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Integration of transcriptomic and metabolic data reveals *hub* transcription factors involved in drought stress response in sunflower (*Helianthus annuus L.*)

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Abstract

Key message **By integration of transcriptional and metabolic profiles we identified pathways and hubs transcription factors regulated during drought conditions in sunflower, useful for applications in molecular and/or biotechnological breeding.**

Abstract Drought is one of the most important environmental stresses that effects crop productivity in many agricultural regions. Sunflower is tolerant to drought conditions but the mechanisms involved in this tolerance remain unclear at the molecular level. The aim of this study was to characterize and integrate transcriptional and metabolic pathways related to drought stress in sunflower plants, by using a system biology approach. Our results showed a delay in plant senescence with an increase in the expression level of photosynthesis related genes as well as higher levels of sugars, osmoprotectant amino acids and ionic nutrients under drought conditions. In addition, we identified transcription factors that were upregulated during drought conditions and that may act as *hubs* in the transcriptional network. Many of these transcription factors belong to families implicated in the drought response in model species. The integration of transcriptomic and metabolomic data in this study, together with physiological measurements, has improved our understanding of the biological responses during droughts and contributes to elucidate the molecular mechanisms involved under this environmental condition. These findings will provide useful biotechnological tools to improve stress tolerance while maintaining crop yield under restricted water availability.

Keywords Sunflower · *Helianthus annuus L.* · Drought · Transcriptomics · Metabolomics · Data integration

Paula Fernandez and Ruth A. Heinz have equally contributed to this work and share last authorship.

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Introduction

Drought is the most important environmental stress that affects agriculture worldwide, particularly in terms of the productivity of field crops (Schmidhuber and Tubiello 2007). The physiological responses to droughts are complex and highly dependent on the severity, duration and timing of the drought as well as on the affected species (Tardieu and Tuberosa 2010). Under these conditions, plant metabolisms are disturbed and should be reconfigured to maintain the essential metabolism and acclimatization thus adopting a novel steady state. The plasticity for a given trait may have important adaptive effects, which minimize deleterious effects of the environment and maximize the survival, growth and reproduction (De Witt et al. 1998; Alpert and Simms 2002; van Kleunen and Fischer 2005; Couso and Fernández 2012).

Drought stress can occur at any stage of plant growth and can affect productivity depending on the timing and duration during crop development. Droughts are generally associated with various morphological and physiological changes in plants including reduced aerial organ expansion, decrease in transpiration and photosynthesis, accumulation of osmotic compounds, activation of detoxifying processes, and transcriptional regulation of many genes (Cramer et al. 2007; Chaves et al. 2009; Thomas 2015). Several efforts have been made to improve crop productivity under drought conditions. However, a large gap remains between yields obtained under optimal and drought conditions (Cattivelli et al. 2008).

Sunflower, the fourth most important oil crop worldwide, is normally susceptible to low temperatures and salinity (Maas and Hoffman 1977; Kratsch and Wise 2000; Huang et al. 2005), but with a relative tolerance to drought conditions because of its highly explorative root system (Connor et al. 1985; Connor and Jones 1985; Sadras et al. 1991). Recently, Andrianasolo et al. (2016) have evaluated genotypic responses to water deficit before and after flowering at the physiological level. These researchers showed that the photosynthetic activity is more sensitive to soil water deficit at the reproductive stage, while the transpiration is more affected at the vegetative stage.

Regarding functional genomics, few research groups have reported about gene expression in response to abiotic stresses in this crop and these studies have been focused on few genes (Ouvrard et al. 1996; Cellier et al. 1998; Giordani et al. 1999; Kiani et al. 2007a, b; Manavella et al. 2008a; Moschen et al. 2014b; Raineri et al. 2015).

Transcriptomic experiments have been developed in sunflower in relation to leaf senescence (Moschen et al. 2016a, b) yield and flooding tolerance (Cabello et al. 2016) as well as to assess the role of NO in root architecture (Corti Monzón et al. 2014). In addition, transcriptomic experiments have been carried out to study seed germination (El-Maarouf-Bouteau et al. 2015), the response to chilling and salt stresses (Fernandez et al. 2008) as well as to water stress (Roche et al. 2007). However, there are no studies to date reporting the analysis of drought response in sunflower integrating transcriptomics, metabolomics and physiological data.

In this study, we characterized transcriptional and metabolic pathways related to drought conditions in sunflower and identified candidate genes and key metabolic pathways involved in the response to early water deficit, through an integrated analysis of transcriptomic and metabolic profiles.

Materials and methods

Plant material and experimental conditions

The experiment was conducted under field conditions at the INTA Balcarce Experimental Station (37°45′S, 58°18′W) during the 2010/11 growing season. The sunflower hybrid VDH 487 (Advanta Seeds, Argentina) was sown under a shelter at a 7.2 plants/m^2 with three biological replicates (plots). Each biological replicate consisted of three randomly selected plants from each plot. Soil fertility, diseases, weeds and insects were adequately controlled. Two experimental conditions were implemented. A control condition in which plants were grown without water and nutritional limitations, with water content close to 40% in the first 60 cm of soil depth. The other condition was a medium-intensive drought in which the volumetric moisture was reduced to about 20% from 20 days post-emergence according to previous studies in sunflower (Pereyra-Irujo et al. 2007) (195°CDAE) (Fig. 1). The water content measurements were performed periodically using a Trase System, Model 6050X1, Soil Moisture Equipment Corp., Santa Barbara, CA, USA.

