregulated (10,100 DECs) (Fig. 4.2). From these observations it seems like that the drought-induced induction of transcriptional activity occurs primarily in the first hours of stress, which might be related to the avoidance strategy above-mentioned (Singh et al., 2015; Verslues et al., 2006). These findings are consistent with results obtained in *Prunus* rootstock roots under hypoxia conditions (Arismendi et al., 2015), and in plants of wheat (Z. Liu et al., 2015) and *Brassica juncea* (Bhardwaj et al., 2015) under drought stress conditions.

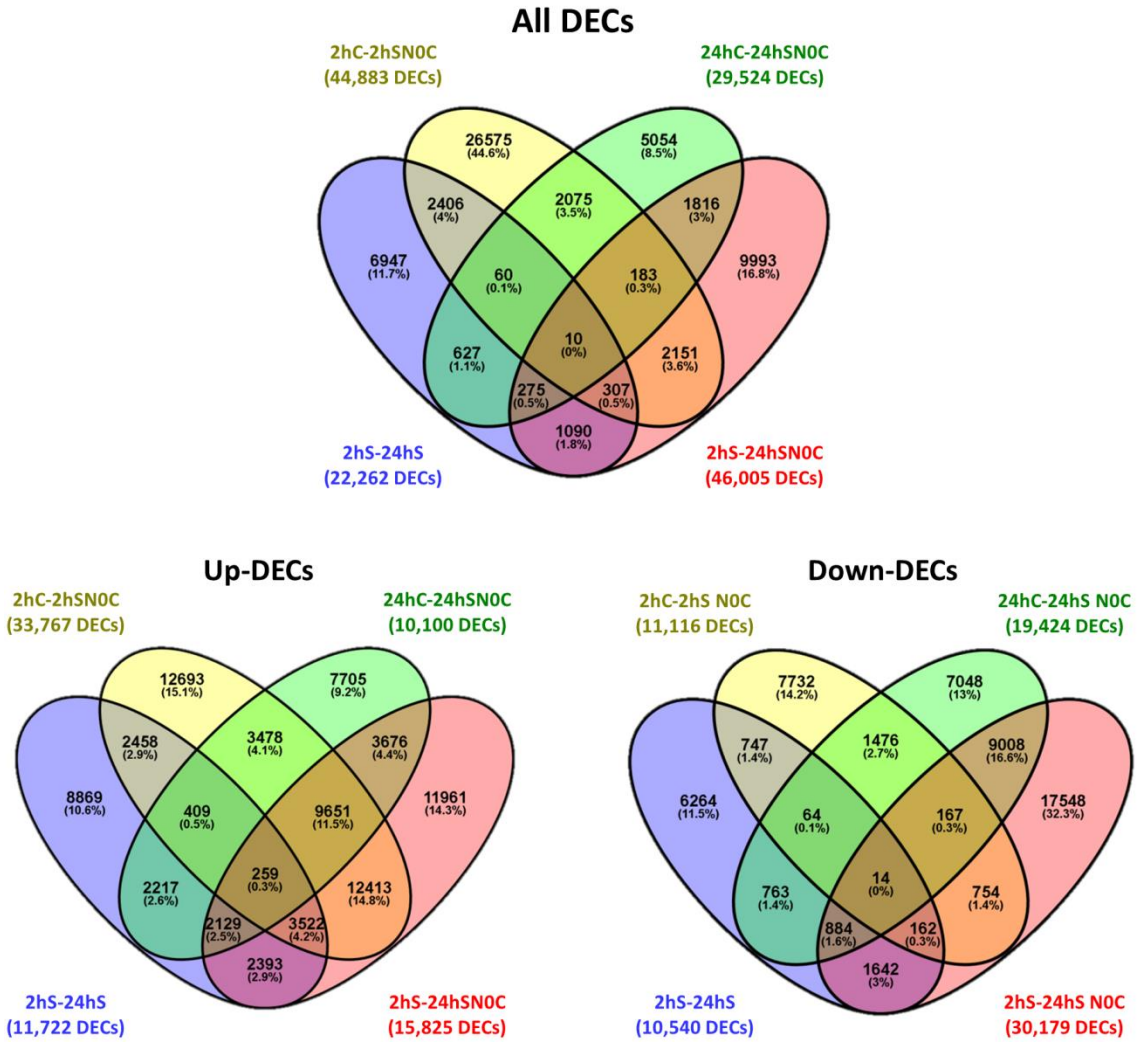

Figure 4.2. Venn Diagrams. Number of DECs (Differentially Expressed Contig) for the four pairwise comparisons between control and stressed samples collected at different time points: 2h stressed vs. 24h stressed (2hS-24hS); 2h control vs. 2h stressed normalized to 0h control (2hC-2hSN0C); 24h control vs. 24h stressed normalized to 0h control (24hC-24hSN0C); and 2h Stressed vs. 24h stressed normalized to 0h control (2hS-24hSN0C).

4.3.4. Functional annotation of the differentially expressed genes (DEGs) and GO term enrichment

Once 83,110 DECs were aligned against the NCBI database with the BLASTX algorithm, a total of 49,521 DEGs of the 83,110 total DEGs showed returned a positive Blast hits. The species distribution for the top Blast hits indicated that 'Garnem' transcripts had the highest similarity with *P. persica* and *P. mume,* with a 21.6% and 11.1% of top matches corresponding to each, respectively (Fig. 4.3). Besides peach and

Japanese apricot, other *Prunus* species, including *P. dulcis*, *P. salicina*, *P. armeniaca* and *P. dulcis × P. persica*, were classified among in the top hits, but in lower number (Fig. 4.3), lending support to our assembly quality as well as to the previous reports that have implicated strong homology among species from *Prunus* genus (Ksouri et al., 2016; J. Wang et al., 2015). These DEGs were mapped functionally mainly with UniProtKB data base (97.8% of GOs), as well as TAIR, GR-protein, PDB and SGN databases, and subjected to InterPro Scan and ANNEX. Finally, 26,700 DEGs were annotated. Then categorized by biological process (BP) (15,870 DEGs), molecular function (MF) (22,595 DEGs) and cellular component (CC) (13,883 DEGs) sets (Fig. 4.4). Of these, 40.68% had a robust homology showing E-values smaller than 1.0E-60, 27.19% had a strong homology with E-values between 1.0E-60 and 1.0E-30, and 29.06% presenting E-values between 1.0E-30 and 1.0E-3 (Zhu et al., 2015). At level 2 of the GO standard classification, DEGs were categorized in 12 functional groups. In the BP category, the largest GO groups were "metabolic process", "cellular process" and "single-organism process", as well as "cellular component organization or biogenesis", "response to stimulus" and "localization" (Fig. 4.4). Within the term "response to stimulus" a greater number of DEGs was identified at 24h (1,208 DEGs) than at 2h (915 DEGs) of drought treatment. Among them, 555 and 812 DEGs were assigned the "response to stress" term. DEGs with MF category were included in two groups: "catalytic activity" and "binding". With respect to CC type, "cell" and "cell part" were the most highly represented GOs, as well as other DEGs including "organelle" and "macromolecular complex" (Fig. 4.4). The same functional groups have also been identified in previous reports, demonstrating that they are important during response to drought. Notably, 1.15% of DEGs were not identifiable, as they did not have either, due to lack of sufficient homology with any gene in the databases, unknown homolog function with their homologs, or they were defined as hypothetical proteins. It is possible that genes with important, but thus far, undefined roles in drought acclimation exist among these unidentified DEGs (Dhanyalakshmi et al., 2016; Luhua et al., 2013). DEGs identified from each pool of pairwise comparisons were subjected to a GO enrichment analysis using Fisher's Exact Test (FDR < 0.05) analysis (Supplementary Material S4.4; Annex 3). GO enrichment analysis provided more further information about our annotated DEGs by identifying the significantly represented GO terms at

each time point of the PEG the treatment. GO terms such as "response to stress", "small molecule metabolic process", "cell wall organization or biogenesis", "signal transduction", "secondary metabolic process", "oxidoreductase activity", "ion binding", "translation factor activity", "RNA binding" and "plasma membrane", among others (Supplementary Material S4.4; Annex 3), were assigned to comprised of specific, corresponding groups of DEGs at 2h of drought stress. The enrichment of these GO terms at 2h indicates that initial drought stress signals transmitted, were recognized initially via cell by wall and membrane sensors. Subsequently trigger downstream stress signaling pathways in response to stress. Then, the synthesis of regulatory proteins and metabolites, including kinases, phosphatases, proteinases, fructosyltransferases, FTs, phytohormones, and calmodulin-binding proteins was affected (Beck et al., 2007; Mahajan and Tuteja, 2005). After 24h of drought stress, DEGs under pertaining to the predominant enriched GO terms related to ontologies included those associated with "nucleic acid binding transcription factor activity", "ATPase activity", "anatomical structure development", "cytoskeleton organization", "cellular amino acid metabolic process", "RNA binding" and "mitochondrion," together with others, were predominant " (Supplementary Material S4.4; Annex 3). These observations indicate that, following initial signal drought stress recognition, regulatory and signal propagating drought stress effectors are activated, including: signal transduction, induction of regulatory proteins, synthesis of functional proteins such as osmolytic enzymes, water channel proteins, membrane transporters, detoxification enzymes, fatty acid metabolic enzymes, and other proteins for the protection of macromolecule integrity would be induced as effectors of drought tolerance protecting against stress (Roychoudhury et al., 2013; Shinozaki and Yamaguchi-Shinozaki, 2007). Interestingly, the GO term "growth" was enriched at the 2h stressed treatment Genes under this GO term may be related to multiple cell wall processes that plants have evolved to adjust to drought stress. One such process, which is implicated by the 'Garnem' data is, the down-regulation of *2-dehydro-3-deoxyphosphooctonate aldolase 1* (*KdsA*), and consequent decrease of *3-Deoxy-D-manno-oct-2-ulosonic acid* (kdo), which would inhibit the formation of new cell walls-just one more stage of multiple mechanisms plants have adapted to adjust themselves to the new stress conditions (Yang et al., 2015). Further genes under the "growth"

term could are likely implicated root growth alterations in response to stress. For example, auxin-related genes, such as auxin-binding proteins (ABP4, ABP-T85) and the enzyme AVP1-pyrophosphatase 1 (Li et al., 2005), are involved in transport of auxin from shoots to roots, thereby promoting root cell elongation in roots in order to enhance the root system and enhanced water uptake from deeper soil layers (Mahajan and Tuteja, 2005).

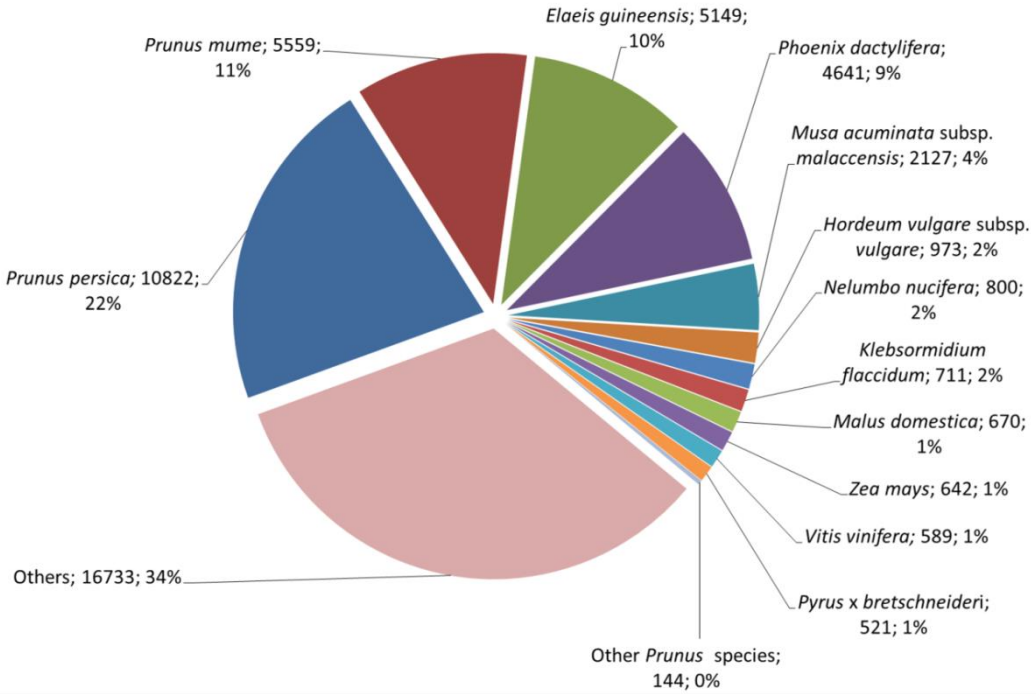

Figure 4.3. Specie distribution of the first 30 BLAST hits per each contig.

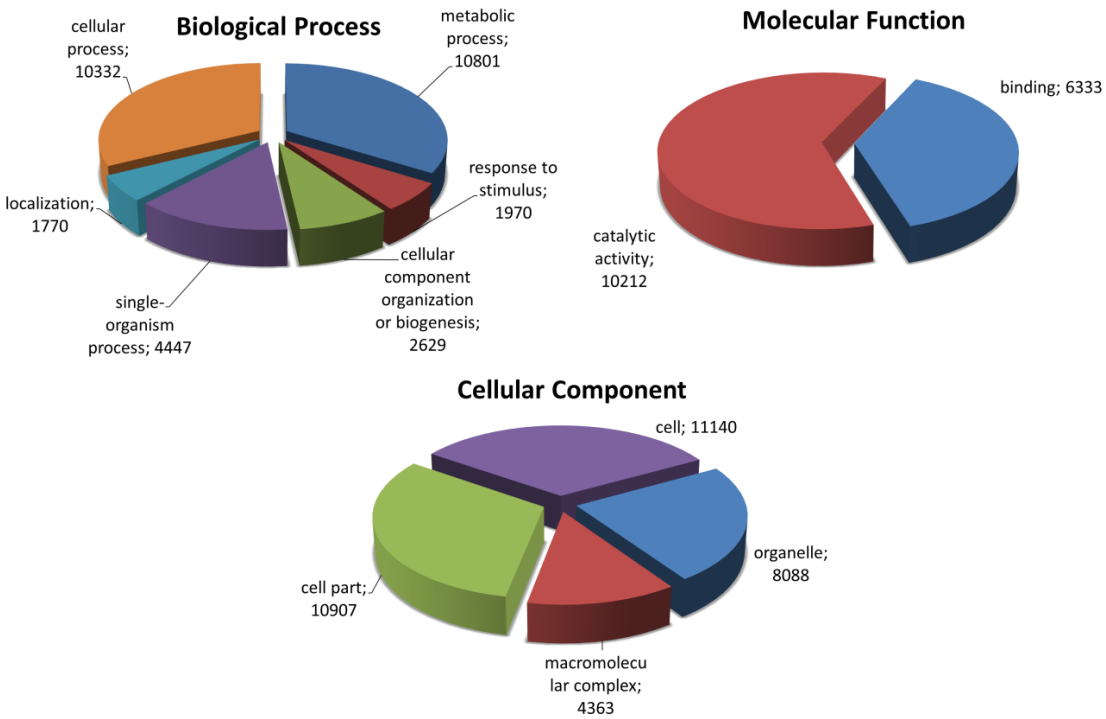

Figure 4.4. Annotated Gene Ontology (GO) term distribution at 2-level for the three GO categories after GO-slim analysis. In total, 26,700 DEGs were annotated and categorized by biological process (BP) (15,870 DEGs), molecular function (MF) (22,595 DEGs) and cellular component (CC) (13,883 DEGs) sets.

4.3.5. Stress perception, signaling cascades, and transcriptional control of drought

Drought tolerance is the result of complex signaling networks, which are triggered after stress is perceived by cell membrane receptors. Next, secondary messengers are activated, initiating a phosphorylation cascade and consequent downstream activation of regulatory genes that modulate expression of drought stress tolerance effector genes, thereby leading to drought adaptation (Mahajan and Tuteja, 2005; Roychoudhury et al., 2013; Yamaguchi-Shinozaki and Shinozaki, 2006). Based on this paradigm, the annotated DEGs identified in the present study were classified into three major groups: (i) Genes involved in signaling cascades and transcriptional control; (ii) Genes involved in acting as cellular protectors against dehydration-related damage; and (iii) Genes implicated in water and ion uptake and transport (Ciarmiello et al., 2011).

DEGs annotated as drought-related genes and discussed in this study have been described in (Supplementary Material S4.3; Annex 3), and the identity of the genes is in agreement with previously published reports (He et al., 2015; Ksouri et al., 2016; Tang et al., 2013; J. Wang et al., 2015).

Genes involved in signaling cascades and transcriptional control

Signal perception: Receptor-like kinases. Perception of drought conditions at the cell membrane is initiated by receptor-like protein kinases (RLKs), two-component histidine kinases, and G-protein-associated receptors (Xiong and Zhu, 2001). A total of 595 DEGs encoding RLKs were identified. These RLKs, which have been implicated in regulation of abiotic stress response, modulation of disease resistance, and signal transduction (Ye et al., 2017) included: chitin elicitor receptor kinases (CERKs), cysteine-rich receptor kinases (CRKs), receptor-like kinases, histidine kinases (HKs), probable leucine-rich repeat receptor kinases (LRR-RLKs), probable L-type lectin-domain containing receptor kinase (LecRKs), serine/threonine-protein kinases (SnRKs), somatic embryogenesis receptor kinases (SERKs), receptor-like cytosolic serine threonine-kinases, wall-associated receptor kinase-like (WAKs), G-type lectin S-receptor-like serine threonine-kinase (GsSRKs), and strubbelig-receptor family kinases (SRFs) (Supplementary Material S4.3; Annex 3).

Calcium-mediated signal transduction. Initial transduction of the drought stress signal is followed by proliferation of secondary messengers, such as calcium (Ca^{2+}), reactive oxygen species (ROS), and inositol phosphates (Mahajan and Tuteja, 2005). Ca^{2+} is responsible for coordination and synchronization of diverse stimuli originating from cellular stress responses (Mahajan and Tuteja, 2005). Following perception of abiotic stress, resulting calcium signaling leads to further rapid increase of cytosolic Ca^{2+} in plant cells (Roychoudhury et al., 2013), a response controlled by Ca^{2+} sensors, pumps, transporters and Ca^{2+} channels (Boudsocq and Sheen, 2010). Ca^{2+} oscillations are involved in regulation of a number of drought response-related physiological processes, such as stomatal closure (Roychoudhury et al., 2013) and ROS regulation (Wilkins et al., 2016). Several studies have discussed the role of Ca^{2+}-dependent protein kinases (CDPKs) in drought tolerance and adaptation, an observation that is

further supported by our analysis.CPK8 and CPK10, for example, enhance tolerance to drought by regulation of stomatal movement in *Arabidopsis* via interaction with CATALASE3 (CAT3) and HSP1 (Heat Shock Protein 1), respectively (Zou et al., 2015, 2010) In the present study, 346 annotated up- and downregulated DEGs involved in Ca^{2+} signaling included several calmodulin coding genes, Ca^{2+} and Ca^{2+}- and calmodulin-dependent serine threonine- kinase-like protein encoding genes, Ca^{2+}- and calmodulin-dependent kinases, Ca^{2+} uniporter mitochondrial proteins, and various Ca^{2+}-binding proteins families including Ca^{2+}-binding EF-hand family proteins, calcineurin B-like proteins (CBLs), CDPKs including CPK8 and CPK10, different Ca^{2+}-transporting ATPases, and the ER Ca^{2+}-binding chaperones calnexin and calreticulin (Supplementary Material S4.3; Annex 3). These findings, consistent with findings in other plant systems, provide further support for the involvement of Ca^{2+} in response to drought stress in 'Garnem' roots (Boudsocq and Sheen, 2010; Wilkins et al., 2016). Notably, in addition to triggering proliferation of calcium responses, changes in Ca^{2+} levels are also involved in activation of phospholipases C and D, thereby triggering lipid signaling pathways (Wilkins et al., 2016). Three DEGs encoding a Ca^{2+}-dependent lipid-binding family protein (CaLB) (Supplementary Material S4.3; Annex 3) were identified, a finding that suggests that CaLB protein may be involved in binding phospholipase D variants and, consequently, in activation of lipid signaling-mediated drought adaptation (Frank et al., 2000).

Kinase cascade-mediated signal propagation. Following initiation of drought-induced Ca^{2+} signaling, downstream Ca^{2+}-activated kinases and phosphatases transduce the drought stress signals via a series of protein phosphorylation cascades, including the aforementioned mitogen-activated protein kinases (MAPKs) and $SnRK_2$ kinases (Mahajan and Tuteja, 2005; L. Wang et al., 2016). MAPK cascades, which consist of three, interlinked protein kinases (Ghorbel et al., 2017; Singh et al., 2010), have been identified in drought response and implicated in crosstalk with both ABA-dependent and ABA-independent signaling pathways (Roychoudhury et al., 2013). Upregulated DEGs encoding MAPKs, MAP2Ks and MAP3Ks (Supplementary Material S4.3; Annex 3) was identified, providing further support for the involvement of MAPK modulated cascade in stress signaling in 'Garnem'. It has previously been suggested

that the 14-3-3 proteins may bind and regulate the activity of MAPK phosphatases (MKPs), which play crucial roles in stress responses in wheat (Ghorbel et al., 2017). Moreover, the large amount of upregulated DEGs encoding 14-3-3 protein isoforms in 'Garnem' suggests that these proteins have a similar, important stress responsive role in *Prunus* (Supplementary Material S4.3; Annex 3).

Phytohormone-mediated signal transduction. Phytohormones regulate growth and development in plants throughout their lifecycle. One of the most important plant hormones with regards to drought response is abscisic acid (ABA), which has been shown to accumulate in plant tissues following onset of drought (Huang et al., 2012). Transcriptomic changes were observed in several DEGs representing key genes involved in ABA biosynthesis (Mahajan and Tuteja, 2005; Xiong and Zhu, 2003; Yamaguchi-Shinozaki and Shinozaki, 2006), including genes encoding *zeaxanthin chloroplastic*, zeaxanthin epoxidase (ZEP), *9-cis*-epoxycarotenoid dioxygenase chloroplastic (NCED) and molybdenum cofactor sulfurase (ABA3). Drought induced accumulation of ABA, followed by ABA-dependent signaling, leads to synthesis of stress-associated genes and secondary metabolites. This, in turn results in induction of physiological responses such as stomatal closure, consequently increasing tolerance to drought (Roychoudhury et al., 2013). Moreover, ABA is involved in a crosstalk with other phytohormones that accumulate during drought response and that work in concert with each other to mediate stress adaptation, including: auxin, ethylene, cytokinin, gibberellic acid, brassinosteroids, salicylic acid (SA) and jasmonic acid (JA) (Zingaretti et al., 2013). While ABA signaling is involved predominantly in stomatal closure and growth cessation responses, auxin signaling, mediated by auxin/indole-3-acetic acid (Aux/IAA) and IAA amido synthetase 3 (GH3), serves to regulate homeostasis as well as ROS signaling and scavenging (Padmalatha et al., 2012). Among the genes involved in auxin homeostasis regulation, GH3 is one of the most important one with regards to the hormone crosstalk that mediates drought stress adaptation (Nobuta et al., 2007; Tognetti et al., 2012). In our data, 3 DEGs encoding a *probable GH3* were found, lending additional support to hormone network-based regulation of stress in 'Garnem'. In addition to GH3, we identified 4 DEGs encoding small auxin up RNA (SAUR) proteins, 11 DEGs encoding various auxin-induced-like proteins, 3 DEGs

encoding auxin-binding proteins (ABPs), 4 DEG's encoding auxin efflux carrier family proteins, and 23 DEGs encoding auxin response factors (ARFs) (Supplementary Material S4.3; Annex 3), of which are related to auxin signal transduction (Zhu et al., 2015). The changes in expression of these genes during water stress in 'Garnem' suggest that regulation of auxin content is also important for response to drought. Closely linked to auxin biosynthesis and signaling, and therefore also key to drought stress response, is ethylene metabolism (Mahajan and Tuteja, 2005). Datta et al., (2015) demonstrated that glutathione, a metabolite characteristically produced during stress responses, induces ethylene biosynthesis by modulation of two key enzymes, 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO). In the present study, a number of DEGs were annotated as ACS and ACO encoding enzymes, indicating the expected activation of ethylene biosynthesis during drought stress (Supplementary Material S4.3; Annex 3). Also, upregulated DEGs were identified encoding ethylene-responsive transcription factor (ERF) families, including ERF1, which plays an important role integrating ethylene and JA-signaling pathways in drought adaptation (Datta et al., 2015) (Supplementary Material S4.3; Annex 3). In addition to ABA, auxin, and ethylene, additional hormones that display a signaling role in response to stress include cytokinins, giberellins, and glutathione-S-transferases. In non-drought conditions, increased cytokinin concentration in xylem causes a decrease ABA-induced stomatal closure (Wilkinson and Davies, 2002). Consistent with this finding, Nishiyama et al., (2011) demonstrated that a reduction of cytokinin content helps to maintain an elevated water level due to reduced stomatal aperture and protection of the membrane structures. Seventeen upregulated DEGs were found encoding laccase enzymes and 13 DEGs encoding cytokinin dehydrogenase-like enzymes (CKXs), both of which are responsible for catalysis of cytokinin degradation (He et al., 2015; Pospíšilová et al., 2016). Gibberellin catabolism, mediated by expression of proteins such as GA 20-oxidase (GA20ox), GA30-oxidase (GA30ox) and DELLA, results in decreased in growth and increased adaptive response to abiotic stress (Zawaski and Busov, 2014; Y. Zhang et al., 2016). 4 upregulated DEGs encoding GA20ox enzymes were identified, as well as 2 upregulated DEGs encoding GA30ox enzymes, and 4 upregulated DEGs corresponding to DELLA proteins (including 2 GA-insensitive proteins and 2 GA repressor proteins)

(Supplementary Material S4.3; Annex 3). In addition to serving as secondary messengers modulating the activity and biosynthesis of other drought related proteins, the above mentioned hormones, along with brassinosteroids, salicylic acid (SA) and jasmonic acid (JA), induce ROS detoxification enzymes, such as glutathione S-transferases (GSTs). Through maintenance of cell redox homeostasis, stress-induced oxidative damage is minimized, resulting in improved tolerance to drought stress (Chen et al., 2012; Huang et al., 2012; Tognetti et al., 2012). We identified 51 DEGs, which were both up- and downregulated, encoding different members of GST family, including: GSTDHAR, GSTF13, GSTF6, GSTL3, GSTT1, GSTU10, GSTU17, GSTU9, GSTZ1, etc. (Supplementary Material S4.3; Annex 3). Taken together, these results suggest that adaptive drought responses in 'Garnem', as in poplar (Zawaski and Busov, 2014), are regulated in concert by the hormone messengers described to reduce water loss through stomata, maintain homeostasis, decrease growth related metabolism, and reduce oxidative damage resulting from drought conditions.

Phospholipid-mediated signal transduction. Drought stress results in alteration of plasma membrane phospholipid composition. In such instances, phospholipids act as precursors for the generation of secondary messenger molecules that mediate adaptive responses to abiotic stress (Bartels and Sunkar, 2005; Wang et al., 2007; Xiong et al., 2002). Four phospholipases have thus far been identified: phospholipase A1 and A2 (PL A1 and PL A2); phospholipase C (PLC); and phospholipase D (PLD) (Bartels and Sunkar, 2005). Drought stress induces overexpression of phosphoinositide-specific phospholipase C (PI-PLC), leading to production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which act as second messengers (Bartels and Sunkar, 2005; Xiong et al., 2002) that function in induction of stomatal closure (Bartels and Sunkar, 2005; Mishra et al., 2006). One DEG have been identified encoding an upregulated PI-PLC, and additional DEGs encoding different PL A1, PL A2 and PLD genes (Supplementary Material S4.3; Annex 3), suggesting the importance of these proteins in drought tolerance adaptation via induction of stomatal closure mechanisms in 'Garnem'.

Transcription factor-mediated signal perpetuation. As a result of drought-induced signaling cascades, kinases and phosphatases target different TFs that recognize *cis*-elements in promoter regions of effector genes, subsequently activating gene expression in response to drought stress. In 'Garnem', the large number of DEGs corresponding to TFs lends support to the importance of transcriptional regulatory elements in maintaining homeostasis during drought stress in *Prunus*. Previous studies have provided further validation for the crucial role of the AP1/ERF TF family comprised of AP2, RAV and ERF groups in stress adaptation (Du et al., 2013a). RAV1 and its homolog RAV2 are negative regulators of ABI5, a bZIP TF that plays a key role in ABA signaling, and thereby play an indirect, but important, role in mediating water loss through stomata (Fu et al., 2014; Skubacz et al., 2016). Upregulated DEGs were found encoding RAV1 and its homolog RAV2, as well as another two DEGs encoding *ABI5* gene at 2h, and at 24h the expression of *ABI5* gene was upregulated (Supplementary Material S4.3; Annex 3). Notably, a DEG encoding an ABI5 homolog, the bZIP TRAB1-like protein, was upregulated at 2h (Supplementary Material S4.3; Annex 3), suggesting that in *Prunus*, RAV TFs may function as negative regulators in ABA signaling. In addition to RAV TFs, multiple studies have indicated that DREB TFs, members of the ERF group of transcription factors, play an important role in enhancing tolerance to multiple abiotic stresses (Mizoi et al., 2012; Sakuma et al., 2006; Sazegari et al., 2015). In our data, 8 DEGs were found encoding DREB1B, DREB2A, DREB2B, DREB2D and DREB3 proteins, the upregulation of which indicates key participation of DREB TFs in drought stress tolerance in *Prunus*. Also upregulated DEGs were identified encoding ERF2 , which have been shown to increase in expression during drought stress (Jin et al., 2010), as well as upregulated DEGs pertaining to the bHLH TFs. Members of this TF family function to regulate stomatal development and cell division / differentiation (SPCH and FAMA), ABA signal transduction (RD22 and MYC2), trichome development and increase of leaf surface boundary layer (GLABRA3) (Castilhos et al., 2014). In 'Garnem' roots under drought stress, DEGs annotated as *SPCH* (1 DEG), *FAMA* (2 DEGs), *MYC2* and *MYC2-like* (6 DEGs) and *GLABRA3* (1 DEG). Furthermore, we identified 57 DEGs encoding bHLH TFs, such as *bHLH84-like*, *bHLH85-like* (Castilhos et al., 2014). The HD superfamily was one of the most highly represented in our data, with 81 DEGs annotated. Changes were also observed in the expression of DEGs

encoding *ATHB-12* and *ATHB12-like* (3 DEGs), *ATHB-6-like* (2 DEGs), and *HAT-5-like* (2 DEGs) genes, which are induced by drought and implicated in both positive and negative regulation of ABA-dependent signaling (Ariel et al., 2007; H. Wang et al., 2015). One of the DEGs identified that we seek to highlight, specifically, is the ALFIN-LIKE 5 PHD finger protein. Previous research has demonstrated that over-expression of *AL5*, a homolog of *ALFIN-LIKE 5*, improves tolerance to drought stress in *Arabidopsis*, thereby inhibiting expression of target genes that function as negative regulators of stress tolerance (Wei et al., 2015). The role of ALFIN-LIKE 5 has not previously been implicated in signaling, but has been shown to act as a downstream component (Wei et al., 2015). *WOX11*, a TF that has been shown to enhance drought tolerance of the root hairs of rice via developmental modulation (Cheng et al., 2016) was upregulated in our dataset (Supplementary Material S4.3; Annex 3). This suggests that WOX11 might also act as a modulator of the 'Garnem' root system under drought conditions. Other important TFs families that play a crucial role in response to drought are WRKY, NAC, NF-YB and Myb TFs (Singh and Laxmi, 2015; Tripathi et al., 2014) each of which has displayed changes in expression in response to drought stress. WRKYs, implicated in ABA-mediated stomatal closure and consequent increase in drought tolerance (Tripathi et al., 2014) which function were discussed later (Supplementary Material S4.3; Annex 3). NAC TFs are implicated in shoot meristem development and auxin signaling, as well as in dehydration response (Olsen et al., 2005). In 'Garnem' we identified upregulated DEGs encoding a NAC domain-containing 19-like, as well as 3 DEGs encoding NAC29 (Supplementary Material S4.3; Annex 3). It is well documented that ANNAC019 binds the *ERD1* (*Early responsive dehydration stress 1*) promoter in response to drought. Furthermore, overexpression of NAC29 has been shown to enhance drought tolerance in wheat (Xu et al., 2015). In addition to WRKY and NAC TFs, seventy DEGs in our dataset were annotated as MYB TFs, 11 of which encode Myb-related Myb4 and Myb4-like proteins (Supplementary Material S4.3; Annex 3). Expression of *Myb4* in the roots is implicated in biosynthesis of lignin, as well as that of solutes, including glucose, sucrose and proline. This leads to a reestablishment and maintenance of osmotic balance during drought stress, consequently conferring improved tolerance (Janiak et al., 2016). NF-YB TFs confer drought tolerance by enhancing WUE, especially *NF-YB7* in poplar (Han et al., 2013). In the 'Garnem'

transcriptome, one DEG annotated as *NF-YB3*, homologous to *PtNF-YB7*, was upregulated at 2h, a finding which suggests that overexpression of this TF might improve WUE in *Prunus* and, consequently, enhance tolerance to drought. In addition to the aforementioned TFs, we identified up- and downregulated DEGs encoding other TFs families whose involvement in drought response has been demonstrated previously, including HSFs in *Pyrus betulaefolia* (K.-Q. Li et al., 2016), PLATZs in *Brassica juncea* (Bhardwaj et al., 2015), Zinc-Finger superfamily TFs: RING finger TFs, A20/AN1 TFs, ZATs, C3HC4 TFs, CCCH TFs and C3HC3 TFs in rice and tomato (Liu et al., 2016; A. C. Rai et al., 2013; Singh and Laxmi, 2015; Vij and Tyagi, 2006), and uspA proteins in *Populus euphratica* (Tang et al., 2013) (Supplementary Material S4.3; Annex 3). These TF's could play similar drought-responsive roles in *Prunus*.

