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Transcriptional Responses in *Prunus persica* Under Conditions of Drought Stress Using RNA Sequencing

By

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Abstract

Peach [*Prunus persica* L. Batsch] is one of the most important stone fruit and it is widely established in arid and semi-arid regions. As a fleshy fruit, a peach contains 87% of water, requiring abundant water supply to ensure proper development and esteemed flavor. However, due to variations in the climate and the increased aridity, drought has become a major constraint, causing crop losses worldwide. The use of drought-tolerant rootstocks in modern fruit production appears to be a useful alternative to alleviate water deficit problems. However, the transcriptomic variation and the major molecular mechanisms that underlie the adaptation of tolerant rootstocks to water shortage remain unclear. Hence, in this study, high throughput sequencing (RNA-seq) was performed to assess the transcriptomic changes and key genes involved in response to drought in root tissues (GF677 rootstock) and leaf tissues (graft, var. Catherina) subjected to 16 days of drought stress. In total 12 RNA libraries were constructed and sequenced. This generated a total of 315M raw reads from root and leaf tissues, which allowed the assembly of 22,079 and 17,854 genes associated with the root and leaf tissues, respectively. Subsets of 500 differentially expressed genes (DEGs) in roots and 236 in leaves were identified and functionally annotated, including 56 Gene Ontology (GO) terms and 103 metabolic pathways, which were mostly associated with phenylpropanoid biosynthesis, and glutathione metabolism. The GO analysis highlighted the biological functions that were exclusive to the root tissue, such as “locomotion” (GO: 0040011), “hormone metabolic process” (GO: 0042445) and “detection of stimulus” (GO: 0051606), indicating the stress-buffering role of the GF677 rootstock. Furthermore, the complex regulatory network involved in the drought response was revealed, involving enzymes that are associated with signaling transduction, hormones regulation, redox homeostasis, and frontline barriers. We identified two poorly characterized genes in *P. persica*: growth regulating factor 5 (GRF5) which may be involved in cellular expansion and AtHB12, which may be involved in root elongation. In order to confirm the reliability of the RNA-seq, the expression patterns of 33 drought-induced genes were validated using RT-qPCR. There was a significant correlation between the results of both technologies ($r = 0.88$ and $r = 0.95$ for the roots and leaves, respectively). The transcriptomic resources generated in this study provide a broad characterization of the acclimation of *P. persica* to drought, shedding light on the major molecular responses to the most important environmental stressor.

Resumen

El melocotonero [*Prunus persica* (L.) Batsch] es uno de los frutales de hueso más importante y ampliamente establecido en regiones áridas y semiáridas. El melocotón es un fruto carnoso con 87% de agua, que exige un suministro constante de agua para abastecer su demanda y mantener su apreciado sabor. Sin embargo, debido a las variaciones climáticas y al aumento de la aridez, la sequía se ha convertido en uno de los estreses ambientales más limitantes causando importantes pérdidas económicas. El uso de porta-injertos tolerantes a la sequía, en la fruticultura moderna, parece ser una alternativa útil para aliviar los problemas de escasez de agua. Sin embargo, no se conocen los cambios moleculares y/o transcriptómicos que modulan esta adaptación en los patrones tolerantes. En este trabajo se ha llevado a cabo una secuenciación de alto rendimiento (RNA-seq), para investigar los cambios transcriptómicos, e identificar los genes candidatos implicados en la respuesta a la sequía, en las raíces (patrón GF677) y las hojas (injerto var. Catherina), sometidos a 16 días de estrés. Se construyeron y secuenciaron doce librerías de RNA en muestras de raíces y hojas que generaron 315M de lecturas crudas, permitiendo el ensamblaje de 22.079 y 17.854 genes en raíz y hoja, respectivamente. Se seleccionaron 500 genes diferencialmente expresados (DEGs) en raíces, y 236 en hojas, y fueron anotados en 56 términos de ontología (GO) y asociados a 103 rutas metabólicas, principalmente la biosíntesis de fenilpropanoides, y el metabolismo del glutatión. El análisis de GO mostró funciones biológicas exclusivas de la raíz, tal como locomoción (GO: 0040011), metabolismo hormonal (GO: 0042445) y detección de estímulos (GO: 0051606), sugiriendo un papel amortiguador del patrón GF677 frente a la sequía. Se reveló una red de regulación compleja involucrada en la respuesta a la sequía, implicando proteínas claves que están asociadas con la transducción de señalización, regulación hormonal, homeostasis redox, y las barreras de defensa. Hemos identificado 2 genes poco descritos hasta el momento en *P. persica*: el factor de regulación del crecimiento 5 (GRF5) que podría estar involucrado en la expansión celular, y el AtHB12, implicado en la elongación de la raíz. Para confirmar la fiabilidad de la técnica RNA-seq, se validó la expresión de 33 genes mediante RT-qPCR. Se encontraron correlaciones significativas entre ambas técnicas (r=0.88 y 0.95 para raíces y hojas, respectivamente). Los recursos transcriptómicos generados en este estudio proporcionan una amplia base sobre la aclimatación de *P. persica* a la sequía arrojando luz sobre las respuestas moleculares frente al factor de estrés más importante.

Résumé

Le pêcher [*Prunus persica* (L.) Batsch] est l'un des fruits à noyau les plus important, largement établie dans les régions arides et semi-arides. Comme un fruit charnu, la pêche contient 87% d'eau exigeant ainsi un approvisionnement abondant en eau pour assurer son développement et sa saveur estimée. Cependant, en raison des variations climatiques et augmentation de l'aridité, la sécheresse est devenue une des contraintes environnementale responsables des pertes économiques importante. L'usage des porte-greffes tolérants à la sècheresse, dans la production de fruits modernes, semble être une alternative utile pour atténuer les problèmes de déficit hydrique. Cependant les variations transcriptomiques et les mécanismes moléculaires qui modulent l'adaptation des portes greffes à la sècheresse demeurent peu claires. Dans ce travail, un séquençage à haut débit (RNA-seq) a été réalisé afin de générer une vaste enquête sur les changements transcriptomiques et les gènes clés impliqués dans la réponse à la sécheresse, dans les racines (porte-greffe GF677) et les feuilles (greffon, var. Catherina) soumis à 16 jours de stress. En total Douze libraires d'ARN des échantillons de racines et feuilles, ont été construites et séquencées. Ceci a généré 315M de lectures brutes permettant l'assemblage de 22,079 et 17,854 gènes. 500 gènes exprimés d'une manière différentielle ont été identifiés dans les racines y 236 dans les feuilles. Ces gènes ont été annotés comprenant 56 termes d'ontologie (GO) et 103 voies métaboliques. L'analyse des ontologies des gènes a mis en exergue des termes biologiques exclusives des racines, comme la locomotion (GO: 0040011), voie métabolique des hormones (GO: 0042445) et la détection de stimulus (GO: 0051606), ce qui suggère un rôle tampon du porte greffe GF677 face à la sècheresse. En outre, un réseau réglementaire complexe intervenant dans la réponse à la sécheresse a été révélé, impliquant des protéines clés qui sont associés avec la signalisation, hormones régulation, l'homéostasie redox, et les barrières morphologiques de défense. Nous avons aussi identifié 2 gènes peu caractérisés en *P. persica:* facteur de régulation de croissance 5 (GRF5) qui pourrait être impliqué dans la modification de l'expansion cellular et AtHB12 qui peut être associé avec l'élongation des racines. La fiabilité de l'ARN-seq a été validée en analysant les profils d'expression de 33 gènes sensibles à la sécheresse en utilisant une RT-qPCR. Les ressources transcriptomiques générées dans cette étude fournissent une large caractérisation de l'adaptation de *P. persica* à la sécheresse tout en mettant en valeur les principales réponses moléculaires à l'un des facteurs environnemental les plus limitant.

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List of abbreviations

BP: Biological Process

CC: Cellular Process

DEGs: Differentially Expressed Genes

ENA: European Nucleotide Archive

FC: Fold Change

FDR: False Discovery Rate

GDR: Genome Database of Rosaceae

GO: Gene Ontology

IPCC: Intergovernmental Panel on Climate Change

MAS: Marker Assisted Selection

Mbp: Mega base pairs

MF: Molecular Function

MPa: Megapascal

RNA-seq: RNA-sequencing

ROS: Reactive Oxygen Species

RPKM: Reads Per Kb of transcripts per Million mapped reads

RT-qPCR: Real-Time quantitative Polymerase Chain Reaction

TF: Transcription Factor

WEGO: Web Gene Ontology Annotation

1. Bibliographic Review

1.1 Introduction to peach

1.1.1 Botanic description and origin

Peach [*Prunus persica* (L.) Batsch], is one of the most prevalent perennial fruit trees belonging to the family *Rosaceae*, *Amygdalus* subgenus and *Prunus* genus. *Prunus* genus is known to be economically important as it encompasses range array of valuable species like almond [*Prunus dulcis* (Mill) D. Webb], apricot [*P. armeniaca* L.], cherry [*P. avium* L.] and plum [*P. domestica* L.], (Figure 1). Originated from warm areas of China, where it was cultivated since the earliest day of Chinese culture, *Prunus persica* was moved through camel-caravan trade routes to western Asia and eventually to Mediterranean, areas. Peach culture spread then from the Mediterranean basin to the American and European countries (Bielenberg et al., 2009). Currently they are more than 3000 peach cultivars in the world of which China has approximately 1000 (Zhao et al., 2015).

Figure 1. Highly valued species within the *Prunus* genus (peach, almond, apricot, cherry and plum). Adopted from Wikipedia.

1.1.2 Peach genetics and genomic resources

Peach has long been one of the genetically best-characterized species within the family *Rosaceae*. Thanks to its advantageous characteristics, it serves as a model species for stone fruit trees (Bielenberg et al., 2009). Indeed, the genetic simplicity (2x = 2n = 16), the small genome size 230 Mbp, the short juvenile phase (2-3 years) and the self-

compatibility, made peach a desirable target for breeders sharing a common goal of tree fruit improvement (Arús et al., 2012). Additionally, availability of molecular markers (see https://www.rosaceae.org/), genetic maps (https://www.rosaceae.org/; Zeballos et al., 2012) and fortunately the full peach genome sequence (https://www.rosaceae.org/) increase the genetic knowledge about peach as a model plant and provide key genetic resources for the scientific community.

1.1.3 Economic and nutritional importance

The family *Rosaceae* includes many species of great economic importance. Particularly, peach ranks in the world the third most important fruit species behind apples and pears (FAOSTAT, 2016). In terms of production, China is the leader worldwide with more than 11.93 million of tones followed by Italy and Spain (Figure 2). In terms of cultivated surface area, China remains the leader followed by Italy then Spain. In Europe, Spain is the second producer of peach with more than one million of tones (FAOSTAT, 2016) and 84,400 hectares in 2013. The main Spanish communities producing peach are Cataluña and Aragón, respectively (Figure 3).

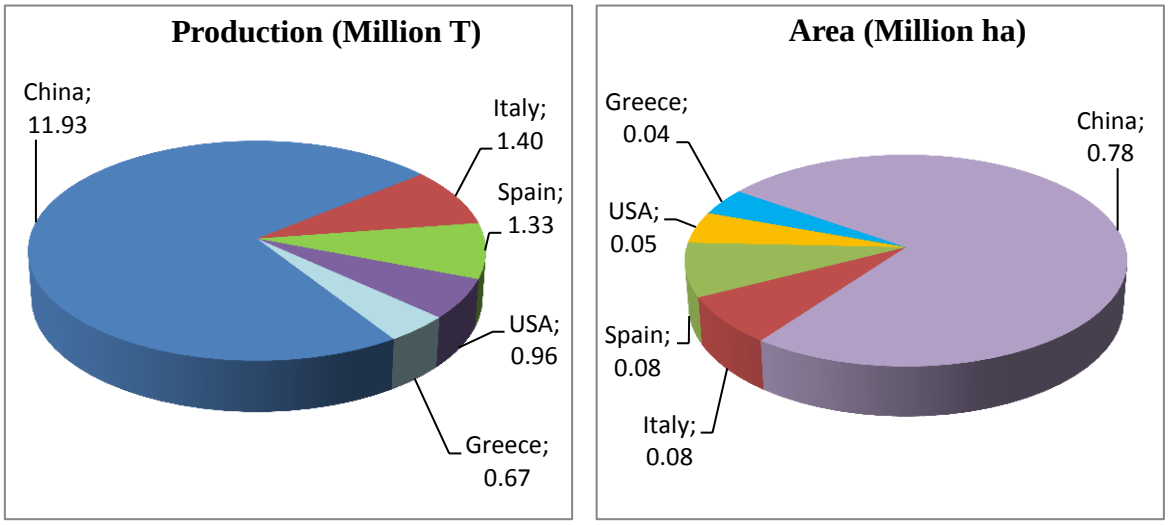

Figure 2. Top peach leader countries worldwide in terms of production and area in 2013. T: Tones, ha: hectares (FAOSTAT, 2016).

Apart from their economic importance, stone fruits have long been highly esteemed for their delicious flavour. They are also excellent source of vitamins and minerals. Due to the high content of vitamins, as well as, other antioxidant and antimicrobial properties, a considerable attention has been given to the potential values of *Prunus* species, in particular peach (Zhao et al., 2015).

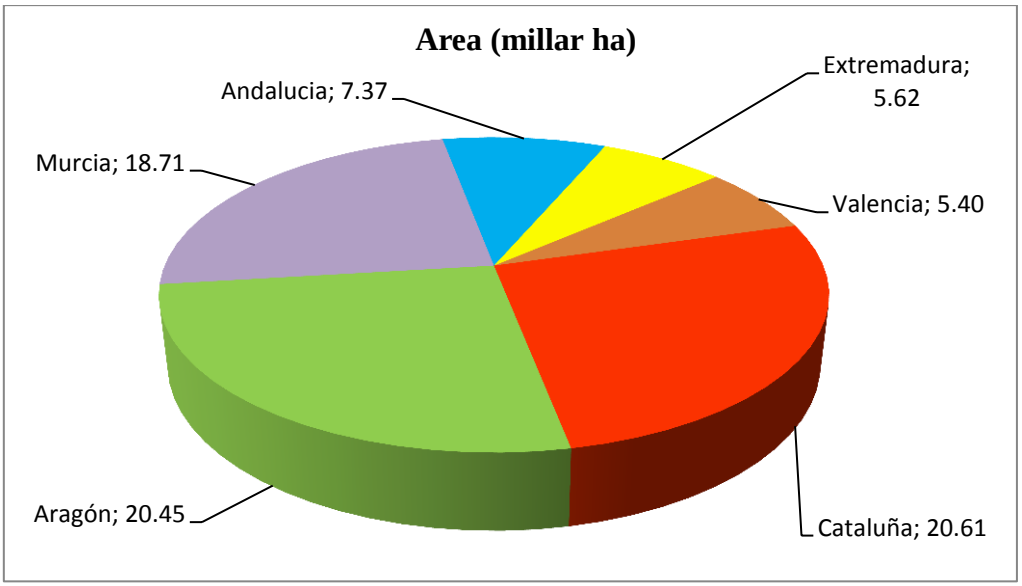

Figure 3. The main Spanish communities producers of peach. ha: hectares (MAGRAMA, 2016).

1.2 Drought stress as a limiting environmental factor: concepts and solutions

1.2.1 Concepts of drought

Drought is known as a complex phenomenon difficult to monitor, and also to precisely define. Depending on the variable used to describe "drought", its definitions may broadly fit into 4 categories (Ngumbi and Kloepper, 2016): (1) meteorological, is a weather- related drought defined as the lack of precipitation over a region for a period of time; (2) hydrological drought, labeled as a significant low water supplies, especially in lakes, streams, reservoirs and groundwater levels, usually after a long period of meteorological drought; (3) agricultural drought, described as a period with declining soil moisture and consequent crop failure and may be the result of combination between the two previous drought-categories; (4) socio-economic drought, known as the incapacity of the water-resource system to satisfy the water demand. Several studies have discussed these four types of drought, however, in this study; we will focus on the agricultural sort.

1.2.2 Scenario of drought

As one of the most important stone crops and widely established in arid and semi-arid regions, *Prunus persica* requires abundant water supply to maintain the appropriate water status and to develop optimal peach flavor. However, under the continuous climatic variations, drought is considered as the most acute stressor, decreasing crop

productivity more than any other environmental factor (Walter et al., 2011). Based on the IPCC fifth assessment report (IPCC, 2014), the global surface average temperature will increase from 1.1˚C to 6.4˚C by the end of this century, then with the global warming effects, intensity and frequency of drought will increase from 1% to 30% by the end of 2100. Such an increase of drought levels will cause inevitably agricultural losses especially in non-developed countries with economies that are highly dependent on agriculture. On the other hand, reduced water resources leads to low productivity and high production costs which in turn result in lower incomes for farmers, increased poverty and aggravated seasonal unemployment rate. However, drought has been intensified not only by climatic factors but also by non-climatic factors such as rapid increases of the world's population leading to many fold increases of water demand, industries and urbanization which contaminate the water supplies, as well as continuously consumption of the ecosystem resources (Yang et al., 2016).

1.2.3 Strategies to cope with drought stress

As sessile organisms, plants have developed specific acclimation and adaptation strategies to overcome low water availability and ensuring their development. Indeed, in response to drought stress, plants exhibit complex mechanisms termed as drought resistance strategies which range from morphological to molecular levels, and may be classified into 4 main aspects (Fang and Xiong, 2015).

- Drought escape: described as the ability of plants to complete their life cycle before the onset of the acute drought. This could be achieved through highly metabolic activity to accelerate growth and flowering time.
- Drought avoidance: relying on phenotypic flexibility and morphological changes to maintain high water status under water shortage conditions. To accomplish drought avoidance, plants generally adopt two main steps: i) reducing water loss by adjusting the stomatal closure and leaves areas, ii) modifying root architecture for enhanced water uptake ability.
- Drought tolerance: defined as the ability of plants to withstand a certain level of water shortage via the regulation of tremendous number of genes and metabolic pathways to protect plant from the resulting stress damage.

- Drought recovery: known as the capacity of plants to restart their growth after exposure to severe drought stress causing loss of turgor pressure and complete leaf dehydration.

It's now accepted that drought pose in near future a veritable threat to climate sensitive economic sectors, especially agriculture. Peach trees, in particular, are mostly cultivated in Mediterranean climate, means that most of vegetative and reproductive phases occur under moderate to severe drought water constraints if irrigation is not applied. Taking together and considering the vulnerability of this limiting factor, many attentions have been focused on useful methods and novel approaches to reduce drought damage and thereby secure the *Prunus* growth. The use of drought-tolerant rootstocks that are able to maintain productivity at low water potential appears to be an efficient alternative to alleviate water deficit problems.

1.3 Importance of rootstocks in modern agriculture

Rootstocks are an essential component in modern fruit production. They are generally used to face market and field limitations, like tree life, biotic and abiotic constraints, fruit maturity and quality, productivity and so on.

It's hence evident that great technical and economic gains can be achieved using suitable rootstocks, selected for their genetic characteristics and environmental adaptations. Indeed, increased demand of new rootstocks with enhanced qualities is observed in nurseries and in markets. This demand has prompted many scientific institutions and public and private organizations, to release new rootstocks that closely satisfy the requirements of the modern fruit growth (Moreno, 2004; Zarrouk et al., 2006; Jiménez et al., 2008; Pinochet, 2009). In Europe, researches have been particularly active in Mediterranean countries such as Italy (Istituto di Coltivazioni Arboree-Pisa), France (INRA-Avignon), and Spain (EEAD-CSIC-Zaragoza; Agromillora S-L., CITA-Aragón, IVIA-Valencia, IRTA-Lérida, and CEBAS-CSIC-Murcia) (Pinochet et al., 1999; Gogorcena et al., 2000; Jiménez, 2006; Byrne et al., 2012).

1.3.1 Types of rootstocks

Rootstocks are of two main types depending on their multiplication methods; (sexual=seeds) and (asexual=clones).

1.3.1.1 Seedling rootstocks

Seedlings arise from the germinated seed of a particular cultivar. In fact, seedling rootstocks have been widely used as the main rootstock source for many fruit trees including peaches, nectarines, apricots, and pears (Durner, 2013). The seed sources can be conveniently divided into two groups: those coming from wild types and those from commercial cultivars. The wild types are usually obtained from peach trees that have escaped domestication and found growing in wild state, while the commercial cultivars are usually those used for commercial processing or drying (Durner, 2013). The seedling rootstocks have certain advantages as they are mostly virus-free, deeper rooted than cloned rootstocks and inexpensive to propagate. However, the major drawbacks of those roots (stocks) are their genetic variability and lack of uniformity which may lead to variability in the growth and performance of the scion (Jiménez, 2006; Gainza et al., 2015). In fact, such variability is likely to occur with seeds from wild-types species of unknown identity, affecting thus important agronomic traits such as vigor and productivity. Nevertheless, this genetic variability can be successfully handled by careful selection of the parental sources and protection from cross pollination.

1.3.1.2 Clonal rootstocks

Clonal are those resulting from vegetative propagation (stool layering, rooted cutting and micro-propagation) of selected individuals (Gainza et al., 2015). Clonal rootstocks have the advantage of preserving uniformity as they generate genetically identical plants that are expected to have identical growth characteristics in a given environment (Byrne et al., 2012). According to Cummins and Aldwinckle (1995) the arising of the first commercial clonal rootstock in the family *Rosaceae* began with apple trees in order to control their vigor and integrate the resistance to apple aphid (*Eriosoma lanigerum* Hausmn.). Then, thanks to its great impact on apple culture, this initiative stimulated the development of similar programs for other species like *Vitis* and *Prunus.* Currently, there are several ongoing efforts to develop clonal rootstocks. Regardless of the new tendency, lesser number of rootstock breeding programs exists compared to the large number of scion breeding programs (Gainza et al., 2015). These may due to the fact that rootstock achievements are planned for long-term and require well-coordinated multidisciplinary effort.

1.3.2 Characteristics of good rootstocks

Once the rootstocks are planted and the orchard is in production, it's not possible to change it without risking serious losses. Thus, the choice of the suitable rootstock is a very crucial step toward successful trees implantation. Indeed the selected rootstock needs to follow these characteristics:

- Have a high degree of compatibility with the scion cultivars.
- Adapted to the adverse climatic and soil conditions of a particular area like cold, drought, heat or salinity.
- Resistant to the most prevalent diseases and pests of the concerned region.
- Exhibit a positive influence on the performance and bearing of the cultivar.
- Possess good nursery potentials like virus-free and germination capacity.