Transcriptomic and metabolic profiles were performed using the 10th leaf (numbered from the bottom to the top of the plant) at three developmental stages, labeled as T1 (young leaf, maximum chlorophyll content), T2 (middle age leaf, pre-anthesis) and T3 (old leaf, post-anthesis) (Fig. 1). The experiment was performed under field conditions. Thus, time was expressed on a thermal time basis by daily integration of air temperature with a threshold temperature of 6 °C. In addition, plant emergence was considered as thermal time origin (°CDAE: *°C Days After Emergence*) as previously proposed for sunflower (Kiniry et al. 1992).

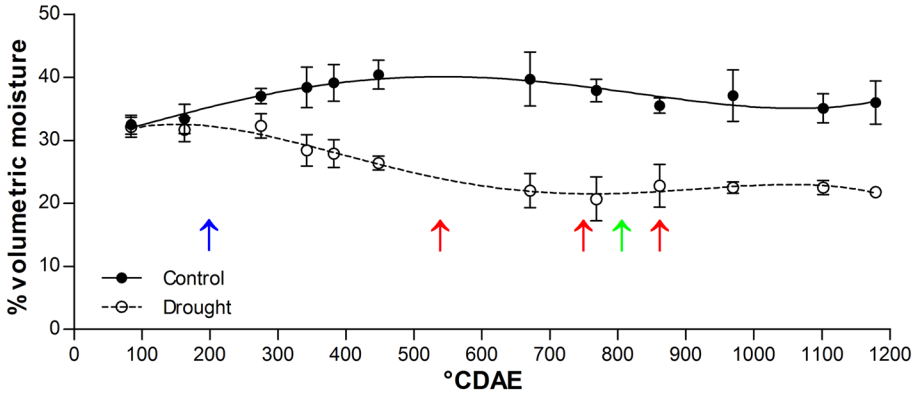

Fig. 1 Soil water content during plant development. Measurements were obtained using a Trase System in the first 60 cm of soil depth. *Solid lines* correspond to control conditions and *dotted lines* correspond to drought conditions. *Red arrows* indicate the three evaluated sampling points (T1, T2 and T3). The *green arrow* shows the anthesis time and the *blue arrow* indicates the initiation of the drought stress treatment. *°CDAE* thermal time (°C days) after emergence

Physiological measurements

Physiological measurements were assessed as previously described (Moschen et al. 2014a) including chlorophyll, total soluble carbohydrates and nitrogen determinations.

Briefly, chlorophyll content of the sampled leaves was measured by chemical extraction with N, N dimethylformamide. 6 disks of 0.5 cm-diameter for each biological replicate were dried with tissue paper and incubated in vials containing 6 ml of *N,N*-dimethylformamide overnight at room temperature in darkness. Absorbance of each sample was measured using a spectrophotometer and chlorophyll content was calculated as:

$$\text{Chlorophyll}\left(\text{mg l}^{-1}\right) = 17.9 * \text{abs}\,(647) + 8.08 * \text{abs}\,(664).$$

Chlorophyll (mg cm^{-2}) = Cl (mg l^{-1})/1.1775 cm^2 (Inskeep and Bloom 1985). For total soluble carbohydrates, 9 disks of 1.2 cm-diameter for each biological replicate were quantified by a colorimetric method using a phenol and sulfuric acid reaction and compared with a standard curve constructed with glucose standard solutions (0–15 µg $µl^{-1}$) (Dubois et al. 1956). The percentage of total nitrogen was measured according to the Dumas method from 60 mg of dry tissue (Dumas 1826).

Additionally, Green leaf area (GLA) was calculated per plant as follows:

$$GLA\left(\text{cm}^2\right) = \frac{(Leaf\ area \times greenness\ \%)}{100}$$

where $Leaf\ area = 1.528 \times (leaf\ width)^{1.7235}$ and *percentage of greenness* was estimated visually, by comparing the relation between green and yellow parts of each leaf (from 100 to 0%) (Aguirrezábal et al. 1996).

Transcriptomic analysis

RNA isolation, quantification and quality controls

The sampled leaves were frozen immediately in liquid nitrogen upon collection and stored at −80 °C until processing. High quality total RNA was isolated from 100 mg of frozen tissue using TriPure, according to the manufacturer´s instructions (Roche, Buenos Aires, Argentina). Genomic DNA was eliminated after treatment with DNase I for 20 min at room temperature using DNase I (Invitrogen, Buenos Aires, Argentina). RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA). The purity and integrity of total RNA was determined by 260/280 nm ratio and by Nano-Bioanalyzer RNA-6000 analysis (Agilent Technologies, Palo Alto, California USA).