Genes involved in acting as cellular protectors against dehydration-related damage

Downstream of transcriptional processes, effector genes play an important role in regulation of pathways involved in cell protective functions and ultimately facilitate adaptation and tolerance to drought. These effector genes include HSPs or chaperones, dehydration responsive genes including LEA proteins and dehydrins, osmoprotectans, ROS-responsive genes, transporters, and cell wall modifying enzymes (Shinozaki and Yamaguchi-Shinozaki, 2007).

HSP effectors of drought adaptation. Several HSP encoding proteins, which assist in the refolding and stabilization of polypeptides and membranes under stress (Padmalatha et al., 2012), were represented among the annotated DEGs in 'Garnem'. In particular, 8 upregulated DEGs were annotated as hsp70-Hsp90 organizing-like proteins (HOPs) (Supplementary Material S4.3; Annex 3). These proteins have previously been implicated in modulation of HSP70/HSP90 interactions and are phosphorylated in roots during drought stress, leading to drought tolerance by binding and stabilizing non-native proteins (Fernández-Bautista et al., 2017; Hao et al., 2015). A group of 'Garnem' root HSP chaperone proteins, represented by 33 DEGs, were identified (Supplementary Material S4.3; Annex 3). The functions of many of these chaperones have been previously documented (Park and Seo, 2015) and are further supported by the results of our functional annotation.

Dehydration responsive gene effectors of drought adaptation. Multiple chaperone machinery-related proteins were identified in our study, as well as 16 DEGs encoding drought-induced chaperonins (Supplementary Material S4.3; Annex 3). Among these were 7 upregulated DEGs annotated as CPN60 and CPN60-like, an observation that is consistent with results of a previous proteomic study in drought stressed cotton (H. Zhang et al., 2016). Several DEGs encoding LEA proteins and dehydrins, which are strongly induced by dehydration (Hundertmark and Hincha, 2008), were upregulated in our dataset, and included DHN2, COR47, ERD4, LEA D34-like, LEA14 and LEA5 (Supplementary Material S4.3; Annex 3). Their functions in chaperone activity and cell membrane protection against water stress, which contribute to enhanced drought tolerance, have been widely reported previously in *Arabidopsis* (Hundertmark and Hincha, 2008), peach (Bassett et al., 2009), *P. mume* (Du et al., 2013b) and *Prunus* rootstocks (Bielsa et al., 2016). Other recent transcriptomic studies have provided further support for the role of these LEA and dehydrin proteins in drought response (He et al., 2015; Ksouri et al., 2016; Magalhães et al., 2016; Padmalatha et al., 2012).

Mediation of drought stress response by ROS-induced effectors. Under drought stress conditions, ROS are produced as signals for induction of stress responses. Ironically, an excessive accumulation of ROS leads to oxidative stress in plants. In order to protect the cell membranes and macromolecules, redox homeostasis and antioxidant signalling processes are induced, thereby striking a balance in the sensitive regulation of drought response (Baxter et al., 2013; Padmalatha et al., 2012; G. K. Rai et al., 2013). A number of DEGs related to accumulation of antioxidant compounds were induced in roots of 'Garnem' under drought (Supplementary Material S4.3; Annex 3). Among them was GST, whose redox homeostasis-maintanence role in drought response was previously discussed. This finding further suggests the presence of a crosstalk between stress homeostasis and phytohormone signalling and metabolism. In addition to GST, we identified 2 upregulated DEGs encoding glutathione reductase (GR) and 4 DEGs associated with glutathione peroxidase (GPX), both of which are regulators of oxidative stress response. Ascorbic acid (AsA), which plays a crucial role in plant growth and development as well as human nutrition, is one

of the most abuntant antioxidants synthesized in plants during drought exposure (G. K. Rai et al., 2013). In our data, various DEGs related to AsA redox system including, 2 downregulated DEGs encoding a dehydroascorbate reductase (DHAR), 12 DEGs encoding for monodehydroascorbate reductases (MDHAR) and MDHAR-like were identified. Additionally, a number of DEGs were annotated as antioxidant enzymes, including superoxide dismutases (SODs), peroxidases (POX), ascorbate peroxidases (APX), and catalases (CAT), which were induced under drought conditions. Additional proteins previously implicated in cell protective and ROS detoxification functions, including ferritins, glutaredoxins, thioredxins and peroxiredoxins, were differentially expressed in the 'Garnem' transcriptome (Ksouri et al., 2016; Tang et al., 2013; Tognetti et al., 2012). Interestingly, the 'Garnem' antioxidant machinery also included one upregulated DEG encoding the alterative oxidase (AOX) enzyme. It is well known that AOX is crucial for limiting ROS production in mitochondria as well as for maintaining redox homeostasis (Noctor et al., 2014). Because of the high capacity for aleviating oxidative stress, this enzyme has been proposed as marker for breeding drought tolerant plant varieties (Padmalatha et al., 2012). The abundance of transcripts related to ROS scavenging enzymes may suggest that under water stress conditions, the ROS detoxification system of 'Garnem', is particularly effective, and may instill improved tolerance to drought.

Osmoprotectant-mediated drought adaptation. The accumulation of compatible osmolytes called osmoprotectants maintains cell turgor and water absortion under drought conditions. This provides greater adaptability and enhances tolerance to drought (Chaves et al., 2003; Singh et al., 2015). Osmoprotectants containing sugars and sugar alcohols were among the DEGs identified in our dataset, specifically, those related to the biosynthesis of trehalose and mannitol. 15 DEGs were annotated as alpha, alpha-trehalose-phosphate synthases (TPS), 2 DEGs as trehalose-phosphate phosphatases (TPP), 7 DEGs as probable TPPs, and 7 DEGs as probable mannitol dehydrogenases (Supplementary Material S4.3; Annex 3) (Loescher et al., 1992; Singh et al., 2015; Valliyodan and Nguyen, 2006). Upregulated DEGs involved in the synthesis of two other carbohydrate-related osmoprotectans were identified, both of which are implicated in drought adaptation: inositol (myo-inositol-1-phosphate

synthase 2, 2 DEGs) and sucrose (sucrose synthase, 9 DEGs) (Singh et al., 2015). Additionally, the upregulation of DEGs encoding galactinol synthase (GolS1) were observed in the 'Garnem' trascriptome data, an enzyme in the raffinose family of oligossacharides (RFOs) that has been shown to accumulate under drought conditions (Zhou et al., 2014) (Supplementary Material S4.3; Annex 3). Furthermore, 13 DEGs encoding enzymes involved proline accumulation, used as indicators of LWP changes following abiotic stress exposure, were upregulated (Roosens et al., 2002; Tang et al., 2013; Yoshiba et al., 1997) (Supplementary Material S4.3; Annex 3), suggesting proline-based osmoprotection during drought stress in 'Garnem' (Singh et al., 2015) (Lambers et al., 2008). The high number of induced osmoprotectant-related transcripts revealed in 'Garnem' suggests their importance in osmotic adjustment under drought exposure. Similar results have been reported in *P. euphratica* subjected to water stress (Tang et al., 2013).

Cell wall protection. Water deprivation triggers an adaptive response in plants, during which cell wall composition is altered to minimize further water loss. The cell's first barrier against dehydration, the cuticle, is composed of cutin and wax, hydrophobic substances that limit the amount of water that can leave the cell (Cui et al., 2016). Several upregulated DEGs related to biosynthesis of cutin and wax accumulation were identified in PEG-stressed 'Garnem' roots (Supplementary Material S4.3; Annex 3), including 3-ketoacyl-synthase-like (KSC), 3-oxoacyl-[acyl-carrier-] synthase chloroplastic-like, and ECERIFERUM enzymes, which are involved in the biosynthesis of protective wax and cutin during drought conditions (Cui et al., 2016; Weidenbach et al., 2014). Additional induced DEGs associated with cell wall strengthening components were identified, including xyloglucan metabolizing enzymes (xyloglucan endotransglucosylase hydrolases (XTHs), a xyloglucan 6-xylosyltransferase (XXT) and α-xylosidases), expansins, chitinases and enzymes related to biosynthesis of pectin (pectinestearases), and cellulose (COBRA and cellulose synthase enzymes). All of these enzymes function as agents controlling cell strength and extension via modification of root structure, and therefore contribute to drought stress adaptation (Houston et al., 2016; D. K. Lee et al., 2017; Padmalatha et al., 2012).

Genes implicated in water and ion uptake and transport

ABC transporter involvement in drought stress response. Transport of metal ions, lipids, sugars and other solutes, and water across the vacuolar and plasma membranes is crucial for maintaing all the functional processes in plants under abiotic stress conditions. In 'Garnem', many of these transporters, important in substrate movement, were induced under drought stress (Padmalatha et al., 2012). Notably, of the transporter groups, ABC transporters were most abundant in 'Garnem' roots, with 123 DEGs annotated as ABC proteins pertaining to seven different families (Supplementary Material S4.3; Annex 3). Previous reports have documented that genes encoding proteins from the ABC-G family are involved in ABA transport in *Arabidopsis*, particularly *AtABCG25* and *AtABCG40* (Nakashima and Yamaguchi-Shinozaki, 2013). ABC proteins also contribute to cutin and wax biosynthesis, both of which facilitate water retention under drought stress conditions. One such protein, ABCG12 is involved in cutin transport and resultant formation of cuticle layers. Additionally, *ABCG7* is a gene that is a precursor to wax synthesis and transport (Cui et al., 2016). Three upregulated DEGs encoding ABC11-like and ABCG7 proteins were annotated in 'Garnem'.

Potassium channel-mediated water retention during drought stress. Efflux of potassium (K^+) ions from guard cells, and consequent increase in ABA concentration, results in membrane depolarization and stomatal closure due to reduced guard cell turgor and volume. K^+ channels implicated in guard cell ion transport include the inward-rectifying K+ channels AKT1, AKT2, KAT1, KAT3, KOR2 and SKOR (Jin et al., 2013). In 'Garnem', more DEGs encoding these K^+ channels were downregulated than upregulated. This may result from accumulation of intracellular Ca^{2+} in guard cells caused by ABA, leading to the downregulation of inward-rectifying K^+ channels and the activation of the other classes of anion channels (S-type and R-type) (Brandt et al., 2012) (Supplementary Material S4.3; Annex 3).

Water potential maintenance by aquaporins. The ability of plants to maintain sufficient water potential under drought conditions requires an increase in root water absorption capacity. Aquaporins play an important regulatory role with regards to

hydraulic conductivity and cytosolic osmoregulation by increasing membrane permeability under water stress conditions, consequently allowing more water to enter the root cells (Bartels and Sunkar, 2005). Thirty six DEGs corresponding to aquaporins from four of the five subfamilies were identified, including NIPs, TIPs, a PIP and a SIP. Most of the DEGs encoding aquaporins were upregulated, with the exception of 5 DEGs, annotated as PIP2;1, PIP-type, TIP1;2, TIP1;3, and NIP2.1-like, which were downregulated (Supplementary Material S4.3; Annex 3). The overexpression of aquaporin encoding genes, together with the drop of LWP during drought period (Table 4.2), lends support to the functionality of aquaporin proteins in maintaining water levels. These findings are consistent with those of previous transcriptomic studies (Ksouri et al., 2016; J. Wang et al., 2015), suggesting that these aquaporin-associated genes may play a role in regulation of drought tolerance (Liu et al., 2013; Pou et al., 2013).

4.3.6. DEGs related to stomatal movement regulation

ABA-induced stomatal regulation. Under drought conditions, ABA regulates these movements, causing changes in turgor of guard cells, thereby modulating stomatal movements and flux of CO_2 and water in plants (Lind et al., 2015). ABA-induced stomatal closure leads to drought adaptation by reducing water loss, stimulating of leaf senescence, downregulating plant growth, and inducing biosynthesis of protective substances (Nishiyama et al., 2011). ABA accumulation is sensed via PYR1/PYL/CAR receptors, which bind to ABA and inhibit the dephosphorylation of $SnRK_2$ kinases, like SNF-1, by PP2C phosphatases. As a result, phosphorylated $SnRK_2$ kinases activate TFs (ABF TFs), which in turn recognize ABRE motifs in the promoter regions of ABA-responsive genes. TF binding triggers induction of these ABA-responsive genes, resulting in stomatal closure (Magalhães et al., 2016; Mishra et al., 2006; Nakashima and Yamaguchi-Shinozaki, 2013). In PEG-stressed 'Garnem', genes encoding PYL2-like and PYL8-like ABA receptors were upregulated, suggesting an increased need for sensing ABA accumulation (Santiago et al., 2012; Zhao et al., 2014). However, PYL4-like receptors, which are recognized by JA (Lackman et al., 2011), were downregulated (Supplementary Material S4.3; Annex 3). Due to the

complexity of the interactions among phytohormones in response to drought, *PYL4* expression may be downregulated through a crosstalk with ABA signaling in 'Garnem' roots. Most of the DEGs encoding PP2C phosphatases were downregulated at 24h of drought stress, indicating inhibition by PYR1/PYL/RCAR receptors. Also upregulated SNF1-related protein kinases (Supplementary Material S4.3; Annex 3), which are key regulators of ABA-induced stomatal movement (Boudsocq et al., 2004) were identified. Several contigs were annotated as SnRK2 substrates in guard cell membranes: the K^+ channel KAT1-like, which was downregulated, and the S-type anion channel SLAH-2-like (homologous to SLAH3), which was upregulated (Supplementary Material S4.3; Annex 3). These observations are supported by findings in *Arabidopsis,* in which SLAH3 impairs the inward-rectifying K^+ channel KAT1 in guard cells, thereby maintaining the stomata closed during drought stress conditions (Y.-F. Wang et al., 2016). In addition to the above-mentioned ABI5, an ARM repeat protein interacting with ABF2 (ARIA) was induced. This protein positively regulates ABA response in *Arabidopsis* by interacting with the ABF2 protein (Kim et al., 2004).

Furthermore, in the 'Garnem' trascriptome data were revealed other DEGs implicated in ABA-mediated stomatal closure in cluding WRKY TFs, which were represented by a number of overexpressed DEGs in 'Garnem' roots and may act as positive or negative regulators of stomatal movements via ABA signaling (Jiang et al., 2014; Tripathi et al., 2014); NAC TFs such us NAC domain-containing 72-like (Olsen et al., 2005; Singh and Laxmi, 2015); *Myb44* gene which acts as repressor of PP2C, a negative regulator of ABA signaling contributing to enhanced drought tolerance via facilitation of drought-induced, ABA-mediated processes like stomatal closure and temporary growth cessation (Li et al., 2015). Interestingly, DEGs annotated as *Myb44* TF in 'Garnem' roots were downregulated suggesting that there may be other regulatory mechanisms inhibiting the expression of this TF in *Prunus* during drought conditions. Additionally, two upregulated DEGs encoding ABCG22 isoform X1 were identified in 'Garnem' roots. The plasma membrane-located protein ABCG22 is required for stomatal regulation via export of ABA to guard cells (Kuromori et al., 2010). Among the osmeoprotectant compounds, two upregulated DEGs in 'Garnem' transcriptome were annotated as a probable trehalase (TRE). The overexpression of

TRE, which hydrolyzes threhalose into glucose, provides improved drought tolerance and regulates stomatal opening via ABA-dependent signaling (Van Houtte et al., 2013) (Supplementary Material S4.3; Annex 3).

Regulation of stomatal closure by genes associated with photosynthesis and sugar metabolism. Hexokinase I, well known for involvement of sugar signaling and metabolism, has also been shown to regulate stomatal closure in citrus, thereby reducing gs and transpiration, resulting in improved (WUE) (Lugassi et al., 2015). We identified DEGs encoding hexokinase I (Supplementary Material S4.3; Annex 3), which may have similar roles in 'Garnem' stomatal regulation during drought adaptation. These findings in the root transcriptome, together with the measured physiological responses 24h, at which gs decreased significantly in the treated plants, suggest that 'Garnem' adapts to drought conditions by reducing transpiration via stomatal closure, therefore reducing water loss and affecting to photosynthetic processes. In 'Garnem' we identified a unique gene encoding a photosynthetic enzyme. This gene, annotated as cytochrome b6-f complex iron-sulfur chloroplastic, was downregulated at 2h of drought stress. Other differentially expressed, photosynthesis-related genes (Supplementary Material S4.3; Annex 3) were both up- and downregulated, suggesting that upregulation of these genes could be due in part to compensation in photosynthetic electron transport or enzyme activity, which would maintain a partially open state of stomata during drought, leading in turn to maintenance of normal root growth (Tang et al. 2013).

4.3.7. Water use efficiency (WUE) as target character for breeding of drought tolerance in *Prunus*

As a physiological consequence of stomatal closure via ABA accumulation, transpiration is reduced and WUE, therefore, improved (Lata and Prasad, 2011). This may be the most important factor associated with plant drought adaptation and tolerance (Blum, 2009; de Almeida Silva et al., 2012). Based on the previous identification of genes directly related to WUE improvement in rice and *Arabidopsis* (Han et al., 2013; Karaba et al., 2007; Xing et al., 2011), three DEGs displaying transcriptomic changes in 'Garnem' will most likely be the appropriate targets for

future investigation in which WUE will be a target characteristic for improving drought tolerance. The target DEGs were *contig_78795*, annotated as *ERF023* TF; *contig_134330* identified as LRR receptor-like serine/threonine-kinase ERECTA; and *contig_128543* encoding for *NF-YB3* TF (Supplementary Material S4.3; Annex 3). *PpERF023* (*ppa026139m*) is homologous to the *AtHARDY* gene (*At2g36450*), an AP2/ERF-like TF. A previous study in rice demonstrated that *AtHARDY* improves WUE by enhancing assimilation of photosynthates and decreasing transpiration, thereby resulting in an improved drought response (Karaba et al., 2007). In 'Garnem', the HARDY gene may play a role in maintenance of root growth processes that are required for drought adaptation. The second selected DEG, *contig_134330*, is homologous to *ppa00847m* (LRR receptor-like serine/threonine-kinase ERECTA isoform X2). Overexpression of *PdERECTA* in transgenic *Arabidopsis* enhanced WUE by eliciting changes in leaf epidermal and mesophyll differentiation, which in turn positively affected growth and accumulation of biomass (Xing et al., 2011). The third selected DEG, *PdNF-YB7,* is a TF induced by osmotic stress and ABA. The overexpression of this gene has been shown to promote primary root elongation and increased photosynthesis, thereby conferring increased WUE and drought tolerance in transgenic *Arabidopsis* lines (Han et al., 2013). In 'Garnem' roots, differentially upregulated *contig_128543* annotated as *NF-YB3-like* TF, which is homologous to *PdNF-YB7*. This finding suggests that *NF-YB3-like* may have similar functions in 'Garnem' to that in poplar and could therefore potentially increase WUE in *Prunus*.

4.3.8. KEGG pathways involved during PEG-induced drought stress

To understand the response and adaptation of 'Garnem' under drought conditions, a better understanding of the complex network of biochemical pathways involved in that response is needed. In order to identify the metabolic pathways involved in PEG-induced drought stress, the annotated DEGs, 655 of which corresponded to enzymes, were mapped to their respective KEGG pathways. Comparing the four DEG pools DEGs belonging to them were mapped in a number of pathways: 2hS-24hS: 718 DEGs in 106 pathways; 2hC-2hSN0C: 2,327 DEGs in 124 pathways; 24hC-24hSN0C: 2,630 DEGs in 139 pathways; and 2hS-24hSN0C: 3,992 DEGS

in 139 pathways. Of the identified DEGs, the most important pathways revealed were purine metabolism (56.42% of DEGs – 51 annotated enzymes), thiamine metabolism (29.56% of DEGs – 6 annotated enzymes), biosynthesis of antibiotics (19.11% of DEGs – 156 annotated enzymes), aminobenzoate degradation (7.62% of DEGs – 6 annotated enzymes), starch and sucrose metabolism (6.44% of DEGs – 32 annotated enzymes) and glycolysis/gluconeogenesis (6.22% of DEGs – 25 annotated enzymes) (Supplementary Material S4.5; Annex 3). Also, it is remarkable that important pathways such as oxidative phosphorylation, carbon fixation in photosynthetic organism, pathways related to osmoprotectants including fructose and mannose metabolism, photosynthesis, and several pathways related to lipid metabolism and amino acid metabolism, among others, were represented under drought response in 'Garnem' roots. Under drought conditions, lipids undergo various changes in their metabolism; these changes serve to maintain cellular homeostasis (Golldack et al., 2014). We annotated enzymes pertaining to several induced pathways related to lipid metabolism such as glicerolipid metabolism, glicerophospholipid metabolism, fatty acid degradation, fatty acid biosynthesis, sphingolipid metabolism, arachidonic acid metabolism, fatty acid elongation, α-linolenic acid metabolism, steroid hormone biosynthesis of unsaturated fatty acids, ether lipid metabolism, steroid degradation, linoleic acid metabolism, steroid biosynthesis cutin, suberin and wax biosynthesis (Fracasso et al., 2016; Golldack et al., 2014; Pan et al., 2016; Wang et al., 2007) (Supplementary Material S4.5; Annex 3). Previous reports have demonstrated that osmotic adjustment (mentioned above) and energy production and preservation, via carbohydrate metabolism, are crucial for plant adaptation to water stress. Enzymes in various key metabolic pathways such as carbon including purine metabolism, starch and sucrose metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, amino sugar and nucleotide sugar metabolism, pentose phosphate pathway, carbon fixation in photosynthetic organisms, fructose and mannose metabolism, pentose and glucuronate interconversions, carbon fixation pathways in prokaryotes, glyoxylate and dicarboxylate metabolism, galactose metabolism, inositol phosphate metabolism and ascorbate and aldarate metabolism were found induced in the transcriptome of 'Garnem' roots (Gong et al., 2010; Pan et al., 2016; Singh et al., 2015) (Supplementary Material S4.5; Annex 3). These findings provide further support for the important roles

of lipids and carbohydrates in the adaptation to drought in 'Garnem'. Similar results have been obtained in previous transcriptomic studies made in sweet potato (Cao et al., 2016), peach (Ksouri et al., 2016) and *L. multiflorum* (Pan et al., 2016).

The annotated enzymes were distributed in 6 major classes namely Hydrolases (55% of DEGs), Transferases (17%) and Oxidoreductases (16%) (Fig.4.5).

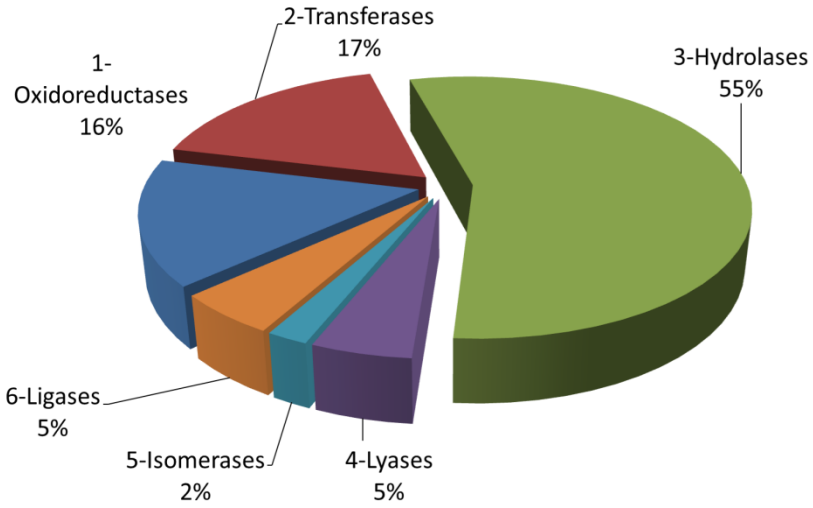

Figure 4.5. Annotated enzyme distribution in the four DEG pools.

4.3.9. qRT-PCR validation

To verify the RNAseq results, 18 genes were randomly selected from the DEGs identified to be involved in drought response for qRT-PCR. Overall, qRT-PCR based expression of 89%, or 16 out of 18 genes, agreed with the RNAseq results indicating robustness of the transcriptome analysis (Fig.4.6). The expression profile of two genes, namely *Myb 108 TF* and *Ca^{2+} Kinase 26*, differed between the RNAseq and qRT-PCR methods (Fig. 4.6E and P). In addition to an 89% correspondence to the RNAseq results, the qRT-PCR results were in accordance with results of previous transcriptomic studies of drought response in peach and Mongolian almond (Ksouri et al., 2016; J. Wang et al., 2015).

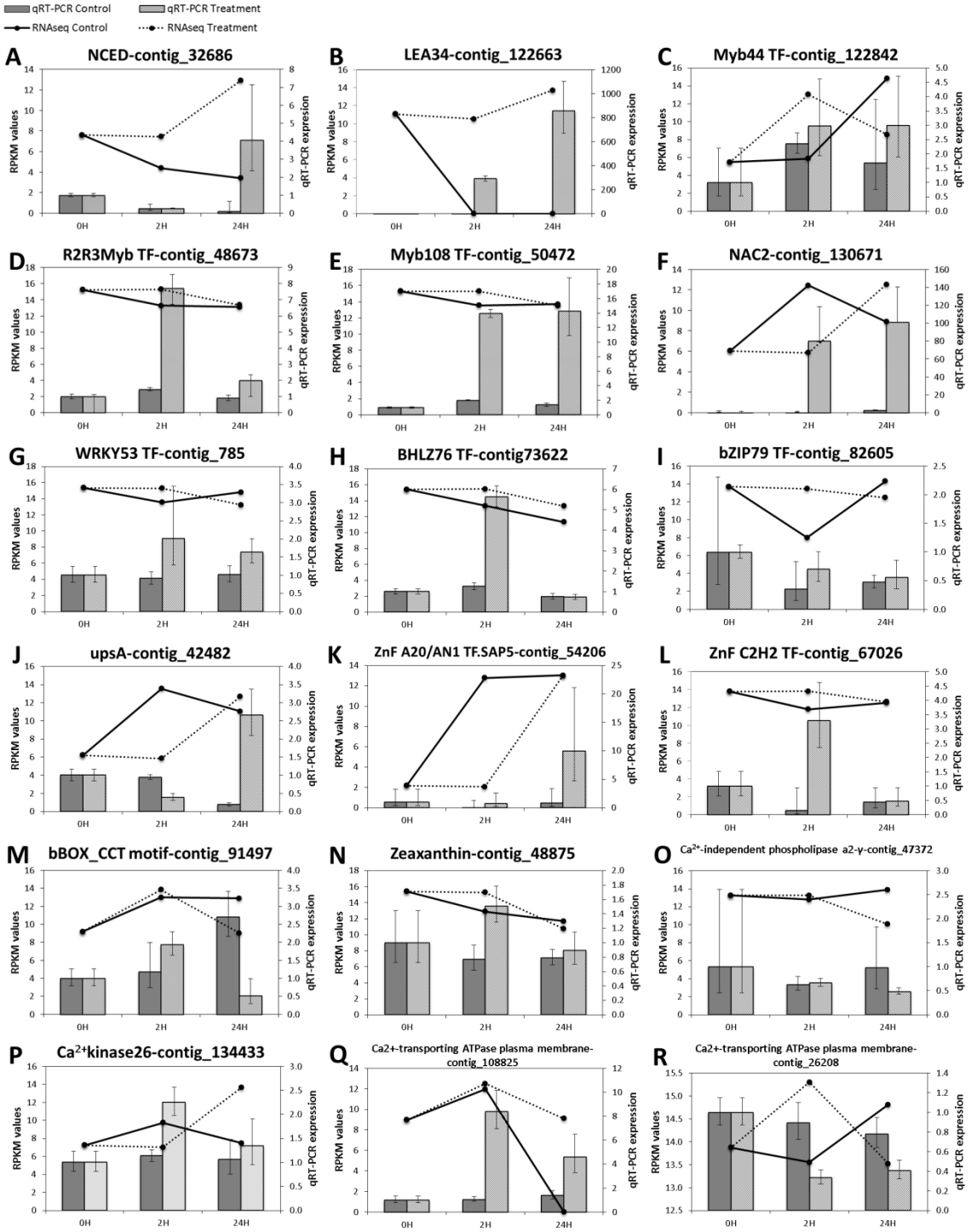

Figure 4.6. qRT-PCR validation of select genes in control and treated plants. The grey-scale bars represent relative gene expression in control (drack grey) and treat plants (light grey) qRT-PCR analysis (right y-axis). qRT-PCR data show the average relative expression of two biological samples with three technical replicates each one. Lines represent RPKM values of the transcripts in control (black line) and treated plants (dotted line) by RNAseq (right y-axis). The error bars represent the standard error between replicates in qRT-PCR analysis.