1.3.3 Peach rootstocks

Peach is compatible with itself and totally graft-compatible with most of the species within its taxonomic subgenus *Amygdalus* which includes *P. persica*, *P. dulcis* (Mill) and *P. davidiana.* So that, most successful peach rootstocks, either seedling or cloned, are coming from interspecific hybrids (Bassi and Layne, 2008). The most interesting hybrids from this group are "peach × almond" and "peach × *P. davidiana*" which have been used as useful tool to overcome the soil-adverse factors and are of great importance in peach growing areas, especially Europe and the Mediterranean basin (Jiménez, 2006; Bassi and Layne, 2008). On the other side, plum and plum hybrid rootstocks are also commonly used, however, they are generally better adapted to waterlogged soils and are less vigorous as compared to peach × almond hybrid rootstocks (Zarrouk et al., 2006). Among the former rootstocks, GF677 has become the most widespread one. It's a naturally hybrid, selected by INRA, France in 1965 and it is clonally micro-propagated (Tsipouridis and Thomidis, 2003). GF677 is also well known for its drought tolerance (Jiménez et al., 2013) and its high vigor (10-15% more vigorous than peach seedlings). GF677 is also adapted to infertile and droughty soils with a well-developed root system making it an extremely popular rootstock with high commercially interest (Gullo et al., 2014). Despite these favorable traits, GF677 induces often excessive scion vigor resulting in delayed precocity and low yield in the first years of growth.

Nonetheless, these deficiencies disappear when the tree achieve the vegetative-reproductive phase and go into the full production (Bassi and Layne, 2008).

1.3.4 Influence of tolerant-rootstocks on peach-drought adaptation

The ability of drought-tolerant rootstocks to confer high tolerance to water scarcity depends on several factors, of which vigor is one of the most important (Corso and Bonghi, 2014). Gambetta et al. (2012) reported that vigorous rootstocks have high hydraulic capacity to deliver water to the scion cultivar due to the pivotal role of aquaporins. On the other hand, root's anatomy and architecture can have a significant impact on the vigor of the rootstocks. In fact, it was reported that in peach, vigorous rootstocks have larger xylem vessels leading thereby to better hydraulic conductance and high water status. Having a well-developed root system may also improve water uptake and nutrient adsorption by exploring more efficiently the resources in the soil. In a similar way, it was found that peach tolerant-rootstocks are likely to form extensive root system than sensitive ones for better water access from deeper soils layers (Beckman and Lang, 2003).

Among the other properties, maintaining the photosynthetic capacity may be a good strategy of drought tolerant rootstocks to alleviate water shortage problems. In this context, it was observed that under water stress conditions, GF677 and ROOTPAC 20, two peach drought tolerant rootstocks, are still maintaining their photosynthetic machinery (Jiménez et al., 2013). Such response could vary according to the rootstock-scion combination, as well as, the level of water deficit experienced. Water loss could be also shifted by limiting the transpiration through the regulation of stomatal conductance. Under water shortage conditions, drought-sensitive rootstocks induce a lower stomatal conductance of the scion, leading to higher reduction in the photosynthetic carbon assimilation rates compared to that of drought-tolerant rootstocks.

The accumulation of osmolytes, mainly proline, sorbitol and raffinose, is a valuable indicator of stress tolerance approach. Indeed proline accumulation has been described as a reactive oxygen species (ROS) scavenger and chaperone molecule to stabilize plant's proteins structure and to face water constraints (Corso and Bonghi, 2014). Raffinose and sorbitol may also act as antioxidants, ameliorating the deleterious effects of drought. In the same context, working under drought stress conditions, GF677

grafted with Catherina variety increased the contents in roots and leaves, raffinose in roots and sorbitol in leaves, which lead to an enhanced water use efficiency (Jiménez et al., 2013).

Generally, drought tolerance responses are controlled at the biochemical and molecular levels. Although the biochemical part is partly elucidated, the molecular side requires deeper investigations to identify the tremendous number of genes controlling these responses. Improvement of this knowledge can be achieved through a deep screening of plant's transcriptome under stress conditions to find out the drought-responsive genes and to explore their functional and molecular basis.

1.4 New approaches to *Prunus* transcriptome analysis

The transcriptome is defined as the complete set of RNA transcripts, whether coding (mRNA), or non-coding (ncRNA) including ribosomal RNA (rRNA), transfer RNA (tRNA), short interfering RNA (siRNA), micro RNA (miRNA), small nucleolar (snoRNA), and Piwi-interacting RNA (piRNA) (Figure 4), expressed in the whole organism (Martínez-Gómez et al., 2011).

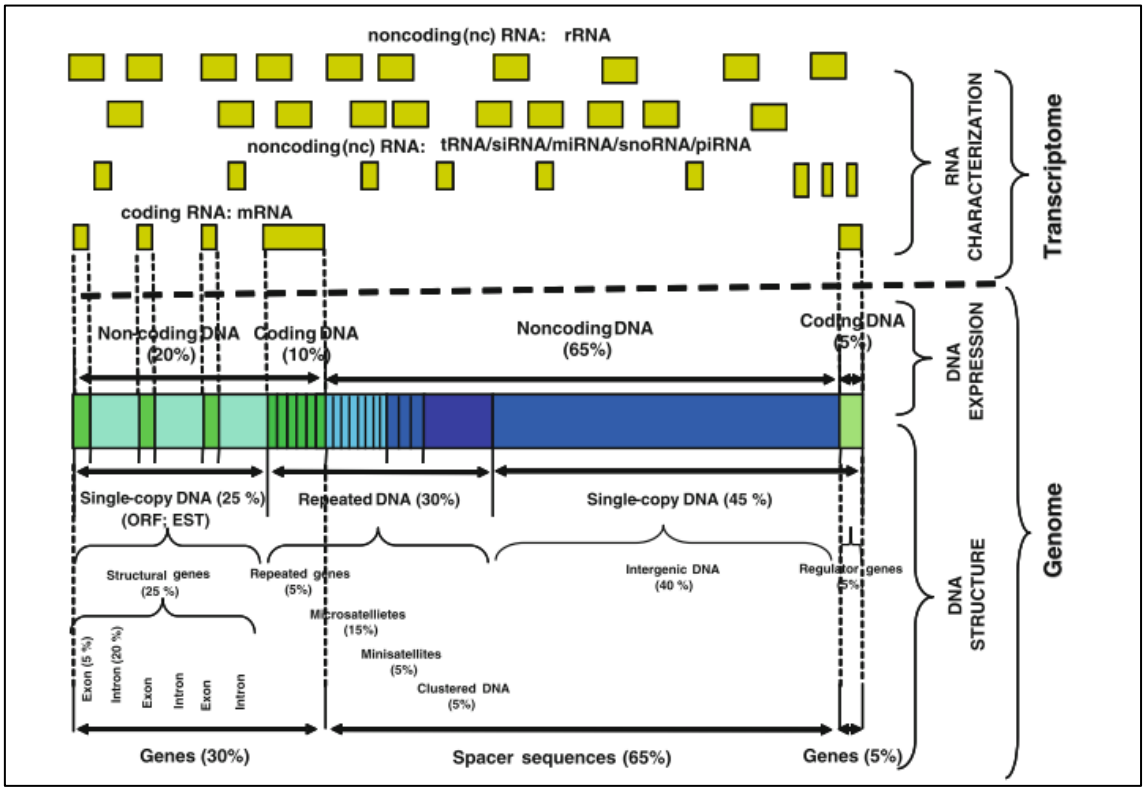

Figure 4. Schematic representation of genome (DNA) organization and transcriptome (RNA) in plants (from Martínez-Gómez et al., 2011).

Over the past decades, huge efforts have been made to develop transcriptomic approaches for highlighting the principle molecular mechanisms of many important plant traits. Among these approaches, expressed sequence tag (EST) and microarrays were largely used representing one of the foremost technological advances in high throughput analysis (Wolf, 2013). Since their arising, they demonstrated a remarkable success in profiling gene expression of different agronomic traits in *Prunus*. In fact, the first transcriptomic analysis in peach was initiated in 2002 with the development and the alignment of the expressed sequences tags (ESTs), that has been applied to other species such as apricot and almond. A collection of more than 100,000 ESTs from different *Prunus* species have been released in public databases and more than 2,500 unigenes have been identified (Martínez-Gómez et al., 2011). As well, EST approach was also assayed for single nucleotide polymorphism detection (SNPs) allowing the detection of more than 6,000 SNPs. Microarray, known also as cDNA biochip, is based on complementary probe hybridization and used to measure the relative abundance of transcripts. It has been employed in peach to obtain meaningful insights into molecular mechanisms underlying complex biological process. Microarrays have been used for the first time on *Prunus* species by 2006, in order to asses fruit quality traits, response to hypoxia and chilling, flower compatibility and other agronomic traits (Martínez-Gómez et al., 2011, references therein). Thousands of genes with different expression patterns were detected using the above-cited technology reinforcing our transcriptomic knowledge.

Despite their value, these platforms display several limitations. In fact, EST technology has been hampered by its low coverage, limiting the detection of low expressed transcripts, its high error rate (as an EST is a single pass sequence) as well as its high cost (Wang et al., 2013). On the other hand, microarray analysis exhibit major drawbacks: its high cross hybridization background noise obstruct the identification of low abundant transcripts. Furthermore, microarrays are closed platforms, relying on the existing knowledge of genomic or EST sequences. Hence, they cannot either cover the presence of novel transcripts or RNA variants like the alternative splicing due to constraints of probe design (Valdés et al., 2013).

Recently, a powerful approach called RNA-sequencing (RNA-seq) has been emerged, overcoming the limitations of the previously described technologies and showing high efficiency of transcriptome profiling.

Since its emergence, RNA-seq has proven itself as a revolutionary transcriptomic approach progressively accepted to examine transcriptional dynamics during various aspects of plant growth, development, and adaptation. However, analysis of RNA-seq must be done with great care, as it not straightforward (Conesa et al., 2016).

1.5 RNA-sequencing

RNA-sequencing, also known as whole transcriptome shotgun sequencing, refers to the use of high throughput sequencing technologies for characterizing the RNA content and revealing the molecular constituent of cells (Valdés et al., 2013). It is a revolutionary tool toward transcriptome assessment, providing insights into fundamental unanswered questions about plant science. This technology is based on cDNA sequencing (complementary DNA), generating thereby randomly decomposed short reads of up to several hundred base pairs (bp). In absence of previously genome or transcriptome information, transcripts need to be reconstruct which is labeled as de novo assembly. In the case of the genome information availability, the obtained reads can be directly aligned on the reference genome. This process is referred as guided reference assembly. The use of RNA-seq in *Prunus* began in 2010 (Martínez-Gómez et al., 2011), permitting a high coverage of the transcriptome which allows more accurate quantification of differential transcript expression as well as the detection of low abundant transcripts and novel genes. It was used for understanding the responses of peach leaves in *Plum pox* Virus infection (Rubio et al., 2014), the responses to drought in Mongolian almond seedlings (Wang et al., 2015), and to hypoxia in *Prunus* roots (Arismendi et al., 2015) as well as the assessment of bud dormancy in pear (Liu et al., 2012) and volatilome peach analysis (Li et al., 2015). Although there are a great number of transcriptome analyses in *Prunus* species, so far no research has been done to investigate the molecular mechanisms of drought adaptation in peach studying roots and leaves at the same time, which make the originality of the present work.

2. Objectives

The final objective of the present study is to shed light on the complex molecular mechanisms that underlie the responses of *Prunus persica* to water deficits and the identification of candidate genes associated with drought tolerance. In order to achieve this goal, various key steps were carefully carried out, starting from:

- Exploration of the transcriptomic variations among well-watered plants (control) and water deprived plants (drought-stressed), using Illumina's HiSeq 2000 Sequencing System to sequence the RNA, in roots (GF677 rootstock) and leaves (graft, var. Catherina).
- Identification of differentially expressed genes (DEGs) and gene expression profiling were performed for deeper understanding of *Prunus persica*· molecular basis in response to drought stress. Theses DEGs could be potential targets of *Prunus* breeding programs as a key step toward improving drought acclimation in the family *Rosaceae* by using advanced marker-assisted selection (MAS).
- Annotation and functional categorization of DEGs were conducted to help shape transcriptomic dynamics at biological, molecular and cellular levels triggered by drought stress.
- Validation of the RNA-seq reliability and the expression level of shortlisted genes chosen for their inferred drought-related functions using RT-qPCR assay.

In contrast to other studies, this work represents the first characterization of drought-related genes in *Prunus persica* that involves assessing both roots and leaves at the same time. These tissues were chosen as the roots are the first plant tissue to perceive drought stress, while leaves are central organs in the control of water loss. Our data contribute to the understanding of drought responses in plants and serve as a publicly available resource for future gene expression, genomic and functional studies in *Prunus* spp.

3. Materials and Methods

3.1 Plant material and drought stress experiment

Clonally propagated plants from the GF677 rootstock *(Prunus dulcis* Miller × *P. persica*), which was selected for its high level of drought tolerance, were acquired from a commercial nursery (Agromillora Iberia, S.L., Barcelona, Spain). The rootstocks were grown for two weeks in 300 cm^3 pots containing a peat substrate, and then they were micrografted with *P. persica* var. Catherina. Subsequently, 30 representative plants were transplanted into 15 L containers with TKS-1, a 1:1 ratio of sand to peat substrate (Floragard, Oldenburg, Germany) and 2 g kg^{-1} Osmocote 14-13-13 (The Scotts Company LLC, Maryville, OH, USA). The plants were grown in an experimental greenhouse Zaragoza, Spain (41º43'N, 0º48'W) under controlled environmental conditions (23°C day/18°C night, 14 h light/10 h dark photoperiod) for 21 days before the start of the experiment. During this period (April 2011 to May 2011) the plants were watered daily until runoff was visible.

The drought stress experiment started on May 14 and continued for 16 days. The 30 plants were randomly separated into two groups: well-watered plants (the control plants) and water-deprived plants (the drought-stressed plants). The control plants were watered daily to field capacity while the stressed plants were watered with 80% of the quantity of water that had been evapotranspired the previous day (Marcelis et al., 2007). The soil water content was measured using time domain reflectometry (TDR), with 20 cm-long probes inserted vertically into the containers (Moret-Fernández et al., 2012). The soil water content and physiological parameters, namely, stem water potential (Ψs), stomatal conductance (g_s), photosynthetic rate (A_N), and intercellular CO_2 concentration (*Ci*), were recorded for both groups. These measurements were taken on days 0, 7, 12, and 16 after the start of the experiment, on clear days between 8:30 and 11:00 (Jiménez et al., 2013). On day 16, leaf and root samples of three randomly selected biological replicates were collected from both the control and drought-stressed plants (12 samples in total). The plant tissues were then frozen in liquid nitrogen and stored at -80°C for later use.

3.2 RNA extraction

The total RNA from the three biological replicates was extracted from the root and leaf samples of the control and water-deprived plants according to the method described by Meisel et al. (2005), which was adapted to mini-preparations (Jiménez et al., 2013). Subsequently, samples were treated with DNase I (Thermo Fisher Scientific, Waltham,

MA, USA) to remove the contaminating genomic DNA. RNA integrity and purity were assessed using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only RNA samples with A260/A280 ratios from 1.9 to 2.1, A260/A230 ratios ≥ 2, and RNA integrity values > 8 were used in the subsequent experiments.

3.3 RNA next generation sequencing

Equal amounts of total RNA of each tissue from each experimental group were used to construct 12 RNA libraries (see Figure 5). Total RNA was submitted to Otogenetics Corporation (Atlanta, GA, USA) for RNA-Seq assays. Briefly, 1-2 μg of cDNA was generated using Clontech Smart cDNA kit (Clontech Laboratories, Inc., Mountain View, CA USA, catalog# 634925) from 100 ng of total RNA. cDNA was fragmented using Covaris (Covaris, Inc., Woburn, MA USA), profiled using Agilent Bioanalyzer, and subjected to Illumina library preparation using NEBNext reagents (New England Biolabs, Ipswich, MA USA, catalog# E6040). The quality, quantity and size distribution of the Illumina libraries were determined using an Agilent Bioanalyzer 2100. The libraries were then submitted for Illumina HiSeq2000 sequencing according to the standard operation. Paired-end 90-100 nucleotide (nt) reads were generated and checked for data quality using FASTQC (Babraham Institute, Cambridge, UK). FASTQ file was sent to customer for downstream analysis. All the raw reads data were deposited with the European Nucleotide Archive (ENA) as part of project PRJEB12334.

3.4 Transcript assembly

The quality of the raw paired-end reads was monitored using FastQC v0.10.0. Low quality segments, erroneous base calls, or adapter fragments were trimmed using ConDeTri Perl script v5.8.9, which discarded data with poor quality scores ($Q < 25$) or read lengths < 35 bp (Smeds and Kunstner, 2011).

Post-processed reads were mapped to the *P. persica* Whole Genome Assembly v1.0 using TopHat software v2.0.3. This software was selected as a mapping tool because it can generate a database of splice junctions based on the gene model annotation (Trapnell et al., 2012). The TopHat parameters were set at < 3 mismatches when mapping reads and a maximum of 20 multiple hits for each library. The resulting aligned sequences were used with the Cufflinks suite v1.3.0. In order to generate assembled transcripts for each tissue type and each experimental group. These

assemblies were then merged together using the Cuffmerge tool to provide a uniform basis for calculating transcript expression. All the assembled transcripts were deposited at the ENA under accession numbers HADJ01000001–HADJ01060423.

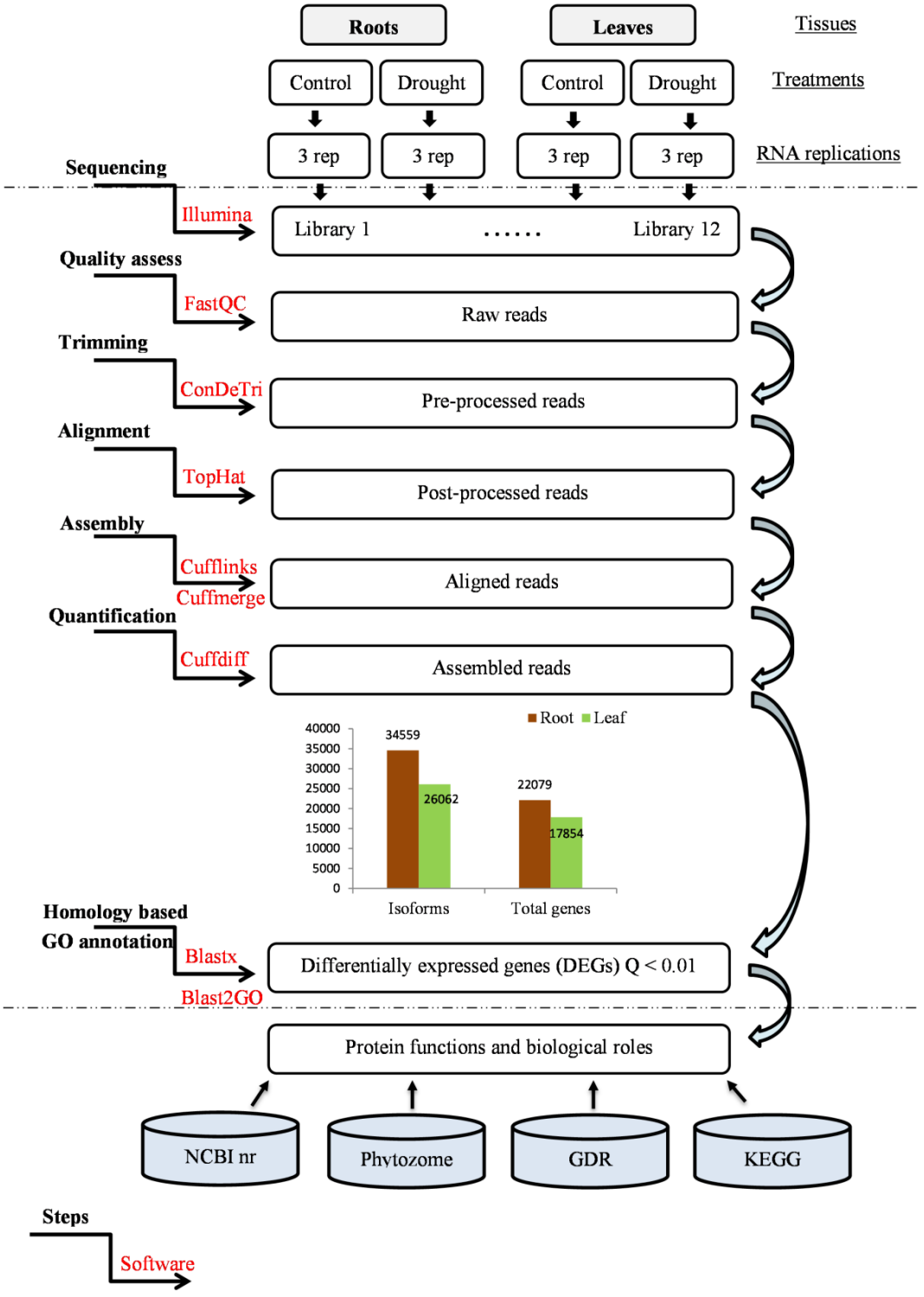

Figure 5. RNA-sequencing pipeline

3.5 Quantification of the levels of gene expression and differential expression analysis

Changes in the relative abundance of transcripts in drought versus control conditions were quantified and normalized to the number of reads per kilobase of transcripts per million mapped reads (RPKM). The gene expression levels were then estimated using the Cuffdiff program from the Cufflinks suite. The statistical significance of the differential expression level of each gene in roots and leaves was determined by initially setting the false discovery rate (FDR)-adjusted *P*-values at 0.05 (which are known as *Q*-values), using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). However, in order to reduce the likelihood of false positives, only DEGs with a *Q* value < 0.01 were considered for further analysis. The complete workflow of the RNA-seq analysis is provided in Figure 5.