Microarray analysis

Transcriptomic profile was performed using a previously developed custom sunflower microarray, Agilent 4 × 44 K (Fernandez et al. 2012). Briefly, data pre-processing was performed using the *Limma* library (Smyth 2005) from the R-package (R Core Team 2012). Background correction was performed using the *rma* algorithm from the *backgroundCorrect* function. Between-array normalization was performed using the *quantile* method from the *normalizeBetweenArray* function. Finally, fold changes were transformed to log2 scale and the information from technical replicates was incorporated by calculating the median parameter.

Differential gene expression analysis

The Sunflower Custom Oligo Microarray includes four arrays per chip; therefore, to accommodate 6 treatments, we planned a hybridization plan following an incomplete block design with four replicates for treatment. Differential gene expression was performed, gene by gene. For this, we estimated a mixed-effects linear model and used *lme* function of the *nlme* library of the R language (Pinheiro et al. 2012). Gene set analysis was conducted according to the Gene Ontology terms (Ashburner et al. 2000) using a logistic regression model (Sartor et al. 2009; Montaner and Dopazo 2010) integrated in the Babelomics suite (Alonso et al. 2015). The p values correction in a multiple test context was performed using the Benjamini-Hochberg method (Benjamini and Hochberg 1995).

Microarray validation: qPCR analysis

The expression of selected genes was evaluated in relation to Elongation Factor −1α (EF−1α), which has been previously selected as a reference gene for expression senescence experiments (Fernandez et al. 2011) (Fig. S1). Specific primer pairs were designed using Primer3 software (Rozen and Skaletsky 2000) with default parameters.

For each sample, 500 ng of RNA were treated with DNase and reverse-transcribed (Superscript III first strand synthesis system, Invitrogen, Buenos Aires, Argentina) using random hexamer primers according to the manufacturer's instructions as previously reported in sunflower (Moschen et al. 2014a).

Metabolic analysis

GC–TOF-MS analysis

Metabolite extraction was performed promoting the extraction of lipophilic and polar compounds according to Roessner-Tunali et al. (2003) with adaptations to sunflower tissue (Peluffo et al. 2010). Samples were derivatised and injected (1 µl) into the GC–TOF-MS system (LECO Corporation, St. Joseph, Michigan, USA). Chromatography was performed on a 30 m SPB-50 column with 0.25 mm inner diameter and 0.25 lm film thickness (Supelco, Belfonte, CA, USA). The temperatures of injection, interface and ion source were set to 230, 250 and 200 °C, respectively. The carrier gas was He at a constant flow ratio of 1 ml m^{-1}. The chromatograms and spectra were evaluated using the ChromaTOF (LECO Corporation, St. Joseph, Míchigan, USA) and TagFinder (Luedemann et al. 2008). Ion spectra were compared to the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/). Metabolite levels were normalized to fresh weight and the internal control ribitol. Changes in metabolite levels were calculated as the fold-change relative to control conditions.

Ion chromatography

Ionic nutrients were assessed by Ion chromatography. A total of 500 µl of 4 °C ULC water (*Ultra Liquid Chromatography*) were added into each metabolite sample extraction. Samples were shaken in vortex 10 s followed by centrifugation 30 min at 14,000 rpm at 4 °C. A total of 480 µl of the supernatant was transferred into glass vials. Serial dilutions of $MgCl_2$, KNO_3, KH_2PO_4, $MgSO_4$, NH_4SO_4 and $CaCl_2$: 100, 50, 25, 12.5, 6.25 and 3.125 µM were used as standard solutions. An ionic nutrient analysis was achieved using Ion Chromatograph Dionex IC3000 (Thermo Scientific, Wisconsin, USA).

Transcriptomic and metabolic integration

MapMan analysis

MapMan software (Thimm et al. 2004) was used to integrate transcriptomic and metabolic profiles. For mapping generation, fasta sequences of *Helianthus annuus* were downloaded from Sunflower Unigene Repository (SUR v1.0) (Fernandez et al. 2012). Annotation was processed through Mercator annotation pipeline (Lohse et al. 2014).

The log2 ratio of fold change (drought/control) was calculated. The expression cutoff was log2 fold change higher or lower than 2 and with a p value lower than 0.05. The resulting data table was used for MapMan analysis.

Weighted gene co-expression network analysis (WGCNA)

WGCNA was performed using the WGCNA R package (v1.51) as described by Langfelder and Horvath (2008). The expression values for 9,684 non-redundant genes were used to construct the network using the following settings: power = 4, minModuleSize = 30, mergeCutHeight = 0.2, maxBlockSize = 10,000, deepSplit = 4, reassignThreshold = 1e−6 and minKMEtoStay = 0.5, networkType = "signed", TOMType = "signed". The network modules were correlated with metabolite levels and differentially expressed transcription factors. Networks were exported for selected modules using the function *exportNetworkToCytoscape* (edge weight threshold of 0.45) and then visualized using Cytoscape (Shannon et al. 2003).

Results

Physiological measurement

Plant development was assessed according to physiological parameters. Progression of GLA reflected the difference in plant size; the control plants showed a maximum GLA of 8000 cm^2, whereas the plants under drought conditions

only reached maximum values of 5000 cm^2. Decrease rate of green leaf area after anthesis was similar under both conditions (Fig. 2a).