4.4. CONCLUSION

The present RNAseq analysis represents a comprehensive, high throughput approach for understanding the transcriptomic changes during the first hours of response to drought stress at functional, biological, and cellular levels in 'Garnem' roots. These results provide insight into the involvement of several genes and interconnectedness of the metabolic pathways induced by water scarcity. Furthermore, several specific drought-responsive genes *ERF023* TF, LRR receptor-like serine/threonine-kinase ERECTA, and *NF-YB3* TF were identified, which are expected to be utilized in future efforts to breed drought tolerance in *Prunus* species

5. PROTEOMIC ANALYSIS REVEALS CHANGES IN ‘GARNEM’ ALMOND × PEACH HYBRID ROOTSTOCK UNDER DROUGHT STRESS

ABSTRACT

Drought affects growth and metabolism in plants. To investigate the changes in root protein abundance involved in the drought-adaptive response, a proteomic analysis in combination to a physiological, as well as a biochemical analysis was performed in plants of 'Garnem', an almond × peach hybrid rootstock, submitted to short-term drought stress conditions. The physiological and biochemical parameters indicated that 'Garnem' triggered it machinery in order to cope drought stress. Abscisic acid (ABA) accumulation levels increased dramatically during the drought exposure inducing the stomata closure, and thus minimizing water losses. These physiological effects were reflected in stomatal conductance and leaf water potential levels. But, surprisingly, 'Garnem' was able to balance its water content as well as maintain an osmotic adjustment in their cell membranes, suggesting a dehydration avoidance strategy. Proteomic analysis revealed significant abundance changes in 29 and 24 spots after 2h and 24h of drought stress, respectively. Out of these, 15 proteins were identified by LC-ESI-MS/MS. These proteins participate in a variety of biological functions including lipid, amino acid and nitrogen metabolism, transcriptional and defense response, protein synthesis and modification, ion transport activity, and control of gene expression regulation. Their abundance changes, influence in drought-responsive mechanisms present in 'Garnem' which would allow enhancing drought tolerance and, thus being able to adapting to drought conditions. Overall, our study may contribute to improve the existing knowledge on the root proteomic changes in response to drought, leading to understand dehydration avoidance and tolerance strategies, and finally, help us to suggest new drought-tolerance breeding approaches.

Keywords: ABA accumulation, Adaptive response, Drought tolerance, Root proteome, Water stress

5.1. INTRODUCTION

In last decades, drought has become in the most severely limiting factor for optimal development and growth in crops. Water scarcity induces a number of alterations at the molecular, cellular, biochemical and physiological levels (Agarwal et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2005).In drought conditions, signaling cascades are triggered, leading to activation or suppression of the expression of specific genes. Many plant species are able to cope with drought stress helped by different strategies including drought escape, avoidance and dehydration tolerance (Varela, 2010). Recent approaches have indicated that the development of these protective strategies allows maintaining cellular homeostasis by modifications in lipid, carbohydrate, nitrogen and amino acid metabolism, carbon fixation processes, and antioxidant and secondary signaling (Fracasso et al., 2016; Golldack et al., 2014; Katam et al., 2016; H. Liu et al., 2015; Pan et al., 2016). Under drought conditions, abscisic acid (ABA) accumulation induces expression of many stress-related genes (Lata and Prasad, 2011; Shinozaki and Yamaguchi-Shinozaki, 2007). ABA is one of the key hormones in drought stress adaptation by regulation of biological processes such as stomatal regulation to minimize water loss, osmotic adjustment, cell membrane stability and regulations in plant growth (Belin et al., 2010; Lind et al., 2015; Verslues et al., 2006).

Currently, transcriptomic studies, whether by microarrays or RNA sequencing, have revolutionized the capacity to elucidate the drought regulatory mechanisms (Fracasso et al., 2016; Gong et al., 2010; Ksouri et al., 2016; Z. Li et al., 2016). However, because of mRNA levels might be not correlated well with protein abundances and functions, due to translation and post-transcriptional modifications such us phosphorylation, glucosylation, ubiquitination and sumoylation (Alam et al., 2010), proteomic approaches are a powerful tool for identification of drought-responsive proteins (H. Liu et al., 2015; X. Wang et al., 2016). These proteins are involved in drought response acting as enzymes or transcriptional factors (TFs), presenting protective functions, interacting with other molecules, playing a role in energy transfer or radicals scavenging pathways (Rodziewicz et al., 2014).

In comparison with transcriptomic analyses, proteomic studies of plants in response to drought are still limited. Most of these studies have been focused on herbaceous crops such as cereals (Hao et al., 2015; Kausar et al., 2013; Ke et al., 2009; H. Liu et al., 2015), legumes (Alam et al., 2010; Bhushan et al., 2007) and others crops as sugarcane (Rahman et al., 2015) or cotton (H. Zhang et al., 2016). In woody plants, drought-response proteomic analyses are more restricted. Several of these researches have been performed in holm oak (Valero-Galván et al., 2013), eucalyptus (Valdés et al., 2013), poplar (Bonhomme et al., 2009) and apple (Macarisin et al., 2009; Wisniewski et al., 2009; Zhou et al., 2015). But studies in *Prunus* species have been mainly focused in other objectives such as effects of fruit ripening (D'Ambrosio et al., 2013), fruit nutrient content (S. Li et al., 2016), self-(in)compatibility (Martínez-García et al., 2015), effects of altitude in fruit quality (Karagiannis et al., 2016), chilling injury and photoperiod (Nilo et al., 2010; Renaut et al., 2008); and heat treatment (Lara et al., 2009). Should be noted that most of these protein identification studies has been focused on leaf tissue. However, on one hand water and nutrients are supplied by roots throughout the whole plant and on the other hand, roots are the first organs to sense water deprivation in the soil. Hence, stress signaling is firstly perceived in roots which transfer the chemical signal towards the shoots, resulting in different biochemical and morphological changes that protect the plant against drought (Janiak et al., 2016).

Prunus L. is a diverse genus whose most of their species are cultivated in the temperate zone and some in the tropical and subtropical regions. The economic importance of this genus consists on its diverse uses as fruit, oil, timber, and ornamentals (Lee and Wen, 2001). Rootstocks are responsible for water and nutrient uptake, resistance to soil-borne pathogens, and tolerance to environmental stresses, to name a few of the more important traits developed in breeding programs (Layne, 1987). Currently, the challenge in *Prunus* rootstock breeding programs is the combination of abiotic tolerances in a new generation of interspecific hybrids resulting from the cross of almond × peach hybrids by plum genotypes. Peach × almond hybrids such as 'Garnem', 'Felinem' and 'Monegro' (which come from the cross 'Garfi' almond

× 'Nemared' peach) show good vigor, nematode resistance, and adaptation to calcareous soils (Felipe, 2009).

To elucidate the response to short-term drought stress for 24h followed by 24h of re-watered period, a proteomic approach was performed in 'Garnem' roots. Several differentially accumulated proteins were identified during drought stress, indicating the changes in root metabolism created in order to cope the stress period that may lead to drought tolerance. Furthermore, physiological responses as well as ABA accumulation in leaves were analyzed in order to obtain a better understanding of 'Garnem' adaptation to drought.

5.2. MATERIALS AND METHODS

5.2.1. Plant material and growth conditions

For the experiment, we considered a total of 28 clonally propagated plants from the drought tolerant almond × peach hybrid [*P. amygdalus* Batsch, syn *P. dulcis* (Mill.) × *P. persica* (L.) Batsch] 'Garnem', which were acquired from the commercial nursery Agromillora Iberia S.L in 2013. (Barcelona, Spain). The plants were maintained in a greenhouse at CITA facilities in Zaragoza, Spain (41°43'28.6''N, 0°48'31.1''W), until drought experiment in 2015. The plants were watered three times a week and fertilized monthly with 15:9:10 N:P:K + 0.2% MgO (Nitricol). Temperatures in the greenhouse during the growth period were in a range of 28 °C and 18 °C, respectively with 12h day /8h night photoperiod. Two months before the experiment, plants were replant in perlite substrate in 20 cm diameter pots in order to be acclimated to the new substrate. During these two months, the frequency of the irrigation and fertilization were increased to three times a week (discharge rate 2 l h^{-1} -dry irrigation system) and twice a month, respectively, for maintaining good water and nutrient status.

5.2.2. Stress conditions and treatment

The experiment design was two randomized block: Control (16 plants) and Treatment (12 plants). The experiment was carried out in a shaded greenhouse located in the CITA facilities in Zaragoza (41°43'N, 0°48'W) from July 29 to July 31, 2015. Whilst the water status of the control plants were watered until field capacity to preserve optimal soil water content along the experiment, the stressed plants underwent a no water supply period of 24h, followed by a re-watering period of 24h. The average climatic conditions during the experimental period were the following: temperature of 22.50 ºC; relative humidity of 64.25%; solar radiation of 19.62 MJ m^{-2} day^{-1}; rainfall of 2.28 mm day^{-1}; and ETo of 5.21 mm day^{-1}. Samples of both, root and leaf tissues, were collected from the control and treated plants at 0h, 2h and 24h along the drought stress period and 24h after re-watering. These samples were immediately frozen at -80 °C for subsequent analysis: roots for proteomic study and leaves for ABA content analysis.

5.2.3. Plant water status

Leaf Water Potential (LWP) was measured in duplicate in leaves using a Scholander-type pressure chamber (Soil Moisture Equipment Corp. Santa Barbara, CA, USA) (Scholander et al., 1964). Stomatal conductance (gs) was also measured from a leaf of each plant with a Leaf Porometer (Decagon Devices Inc. Pullman, WA, USA). Relative Water Content (RWC) was measured in duplicate as per previously published methods (Barrs and Weatherley, 1962). Briefly, three 1 cm diameter leaf discs were weighed (W) and rehydrated to their turgid weight (TW) by floating them in *petri* plates containing deionized water for 4h at room temperature. The dry weight (DW) was obtained after 24h at 80 °C in an oven. RWC was calculated following the equation:

$$\mathrm{RWC}\ \% = \frac{\mathrm{W} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}} \times 100$$

Electrolyte Leakage (EL) was calculated from Cell Membrane Stability (CMS) rate. CMS was evaluated in duplicate following previously published protocols (Blum

and Ebercon, 1981). Briefly, three 1 cm diameter leaf discs, previously cleaned twice with deionized water to remove surface-bound electrolytes, were submerged in a 50 ml vial containing 10 ml of deionized water and incubated in the dark for 24h at room temperature. Conductance was then measured with a conductivity meter (CRISON micro CM 2201, Barcelona, Spain). This measurement was taken as C1 (control samples) and as T1 (treated samples). After the measurement, the vials with the samples were autoclaved for 15 min at 121 °C. When the samples reached room temperature, a second reading was recorded (C2 for control samples; T2 for treated samples). CMS and EL were calculated according the following formulas:

$$\text{CMS } \% = \frac{1-\frac{T1}{T2}}{1-\frac{C1}{C2}} \times 100 \qquad \text{EL } \% = 100 - \text{CMS } \%$$

Each of the parameters described above were measured and recorded at 0, 2 and 24h of treatment and 24h after re-watering both, control and treated plants.

5.2.4. Abscisic acid determination

ABA extraction

Lyophilized leaf samples were homogeneously grinded in a 6875 Freezer/Mill® High Capacity Cryogenic Grinder (SPEX® SamplePrep, INC., UK) with liquid nitrogen. A volume of 3 ml of a buffer containing acetone:water:formic acid (80:19:1, v/v/v) were added to 0.05 g of powdered leaf sample, shaken to 2,000 rpm for 30 min in a test tube shaker (Multi Reax Shaker, Heidolph Instruments, Schwabach, Germany), and after centrifugation for 10 min at 4 °C, the supernatant was recover in a new tube. This step was repeated twice. The acetone was evaporated from samples with a nitrogen stream using a Sample Concentrator (SBHCONC/1 model; Stuart, Fisher Scientific Bioblock, Illkirch, France) until a volume less than 1 ml approximately, and then the samples were adjusted to 1 ml with milliQ water (Millipore). The extract was filtered [0.45-µm, 13 mm Nylon filter (Sartorius)] and 10ng μl^{-1} [$^2H_6$]-ABA was added as internal standard, which was prepared according to Gómez-Cadenas et al., (2002), before the UPLC system injection.

ABA determination by mass spectrometry

ABA determination was performed in a UPLC-TQD (ACQUITY, Waters). An Excel 2 C18-AR column (50 × 2.1 mm, ACE, UK) was used stabilized to 40 °C. The mobile phase was constituted by 70% methanol (solvent A) and 90% acetonitrile (AN) (solvent B) which contain 0.1% formic acid. The gradient was programmed to change linearly: 0-1min, 100% A; 1-2.5 min, 100-50% A; 2.5-2.8 min, 50% A; and 2.8-3 min, 100% A, with 2 min of equilibration before the following injection. The solvent flow level and the volume injection were adjusted to 0.15 ml min^{-1} and 20 µl, respectively.

For ABA identification and quantification, mass spectrometry was performed by ACQUITY-TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The electrospray conditions were de following: Polarity ES, capillary voltage 3.0 kV, source temperature 120 °C, desolvation gas temperature 350 °C. High-purity nitrogen was used as auxiliary gas and the nebulizer, and argon was used as the collision gas. Cone gas flow was set on 90 L h^{-1}, and desolvation gas flow was set on 900 L h^{-1}.

ABA analysis was carried out in MRM (Multiple Reaction Monitoring) mode, monitoring the transactions for [$^2H_6$]ABA and ABA at *m/z* 269→159, 225; and 263→ 153, 219, respectively.

The cone voltage (V) and collision energies (eV) were optimized to obtain the maximum signal, resulting: 20 V and 12 eV for [$^2H_6$]ABA, and 15 V, 10 eV, and 12 eV for ABA. The raw data were collected and processed with a MassLynx 4.1 software. Quantification was performed using calibration curves based on the ABA/[$^2H_6$]ABA ratio of standard solutions.

Calibration curves preparation

Solutions of 1, 4, 10, 25, 50 and 120 ng ml^{-1} in 30% AN with 0.1% formic acid were prepared from an initial solution of 1,000 µg mL^{-1} ABA in AN. Aliquots for each point of 450 µl were passed into 2 ml vials with 50 µl of 10 ng $µl^{-1}$ [$^2H_6$]ABA before the UPLC system injection.

Statistical analysis

Statistical analyses were performed with SPSS 21 software package (IBM SPSS Statistics, USA) for gs, LWP, RWC and EL, as well as ABA content. Before carrying out any statistical analysis, the normality of all the data was studied using the Kolmogorov-Smirnov test. Data following a Normal distribution were subjected to ANOVA to test for significant differences between treatments and among hours. The significant difference was assessed with Duncan's test ($p \leq 0.05$). In case the hypothesis of normality was discarded at the 95 % confidence level, the data were subjected to non-parametric data Kruskal-Wallis' test ($p < 0.05$). Besides, the statistical differences between treatments for each time point were determined by the Student's *t*-test ($p \leq 0.05$).

5.2.5. Proteome analysis

Root samples were sent to Naxxis Biotech S.L., Parc Ciencific de Barcelona (Barcelona, Spain) for protein extraction, 2-D electrophoresis and protein identification by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis.

Protein extraction

'Garnem' roots (approximately 0.5 g) were homogenized in a mortar and pestle with liquid nitrogen and re-suspended in 1.2 ml of lysis buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 18 mM Tris-HCl, pH 8.0 and 0.2 v/v Triton X-100, supplemented with 53 u ml^{-1} DNase I, 4.9 u ml^{-1} RNase and a cocktail of protease inhibitors (1 mM PMSF, 1 µM pepstatin, 50 µM leupeptine, 10 µM E-64 and 10 µg ml^{-1} aprotinine). After 10 min of incubation at 4 °C, 14 mM dithiothreitol (DTT) was added. The protein extracts were centrifuged (35,000 × g, 10 min, 4 °C) repeating the 14 mM DTT addition step until the supernatant was clarified, and the protein extract isolated. Finally, total protein content was estimated by the Bradford method (Bradford, 1976).

2-D electrophoresis and image analysis

Samples containing 200 µg of total protein were diluted in rehydration solution (8 M urea, 2% w/v CHAPS, 0.5% v/v IPG buffer and bromophenol blue) including 7 µl DeStreak Reagent (GE Healthcare, Barcelona, Spain) and loaded onto 18 cm immobilized pH gradient (IPG) strips (pH 4-7) (GE Healthcare, Barcelona, Spain). Strips were rehydrated at room temperature, followed by 10h at 50 V. Isoelectric focusing was performed by gradient for 500 V (1.5h), 1,000 V gradient (1.5h), 2,000 V gradient (1.5h), 4,000 V gradient (1.5h), 8,000 V gradient (2h) and 8,000 V for 6h using an Ettan™ IPGphor™ Isoelectric Focusing System (GE Healthcare). For the second dimension, the strips were equilibrated previously with equilibration buffer (6 M urea, 50 mM Tris-HCl (pH 8.8), 30% v/v glycerol, 2% v/v SDS, bromophenol blue and 10 mg mL^{-1} DTT during 15 min, followed by a second equilibration step with 25 mg mL^{-1} 2-iodoacetamide for 15 min. Then, the strips were loaded on SDS-PAGE 12% polyacrylamide gel (20 × 25 × 0.1 cm) and run at 15 W for 30 min, then at 100 W for 4h. The 2-DE gels were run by duplicated (technical replicates) with two biological replicates by each sample. After protein separation, the gels were stained with silver nitrate for protein visualization (Shevchenko et al., 1996).

The 2-DE gels stained with silver nitrate were scanned using the ImageScanner desktop instrument and the LabScan application (GE Healthcare). Images were analyzed using the ImageMaster™ 2-D Platinum 5.0 Software (GE Healthcare) as described by Farinha et al., (2011). In order to compare the root proteome of the different time points and treatments, automatic spot matching was established between synthetic gel images. Also, a careful visual inspection was carried out to confirm correct spot matching.

Statistical analysis

The statistical analysis of the protein expression among time points and between treatments was performed as described by Farinha et al., (2011). Coefficient of correlation (*r*) and coefficient of variation (CV) were calculated after pairwise comparisons between technical and biological gel replicates. The normalized protein

spot volumes of all spots matching between technical replicates were considered for calculating average *r* and CV, whereas a mean of the spots (covering all ranges of normalized spot volumes, i.e. from low to high abundant protein spots) was used to determine biological variation. The statistical evaluation of proteins expression differences among treatments was performed as previously described (Farinha et al., 2011; Jorrin-Novo, 2009). Spots showing a quantitative variation or their relative spot volume (≥ ratio 1.5) and positive GAP (statistical parameters IMAGEMASTER 2-D PLATINUM 5.0 software) were selected as differentially expressed. Significant protein abundance was validated by Student's *t*-test ($p < 0.05$).

Protein identification

Selected protein spots differentially accumulated at least 1.5-fold in the pairwise comparisons studied were excised from 2-DE gels, de-stained and digested for further identification by LC-ESI-MS/MS.

Excised gel spots were washed with 500 µl of deionized water three times for 15 min each at room temperature under soft shaking, followed by a stain reduction with 50 mM $Na_2S_2O_3$ and 15 mM $K_3Fe(CN)_6$ for 5 min. Protein spots were washed with 500 µl of MilliQ water three times for 5 min each at room temperature under soft shaking again. Then, spots were equilibrated by treatment with 200 mM NH_4HCO_3 (BA) for 20 min at room temperature and soft shaking. After these steps, 50 µl of a buffer containing 10 mM DTT in 200 mM BA were added to the protein spots and shaken, followed by incubation for 20 min at 37 °C, and then, the supernatant was removed. Spots were washed with 50 µl of a buffer containing 55 mM 2-iodoacetamide in 200 mM BA and incubated for 20 min at 37 °C under dark conditions. The excess of 2-iodoacetamide was removed and 100 µl of 100% AN was added followed by an incubation during 5 min at 37 °C. Supernatant was removed. Newly, spots were washed with 50 µl of 100% AN by incubation for 5 min at 37 °C, then the excess of solution was removed. After drying the protein spots during 10 min at room temperature, these spots were placed on ice and rehydrated in a digestion buffer (50 mM BA and 10% v/v AN) including 0.1 µg μl^{-1} of trypsin and incubating on ice during 45 min. After overnight incubation (8-16h) at 37 °C, the supernatant was transferred to a

new Eppendorf tube and peptides were extracted from the gel matrix with a buffer containing 60% v/v AN and 0.1% v/v formic acid. Finally, the extracts were evaporated in SpeedVac concentrator at room temperature and re-suspended in 12 µl of 0.1% v/v formic acid.

The digests were analyzed by LC-ESI-MS/MS using a QExactive plus Orbitrap MS (Thermo Fisher Scientific) that was coupled to an Easy-nLC1000 (Proxeon Biosystems). Peptides were separated on a C18 analytical column (75 µm × 50 cm) at a 200 nl min^{-1} flow rate. The elution gradient was from buffer A (0.1% formic acid in MiliQ water) to buffer B (0.1% Formic Acid in 99% AN) as follows: from 100% buffer A to 27% of buffer B for 240 min, from 27% to 90% of buffer B for 9 min and from 90% to 2% of buffer B for 33 min. The spray voltage was set to 2.1 kV, and the temperature of the heated capillary was 270 °C. The MS scanned a mass range of 200 to 2,000 Da, with MS resolution 70,000 and 60 ms maximum injection time. The data on the top 15 most abundant peptides (ions) were analyzed in data-dependent scan mode. The normalized collision energy was adjusted to 28%, and the dynamic exclusion was set to a repeat count of 1, repeat duration of 20 s, and ± 2 *m/z* exclusion mass width. The result was performed by spectral counting analysis using the Proteome Discoverer software 1.4 (Thermo Fisher Scientific).

5.3. RESULTS

5.3.1. Physiological response to drought stress

In order to confirm the exposure of drought conditions, different physiological parameters were measured during the experiment. Treated plants registered gs and LWP values lower than control plants along the trial period (Fig. 5.1A and B, Table 5.1), indicating a response to drought stress. However, statistical differences were only observed for gs at 24h after re-watering (Fig. 5.1A), when treated-plant values were 44.82% lower than well-watered-plant values (Table 5.1); and for LWP at 2h of drought exposure (Fig. 5.1B), time when LWP for treated plants were 25.78% lower than well-watered plants value (Table 5.1). This minimum LWP value reached for treated plants at 2h confirmed the response to a reduction in the water soil content. In addition, RWC

and EL were also estimated, obtaining high rates both, RWC and EL, for treated plants (Fig. 5.1C and D). Although RWC rates were not statistically different both, between treatments and among hours (Table 5.1), a slight decrease was observed between treated and well-watered plants at 2h (Fig. 5.1C). But at 24h of treatment and also after recovery time, RWC of treated plants were higher than well-watered plants (Fig. 5.1C). EL can be used to quantify the extent of cellular damage caused by stress (Verslues et al., 2006). EL rate in 'Garnem' leaves decreased significantly throughout the time exposure (Fig. 5.1C) from 2.83% at time 0 to 0.510% after 24h of drought stress (Table 5.1), indicating that cell membrane held stable during water dissection.

Table 5.1. ANOVA results from stomatal conductance (gs), leaf water potential (LWP), relative water content (RWC), electrolyte leakage (EL) and ABA content in leaves during the drought treatment of 'Garnem' rootstock. Same letter values indicate a no significant difference ($p \leq 0.05$) following Duncan's post hoc test for each treatment (control and treatment) among hours. (h = hour, R = Re-watering, DW = Dry weight).

	Stomatal Conductance (gs) (mmol m^{-2} s^{-1})							
	0h		**2h**		**24h**		**24h R**	
Control	86.025±10.935	ab	117.475±14.551	b	65,45±9.651	a	65,425±3.214	a
Treatment	86.025±10.935	b	99.975±14.871	b	39.35 ±6.208	a	36.100±2.357	a
	Leaf Water Potential (LWP) (MPa)							
	0h		**2h**		**24h**		**24h R**	
Control	-0.962±0.162	b	-1.618±0.074	a	-0.612±0.096	c	-1.000±0.081	b
Treatment	-0.962±0.162	b	-2.187±0.189	a	-0.906±0.137	b	-0.893±0.109	b
	Realtive Water Content (RWC) (%)							
	0h		**2h**		**24h**		**24h R**	
Control	82.536±4.902	a	79.869±1.765	a	79.242±1.759	a	71.415±1.487	a
Treatment	82.536±4.902	a	76.336±1.935	a	83.816±2.028	a	80.246±4.007	a
	Electrolyte Leakage (EL) (%)							
	0h		**2h**		**24h**		**24h R**	
Treatment	2.837±1.410	b	1.697±0.694	ab	0.510±0.449	a	0.152±0.152	a
	ABA content (ng g^{-1}) DW							
	0h		**2h**		**24h**		**24h R**	
Control	205.176±73.508	a	137.090±22.126	a	176.816±11.779	a	220.122±30.150	a
Treatment	205.176±73.508	a	299.486±48.113	a	573.369±69.095	b	254.191±20.206	b

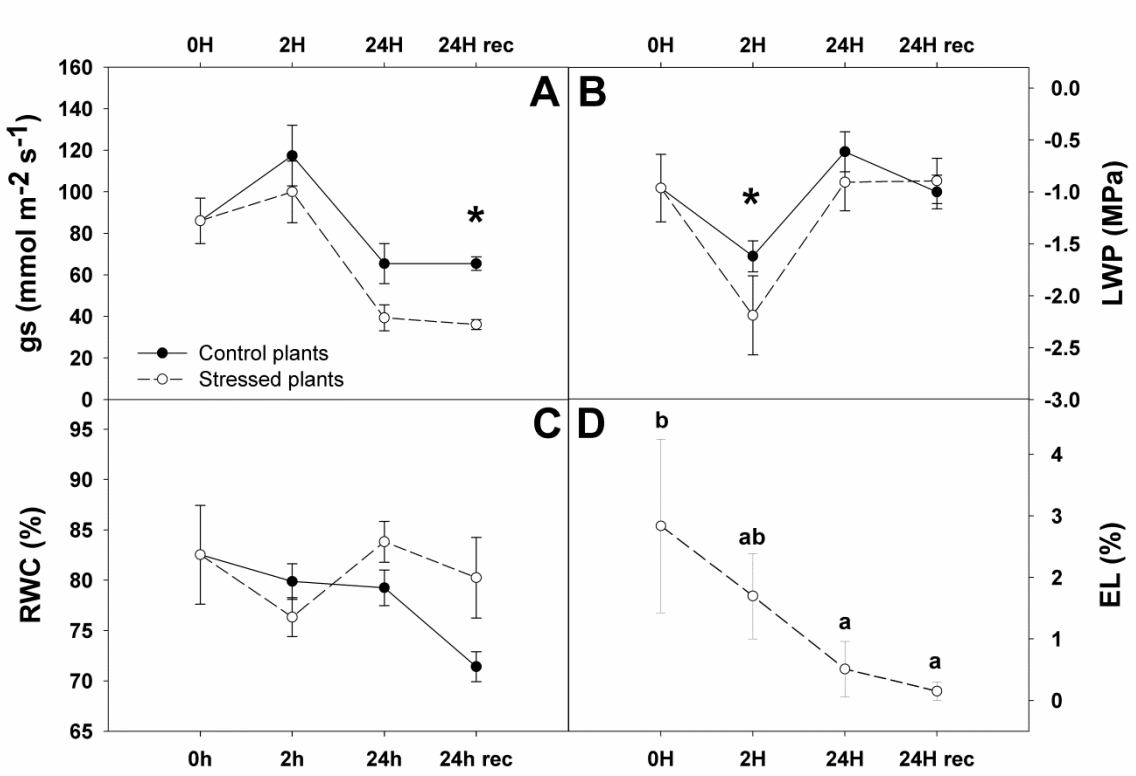

Figure 5.1. Stomatal conductance (gs) (A), leaf water potential (LWP) (B), relative water content (RWC) (C) and electrolyte leakage (EL) (D) during the drought experiment for control and treated plants of 'Garnem'. Continuous lines indicate well-watered plants, while dash lines indicate stressed plants. (H = Hours, rec = recovery). Error bars represent the standard error of the mean. Asterisks represent significant differences ($p \leq 0.05$) between treatments (control and stressed) for each time point of the experiment. Similar letter values indicate no significant difference ($p \leq 0.05$) following Duncan's post-hoc test, among time points of drought experiment.

5.3.2. Changes in ABA accumulation under drought stress

During drought stress period, treated plants showed a significant and exponential increase in their ABA content from 205.18 ng g^{-1} to 573.37 ng g^{-1} at 0h and 24h of treatment, respectively (Table 5.1). However, when water status was restored, ABA content dropped dramatically until reaching similar levels than well-watered plants (Fig. 5.2). On the other side, ABA content in well-watered plants was constant without statistical differences throughout the experiment, presenting an average ABA level of 184 ng g^{-1} (Fig. 5.2).

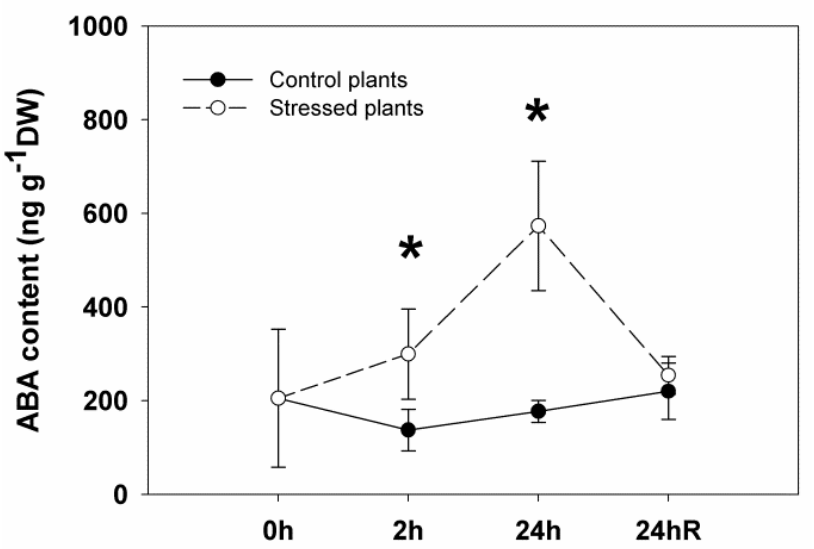

Figure 5.2. Abscisic acid (ABA) content in leaves of 'Garnem' during the drought experiment in control and treated plants. Continuous lines indicate well-watered plants, while dash lines indicate stressed plants. (H = Hours, rec = recovery). Error bars represent the standard error of the mean. Asterisks represent significant differences ($p \leq 0.05$) between treatments (control and stressed) for each time point of the experiment.

5.3.3. Proteome profiling of 'Garnem' roots under drought and gel analysis

A comparative proteome study was performed using roots of 'Garnem' rootstock exposed to a drought stress period of 24h followed by 24h of recovery. Two plant conditions were evaluated: well-watered (control) and treated (drought) in order to understand the response and adaptability mechanisms under drought stress in this genotype. A total of 24 2-DE gels were run from two technical replicates for each two biological replicates. The 2-DE gels representing the root proteome response to drought of each time point in control and treated conditions showed a similar spot-pattern distribution along the separation range of p*I* 4-7 and molecular weight 14-66 kDa (Fig. 5.3). Most of the spots were concentrated within a p*I* range from 5 to 6.

The number of spots found was different among treatments. Control gels showed more spots (1,021-0h, 1,123-2h, 1,182-24h) than treatment gels (1,048-2h and 950-24h and 1,109-24h recovery). Technical replicates showed a homology ranged from 69 to 72% of homology, and a linear regression higher than 0.82. While the homology between biological replicates ranged from 69 to 72%. After the pairwise comparisons between all gels, only two comparisons revealed detected protein spots

with at least a 1.5-fold based on their abundance: control at 2h vs. treated at 2h comparison (C2h-T2h) and control at 24h vs. treated at 24h comparison (C24h-T24h). In C2h -T2h, the percentage of shared proteins between control and treated plants was of 66% (773 spots), while at 24h this percentage was of 64% of shared proteins (760 spots)

Once the representative 2-DE gel images were analyzed, the detected spots with significant accumulation were established. A total of 29 differentially abundant spots with a CV of 2.3% and 24 differentially abundant spots with a CV of 3.2% were found in C2h-T2h and C24h-T24h comparisons, respectively. Specifically, focusing on spots from the proteome comparison at 2h, 8 from the 29 differenced spots were only accumulated in control plants, whereas 3 spots were unique for treated plants (Table 5.2). Out of those, 18 differenced spots were matched in both control and treated conditions. Among these, 13 spots were more accumulated in control than in treated plants (down-regulated) and 5 spots were more accumulated in treated than in control plants (up-regulated) (Table 5.2). On the other hand, when proteomes at 24h were compared, 7 differenced spots were only accumulated in control plant and 1 differenced spot was unique for treated plants at 24h of drought exposure. Besides, 16 differenced spots were shared between treatments, being 13 of these spots down-regulated and 3 spots up-regulated (Table 5.2). In addition, two differentially abundant spots were found down-regulated at both 2h and 24h of drought exposure (spot 233-219 and 1156-1134) (Table 5.2).