3.6 Homology search and functional annotation

Significant differentially expressed genes were annotated using BLASTX by scanning four standard resources: the NCBI nr database (www.ncbi.nlm.nih.gov), Phytozome11 (https://phytozome.jgi.doe.gov/pz/portal.html), the Genome Database of Rosaceae (GDR) (https://www.rosaceae.org/), and the Kyoto Encyclopedia of Genes and Genomes (KEGG). The E-value cut-off was set to 1E-5 and a 70% query coverage threshold was used to discard partial/single-domain protein matches. A gene ontology (GO) analysis was performed using standalone Blast2GO v3.2 with the same E-value cut-off. This software assigned GO terms to each DEG to allow their putative functions to be predicted in terms of molecular functions (MF), biological processes (BP), and cellular components (CC) (Gotz et al., 2008). These annotations were enhanced by merging them with InterProScan-assigned GO terms, and then running the annotation augmentation module (Annex). The resulting GO terms were plotted and visualized with the Web Gene Ontology Annotation Plot (WEGO) tool (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) (Ye et al., 2006).

3.7 Gene ontology (GO) enrichment

GO enrichment was carried out for the DEGs associated with both tissues using the singular enrichment analysis (SEA) function of the web-based tool AgriGO (http://bioinfo.cau.edu.cn/agriGO/). The input list consisted of the whole set of DEGs, while the annotation of Peach Genome (v1.0 Joint Genome Institute) was used as a pre-

computed background. Overrepresented terms in the three main categories (BP, MC, and CC) were filtered using Fisher's exact test and the Benjamini-Hochberg multiple testing correction (Q-value < 0.05).

3.8 RT-qPCR validation

In order to confirm the reliability and accuracy of the RNA-seq analysis, RT-qPCR was performed on a set of 33 root and leaf genes selected for their putative drought-related functions, including 16 up-regulated genes ($Log_2FC > 2$), seven unchanged genes ($|Log_2FC| < 2$), and ten down-regulated genes ($Log_2FC < -2$). In these expressions, FC is the fold change ratio between the drought-stressed and control group RPKM expressions (Table 1).

Table 1. Genes from roots (GF677 rootstock) and leaves (graft, var. Catherina) selected for RT-qPCR validation. The RPKM values are indicated for the control and drought-stressed plants. The fold change (FC) was calculated as the ratio between the drought-stressed and control plants. The gene ID is the same as that deposited in the ENA. The grey rows indicate genes with $|Log_2FC| < 2$.

Tissues	Gene ID	Description	Abbreviation	RPKM		Log_2FC
				Control	Drought	
Root (stock GF677)	GF677_18885	Dehydrin Rab 18	Rab18	1.59	45.34	4.83
	GF677_10293	Unknown protein	Unknown	0.23	6.03	4.72
	GF677_9678	Metalloendoproteinase 1-like	MMP1	0.44	9.05	4.36
	GF677_15114	Phosphatase 2C 24	PP2C.24	0.41	7.78	4.25
	GF677_18824	at5g66780 mud21_2	at5g66780	1.40	21.05	3.91
	GF677_2910	Probable nucleoredoxin 2	NRX2	2.90	24.53	3.08
	GF677_10709	Gibberellin2 beta dioxygenase 2	GA2OX2	1.32	11.19	3.08
	GF677_10265	Transcription repressor MYB6	MYB6	3.32	25.32	2.93
	GF677_9569	NAC domain containing protein100	NAC100	2.79	18.90	2.76
	GF677_8702	Ribulose biphosphate carboxylase small chloroplastic	RBCS	5.95	36.21	2.61
	GF677_6534	Homeobox- leucine zipper AtHB12_like	AtHB12	25.00	117.06	2.23
	GF677_3749	Dehydration-responsive protein RD22	RD22	8.89	40.23	2.18
	GF677_10556	LRR receptor-like serine/threonine-protein kinase	LRR	4.21	8.89	1.08
	GF677_18270	Probable glutamate carboxypeptidase 2	GCPII	10.21	15.11	0.57
	GF677_721	Cinnamoyl-CoA reductase 2-like	CCR2	30.51	14.60	-1.06
	GF677_17720	Ethylene-responsive transcription factor RAP2-11	RAP2-11	40.94	8.14	-2.33
	GF677_7066	Abscisic acid receptor PYL4-like	PYL4	59.46	5.52	-3.43
	GF677_17672	2-Aminoethanethiol Dioxygenase	ADO	100.52	5.94	-4.08
	GF677_300	1-aminocyclopropane-1carboxylate oxidase homolog 1	ACO1	12.70	0.64	-4.31
	GF677_14474	Germin-like	GLP	58.36	1.42	-5.36
	GF677_2725	Lignin-forming anionic peroxidase 4-like	APRX4	20.68	0.29	-6.15
Leaf (graft, var. Catherina)	cvCatherina.11767	Purple acid phosphatase 17 like	PAP17	2.94	70.81	4.59
	cvCatherina.14437	Alpha-xylosidase 2	XYL2	1.67	22.09	3.73
	cvCatherina.15841	SPX domain-containing 1-like	SPX1	30.42	321.97	3.40
	cvCatherina.15894	Monogalactosyldiacylglycerol synthase 2, chloroplastic	MGD2	2.95	18.68	2.66
	cvCatherina.11558	Glycerophosphoryl diester phosphodiesterase 3	SHV3	52.65	110.59	1.07
	cvCatherina.6050	Abscisic acid receptor PYL8	PYL8	38.04	50.06	0.40
	cvCatherina.13766	Pyroglutamyl-peptidase 1-like	PGPEP1	82.33	67.07	-0.30
	cvCatherina.370	Pectate lyase 1 related	PEL1	14.26	7.17	-0.99
	cvCatherina.5807	Ethylene-responsive transcription factor ERF106	ERF106	11.10	2.22	-2.32
	cvCatherina.12386	Aquaporin TIP1.2	TIP1.2	7.48	0.86	-3.13
	cvCatherina.5855	Pectin methylesterase inhibitor	PMEI	17.60	1.53	-3.53
	cvCatherina.7039	Probable FBOX protein at5g04010	at5g04010	21.19	0.20	-6.72

The total RNA samples (1.5 μg) were treated with DNAse I to remove the contaminating genomic DNA. Subsequently, the samples were reverse transcribed using oligo $(dT)_{18}$ as a primer with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). The RT-qPCR reactions were performed with the 7500 Fast Real-Time PCR System v2.0.1 (Applied Biosystem by Life Technologies, Grand Island, NY, USA) using bi-technical replicates and tetra-biological replicates for each tissue-experimental group (two of the biological replicates were from the same plants used in the RNA-seq analysis). The reactions were performed using 10 μl of SYBR Green Master Mix (Kapa Biosystems, Boston, Massachusetts, USA), 1 μl of each primer (making a total of 4 μM), and 5 μl of diluted cDNA in a final volume of 20 μl. Control cDNA and control primer were included for each run. The primers were designed using NCBI primer-BLAST software (http:/www.ncbi.nlm.nih.gov/tools/primer-blast/) according to the following criteria: primer size of 18–22 bp, GC content between 40 and 60%, amplicon size of 90–160 bp, and annealing temperatures from 57°C to 62°C. Moreover, the primers were aligned to the target gene sequence using BioEdit software v7.2 to ensure specific annealing. The likely secondary structures were also assessed to avoid hairpins and primer dimers. A BLASTN scan of the theoretical amplicon was carried out to test the homology to the target genes. Finally, each of the products underwent gel electrophoresis to confirm the presence of a single amplicon of the expected length. The primers features are listed in Table S1 and primers sequences will be available in the online published manuscript. The efficiencies and quantification cycle (Cq) for each gene were calculated using the LinRegPCR program (Ruijter et al., 2009). Gene expression measurements were determined using the gene expression difference (GED) formula (Schefe et al., 2006). Actin 2 and AGL-26 were used as reference genes for data normalization. The relative expression was calculated with respect to the GF677 rootstock control group. A correlation analysis between the levels of gene expression according to the RNA-seq and RT-qPCR analyses was performed using SPSS v23.0.

4. Results

4.1 Phenotypic and physiological response to drought stress

After a period of drought stress of 16 days, the main visible effects on the plants were wilting and slight defoliation due to the decreased turgor pressure and the shrinkage of the leaf cells. In order to verify that the visual symptoms were indicative of exposure to water deficit conditions, the soil water content and standard physiological parameters of drought-induced effects on leaves were measured (Table 2). The soil water content dropped remarkably from 26.63% in the control plants to 10.69% in the drought-stressed plants, which indicated the presence of decreased turgor pressure and therefore explained the wilting. A decrease was also observed for the stem water potential (Ψs) in response to the reduction in soil water content, further confirming that the plants experienced drought stress. In addition, as leaf water status is considered to be a reliable indicator of plant water balance, stomatal conductance (g_s), intercellular CO_2 concentration (*Ci*), and the net photosynthetic rate (A_N) were measured. The results revealed that the drought-stressed plants exhibited a lower stomatal conductance compared to the control plants. This reduction led to a significant decline in intracellular CO_2 concentrations, which decreased from 277.30 μmol CO_2 mol^{-1} to 261.2 μmol CO_2 mol^{-1}. Taken together, these results explain the observed slowdown of the photosynthetic machinery (A_N) as a result of the drought conditions.

Table 2. Soil water content (SWC), stem water potential (Ψs), stomatal conductance (g_s), CO_2 concentration (*Ci*), and photosynthetic rate (A_N) in leaves (graft, var. Catherina) in control and drought-stressed plants after 16 days.

Treatments	SWC %	Ψs MPa	g_s mol H_2O $m^{-2}s^{-1}$	*Ci* μmol CO_2 mol^{-1}	A_N μmol CO_2 m^{-2} s^{-1}
Well-watered	26.63 ± 0.18	-0.73 ± 0.05	0.56 ± 0.03	277.30 ± 2.17	20.82 ± 0.54
Water-deprived	10.69 ± 0.28	-1.08 ± 0.02	0.37 ± 0.03	261.27 ± 4.27	18.35 ± 0.42

MPa: megapascal. The means were compared using t-Student test and were found to be significant in all cases ($P < 0.05$).

4.2 RNA-sequencing and transcriptomic profiles

As roots are the first organs to be exposed to drought, and leaves are the first to sense water loss, both tissues were sampled from the control and the drought-stressed plants and used for transcriptome analysis to obtain an overview of the responses of *P. persica* during water deprivation. Three biological replicates were processed in order to construct 12 RNA libraries (Figure 5). A total of 315M paired 100 bp reads were

generated, ranging from 20.81 to 61.30M raw reads per library. Among those, more than 188M (59.85%) high-quality sequences were retained after pre-processing and filtering out reads containing adaptors, short reads (< 35 bp), and reads with low quality scores (Q < 25). The remaining 136M single and paired reads (72.34% of all the high-quality sequences) were mapped to the *Prunus* reference genome (from 10.18M to 14.78M reads per library), which showed that the quality of these mapped genes was good enough to conduct the subsequent analysis. A summary of the raw data generated, and the trimmed and mapped reads, is summarized in Table 3.

Consequently, the aligned sequence reads were used for reference-guided assembly and thereafter merged using the Cufflinks-Cuffmerge workflow. Overall, 34,559 and 26,062 transcript isoforms were obtained from roots and leaves, respectively, which correspond to 22,079 and 17,854 genes, respectively (Figure 5).

Table 3. Summary of RNA sequencing data in million (M) reads from 12 RNA libraries of control and drought-stressed roots (GF677 rootstock) and leaves (graft, var. Catherina) after 16 days of drought.

Libraries	Raw reads (M)	Clean reads (M)			Mapped reads (M)		
		Paired	Unpaired	Total	Paired	Unpaired	Total
RC 1	61.30	28.54	12.31	40.85	10.71	4.16	14.78
RC 2	25.52	10.10	5.15	15.25	7.83	4.07	11.90
RC 3	22.69	8.48	4.75	13.23	7.03	3.95	10.98
RD 1	24.97	9.37	5.04	14.41	7.49	4.05	11.54
RD 2	24.49	7.76	5.47	13.23	5.89	4.29	10.18
RD 3	20.81	8.14	4.79	12.93	6.51	3.81	10.32
LC 1	23.12	8.72	5.44	14.16	7.14	4.57	11.71
LC 2	23.02	9.05	5.30	14.35	7.48	4.51	11.99
LC 3	22.30	8.01	4.76	12.77	6.63	4.07	10.70
LD 1	22.00	8.33	4.42	12.75	6.77	3.75	10.84
LD 2	22.86	6.20	5.51	11.71	5.37	4.93	10.30
LD 3	22.00	8.63	4.33	12.96	7.20	3.78	10.38
Total	315.08	121.33	67.27	188.60	86.05	49.94	135.99

RC: root control; RD: root drought-stressed; LC: leaf control; LD: leaf drought stressed.

4.3 Analysis of differentially expressed genes (DEGs)

In order to explore the transcriptional response to drought stress, genes in both roots and leaves were tested for differential expression between the control and drought conditions. Expression levels for each gene were calculated and normalized to RPKM values. Initially, the multiple testing corrections involved a Q-value < 0.05 and a total of

1,171 genes were found to exhibit differential expression (813 in the roots and 358 in the leaves). Subsequently, a more stringent *Q*-value was applied (< 0.01) in order to identify the most reliable DEGs. In this analysis, 500 DEGs were identified in the roots and 236 in the leaves. The distribution of these genes is provided in Figure 6.

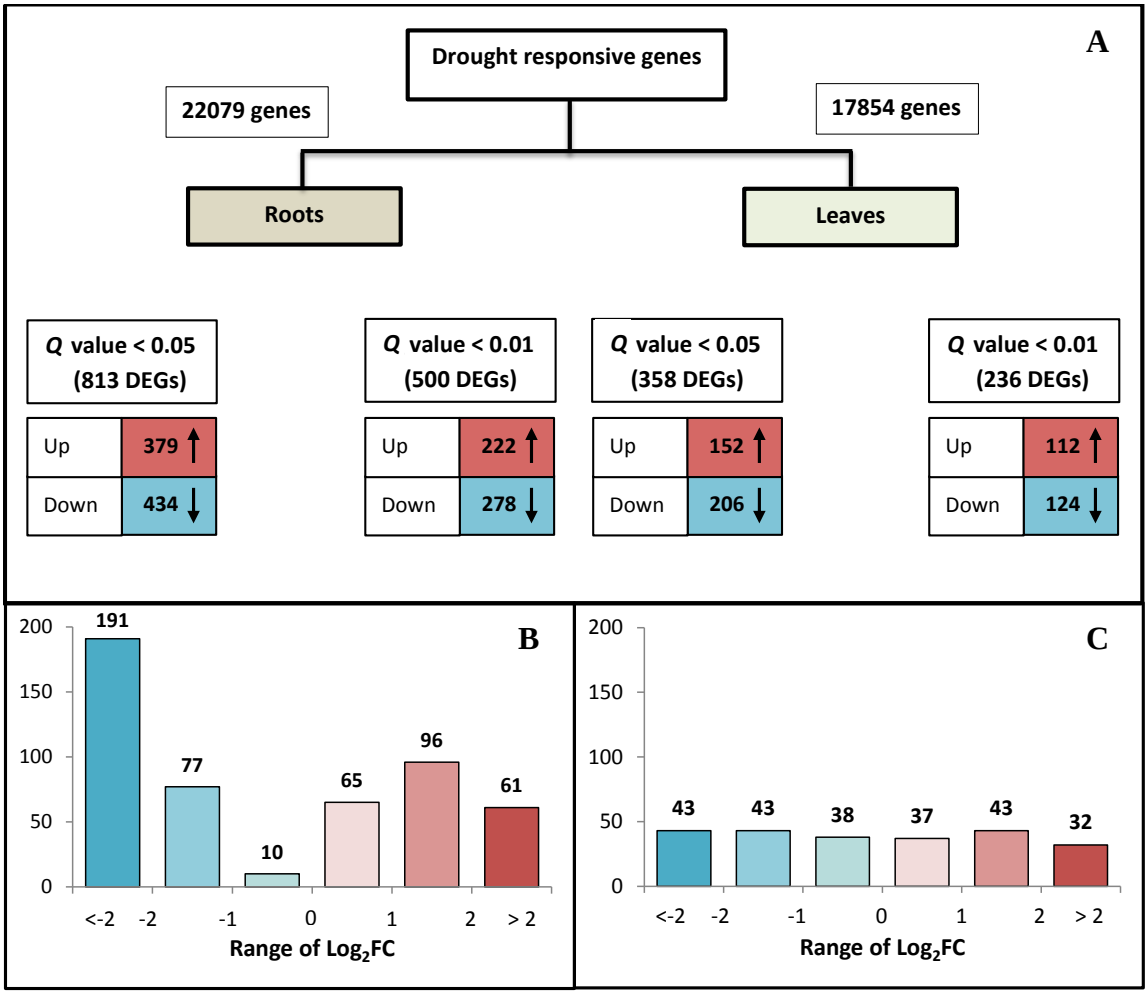

Figure 6. (A): Number of total and differentially expressed genes (DEGs) showing either up-regulation or down-regulation at different *Q*-values in roots (GF677 rootstock) and leaves (graft, var. Catherina). Up: up-regulated genes (red); down: down-regulated genes (blue). (B), (C): Relative expression of DEGs selected at *Q*-value < 0.01 in roots and leaves. The color intensity indicates the level of the change in expression: a darker color represents a larger change in expression. The *x*-axis indicates the range of Log_2FC. The fold change (FC) was calculated as the ratio between the drought-stressed and control plants, while the *y*-axis indicates the number of detected DEGs.

As illustrated, under drought stress conditions, the number of down-regulated genes was slightly higher than the number of up-regulated genes. Furthermore, the analysis revealed that there were approximately twice the number of DEGs in the roots than in the leaves, confirming the expectations that i) the root is the first organ that senses and

is affected by drought stress and ii) roots respond faster to stress than leaves, undergoing more complex gene regulation during water deprivation. These results further highlight the key role of rootstocks as stress buffers.

4.4 Annotation of differentially expressed genes (DEGs)

In order to assign putative functions to the DEGs, all identified transcripts with a *Q*-value < 0.01were subjected to Blast2GO annotation. After enhancing the annotation by running InterProScan and Annex, 1,904 root annotations and 815 leaf annotations were obtained. Out of the 500 DEGs in the roots, 361 (72.2%) were successfully annotated; however, 52 (10.4%) DEGs did not match any of the sequences characterized in the databases, which may indicate the presence of novel genes. Among the root sequences, 53 (14.7%) were either hypothetical or uncharacterized proteins. Regarding the leaves, 169 out of 236 sequences (71.6%) were annotated, including 19 (11.2%) with hypothetical or uncharacterized functions, while 34 (14.41%) sequences had no significant hits. A summary is presented in Figure S1. Regarding the matched species, the majority of the highest-scoring hits were from *P. persica* (70.49% in the root and 79.69% in the leaves) and *P. mume* (24.63% in the roots and 16.75% in the leaves), which belongs to the family *Rosaceae*. These results confirm the quality of our data and the assembly process. A graph displaying the species distribution and the top BLASTX hits is provided in Figure S2.

The entire set of DEGs was subjected to GO analysis in order to achieve a broader functional characterization. As a result, 500 DEGs in the roots and 236 in the leaves were classified into 56 subcategories within three main categories (BP, MF, and CC). In total, 283 DEGs in the roots and 137 in the leaves were associated with BP terms, 298 root DEGs and 139 leaf DEGs were associated with MF terms, and 211 root DEGs and 108 leaf DEGs were annotated with CC terms. Note that in many cases the same sequence can be assigned to more than one category. In both tissues, the most represented BP subcategories were “metabolic process”, followed by “cellular process” and “response to stimulus” (Figure S3). As for MF, the major subcategories were “binding” and “catalytic activity”. Finally, among the CC terms, “membrane” was the most dominant subcategory for the roots, followed by “cell” and “cell part”, which were associated with the leaves. These results, with a comprehensive list of GO subcategories, are plotted in Figure 7.

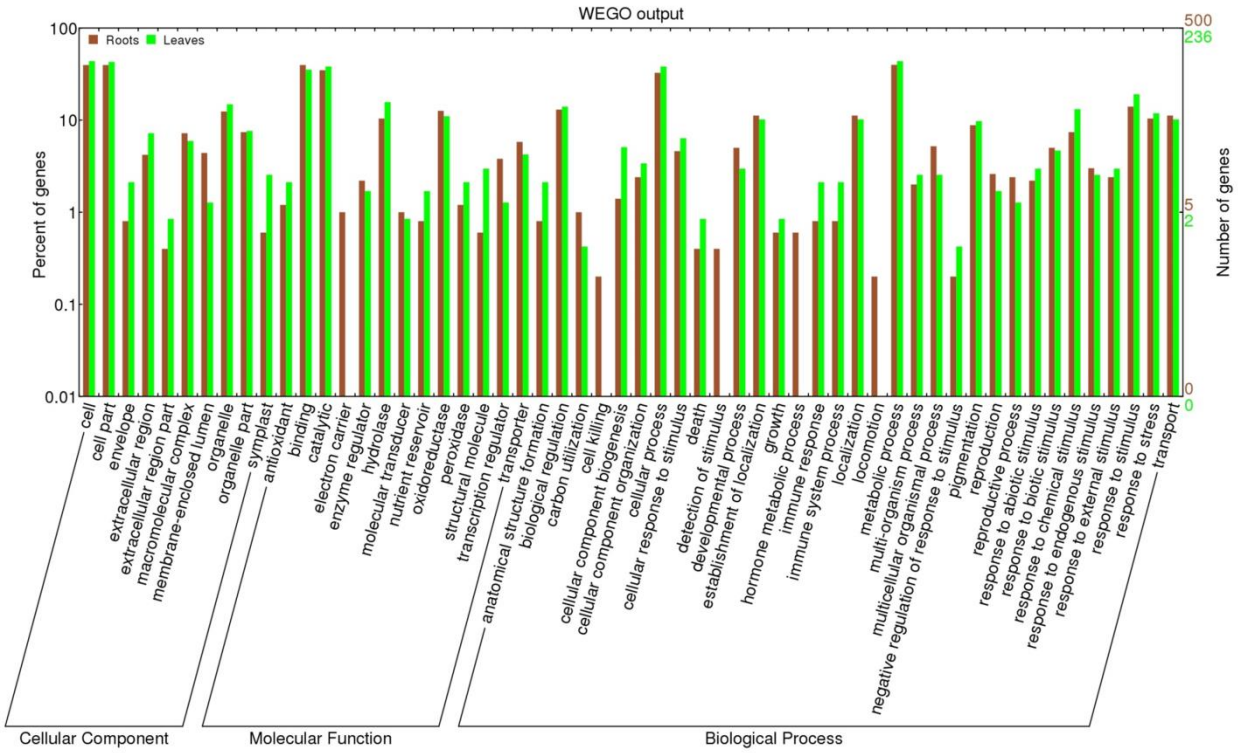

Figure 7. Histogram of gene ontology (GO) terms assigned to differentially expressed genes (DEGs) in roots (GF677 rootstock, n = 500) and leaves (graft, var. Catherina, n = 236). The DEGs are categorized into three main groups: cellular components (CC), molecular functions (MF), and biological processes (BP). Note that the vertical axes use a logarithmic scale.