Chlorophyll content in the control leaf showed maximum values, which were near to 0.03 mg/cm^2 at 560°CDAE. From this point, the chlorophyll declined slowly up to flowering and more steeply from this point up to 900°CDAE. In the drought condition, the sampled leaf always presented higher values than the control, with a maximum value of 0.035 mg/cm^2 at 560°CDAE and a slower decrease up to 970°CDAE from emergence (Fig. 2b). Likewise, the drought treatment affected soluble sugar content with higher values during all leaf development but a mild increase toward the end of leaf development (Fig. 2c). However, nitrogen content did not show any differences between treatments throughout leaf development (Fig. 2d).

Transcriptomic analysis

We analyzed 9684 non-redundant genes from the microarray experiment and detected 3434 differentially expressed genes between drought vs. control conditions at the three sampling time points (T1 = 512; T2 = 1845 and T3 = 2250 genes). Values were considered significant with p values lower than 0.05 and with a fold change higher or lower than 2. The Venn diagram in Fig. 3 shows that 101 genes were downregulated and 41 were upregulated at all the sampling points (Table S1).

Microarray validation through qPCR analysis yielded similar expression profiles in all the evaluated transcripts in comparison with microarray data (Fig. S1). To analyze major effects in gene regulation under drought conditions, we selected the top up- and downregulated genes with a fold change higher or lower than 4 in at least one of the evaluated conditions. This analysis showed 602 genes differentially expressed in at least one of the three evaluated sampling points (Table S2). The most represented functional categories correspond to amino acid metabolism, cell wall modification and organization, hormone, lipid and sugar metabolism, protein degradation and modification, transcription regulation, secondary metabolism and transport.

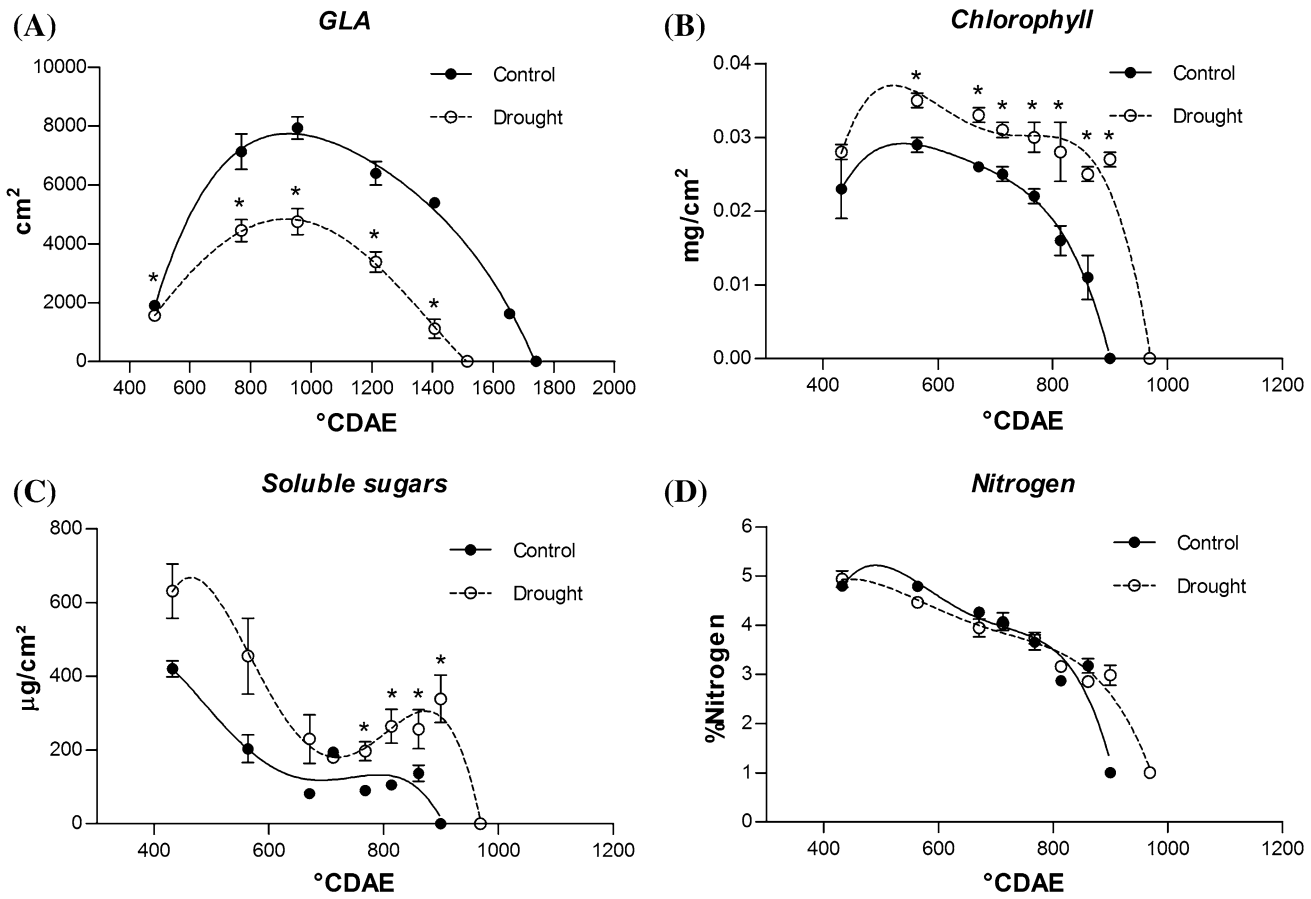

Fig. 2 Physiological measurements of experimental conditions. **a** Green leaf area (GLA); **b** chlorophyll content; **c** soluble sugars and **d** nitrogen content. *Solid lines* correspond to control conditions and *dotted lines* correspond to drought conditions. *Error bars* correspond to standard errors and *asterisks* indicate significant differences ($P \leq 0.05$). °*CDAE* thermal time (°C days) after emergence