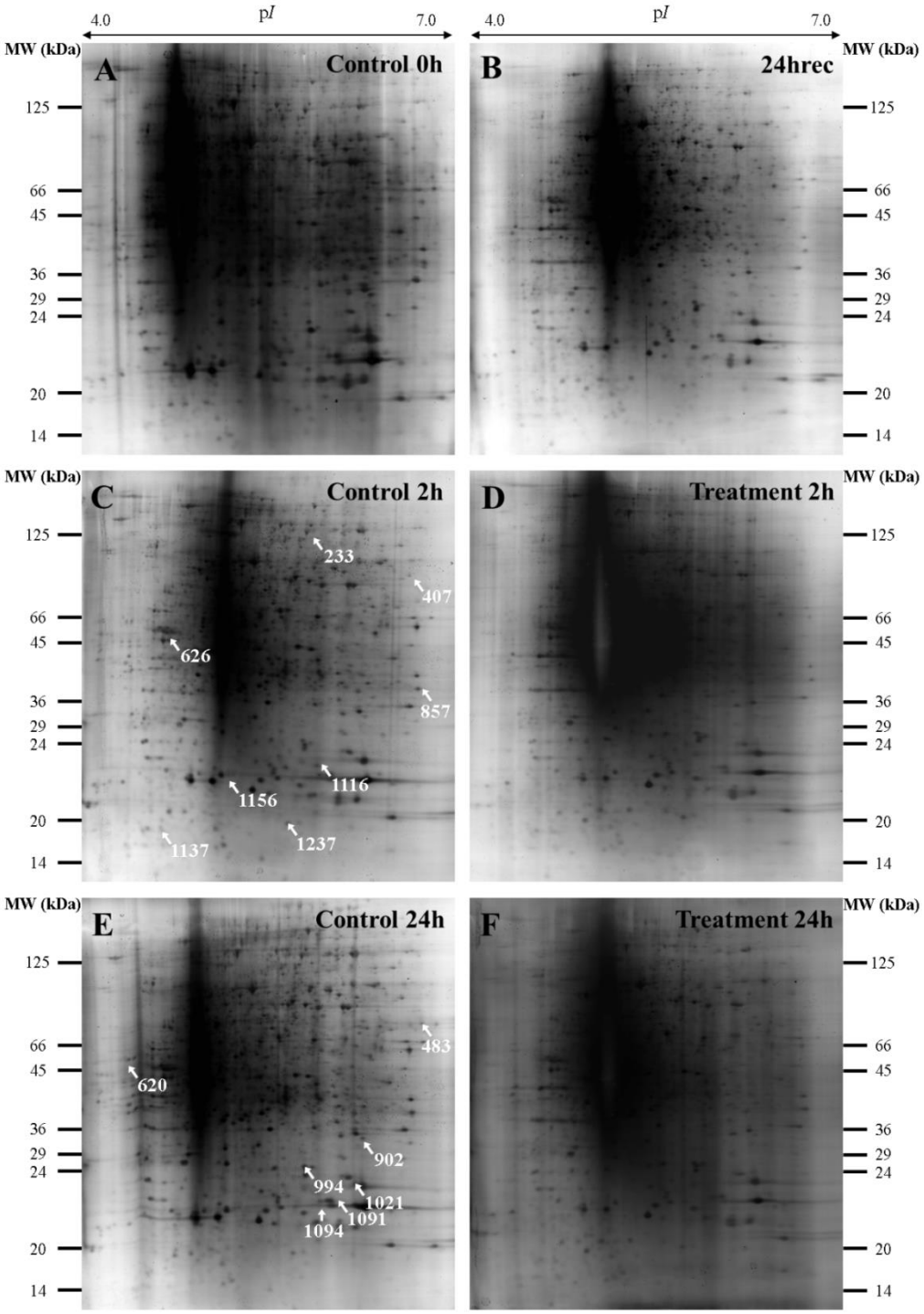

Figure 5.3. Representative 2-DE gel images of root proteins in control and treated 'Garnem' plants during drought experiment. Spots whose abundance differed significantly ($p < 0.05$; 1.5-fold of change) between control and treated plants which were identified by LC-EMSI-MS/MS, are represented by arrows.

Table 5.2. Drought-induced spots whose abundance is significantly different ($p < 0.05$; 1.5-fold of change) in response to drought in 'Garnem' roots after pairwise comparisons between representative 2-DE gel images. Asterisks represent proteins identified by LC-EMSI-MS/MS. Spot ID in bold indicates the differential spot found at both, 2h and 24h of drought stress. (↑ represents higher protein abundance in treated plants; ↓ represents lower protein abundance in treated plants).

	Control 2h		Treatment 2h				Control 24h		Treatment 24h		
Spot ID	%vol	Ratio	%vol	Ratio		Spot ID	%vol	Ratio	%vol	Ratio	
626*	0.242±0.054	3.594	0.022±0.003	-3.594	↓	**219***	0.123±0.045	1.000	0.008±0.007	1.000	↓
1137*	0.009±0.003	-3.082	0.082±0.015	3.127	↑	931	0.026±0.004	3.326	0.004±0.005	-2.361	↓
1237*	0.014±0.010	-2.688	0.102±0.011	2.688	↑	902*	0.186±0.106	2.243	0.020±0.016	0.020	↓
1140	0.124±0.005	2.927	0.039±0.009	-2.464	↓	640	0.132±0.053	2.362	0.023±0.018	-1.955	↓
835	0.071±0.015	2.232	0.018±0.007	-2.232	↓	**1134***	0.313±0.040	1.920	0.110±0.032	-2.275	↓
1156*	0.494±0.171	2.275	0.132±0.033	-1.953	↓	563	0.051±0.011	1.915	0.009±0.012	-4.208	↓
857*	0.230±0.075	1.952	0.067±0.012	-1.952	↓	1091*	0.206±0.061	1.803	0.046±0.035	-1.803	↓
934	0.049±0.006	1.944	0.009±0.003	-1.944	↓	483*	0.158±0.073	1.791	0.024±0.023	-1.791	↓
260	0.070±0.020	1.935	0.023±0.003	-1.935	↓	1021*	0.160±0.057	1.787	0.024±0.034	-1.787	↓
407*	0.044±0.015	-1.896	0.248±0.136	1.896	↑	617	0.026±0.010	1.714	0.004±0.006	-1.714	↓
612	0.076±0.030	1.891	0.013±0.011	-1.891	↓	374	0.067±0.023	1.705	0.025±0.001	-1.705	↓
1116*	0.119±0.049	1.891	0.015±0.003	-1.891	↓	372	0.045±0.012	1.000	0.009±0.012	-1.621	↓
1068	0.013±0.010	-1.799	0.056±0.015	1.799	↑	**994***	0.176±0.091	1.509	0.033±0.023	-1.509	↓
688	0.009±0.003	1.713	0.014±0.008	-1.713	↓	620*	0.038±0.007	-1.637	0.173±0.069	1.995	↑
224	0.172±0.058	1.695	0.041±0.026	-1.695	↓	1094*	0.014±0.003	-1.611	0.143±0.059	2.072	↑
205	0.125±0.042	1.648	0.039±0.011	-1.648	↓	869	0.196±0.063	-1.528	0.449±0.121	1.852	↑
910	0.110±0.023	1.537	0.033±0.024	-1.537	↓	150	0.040±0.025	1.000	-	-1.000	↓
1000	0.034±0.013	1.530	0.007±0.003	-1.823	↓	238	0.014±0.012	1.000	-	-1.000	↓
186	0.201±0.110	1.000	-	-1.000	↓	369	0.017±0.012	1.000	-	-1.000	↓
344	0.089±0.007	1.000	-	-1.000	↓	377	0.010±0.008	1.000	-	-1.000	↓
233*	0.086±0.036	1.000	-	-1.000	↓	427	0.019±0.015	1.000	-	-1.000	↓
173	0.064±0.036	1.000	-	-1.000	↓	557	0.027±0.019	1.000	-	-1.000	↓
107	0.040±0.039	1.000	-	-1.000	↓	238	0.014±0.012	-1.000	-	1.000	↓
53	0.030±0.024	1.000	-	-1.000	↓	950	-	-1.000	0.065±0.081	-0.077	↑
89	0.071±0.019	1.000	-	-1.000	↓						
56	0.016	1.000	-	-1.000	↓						
861	-	-1.000	0.029±0.017	1.000	↑						
893	-	-1.000	0.106±0.036	1.000	↑						
1081	-	-1.000	0.031±0.003	1.000	↑						

5.3.4. Identification of proteins differentially accumulated by MS

Out of those spots with significant differences of accumulation between control and treated plants at 2h and 24h, a total of 15 protein spots with differentially abundance were selected for identification by LC-ESI-MS/MS. Among these, the down-regulated protein spots were spots 626, 857 and 1116 at 2h, and spots 483, 902, 994, 1021 and 1091 at 24h. In contrast, up-regulated protein spots were spots 407, 1137 and 1237 at 2h, and spots 620 and 1094 at 24h. Also, spots 233-219 and 1156-1134

were selected for identification due to their significant accumulation in control than in treated plants at both, 2h and 24h.

Protein sequence searching was performed by a BLAST search against the non-redundant NCBI and UniProt databases using their p*I* and their calculated and expected molecular masses, as well as the protein coverage, peptide number and the number of unique peptides, as searching parameters. Thus, the 15 spots were identified as proteins implicated in several biological functions including lipid metabolism: the enzyme 1-acyl-sn-glycerol-3-phosphate acyltransferase (LPAT - spot 626) and a lipoxygenase (spot 233); amino acid metabolism: the prunasine hydrolase (spot 407); nitrogen metabolism: nitrite reductase (NIR - spot 994); ion transport activity: Putative S-adenosyl-methionine 3-amino-3-carboxypropyl transferase (spot 1116); carbon metabolism: Putative NAD dependent malic enzyme (NAD-ME - spot 1091); protein synthesis and modification processes: putative dnaK-type molecular chaperone hsc70,1 (spot 1156); transcriptional response: C-repeat binding factor 1 (CBF1 - spot 1041) and CBF2 (spot 902); defense response: a putative allergen Pru du 1.06B (spot 1237); hormone response: two S locus F-box proteins d (SLFd - spots 620 and 857); RNA processes: maturase k (matK - spot 483); cell cycle regulation: Cyclin-dependent kinase type A (Cdc2a - spot 1021), and finally, a putative uncharacterized protein (spot 1137) (Table 5.3).

Table 5.3. Drought-induced differentially abundance proteins identified in 'Garnem' roots by LC-EMI-MS/MS. (↑ represents higher protein abundance in treated plants; ↓ represents lower protein abundance in treated plants). Spot ID in bold indicates the differential spot found at both 2h and 24h of drought stress.

Spot ID	Protein name	Species	Accession no.	Gene name	Coverage (%)	Theor. Mw	Exper. Mw	Theor. pI	Exper. pI	
Lipid metabolism										
626	1-acyl-sn-glycerol-3-phosphate acyltransferase	*Prunus dulcis*	Q9SDN3	N/A	40.20	34		9.73		↓
233	Lipoxygenase	*Prunus dulcis*	Q9LEA9	*lox*	98.72	98		5.92		↓
Amino acid and nitrogen metabolism										
407	Prunasin hydrolase	*Prunus dulcis*	H9ZGE0	*Ph691*	79.96	61.9		6.48		↑
994	Putative nitrite reductase	*Prunus dulcis*	A7Y7M0	N/A	82.29	30.2		5.21		↓
Ion transport activity										
1116	Putative S-adenosyl-methionine 3-amino-3-carboxypropyl transferase	*Prunus dulcis*	A7Y7I9	N/A	100.00	24.3		6.79		↓
Carbon metabolism										
1091	Putative NAD dependent malic enzyme	*Prunus dulcis*	A7Y7M4	N/A	98.27	26.1		9.85		↓
Protein synthesis/modification										
1156	Putative dnaK-type molecular chaperone hsc70,1	*Prunus dulcis*	A7Y7I0	*hsc70.1*	99.44	20.1		5.16		↓
Transcriptional response										
902	C-repeat binding factor 2	*Prunus dulcis*	I6QEL8	*CBF2*	100.00	26.6		5.44		↓
1094	C-repeat binding factor 1	*Prunus dulcis*	A0A0H3U	*CBF1*	98.35	27.4		7.87		↑
Defense response										
1237	Putative allergen Pru du 1,06B	*Prunus dulcis*	B6CQS7	*Pru du 1,(*	100.00	17.4		5.25		↑
Hormone response										
857	S locus F-box protein d	*Prunus dulcis*	Q84KJ8	*SLFd*	27.38	47.1		4.73		↓
620	S locus F-box protein d	*Prunus dulcis*	Q84KJ8	*SLFd*	46.94	47.1		4.73		↑
Other functional proteins										
483	Maturase K	*Prunus dulcis*	Q8WJP3	*matK*	97.30	60.2		9.38		↓
1021	Cyclin-dependent kinase type A	*Prunus dulcis*	A3QNN7	*Cdc2a*	99.66	33.9		7.27		↓
1137	Putative uncharacterized protein	*Prunus dulcis*	Q9XH11	N/A	18.54	22.6		6.38		↑

5.4. DISCUSSION

5.4.1. Effects of water stress on physiological response

Under drought, plants develop different strategies in order to survive, being able to adapt to the new unfavorable environmental conditions. These strategies are mainly associated to plant modifications, which are promoted by accumulation of the phytohormone ABA (Belin et al., 2010). ABA accumulation triggers different mechanisms of response, such as stomatal regulation to minimize water loss, osmotic adjustment, cell membrane stability and regulations in plant growth (Belin et al., 2010; Lind et al., 2015; Verslues et al., 2006). In our study, ABA content in leaves of 'Garnem' was exponentially increased by 31.5% at 2h and by 64.2% after 24h of drought exposure in treated plants (Fig. 5.2). This rapid increment of ABA content in leaves, suggest a rapid long-distance hydraulic signal from roots to shoots inducing an instantaneously drought response in 'Garnem' leaves as stomata closure (Christmann et al., 2013; Osakabe et al., 2014).

To evaluate the effects of short-term drought stress on 'Garnem' plants, gs, LWP, RCW and EL were determine in leaves at 0h, 2h, and 24h of drought treatment and after 24h of re-watering. The lower values of LWP and gs in treated plants under drought conditions (Fig. 5.1A and B) confirmed that water scarcity causes drought stress response in 'Garnem'. Due to the lack of soil water content, LWP decreased during the drought treatment (Davies et al., 1994; Gollan et al., 1992). Under low LWP conditions, 'Garnem' reduced gs in order to decrease the loss of water via transpiration by ABA-induced stomatal closure, which led to a decrease in photosynthesis by reduction in stomatal CO_2 uptake (Negin and Moshelion, 2016; Verslues et al., 2006). RWC was not significantly lower in treated plant under drought exposure (Fig. 5.1C), indicating that the water content was not affected by drought conditions. 'Garnem' might response balancing water uptake and water loss as dehydration avoidance strategy, which allows it to maintain a high water content despite a decreased LWP (Verslues et al., 2006). This strategy can be carry on by solute accumulation as proline, sugars or betaines to prevent the water loss (Singh et al., 2015). Another avoidance mechanism is related to preserve the properties of the cell

walls. In our results, it was observed that EL decreased until being close to 0% after 24h of drought stress (Fig. 5.1D). Accumulation of proline, as well as the expression of ABA-related genes, including aquaporins, LEA proteins or anion transporters could be act protecting membrane structure of 'Garnem' cells against dehydration damages (Nishiyama et al., 2011; Shinozaki and Yamaguchi-Shinozaki, 2007; Verslues et al., 2006). When water supply was restored, both ABA accumulation and LWP reached similar levels in treated than control plants (Fig. 5.1B and 5.2), suggesting a fast water status recovering in 'Garnem'.

5.4.2. Effects of water stress on proteome profile

Proteomics provide a global study of protein changes at structural, functional and abundance level, as well as their interactions at a specific time point (Ghosh and Xu, 2014). The importance of proteomics approaches reside on the fact that proteins are the result of transcriptional activities, and the link between transcriptome and metabolome, being the main players in the most of cellular events (H. Liu et al., 2015; X. Wang et al., 2016).

In our study, the large number of differentially accumulated proteins identified in 'Garnem' roots as result of drought stress, suggested the importance of root system in the drought response. Roots, as first organ on sensing the effects of soil water scarcity, would trigger the metabolism alterations in order to cope the stress period. Thus, among the total of proteins altered by at least 1.5-fold under drought treatment, 15 spots were identified by MS as drought-related proteins. One of these proteins, SLFd protein, was spotted in two different locations with distinct p*I*, spots 620 and 857 (Fig. 5.3). This multiple identification may suggest different isoforms, or a possible post-translational modification of this protein in 'Garnem' (Alam et al., 2010; Katam et al., 2016).

The involvement of these drought-induced proteins in the different functions during the response of 'Garnem' to drought has been discussed.

Lipid metabolism

Cell membrane stability is a key target under drought stress. Lipids, as a main component of cell membranes, help to preserve cell membrane integrity as response to drought (Gigon et al., 2004). In our study, two proteins related to lipid metabolism were identified, the lipoxygenase (LOX) 9-LOX1 (spot 233) and a 1-acyl-sn-glycerol-3-phosphate acyltransferase (LPAT-spot 626) (Table 5.3). LOXs are enzymes involved in oxylipin biosynthesis via lipid degradation, having linoleic and linolenic acids as substrates (Lim et al., 2015; Sofo et al., 2004). As result, a loss of cell membrane integrity as produced (Mita et al., 2001). Most of the reports have been demonstrated that LOX activity was induced under drought conditions in several species as *Arabidopsis* (Ashoub et al., 2015), pepper (Lim et al., 2015), olive (Sofo et al., 2004). Ashoub et al., (2013) determined that in drought-tolerant barley genotype, LOX activity was not induced under drought. In 'Garnem' roots, LOX abundance was significantly higher in control than in treated plants at both 2h and 24h (Table 5.2). This lack of LOX activity under drought stress associated to the results in EL (Fig. 5.1D), might suggest that 'Garnem' cell membrane integrity would be preserved during drought stress conditions. The second lipid-metabolism-related protein identified in 'Garnem' was a LPAT. This enzyme is involved in phospholipid metabolism and in triacylglycerol (TAG) synthesis (Campalans et al., 2001; Chi et al., 2015; Kim et al., 2012). It have been reported that LPAT protein was up-regulated in root of both, peanut and *Brachypodium distachyon*, but down-regulated in leaf tissue under short-term salt and drought conditions (Chi et al., 2015; Kim et al., 2012). Campalans et al., (2001) studied the LPAT expression level in eight, not tolerant water-deficit-stress, almond cultivars after a drought exposure of 7 days. These authors described the induction of LPAT in response to drought, but this protein was also induced in leaves of control plants from two of the studied cultivars. In 'Garnem' roots, the abundance of LPAT protein was significantly lower in treated than in control plants (Table 5.2). This response combined with the results of previous reports above mentioned may suggest that LPAT induction would be dependent of the expression of other genes. Drought response is a complex network in which are involved many genes. Besides, the duration of the drought exposure may be other induction factor in LPTA expression. It

is noteworthy that LPAT abundance increased at 24h of drought exposure in our experiment (Supplementary Material S5.1; Annex 4).

Amino acid and nitrogen metabolism

During drought, an osmotic adjustment is performed by accumulation of solutes to prevent water loss including ions, nitrogen-containing compounds, polyamines and ammonium compounds (Reddy et al., 2004). In our analysis, the levels of two proteins were identified with significantly different abundance under drought stress at different time points of drought exposure: prunasin hydrolase (spot 407) and a putative nitrite reductase (spot 994) (Tables 5.2 and 5.3). Prunasin hydrolase belongs to β-glucosidase enzymes. β-glucosidases are involved in functions including glycoside metabolism, defense, cell wall lignification, cell wall β-glucan turnover and phytohormone activation (Cairns and Esen, 2010). This enzyme is involved in the cyanoamino acid metabolism, hydrolyzing prunasin to release (R)-Mandelonitrile and, after other reaction, hydrogen cyanide (HCN) is produced. Finally, HCN can be catalyzed in different secondary metabolites such as alanine, asparagine, and glutamate. These results might suggest that, as drought adaptive response, an accumulation of these amino acids would be performed in 'Garnem' roots at 2h of drought stress, playing an important role as osmoprotectants (H. Liu et al., 2015). Nitrogen assimilation is also affected by water stress. When nitrate is absorbed, it is transformed in ammonium by nitrate reductase (NR) and nitrite reductase (NIR), and then assimilated in amino acids (X. Wang et al., 2016). In our results, the protein identified as NIR (spot 994) abundance was significantly lower in treated than in control plants at 24h of drought treatment (Table 5.2). These results are in accordance with Ashoub et al., (2015), indicating that nitrogen assimilation was rapidly inhibited by drought in 'Garnem' roots.

Ion transport activity

The enzyme putative S-adenosyl-methionine 3-amino-3-carboxypropyl transferase (spot 1116), also called nicotianamine synthase (NAS), was significantly lower abundant in treated than in well-watered plants. But, after 24h of drought

exposure, this abundance reached a higher level than in control plants, but not significant (Table 5.2). This enzyme plays a key role in the synthesis of mugienic acid family phytosiderphores, which are chelators to solubilize iron for efficient uptake from roots (Ergen et al., 2009; Zhang and Zheng, 2008). It was demonstrated that NAS enhances drought tolerance in ryegrass after 14 days without watering (Zhang and Zheng, 2008). D.-K. Lee et al., (2017)confirmed that overexpression of *OsNAS1* and *OsNAS2,* two direct targets of *OsNAC6*, which is a drought-responsive TF that regulates root development and confers drought tolerance, after 5 days of drought stress. Based on those reports and our results, we could suggest that the induction of this enzyme might be produced after 24h of drought exposure in 'Garnem' roots, playing a role in drought tolerant mechanism at long-term stress.

Carbon metabolism

NAD-dependent malic enzyme (NAD-ME) is a regulatory enzyme for the malate metabolism in mitochondria (Artus and Edwards, 1985). This enzyme catalyzes malate to release pyruvate, CO_2 and NADH. Then pyruvate is converted to acetyl-CoA, and thus producing ATP and carbon skeletons through TCA cycle and the CO_2 is utilized for mitochondrial respiration (Tronconi et al., 2008). In our results, NAD-ME (spot 1091) was identified showing a significantly decrease in its abundance in treated plants at 24h of drought treatment (Table 5.2). It is known that malate is involved in stomatal conductance and osmotic potential via stomatal regulation under stress (Lee et al., 2008). Jia et al., (2016) observed that in drought-tolerant plant, *A. mongolicus*, malic acid content increased under drought conditions. It could be in accordance with our results. As result of a down-regulation of NAD-ME, the protein abundance was lower under drought stress, thus the content of malate might be increased in 'Garnem' playing an important role in stomatal regulation for drought adaptation.

Protein synthesis and modification

The levels of a putative dnak-type molecular chaperone, hsc70.1 (heat shock cognate-spot 1156) were significantly lower in treated plants, both 2h and 24h after drought treatment in 'Garnem' (Table 5.2). It is widely known that HSP (heat shock

proteins) are important proteins that function as chaperones, helping in protein refolding and stabilizing polypeptides and membranes under stress (Padmalatha et al., 2012), but also play key roles regulating several physiological responses (Clèment et al., 2011). Among these proteins, HSC70 chaperones are found. In previous investigations indicated that hsp70 can be expressed in cell of well-watered plants, playing an crucial role in the maintenance of normal cell functions (Storozhenko et al., 1996). Furthermore, Clement et al., (2011) demonstrated that overexpression of *hsc70.1* causes a negative effect in ABA-mediated stomata closure leading to a water loss in drought conditions. According to our results, the decrease of abundance of chaperone hsc70.1 might suggest a drought response in 'Garnem' roots, which would allow stomatal closure via ABA signaling, and thus leading to drought stress tolerance in this rootstock.

Transcriptional response

One of the signaling stages under drought stress implicates the work of TFs, which are involved in a most important role in response to water stress. They are responsible of recognize *cis*-elements, found in promoter region of effector genes, and activate their expression (Nakashima and Yamaguchi-Shinozaki, 2013). C-repeat binding factor (CBF)/dehydration-responsive element-binding factor (DREB) proteins belongs to CBF/DREB sub-family within the APETALA2/Ethylene responsive factor (AP2/ERF) superfamily of TFs (Nakano et al., 2006). CBFs/DREBs has been extensively studied in abiotic stress responses in different species as *Arabidopsis* (Xu et al., 2014), herbaceous crops (Tavakol et al., 2014; Wang et al., 2008) and woody plants including poplar (Chu et al., 2014), citrus (He et al., 2016), apple (Wisniewski et al., 2011), peach (Artlip et al., 2013) and almond (Barros et al., 2012). At 24h after drought conditions, two proteins with significant abundance (spots 902 and 1094) were identified as members of CBF/ DREB: CBF2 and CBF1 TFs, respectively (Table 5.3). The CBF2 abundance level was significantly lower in plants submitted to drought than in control plants, whereas CBF1 abundance was significantly higher in treated than in well-watered plants (Table 5.2). It has been confirmed that while CBF2 protein is only induced by exposure to both, cold and ABA application, but not by drought (Artlip et

al., 2013; Barros et al., 2012; Liu et al., 1998; Novillo et al., 2004), CBF1 is induced by cold, drought and ABA application (Artlip et al., 2013; Barros et al., 2012; Novillo et al., 2004). It is noteworthy that Novillo et al., (2004) also indicated that *CBF2* negatively regulates *CBF1* expression under low temperature. Our results were in agreement with these previous reports, suggesting the key role of the DREB TFs in the response of 'Garnem' roots to drought stress.

Defense response

The induction of defense-related proteins leads to trigger self-defense mechanisms as response to abiotic and biotic stresses (Xiao et al., 2009). In the present study, one pathogen-related protein was represented with a higher significant abundance in treated plants at 2h after drought stress: a putative allergen Pru du 1.06B protein (spot 1237) (Table 5.2). This protein belongs to the birch allergen Bet v 1 homologous within the pathogenesis-related protein 10 (PR 10) super-family (Breiteneder and Ebner, 2000). Proteomic changes, in the induction of allergen proteins, has been observed in peach and nectarine fruits under chilling and heat stress (Giraldo et al., 2012; Lara et al., 2009; Li et al., 2012; Nilo et al., 2010; Zhu et al., 2015), and also in apricot fruit ripening (D'Ambrosio et al., 2013). Allergen proteins are also induced in grape under drought stress (Grimplet et al., 2009). Our results may suggest that allergen proteins are stimulate not only in fruit tissues under abiotic stress, also in roots, playing a role in root protection against drought stress, helping to improve 'Garnem' acclimation to drought.

Other functional proteins and one uncharacterized protein

Two spots were identified as a SLFd protein: the spot 857, which showed a lower significant abundance in treated plants at 2h. In contrast, after 24h of no water supply, its abundance was increased, but not significantly; and the spot 620, which abundance was significant higher in treated plants at 24h after drought exposure (Table 5.2). SLFd proteins are a subunit of SCF (Skp1-RBx1-Cull-F-box protein) E3 ubiquitin ligases, involved in plant growth and organ development, photomorphogenesis, circadian clock, flowering time, and phytohormone regulators

(Song et al., 2015). Its implication in abiotic stresses has been demonstrated. Song et al., (2015) identified 972 putative F-box proteins with diverse responses to salt, heavy metals, drought and ABA application. Moreover, Zhang et al., (2008) reported that a F-box protein DOR is involved in ABA-induced stomatal closure under drought conditions. In line with these investigations, SLFd protein might play a role in the response to drought stress in roots, regulating the ABA signaling pathway after 24h of drought exposure in 'Garnem' roots.

Under drought conditions, cell cycle and DNA replication, RNA processing and RNA editing mechanisms are also affected. In 'Garnem' roots a Cdc2a kinase protein (spot 1021) was found with lower significant abundance in stressed plants after 24h of stress (Table 5.2). This kinase is an important regulator of the cell cycle and DNA replication. Its low abundance during drought period may suggest that stress condition can inhibit the cell cycle process, decreasing cell division and differentiation (Setter and Flannigan, 2001; Zhu, 2002). Our results was in accordance to previous reports in cotton and *Arabidopsis* under drought stress conditions (Padmalatha et al., 2012; Su et al., 2013). The spot 483, which showed a lower abundance in treated plants after 24h drought exposure, was classified as a matk enzyme (Tables 5.2 and 5.3). This enzyme is involved in RNA processes modulating mRNA splicing (Xu et al., 2016). In herbaceous species, this enzyme was induced by drought in safflower (Thippeswamy et al., 2013), by salt stress in wheat (Xu et al., 2016) and by freezing in *Saussurea laniceps* (Huang et al., 2016). However, in a dry-region poplar population, the expression of *matk* was down-regulated under drought conditions. These evidences, together with our results, may indicate that post-transcriptional regulation was affected by abiotic stresses, showing a diverse and complex response depending on the plant species.

Among the identified proteins, one spot was classified as a putative uncharacterized protein, spot 1137 (Table 5.3). These are proteins whose functional role is still unknown, but by homology criteria their function can be predicted. The uncharacterized protein is homologous to M5XCS7 protein of *P. persica*, which is encoded by *ppa003088m* gene. This gene is classified as a pentatricopeptide repeat (PPR)-containing protein family according to Phytozome v12.0

(https://phytozome.jgi.doe.gov/pz/portal.html). PPR proteins are implicated in gene expression regulation including RNA cleavage, splicing and translation at post-transcriptional level (Rahman et al., 2015). In 'Garnem' this protein was significantly induced in stressed plants after 2h of drought stress exposure (Table 5.2), which might suggest that gene expression regulation processes could be maintained in order to response to drought allowing the induction of drought-responsive genes and, thus the 'Garnem' drought acclimation.

In conclusion, alterations at physiological, biochemical level, as well as at proteomic level were determined in the roots of 'Garnem' hybrid rootstock, revealing a response to short-term drought stress conditions. The physiological and biochemical parameters indicated that 'Garnem' triggered it machinery in order to cope drought stress. ABA accumulation levels increased dramatically during the drought exposure inducing the stomata closure, and thus minimizing water losses. As a consequence, gs decreased, as well as LWP. But, surprisingly, RWC was maintained along the drought treatment. 'Garnem' was able to balance its water content, following a dehydration avoidance strategy. The lack of cell membrane injury determined in 'Garnem' under our drought stress conditions would suggest an osmotic adjustment in 'Garnem' cell membranes, confirming that avoidance strategy above-mentioned. Proteomic analysis revealed significant changes in 29 and 24 spots after 2h and 24h of drought stress, respectively. The proteins identified in our study participate in a variety of biological functions including lipid, amino acid and nitrogen metabolism, transcriptional and defense response, protein synthesis and modification, ion transport activity, and control of gene expression regulation. Their abundance changes, influence in drought-responsive mechanisms present in 'Garnem', which would allow enhancing drought tolerance and, thus being able to adapting to drought conditions. Overall, our study may contribute to improve the existing knowledge on the root proteomic changes in response to drought, leading to understand dehydration avoidance and tolerance strategies, and finally, help us to suggest new drought-tolerance breeding approaches.