The annotations within the BP category were the most informative, as they are easier to interpret in the context of drought responses. The list of DEGs annotated with "metabolic process" is an important resource for the identification of novel genes involved in drought acclimation. In addition, Figure 7 shows that "locomotion" (GO: 0040011), "hormone metabolic process" (GO: 0042445), "detection of stimulus" (GO: 0051606), and "cell killing" (GO: 0001906) were exclusively associated with root DEGs, thus highlighting the essential role of roots in plants' responses to drought. Moreover, DEGs involved in "response to stimulus" (GO: 0006950) seem to play a pivotal role in drought sensing and the responses.

The GO enrichment analysis was performed using a *Q*-value < 0.05. The results revealed that, in the analysis of the root tissue, there were significant differences in the 26 GO terms between the DEGs and the genome reference, while only three molecular GO terms were enriched in the analysis of the leaves ("heme", "iron," and "tetrapyrrole binding") (data not shown). In the analysis of the roots, the enriched GO terms were related to BP and MF (12 and 14 GO terms, respectively, as illustrated in Figure S4).

The most significant BP terms, such as “responses to stimulus” (GO: 0050896), “responses to stress” (GO: 0006950), and “biotic stimulus” (GO: 0009607), are shown in Figure 8.

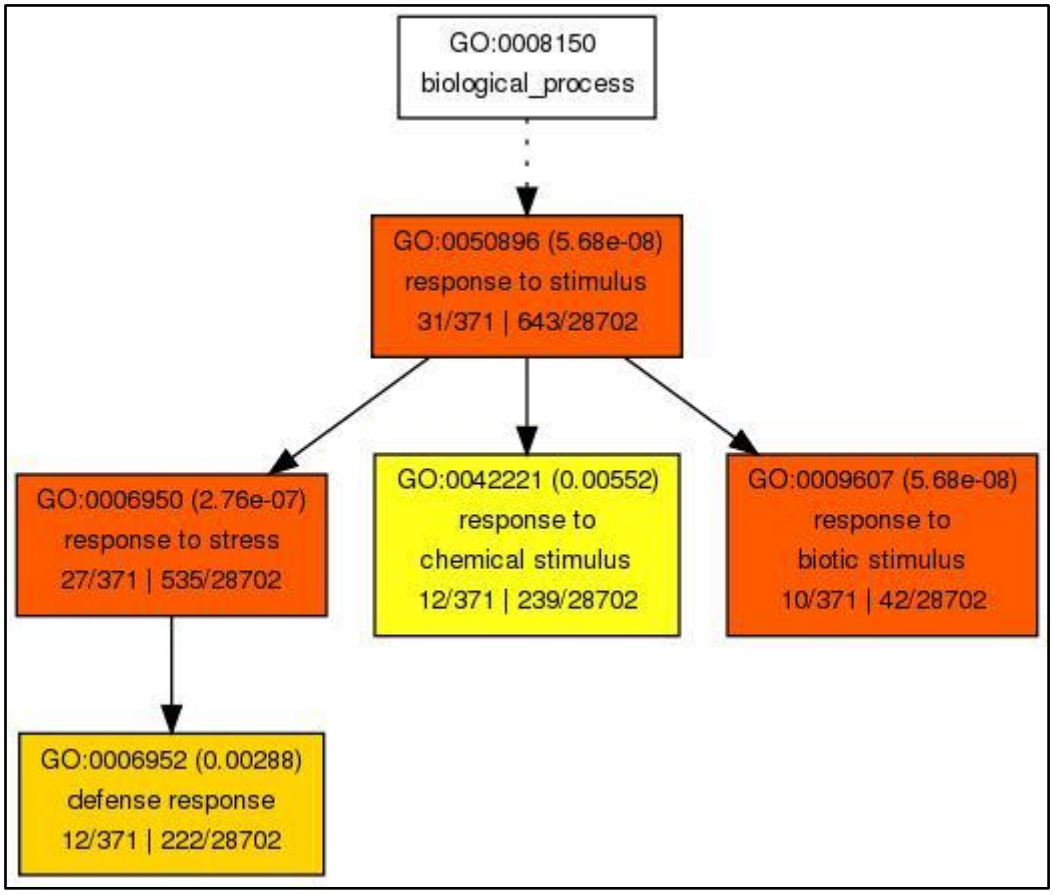

Figure 8. The top enriched gene ontology (GO) terms in the biological processes (BP) category in roots (GF677 rootstock) after a singular enrichment analysis (SEA) in AgriGO. Each box indicates the GO number and a full description, with the *Q*-value in parenthesis. The numbers on the left side indicate the fraction of DEGs sharing the GO term. The numbers in the right side indicate the background numbers of genes associated with the GO term in the *P. persica* genome. The significance of the enrichment is displayed using a color scale from yellow to red. The complete set of enriched GO terms is illustrated in Figure S4A.

A heat map of DEGs involved in the “response to stimulus” is shown in Figure 9 (see list in Table S2). These genes were clustered into five clades according to their expression patterns. The genes in clusters C1, C4, and C5 had higher levels of expression in the control plants than in the drought stressed plants; and they mainly encode peroxidases and proteins related to the responses to biotic stress, such as major allergen proteins, which indicates that drought turns off this machinery. The remaining clusters (C2 and C3) comprised genes with higher levels of expression in the drought-

stressed plants than in the control plants and they included kinases, transcription factors (TFs), and genes related to phosphate starvation.

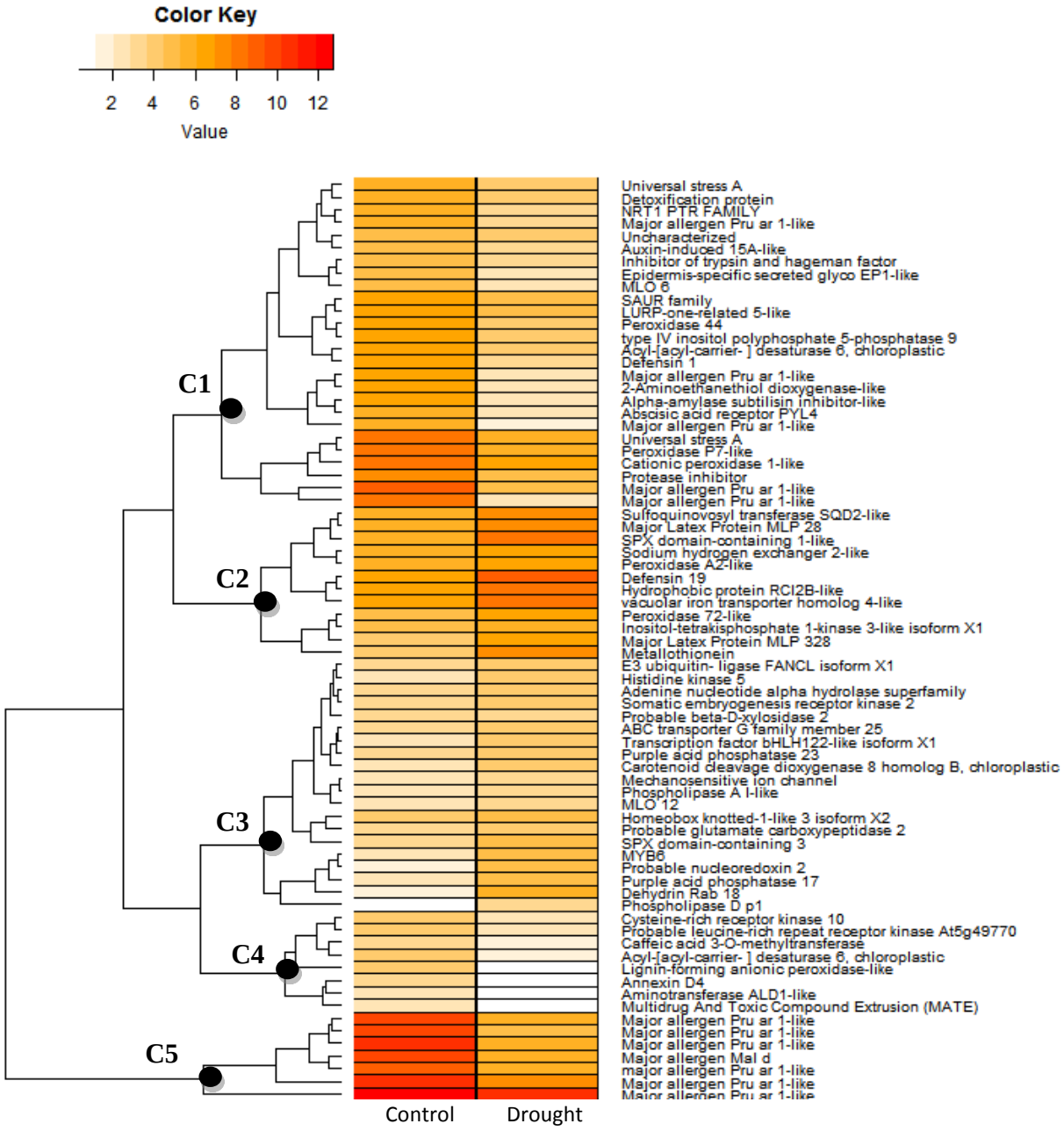

Figure 9. Heat map of the differentially expressed genes (DEGs) in roots (GF677 rootstock) that are involved in "response to stimulus" (GO: 0050896). The colors indicate the abundance of transcripts calculated as Log_2 (RPKM+1) in the control and drought-stressed plants (see color key). The main gene clusters are numbered from C1 to C5. Further information about each gene is provided in Table S2, listed, and grouped in clusters.

4.5 Classification of drought-inducible genes in GF677 rootstock budded with the Catherina cultivar

After annotation, the DEGs in both tissues were classified into two major categories: regulatory genes (genes implicated in signaling and transcriptional regulation) and functional genes (genes that encode proteins that are directly involved in cell protection and damage repair). These genes are described in the next sections and they are further detailed in Tables S3.A and S3.B.

4.5.1 Expression of drought stress regulatory genes

Regulatory genes play an important role in eliciting responses to abiotic stress. In this study, we detected 103 DEGs involved in signaling and regulation, of which only 15 were leaf DEGs (Figure 10.A and Table S3.A). These DEGs included receptors and protein kinases (32), calcium sensors (7), phospholipases (2), phosphatases (4), transcription factors (30), and hormone-related genes (28). The identification of such a large number of genes indicates that plants use a large array of signaling mediators and complex pathways to combat drought stress.

Amongst the genes of the protein kinases, the receptor-like kinase (RLK) gene and the leucine-rich repeat receptor-like kinase (LRR-RLK) gene were the most redundant largely exhibited down-regulation under conditions of drought (Figure 10.A and Table S3.A). Likewise, the genes of the cysteine-rich receptor-like kinases (CRKs) exhibited down-regulation. In contrast, the genes of kinases groups (serine-threonine kinases (STKs) and protein kinases (PKs)) were all up-regulated with the exception of cvCatherina.11364. Regarding the phospholipases (Phospholipase A and D), they were up-regulated exclusively in the roots.

A substantial number of the DEGs were TF genes, which were distributed into eight major families, based on their DNA-binding domains: bHLH (6), NAC (5), ERT (5), HD-ZIP (4), ORG2-like (3), WRKY (3), MYB (3), and growth-regulating factor 5 (GRF5) (1). As a result of the pivotal role of hormones as regulatory compounds, several of the DEGs were found to be hormone-related genes, which were related to the following hormones: abscisic acid (ABA), auxins, ethylene, gibberellins (GA), and brassinosteroids (BRs); these hormones exhibited varying expression patterns. These results indicate that drought stress drives changes in the expression of many regulatory genes which serve as key components of signal transduction pathways.

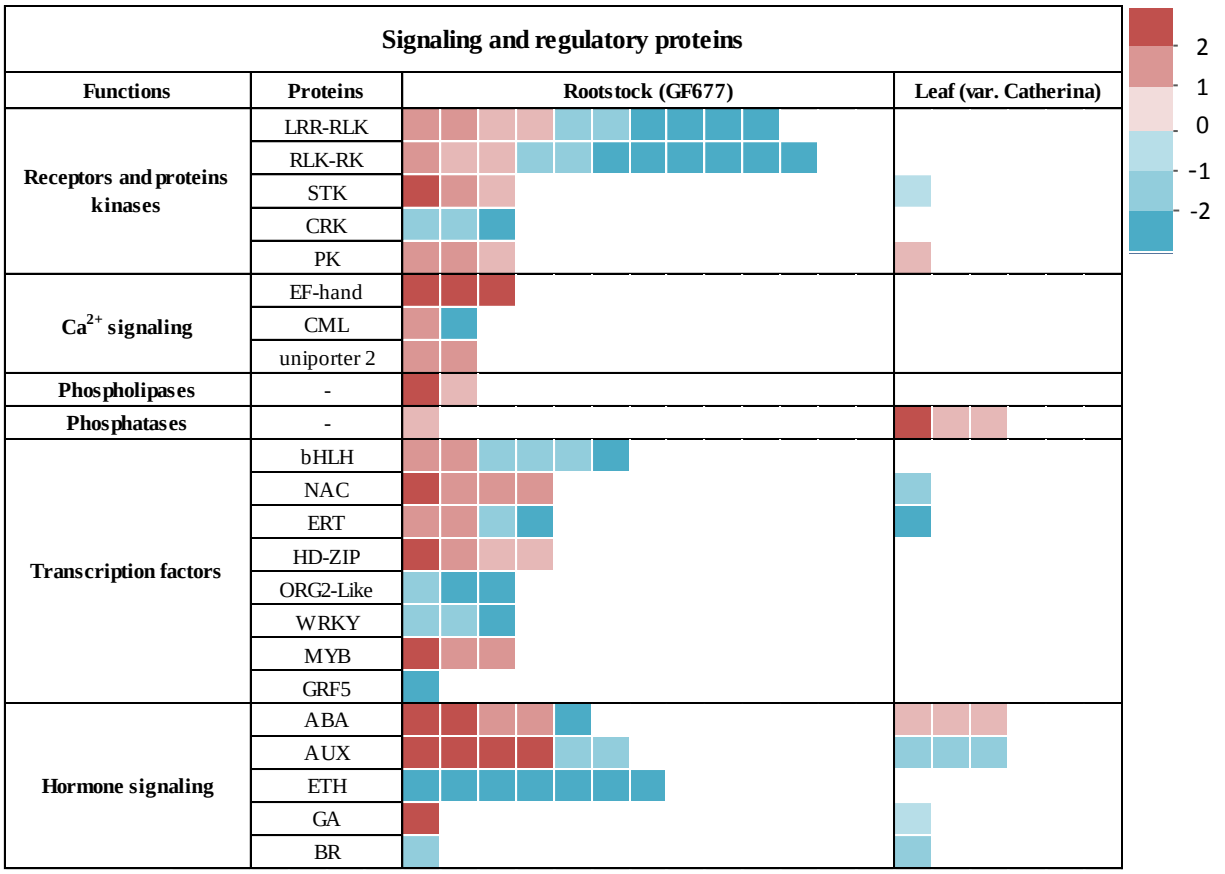

Figure 10.A. Levels of expression of differentially expressed genes (DEGs) in roots (GF677 rootstock) and leaves (graft, var. Catherina) involved in signaling and regulatory processes. Details are provided in Table S3.A. The scale bar on the right represents the observed changes in expression in terms of Log_2FC from up-regulation (red squares) to down-regulation (blue squares). The fold change was calculated as the ratio between the drought-stressed and control plants. ERT: ethylene-responsive transcription factor, GRF5: growth regulating factor 5, ABA: abscisic acid, ETH: ethylene, AUX: auxin, GA: gibberellin, BR: brassinosteroid.

4.5.2 Expression of drought stress functional genes

We identified 92 DEGs involved in functional processes, of which 39 were leaf DEGs (Figure 10.B and Table S3.B). One of the inevitable consequences of drought stress is the enhanced production of reactive oxygen species (ROS). Five electron carriers were detected only in the roots, providing evidence about the initiation of redox signaling, which was also highlighted in the GO analysis. However, in addition to the role of ROS products as secondary messengers during drought, they can also induce oxidative damage. Plants have evolved several enzymatic compounds in order to maintain redox homeostasis. The DEGs were founds to encode three types of these enzymes: glutathione S transferases (GSTs, 9), peroxidases (7), and ascorbate peroxidases (APXs, 3), and non-enzymatic machinery including ferritins (4), and thioredoxin (1).

As shown in Figure 10.B, the non-enzymatic genes all exhibited up-regulation while some enzymatic compounds were down-regulated, and seven of the DEGs were expressed in the leaves only under control conditions.

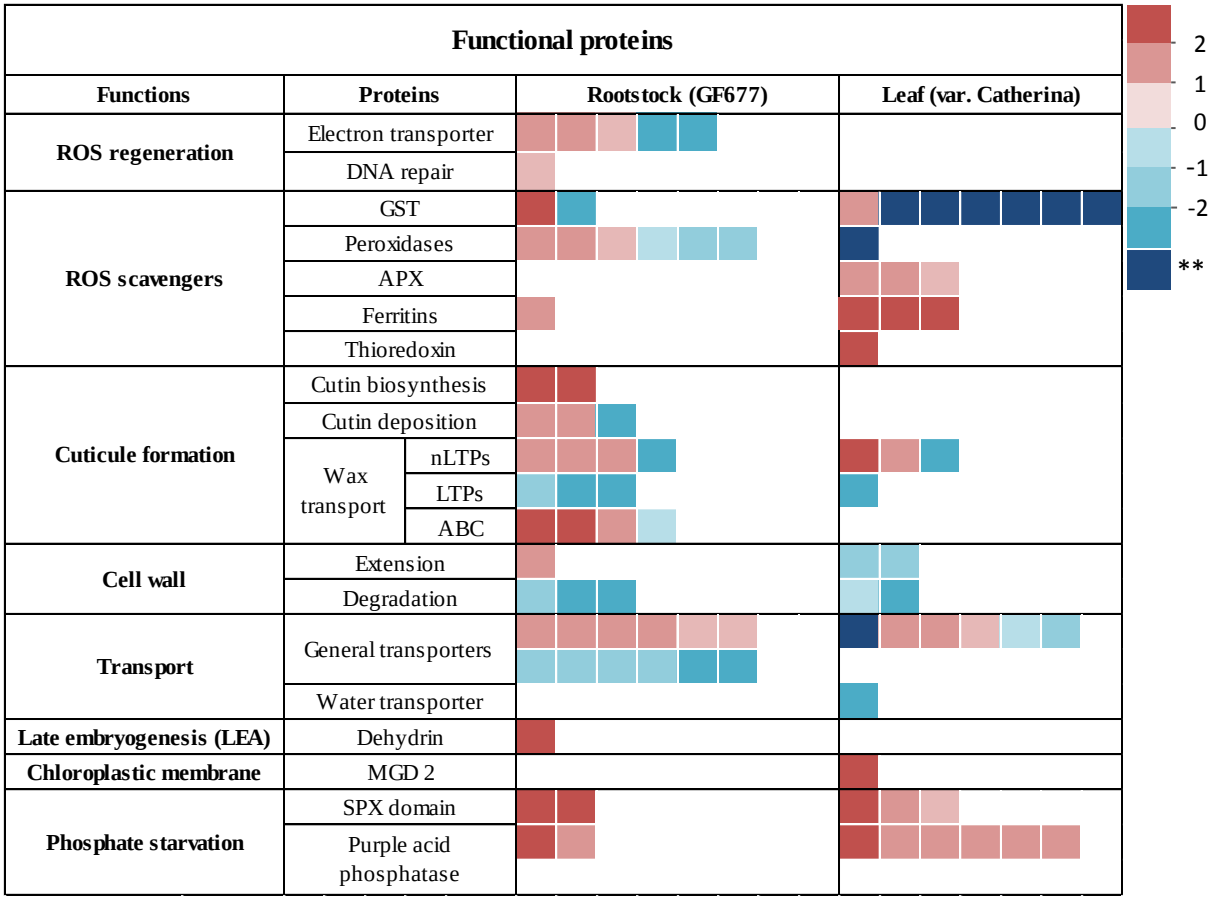

Figure 10.B. Levels of expression of differentially expressed genes (DEGs) in roots (GF677 rootstock) and leaves (graft, var. Catherina) involved in functional process. Details are provided in Table S3.B. The scale bar on the right represents the observed changes in expression in terms of Log_2FC from up-regulation (red squares) to down-regulation (blue squares). The dark blue pattern with stars indicates genes uniquely expressed in leaves in the control group. The fold change was calculated as the ratio between the drought-stressed and control plants. APX: ascorbate peroxidase, nLTPs: non-specific-lipid transfer proteins, LTPs: lipid transfer proteins, ABC: ATP binding cassette.

In addition, DEGs involved in cuticle formation were found, including genes involved in cutin biosynthesis and deposition (5) and wax transport (15). These large numbers of DEGs imply that the cuticle may undergo extensive remodeling during drought as part of the plant's adaptive survival mechanism (Figure 10.B and Table S3.B). Furthermore, we identified several DEGs that were involved in cell wall extension (3) and

degradation (5), which highlights the fact that plant cell walls constitute a major frontline of the plant defense system.

A total of 19 DEGs were annotated as transporters, including an aquaporin gene that was down-regulated in the leaves (cvCatherina.12386, see Table S3.B). Late embryogenesis-abundant (LEA) genes are commonly induced during drought stress, thus we identified the LEA gene GF677_18885, which corresponds to the dehydrin Rab18. Based on the pivotal role of phosphorous in plant life, we identified 13 genes related to phosphate starvation, all of which were up-regulated in-both the roots and the leaves.