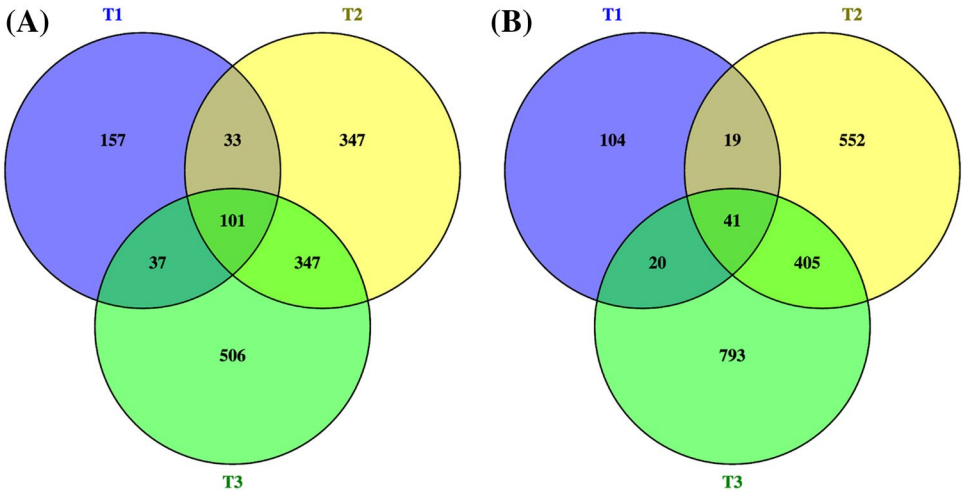

Fig. 3 Venn diagram of differentially non-redundant expressed genes between drought versus control conditions at the three sampling points. *T1* 512 probes (young leaf, maximum chlorophyll content), *T2* 1845 probes (middle age leaf, pre-anthesis) and *T3* 2250 probes (old leaf, post-anthesis). **a** Downregulated genes and **b** upregulated genes

Functional analysis

Through gene set analysis methodology, we detected differentially enriched functional categories represented on the microarray. Indeed, the number of downregulated GO categories was higher than the number of upregulated categories, under drought conditions (Fig. 4). The enriched upregulated GO categories contained genes mainly related to isoprenoid, glycogen and RNA metabolic processes, chemical stimulus, carbon fixation, cellular response to stress and DNA repair (Fig. S2). On the other hand, the enriched downregulated GO categories contained genes mainly related to macromolecule and organic substance metabolic processes, asparagine and ATP biosynthetic process, GTP catabolic process and genes related to cellular localization (Fig. S2).

Transcription factors analysis

We then identified Sunflower Transcription Factors (TFs) by comparing approximately 23,000 sequences of TFs from *Arabidopsis lyrata, A. thaliana, Oryza sativa, Populus trichocarpa, Vitis vinifera* and *Zea mays* available at the plant TF database (http://plntfdb.bio.uni-potsdam.de/v3.0/) (Pérez-Rodríguez et al. 2010) with SUR v1.0 database (Fernandez et al. 2012). Their expression analysis revealed that 42 TFs were differentially expressed under drought conditions, with a log2 fold change higher or lower than 2 in at least one of the three sampling times (Table S3). Among the downregulated TFs, we found that most of them correspond to the AP2/EREBP, WRKY, MYB and NAC TF families. Furthermore, members of zf-HD, AP2/DREB and Sigma70-like TF families were upregulated during drought conditions.

Metabolic analysis

Primary metabolite analysis

By GC–TOF-MS analysis, we detected 54 primary metabolites, including different amino acids, organic acids, sugars and sugar alcohols (Fig. 5). This analysis displayed two distinctly separated groups of metabolites that were up or downregulated during drought conditions. Most of the amino acid metabolites showed lower levels under drought