6. RESPONSE OF PEACH CULTIVARS THROUGHOUT DROUGHT AND RE-WATERING PERIOD IN AN WARM AND ARID REGION

ABSTRACT

Two peach cultivars with different chilling hour, 'Ufo-3' and 'Fergold', grafted onto the interspecific hybrid almond x peach rootstock 'Garnem', were evaluated for drought response in an experimental plot located at IMIDA facilities in El Jimenado, Murcia (Spain) during summers 2015 and 2016. After fruit harvest, the stressed trees were subjected to a no-water period of 5 days, followed by a re-watering period of 5 days with the same irrigation regimen that the well-watered trees. During the experiment tree water status and gas exchange was evaluated as well as the abscisic acid (ABA) accumulation in leaves. Drought conditions originated a drop of the leaf water potential (LWP) as soil water content (SWC) decreased in both peach cultivars. As consequence, stomatal closure was induced, causing a decline in all gas exchange rates. After the re-watering period, the stressed trees recovered their plant water status, restoring their homeostasis functions. High correlation between gas exchange rates was found. Besides, a negative strong correlation between LWP and intrinsic water use efficiency (WUE) was observed only in 2015. This might indicate a drought tolerant strategy that allowed maintaining the photosynthetic capacity in spite of low stomatal conductance (gs) and LWP values under drought conditions. ABA levels during stress period did not change in relation with the ABA basal levels, thus may indicate that both cultivars were not experienced a severe drought stress. Under Murcia edaphoclimatic conditions, trees presented a physiological response after 5 days without irrigation, but probably for a biochemical response, it could be necessary a stronger drought stress period. This agronomic evaluation will allow understand the influence of 'Garnem' rootstock on the physiological and biochemical adaptation of the grafted cultivars to drought stress on arid field conditions.

Keywords: Gas exchange, Leaf water potential, *Prunus* rootstock, Water stress, Water use efficiency

6.1. INTRODUCTION

Prunus L. is a diverse genus that grows mainly in the temperate zone and some in the tropical and subtropical regions. This genus is economically important due to its diverse uses as fruit, oil, timber, and ornamentals (Lee and Wen, 2001). Spain is a big producers for peach and almond, behind China for peach, and behind the US and Australia for almond production (FAOSTAT, 2017). In Spain, the production of peach and nectarine are focused in Ebro Valley and Murcia; while almond is produced mainly in regions of Andalusia, Ebro Valley and Mediterranean regions including Community of Valencia and Murcia (MAPAMA, 2017). These semi-arid Spanish regions stand out by its characteristic climatology with long dry periods with high irradiance and temperature levels (Barradas et al., 2005), favorable for the development of different environmental stresses such as drought.

Under drought conditions, plants trigger strategies of escape, avoidance and dehydration tolerance (Varela, 2010). When plant water status is declined by the lack of soil water, different mechanisms have been triggered. These mechanisms are identified as drought adapted and tolerance responses that are associated with regulation of physiological and biochemical processes such as stomatal regulation, osmotic adjustment, cell membrane stability and regulations on plant growth (Belin et al., 2010; Lind et al., 2015; Verslues et al., 2006). Abscisic acid (ABA) is one of the crucial hormones involved in stomatal regulation under water deficit (Kim et al., 2010). When ABA accumulation is produced, the expression of stress-related genes are induced (Lata and Prasad, 2011; Shinozaki and Yamaguchi-Shinozaki, 2007). As a physiological consequence of ABA accumulation, water loss is reduced by leaf stomata closure. Partial or complete stomatal closure maintain a favorable water balance while limiting the carbon gain (Kim et al., 2010). Stomatal conductance (gs) is decreased as result of the leaf water potential (LWP) decrease (Romero and Botía, 2006). As consequence, photosynthesis capability is affected by the decline of CO_2 availability due to diffusion restrictions through the stomata and the mesophyll (Chaves et al., 2009), as well as transpiration mechanism is impaired. One of the most crucial factor

associated with plant drought adaptation is water use efficiency (WUE) (Blum, 2009). Tolerant plants increase their WUE under water stress as result of the non-linear relationship between CO_2 assimilation in photosynthesis and loss of water transpired (Bassett et al., 2014; Condon et al., 2004; Tomás et al., 2012) and it is related to the productivity of plants under drought conditions (Melisova et al., 2015).

Root system is the responsible for water and nutrient uptake. The influence of rootstock on changes in stomata size and regulation, transpiration and grafted tree water potential must be consider in order to select drought adapted rootstocks which confer tolerance to the grafted cultivar (Hajagos and Végvári, 2013). This influence has been evaluated under different water deficits in previous reports in woody plants such as pistachio (Gijón et al., 2010), peach (García Brunton et al., 2004; Jiménez et al., 2013; Martinazzo et al., 2011; Rickes et al., 2017), cherry (Hajagos and Végvári, 2013), and almond (Isaakidis et al., 2004). Several species of *Prunus* such as *P. amygdalus* Batsch, *P. persica* (L.) Batsch, *P. cerasifera* Ehrh., *P. davidiana* (Carr.) Franch, *P. mira* Koehne kov et. kpst, *P. domestica* L. and *P. insititia* L. are utilized as rootstocks. Currently, the challenge in rootstock breeding programs is the combination of abiotic tolerances in a new generation of interspecific hybrids resulting from the cross of almond × peach hybrids by plum genotypes (Bielsa et al., 2014; Byrne et al., 2012; Felipe, 2009; Lecouls et al., 2004). Peach × almond hybrids such as 'Garnem', 'Felinem' and 'Monegro' (which come from the cross 'Garfi' almond × 'Nemared' peach) show good vigor, nematode resistance, and adaptation to calcareous soils (Felipe, 2009).

In last years, generation of high-throughput *-omics* technologies have facilitated the identification of new candidate genes and have allowed a better understanding of the molecular drought response mechanisms. This molecular information is supported by a physiological drought response, which is usually obtained under specific and controlled experimental conditions. It is known that trees under field conditions have a different behavior. Thus, Arndt et al., (2000) found a degree of drought tolerance in peach trees under drought on field conditions, in contrast to the lack of osmotic adjustment found in potted peach plants on greenhouse conditions. Understanding the physiological response on field is a crucial

issue to select drought tolerant lines in breeding. Under this frame, previous reports have been focused on the study of the effect of water starvation on plant water status and gas exchange rates in different fruit woody species including pistachio (Gijón et al., 2010; Memmi et al., 2016), olive (Boussadia et al., 2008), grapevine (Rodriguez-Dominguez et al., 2016; Tombesi et al., 2015), almond (Espadafor et al., 2017; Yadollahi et al., 2011) and different *Prunus* rootstocks (García Brunton et al., 2004; Jiménez et al., 2013; Martinazzo et al., 2011; Rickes et al., 2017).

In this context, the aim of our study was to determine the physiological and biochemical response under field conditions, as well as the influence of the rootstock under the scion subjected to drought stress for 5 days followed by 5 days of re-watering. In particular, we evaluated the regulation of gas exchange and plant water status, as well as the ABA accumulation in two peach cultivars with different chill requirement. In particular, we studied the flat peach, 'Ufo-3', and the yellow canning peach, 'Fergold'; grafted on the interspecific hybrid almond × peach rootstock, 'Garnem'. The experiment was carried out after the fruit harvest due to that phase is crucial for upholding the uptake nutrients, as well as the photoassimilates production that will be used in the next budding and flowering period (Timm, 2007).

6.2. MATERIALS AND METHODS

6.2.1. Plant material and field trial design

For drought response evaluation, two peach cultivars with different chill requirement 'Ufo-3' [*Prunus persica* (L.) Batsch var. *platycarpa*] (500 c.u.) and 'Fergold' (750 c.u.), grafted onto the interspecific hybrid almond x peach rootstock [*P. amygdalus* Batsch, syn *P. dulcis* (Mill.) x *P. persica* (L.) Batsch] 'Garnem' were considered. The cultivars were grafted during the summer of 2013. In 2014, trees were established in a randomized complete-block design with 10 replicates and 2 guards for each cultivar and treatment block. The tree spacing was 5 × 2.5 m. The experimental plot was located at IMIDA facilities in El Jimenado, (Torre Pacheco) Murcia (37° 45' 33.5'' N, 1° 01' 33.5'' W). The installation of the drip irrigation system included an automated

system for the application of water in each treatment, including two irrigation lines per row of stress and four drippers for each trees (discharge rate 2 l h^{-1}).

The drought experiment was carried out on July 2015 and September 2016. Then, after fruit harvest, two different irrigation treatments were applied: control and stressed trees. Control trees were watered until field capacity to preserve optimal soil water content (SWC) along the experiment, whilst the stressed trees underwent a no water supply period of 5 days. Once the stress period was completed, trees were re-watered with the same irrigation regimen of the control trees. The average climatic conditions during the two experimental periods were the following: average temperature of 26.65 °C; relative humidity of 64.3%; solar radiation of 28.66 MJ m^{-2} day^{-1}; no rainfall; and ETo of 6.12 mm day^{-1} for 2015 period; and temperature of 21.89 °C; relative humidity of 63.33%; solar radiation of 19.25 MJ m^{-2} day^{-1}; no rainfall; and ETo of 3.70 mm day^{-1} for 2016 period. Soil moisture was measured using a portable capacitance sensor system (Diniver, 2000, Sentek Pty. Ltd., Australia). One access tube was placed in the middle furrow for each water treatment block. Measurements were taken at 1-m intervals with maximal soil depth of 100 m at 0 and 5 days after starting the drought experiment and 5 days after re-watering period.

6.2.2. Tree water status

Leaf water potential (LWP) was measured in triplicated in leaves using a Scholander-type pressure chamber (Soil Moisture Equipment Corp. Santa Barbara, CA, USA) (Scholander et al., 1964), between 12:00 and 13:00 h at 0 and 5 days after starting the drought experiment and 5 days after re-watering period. Relative Water Content (RWC) was measured in duplicate as previously published methods (Barrs and Weatherley, 1962). Leaf samples for RWC calculation were picked up at 09:00 h. Briefly, three 1 cm diameter leaf discs were weighed (W) and rehydrated to their turgid weight (TW) by floating them *in petri* plates containing deionized water for 4h at room temperature. The dry weight (DW) was obtained after 24h at 80 °C in an oven. RWC was calculated following the equation:

$$\% \text{ RWC} = \frac{\text{W} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

6.2.3. Photosynthetic parameter measurement

Gas exchanges rates including: stomatal conductance (gs), net photosynthesis or net CO_2 assimilation (An), transpiration rate (E) and intracellular CO_2 concentration (Ci) were measured in duplicated using a portable photosynthesis system (Model Li-6400XT, Li-Cor Biosciences, Lincoln, NE) on leaves similar to those used for LWP and RCW measurements. WUE index was calculated as a relation of An / gs. Data collection was performed between 12:00 and 13:00 h at 0 and 5 days after starting the drought experiment and 5 days after re-watering period.

6.2.4. Abscisic acid content determination

ABA extraction

Leaves were sampled in duplicated for each water treatment block at 0 and 5 days of drought exposure and 5 days after re-watering. Then, lyophilized leaf samples were homogeneously grinded in a 6875 Freezer/Mill® High Capacity Cryogenic Grinder (SPEX®SamplePrep, INC., UK) with liquid nitrogen. A volume of 3 ml of a buffer containing acetone:water:formic acid (80:19:1, v/v/v) were added to 0.05 g of powdered leaf sample, shaken to 2,000 rpm for 30 min in a test tube shaker (Multi Reax Shaker, Heidolph Instruments, Schwabach, Germany), and after centrifugation for 10 min at 4 °C, the supernatant was recover in a new tube. This step was repeated twice. The acetone was evaporated from samples with a nitrogen stream using a Sample Concentrator (SBHCONC/1 model; Stuart, Fisher Scientific Bioblock, Illkirch, France) until a volume less than 1 ml approximately, and then the samples were adjusted to 1 ml with milliQ water (Millipore). The extract was filtered [0.45-µm, 13 mm Nylon filter (Sartorius)] and 10ng μl^{-1} [$^2H_6$]-ABA was added as internal standard, which was prepared according to Gómez-Cadenas et al., (2002), before the UPLC system injection.

ABA determination by mass spectrometry

ABA determination was performed in a UPLC-TQD (ACQUITY, Waters). An Excel 2 C18-AR column (50 × 2.1 mm, ACE, UK) was used stabilized to 40 °C. The mobile

phase was constituted by 70% methanol (solvent A) and 90% acetonitrile (AN) (solvent B) which contain 0.1% formic acid. The gradient was programmed to change linearly: 0-1min, 100% A; 1-2.5 min, 100-50% A; 2.5-2.8 min, 50% A; and 2.8-3 min, 100% A, with 2 min of equilibration before the following injection. The solvent flow level and the volume injection were adjusted to 0.15 ml min^{-1} and 20 µl, respectively.

For ABA identification and quantification, mass spectrometry was performed by ACQUITY-TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The electrospray conditions were the following: Polarity ES, capillary voltage 3.0 kV, source temperature 120 °C, desolvation gas temperature 350 °C. High-purity nitrogen was used as auxiliary gas and the nebulizer, and argon was used as the collision gas. Cone gas flow was set on 90 l h^{-1}, and desolvation gas flow was set on 900 l h^{-1}.

ABA analysis was carried out in MRM (Multiple Reaction Monitoring) mode, monitoring the transactions for [$^2H_6$]ABA and ABA at *m/z* 269→159, 225; and 263→ 153, 219, respectively.

The cone voltage (V) and collision energies (eV) were optimized to obtain the maximum signal, resulting: 20 V and 12 eV for [$^2H_6$]ABA, and 15 V, 10 eV, and 12 eV for ABA. The raw data were collected and processed with a MassLynx 4.1 software. Quantification was performed using calibration curves based on the ABA/[$^2H_6$]ABA ratio of standard solutions.

Calibration curves preparation

Solutions of 1, 4, 10, 25, 50 and 120 ng ml^{-1} in 30% AN with 0.1% formic acid were prepared from an initial solution of 1,000 µg ml^{-1} ABA in AN. Aliquots for each point of 450 µl were passed into 2 mL vials with 50 µl of 10 ng $µl^{-1}$ [$^2H_6$]ABA before the UPLC system injection.

6.2.5. Statistical analysis

Statistical analyses were performed with SPSS 21 software package (IBM SPSS Statistics, USA). Before carrying out any statistical analysis, the normality of all the data

was studied using the Kolmogorov-Smirnov test. Data following a Normal distribution were subjected to ANOVA to test for significant differences between treatments. The significant difference was assessed with Duncan's test ($p \leq 0.05$). In case the hypothesis of normality was discarded at the 95 % confidence level, the data were subjected to non-parametric data Kruskal-Wallis' test ($p < 0.05$). The experiment consisted of four independent factors (i) cultivar, (ii) treatment, (iii) day, and (iv) year. Besides, the statistical differences between genotypes and between treatments for each day were determined by the Student's *t*-test ($p \leq 0.05$). The statistical analyses were carried out for LWP, gs, An, E, Ci, RWC, SWC and ABA content. Pearson's correlations for parametric data and Spearman's Rho test for non-parametric data ($p < 0.01$), and regression analysis were used to determine the association between the gas exchange parameters with themselves and also with LWP.

6.3. RESULTS

Environmental conditions were typical for Mediterranean climate in the region of Murcia for the days in which drought experiment was performed both 2015 and 2016. Even thought, because of the experiments were carried out in two different months each year (on July in 2015 and on September in 2016), these weather parameters changed between years, mainly average temperatures, solar radiation and ETo values. Those variations were reflected in the statistical analysis. Physiological parameters presented significant variations between 2015 and 2016, except for SWC (Table 6.1). Nevertheless, the results in the interactions showed that there was no interaction by the year in the other three factors: cultivar, treatment and day (Table 6.1). In our experiment the only significant interactions observed were by cultivar, treatment and day. The differences observed between years did not have significant influence in the response to drought stress in any peach cultivar grafted onto 'Garnem' rootstock.

Table 6.1. Significances after analysis of variance for leaf water potential (LWP), stomatal conductance (gs), net photosynthesis (An), transpiration (E), intracellular CO_2 concentration (Ci), soil water content (SWC) and relative water content (RWC) measured during drought experiment. One-way ANOVA was performed for data with a Normal Distribution. The significant difference was assessed with Duncan's test ($p \leq$

0.05). Data without a Normal Distribution were subjected to non-parametric data Kruskal-Wallis' test ($p < 0.05$). Significance: * $p \leq 0.05$; ns indicates not significant; — indicates no analyzed.

Significances ($p \leq 0.05$)

	LWP	gs	An	E	Ci	SWC	RWC
Cultivar (c)	ns	*	*	*	ns	ns	ns
Treatment (t)	*	*	*	*	*	ns	ns
Day (d)	*	*	*	ns	*	ns	*
Year (y)	*	*	*	*	*	ns	—
t x d	*	*	*	*	*	ns	ns
c x d	ns	*	ns	*	*	ns	ns
d x y	ns	ns	ns	ns	ns	ns	—
c x t	ns	ns	ns	ns	ns	ns	ns
t x y	ns	ns	ns	ns	ns	ns	—
c x y	ns	ns	ns	ns	ns	ns	—
c x t x d	ns	*	*	*	*	ns	ns
c x d x y	ns	ns	ns	ns	ns	ns	—
c x t x y	ns	ns	ns	ns	ns	ns	—
d x t x y	ns	ns	ns	ns	ns	ns	—
cx t x d x y	ns	ns	ns	ns	ns	ns	—

6.3.1. Effects of drought stress in water status

When drought stress period was started, the mean of SWC accumulated in the first 30 cm of depth, decreased in 26% and 27% during the drought period in 2015 and 2016, respectively for 'Ufo-3' stressed cultivar (Fig. 6.1A and B). In case of 'Fergold' stressed tress, this decrease was slightly higher for the same stress period, within 28% and 30% of SWC accumulation less in 2015 and 2016, respectively (Fig. 6.1A and B). After 5 days of re-watering period, SWC both 2015 and 2016 reached similar values than well-watered tress. However, no significant differences were showed between treatments, cultivars and days, and in the interaction among them (Tables 6.1 and 6.2).

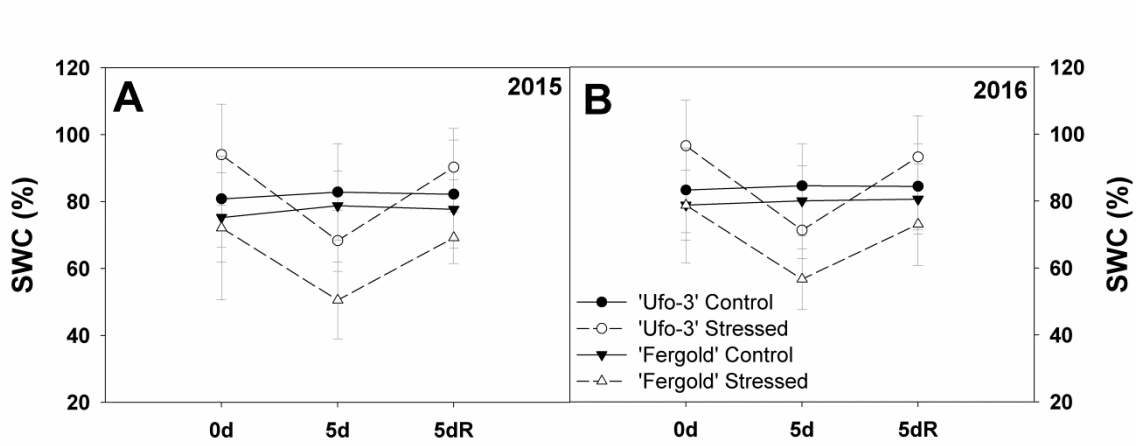

Figure 6.1. Evolution of soil water content (SWC) accumulated in the first 30 cm of depth (A) in 2015 and (B) in 2016 throughout drought experiment. Error bars represent the standard error of the mean. (d = days, R = recovery).

We found significant differences in LWP between treatments and days, and in days x treatment interaction, but not significance differences were observed between cultivars (Table 6.1). LWP values of well-watered trees ranged between -2.46 MPa and -2.35 MPa in 2015 and between -2.30 MPa and -2.14 MPa in 2016 (Table 6.2). Not significant differences were found neither between cultivars nor among days in these well-watered trees (Table 6.2 and Fig. 6.2A and B). Drought stressed trees exhibited a significant decline in LWP values after 5 days of drought exposure as result of the SWC reduction in both cultivars and years (Table 6.2). Stressed LWP values dropped to -3 MPa, approximately, showing significant differences with respect to well-watered LWP values (Table 6.2 and Fig. 6.2A and B). When stressed trees were re-watered, their LWP values were similar to control trees, suggesting that these trees recovered their water status after 5 days of irrigation (Table 6.2 and Fig. 6.2A and B).

Table 6.2. ANOVA results from leaf water potential (LWP), stomatal conductance (gs), net photosynthesis rate (An), transpiration rate (E), intracellular CO_2 concentration (Ci), soil water content (SWC) during the drought treatment for years 2015 and 2016; and relative water content (RWC) only for 2015. Same letter values indicate a no significant difference ($p \leq 0.05$) following Duncan's post hoc test for each genotype ('UFO 3' and 'Fergold'), and treatment (control and treatment) among day. (d = day, R = Re-watering).

		Year 2015			Year 2016		
		Leaf Water Potential (LWP) (MPa)			**Leaf Water Potential (LWP) (MPa)**		
		0d	**5d**	**5dR**	**0d**	**5d**	**5dR**
Control	**UFO 3'**	-2,352±0,055 a	-2,462±0,056 a	-2,442±0,057 a	-2,180±0,050 a	-2,202±0,048 a	-2,302±0,059 a
	Fergold'	-2,359±0,054 a	-2,449±0,033 a	-2,383±0,071 a	-2,191±0,042 a	-2,176±0,044 a	-2,141±0,047 a
Treatment	**UFO 3'**	-2,362±0,036 b	-2,951±0,060 a	-2,424±0,0,53 b	-2,268±0,053 b	-2,828±0,040 a	-2,203±0,052 b
	Fergold'	-2,417±0,030 b	-2,964±0,057 a	-2,466±0,056 b	-2,199±0,054 b	-2,863±0,030 a	-2,270±0,053 b
		Stomatal Conductance (gs) (mmol m^{-2} s^{-1})			**Stomatal Conductance (gs) (mmol m^{-2} s^{-1})**		
		0d	**5d**	**5dR**	**0d**	**5d**	**5dR**
Control	**UFO 3'**	418,854±47,968 b	262,510±21,788 a	265,189±18,774 a	479,537±27,090 b	261,861±18,736 a	289,203±9,182 a
	Fergold'	261,376±52,966 a	260,607±16,692 a	291,956±17,965 a	350,994±25,970 b	231,377±14,847 a	303,176±4,170 b
Treatment	**UFO 3'**	383,136±18,617 c	139,271±7,897 a	284,764±28,622 b	397,522±13,777 c	201,911±5,591 a	306,637±12,858 b
	Fergold'	324,261±51,416 b	140,017±18,671 a	186,933±35,616 a	345,983±43,702 b	188,618±17,972 a	276,422±11,231 b
		Net Photosynthesis (An) (µmol m^{-2} s^{-1})			**Net Photosynthesis (An) (µmol m^{-2} s^{-1})**		
		0d	**5d**	**5dR**	**0d**	**5d**	**5dR**
Control	**UFO 3'**	18,751±1,189 a	19,599±0,937 a	19,991±1,006 a	20,640±0,352 b	19,025±0,414 a	21,663±0,779 b
	Fergold'	15,172±1,789 a	19,192±1,041 ab	21,223±0,494 b	19,835±0,314 a	18,425±0,689 a	21,983±0,565 b
Treatment	**UFO 3'**	20,062±0,677 b	13,527±0,622 a	21,807±1,371 b	20,824±0,202 b	15,027±0,247 a	21,882±0,643 b
	Fergold'	18,022±1,181 a	13,602±1,256 a	15,935±2,146 a	19,604±0,583 b	16,402±0,557 a	20,776±0,775 b
		Transpiration (E) (mmol m^{-2} s^{-1})			**Transpiration (E) (mmol m^{-2} s^{-1})**		
		0d	**5d**	**5dR**	**0d**	**5d**	**5dR**
Control	**UFO 3'**	7.175±0.426 ab	8.195±0.528 b	6.474±0.369 a	7.999±0.394 a	8.124±0.414 a	7.223±0.253 a
	Fergold'	4.657±1.053 a	8.375±0.324 b	7.057±0.216 b	6.311±0.697 a	8.335±0.278 b	8.033±0.330 b
Treatment	**UFO 3'**	6.850±0.520 b	5.352±0.218 a	7.033±0.544 b	7.582±0.431 b	5.457±0.325 a	7.859±0.219 b
	Fergold'	5.193±0.760 a	5.395±0.584 a	4.880±0.748 a	5.596±0.708 a	5.961±0.438 a	6.313±0.259 a
		Intracellular CO_2 Concentration (Ci) (µmol m^{-2} s^{-1})			**Intracellular CO_2 Concentration (Ci) (µmol m^{-2} s^{-1})**		
		0d	**5d**	**5dR**	**0d**	**5d**	**5dR**
Control	**UFO 3'**	268.316±3.103 b	215.818±5.070 a	219.291±3.413 a	284.407±8.934 b	213.461±4.007 a	222.588±1.384 a
	Fergold'	241.944±12.589 a	219.340±3.575 a	221.280±4.277 a	299.212±11.465 c	215.700±2.252 a	226.792±2.710 b
Treatment	**UFO 3'**	257.888±2.660 c	190.450±5.376 a	212.520±3.179 b	268.788±7.083 c	193.176±7.178 a	220.724±1.197 b
	Fergold'	249.647±11.934 b	176.500±10.626 a	194.975±11.371 a	273.404±11.234 c	175.383±12.707 a	218.963±1.884 b
		Soil Water Content (SWC) (%)			**Soil Water Content (SWC) (%)**		
		0d	**5d**	**5dR**	**0d**	**5d**	**5dR**
Control	**UFO 3'**	80.830±14.536 a	82.820±14.476 a	82.177±16.200 a	83.280±12.717 a	84.534±12.447 a	84.407±12.811 a
	Fergold'	75.210±13.409 a	78.780±10.244 a	77.663±8.929 a	78.812±17.204 a	80.069±9.112 a	80.559±12.272 a
Treatment	**UFO 3'**	93.967±15.129 a	68.293±9.144 a	90.257±11.563 a	96.467±10.461 a	71.267±10.394 a	93.165±10.459 a
	Fergold'	72.123±21.520 a	50.523±11.481 a	69.200±7.648 a	78.724±17.204 a	56.645±9.112 a	73.055±12.272 a
		Realtive Water Content (RWC) (%)					
		0d	**5d**	**5dR**			
Control	**UFO 3'**	64.504±1.281 a	66.943±2.114 a	91.202±1.819 b			
	Fergold'	61.415±1.785 a	68.070±0.880 b	92.121±3.709 c			
Treatment	**UFO 3'**	61.638±1.495 a	66.835±1.495 a	92.883±1.460 b			
	Fergold'	65.237±1.500 a	71.015±1.760 b	96.685±1.706 c			

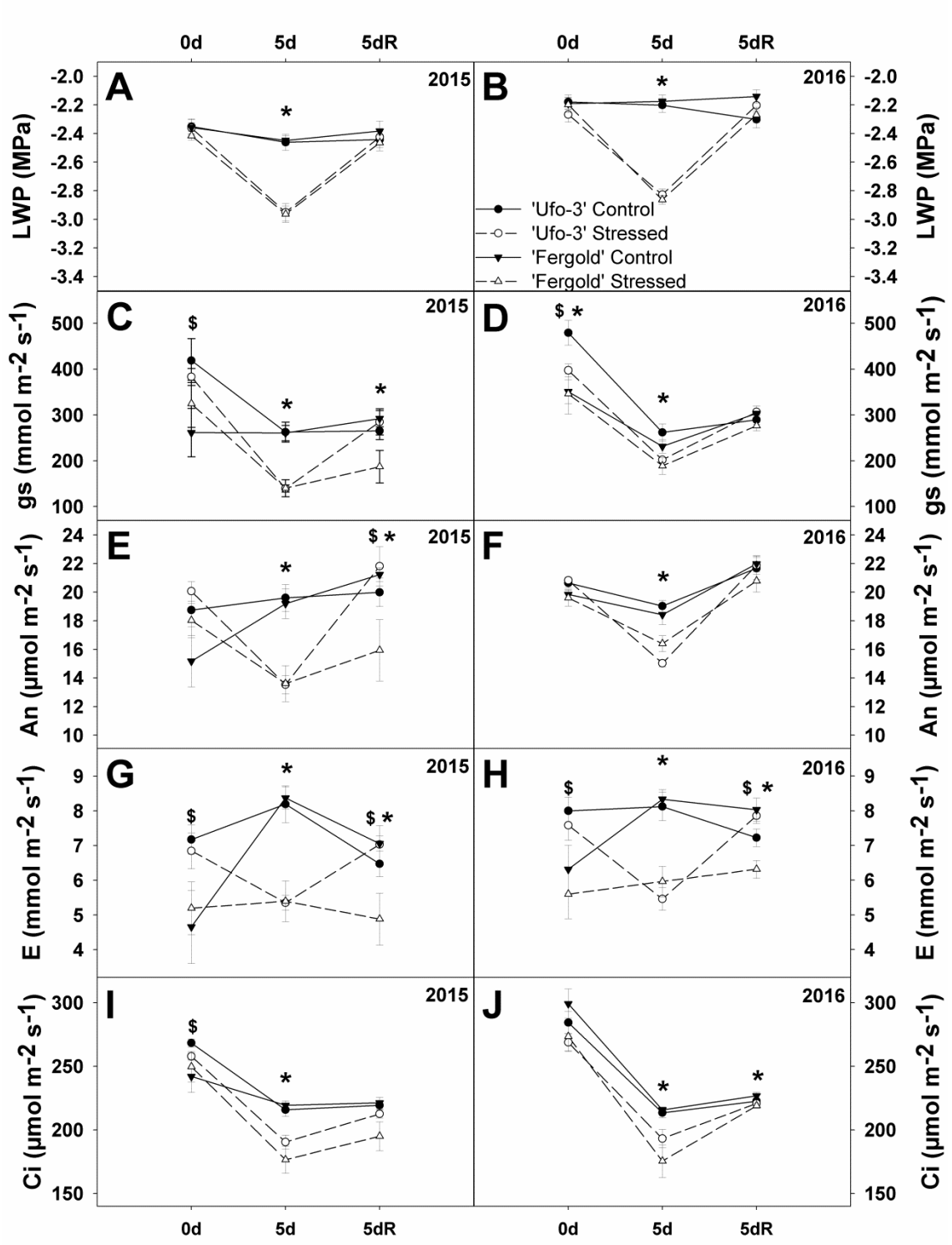

Figure 6.2. Evolution of (A and B) leaf water potential (LWP), (C and D) stomatal conductance (gs), (E and F) net photosynthesis (An), (G and H) transpiration (E) and (I and J) intracellular CO_2 concentration (Ci) during the drought experiment in the years 2015 and 2016. Continuous lines indicate well-watered trees, while dash lines indicate stressed trees. Error bars represent the standard error of the mean. Asterisks represent significant differences ($p \leq 0.05$) between treatments (control and stressed) for each time point of the experiment; $ symbols indicate differences ($p \leq 0.05$) between cultivars ('Ufo-3' and 'Fergold') for each time point of the experiment. (d = days, R = recovery).

RWC was only estimated in the first year. RWC in leaves increased during the drought exposure, as well as after 5 days of re-watering, when trees reached the maximum rates with a range from 91.20 % for well-watered 'Ufo-3'cultivar to 96.69% stressed 'Fergold' cultivar (Table 6.2). We only found significant differences among days, but not between treatments and cultivars, and their interactions (Tables 6.1 and 6.2). RWC rate of 'Ufo-3'-stressed trees only showed significant differences after 5 days of re-watered period. However, 'Fergold' cultivar showed significantly different RWC rates among the three time points (Table 6.2).