4.6 KEGG pathway analysis

In order to look into the pathways that the DEGs were involved in, KEGG analysis was carried out. Of the 500 DEGs in the roots, 159 had significant matches in the KEGG database (137 enzymes) and they were classified into 65 pathways (Table S4.A). These enzymes were further classified as oxidoreductases, transferases, hydrolases, lyases, and isomerases, with the oxidoreductases and transferases being the most dominant enzymes (data not shown). In the leaves, 92 genes were assigned to 38 KEGG pathways and associated with 53 enzymes, of which the most common were hydrolases followed by oxidoreductases (data not shown). The complete set of matched pathways is summarized in Table S4. The major pathways identified in the roots were phenylpropanoid biosynthesis (9 DEGs and 3 enzymes) and aminobenzoate degradation (9 DEGs 378 and 4 enzymes). In the leaves, glutathione metabolism (10 DEGs and 4 enzymes), aminobenzoate degradation (10 DEGs and 2 enzymes), and drug metabolism associated with cytochrome P450 (6 DEGs and 1 enzyme) had the highest levels of differential expression.

4.7 RT-qPCR validation of DEGs from the RNA-seq analysis

In order to further confirm the accuracy of the RNA-seq expression estimates, a total of 33 candidate genes were selected for RT-qPCR validation according to their RPKM transcript abundance and Log_2FC. As illustrated in Figure 11, the expression values of the selected DEGs in both tissues significantly correlated with the RPKM values, with the exception of the chloroplastic ribulose bisphosphate carboxylase small chain (RBCS) gene, which may be a result of the unstable expression of this chloroplastic gene.

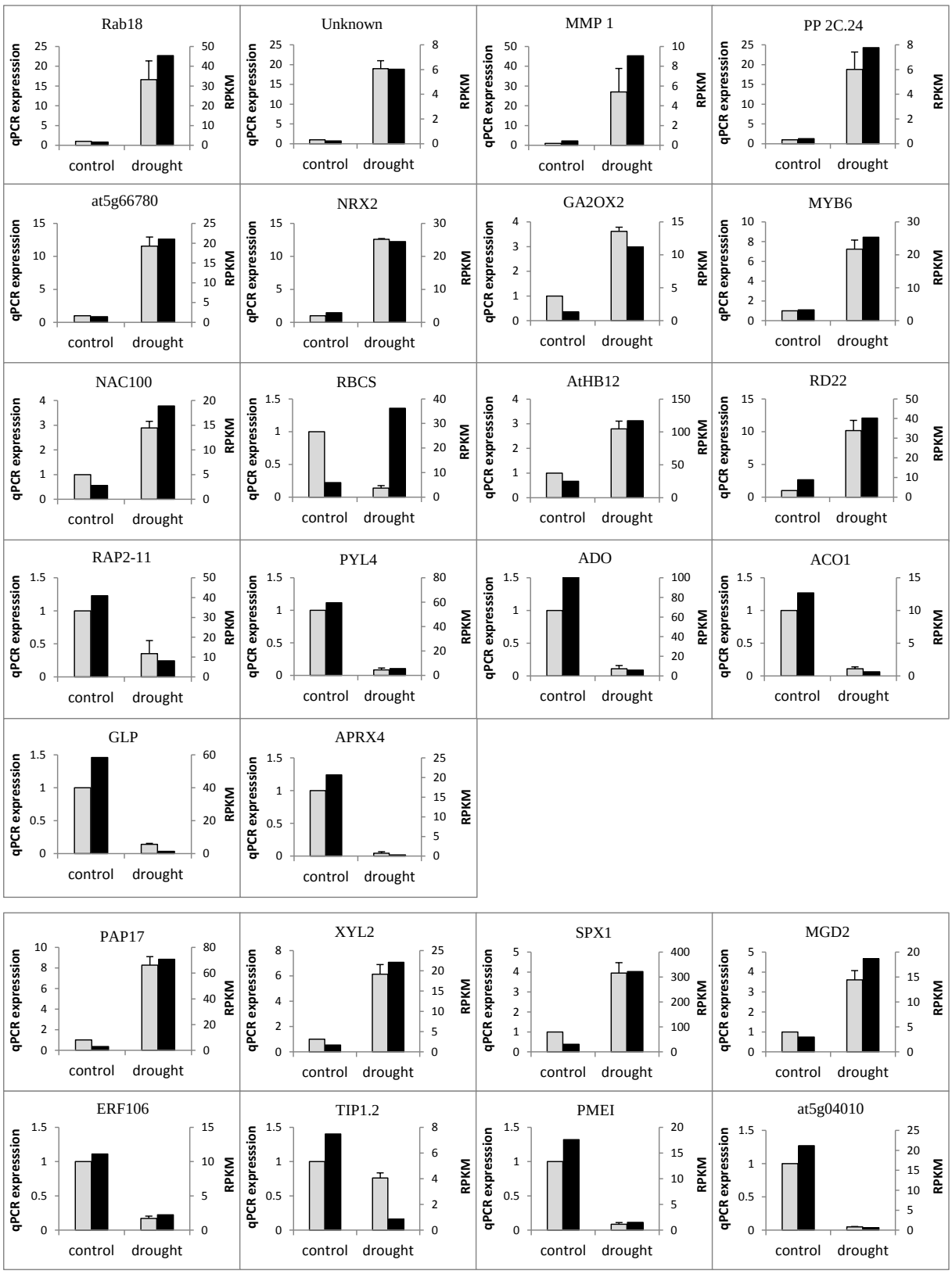

Figure 11. RT-qPCR validation of selected genes in roots (GF677 rootstock) and leaves (graft, var. Catherina) in the control and drought-stressed plants. The grey bars represent the relative expression determined by RT-qPCR (left *y*-axis) and the black bars represent the level of expression (RPKM) of the transcripts (right *y*-axis). The relative expression in the RT-qPCR analysis was normalized to the level in the GF677 rootstock of the control plants. The error bars indicate the standard error of tetra-biological and bi-technical replicates. See abbreviations and further information about each gene in Table 1 and Table S1.

The correlation between the RNA-seq and RT-qPCR measurements was evaluated using linear regression, based on the following equation: RT-qPCR value = b (RNA-Seq value) + a (Figure 12). Interestingly, the linear regression analysis showed a highly significant correlation between the methods, indicating a general agreement regarding the transcript abundance determined by both methodologies (r=0.88 and r=0.95 for root and leaf DEGs, respectively). In conclusion, the obtained results confirm the reliability of the transcriptomic profiling data estimated from RNA-seq data.

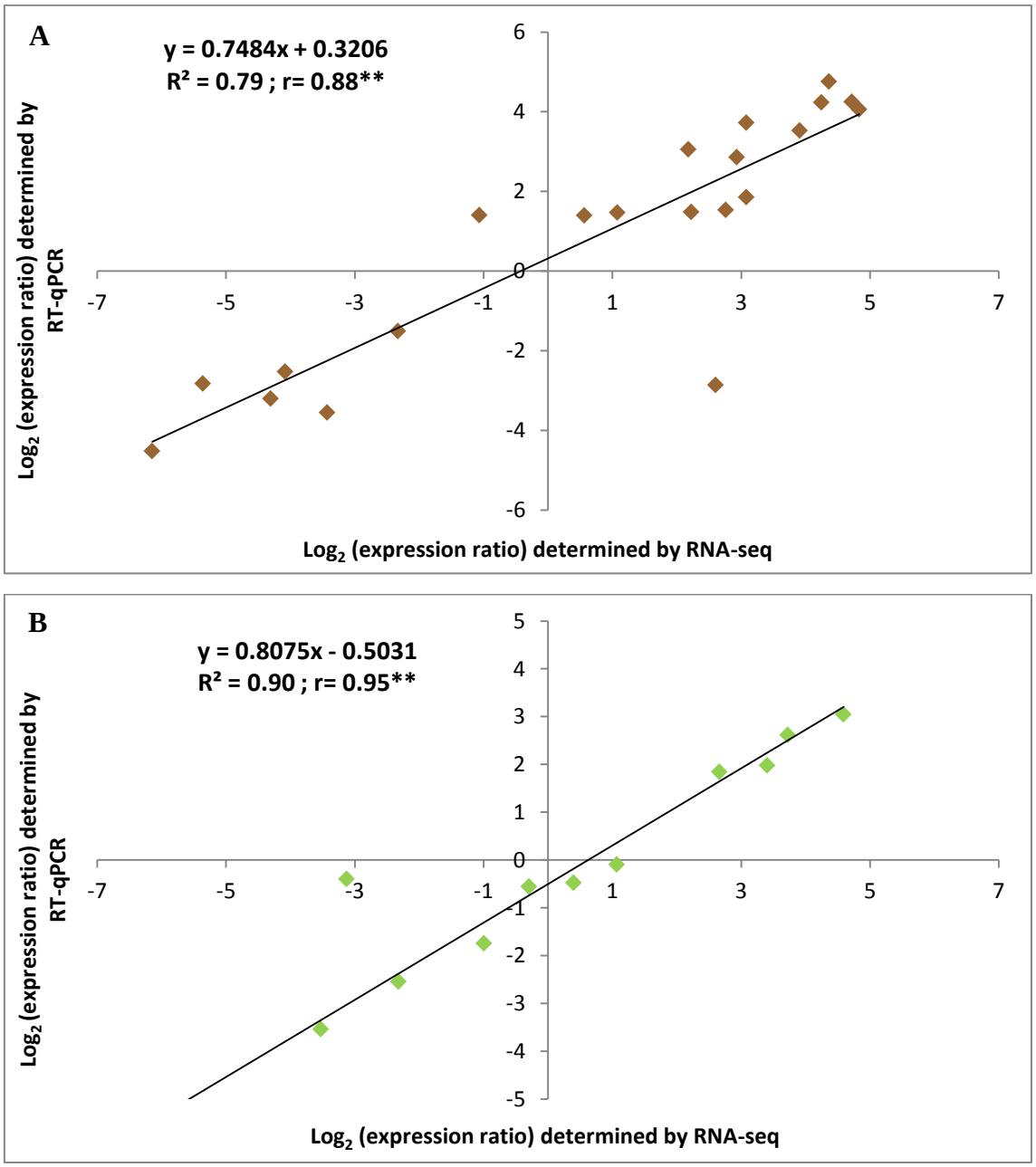

Figure 12. Linear regressions involving the RNA sequencing data and the RT-qPCR validation data expressed in term of Log_2FC. The fold change (FC) was calculated as the ratio between the drought stressed and control plants. (A) and (B) indicate roots and leaves, respectively. **Significant Pearson's correlation coefficient at $P \leq 0.01$.

5. Discussion

5.1 Physiological responses to drought

As sessile organisms, plants are unable to escape when environmental conditions become unfavorable. Nevertheless, they can successfully deploy complex physiological and molecular strategies to cope with environmental stresses. In this study, the physiological measurements confirmed that the plants were effectively subjected to a water deficit, and the plants elicited physiological responses to combat it. In fact, when water availability is limited, plants change their biochemistry in order to be able to retain as much water as possible and increase their chances of survival. One of the earliest responses to minimize water loss is the reduction of stomatal conductance, which leads to a reduction in CO_2 diffusion through the stomata pores. The results concurred with the findings of several other studies that showed that water scarcity significantly reduces the rate of photosynthesis (Jiménez et al., 2013) by affecting the CO_2 balance and stomatal status (Rahmati et al., 2015).

5.2 Insight into the *Prunus* spp. transcriptome

In order to investigate the dynamic changes in gene expression in the roots of GF677 rootstock and the leaves of Catherina cultivar budded together, RNA-seq was employed using the Illumina platform. Surprisingly, after quality control, the total number of clean reads generated from the RNA libraries of the drought-stressed plants was lower than the number for the control plants (Table 3). This is in contrast with previous studies, which have reported an activated plant transcriptome in response to drought (Tang et al., 2013; Garg et al., 2016). We thus hypothesize that a drought period of 16 days may be too short to drive the full upregulation of the *P. persica* genome, especially as the rootstock used was selected for its tolerance to drought (Jiménez et al., 2013).

The most frequent BLASTX top hits in our sequence homology searches were from *P. persica* and *P. mume*. These similarities highlight the quality of the assembly process. The annotation of the DEGs revealed a considerable number of hypothetical or uncharacterized protein functions, and some of these genes had large changes in expression. These genes could provide a good starting point for further experimental characterization. Generally, proteins with unknown functions are widespread across species, even in model plants. Indeed, in *Arabidopsis thaliana*, 13% of the genes encode proteins with unknown functions (Luhua et al., 2013). In spite of this, such genes are potentially interesting as they may encode proteins that would be valuable to breeders.

Elucidating their biological roles in *Prunus* spp. is thus an important challenge, which we aim to achieve in future studies.

After GO annotation, the DEGs were labeled with 56 terms within three main categories (BP, MF, and CC). The most dominant terms, illustrated in Figure S3, concur with the findings of previous research, confirming their universal involvement in the response to drought stress conditions (Dong et al., 2014; Li et al., 2016). In addition, some GO terms such as "locomotion", "hormone metabolic process", "detection of stimulus," and "cell killing" were exclusively associated with root DEGs (Figure 7), indicating that despite being sessile, *P. persica* roots exhibit dynamic changes in architecture in response to water scarcity. An induced phospholipase D (PLD), associated with the regulation of cell migration and root development, is likely to be involved in locomotion. This observation reveals that, under conditions of drought stress, roots move downward in order to find water and escape from the harmful external factors. In fact, PLD hydrolyzes lipids, which results in the formation of phosphatidic acid (PA), a compound that is responsible of inducing cell proliferation and primary root growth (McLoughlin and Testerink, 2013). Functions related to the detection of stimuli were found to be increased in the roots during the drought responses of *P. persica*, confirming that these organs are responsible for sensing water deprivation. According to previous studies, the main steps for handling any type of abiotic stress are signal perception, signal transduction, and expression of stress-inducible genes. Thus, we propose that root cells first perceive drought through sensors located in the cell wall and/or membrane and then they convey the signals to other organs.

Indeed, we have found that "membrane" and "cell" were the most dominant GO terms in the CC category, particularly regarding root DEGs. Furthermore, the transcripts GF677_1518 and GF677_20344, which code for defensins (Table S2), were associated with "cell killing of invasive organisms" (GO: 0031640, Table S2). The "hormone metabolic processes" (GO: 0042445) associated with the root DEGs will be further discussed in section 6.3.1.

5.3 Generic signaling pathways involved in *Prunus* spp. during drought stress

We observed that the initiation of drought stress triggered a wide range of responses, which implies that there are many genes and mechanisms involved in drought tolerance in *P. persica*. According to their associated proteins, we classified the DEGs as signaling and regulatory or functional.

5.3.1 Signaling and regulatory proteins

5.3.1.1 Receptor kinases

Stress perception is the first step involved in the activation of adaptive responses to ensure plant survival. The detection of extracellular stress signals is generally carried out via the receptor kinases on the cell walls and membranes, which bridge the gap between the perception of stress and signal transmission to the target genes. The majority of differentially expressed receptor kinases were found in the roots, supporting the notion that these organs are the primary sensors of drought stress. RLKs, including LRR-RLKs, formed the largest gene family in the data. However, most of them were strongly down-regulated, whereas the remainders were slightly up-regulated (Figure 10.A, Table S3.A). It is well-documented that LRR-RLKs play roles in both biotic and abiotic stress responses (Osakabe et al., 2014); thus, we hypothesize that drought stress down-regulates the biotic-response machinery, as shown in Figure 9 (see, for instance, C5). This negative feedback could potentially be due to the repression of some LRR-RLKs, which was also observed for CRKs.

5.3.1.2 Ca^{2+} signaling

Following signal perception, the signals are relayed to downstream secondary messenger molecules, which are mainly calcium ions (Ca^{2+}), ROS, and phytohormones, in order to initiate the signal transduction pathway. Ca^{2+} serves as versatile signaling messenger in response to various abiotic stimuli. The cytosolic concentration of Ca^{2+} has been found to increase due to the activation of Ca^{2+} channels during drought and salinity stress (Knight et al., 1997). Perturbations in the cytosolic concentration of Ca^{2+} are recognized by calcium-binding proteins (CBPs) that function as Ca^{2+} sensors, of which EF-hand CBPs are the major type (Batistič and Kudla, 2012). The DEGs involved Ca^{2+} signaling were strongly induced in the roots except for GF677_3137 (Table S3.A). The up-regulation of calcium uniporters, which transport Ca^{2+} from the cytosol to the mitochondrial matrix, suggests that drought stress could increase the Ca^{2+} concentration in *Prunus* spp. as way of maintaining the structural rigidity of the cell wall, which is in agreement with the results of previously cited studies (Knight et al., 1997; Batistič and Kudla, 2012). Furthermore, the up-regulation of CBPs indicates that there is an enhancement of the intracellular signal transduction in *Prunus* spp. roots that are exposed to drought. The results may imply that EF-binding CBPs have a key role in

sensing the Ca^{2+} signals and relaying the information to the rest of plant regulatory system.

5.3.1.3 Protein kinases and phospholipases

In contrast to the CBPs, PKs are sensor responders (Batistič and Kudla, 2012) that initiate phosphorylation cascades and thereby play important roles in responses to drought stress (Singh and Laxmi, 2015). The genes of STKs and PKs were up-regulated which indicates the initiation of phosphorylation cascades. A notable DEG that was up-regulated during conditions of drought was the inositol-tetrakisphosphate 1-kinase gene (GF677_21039, see Table S3.A). This member of the inositol pyrophosphate (IP) family has been reported to catalyze the production of inositol 1,3,4,5,6 pentakisphosphate IP_5, which acts as a secondary messenger during environmental stress (Worley et al., 2013). On the other hand, we identified induced genes that encoded diacylglycerol kinase (DKG, cvCatherina.7529) and phospholipase D (PLD, GF677_17117), which are considered to be key generators of PA, a major root lipid signaling molecule during conditions of drought (Arisz et al., 2009; Hong et al., 2009; McLoughlin and Testerink, 2013).

5.3.1.4 Transcription factors (TFs)

At the end of signaling cascades, TFs, broadly categorized as early-induced genes, are targeted by PKs and phosphatases (Hussain Wani et al., 2016). In the present study, drought significantly influenced transcription regulation, especially in the roots (in contrast, only two leaf TFs were annotated). This suggests that the transcriptional reprogramming of stress-responsive genes is initiated in the roots, reflecting their pivotal regulatory role.

Amongst the genes of the TFs, the bHLH genes were the most redundant largely exhibited down-regulation under conditions of drought. However, bHLH122 was found to be induced, which concurs with its previously reported role in drought tolerance in *A. thaliana*, where it increases levels of cellular ABA by repressing the catabolism of ABA (Liu et al., 2014). NAC factors are known to play diverse roles in stress responses (Bianchi et al., 2015). In particular, GF677_17765 may encode NAC29, which was found to delay senescence and boost primary root elongation in transgenic *A. thaliana* roots (Huang et al., 2015). Similarly, HD-ZIP and MYB TFs were strongly induced during drought, shedding light on their putative roles as mediators of drought signaling

(Chew et al., 2013; Chen et al., 2014). In particular, the gene encoding transcript GF677_6534 is a putative orthologue of the ABA-dependent AtHB12 (a HD-Zip gene) found in *A. thaliana*, which promotes root elongation during mild drought stress (Ré et al., 2014). Thus, after considering the previously discussed "locomotion" annotation (Figure 7), we hypothesize that GF677_6534 may control root elongation during conditions of drought. This would contribute to the annotation of members of the HD-Zip family in *Prunus* spp., of which only the AtHB8 gene has been functionally characterized (Zhang et al., 2014). The repression of WRKY70 is in agreement with previous reports that have highlighted its role as a negative regulator of cell senescence (Griffiths et al., 2014). Interestingly, among the TFs, we also found that GRF5 was repressed in the roots. The family of GRFs comprises ten TFs in *P. persica*, but the description of their functions is still incomplete (see annotations, for instance at (http://planttfdb.cbi.pku.edu.cn/search.php). According to studies on *A. thaliana*, GRF is involved in leaf and root expansion (Omidbakhshfard et al., 2015), although its regulatory effect over pivotal and lateral roots remains unclear.

5.3.1.5 Hormone signaling (Phytohormones)

One of the major signaling molecules used during drought is ABA. The key step in its synthesis is catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED3). The transcript GF677_11309 encodes NCED3 and it was significantly induced in roots, confirming that a water deficit enhances the synthesis of ABA (Table S3.A). In agreement with previous reports, protein phosphatases PP2C, which are major ABA regulators, were also up-regulated (Tang et al., 2013; Yang et al., 2015; Iovieno et al., 2016; Magalhães et al., 2016). The accumulation of ABA leads to activation of ABA-dependent TFs, such as MYB factors, as was shown in this study. For instance, the promoter of the drought-inducible gene RD22 (GF677_3749, which was validated by RT-qPCR) is known to harbor cis-elements that can be bound by MYB TFs in *A. thaliana* (Yamaguchi-Shinozaki and Shinozaki, 1993). Furthermore, in the roots we found three indole-3-acetic-acid-amido synthetases with $Log_2FC > 2$ that adjust cell auxin levels via the inactivation of indole-3-acetic acid (IAA), one of the major forms of auxins in plants (Böttcher et al., 2012). The up-regulation of these genes occurred with the down-regulation of auxin-responsive proteins (auxin-binding protein family and the SAUR family) (Table S3.A). Thus, we propose that the inhibition of auxin biosynthesis is an

adaptive survival strategy that reduces cell division and helps to avoid water loss, particularly in the leaves.

Seven root DEGs encoding aminocyclopropane-1-carboxylate oxidase (ACO1), a key enzyme in the ethylene biosynthesis pathway, were strongly down-regulated. Likewise, most of the ethylene responsive transcription factors (ERTs) followed the same pattern, as they are sensors of ethylene (Müller and Munné-Bosch, 2015). As ethylene has been linked to the promotion of cell senescence (Griffiths et al., 2014), we propose that the inhibition of ethylene biosynthesis is a mechanism to reduce the effects of drought, probably in coordination with NAC29, which also delays senescence (Huang et al., 2015).

In this study, GA2OX2 (GF677_10709), an important regulator of GA levels, was strongly up-regulated (Table S3.A, Figure 10.A), leading to the deactivation of bioactive GA (Busov, 2014). Reduced GA levels in the roots may be another mechanism used to economize water use by reducing the plant's growth, as previously reported in *Populus* (Busov, 2014).

The data indicate that there were two repressed BR-responsive genes in both tissues, which supports the findings of molecular studies that have reported that there is crosstalk between BR and other hormones (GA, auxin, and ethylene) (Bajguz and Hayat, 2009). Overall, our results indicate the prominent role of ABA-regulated responses to drought, while the all other major hormones and related pathways are generally down-regulated.