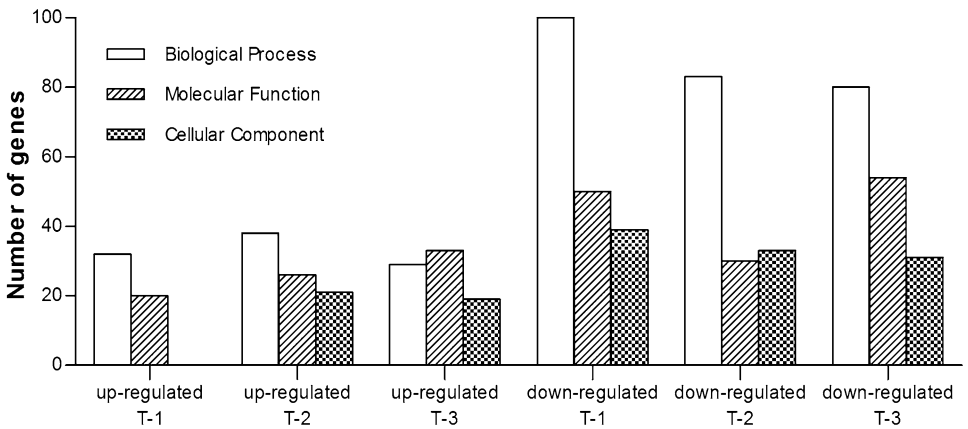

Fig. 4 Distribution of significant GO terms: biological process, molecular function and cellular component at the three evaluated sampling points, *T1* young leaf, maximum chlorophyll content, *T2* middle age leaf, pre-anthesis and *T3* old leaf, post-anthesis

conditions. Nevertheless, the amino acids and derivatives proline, tyramine, glycine, malonate and γ-aminobutyrate showed higher levels under drought conditions, thus indicating a putative role of these metabolites during stress response in sunflower. By contrast, glycolysis and tricarboxylic acid cycle (TCA) metabolites and all the detected carbohydrates showed higher levels under drought conditions.

Ionic nutrient analysis

By ion chromatography, we detected four anionic (chloride, nitrate, sulphate and phosphate) and cationic (sodium, ammonium, potassium, magnesium and calcium) nutrients (Fig. 6). Among the anionic analysis, we found an accumulation of chloride and decrease of nitrate in response to drought. By contrast, the entire cationic nutrients showed an accumulation in leaf under this condition with significant differences in T3 after a long drought treatment.

Integrative analysis

In addition, we characterized the sunflower drought response by integrating transcriptomic and metabolic data and using MapMan (Thimm et al. 2004) (Fig. 7). We were able to detect the most important biological processes affected by drought in sunflower. Among the studied stages (T1, T2 and T3), T2 and T3 showed the higher differences between treatments, with a better clustering of data on