6.3.2. Effects of drought stress in gas exchange

Significant differences were found between treatments, days and in their interactions, as well as in the interaction among these two factors and the cultivars in gs, An, E and Ci values during drought experiment. Also, we found significant differences between the cultivars in gs, An and E, except in Ci values. Similarly, between days in gs, An and Ci, but not in E values. Finally, there was a significant interaction between the cultivars and the days in gs, E and Ci values, but not in An values (Table 6.1). After 5 days of drought exposure, stressed trees from both cultivars exhibited a significant decline in gs, An, E and Ci values with respect to well-watered tress in both years, without showing significant differences between 'Ufo-3' and 'Fergold' cultivars (Fig. 6.2C-J). This decrease was also significantly different with respect to day 0 in gs, An, E and Ci in 'Ufo-3'-stressed trees in both years. On the contrary, 'Fergold'-stressed trees showed significantly differences with respect to day 0 in gs and Ci in both years, and only in An in 2016 (Table 6.2). When stressed trees recovered their water status, gs, An, E and Ci values increased, but not reaching similar values as control tress (Table 6.2 and Fig. 6.2C-J). It was noteworthy that significant differences between cultivars were found in gs and E in both years, and also in Ci only in 2015. These significant differences were continued once irrigation was re-stored only in E in both years. In addition, An rate showed also significant differences in 2015 (Fig. 6.2C, D, E, G, H and I).

When the evolution of the studied parameters of well-watered trees were analyzed among the days, gs, An, E and Ci values were not maintained statistically

similar in well-watered trees of both 'Ufo-3' and 'Fergold' cultivars in both years, except for gs and An values in 'Fergold' and 'Ufo-3', respectively in 2015, and E values in 'Ufo-3' in 2016 (Table 6.2). Pearson's correlation coefficients showed good correlations between the gas exchange parameters. A high positive correlation between An and gs (r = 0.83 in both years, $p < 0.01$), between An and E (r = 0.79 in 2015 and r = 0.77 in 2016, $p < 0.01$), and between gs and Ci (r = 0.94 in 2015 and r = 0.91 in 2016, $p < 0.01$) were found in our study (Fig. 6.3B-C).

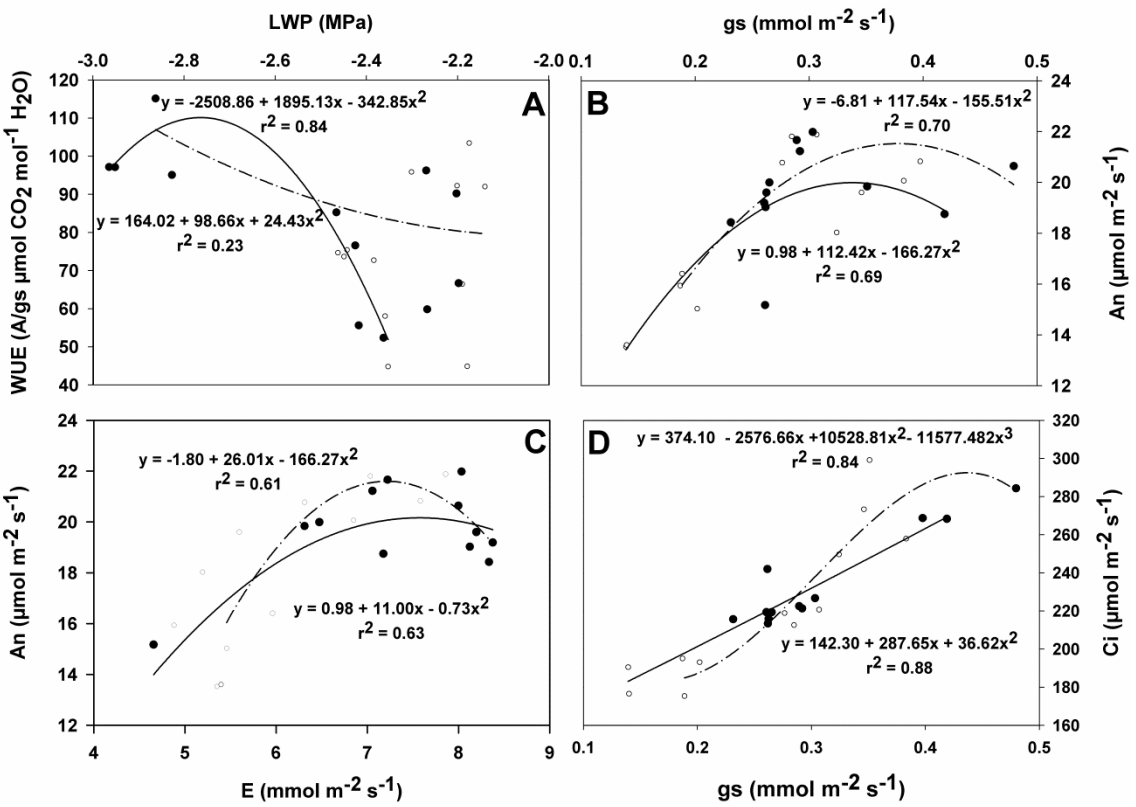

Figure 6.3. Relationship between (A) intrinsic water use efficiency (WUE) and leaf water potential (LWP); (B) Relationship between net photosynthesis (An) and stomatal conductance (gs); (C) Relationship between An and transpiration (E); and (D) Relationship between intracellular CO_2 concentration (Ci) and gs in both years 2015 and 2016. Each value is a single measurement. Black dots represent well-watered trees; and white dots represent stressed trees. Solid line represents regression curve for year 2015; Dash-dot line represents regression curve for year 2016.

When intrinsic WUE was calculated as a relation of An and gs (An / gs), we found that WUE was higher in stressed trees (97.13 %) than in well-watered (74.13%) after 5 days of drought exposure (97.13 %) in 2015(Fig. 6.4A). However, in 2016, significant differences were not found between treatments after 5 days of drought stress, while mean WUE ratio was 76.14 % and 80.69 % in well-watered and stressed

trees, respectively (Fig. 6.4B). However, we found significant differences both treatments and cultivars, at initial time point in 2016, standing out a lower WUE in 'Ufo-3' (43.04 %) than in 'Fergold' trees (56.51 %) (Fig. 6.4B). After irrigation period, WUE was higher in both treatments than initially in both years. But not significant differences were found between cultivars or treatments for that time point (Fig. 6.4). When the relationship between WUE and LWP was studied, it was observed that the more negative LWP values were, the higher WUE values became (Fig. 6.3A). These two parameters showed a significant negative correlation ($r = -0.91$, $p < 0.01$) in 2015. But this negative association between WUE and LWP was moderate, although significant, in the second year ($r = -0.47$, $p < 0.01$) (Fig. 6.3A).

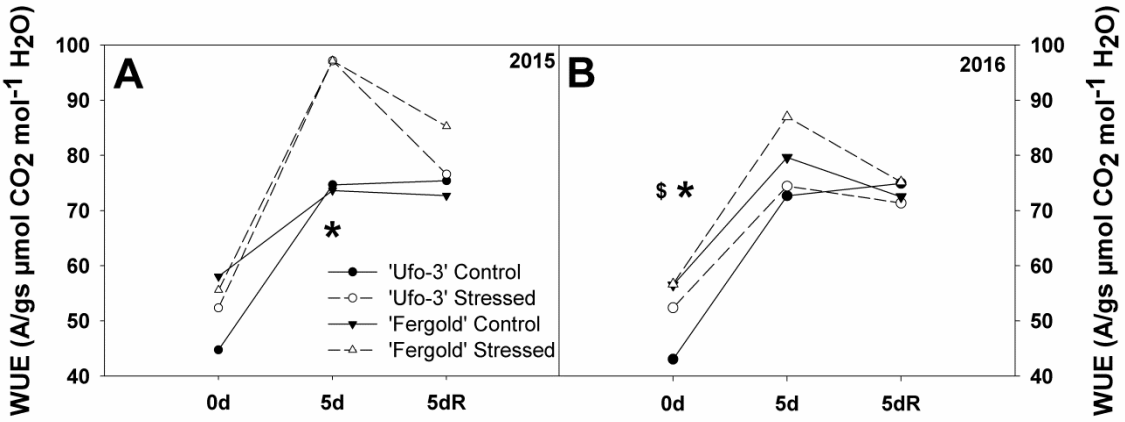

Figure 6.4. Evolution of intrinsic water use efficiency (WUE) calculated from An / gs relationship (A) in 2015 and (B) in 2016 throughout drought experiment. Asterisks represent significant differences ($p \leq 0.05$) between treatments (control and stressed) for each time point of the experiment; $ symbol indicates differences ($p \leq 0.05$) between cultivars ('Ufo-3' and 'Fergold') for each time point of the experiment. (d = days, R = recovery).

6.3.3. ABA accumulation in tress

When foliar ABA content was analyzed, no significant differences were found (Fig. 6.5). ABA values ranged from 414.62 to 632.00 ng g^{-1} and from 450.40 to 553.49 ng g^{-1} in 'Ufo-3' and 'Fergold' cultivars, respectively in control trees. The stressed trees reached ABA values of 698.10 ng g^{-1} in 'Ufo-3' and 478.80 ng g^{-1} in 'Fergold' (Fig. 6.5). Foliar ABA content in 'Ufo-3' control trees was decreased after 5 days of drought and then increased after the re-watering period, while foliar ABA content in 'Fergold' control trees increased first, and then experienced a lightly decline (Fig. 6.5). Only 'Ufo-

3' stressed trees reached higher values than control trees after 5 days under no-irrigation conditions, as opposite to 'Fergold' (Fig. 6.5). Even thought, the ABA content in both stressed cultivars decreased after 5 days of re-watering, reaching lower values than control trees at that time point (Fig. 6.5). Correlation coefficients were calculated in order to find a relation between foliar ABA accumulation and physiological parameters including stomatal conductance, tree water status (LWP) and soil water status (SWC), but no correlation were found.

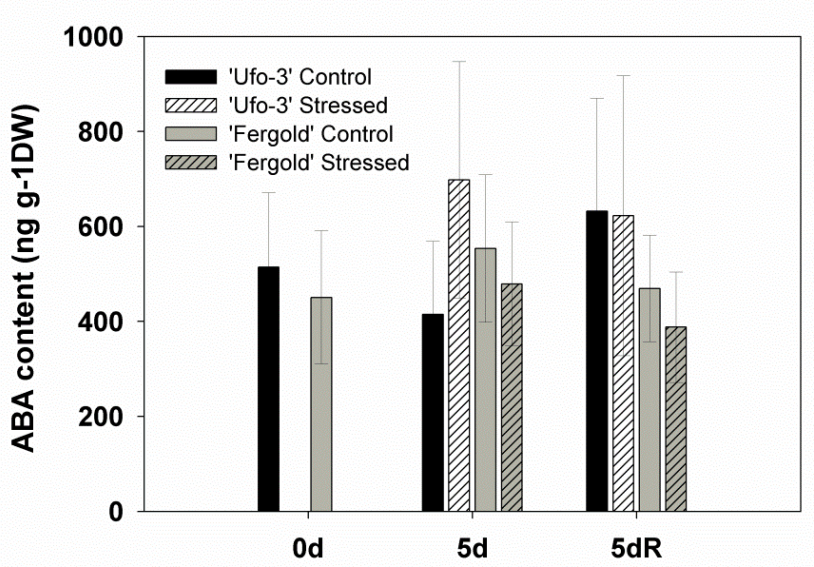

Figure 6.5. Abscisic acid (ABA) content during the drought experiment in 2015. Black bars: 'Ufo-3' control tress; White striped bars: 'Ufo-3' Stressed trees; Gray bars: 'Fergold' control tress; Gray striped bars: 'Fergold' stressed tress.

6.4. DISCUSSION

Currently, with the advances of novel -*omics* techniques, scientific community are focused on study plant drought responses at molecular level, searching candidate genes and markers in order to use them in breeding programs. However, although this molecular information obtained under specific and controlled experimental conditions, could be compare with field conditions, it is also necessary relating these molecular results with the physiological drought responses. Reports as Arndt et al., (2000) bring to light the importance of field studies. These authors observed a degree of drought tolerance in peach trees under drought on field conditions, in contrast to the lack of osmotic adjustment found in potted peach trees on greenhouse conditions. So, understanding the physiological behavior to drought under field conditions is a key

issue in order to developing drought tolerant lines in breeding. In last decades, different drought studies have been performed in a number of woody plants such as olive, almond, peach, grapevine, pistachio and different *Prunus* rootstocks, in which the tree water relations and its gas exchange mechanisms were reported (Boussadia et al., 2008; Espadafor et al., 2017; Martinazzo et al., 2011; Memmi et al., 2016; Rickes et al., 2017; Rodriguez-Dominguez et al., 2016; Solari et al., 2006; Tombesi et al., 2015; Yadollahi et al., 2011). In our study, two peach cultivars with different chill requirement, 'Ufo-3' and 'Fergold', grafted onto the interspecific hybrid almond × peach rootstock, 'Garnem', were submitted to different irrigation levels (well-watered and no-watered) on a field experiment to evaluate the physiological response to water stress considering the influence of 'Garnem' on the response of different peach cultivars used as scion, as well as to study the role played by ABA in response to drought stress.

Different responses were observed between control and stressed trees during the drought period. LWP and SWC values were maintained stable throughout the drought experiment in well-watered trees, indicating good water supplies for this group of trees. Trees submitted to drought stress experienced a drop in both parameters, LWP and SWC (Fig. 6.1 A and B). LWP dropped reaching similar values in both cultivars, which confirmed changes in water availability. However, the decrease of SWC in 'Fergold' cultivar was higher than in 'Ufo-3' (Fig. 6.1A and B). The different water requirements for each cultivar based on their different ripening date, might explain the differences found in SWC. 'Ufo-3' ripen one month earlier than 'Fergold', so that, its water consumption would be lower at the time of the experiment, and then the decline SWC would be less pronounced than in 'Fergold' cultivar. This values were in accordance to Jiménez et al., (2013). It was unexpected that control trees from the two studied cultivars showed significant changes in their gas exchange rates throughout the experiment in both years, but mainly in the first year (Table 6.2 and Fig 6.2). It might suggest that there were other factors, as well as plant water status, such as environment conditions: light, humidity, temperature, CO_2, and phytohormones which regulate stomata movements, generating changes in transpiration and photosynthesis mechanisms (Assmann and Shimazaki, 1999). However, although

control trees gas exchange rate values were not constant on the experiment time, when drought treatment was imposed, their values were higher than in stressed trees (Fig. 6.2), indicating a good performance of the transpiration and photosynthesis capabilities. After 5 days under drought conditions, stressed trees exhibited a remarkable drop in SWC and LWP values, which supported the statement that changes in LWP were induced by soil water conditions (Davies et al., 1994; Gollan et al., 1992). A decline in LWP causes hydraulic failure due to a probable embolism induction in xylem vessels. To prevent the hydraulic failure, gas exchange is regulated by stomata closure in order to control water losses (Jones and Sutherland, 1991). In our experiment, gs, An, E and Ci dropped to significant lower values after 5 days under drought conditions. The gs reduction would be caused by the stomatal closure, which would allow to maintain the water status leading to an osmotic adjustment (Jones and Sutherland, 1991). Trends of An, E and Ci coincided with the decline in gs in stressed trees. In addition, the significant relationships found between gas exchange parameters (Fig. 6.3 B-C) would confirm that stomatal regulation might constrain the influx of CO_2, as well as limit the water loss though transpiration and photosynthesis processes (Negin and Moshelion, 2016; Verslues et al., 2006). Our results were in agreement with previous reports in almond, peach and hybrid *Prunus* rootstocks (Espadafor et al., 2017; Jiménez et al., 2013; Martinazzo et al., 2011; Rickes et al., 2017; Romero et al., 2004; Torrecillas et al., 1996). When irrigation was restored, stressed trees reached similar LWP, SWC and gas exchange rate values as well-watered trees, except for An and E rates in 'Fergold' cultivar (Fig. 6.2 E, G and H). Differences found in An and E rates between control and stressed trees belong to 'Fergold' might indicate an irreversible damage on photosynthesis apparatus during stress period, which did not allow it reestablished its CO_2 uptake as in 'Ufo-3' stressed trees (Romero et al., 2004). 'Ufo-3' grafted onto 'Garnem' showed a better recovery of plant water status and photosynthetic functions than 'Fergold' grated onto 'Garnem', which represents a characteristic response of trees with drought tolerance strategy (Romero et al., 2004; Torrecillas et al., 1996). In previous reports similar physiological responses was observed in almond (Gomes-Laranjo et al., 2006; Romero et al., 2004) and peach trees (Mellisho et al., 2011; Rickes et al., 2017) and also in hybrid *Prunus* rootstocks (Jiménez et al., 2013), which were in accordance with our results.

During drought experiment, RWC presented high rates, but without significant differences either between treatments or between cultivars (Table 6.2). It was inconsistent with previous studies in which RWC decreased by drought exposure in different species such as sugarcane (Almeida et al., 2013), tobacco (Rabara et al., 2015), *Arabidopsis* (Gigon et al., 2004), as well as in fruit trees as lemon (Pérez-Pérez et al., 2009), apple and quince rootstocks (Bolat et al., 2014), and almond trees (Romero et al., 2004). Rodriguez-Gamir et al., (2010) suggested that under, their water stress conditions, the smaller reductions in leaf RCW might be consequence of an osmotic adjustment which was related to the tolerance showed by the citrus rootstock evaluated. However, Mellisho et al., (2011) evaluated the response to drought of peach cultivars grafted onto the peach × almond 'GF-677', finding higher RWC rates in stressed than in control trees. Similar results were found in 'Ramillete' almond by Torrecillas et al., (1996), suggesting that this behavior is a characteristic response of xeromorphic plants as almond. In our experiment, the studied cultivars grafted onto 'Garnem' might balance their water uptake and water loss avoid as drought-avoidance strategy, which might allow better osmotic adjustment and the maintenance of a high water content despite a low LWP values (Verslues et al., 2006).

WUE is considered one of the most important component of plant drought tolerance strategy (Blum, 2009). Tolerant plants balance their gas exchange maximizing the CO_2 uptake for photosynthesis, minimizing the water loos and then, maximizing soil water-use for transpiration (Blum, 2009; Lawson and Blatt, 2014). Intrinsic WUE resulted significantly higher in stressed than in well-watered trees from day 0 (Fig. 6.4), being in accordance with previous reports in grapevine (Medrano et al., 2015) and in different *Prunus* rootstocks (Jiménez et al., 2013). Trees with water availability do not need to regulate their resources because they have them. Thus, when environmental conditions are adverse, instead of closing the stomata to maintain their reserves, well-watered trees will remain them open to continue their photosynthetic activity at the expense of spending water. Hence, their WUE rate will be low. On the contrary, stressed trees with no water supply, which present a drought-tolerant strategy, will attempt to do an efficient use of the limited soil water, closing their stomata for avoiding water loss by transpiration, at the cost of reducing their

photosynthetic capacity. (Blum, 2009). It would explain why stressed trees presented WUE rate higher than well-watered in our experiment (Fig. 6.4). When relationship between intrinsic WUE and LWP was studied, a negative high correlation was found between both parameters in the studied trees in 2015, but not in 2016. The different relation along the years would be caused by differences on field environment conditions between 2015 and 2016. As above-mentioned, gas exchange rates might be induced by different factors such as environmental parameters (Assmann and Shimazaki, 1999). In contrast to our negative association for WUE and LWP, Romero et al., (2004) found a positive linear correlation in almond trees. Under our field-grown conditions, as the LWP decrease by the water scarcity, WUE was higher in the two peach cultivars grafted onto 'Garnem' rootstock, suggesting that this good performance could be related to the influence of 'Garnem', a vigorous rootstock, over the cultivars. 'Garnem' could present a drought tolerant strategy, allowing the maintenance of the photosynthetic capacity in spite of low gs and LWP values for the whole tree (Hajagos and Végvári, 2013; Jiménez et al., 2013).

Under a reduced soil water content, roots perceive the water scarcity and ABA biosynthesis is stimulated, accumulating in roots. Then, ABA is transported by xylem vessels to leaves, where is translocating to guard cells. Finally, the high ABA levels accumulated in leaves trigger stomata closure in order to maintain cell turgor (Sauter et al., 2001). As consequence, stomatal conductance, CO_2 assimilation and transpiration are reduced (Jones and Sutherland, 1991). The foliar ABA values in both control and stressed trees throughout the experiment period indicated that these trees did not experience severe stress during the no-irrigation period (Fig. 6.5). Evidences on the increase of ABA content in the response to water stress has been studied in several species including *Arabidopsis* (Christmann et al., 2005), cereals (Jacobsen et al., 2009; Seiler et al., 2014; Wang et al., 2003), forest trees (Sancho-Knapik et al., 2017) and fruit trees as citrus (de Ollas et al., 2013; Forner-Giner et al., 2011; Zandalinas et al., 2016), mulberry (Huang et al., 2013) and grapevine (Stoll et al., 2000). However, these reports have mostly been performed in potted plants under controlled conditions in a greenhouse, but not under field-grown conditions. The unexpected results on leaf ABA content both in control and stressed trees, together

with the lack of correlation between biochemical response and physiological response, evidence the clear different behavior between experiments in which potted plants are submitted to controlled conditions and experiments with trees on field-grown conditions (Arndt et al., 2000). In a dry and arid environment as Murcia, where irrigated trees reached LWP values near to -2 MPa, a no-water supply period produces a rapid physiological response but not visible biochemical response is produced. For a biochemical response, it might be necessary a more severe drought stress level, due to the fact that leaf ABA basal level in our tress was not reach during the 5 days of drought stress treatment. A similar performance were found by (Huang et al., 2013) in mulberry stressed plants, demonstrating that under drought stress, mulberry stressed trees were less sensitive to water deficit, then produced less ABA and presented a stronger adaptability and drought resistance.

We concluded that no-watered trees performed a physiological response declining their gas exchange rates at low LWP values in order to preserve an osmotic adjustment. This response was reflected in the RWC values. Then, these stressed trees were able to use their lower soil and plant water sources. This fact was reflected in the higher WUE rate presented by the stressed tress. Then, both 'Ufo-3' and 'Fergold' cultivars grafted on 'Garnem' rootstock showed a drought adaptability and tolerance. This adaptive response was confirmed by the lack of variability in foliar ABA accumulation under no-water conditions between control and stressed trees.

7. APPROACH FOR THE IDENTIFICATION OF ALMOND GENOMIC REGIONS IN FOUR INTERSPECIFIC HYBRID PROGENIES BY SSRS

ABSTRACT

The challenge in rootstock breeding programs is the combination of abiotic stress tolerances in new interspecific hybrids including crosses combining almond, peach and plum genotypes in order to obtain rootstocks adapted to a wide range of soil conditions. We analyzed 49 individuals belonging to four 3-way interspecific hybrid progenies and their parental genotypes (two myrobalan plums (*P. cerasifera* Ehrh.) 'P.2175' and 'P.2980', the almond-peach hybrids [*P. amygdalus* Batsch × *P. persica* (L.) Batsch] 'Garnem' and 'Felinem', 'Garfi' almond *P. amygdalus* Batsch and 'Nemared' peach *P. persica* (L.) Batsch). Forty-eight polymorphic SSRs in the parental genotypes were screened along the eight linkage groups obtained from several *Prunus* reference maps. The UPGMA dendrogram generated using the genetic variability observed, classified the genotypes in five different clusters, allowing us to differentiate the almond genomic regions from the peach and plum background in our progenies. The study of specific candidate drought-tolerance-related genes located in those regions will be accomplished as well as comparative genomic analysis once the almond genome will be available.

Keywords: Genetic Diversity, *Prunus*, Rootstock, Microsatellite markers

7.1. INTRODUCTION

Rootstocks are a crucial factor in fruit production due to their responsibility for water and nutrient uptake, as well as their ability to adapt to diverse environmental conditions and cultural practices (Gainza et al., 2015). Thus, rootstocks provide traits absent in scion such us vigor control, anchorage, resistance to soil-borne pathogens, and tolerance to drought, salinity and waterlogging, to name a few of the more important traits developed in breeding programs (Gainza et al., 2015; Layne, 1987).

Due to the advance in water scarcity along Mediterranean Area, selection of drought adapted rootstocks has become essential. Almond seedlings are the best adapted to a wide range of soil water scarcity (Isaakidis et al., 2004), and have been used during centuries in non-irrigated conditions in most of the Mediterranean orchards. However, these rootstocks are susceptible to root asphyxia and nematodes (Rubio-Cabetas, 2016). Interspecific hybrid crosses between wild-relative species, offers an extensive range of opportunities in *Prunus* breeding programs to compile traits together. These interspecific crosses have been performed for almond and peach rootstock breeding, mainly almond (*P. amygdalus* Batsch) × peach [*P. persica* (L.) Batsch], but also peach × *P. davidiana* and *P. webbii* × almond, due to their natural abiotic and biotic resistances and source of new genes to introgress in cultivated *Prunus* rootstocks (Alimohammadi et al., 2013; Bielsa et al., 2014; Byrne et al., 2012; Felipe, 2009; Gradziel et al., 2001; Lecouls et al., 2004). According to peach × almond hybrids, 'GF-677' has been one of the most used clonal rootstock (Rubio-Cabetas et al., 2005). In last decades, new selections were released, from 'Garfi' × 'Nemared' (G×N) series (Felipe, 2009). These parentals were selected by the good propagation via hardwood cutting presented in 'Garfi' almond and by the resistance to nematodes presented in 'Nemared' (Socias i Company et al., 2009). From this crossing three clones were selected: 'Garnem', 'Felinem' and 'Monegro'. These three new rootstocks show resistance to root-knot nematodes from *Meloidogyne* spp., tolerance to chlorosis similar to 'GF-677', tolerance to drought, as well as good performance in replanting conditions, and provide also a good vigor (Felipe, 2009). They were selected primarily for almond, but these hybrids are also use for peach due to their good graft

compatibility and better performance for low chilling varieties. However, to confer tolerance to waterlogging new crosses between myrobalan plums (*P. cerasifera* Ehrh) and G×N hybrids were created and are under evaluation (Amador et al., 2012; Xiloyannis et al., 2007).

Simple Sequence Repeat (SSR) technology have been used widely for many genome analysis including genetic diversity and structure analyses, genetic characterization, identification and cultivar certification in *Prunus* species (Bouhadida et al., 2009; Cheng et al., 2013; Dondini et al., 2007; Fernández i Martí et al., 2015; Gasic et al., 2009; Martínez-Gómez et al., 2003a; Xie et al., 2010; Zeinalabedini et al., 2008). The extensive use of these markers has been due to their abundance, multi-allelic nature, codominant inheritance, reproducibility, transferability over genotypes and widely genome coverage (Gasic et al., 2009). In last decade, SSRs have been used for saturation of existent reference *Prunus* linkage maps (Aranzana et al., 2003; Donoso, 2014; Howad et al., 2005; Joobeur et al., 1998) and generating new maps such as in myrobalan plum 'P.2175' and in the almond × peach hybrid 'GN22' (Dirlewanger et al., 2004). More recently, developments in sequencing and genotyping techniques provide reference genomes in *Prunus* genus, such as peach (Verde et al., 2013) and Japanese apricot (Zhang et al., 2012), and many other available genomes representing a new tool for breeding in woody plants (Badenes et al., 2016).

This study represents a preliminary approach for genotyping the almond genomic regions in four myrobalan plum × (G×N) progenies with several resistances to biotic and abiotic stresses within a rootstock breeding program using high polymorphic SSRs. Taking in account the peach reference genome (Verde et al., 2013) as well as, the almond reference genome, once it will be available, these results would represent an important key link between *Prunus* genetic reference maps and the new physical reference maps. Our approach would help to find candidate drought-tolerance-related genes that might be located physically in those regions of the specific linkage groups, as drought-tolerance trait might be inherited by almond parental.

7.2. MATERIALS AND METHODS

7.2.1. Plant material and DNA isolation

Four 3-way interspecific hybrid progenies (a total of 43 individuals) obtained from crosses between two myrobalan plums 'P.2175' and 'P.2980' (*P. cerasifera* Ehrh) as female parentals, and the almond × peach hybrids, 'Garnem' and 'Felinem' [*P. amygdalus* Batsch, syn *P. dulcis* (Mill.) × *P. persica* (L.) Batsch], as male parentals, together with these 6 parentals, were genotyped (Table 7.1). These individuals were included in the CITA (Centro de Investigación y Tecnología Agroalimentaria de Aragón) rootstock collection, located at the CITA facilities in Zaragoza, Spain (41°43'N, 0°48'W). Conventional orchard practices were used in tree training and weed control. Water requirements were supplied by surfaced irrigation.

Table 7.1. List of plant material analyzed for characterization with SSRs.

Parental Genotypes			**Code**
'Garfi' [*P. amygdalus Batsch* , syn *P. dulcis* (Mill.)]			
'Nemared' [*P. persica* (L.) Batsch]			
'Garnem' (GN15) ('Garfi' × 'Nemared')			15
'Felinem' (GN22) ('Garfi' × 'Nemared')			22
Myrobalan 'P.2175' (*P. cerasifera* Ehrh)			A
Myrobalan 'P.2980' (*P. cerasifera* Ehrh)			B
Hybrid populations	♀	♂	**Clone number**
A_15_02	'P.2175'	'Garnem'	2
A_15_03	'P.2175'	'Garnem'	3
A_15_04	'P.2175'	'Garnem'	4
A_15_05	'P.2175'	'Garnem'	5
A_15_06	'P.2175'	'Garnem'	6
A_15_07	'P.2175'	'Garnem'	7
A_15_08	'P.2175'	'Garnem'	8
A_15_09	'P.2175'	'Garnem'	9
A_15_10	'P.2175'	'Garnem'	10
A_15_12	'P.2175'	'Garnem'	12
A_15_13	'P.2175'	'Garnem'	13
A_15_16	'P.2175'	'Garnem'	16
A_15_17	'P.2175'	'Garnem'	17
A_15_18	'P.2175'	'Garnem'	18
A_15_20	'P.2175'	'Garnem'	20
A_15_22	'P.2175'	'Garnem'	22
A_15_25	'P.2175'	'Garnem'	25
A_15_26	'P.2175'	'Garnem'	26
A_15_27	'P.2175'	'Garnem'	27
A_15_28	'P.2175'	'Garnem'	28
A_22_01	'P.2175'	'Felinem'	1
A_22_05	'P.2175'	'Felinem'	5
A_22_07	'P.2175'	'Felinem'	7
A_22_08	'P.2175'	'Felinem'	8
A_22_10	'P.2175'	'Felinem'	10
A_22_14	'P.2175'	'Felinem'	14
A_22_16	'P.2175'	'Felinem'	16
A_22_51	'P.2175'	'Felinem'	51
A_22_78	'P.2175'	'Felinem'	78
A_22_87	'P.2175'	'Felinem'	87
A_22_93	'P.2175'	'Felinem'	93
A_22_114	'P.2175'	'Felinem'	114
A_22_115	'P.2175'	'Felinem'	115
A_22_116	'P.2175'	'Felinem'	116
A_22_117	'P.2175'	'Felinem'	117
A_22_132	'P.2175'	'Felinem'	132
A_22_142	'P.2175'	'Felinem'	142
B_15_03	'P.2980'	'Garnem'	3
B_15_05	'P.2980'	'Garnem'	5
B_15_09	'P.2980'	'Garnem'	9
B_22_06	'P.2980'	'Felinem'	6
B_22_10	'P.2980'	'Felinem'	10
B_22_23	'P.2980'	'Felinem'	23

Genomic DNA was isolated from 0.5 g of young leaf tissue using the DNeasy® Plant Mini Kit (Quiagen Inc. Valencia, CA) following the manufacturer's instructions. Then, the DNA was quantified and stored for PCR amplifications.