5.3.2 Functional proteins

Electron transporters are the major site of ROS production (Cruz de Carvalho, 2008). Up-regulation of the DEGs associated with electron transporters increases the electron flux, thereby increasing ROS production and disturbing the ROS balance. Overproduction of ROS is extremely harmful to plants as it causes lipid oxidation, DNA damage, and programmed cell death (Das and Roychoudhury, 2014). Interestingly, in the roots, we identified an induced E3 ubiquitin-protein ligase FANCL (previously described in studies on humans), which is involved in DNA repair (GF677_16223, Table S3.B). Based on this, we suggest that this protein may repair oxidative DNA damage. Furthermore, ROS scavengers were mainly expressed in the leaves, which confirm that this tissue is more susceptible to oxidative damage than root tissue. Indeed, it has been documented that ROS generation mainly occurs in photosynthetic tissues, in

chloroplasts and mitochondria (Das and Roychoudhury, 2014). Moreover, non-enzymatic scavenging genes were up-regulated, highlighting the need to protect against oxidative stress. Although studies have reported high levels of GST activity during drought (Liu et al., 2015; Garg et al., 2016), six of the genes associated with GST were exclusively expressed in the leaves of the control plants and they were not detected in the drought-stressed plants. These findings suggest that the ROS system was not activated in the leaves, which may be a result of the effect of the drought-tolerant GF677 rootstock masking the effects of the drought. The fact that GSTs serve as auxin-binding proteins (Marrs, 1996) may also explain our findings as this hormone was found to be down-regulated in both tissues.

The role of detoxification enzymes in cell protection has been well-documented in other plants (Sappl et al., 2009; Das and Roychoudhury, 2014), as well as the roles of proteins such as nucleoredoxin, multidomain thioredoxin, and ferritin (Kang and Udvardi, 2014; Li and Wei, 2016). In this study ferritins were found to be up-regulated in the drought-stressed plants, potentially in order to sequester free iron that would otherwise catalyze the Fenton reaction and produce highly reactive hydroxyl radicals.

The cuticle is composed of two layers, an inner layer of cutin and an out layer of wax, to ensure that the plant has hydrophobic protection against water loss (Yeats and Rose, 2013). Genes involved in cutin biosynthesis and deposition were expressed at high levels in the roots of drought-stressed plants. This may help to reinforce plants' first-line barrier as drought can weaken roots, making them more susceptible to biotic attack. DEGs encoding wax transporters showed variable expression patterns: while lipid transfer proteins (LTPs) were repressed, nonspecific lipid transfer proteins (nLTPs) and ABC (ATP-binding cassette) transporters were mostly activated. Based on these findings, we suggest that although drought negatively affected LTPs, the other transporters may play an important role in wax accumulation, and thus enhance the rigidity of the plant, as reported in coffee undergoing drought stress (Mofatto et al., 2016).

Plants experiencing low water availability face the challenge of reducing their leaf area while maintaining their root growth. Expansins play a role in this process, by extending the cell walls, and they are known to be regulated by auxin (Perrot-Rechenmann, 2010). The results showed that there was one induced expansin and two repressed expansins in the roots and leaves, respectively. These observations suggest that the roots continue to grow under conditions of drought, while leaves reduce their levels of water loss. This

model is consistent with the previously discussed auxin-responsive pathways, which appear to be down-regulated, and the activation of AtHB12, which is thought to be involved in root elongation. In addition, DEGs encoding enzymes involved in cell wall degradation were strongly down-regulated in both tissues, which appear to be an adaptive way to increase cell wall rigidity. Transporter genes showed changes in expression in both directions. The most interesting example is perhaps the aquaporin TIP1.2, which was found to be down-regulated in the leaves ($Log_2FC = -3.13$, see Table S3.B). This expression pattern in the leaves is consistent with the measured levels of stomatal conductance (see Table 2), as suggested by Pou et al. (2013). In contrast, the dehydrin Rab18, an LEA protein, was significantly up-regulated in the roots, which concurs with its expected role in protecting cellular components from dehydration (Graether and Boddington, 2014).

Regarding the integrity of the chloroplasts, we found that monogalactosyldiacylglycerol synthase (MGD2) was up-regulated in the leaves; this molecule is known to be involved in the biosynthesis of galactolipid, a molecule that stabilizes the chloroplast membrane, thereby ensuring the photosynthetic can be maintained (Wang et al., 2014).

Finally, transcripts encoding proteins that are involved in phosphate starvation were overexpressed in both tissues, which highlights the role of phosphate in many vital pathways, in particular, photosynthesis, signaling, and growth (Dos Santos et al., 2006).

6. Conclusions

Drought tolerance is a complex trait that is controlled by multiple genes, and the identification of drought-inducible genes in this study provides an insight into the major mechanisms adopted by *P. persica* to tolerate periods of drought. This study is the first to assess drought effects on both root and leaf tissues in *Prunus* spp. It has showed that under water deficit constraints *P. persica* react at the molecular, cellular and biological levels.

- We can conclude that RNA-seq is an efficient high-throughput method for exploring the wide diversity of genes expressed under drought stress. This technology is a valuable tool to enhance our understanding on the genomics underlying drought stress tolerance in *P. persica*.
- We have obtained a valuable dataset of differential expression genes (DEGs); 500 DEGs in roots and 236 DEGs in leaves at *Q*-value < 0.01. DEGs identified in roots are twice those identified in leaves, indicating that roots undergo more genetic complexity in response to water deficit stress and highlighting the role of the rootstock as stress buffer.
- We reported some exclusive functions of the root organ (locomotion, detection of stimulus and hormone metabolism process) reflecting their major role as stress sensors and reinforce our idea that the root system can improve water uptake and transport as well as detecting soil water deficit and send signals to the rest of the organs.
- We annotated and categorized the DEGs as signaling and regulatory and functional genes which are useful in expanding our knowledge of the fundamental aspects of drought responses. This large number of responsive genes was found to play different functions related to signal perception and transduction, regulation of transcription, hormones biosynthesis and frontline barriers modification which indicate that drought responses in *P. persica* is the consequence of interactions among multiple genes and pathways ranging from morphological to molecular levels.
- Overall, the results shed light on the prominent role of ABA as the major drought-induced hormone in response to drought, while the other hormones and related pathways were shown to be down-regulated.
- We interestingly identified two drought-responsive genes in root tissue, “GRF5” and “AtHB12” that are potentially involved in drought adaptations providing

thus a good starting point for investigations of poorly characterized genes in *P. persica.* However, when assessing the genes that are potentially involved in drought responses, it should be taken into account that plant responses depend largely on the severity and duration of the water deficit scenario.

7. References

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8. Supplementary Materials

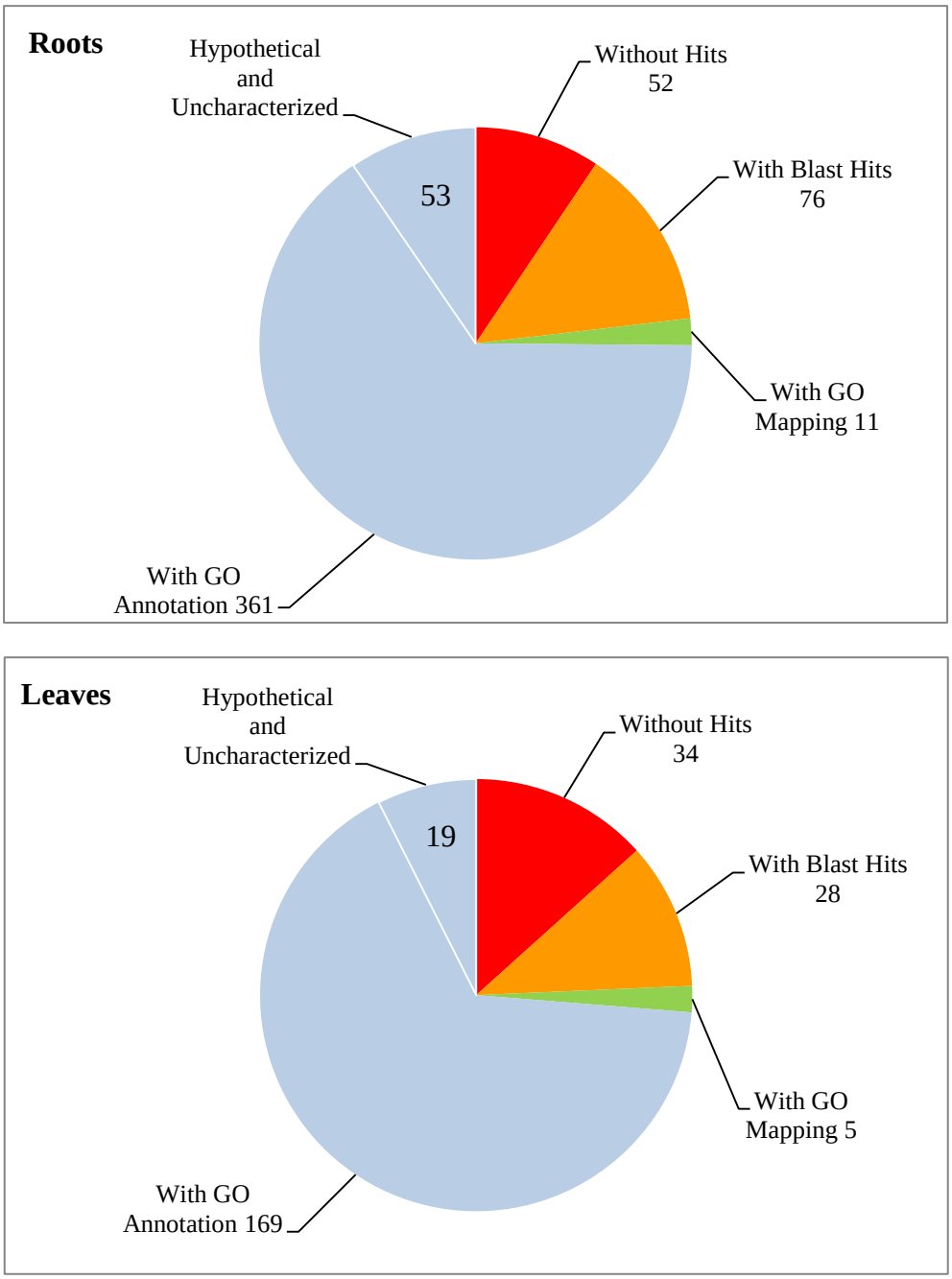


Figure S1. Annotation summary of differentially expressed genes (DEGs; *Q*-value < 0.01) in root and leaf tissues (GF677 rootstock budded with var. Catherina). GO mapping indicates genes that were mapped but not annotated.

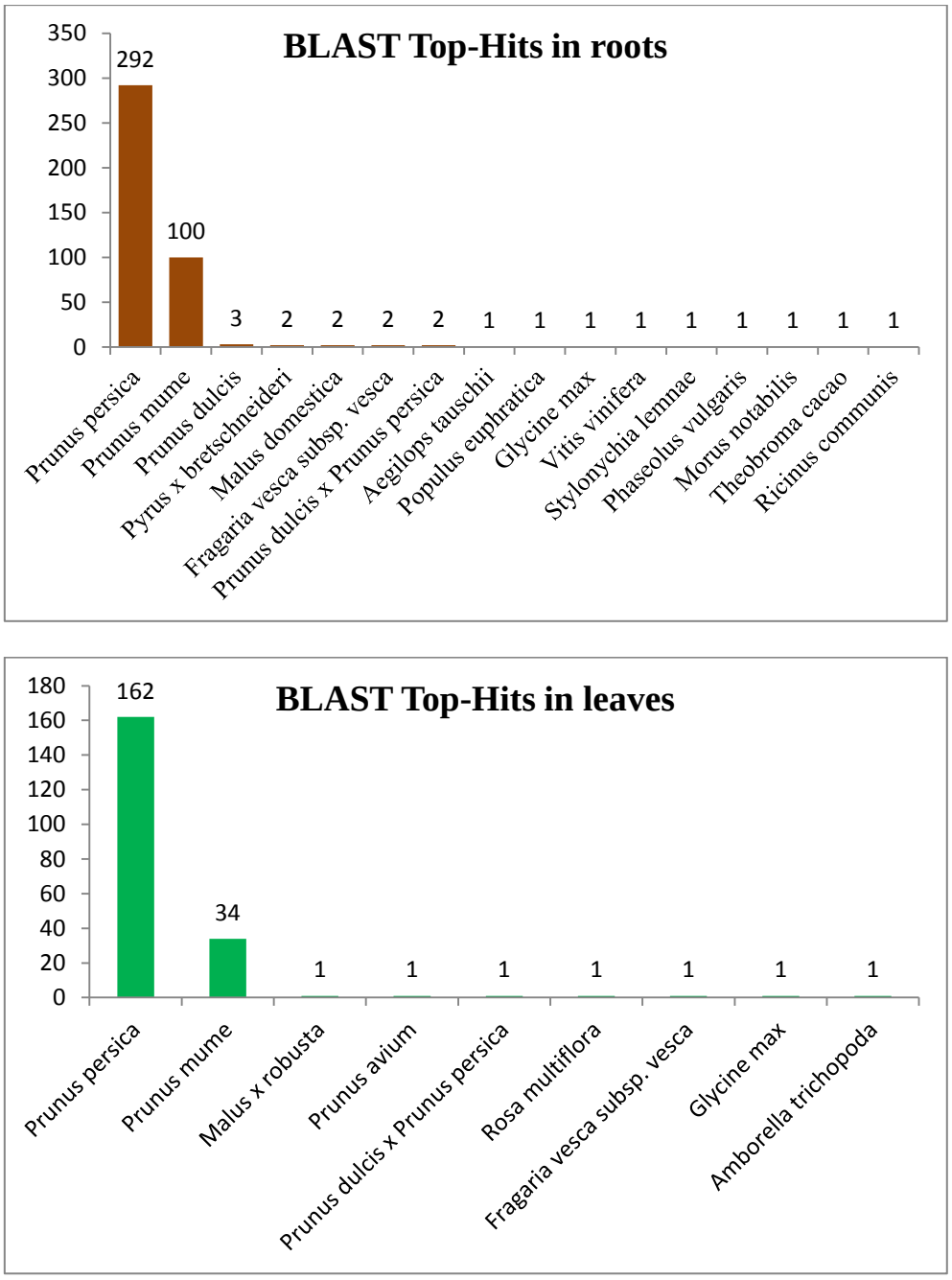


Figure S2. Species distribution according to the BLASTX top hits in roots (GF677 rootstock) and leaves (graft, var. Catherina).

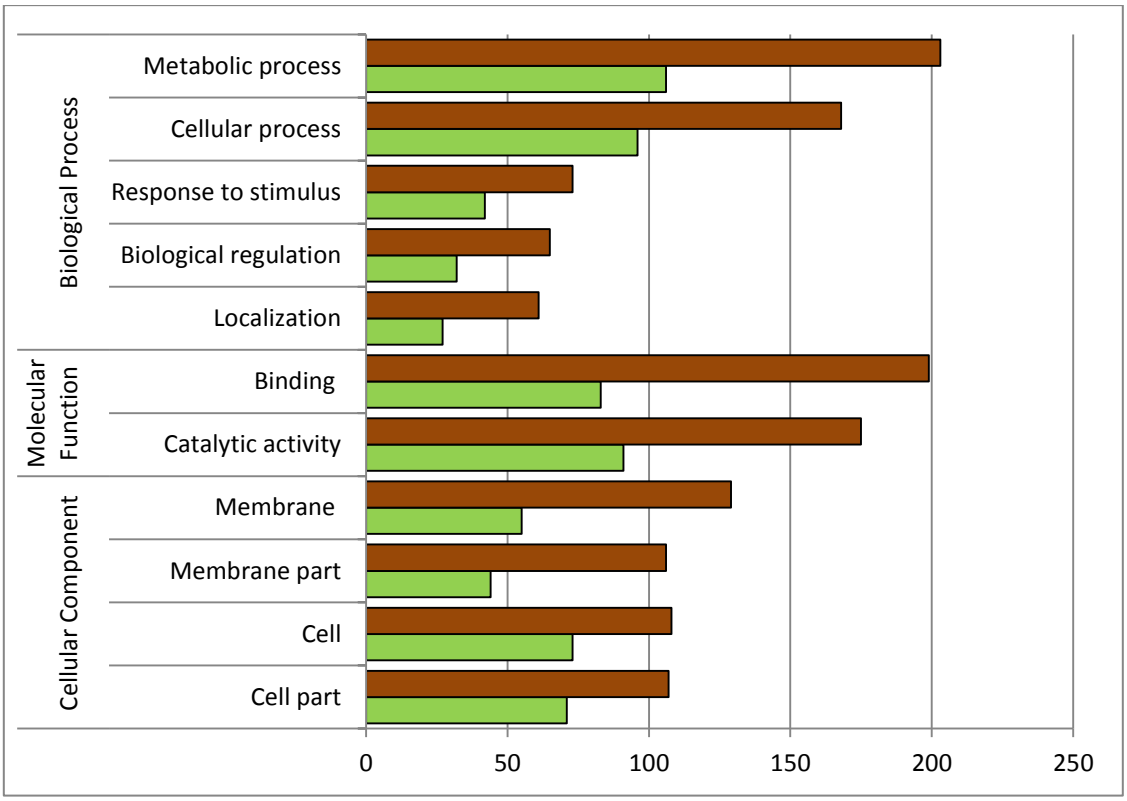
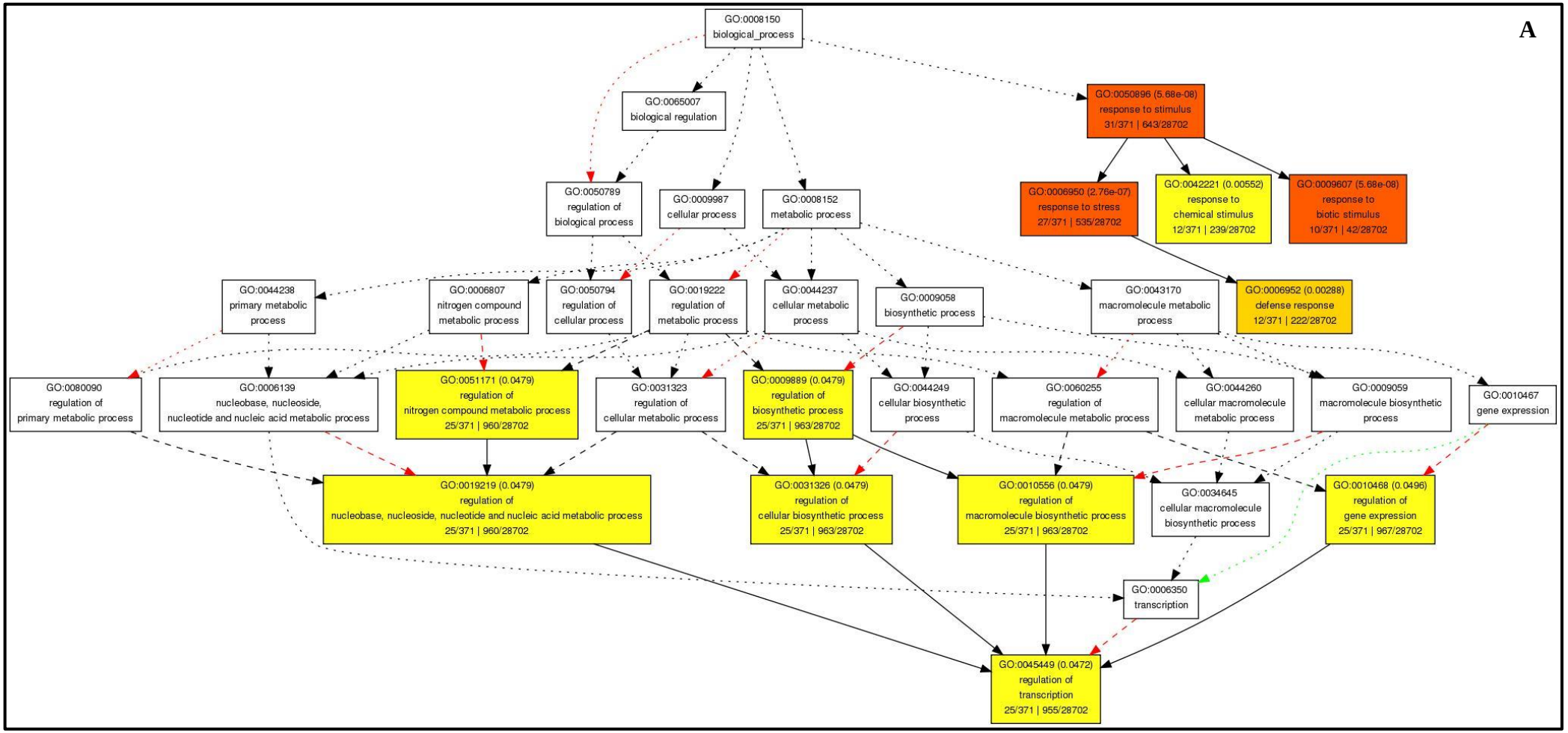


Figure S3. Most frequent gene ontology (GO) terms assigned in the three main categories: biological processes (BP), molecular functions (MF), and cellular components (CC). The *x*-axis indicates the number of differentially expressed genes (DEGs) in each category. Brown color corresponds to roots (GF677 rootstock) and green color to leaves (graft, var. Catherina).