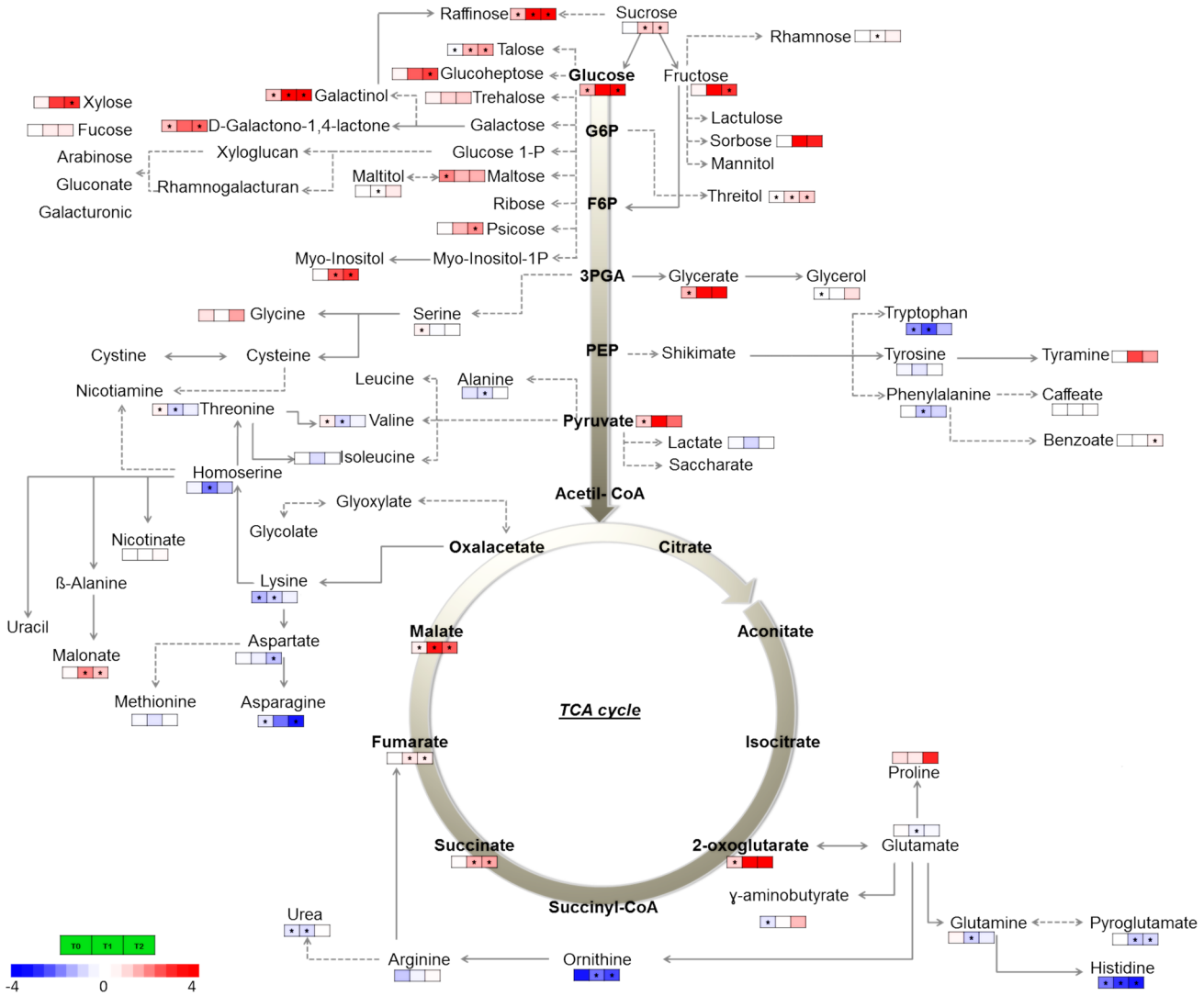

Fig. 5 Metabolic profile analysed by GC–TOF-MS. Each graph represents the (*Log2*) fold change drought versus control condition; *columns* represent the three evaluated sampling points for each metabolite. Colour intensity corresponds to the expression ratio at logarithmic scale (*red* upregulated, *blue* downregulated). The *asterisk* within each graph indicates significant differences (P value <0.05)

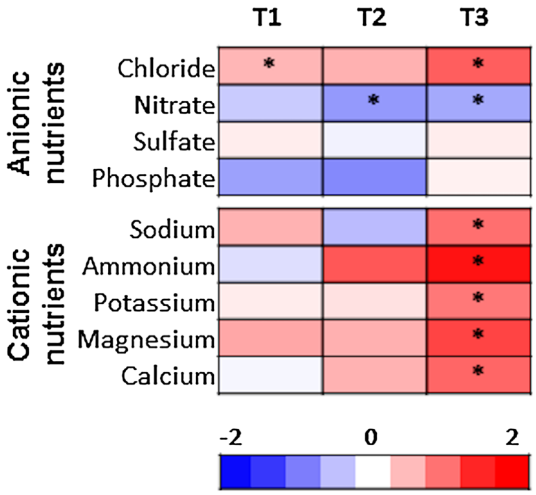

Fig. 6 Ionic nutrient analysis. Each graph represents the (*Log2*) fold change drought versus control condition; *columns* represent the three evaluated sampling points for each nutrient. Colour intensity corresponds to the expression ratio at logarithmic scale (*red* upregulated, *blue* downregulated). The *asterisk* indicates significant differences (P value <0.05)

biological processes (Fig. 7 a, b). By contrast, T1 did not display a marked differentiation between biological processes (data not showed). We detected higher expression levels at T2 (middle age leaf, pre-anthesis). This finding evidences an early activation of drought tolerance mechanisms before anthesis. Under drought conditions imposed at early developmental stage (Fig. 1), the expression levels of genes related to photosynthesis and photorespiration as well as those involved in sugar synthesis and starch degradation increased. This lead to a significant increase in sugars content. Likewise, the expression levels of genes related to sucrose degradation decreased under drought. Glycolysis related genes and TCA cycle metabolites also showed an overexpression under drought conditions. Ascorbate, glutathione cycle genes displayed high expression levels, especially at T2, thus demonstrating an active detoxification process under drought conditions in sunflower.

Regarding cell wall related genes, we identified high levels of several genes associated to cell wall modification, cellulose synthesis, pectase lyases and polygalacturonases. Lipid metabolism related genes also exhibited high expression levels, especially those related to omega 3 desaturase and steroids metabolism. Similarly, the genes associated to flavonoid and terpene metabolism were upregulated during drought.

We performed WGCNA analysis to find co-expressed genes related to biological processes that we demonstrated to be involved in the drought response. WGCNA allocated 7021 genes to 12 modules (Table S4); each module is represented by a color and contained between 57 and 1836 genes. The remaining genes were allocated to the unassigned grey module.

We then searched for highly connected transcription factors related to differentially expressed genes and biological processes of interest. For this purpose, we correlated each module with metabolite levels (Fig. S3). In addition, we determined the distribution of the 93 upregulated and 95 downregulated TFs (Table S4). The brown and blue modules were positively correlated with sugar metabolites and contained upregulated transcription factors (mainly in the pre-anthesis sampling). By contrast, the turquoise module contained most of the downregulated transcription factors and showed a negative correlation with sugars and a corresponding high positive correlation with amino acid (except for the amino acids and derivatives mentioned above) (Fig. S3).

We then exported the selected modules and visualized them by using Cytoscape (Shannon et al. 2003) to find highly connected TFs associated to drought in sunflower (Fig. 8). This analysis showed 12 upregulated and 19 downregulated TFs with high numbers of connections (degree of >15 and >20 respectively) and therefore are potentially acting as hubs in the gene network. Transcription factor classification showed that members of sigma like factors, zf-HD and HD-Zip TFs families displayed higher expression levels. On the other hand, the members of NAC, AP2-EREBP and bZIP TFs families showed the lower expression levels. These findings postulate these transcription factors as potential *hub* genes regulated during drought in sunflower.