7.2.2. SSR Amplification

In order to have a wide cover of the genome, 48 SSRs markers (Table 7.2) distributed along the eight linkage groups and obtained from several *Prunus* reference maps (Dirlewanger et al., 2004; Donoso, 2014; Howad et al., 2005) were screened in both parentals and hybrids individuals. Genotyping was performed at Centre de Recerca en Agrigenòmica (CRAG) in Bellaterra, Barcelona, Spain. PCR reactions were performed in a 20-mL volume and the reaction mixture contained 1x PCR buffer (Invitrogen, Barcelona, Spain), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer, one unit of Taq DNA Polymerase (Invitrogen), and 20 ng of genomic DNA. The cycling parameters consisted in a denaturation during 1 min at 94 °C, 35 cycles of 15 s at 94 °C, 15 s for the specific annealing temperatures for the different primers used (data not shown), and 1 min at 72 °C, followed by a final extension of 2 min at 72 °C. The PCR reactions were carried out in a 96-well block Thermal cycler (Applied Biosystems, Madrid, Spain). PCR products were detected using an ABI PRISM 3130 Genetic Analyzer and GeneMapper analysis software (Applied Biosystems). Each reaction was repeated and analyzed twice for confirmation. For capillary electrophoresis detection, forward SSR primers were labeled with 5#-fluorescence dyes PET, NED, VIC, and 6-FAM and the size standard used in the sequencer was Gene Scan™ 500 Liz® (Applied Biosystems).

Table 7.2. *Prunus* SSR markers from different *Prunus* species studied in the interspecific hybrid progenies and in their parentals. Underlined marker showed monomorphism.

SRR name	Linkage Group	Alleles Observed no.	Size range (bp)	Species origin	Reference
UDP-96-018	G1	4	230-235	*P. persica*	Cipriani et al 1999
CPPCT027	G1	4	69-111	*P. persica*	Aranzana et al., 2002
UDP96-005	G1	4	126-173	*P. persica*	Cipriani et al 1999
CPPCT026	G1	5	155-178	*P. persica*	Aranzana et al., 2002
CPPCT019_1	G1	5	179-215	*P. persica*	Aranzana et al., 2002
CPPCT019_2	G1	7	170-215	*P. persica*	Aranzana et al., 2002
BPPCT028	G1	7	157-181	*P. persica*	Dirlewanger et al. 2002
CPPCT044	G2	4	165-187	*P. persica*	Aranzana et al., 2002
UDP98-025	G2	7	89-130	*P. persica*	Cipriani et al 1999
BPPCT002	G2	6	189-230	*P. persica*	Dirlewanger et al. 2002
UDP96-013	G2	7	144-199	*P. persica*	Cipriani et al 1999
pchgms1	G2	7	164-193	*P. persica*	Sosinski et al., 2000
UDA-023	G2	7	129-189	*P. dulcis*	Testolin et al., 2004
EPPCU5990	G3	3	188-201	*P. persica*	Howad et al 2005
BPPCT007	G3	7	126-145	*P. persica*	Dirlewanger et al. 2002
BPPCT039	G3	7	126-151	*P. persica*	Dirlewanger et al. 2002
CPPCT002	G3	4	90-99	*P. persica*	Aranzana et al., 2002
UDP96-008	G3	3	119-132	*P. persica*	Cipriani et al 1999
EPPCU0532	G3	6	164-187	*P. persica*	Howad et al 2005
BPPCT010	G4	7	121-165	*P. persica*	Dirlewanger et al. 2002
CPPCT005	G4	7	117-153	*P. persica*	Aranzana et al., 2002
CPDCT045	G4	6	121-167	*P. dulcis*	Mnejja et al., 2005
M12a	G4	8	174-224	*P. persica*	Yamamoto et al 2002
UDP97-402	G4	6	130-144	*P. persica*	Cipriani et al 1999
PS12a2	G4	6	154-187	*P. avium*	Joobeur et al., 2000
CPPCT040	G5	4	185-209	*P. persica*	Aranzana et al., 2002
UDP97-401	G5	5	102-141	*P. persica*	Cipriani et al 1999
PaCITA021	G5	6	224-246	*P. armeniaca*	Lopez et al., 2002
CPPCT013	G5	2	148	*P. persica*	Aranzana et al., 2002
BPPCT038	G5	6	123-162	*P. persica*	Dirlewanger et al. 2002
BPPCT014	G5	4	194-207	*P. persica*	Dirlewanger et al. 2002
CPPCT008	G6	6	148-167	*P. persica*	Aranzana et al., 2002
UDP96-001	G6	5	100-136	*P. persica*	Cipriani et al 1999
CPSCT012	G6	5	148-161	*P. salicina*	Mnejja et al., 2004
BPPCT025	G6	8	154-193	*P. persica*	Dirlewanger et al. 2002
UDP98-412	G6	6	98-124	*P. persica*	Vilanova et al., 2003
CPPCT021	G6	3	179-191	*P. persica*	Aranzana et al., 2002
CPSCT004	G7	3	123-125	*P. salicina*	Mnejja et al., 2004
CPSCT004_a	G7	7	182-201	*P. salicina*	Mnejja et al., 2004
pchgms6	G7	4	176-212	*P. persica*	Sosinski et al., 2001
UDAp-407	G7	5	81-112	*P. armeniaca*	Messina et al., 2004
CPPCT033	G7	6	128-157	*P. persica*	Aranzana et al., 2002
pchcms2	G7	6	167-185	*P. persica*	Sosinski et al., 2000
Ps5c3	G7	8	100-128	*P. avium*	Joobeur et al., 2000
CPSCT018	G8	4	150-169	*P. salicina*	Mnejja et al., 2004
CPPCT058	G8	4	119-123	*P. persica*	Aranzana et al., 2002
CPPCT035	G8	6	132-177	*P. persica*	Aranzana et al., 2002
BPPCT012	G8	3	137-158	*P. persica*	Dirlewanger et al. 2002
M6a	G8	7	182-218	*P. persica*	Yamamoto et al 2003
UDP98-409	G8	5	124-141	*P. persica*	Cipriani et al 1999

7.2.3. Data analysis

The data generated with the SSR genotyping was used for the analysis of the genetic similarity relationships among the individuals. The genetic distances between genotypes were calculated with NTSYSpc v2.1 software (Exeter Software, Stauket, NY). Then, a dendrogram was generated using the unweighted pair group method average (UPGMA) cluster analysis based on the Nei and Li, (1979) similarity index.

7.3. RESULTS AND DISCUSSION

7.3.1. SSR amplification

Out of the 48 SSR markers analyzed in the cross parentals and in the four progenies, 9 SSRs (19% of all SSRs analyzed) did no amplify in plum genome regions, and other 8 SSRs (17% of all SSRs analyzed) presented a weak signal. These markers belonged to UDP (5 SSRs), EPPCU (1 SSR), CPPCT (8 SSRs) series, which were isolated from *P. persica* (Table 7.2), and last one belonged to PaCITA serie, isolated from *P. armeniaca*. This results could suggest a low degree of transportability for plum species or due to the length of the SSR repeats (Dondini et al., 2007). However, in our study a higher transportability of the SSR markers from peach to almond was observed 47 SSRs from a total of 48 SSR loci were successful amplified in 'Felinem' and 'Garnem' genome regions, and in their parentals 'Garfi' and 'Nemared'. These SSRs markers were useful to distinguish almond from peach and plum genome regions in all progenies. It was noteworthy that 5 polymorphic SSR markers (BPPCT007, BPPCT039, UDP96-001, BPPCT025 and M6a) showed different alleles between 'Felinem' and 'Garnem' genotypes, and could be useful to difference them in genetic characterization and fraud analysis for Plant Breeder Right (PBR). The number of alleles per locus varied from 2 for CPPCT013 marker, to 8 for M12a, BPPCT025 and Ps5c3 markers (Table 7.2), with an average value of 5.44.

7.3.2. Clustering of individuals

A dendrogram of 49 individuals was created based on genetic similarity by 47 polymorphic SSRs (Fig. 7.1). Individuals were classified in five different clusters. The almond 'Garfi' was the genotype with the lowest similarity coefficient, classifying in a single-individual cluster A. Cluster B grouped both peach 'Nemared' and the G×N hybrids 'Felinem' and 'Garnem', confirming that these two hybrids are close to peach than almond. The female parental myrobalan 'P.2175' was grouped in the cluster C, with the tri-hybrid 'P.2175' × 'Garnem' - clone 9. The 95% of hybrid individuals from 'P.2175' myrobalan female parent. were classified in cluster D, being the biggest group with 36 individuals at total. However, one the clone 23, from the cross between 'P.2980' × 'Felinem', was also clustered in this group. Cluster D was characterized by the highest genetic diversity over the other clusters. Finally, the myrobalan 'P.2980' female parent and its progeny were grouped into the cluster E, as well as the clone 5 from 'P.2175' × 'Garnem' cross. In this cluster E, the individuals were clearly separated in two sub-groups based on their male parent: 'Garnem' for sub-group E1 and 'Felinem' for sub-group E2 respectively (Fig. 7.1). The different clustering found in the parental material was in agreement with previous reports such us Bortiri et al., (2006). These authors classified 37 species of *Prunus* and eight other genera of Rosaceae based on molecular and morphological data. In their classification *P. dulcis* and *P. persica* were more related between them than between *P. cerasifera*.

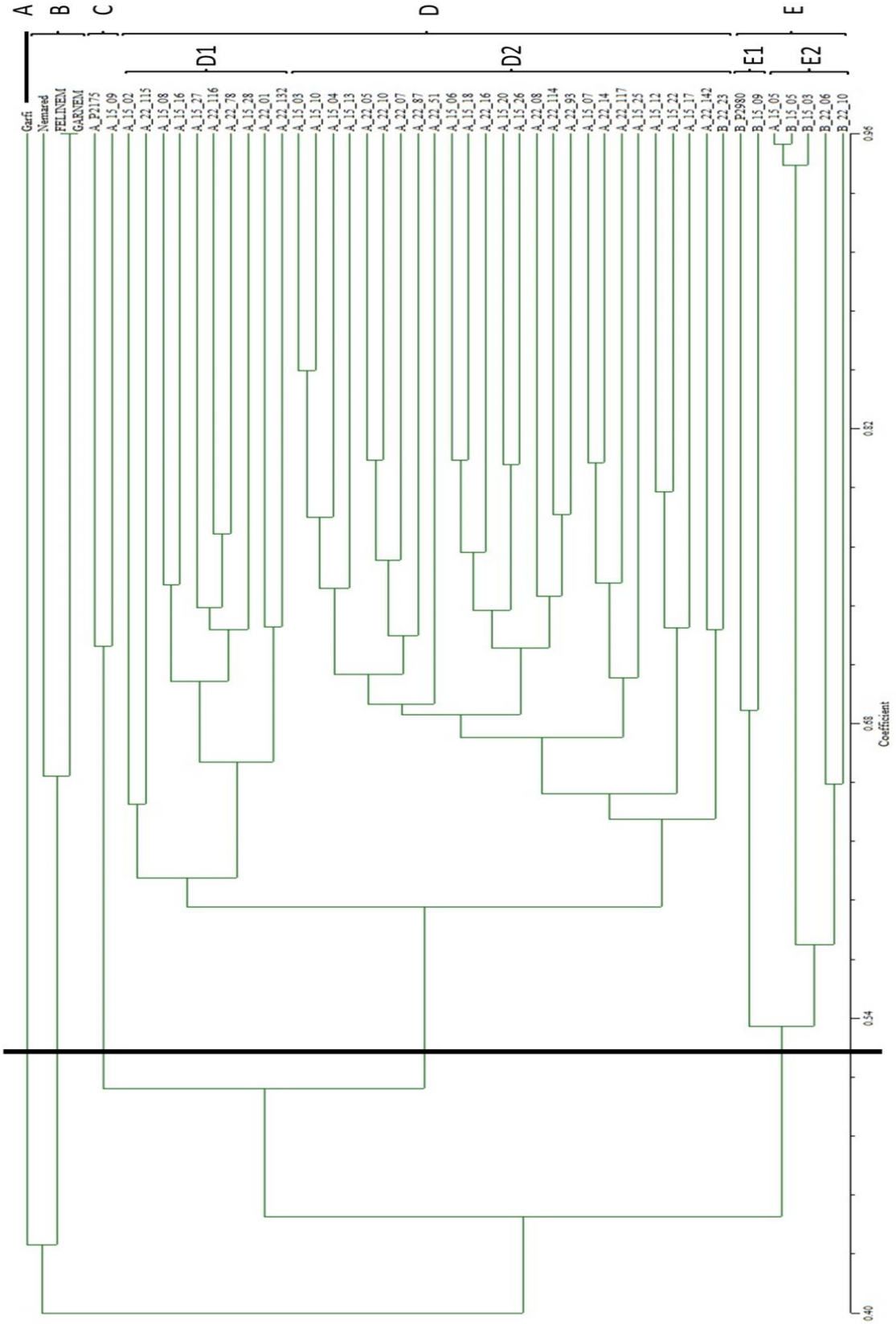

Figure 7.1. Dendrogram represented the diversity analysis of the six parentals and their four progenies based on UPGMA analysis after amplification with 48 SSRs.

It was unexpected to find three individuals away from the progeny that they were supposed to belong to, the tri-hybrids ‘P.2175’ × ‘Garnem’ - clone 9 in cluster C, ‘P.2980’ × ‘Felinem’ - clone 23 in cluster D and ‘P.2175’ × ‘Garnem’ - clone 5 in cluster E. The paternity of both individuals might be questioned, which might confirm the wrong crossing direction of these individuals.

7.3.2. Identification of the almond genomic regions

As result of the genetic analysis, it was possible to identify possible almond genome regions present along the eight linkage groups within our progenies and discriminate them from peach and plum genome regions (Fig. 7.2). The most conserved areas were observed in five linkage groups (1, 2, 3, 4, 5) (Fig. 7.2, red rectangle) rather than in the other three (6, 7, 8) in all individuals. Only the tri-hybrids belonging to cluster C and E1, showed their genome similar to plum in both of them. A region with a high level of crossovers was observed in linkages groups 6, 7 and 8 in every individual of clusters D1, D2 and E2 (Fig. 7.2). It is noteworthy that the locus screened in linkage group 7, with the CPSCT004 SSR marker showed only almond alleles in the cluster D individuals (Fig. 7.2, black rectangle). Based on these results, the regions belonging to linkage groups 1, 2, 3, 4 and 5 with more conserved areas could be the region to identify interesting traits from almond.

Figure 7.2. Graphical representation of the 6 parentals and their progenies identifying the almond, peach and plum regions along the eight LG. Plum genome is represented by blue color. Peach genome is represented by green color. Almond genome is represented by red color. White color represents no amplification. More conserved region was marked by red rectangle. Loci for CPSCT004 was marked by black rectangle.

In conclusion, this preliminary approach would be useful for increase the efficiency on the identification of candidate areas codifying traits of interest as drought tolerance in these specific genomic regions once almond genome will be available.

8. DISCUSIÓN GENERAL

La tolerancia al estrés hídrico es un carácter cuantitativo. La respuesta a dicho estrés hace que la planta desencadene un gran número de procesos fisiológicos, bioquímicos y moleculares permitiendo su adaptación. Puesto que todos esos procesos son de una gran complejidad, el estudio de los mecanismos que los desencadenan resulta de gran interés. Bajo esta premisa, los estudios realizados en esta tesis doctoral tienen como objetivo elucidar y comprender la respuesta a sequía en genotipos pertenecientes al género *Prunus* L., además de identificar genes candidatos implicados en dicha respuesta y relacionados con la mejora en el uso eficiente del agua (UEA), para su posterior aplicación en un programa de mejora para la selección de portainjertos tolerantes a sequía.

8.1. Fenotipado del uso eficiente del agua (UEA)

La obtención de portainjertos con un alto nivel de UEA asegura la producción y la rentabilidad en una plantación frutal. Por ello, se evaluó de forma paralela en los ensayos de estrés hídrico, el rango de UEA en una colección de individuos pertenecientes a *Prunus* L. (Capítulo 3). Esta población formada por portainjertos híbridos, sus parentales y una colección de especies silvestres relacionadas con el almendro, se fenotipó midiendo el contenido foliar en cenizas y la discriminación del isótopo 13C ($\Delta^{13}C$), ambos relacionados directamente con el UEA. Este concepto se basa en el transporte pasivo de los minerales vía xilema y su acumulación en los tejidos. Así, cuanto mayor es la transpiración, mayor el transporte de minerales. Como consecuencia, el contenido en cenizas aumenta, disminuyendo el UEA (Blum, 2011; Glenn, 2014; Masle et al., 1992). Los resultados indicaron una correlación positiva entre ambos parámetros en todos los genotipos estudiados. El mayor UEA se observó en las especies silvestres: *P. davidiana, P. bucharica*, *P. gorki*, *P. kotschii*, *P. orientalis*, *P. vavilovi* y *P. zabulica*. Estos genotipos cuyo origen es principalmente de zonas áridas y montañosas muestran una adaptación genética a las condiciones climáticas extremas (Gradziel, 2009; Kester y Gradziel, 1996; Wang, 1985). Por tanto, estos genotipos son fuente potencial de genes relacionados con un mejor UEA y para la mejora de patrones tolerantes a la sequía.

Aunque, en el ensayo de sequía a largo plazo (0d - 10d - 15d- 10dR - 15dR) se estudió el contenido mineral en hojas, no se observaron cambios a lo largo del experimento (Capítulo 2), ya que, como mínimo, sería necesaria una evaluación estacional o anual. En este caso, el periodo de ensayo no fue suficiente para encontrar variaciones significativas en el contenido de cenizas (Bielsa et al., 2016).

8.2. 'Garnem': respuestas fisiológica y bioquímica. Hacia la evitación del estrés por sequía

El comportamiento fisiológico de 'Garnem', portainjerto tolerante a sequía (Felipe, 2009), fue estudiado en los diferentes experimentos de esta tesis. En primer lugar, en el ensayo a largo plazo (0d - 10d - 15d- 10dR - 15dR) junto a dos híbridos: 'Tri-híbrido-3' y OP-'P.2175' (Capítulo 2). En segundo lugar, se realizaron dos ensayos a corto plazo: en las plantas sometidas a un tratamiento osmótico con PEG6000 en condiciones controladas durante (0h - 2h - 24h) (Capítulo 4); y en plantas sometidas a estrés hídrico en el invernadero (0h - 2h - 24h - 24hR) (Capítulo 5).

Tanto a largo plazo (Capítulo 2) como a corto (Capítulos 4 y 5) se producía un descenso del potencial hídrico foliar (LWP) y la conductancia estomática (gs), que provocó el cierre estomático, impidiendo las pérdidas de agua, lo que se interpretaría como un mecanismo de evitación en respuesta al estrés y así, poder realizar un posterior ajuste osmótico (Varela, 2010; Verslues et al., 2006).

A largo plazo (Capítulo 2), además del cierre estomático y el ajuste osmótico, se evaluó la epinastia como otro mecanismo de evitación. Los resultados indicaron que tanto 'Garnem' como sus híbridos, 'Tri-híbrido-3' y OP-'P.2175', redujeron su área foliar y biomasa mediante el enrollamiento y la abscisión foliar, respectivamente. Con este mecanismo se mantiene el potencial hídrico tanto en los meristemos como en las raíces (Engelbrecht y Kursar, 2003; Kozlowski y Pallardy, 2002). Este ensayo reflejó asimismo, una relación entre el vigor y la respuesta adaptativa al estrés hídrico. 'Garnem', el genotipo más vigoroso de los tres, necesitaría un requerimiento de agua mayor para cubrir su consumo de agua y disminuyó más rápidamente sus recursos hídricos (Bielsa et al., 2015). Además, se observaron las diferentes estrategias

adoptadas por cada genotipo. 'Garnem' se comportó durante los primeros días de estrés (10d) como una planta derrochadora, que consume rápidamente sus reservas hídricas para mantener la tasa de crecimiento; que se transforma en planta ahorradora cuando el estrés fue más severo (15d) y así, controlar a bajos potenciales la pérdida de conductividad hidráulica (Jones y Sutherland, 1991). Sin embargo, la estrategia adoptada por los otros dos híbridos 'Tri-híbrido-3' y OP-'P.2175' fue de plantas ahorradoras desde el primer momento (Jones y Sutherland, 1991). Este comportamiento podría indicar una mejor adaptación de estos, destacando OP-'P.2175'. La recuperación de los valores de LWP y gs**,** además del rebrote foliar durante el periodo de recuperación de 15 días, sugirió que los tres genotipos presentaban un rápido restablecimiento de sus funciones homeostáticas.

En los ensayos a corto plazo (Capítulos 4 y 5), los mecanismos de evitación explicados anteriormente, fueron confirmados con los cálculos del contenido relativo de agua (RWC) y de la estabilidad de la membrana citoplasmática a través de la fuga de electrolitos (EL). Ambos parámetros explican de nuevo la adaptación de 'Garnem' a las condiciones de sequía. Por tanto, 'Garnem' a bajos potenciales hídricos, sería capaz de mantener altos sus valores de RWC y baja su tasa de (EL), lo cual confirmaría ese ajuste osmótico mediante la acumulación de osmolitos, mencionado anteriormente (Singh et al., 2015; Verslues et al., 2006).

Para determinar la primera señal del estrés a corto plazo (Capítulo 5), se analizó el contenido de ácido abscísico (ABA) en las hojas. La acumulación exponencial de ABA en las hojas de 'Garnem' desencadenaría los mecanismos ya observados como el cierre estomático, además del ajuste osmótico, la estabilidad de la membrana y regulaciones en el crecimiento (Belin et al., 2010; Lind et al., 2015; Verslues et al., 2006). Este incremento en las hojas (Fig. 5.2) sugiere una rápida señal desde las raíces, primer órgano en percibir el estrés, hasta las hojas induciendo el cierre estomático (Christmann et al., 2013; Osakabe et al., 2014). El descenso del nivel de ABA a las 24h de recuperación, indica así mismo un rápido restablecimiento en la homeostasis de 'Garnem' observado ya en su respuesta fisiológica en el ensayo a largo plazo (Capítulo 2).

El estrés hídrico afecta de diferente forma a plantas en condiciones controladas (Capítulos 2 4 y 5) o en campo (Capítulo 6). Esto se observó analizando la respuesta fisiológica y bioquímica en árboles de 'Garnem' injertados con variedades de melocotonero 'Ufo-3' y 'Fergold' sometidas a estrés en postcosecha**.** Los árboles control presentaban valores de LWP cercanos a -2 MPa, encontrándose diferencias en el contenido hídrico del suelo (SWC) entre las dos variedades (Fig. 6.1). Las diferencias, podrían deberse a la distinta necesidad hídrica por la fecha de cosecha. 'Fergold', al madurar un mes más tarde, consumiría mayor cantidad de agua durante el periodo del experimento.

Las tasas de intercambio gaseoso (gs, E, An, Ci) así como los valores de LWP, RWC y variaron entre los árboles control y los estresados. Las dos variedades injertadas sobre 'Garnem' fueron capaces de mantener su contenido hídrico y su turgencia, a pesar de los bajos potenciales, presentando esa estrategia de evitación ya mencionada (Singh et al., 2015; Verslues et al., 2006). Los árboles estresados presentaron valores de UEA intrínseco mayores incluso que en los árboles control, estando en concordancia con estudios anteriores (Jiménez et al., 2013; Medrano et al., 2015). Lo que volvió a poner de manifiesto la estrategia de tolerancia presentada por 'Garnem' en todos los ensayos realizados. Sin embargo, en este caso, esta respuesta fisiológica no fue debida a los mecanismos de señalización en los que el ABA está implicado (Sauter et al., 2001), como se observó a corto plazo (Capítulo 5). La falta de correlación entre la respuesta bioquímica y fisiológica sugirió que bajo condiciones de campo los cambios fisiológicos permiten la evitación al estrés. El mecanismo de señalización del ABA a las hojas necesitaría un nivel de estrés más severo que el experimentado durante estos 5 días en estas condiciones edafoclimáticas.

8.3. 'Garnem' y su respuesta molecular. Hacia la tolerancia a la sequía

La tolerancia a la sequía es el resultado de un entramado de señalización complejo que es activado una vez que la planta percibe el estrés (Mahajan y Tuteja, 2005). El estudio a largo plazo (Capítulo 2), además de comprender la respuesta fisiológica, ha permitido determinar el nivel de respuesta génica. Durante el periodo de

estrés, los tres genotipos mostraron una inducción en la expresión tanto de los **f**actores transcripción (FTs), un dedo de zinc AN20/AN1 y un bZIP, como activadores de los genes efectores, los cuales protegen la homeostasis de la célula y permiten la adaptación (Mahajan y Tuteja, 2005; Roychoudhury et al., 2013; Yamaguchi-Shinozaki y Shinozaki, 2006), con niveles de expresión mayores en OP-'P.2175' que 'Garnem' y 'Tri-híbrido-3' (Fig. 2.4). La mayor respuesta génica en tejido de raíz que en floema sugiere que la respuesta primaria al estrés hídrico es producida en el tejido radicular (Aguado et al., 2014; Wisniewski et al., 2004). En particular, la expresión de la proteína LEA fue mayor que la de *PpDhn1*, sugiriendo el importante papel osmoprotector de esta proteína en la respuesta de tolerancia a sequía (Babu et al., 2004; Battaglia et al., 2008).

El estudio en la colección de híbridos, sus parentales y especies silvestres de las regiones promotoras de dos genes, *PpDhn2* y *DREB2B* (Capítulo 3) ha permitido determinar los elementos *cis* reguladores (CREs) que hacen posible la activación de ambos genes en respuesta a sequía y cuyo papel en la mejora del UEA bajo condiciones de estrés hídrico ha sido demostrada en otras especies (Khan, 2011; Melisova et al., 2015). Los resultados revelaron una mayor diversidad en la región promotora de *PpDhn2*, que en *DREB2B* donde todos los individuos compartieron CREs similares. El CRE más común fue el elemento ABRE (*ABA-responsive element*), lo cual indicaría que además de *PpDhn2*, *DREB2B* también tendrían un papel regulador en la ruta de señalización ABA-dependiente (Sazegari et al., 2015; Yamaguchi-Shinozaki y Shinozaki, 2005). Otros CREs identificados en ambos genes fueron los elementos MYB y los lugares de reconocimiento MYC, fundamentales en la respuesta sensible a ABA y a deshidratación (Abe et al., 1997; Roychoudhury et al., 2013). En particular, los resultados revelaron CREs de la región promotora de *PpDhn2* específicos de las especies silvestres del grupo VI, HSE (*heat sock element*) y LTRE (*low-temperature-responsive element*) (Fig. 3.4) lo que pondría de manifiesto el papel de *PpDhn2* en la respuesta a las bajas temperaturas, además de a la sequía en estas especies (Garcia-Bañuelos et al., 2009; Zolotarov y Strömvik, 2015). En este grupo VI destacó la caja GT3, un regulador negativo de UEA mediante la inactivación del gen *SDD1* (Yoo et al., 2010), región a ser considerada en futuros estudios para dilucidar su potencial papel.

En la región promotora de *DREB2B* (Fig. 3.5) se encontraron en todos los individuos elementos LTRE, algo que contradice estudios anteriores ya que este TF no se induce por bajas temperaturas (Bartels y Sunkar, 2005; Lata y Prasad, 2011; Liu et al., 1998).

El análisis transcriptómico realizado en raíces de 'Garnem' a corto plazo (Capítulo 4) reveló la expresión diferencial de un total de 83,110 DECs (*Differentially Expressed Contigs*), destacando el mayor número de DECs sobreexpresados a las 2h que a las 24h de estrés (Fig. 4.2) de los cuales se anotaron funcionalmente 26,700 DEGs (*Differencially Expressed Genes*). Se identificaron DEGs actuando en cada una de las etapas iniciales de respuesta al estrés hídrico como son kinasas, fosfatasas y DEGs relacionados con la acumulación de Ca^{2+} y otros segundos mensajeros, DEGs relacionados con las cascadas de fosforilación y un gran número de FTs. Estos FTs activan genes efectores, entre los que se identificaron DEGs que inducen la síntesis de proteínas reguladoras, enzimas implicadas en la biosíntesis de osmolitos, proteínas relacionadas con el transporte de agua e iones, enzimas antioxidantes, etc. Las principales funciones de estos DEGS son la estabilización de las proteínas para evitar su desnaturalización y la protección de la membrana celular con el fin de mantener la homeostasis de la célula (Beck et al., 2007; Mahajan y Tuteja, 2005; Roychoudhury et al., 2013; Shinozaki y Yamaguchi-Shinozaki, 2007). Así mismo, se identificaron DEGs relacionados con la ruta de señalización ABA-dependiente, evidenciando la importancia de esta fitohormona en la regulación del cierre estomático (Lind et al., 2015). Este estudio reveló tres DEGs directamente relacionados con la mejora del UEA: el FT *ERF023*, el *LRR receptor-like serine/threonine-kinase* ERECTA, y el FT *NF-YB3* (Han et al., 2013; Karaba et al., 2007; Xing et al., 2011) en la raíz de 'Garnem'. Debido a que el UEA es uno de los componentes más importantes en la estrategia de tolerancia a sequía (Blum, 2009), estos tres genes podrían ser genes candidatos para la selección de portainjertos tolerantes a sequía a través de la mejora en el UEA.

El estudio proteómico realizado en raíces de 'Garnem' a corto plazo (Capítulo 5), confirmó la importancia de varias de las rutas metabólicas identificadas en el estudio transcriptómico anterior. Del total de *spots* diferencialmente abundantes encontrados a las 2 y 24h, se pudieron identificar 15 proteínas implicadas en

diferentes procesos. Estos son el metabolismo de los lípidos, que mantienen la estabilidad de la membrana celular (Gigon et al., 2004); el metabolismo del nitrógeno y los aminoácidos implicados en la acumulación de solutos que previenen las pérdidas de agua como iones, compuestos nitrogenados, poliaminas y compuestos amonio actuando como osmoprotectores (Reddy et al., 2004); proteínas relacionadas con la actividad de transporte de iones implicados en la síntesis de compuestos que regulan el crecimiento radicular y la tolerancia a sequía (D.-K. Lee et al., 2017); proteínas involucradas en el metabolismo del carbono y el cierre estomático (Lee et al., 2008); en procesos de mantenimiento de la naturaleza proteínica que actúa como chaperona (Clèment et al., 2011; Padmalatha et al., 2012); proteínas implicadas en procesos de respuesta transcriptómica; proteínas con un papel en procesos de respuesta defensiva, poniendo de manifiesto la interconexión entre los mecanismos de respuesta a estreses bióticos y abióticos (Xiao et al., 2009), además de otras proteínas implicadas en la respuesta ABA-dependiente (Zhang et al., 2008); en procesos de modulación y *splicing* del ARN (Xu et al., 2016); y finalmente una proteína no caracterizada, la cual también puede poseer un papel importante en la respuesta adaptativa de 'Garnem' a la sequía (Rahman et al., 2015).

La identificación preliminar de las regiones genómicas de almendro en cuatro progenies tri-híbridas a través del análisis por marcadores microsatélites (SSRs) (Capítulo 7) permitió determinar los grupos de ligamiento en los que el genoma de almendro está más conservado. Este resultado facilitaría la búsqueda de genes relacionados con la tolerancia a la sequía en estudios futuros, asumiendo que la tolerancia a la sequía es mayor en el almendro que en el melocotonero y el ciruelo, para la selección de portainjertos tolerantes a distintos estreses abióticos que permitan abarcar un amplio rango de adaptación.

9. CONCLUSIONES

1. La correlación entre el contenido de cenizas y la discriminación del isótopo 13C ($\Delta^{13}C$) ha permitido identificar 7 especies silvestres con un mejor uso eficiente del agua (UEA) en una colección de *Prunus* con diferente origen, lo que muestra su adaptación genética a condiciones severas de estrés hídrico.

2. 'Garnem' se comporta como una planta derrochadora en los primeros días de estrés hídrico, debido a su vigor, mientras que se comporta planta ahorradora, cuando los recursos hídricos se han agotado.