A
GO:0008150
biological_process
GO:0065007
biological regulation
GO:0050896 (5.68e-08)
response to stimulus
31/371 | 643/28702
GO:0050789
regulation of
biological process
GO:0009987
cellular process
GO:0008152
metabolic process
GO:0006950 (2.76e-07)
response to stress
27/371 | 535/28702
GO:0042221 (0.00552)
response to
chemical stimulus
12/371 | 239/28702
GO:0009607 (5.68e-08)
response to
biotic stimulus
10/371 | 42/28702
GO:0044238
primary metabolic
process
GO:0006807
nitrogen compound
metabolic process
GO:0050794
regulation of
cellular process
GO:0019222
regulation of
metabolic process
GO:0044237
cellular metabolic
process
GO:0009058
biosynthetic process
GO:0043170
macromolecule metabolic
process
GO:0006952 (0.00288)
defense response
12/371 | 222/28702
GO:0080090
regulation of
primary metabolic process
GO:0006139
nucleobase, nucleoside,
nucleotide and nucleic acid metabolic process
GO:0051171 (0.0479)
regulation of
nitrogen compound metabolic process
25/371 | 960/28702
GO:0031323
regulation of
cellular metabolic process
GO:0009889 (0.0479)
regulation of
biosynthetic process
25/371 | 963/28702
GO:0044249
cellular biosynthetic
process
GO:0060255
regulation of
macromolecule metabolic process
GO:0044260
cellular macromolecule
metabolic process
GO:0009059
macromolecule biosynthetic
process
GO:0010467
gene expression
GO:0019219 (0.0479)
regulation of
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
25/371 | 960/28702
GO:0031326 (0.0479)
regulation of
cellular biosynthetic process
25/371 | 963/28702
GO:0010556 (0.0479)
regulation of
macromolecule biosynthetic process
25/371 | 963/28702
GO:0034645
cellular macromolecule
biosynthetic process
GO:0010468 (0.0496)
regulation of
gene expression
25/371 | 967/28702
GO:0006350
transcription
GO:0045449 (0.0472)
regulation of
transcription
25/371 | 955/28702

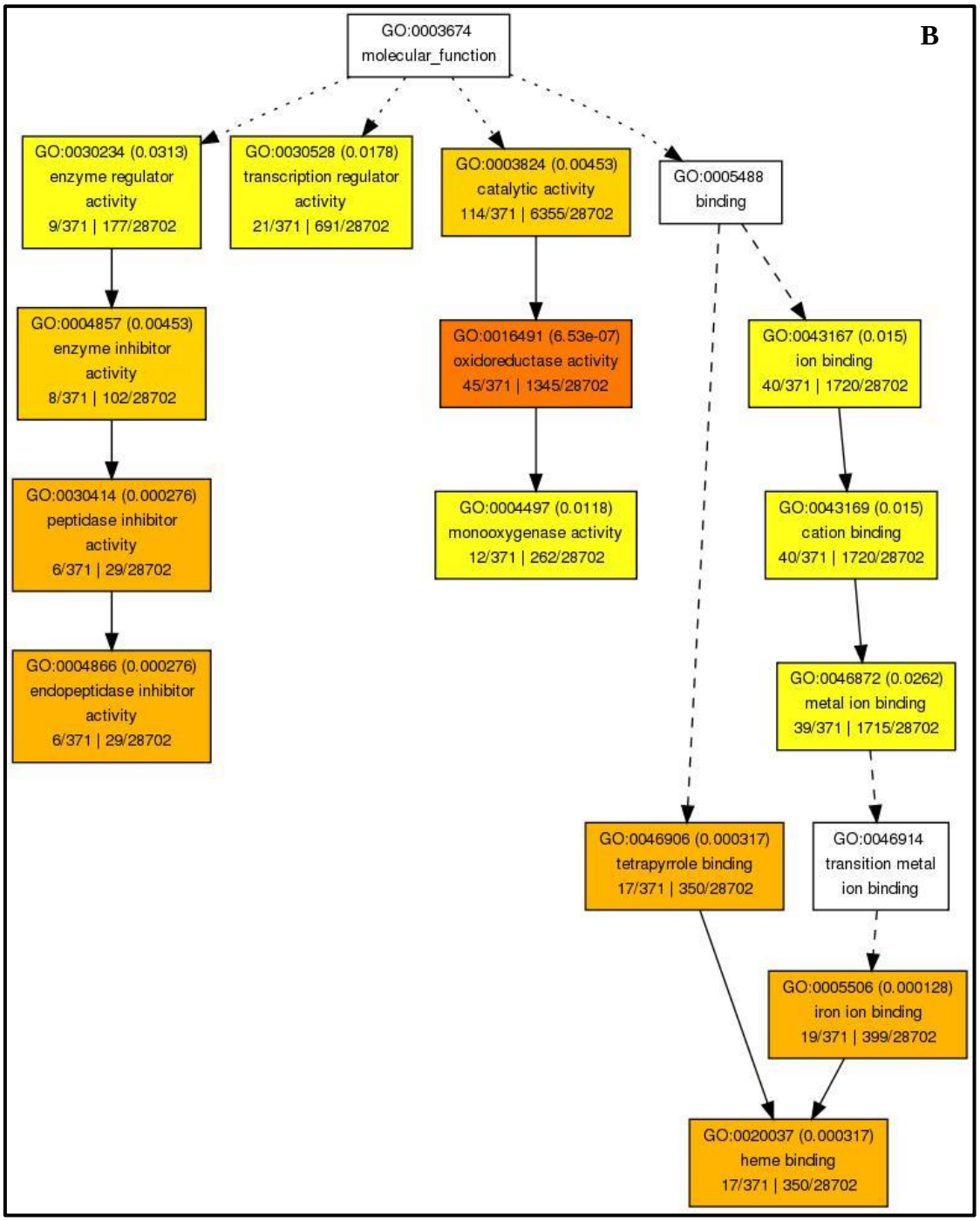


Figure S4. Significant gene ontology (GO) terms from biological processes (A) and molecular functions (B) enriched in roots (GF677 rootstock), after a singular enrichment analysis (SEA) in AgriGO. Each box indicates the GO number and full description with *Q*-values in parenthesis. The numbers on the left side indicate the fraction of DEG genes sharing each GO term. The numbers on the right side indicate the background numbers of genes associated with the GO term in the *P. persica* genome. The significance of the enrichment is displayed using a color scale from yellow to red. The white boxes show GO terms with adjusted *P*-values > 0.05.

Table S1. Primers list and features of differentially expressed genes (DEGs) in roots (GF677 rootstock) and leaves (graft, var. Catherina) selected for RT-qPCR validation. The gene ID is the same as that deposited in the ENA. The grey rows indicate genes with $|Log_2FC| < 2$. GDR: Genome Database of Rosaceae.

Genes differentially expressed in roots

Gene ID	ID in GDR database	Locus	Description	Abbreviations
GF677_18885	ppa011637m	scaffold_7:17140290-17142422	Dehydrin rab 18	Rab18
GF677_10293	ppa009829m	scaffold_3:21224913-21415329	Unknown protein	Unknown
GF677_9678	ppa016355m	scaffold_3:20151304-20170331	Metalloendoproteinase 1-like	MMP1
GF677_15114	ppa006696m	scaffold_6:4698391-4700540	Phosphatase 2C 24	PP2C.24
GF677_18824	ppa013228m	scaffold_7:16790537-16791344	at5g66780 mud21_2	at5g66780
GF677_2910	ppa006064m	scaffold_1:32257654-32261879	Probable nucleoredoxin 2	NRX2
GF677_10709	ppa008211m	scaffold_4:3917370-3919509	Gibberellin2 beta dioxygenase 2	GA2OX2
GF677_10265	ppa007438m	scaffold_4:1665468-1667309	Transcription repressor MYB6	MYB6
GF677_9569	ppa007883m	scaffold_3:19548129-19549934	NAC domain containing protein100 like	NAC100
GF677_8702	ppa012123m	scaffold_3:12230346-12231657	Ribulose bisphosphate carboxylase small chain chloroplastic	RBCS
GF677_6534	ppa010647m	scaffold_2:19683395-19685063	Homeobox- leucine zipper AtHB12_like	AtHB12
GF677_3749	ppa005699m	scaffold_1:37149693-37151803	Dehydration-responsive protein RD22	RD22
GF677_10556	ppa002450m	scaffold_4:3002171-3008328	LRR receptor-like serine/threonine-protein kinase At5g45840	LRR
GF677_18270	ppa026745m	scaffold_7:12589869-12593343	Probable glutamate carboxypeptidase 2	GCPII
GF677_721	ppa008797m	scaffold_1:7495645-7497762	Cinnamoyl-CoA reductase 2-like	CCR2
GF677_17720	ppa016109m	scaffold_7:4278166-4279839	Ethylene-responsive transcription factor RAP2-11	RAP2-11
GF677_7066	ppa025240m	scaffold_2:23277709-23279257	Abscisic acid receptor PYL4-like	PYL4
GF677_17672	ppa009537m	scaffold_7:3370462-3373793	2-Aminoethanethiol Dioxygenase	ADO
GF677_300	ppa023251m	scaffold_1:2553496-2555109	1-aminocyclopropane-1-carboxylate oxidase homolog 1	ACO1
GF677_14474	ppa016616m	scaffold_5:18137563-18138859	Germin-like	GLP
GF677_2725	ppa023604m	scaffold_1:31141962-31143686	Lignin-forming anionic peroxidase 4-like	APRX4

Genes differentially expressed in leaves

Gene ID	ID in GDR database	Locus	Description	Abbreviations
cvCatherina.11767	ppa008418m	scaffold_5:17984196-17986682	Purple acid phosphatase 17 like	PAP17
cvCatherina.14437	ppa001232m	scaffold_7:6344692-6348608	Alpha-xylosidase 2	XYL2
cvCatherina.15841	ppa009473m	scaffold_7:21242038-21244975	SPX domain-containing 1-like	SPX1
cvCatherina.15894	ppa006453m	scaffold_7:21656515-21659600	Monogalactosyldiacylglycerol synthase 2, chloroplastic	MGD2
cvCatherina.11558	ppa006823m	scaffold_5:16453361-16456966	Glycerophosphoryl diester phosphodiesterase 3	SHV3
cvCatherina.6050	ppa011927m	scaffold_2:25645720-25648634	Abscisic acid receptor PYL 8	PYL8
cvCatherina.13766	ppa011255m	scaffold_6:25840498-25843531	Pyroglutamyl-peptidase 1-like	PGPEP1
cvCatherina.370	ppa005761m	scaffold_1:4287856-4293128	Pectate lyase 1 related	PEL1
cvCatherina.5807	ppa022802m	scaffold_2:23997510-23998782	Ethylene-responsive transcription factor ERF106	ERF106
cvCatherina.12386	ppa010367m	scaffold_6:5724911-5726097	Aquaporin TIP1.2	TIP1.2
cvCatherina.5855	ppa011607m	scaffold_2:24339289-24340181	Pectin methylesterase inhibitor	PMEI
cvCatherina.7039	ppa025502m	scaffold_3:11265705-11266708	Probable FBOX protein at5g04010	at5g04010

Reference genes

Gene ID	ID in GDR database	Locus	Description	
__	ppa010708m	scaffold_2:9,706,751..9,710,146	AGL-26 LIKE	
__	ppa007238m	scaffold_5:12,941,307..12,944,36	Actin 2	

Table S2. List of differentially expressed genes in roots (stock GF677) involved in response to stimulus (GO: 0050896), with their homology-based predicted functions, RPKM in both conditions and Log_2 (ratio). Fold change (FC) is calculated simply as the ratio (drought/control). Gene ID is the same deposited at ENA archive.

Clusters	Gene _ID	Functions	RPKM control	RPKM drought	Log_2 FC	Regulation
C1	GF677_8275	Universal stress A	59.76	16.40	-1.87	Down
	GF677_5910	Detoxification protein	44.59	17.45	-1.35	Down
	GF677_12859	NRT1 PTR FAMILY	54.17	11.15	-2.28	Down
	GF677_956	Major allergen Pru ar 1-like	49.64	9.34	-2.41	Down
	GF677_7420	Uncharacterized	29.97	13.13	-1.19	Down
	GF677_21081	Auxin-induced 15A-like	33.26	9.89	-1.75	Down
	GF677_272	Inhibitor of trypsin and hageman factor	38.20	6.96	-2.46	Down
	GF677_11410	Epidermis-specific secreted glyco EP1-like	34.71	5.96	-2.54	Down
	GF677_16447	MLO 6	27.10	5.54	-2.29	Down
	GF677_7887	SAUR family	94.81	24.76	-1.94	Down
	GF677_16970	LURP-one-related 5-like	77.01	25.33	-1.60	Down
	GF677_17639	Peroxidase 44	84.19	15.58	-2.43	Down
	GF677_2691	Type IV inositol polyphosphate 5-phosphatase 9	74.94	16.97	-2.14	Down
	GF677_20004	Acyl-[acyl-carrier-] desaturase 6, chloroplastic	105.81	12.82	-3.05	Down
	GF677_1518	Defensin 1	89.47	10.17	-3.14	Down
	GF677_985	Major allergen Pru ar 1-like	121.19	5.52	-4.46	Down
	GF677_17672	2-Aminoethanethiol dioxygenase-like	100.52	5.94	-4.08	Down
	GF677_11339	Alpha-amylase subtilisin inhibitor-like	70.79	5.83	-3.60	Down
	GF677_7066	Abscisic acid receptor PYL4	59.46	5.52	-3.43	Down
	GF677_980	Major allergen Pru ar 1-like	65.82	1.93	-5.09	Down
	GF677_17088	Universal stress A	283.06	46.96	-2.59	Down
	GF677_14803	Peroxidase P7-like	295.23	40.24	-2.88	Down
	GF677_16877	Cationic peroxidase 1-like	258.69	75.04	-1.79	Down
	GF677_5072	Protease inhibitor	173.14	29.25	-2.57	Down
	GF677_993	Major allergen Pru ar 1-like	455.64	21.44	-4.41	Down
	GF677_994	Major allergen Pru ar 1-like	283.21	6.09	-5.54	Down
C2	GF677_16722	Sulfoquinovosyl transferase SQD2-like	49.99	189.76	1.92	Up
	GF677_2591	Major Latex Protein MLP 28	54.09	174.13	1.69	Up
	GF677_19583	SPX domain-containing 1-like	50.44	239.40	2.25	Up
	GF677_1088	Sodium hydrogen exchanger 2-like	50.44	119.84	1.25	Up
	GF677_15065	Peroxidase A2-like	53.96	105.24	0.96	Up
	GF677_20344	Defensin 19	98.10	412.72	2.07	Up
	GF677_17001	Hydrophobic protein RCI2B-like	91.80	353.57	1.95	Up
	GF677_20074	vacuolar iron transporter homolog 4-like	84.76	266.96	1.66	Up
	GF677_18894	Peroxidase 72-like	26.43	71.05	1.43	Up
	GF677_21039	Inositol-tetrakisphosphate 1-kinase 3-like isoform X1	24.69	62.77	1.35	Up
	GF677_2590	Major Latex Protein MLP 328	18.21	71.77	1.98	Up
	GF677_11520	Metallothionein	13.28	173.29	3.71	Up
C3	GF677_16223	E3 ubiquitin- ligase FANCL isoform X1	7.05	14.68	1.06	Up
	GF677_12109	Histidine kinase 5	6.11	14.34	1.23	Up
	GF677_2289	Adenine nucleotide alpha hydrolase superfamily	7.81	12.90	0.72	Up
	GF677_20261	Somatic embryogenesis receptor kinase 2	7.24	12.11	0.74	Up
	GF677_22052	Probable beta-D-xylosidase 2	6.57	10.54	0.68	Up
	GF677_5054	ABC transporter G family member 25	6.38	17.00	1.41	Up
	GF677_15000	Transcription factor bHLH122-like isoform X1	5.83	17.41	1.58	Up
	GF677_438	Purple acid phosphatase 23	6.49	19.72	1.60	Up
	GF677_3709	Carotenoid cleavage dioxygenase 8 homolog B, chloroplasti	4.51	18.20	2.01	Up
	GF677_5813	Mechanosensitive ion channel	4.92	9.05	0.88	Up
	GF677_10190	Phospholipase A I-like	4.98	8.35	0.74	Up
	GF677_11900	MLO 12	3.62	10.47	1.53	Up
	GF677_19674	Homeobox knotted-1-like 3 isoform X2	14.57	21.85	0.59	Up
	GF677_18270	Probable glutamate carboxypeptidase 2	10.21	15.11	0.57	Up
	GF677_6071	SPX domain-containing 3	7.44	36.98	2.31	Up
	GF677_10265	MYB6	3.32	25.32	2.93	Up
	GF677_2910	Probable nucleoredoxin 2	2.90	24.53	3.08	Up
	GF677_14452	Purple acid phosphatase 17	4.12	31.65	2.94	Up
	GF677_18885	Dehydrin Rab 18	1.59	45.34	4.83	Up
	GF677_17117	Phospholipase D p1	1.06	6.67	2.66	Up
C4	GF677_10209	Cysteine-rich receptor kinase 10	15.39	3.84	-2.00	Down
	GF677_16990	Probable leucine-rich repeat receptor kinase At5g49770	12.97	3.27	-1.99	Down
	GF677_13608	Caffeic acid 3-O-methyltransferase	10.73	2.11	-2.35	Down
	GF677_20005	Acyl-[acyl-carrier-] desaturase 6, chloroplastic	13.77	1.33	-3.37	Down
	GF677_2725	Lignin-forming anionic peroxidase-like	20.68	0.29	-6.15	Down
	GF677_5236	Annexin D4	7.74	0.47	-4.04	Down
	GF677_4609	Aminotransferase ALD1-like	4.71	0.34	-3.81	Down
	GF677_18051	Multidrug And Toxic Compound Extrusion (MATE)	4.88	1.09	-2.16	Down
C5	GF677_989	Major allergen Pru ar 1-like	1,039.98	50.45	-4.37	Down
	GF677_986	Major allergen Pru ar 1-like	965.52	37.81	-4.67	Down
	GF677_981	Major allergen Pru ar 1-like	1,363.10	44.12	-4.95	Down
	GF677_990	Major allergen Mal d	757.25	50.08	-3.92	Down
	GF677_15343	major allergen Pru ar 1-like	530.08	54.64	-3.28	Down
	GF677_987	Major allergen Pru ar 1-like	2,075.70	212.63	-3.29	Down
	GF677_983	Major allergen Pru ar 1-like	6,681.83	1319.04	-2.34	Down

Table S3. Functional classification of annotated differentially expressed genes (DEGs) in roots (GF677 rootstock) and leaves (graft, var. Catherina). The gene ID is the same as that deposited in the ENA. GDR: Genome Database of Rosaceae. The color bar indicates the transition of the expression pattern in terms of Log_2FC from upregulation (red squares) to downregulation (blue squares). The dark blue pattern with stars corresponds to genes uniquely expressed in the leaves of the control plants. The genes that were validated using RT-qPCR are in bold. The fold change (FC) was calculated as the ratio between the drought-stressed and control plants. (A) Signaling and regulatory proteins. (B) Functional proteins

A- Signaling and regulatory proteins

Functions		Gene ID	ID in GDR	Annotation	Tissues	Log_2FC
Receptors and proteins kinases	LRR-RLK receptor kinases	GF677_7143	ppa000889m	Probably inactive LRR protein kinase At3g28040	root	1.59
		GF677_10556	**ppa002450m**	**LRR receptor-like serine threonine protein-kinase**	**root**	**1.08**
		GF677_20261	ppa002871m	Somatic embryogenesis receptor kinase 2	root	0.74
		GF677_19662	ppa000884m	LRR receptor kinase TDR	root	0.64
		GF677_11379	ppa003884m	Probable LRR receptor-like serine threonine- kinase At3g14840	root	-1.65
		GF677_16990	ppa000921m	Probable LRR receptor kinase At5g49770	root	-1.99
		GF677_11080	ppa017185m	Probable LRR receptor-like serine threonine- kinase At4g36180	root	-2.25
		GF677_11081	ppa017185m	Probable LRR receptor-like serine threonine- kinase At4g36180	root	-2.65
		GF677_5955	ppa020571m	Probable LRR receptor-like serine threonine- kinase At3g47570	root	-2.79
		GF677_11863	ppa017796m	Probable LRR receptor kinase At5g49770	root	-3.82
	RLK and RK receptor kinases	GF677_17252	ppa006797m	Receptor-like cytosolic serine threonine-protein kinase rbk2	root	1.19
		GF677_8772	ppa000942m	Receptor protein kinase TMK1	root	0.67
		GF677_12937	ppa006942m	Probable receptor-like protein kinase At5g47070	root	0.53
		GF677_5741	ppa003241m	Probable receptor kinase At1g67000	root	-1.11
		GF677_5727	ppa004124m	Probable receptor kinase At1g67000	root	-1.71
		GF677_3514	ppa027139m	Receptor kinase 1	root	-2.21
		GF677_15053	ppb015337m	G-type lectin S-receptor-like serine threonine- kinase RLK1	root	-2.48
		GF677_15022	ppb016806m	G-type lectin S-receptor-like serine threonine- kinase RLK1	root	-2.72
		GF677_8917	ppa019342m	Wall-associated receptor kinase-like 1	root	-3.06
		GF677_8592	ppa018157m	Probable receptor kinase At1g67000	root	-4.56
		GF677_3515	ppa027139m	Receptor kinase 1	root	-4.79
	STK	GF677_19962	ppa023131m	Serine/threonine-protein kinase At5g01020-like	root	2.84
		GF677_8729	ppa004124m	Serine threonine- kinase CDL1	root	0.96
		GF677_19063	ppa007710m	Probable serine threonine- kinase Cx32, chloroplastic	root	0.79
		cvCatherina.11364	ppa007019m	Serine threonine- kinase HT1-like	leaf	-0.52
	CRK	GF677_10211	ppa002309m	Cysteine-rich receptor kinase 29	root	-1.46
		GF677_10210	ppa002615m	Cysteine-rich receptor kinase 15	root	-1.81
		GF677_10209	ppa022109m	Cysteine-rich receptor kinase 10	root	-2.00
	Protein kinases (PK)	GF677_21039	ppa021570m	Inositol-tetrakisphosphate 1-kinase 3-like isoform X1	root	1.35
		GF677_12109	ppa000361m	Histidine kinase 5	root	1.23
		GF677_9801	ppa002420m	Kinase PVPK-1	root	0.80
		cvCatherina.7529	ppa004529m	Diacylglycerol kinase 5	leaf	0.50
Ca^{2+} signaling	EF-hand family	GF677_3581	ppa016083m	Calcium-binding EF-hand family	root	2.82
		GF677_3574	ppa026792m	Calcium-binding EF-hand family	root	2.78
		GF677_3573	ppa026792m	Calcium-binding EF-hand family	root	2.28
	Calcium-binding CML	GF677_3582	ppa013983m	Calcium-binding CML10	root	1.94
		GF677_3137	ppa021408m	Probable calcium-binding CML41	root	-2.50
	Uniporter	GF677_18778	ppa006923m	Calcium uniporter 2, mitochondrial	root	1.95
		GF677_18777	ppa006923m	Calcium uniporter 2, mitochondrial	root	1.90
Phospholipases	-	GF677_17117	ppa000572m	Phospholipase D p1	root	2.66
		GF677_10190	ppa000303m	Phospholipase A I-like	root	0.74
Phosphatases	-	GF677_16384	ppa011449m	Acylphosphatase	root	0.60
		cvCatherina.6526	ppa009904m	Inorganic pyrophosphatase 1-like	leaf	2.61
		cvCatherina.7411	ppa011399m	Probable tyrosine- phosphatase At1g05000	leaf	0.97
		cvCatherina.8503	ppa022650m	Probable 2-carboxy-D-arabinitol-1-phosphatase	leaf	0.32

Table S3 (A) Signaling and regulatory proteins (continued).

Functions		Gene ID	ID in GDR	Annotation	Tissues	Log_2FC
Transcription factors	bHLH	GF677_251	ppa017791m	Transcription factor bHLH92	root	1.80
		GF677_15000	ppa006295m	Transcription factor bHLH122-like isoform X1	root	1.58
		GF677_10526	ppa021837m	Transcription factor bHLH30-like	root	-1.11
		GF677_16183	ppa007656m	Transcription factor bHLH93	root	-1.55
		GF677_4929	ppa009461m	Transcription factor bHLH96-like	root	-1.65
		GF677_4782	ppa016514m	Transcription factor bHLH93-like	root	-2.31
	NAC	**GF677_9569**	**ppa007883m**	**NAC domain-containing 100-like**	**root**	**2.76**
		GF677_11557	ppa008301m	NAC domain-containing 72	root	1.98
		GF677_17765	ppa007445m	NAC transcription factor 29-like	root	1.87
		GF677_19364	ppa004775m	NAC domain containing 75 isoform 1	root	1.01
		cvCatherina.5318	ppa019780m	NAC domain-containing 72-like	leaf	-1.23
	ERT	GF677_16383	ppa003783m	AP2-like ethylene responsive transcription factor	root	1.25
		GF677_16245	ppa002612m	AP2-like ethylene-responsive transcription factor ANT	root	1.00
		GF677_21629	ppa023839m	Ethylene-responsive transcription factor 1B-like	root	-1.90
		GF677_17720	**ppa016109m**	**Ethylene-responsive transcription factor RAP2-11-like**	**root**	**-2.33**
		cvCatherina.5807	**ppa022802m**	**Ethylene-responsive transcription factor ERF106-like**	**leaf**	**-2.32**
	HD-ZIP	**GF677_6534**	**ppa010647m**	**Homeobox-leucine zipper AtHB12-like**	**root**	**2.23**
		GF677_18786	ppa011343m	Homeobox-leucine zipper protein athb-40	root	1.68
		GF677_3702	ppa001386m	Homeobox-leucine zipper AtHB-8	root	0.78
		GF677_19674	ppa007698m	Homeobox knotted-1-like 3 isoform X2	root	0.59
	ORG2	GF677_17863	ppa018670m	Transcription factor ORG2-like	root	-1.50
		GF677_17858	ppa024966m	Transcription factor ORG2-like	root	-2.15
		GF677_17857	ppa016095m	Transcription factor ORG2-like	root	-3.28
	WRKY	GF677_16920	ppa020736m	Probable WRKY transcription factor 70	root	-1.81
		GF677_16916	ppa020736m	Probable WRKY transcription factor 70	root	-1.93
		GF677_9903	ppa016459m	Probable WRKY transcription factor 75	root	-2.26
	MYB	**GF677_10265**	**ppa007438m**	**Transcription factor MYB 6**	**root**	**2.93**
		GF677_4292	ppa026006m	Transcription factor MYB39	root	1.97
		GF677_16023	ppa019923m	Transcription factor MYB39-like	root	1.53
	GRF	GF677_7962	ppa017593m	Growth-regulating factor 5-like	root	-2.71
Hormone signaling	ABA	**GF677_15114**	**ppa006696m**	**Probable phosphatase 2C 24**	**root**	**4.25**
		GF677_19254	ppa005286m	Phosphatase 2C 77	root	3.13
		GF677_11309	ppa002804m	9-cis-epoxycarotenoid dioxygenase NCED3	root	1.95
		GF677_19391	ppa006926m	Phosphatase 2C 56-like	root	1.85
		GF677_7066	**ppa025240m**	**Abscisic acid receptor PYL4**	**root**	**-3.43**
		cvCatherina.6471	ppa004252m	Phosphatase 2C 16-like	leaf	0.74
		cvCatherina.15676	ppa006926m	Phosphatase 2C 56-like	leaf	0.68
		cvCatherina.6050	**ppa011927m**	**Abscisic acid receptor PYL8**	**leaf**	**0.40**
	AUX	GF677_19295	ppa023151m	Auxin-induced in root cultures 12-like	root	2.93
		GF677_11622	ppa002986m	Indole-3-acetic acid-amido synthetase	root	2.76
		GF677_20924	ppa003134m	Probable indole-3-acetic acid-amido synthetase	root	2.71
		GF677_20923	ppa003134m	Probable indole-3-acetic acid-amido synthetase	root	2.01
		GF677_21081	ppa017462m	Auxin-induced 15A-like	root	-1.75
		GF677_7887	ppa013543m	SAUR family (Small auxin up RNA)	root	-1.94
		cvCatherina.6444	ppa011467m	Auxin-binding ABP19a	leaf	-1.65
		cvCatherina.6397	ppa013543m	SAUR family (Small auxin up RNA)	leaf	-1.81
		cvCatherina.6446	ppa011467m	Auxin-binding ABP19a	leaf	-1.98
	ETH	GF677_21642	ppa026451m	1-aminocyclopropane-1-carboxylate oxidase homolog 1-like	root	-2.14
		GF677_19946	ppa016953m	1-aminocyclopropane-1-carboxylate oxidase-like	root	-3.32
		GF677_19306	ppa009228m	1-aminocyclopropane-1-carboxylate oxidase 1	root	-3.92
		GF677_301	ppa022672m	1-aminocyclopropane-1-carboxylate oxidase homolog 1-like	root	-3.95
		GF677_302	ppa015518m	1-aminocyclopropane-1-carboxylate oxidase homolog 1-like	root	-4.22
		GF677_300	**ppa023251m**	**1-aminocyclopropane-1-carboxylate oxidase homolog 1-like**	**root**	**-4.31**
		GF677_21639	ppa022472m	1-aminocyclopropane-1-carboxylate oxidase homolog 1-like	root	-5.36
	GA	**GF677_10709**	**ppa008211m**	**Gibberellin 2-beta-dioxygenase-like**	**root**	**3.08**
		cvCatherina.2406	ppa013714m	Gibberellin-regulated 1-like	leaf	-0.95
	BR	GF677_8842	ppa020825m	Brassinosteroid-regulated protein BRU1-like	root	-1.20
		cvCatherina.4169	ppa009792m	Brassinosteroid-regulated BRU1-like	leaf	-1.55

Table S3 (B) Functional proteins.

B- Functional proteins

Functions			Gene ID	ID in GDR	Annotation	Tissues	Log_2FC	
ROS generation	Electron transporter		GF677_7191	ppa017806m	Mavicyanin-like	root	1.84	
			GF677_9227	ppa017076m	Blue copper protein	root	1.74	
			GF677_6176	ppa026142m	Cucumber peeling cupredoxin-like	root	0.73	
			GF677_19596	ppa009089m	Blue copper protein	root	-2.16	
			GF677_19598	ppa011524m	Blue copper protein like	root	-2.58	
	DNA repair		GF677_16223	ppa017844m	E3 ubiquitin- ligase FANCL isoform X1	root	1.06	
ROS scavengers	Glutathione S-transferases (GST)		GF677_11293	ppa011202m	Probable glutathione S-transferase	root	2.31	
			GF677_14079	ppa019399m	Glutathione S-transferase U10-like	root	-2.91	
			cvCatherina.9155	ppa011202m	Probable glutathione S-transferase	leaf	1.69	
			cvCatherina.13478	ppa018112m	Probable glutathione S-transferase	leaf	-1.80e+308	**
			cvCatherina.17367	ppa024281m	Probable glutathione S-transferase	leaf	-1.80e+308	**
			cvCatherina.1098	ppa022301m	Probable glutathione S-transferase	leaf	-1.80e+308	**
			cvCatherina.11474	ppa027220m	Glutathione S-transferase U9-like	leaf	-1.80e+308	**
			cvCatherina.351	ppa024247m	Probable glutathione S-transferase	leaf	-1.80e+308	**
			cvCatherina.266	ppa011087m	Probable glutathione S-transferase	leaf	-1.80e+308	**
	Peroxidases		GF677_20376	ppa023313m	Peroxidase 2	root	1.64	
			GF677_18894	ppa008349m	Peroxidase 72-like	root	1.43	
			GF677_15065	ppa007826m	Peroxidase A2-like	root	0.96	
			GF677_16877	ppa008642m	Cationic peroxidase 1-like	root	-1.79	
			GF677_17639	ppa009513m	Peroxidase 44	root	-2.43	
			GF677_14803	ppa018701m	Peroxidase P7-like	root	-2.88	
			cvCatherina.473	ppa008569m	Peroxidase 16	leaf	-1.80e+308	**
	Ascorbate peroxidase (APX)		cvCatherina.12438	ppa010431m	Cytosolic ascorbate peroxidase	leaf	1.36	
			cvCatherina.13321	ppa010426m	L-ascorbate peroxidase 2, cytosolic	leaf	1.22	
			cvCatherina.3325	ppa006270m	Probable L-ascorbate peroxidase 6, chloroplastic isoform X1	leaf	0.20	
	Ferritins		GF677_16815	ppa010086m	Ferritin-4	root	1.66	
			cvCatherina.5689	ppa009703m	Ferritin-3, chloroplastic-like	leaf	2.82	
			cvCatherina.13605	ppa010086m	Ferritin-4, chloroplastic-like	leaf	2.35	
			cvCatherina.13603	ppa008598m	Mitoferrin-like	leaf	2.27	
	Thioredoxin		**GF677_2910**	**ppa006064m**	**Probable nucleoredoxin 2**	**root**	**3.08**	
Cuticule formation	Cutin biosynthesis		GF677_7917	ppa004479m	3-oxoacyl-[acyl-carrier-protein] synthase II (KAS II)	root	2.54	
			GF677_223	ppa004243m	3-ketoacyl- synthase 11-like (KCS)	root	2.20	
	Cutin deposition		GF677_15537	ppa007967m	GDSL esterase lipase At5g22810	root	1.61	
			GF677_18716	ppa021185m	GDSL esterase lipase At2g23540	root	1.46	
			GF677_7885	ppa026827m	GDSL esterase lipase 2-like	root	-2.87	
	Wax transport	nLTPs	GF677_5697	ppa013464m	Non-specific lipid-transfer 8-like	root	1.84	
			GF677_21746	ppa021933m	Non-specific lipid-transfer At2g13820	root	1.63	
			GF677_21748	ppa011432m	Non-specific lipid-transfer At2g13820	root	1.48	
			GF677_14820	ppa013519m	Non-specific lipid-transfer 1-like	root	-4.95	
			cvCatherina.12046	ppa013519m	Non-specific lipid-transfer 1-like	leaf	2.47	
			cvCatherina.12044	ppa023836m	Non-specific lipid-transfer 1-like	leaf	1.12	
			cvCatherina.4632	ppa013464m	Non-specific lipid-transfer 8-like	leaf	-4.06	
		LTPs	GF677_6936	ppa014695m	Lipid-transfer DIR1	root	-1.93	
			GF677_20656	ppa021237m	Lipid-transfer DIR1	root	-2.64	
			GF677_11835	ppa016471m	Lipid-transfer DIR1	root	-3.00	
			cvCatherina.1090	ppa013722m	Lipid-transfer DIR1	leaf	-2.73	
		ABC	GF677_2380	ppa002351m	ABC transporter G family member 21	root	2.75	
			GF677_19512	ppa000236m	ABC transporter G family member 32	root	2.14	
			GF677_5054	ppa016652m	ABC transporter G family member 25	root	1.41	
			GF677_12006	ppa022260m	ABC transporter C family member 3-like	root	-0.56	
Cell wall	Extension		GF677_12964	ppa010376m	Expansin-like B1	root	1.91	
			cvCatherina.12041	ppa010314m	Expansin-A4-like precursor	leaf	-1.10	
			cvCatherina.17131	ppa010171m	Expansin-like A2	leaf	-1.23	
	Degradation		GF677_2000	ppa003156m	U-box domain-containing 19-like	root	-1.62	
			GF677_13579	ppa004653m	Endoglucanase 1	root	-2.64	
			GF677_2725	**ppa023604m**	**Lignin-forming anionic peroxidase-like**	**root**	**-6.15**	
			cvCatherina.370	**ppa005761m**	**Pectate lyase 1 related**	**leaf**	**-0.99**	
			cvCatherina.5855	**ppa011607m**	**Pectin methylesterase inhibitor**	**leaf**	**-3.53**	

Table S3 (B) Functional proteins (continued).

Functions		Gene ID	ID in GDR	Annotation	Tissues	Log_2FC
Transport	General transporters	GF677_20074	ppa010996m	Vacuolar iron transporter homolog 4-like	root	1.66
		GF677_12537	ppa025037m	Probable inorganic phosphate transporter 1-9	root	1.26
		GF677_7237	ppa004254m	Glycerol-3-phosphate transporter 1	root	1.23
		GF677_4246	ppa002542m	Sulfate transporter 1.3 like	root	1.17
		GF677_21626	ppa003959m	Inorganic phosphate transporter 1-4	root	0.78
		GF677_16142	ppa001860m	Nucleobase-ascorbate transporter 11 isoform X1	root	0.72
		GF677_5963	ppa017165m	Bidirectional sugar transporter SWEET17-like	root	-1.13
		GF677_5742	ppa027079m	GABA transporter 1-like	root	-1.16
		GF677_15477	ppa021664m	Nitrate transporter	root	-1.47
		GF677_10035	ppa001984m	Oligopeptide transporter 3	root	-1.71
		GF677_11103	ppa025144m	Organic cation carnitine transporter 3-like	root	-2.03
		GF677_13549	ppa022258m	Aluminum-activated malate transporter 10-like	root	-2.03
		cvCatherina.1306	ppa016701m	Potassium transporter 5-like	leaf	-1.80e+308 **
		cvCatherina.5846	ppa004254m	Glycerol-3-phosphate transporter 1	leaf	1.86
		cvCatherina.17465	ppa003959m	Inorganic phosphate transporter 1-4	leaf	1.14
		cvCatherina.7429	ppa003996m	Glucose transporter 1	leaf	0.55
		cvCatherina.5372	ppa003746m	Vacuolar amino acid transporter 1	leaf	-0.94
		cvCatherina.8185	ppa001984m	Oligopeptide transporter 3	leaf	-1.34
	Water transporter	**cvCatherina.12386**	**ppa010367m**	**Aquaporin TIP1.2**	**leaf**	**-3.13**
LEA	Late embryogenesis	GF677_18885	ppa005514m	Dehydrin Rab 18	root	4.83
Chloroplast	Chloroplastic membrane	**cvCatherina.15894**	**ppa006453m**	**Monogalactosyldiacylglycerol synthase 2, chloroplastic (MGD2)**	**leaf**	**2.66**
Phosphate starvation	SPX domain	GF677_6071	ppa010111m	SPX domain-containing 3	root	2.31
		GF677_19583	ppa009473m	SPX domain-containing 1-like	root	2.25
		cvCatherina.15841	**ppa009473m**	**SPX domain-containing 1-like**	**leaf**	**3.40**
		cvCatherina.7759	ppa008419m	SPX domain-containing 4	leaf	1.86
		cvCatherina.3224	ppa009366m	SPX domain-containing 1-like	leaf	0.91
	Purple acid phosphatase	GF677_14452	ppa008456m	Purple acid phosphatase 17	root	2.94
		GF677_438	ppa003722m	Purple acid phosphatase 23	root	1.60
		cvCatherina.11767	**ppa008418m**	**Purple acid phosphatase 17**	**leaf**	**4.59**
		cvCatherina.374	ppa003722m	Purple acid phosphatase 23	leaf	1.68
		cvCatherina.2459	ppa005293m	Purple acid phosphatase 2	leaf	1.55
		cvCatherina.7049	ppa014823m	Probable inactive purple acid phosphatase 16	leaf	1.54
		cvCatherina.4274	ppa002700m	Probable inactive purple acid phosphatase 27	leaf	1.28
		cvCatherina.5326	ppa006786m	Probable inactive purple acid phosphatase 29	leaf	1.14

Table S4. KEGG metabolic pathways identified and number of differentially expressed (DEGs) and enzymes involved in each pathway. (A) Roots (GF677 rootstock). (B) Leaves (graft, var. Catherina).

A-Roots

ID	Pathways	Number of DEGs	Number of enzymes
map00940	Phenylpropanoid biosynthesis	9	3
map00627	Aminobenzoate degradation	9	4
map00730	Thiamine metabolism	5	1
map00230	Purine metabolism	5	2
map00710	Carbon fixation in photosynthetic organisms	5	2
map00270	Cysteine and methionine metabolism	5	7
map00982	Drug metabolism - cytochrome P450	5	4
map00500	Starch and sucrose metabolism	5	6
map00520	Amino sugar and nucleotide sugar metabolism	4	5
map00980	Metabolism of xenobiotics by cytochrome P450	4	3
map00140	Steroid hormone biosynthesis	4	2
map04660	T cell receptor signaling pathway	4	2
map00941	Flavonoid biosynthesis	4	3
map00130	Ubiquinone and other terpenoid-quinone biosynthesis	3	2
map00250	Alanine, aspartate and glutamate metabolism	3	2
map00562	Inositol phosphate metabolism	3	4
map00360	Phenylalanine metabolism	3	5
map00630	Glyoxylate and dicarboxylate metabolism	3	1
map00350	Tyrosine metabolism	3	5
map00830	Retinol metabolism	3	3
map00071	Fatty acid biosynthesis	3	3
map00260	Glycine, serine and threonine metabolism	3	3
map00620	Pyruvate metabolism	3	2
map00330	Arginine and proline metabolism	2	1
map00564	Glycerophospholipid metabolism	2	3
map00960	Tropane, piperidine and pyridine alkaloid biosynthesis	2	4
map00400	Phenylalanine, tyrosine and tryptophan biosynthesis	2	4
map00401	Novobiocin biosynthesis	2	4
map00232	Caffeine metabolism	2	1
map00590	Arachidonic acid metabolism	2	2
map00591	Linoleic acid metabolism	2	1
map00950	Isoquinoline alkaloid biosynthesis	2	3
map04070	Phosphatidylinositol signaling system	2	2
map01040	Biosynthesis of unsaturated fatty acids	2	1
map00300	Lysine biosynthesis	2	2
map00220	Arginine biosynthesis	2	1
map00460	Cyanoamino acid metabolism	2	2
map00340	Histidine metabolism	2	1
map00983	Drug metabolism - other enzymes	2	1
map00984	Steroid degradation	2	1
map00061	Fatty acid degradation	2	1
map00380	Tryptophan metabolism	2	1
map00053	Ascorbate and aldarate metabolism	1	1
map00010	Glycolysis / Gluconeogenesis	1	1
map00450	Selenocompound metabolism	1	2
map00051	Fructose and mannose metabolism	1	2
map00052	Galactose metabolism	1	1
map00930	Caprolactam degradation	1	1
map00561	Glycerolipid metabolism	1	1
map00680	Methane metabolism	1	1
map00040	Pentose and glucuronate interconversions	1	2
map00480	Glutathione metabolism	1	1
map00920	Sulfur metabolism	1	1
map00565	Ether lipid metabolism	1	1
map00592	alpha-Linolenic acid metabolism	1	1
map00030	Pentose phosphate pathway	1	1
map00906	Carotenoid biosynthesis	1	1
map00904	Diterpenoid biosynthesis	1	1
map00190	Oxidative phosphorylation	1	1
map00860	Porphyrin and chlorophyll metabolism	1	1
map00626	Naphthalene degradation	1	1
map00945	Stilbenoid, diarylheptanoid and gingerol biosynthesis	1	1
map00943	Isoflavonoid biosynthesis	1	1
map00900	Terpenoid backbone biosynthesis	1	2
map00625	Chloroalkane and chloroalkene degradation	1	1

Table S4 (continued).

B-Leaves

ID	Pathways	Number of DEGs	Number of enzymes
map00480	Glutathione metabolism	10	4
map00627	Aminobenzoate degradation	10	2
map00982	Drug metabolism - cytochrome P450	6	1
map00980	Metabolism of xenobiotics by cytochrome P450	6	1
map00940	Phenylpropanoid biosynthesis	5	1
map00230	Purine metabolism	4	2
map00730	Thiamine metabolism	4	1
map00564	Glycerophospholipid metabolism	4	4
map00620	Pyruvate metabolism	4	1
map00860	Porphyrin and chlorophyll metabolism	3	2
map04660	T cell receptor signaling pathway	3	1
map00983	Drug metabolism - other enzymes	3	1
map00630	Glyoxylate and dicarboxylate metabolism	2	1
map00053	Ascorbate and aldarate metabolism	2	1
map00561	Glycerolipid metabolism	2	2
map00040	Pentose and glucuronate interconversions	2	3
map00750	Vitamin B6 metabolism	1	1
map00430	Taurine and hypotaurine metabolism	1	1
map00592	alpha-Linolenic acid metabolism	1	1
map00350	Tyrosine metabolism	1	1
map00010	Glycolysis / Gluconeogenesis	1	1
map00590	Arachidonic acid metabolism	1	1
map00250	Alanine, aspartate and glutamate metabolism	1	1
map00591	Linoleic acid metabolism	1	1
map00910	Nitrogen metabolism	1	2
map04070	Phosphatidylinositol signaling system	1	1
map00190	Oxidative phosphorylation	1	2
map00520	Amino sugar and nucleotide sugar metabolism	1	1
map00020	Citrate cycle (TCA cycle)	1	1
map00460	Cyanoamino acid metabolism	1	1
map00260	Glycine, serine and threonine metabolism	1	1
map00140	Steroid hormone biosynthesis	1	1
map00965	Betalain biosynthesis	1	1
map00941	Flavonoid biosynthesis	1	1
map00400	Phenylalanine, tyrosine and tryptophan biosynthesis	1	1
map00565	Ether lipid metabolism	1	1
map00500	Starch and sucrose metabolism	1	2
map00720	Carbone fixation pathways	1	1