3. Los parámetros fisiológicos monitorizados en los experimentos, indican que los genotipos estudiados presentan una respuesta de evitación a la sequía. El cierre estomático impide las pérdidas de agua retrasando el efecto del estrés hídrico. Además, el mínimo porcentaje de fuga de electrolitos (EL) revela un ajuste osmótico en 'Garnem' que le permite mantener la turgencia.

4. El ácido abscísico (ABA) desencadenó una rápida señalización en 'Garnem' regulando el cierre estomático y permitiendo mantener sus funciones homeostáticas durante el estrés hídrico.

5. En las condiciones de campo estudiadas no se observó correlación entre las respuestas fisiológica y bioquímica, sugiriendo que el nivel de estrés no fue suficiente para que se desencadene el mecanismo de señalización de ABA.

6. Los genes *PpDhn2* y *DREB2B* presentan en sus regiones promotoras elementos *cis* relacionados tanto con la ruta de señalización ABA-dependiente como con la ABA-independiente, permitiendo determinar la implicación de estos genes en la respuesta de tolerancia a la sequía. Igualmente se han identificado otros elementos *cis* relacionados con el estrés a bajas temperaturas en ambos genes, sugiriendo su participación en la respuesta a este estrés.

7. El análisis transcriptómico en raíz de 'Garnem' ha permitido anotar funcionalmente 26,700 genes expresados diferencialmente (DEGs) que participan en la respuesta a estrés hídrico, desde la percepción del estrés hasta la adaptación al mismo.

8. El estudio transcriptómico ha revelado más DEGs a las 2h que a las 24h, siendo estos la primera vez que se identifican en raíz de un *Prunus*, indicando el papel esencial en la percepción y señalización del estrés.

9. Se han identificado un gran número de DEGs relacionados con la regulación estomática inducida por ABA lo que pone de manifiesto el importante papel de esta hormona en la respuesta adaptativa y de tolerancia en 'Garnem'.

10. Ocho genes candidatos podrían ser elegidos como biomarcadores de tolerancia a la sequía en portainjertos de *Prunus*: Los DEGs *ERF023*, *LRR receptor-like serine/threonine-kinase* ERECTA y *NF-YB3*; el gen de la proteína LEA, y los genes *PpDhn1*, *PpDhn2* y *DREB2B*, por su relación con el mejor UEA y por último el DEG *Myb44,* represor de la fosfatasa PPC2, validado mediante qRT-PCR.

11. Las 15 proteínas identificadas mediante LC-ESI-MS/MS, relacionadas con la respuesta adaptativa al estrés hídrico corroboró la importancia de los procesos metabólicos previamente descritos en el análisis transcriptómico en raíz de 'Garnem'.

12. El análisis de tri-híbridos con microsatélites (SSRs) ha diferenciado una región genómica de almendro en cinco grupos de ligamiento (GL) del mapa genético de *Prunus*, que resultará de utilidad en estudios de genómica comparativa para estudiar caracteres de interés procedentes del almendro.

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11. ANEXOS

Supplementary Table S2.1. Daily environmental data along the experimental period.

	DATE	TMED	TMAX	TMIN	HRMED	HRMAX	HRMIN	VVMED	DVMED	VVMAX	DVMAX	RSOLMED	PREC	ET0
day 0	**July 5, 2011**	**24,4**	**34,1**	**13**	**53**	**92,9**	**21,2**	**1,3**	**35**	**6**	**252**	**30,3**	**0**	**6,5**
	July 6, 2011	23,9	31,7	16,7	52,5	75,5	29,1	3,2	271	7,7	303	30,3	0	7,7
	July 7, 2011	21,7	30,4	14,5	54,1	80,5	28	3,1	264	7,9	282	29,9	0	7,3
	July 8, 2011	22,4	31,2	13,8	43,9	71,1	17	2	262	7	256	30,8	0	7,1
	July 9, 2011	25	33,8	16,1	53,2	81,4	21,8	1,7	39	6,5	134	29,9	0	6,9
	July 10, 2011	25	32,5	17,6	60,2	90,9	33,4	1,7	248	5,8	346	27,8	0	6,2
	July 11, 2011	25,3	34,1	16,2	56,9	93,1	27,7	1,9	137	6,4	143	29,4	0	6,9
	July 12, 2011	24	32,8	16,5	62	89	38,5	2,7	234	13,1	319	23,4	0,7	6,3
	July 13, 2011	18,5	21	15,8	51,9	58,8	44	6	297	12,4	309	0,2	0	4,3
day 10	**July 14, 2011**	**21,2**	**27,8**	**14,6**	**50,8**	**72,8**	**29,3**	**3,4**	**290**	**12,5**	**267**	**30**	**0**	**7**
	July 15, 2011	22,1	31,3	11,1	53,9	90,8	22,4	1,5	8	6,7	286	30,5	0	6,2
	July 16, 2011	25,8	34,6	16,6	53,3	84,4	24,1	1,3	126	6,6	134	28,4	0	6,3
	July 17, 2011	21,3	24,6	15	52	75,2	31,9	5	301	11	325	30,4	0	7
	July 18, 2011	20,1	28,4	12,6	51	78,5	22,9	2,1	300	6,4	316	29	0	6,3
day 15	**July 19, 2011**	**19,4**	**23**	**15,1**	**56,2**	**94,1**	**34**	**5**	**314**	**13,4**	**310**	**27,7**	**1,1**	**5,9**
	July 20, 2011	20,2	28,1	12,2	52,9	77,7	31,5	3	305	9,4	333	30,3	0	6,7
	July 21, 2011	21,4	27,4	15,1	54,6	86,2	31,4	3,5	288	8,4	311	29,9	0	6,7
	July 22, 2011	19,5	25	14,9	50	73,2	29,8	3,9	294	8,8	290	26,6	0	6,5
	July 23, 2011	19	25,2	13,4	48,4	68,4	28,1	4,6	300	9,9	290	29,6	0	7,1
	July 24, 2011	20,3	25,2	15,3	54,8	69	40,6	6	300	12,8	305	28,5	0	7
	July 25, 2011	22,9	27,5	17,5	57,2	79,3	40,8	4,8	308	11,1	4	25,1	0	6,6
	July 26, 2011	23,1	28,1	18,8	56,6	79,8	35,6	4,5	293	11	295	21,6	0	6,5
	July 27, 2011	22,2	29,2	15,9	53,6	75	29,5	3,2	296	7,5	282	27,8	0	6,9
day 10 R	**July 28, 2011**	**21,7**	**30**	**13,2**	**59,1**	**90,7**	**25,1**	**1,9**	**255**	**7,6**	**241**	**28,4**	**0**	**6,1**
	July 29, 2011	23	31,9	14,8	55	84,9	16,7	2,2	242	6,5	293	27,6	0	6,7
	July 30, 2011	23,7	31,9	15,4	54,6	86,3	21,6	2,6	305	8,3	243	28,2	0	7
	July 31, 2011	22,5	30,6	14	58,9	90,7	26,4	1	163	4,3	95	27,1	0	5,3
	August 1, 2011	24,6	32,7	16,2	65,9	90,2	38,8	1,9	123	7,3	110	23,3	0,3	5,6
day 15 R	**August 2, 2011**	**25,2**	**32,9**	**18,8**	**63,6**	**92,5**	**28,4**	**1,6**	**99**	**5,9**	**1**	**20,2**	**2**	**5,2**

Legend

TMED	Daily air temperature average at 1.5 m above the ground, ºC
TMAX	Daily maximum aire temperature at 1.5 m above the ground, ºC
TMIN	Daily minimum aire temperature at 1.5 m above the ground, ºC
HRMED	Daily Relative Humedity average at 1.5 m above the ground, %
HRMAX	Daily maximum Relative Humedity at 1.5 m above the ground, %
HRMIN	Daily minimum Relative Humedity at 1.5 m above the ground, %
VVMED	Daily wind speed average at 2 m above the ground, m s-1
DVMED**	Daily wind direction average at 2 m abave the ground
VVMAX	Maximun daily wind speed at 2 m above the ground, m s-1
DVMAX**	Direction of maximum gust of wind to 2 m above the ground
RSOLMED	Daily global solar radiation average, MJ m-2 day-1
PREC	Daily rainfall, mm day-1
ETo	Reference Evapotranspiration (FAO Penman-Monteith method), mm day-1

Supplementary Material S2.2. RNA isolation protocol by Meisel et al. (2005) with some modifications (Chang et al., 1993; Salzman et al., 1999; Zeng and Yang, 2002).

All solutions were made using distilled water treated with diethylpyrocarbonate (DEPC) and autoclaved. First of all, 6.5 ml of extraction buffer (100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% (w/v) CTAB, 0.4 g/l Spermidine, 2% β-mercaptoethanol, 2% (w/v) PVP-40) were preheated at 65 °C in a water bath. 0.5 g of frozen root at -80 °C were grounded in a mortar with liquid N_2. The powder was quickly transferred to a 13 ml polypropylene-rounded-tip tube with 6.5 ml of warmed extraction buffer, homogenized by vortexing and then incubated at 65 °C for 15 min. After this time was added 6.5 ml of chloroform-isoamyl alcohol (24: 1, v/v). Once the sample was homogenized, it was centrifuged at 9500 rpm and 4 °C for 20 min. After centrifugation, the supernatant was transferred to a 15 ml polypropylene-conical-base tube and equal volume (6.5 ml) of chloroform-isoamyl alcohol (24: 1, v/v) was added. It was again mixed and centrifuged at 9500 rpm and 4 °C for 20 min again. The supernatant was transferred to a new 15 ml polypropylene-conical-base tube and 0.25 volumes of 10 M LiCl were added. The sample was well mixed and incubated overnight at 4 °C to precipitate the RNA. The next day, the sample was centrifuged at 9000 rpm and 4 °C for 30 min. The supernatant was discarded and the pellet was re-suspended in 50 µl of SSTE (10 mM Tris HCL (pH 8.0), 1 mM EDTA (pH8.0), 1 M NaCl, 0.5% SDS) and 450 µl of DEPC-treated water. Then, the re-suspended pellet was transferred to a 1.5 ml Eppendorf tube and 500 µl of chloroform-isoamyl alcohol (24: 1, v/v) were added. Mixed sample was centrifuged at 12,000 rpm and 4 °C for 10 min. After this step, the supernatant was transferred to a new Eppendorf tube, 2 volumes of 100% ethanol were added and the sample was incubated at -80 °C for 45 min. The sample was centrifuged at 12000 rpm and 4 °C for 30 min. The supernatant was discarded and the pellet was washed with 1 ml 70% ethanol and centrifuged again at 12,000 rpm and 4 °C for 20 min. Finally, the supernatant is removed and the pellet was dried at room temperature. RNA was re-suspended in 30 µl of DEPC-treated water and stored at -80 ºC until use.

Supplementary Material S2.3. ANOVA results from Leaf Water Potential (LWP) and Stomatal Conductance (gs) during the drought experiment for the studied genotypes. Same letter values indicate a no significant difference (p≤0.05) following Tuckey's post hoc test. (d=days, R= Recovery).

		Leaf Water Potential (LWP) (Mpa)									
		0d		10d		15d		10d R		15d R	
Control	'Garnem'	-0.643±0.047	b	-0.593±0.052	b	-0.600±0.029	b	-0.517±0.060	c	-0.487±0.058	b
Control	'Tri-hybrid-3'	-0.858±0.008	a	-0.800±0.050	a	-0.725±0.014	a	-0.783±0.044	b	-0.842±0.110	a
Control	OP-'P.2175'	-0.933±0.017	a	-0.683±0.033	ab	-0.817±0.033	a	-1.117±0.073	a	-0.733±0.033	ab
Treatment	'Garnem'	-0.627±0.023	b	-3.800±0.126	a	-4.300±0.000	a	-0.400±0.050	b	-0.300±0.000	b
Treatment	'Tri-hybrid-3'	-0.733±0.017	ab	-1.650±0.104	c	-4.283±0.044	a	-0.675±0.025	ab	-0.563±0.088	a
Treatment	OP-'P.2175'	-0.833±0.033	a	-2.567±0.017	b	-4.000±0.104	b	-0.817±0.093	a	-0.717±0.044	a

		Stomatal Conductance (gs) (mmol $m^{-2}s^{-1}$)									
		0d		10d		15d		10d R		15d R	
Control	'Garnem'	82.200±15.264	a	118.233±30.863	a	116.567±20.955	a	41.133±13.919	a	128.333±19.932	a
Control	'Tri-hybrid-3'	91.733±11.307	a	91.733±12.663	a	129.767±9.813	a	58.350±6.988	a	102.933±11.178	a
Control	OP-'P.2175'	90.717±8.809	a	90.717±12.282	a	113.183±13.590	a	52.317±6.151	a	117.900±12.339	a
Treatment	'Garnem'	148.667±13.372	a	9.433±3.979	a	8.333±6.099	a	51.458±5.392	a	133.833±8.464	a
Treatment	'Tri-hybrid-3'	155.383±14.735	a	18.717±5.357	a	3.883±1.134	a	102.475±7.672	b	154.550±17.032	a
Treatment	OP-'P.2175'	139.000±18.427	a	16.783±5.377	a	3.960±1.509	a	88.733±6.289	b	163.733±10.736	a

Supplementary Material S2.4. ANOVA results from Relative Gene Expression during the drought experiment for the studied genotypes. Same letter values indicate a no significant difference (p≤0.05) following Tuckey's post hoc test among genotypes for each tissue and each day of treatment. (d=days, R= Recovery).

A20/AN1 Zinc Finger TF (*ppa012373m*)										
Genotype	**Tissue**	**0d**	**10d**		**15d**		**10d R**		**15d R**	
'Garnem'	Phloem	1±0	a	1.211±0.321	a	1.628±0.643	a	1.468±0.866	a	2.266±0.066	a
'Tri-hybrid-3'	Phloem	1±0	a	2.366±0.876	a	3.394±0.333	b	0.621±0.358	a	2.581±0.525	a
OP-'P.2175'	Phloem	1±0	a	1.260±0.232	a	2.340±0.038	ab	1.224±0.288	a	1.639±0.122	a
'Garnem'	Root	0.747±0.047	a	1.053±0.131	a	2.043±0.501	a	1.135±0.178	a	1.073±0.308	a
'Tri-hybrid-3'	Root	1.983±0.702	a	3.666±1.146	ab	7.334±0.993	a	2.640±0.053	b	5.726±0.876	b
OP-'P.2175'	Root	2.078±0.805	a	7.653±1.131	b	24.722±2.772	b	1.298±0.221	a	6.452±0.597	b

bZIP TF (*ppa013046mm*)										
Genotype	**Tissue**	**0d**	**10d**		**15d**		**10d R**		**15d R**	
'Garnem'	Phloem	1±0	a	0.494±0.194	a	1.528±0.188	a	0.265±0.154	a	0.578±0.208	a
'Tri-hybrid-3'	Phloem	1±0	a	0.973±0.345	a	1.109±0.105	a	0.320±0.155	a	1.052±0.388	a
OP-'P.2175'	Phloem	1±0	a	1.032±0.235	a	2.256±0.513	a	0.903±0.104	a	0.652±0.031	a
'Garnem'	Root	2.007±0.335	a	1.375±0.035	a	1.038±0.500	a	1.305±0.199	a	1.697±0.592	a
'Tri-hybrid-3'	Root	2.054±0.153	a	3.170±0.277	ab	3.171±1.113	a	2.562±0.702	a	1.014±0.043	a
OP-'P.2175'	Root	2.232±0.260	a	6.201±0.579	b	10.521±0.865	b	1.098±0.0231	a	1.361±0.444	a

Dehydrin (*ppa005514m*)										
Genotype	**Tissue**	**0d**	**10d**		**15d**		**10d R**		**15d R**	
'Garnem'	Phloem	1±0	a	2.435±0.071	a	2.356±0.202	a	2.468±1.538	a	0.376±0.054	a
'Tri-hybrid-3'	Phloem	1±0	a	1.854±0.290	a	6.203±0.806	b	0.313±0.140	a	0.738±0.364	a
OP-'P.2175'	Phloem	1±0	a	2.226±0.079	a	5.059±0.293	ab	0.521±0.012	a	0.640±0.209	a
'Garnem'	Root	0.139±0.045	a	3.123±0.435	a	3.259±0.495	a	0.093±0.001	a	0.073±0.007	a
'Tri-hybrid-3'	Root	0.766±0.502	a	0.996±0.130	a	12.978±1.586	b	0.081±0.037	a	0.285±0.099	a
OP-'P.2175'	Root	0.676±0.105	a	2.592±0.483	a	15.351±1.756	b	0.407±0.189	a	1.703±0.368	b

LEA protein (*ppa008651m*)										
Genotype	**Tissue**	**0d**	**10d**		**15d**		**10d R**		**15d R**	
'Garnem'	Phloem	1±0	a	21.358±0.677	a	53.432±1.053	a	16.549±2.274	b	2.703±0.361	a
'Tri-hybrid-3'	Phloem	1±0	a	11.686±0.707	a	48.654±0.980	a	6.968±0.370	a	0.835±0.033	a
OP-'P.2175'	Phloem	1±0	a	133.562±4.960	b	311.100±9.470	b	3.003±0.742	a	2.154±0.608	a
'Garnem'	Root	0.912±0.112	a	28.290±3.534	a	23.409±2.907	a	1.070±0.057	a	0.517±0.046	a
'Tri-hybrid-3'	Root	5.545±0.190	b	13.321±0.570	a	80.994±2.379	a	0.735±0.094	a	2.461±0.526	a
OP-'P.2175'	Root	2.791±0.608	a	24.725±3.176	a	362.892±19.129	b	2.068±0.345	a	4.686±1.374	a

ANEXO 2

Supplementary Material S3.1. *Cis*- regulatory elements of *PpDhn2* promoter gene region in each of the cluster-representative individual. Cells in grey color, CREs outside of the first 1,000 pb 5' of the translation start site. Due to the extension of this data set, this information is only attached in the extended data in electronic version of annex section.

Supplementary Material S3.2. CREs of *DREB2B* promoter gene region in each of the cluster-representative individual. Cells in grey color, CREs outside of the first 1,000 pb 5' of the translation start site. Due to the extension of this data set, this information is only attached in the extended data in electronic version of annex section.

ANEXO 3

Supplementary Material S4.1. Physiological monitoring of LWP in acclimation period of PEG-treated plants. Due to the extension of this data set, this information is only attached in the extended data in electronic version of annex section.

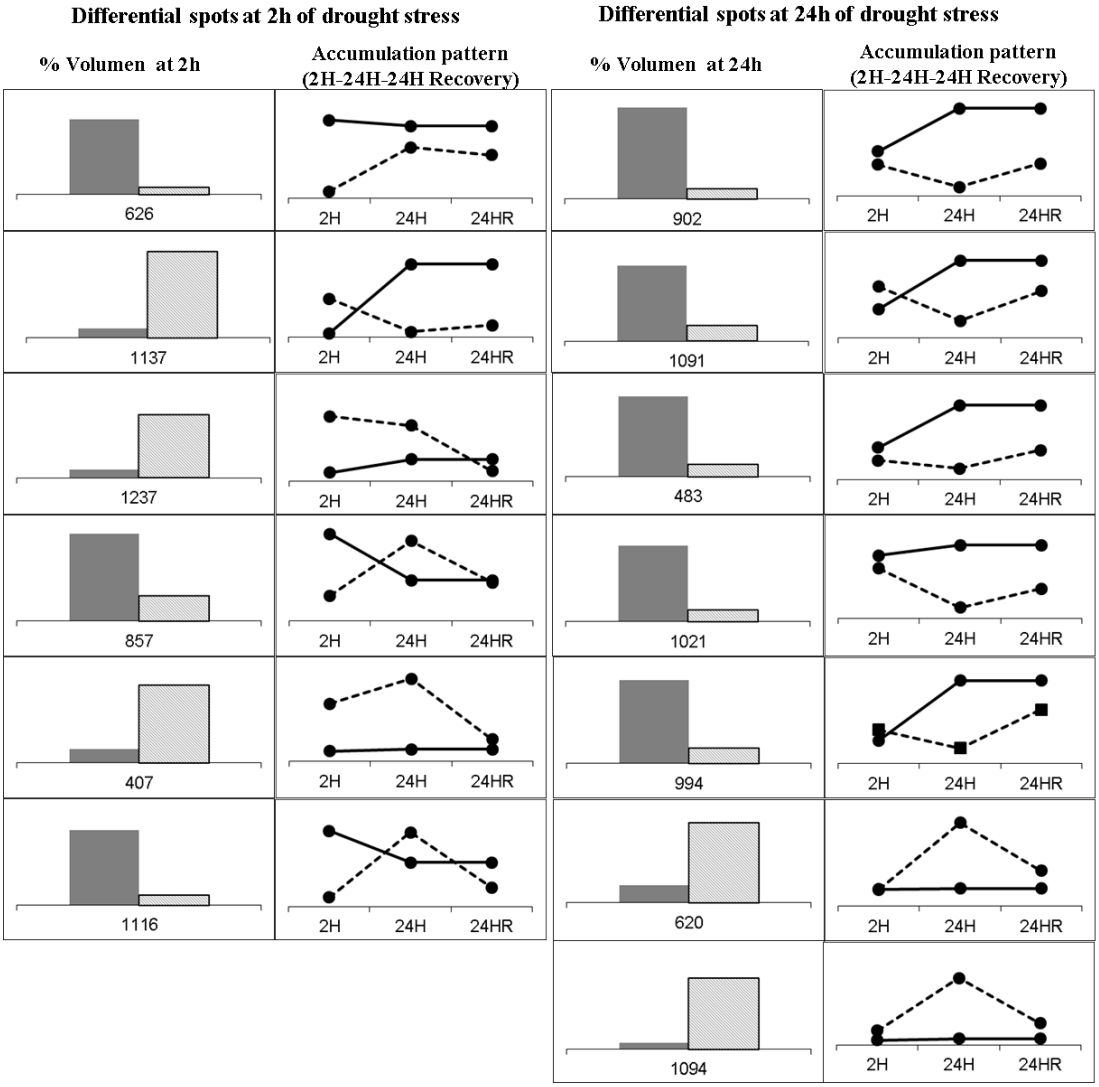
Supplementary Material S4.2. Primers used in validation gene by qRT-PCR.

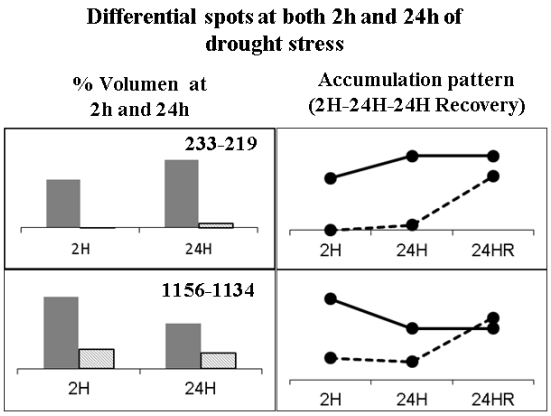
Gene	Primer	Sequence 5'-->3'	Annealing Temperature(º C)	Size
9-cis-epoxycarotenoid dioxygenase chloroplastic-like	**GN_NCED_qPCR_fwd**	TCCAAACCGAGACGTCTTTT	59.71	158
	GN_NCED_qPCR_rev	TGGACATCACGCTAGACCAA	60.26	
Late embryogenesis abundant protein d-34-like	**GN_LEA34_qPCR_fwd**	TCTCGGGTGCTACTGAGAAA	58.6	181
	GN_LEA34_qPCR_rev	ACGGCAGGAAATCACATCTT	59.56	
Transcription factor myb44-like	**GN_Myb44_qPCR_fwd**	TGTGCTGTGGAGATGGAAGA	60.4	154
	GN_Myb44_qPCR_rev	AGCGTTACGGATCATTTTGG	59.96	
R2R3 Myb transcription factor	**GN_R2R3MYBTF_qPCR_fwd**	TACAAATGGGCATCGTCTCA	60.07	154
	GN_R2R3MYBTF_qPCR_rev	CCAAAGTGCATGAGATTCCA	59.65	
Transcription factor myb108	**GN_Myb108_qPCR_fwd**	TGTTGCTGAGCTGCTCTTGT	59.93	152
	GN_Myb108_qPCR_rev	TGACCAGTGTTTCAATCCAG	57.49	
NAC domain-containing protein 2	**GN_NAC2_qPCR_F2**	CCAAGCAAACAAACTCAGCA	60.03	132
	GN_NAC2_qPCR_R2	TAACTCAGGCGAGCTTCCAT	59.98	
Probable WRKY transcription factor 53	**GN_WRKY53_qPCR_fwd**	TTTCCGTCTTCCTCTCATGG	60.19	132
	GN_WRKY53_qPCR_rev	ATGACCTGTGGGCAGTTGTT	60.43	
Transcription factor bhlh36-like	**GN_BHLH36-like_qPCR_fwd**	TATCAAGGGAAAGCGTTCGT	59.71	124
	GN_BHLH36-like_qPCR_rev	CGAAGACGTCCCGATCTTTA	60.21	
bzip transcription factor bzip79	**GN_bZIPTF79_qPCR_fwd**	TGGTGCATTGATCTCCACAT	59.93	131
	GN_bZIPTF79_qPCR_rev	GGGTAAATTGTCGCCATCAG	60.33	
Universal stress protein a-like protein	**GN_uspA_qPCR_fwd**	AGCCTCCCAAAGCTACCAAT	60.1	135
	GN_uspA_qPCR_rev	TATCAAGAACCTCCGGATCG	60.03	
Zinc finger A20 and AN1 domain-containing stress-associated protein 5-like	**GN_ZFA20/AN1_SAP5_qPCR_F2**	TCAATCATCCACACTCACGAA	60.10	146
	GN_ZFA20/AN1_SAP5_qPCR_R2	CGGGTTGTTGTGCATTCTCT	61.10	
Zinc finger family protein (C2H2 ZnF)	**GN_ZnF_C2H2_qPCR_fwd**	AGTATGGCCGACGATGATGT	58.96	134
	GN_ZnF_C2H2_qPCR_rev	CATTCGTGCACCTTCGCTTT	59.76	
zinc finger protein constans-like 13 (COL-ZnF)	**GN_Bbox_CCT motif_qPCR_fwd**	TGTGCTTGGAGAAAAGCTGA	59.72	130
	GN_Bbox_CCT motif_qPCR_rev	CACCAAAAGCGTCTCTGTGA	60.02	
Zeaxanthin chloroplastic-like	**GN_Zeaxanthin_qPCR_fwd**	AGCAGCGAAACATGAAGGAT	59.84	137
	GN_Zeaxanthin_qPCR_rev	CATTCTCGTCAATGGCTTCC	60.6	
Calcium-independent phospholipase a2-γ	**GN_Ca_Phosa2-gamma_qPCR_fwd**	TGATTAAGGTGAGCACGTGGA	59.38	120
	GN_Ca_Phosa2-gamma_qPCR_rev	ATTGTTTGCCACTATTGCACCA	59.37	
Calcium-dependent protein kinase 26-like	**GN_CaKinase26_like_qPCR_F2**	GGTGCATCCCTCATGCTTAT	59.92	123
	GN_CaKinase26_like_qPCR_R2	TGATGGGCGCATAGATTACA	60.06	
Calcium-transporting atpase plasma membrane-type-like	**GN_CaATPase_qPCR_F2**	GCAACCTCTGTTCCTGCAAT	60.26	120
	GN_CaATPase_qPCR_R2	ACAGTTGAGGGATGGGTTCA	60.36	
Calcium-transporting atpase plasma membrane-type	**GN_CaATPase_4_qPCR_F2**	CCAGCACCAGCATCAATAAA	59.69	120
	GN_CaATPase_4_qPCR_R2	GGACTATGATTGTCATCCGTTT	57.93	

Supplementary Material S.4.3. Total DECs found in the four pairwise comparisons including the Reads Per Kilobase per Million (RPKM) values, as well as the Log fold change (FC) values. List of the total annotated DEGs involved in PEG-treated experiment. DEG classification based on its involvement in the stress response. Due to the extension of this data set. This information is only attached in the extended data in electronic version of annex section.

Supplementary Material S.4.4. Significantly enriched GOs for the four pools of differentially expressed genes (DEGs). Due to the extension of this data set. This information is only attached in the extended data in electronic version of annex section.

Supplementary Material S.4.5. List of total metabolic pathways for the four pools of DEGs involved in PEG-treated experiment. Due to the extension of this data set. This information is only attached in the extended data in electronic version of annex section.





Supplementary Material S.5.1. Accumulation patterns throughout drought stress experiment for each differential spots identified by LC-ESI-MS/MS. Control plants: Dark grey bars and black solid lines; stressed plants: Light grey striped bars and black dash lines.

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Physiological characterization of drought stress response and expression of two transcription factors and two LEA genes in three *Prunus* genotypes

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ABSTRACT

Global warming has led to a progressive decrease in rainfall, which is reflected by a reduction of water resources in the soil and a negative effect on crop production in Mediterranean areas. Under drought stress, many plants react by inducing a different series of responses at both physiological and molecular levels, allowing them to survive for a variable period of time. Therefore, in order to understand the response of roots to drought conditions, the genotypes peach × almond 'Garnem' [*P. amygdalus* Batsch × *P. persica* (L.) Batsch] and their progeny, the hybrid 'P.2175' × 'Garnem'-3 and OP-'P.2175' (*P. cerasifera* Ehrh.) were subjected to a period of water deficit. Drought conditions with a subsequent re-watering period were tested for potted plants for one month. Stomatal conductance and leaf water potential were measured to monitor the plant physiological responses. Significant differences among the drought stress and drought stress recovery treatments and among the genotypes were observed. In addition, four genes related to the ABA biosynthesis pathway were studied for their expression by RT-qPCR: an AN20/AN1 zinc finger protein (*ppa012373m*); a bZIP transcription factor (*ppa013046m*); a dehydrin (*ppa005514m*) and a LEA protein (*ppa008651m*). Their expression profiles correlated with our physiological results of drought response, being higher in roots than in phloem tissue. In general, the expression of the four studied genes was higher after 15 days under drought conditions. Under drought and recovery conditions, the zinc finger and bZIP transcription factors showed significant differences in their relative expression levels from LEA and dehydrin. These results suggest the role of LEA and dehydrin in the regulatory response to drought stress in *Prunus* genotypes. Therefore, the dehydrin and the protein LEA might be potential biomarkers to select rootstocks for tolerance to drought conditions.

1. Introduction

Stress can be defined as a physiological deviation from normal plant functions that can damage or cause irreversible damage to the plant (Nagarajan, 2010), negatively affecting crop growth and yield. Drought stress is one of the biggest problems in agriculture, especially in arid and semi-arid climates (Bartels and Sunkar, 2005) in the Mediterranean region where water availability is the most important factor for plant survival. Since Mediterranean countries are the main stone fruit producers (FAO, 2014), the use of adapted rootstocks is necessary for such limited edaphoclimatic conditions. Currently, the challenge in rootstock breeding programs is the combination of abiotic tolerances in a new generation of interspecific hybrids resulting from the cross of almond × peach hybrids by plum genotypes. Peach × almond hybrids such as 'Garnem', 'Felinem' and 'Monegro' (which come from the cross 'Garfi' almond × 'Nemared' peach) show good vigour, nematode resistance, and adaptation to calcareus soils (Felipe, 2009). Myrobalan plums such as 'P.2175' provide a wide spectrum of root-knot nematode resistance (Rubio-Cabetas et al., 2000) and tolerance to waterlogging (Amador et al., 2012).

During the stress period, plants undergo some morphological and physiological changes due to hormones such as abscisic acid (ABA) and ethylene (Bruce et al., 2002; Munns, 2002). ABA accumulation under water deficit conditions activates different genes linked to stress (Narusaka et al., 2003). The ABA-inducible genes have *cis*-elements in their promoter regions including *ABA-responsive elements* (ABRE) (Yamaguchi-Shinozaki and Shinozaki, 2005). The activation of these elements through different transcription factors (TFs) ABA-responsive element binding proteins,

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