Discussion

Sunflower is susceptible to low temperatures and salinity (Maas and Hoffman 1977; Kratsch and Wise 2000; Huang et al. 2005), but shows a relatively high tolerance to drought thanks to its highly explorative root system (Connor et al. 1985; Connor and Jones 1985; Sadras et al. 1991).

Drought was triggered in the early stages of plant development (Fig. 1) which allowed the plants to adapt. This resulted in smaller plants with smaller leaves. In previous studies, Rousseaux et al. (1996) reported that different levels and quality of PAR radiation absorbed by leaves affect the duration of green leaf area during the pre- and post-anthesis phases.

The 10th leaf was selected for this study, as it develops in a lower crop stratum of plants and is therefore affected by an increased shading by the upper leaves, decreasing the red/far red relationship. Under drought conditions, this shading effect is lower owing to the minor leaf area intercepting more quantity and quality of PAR radiation (Fig. 1a). As a result, senescence was delayed and chlorophyll content showed high levels under drought (Fig. 1b).

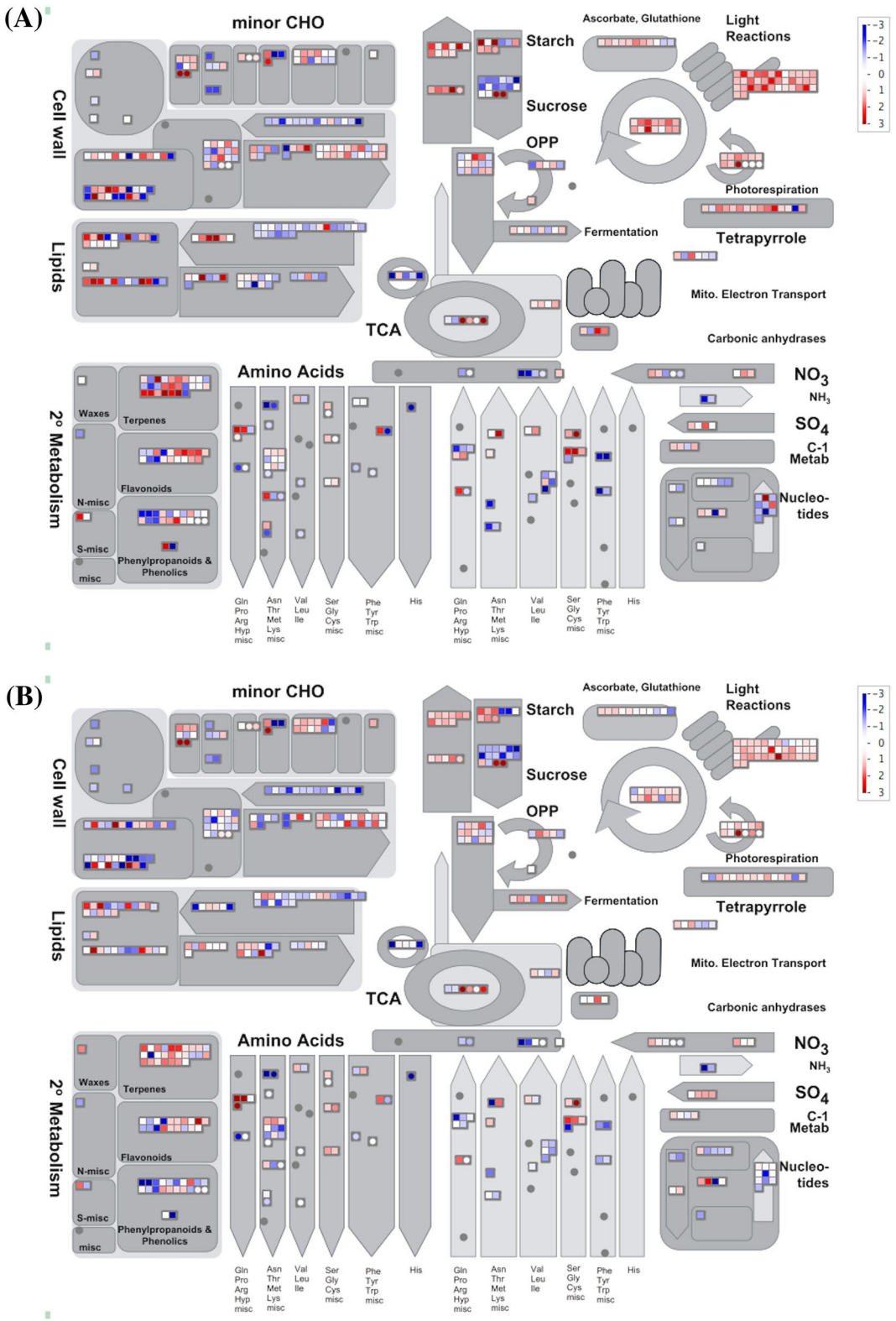

Fig. 7 Metabolism overview in the field experiment. **a** (*Log2*) fold change drought versus control condition at pre-anthesis time (T2) and **b** (*Log2*) fold change drought versus control condition at post-anthesis time (T3). Genes and metabolites are represented by *squares* and *circles*, respectively. Colour intensity corresponds to the expression ratio at logarithmic scale (*red* upregulated, *blue* downregulated)

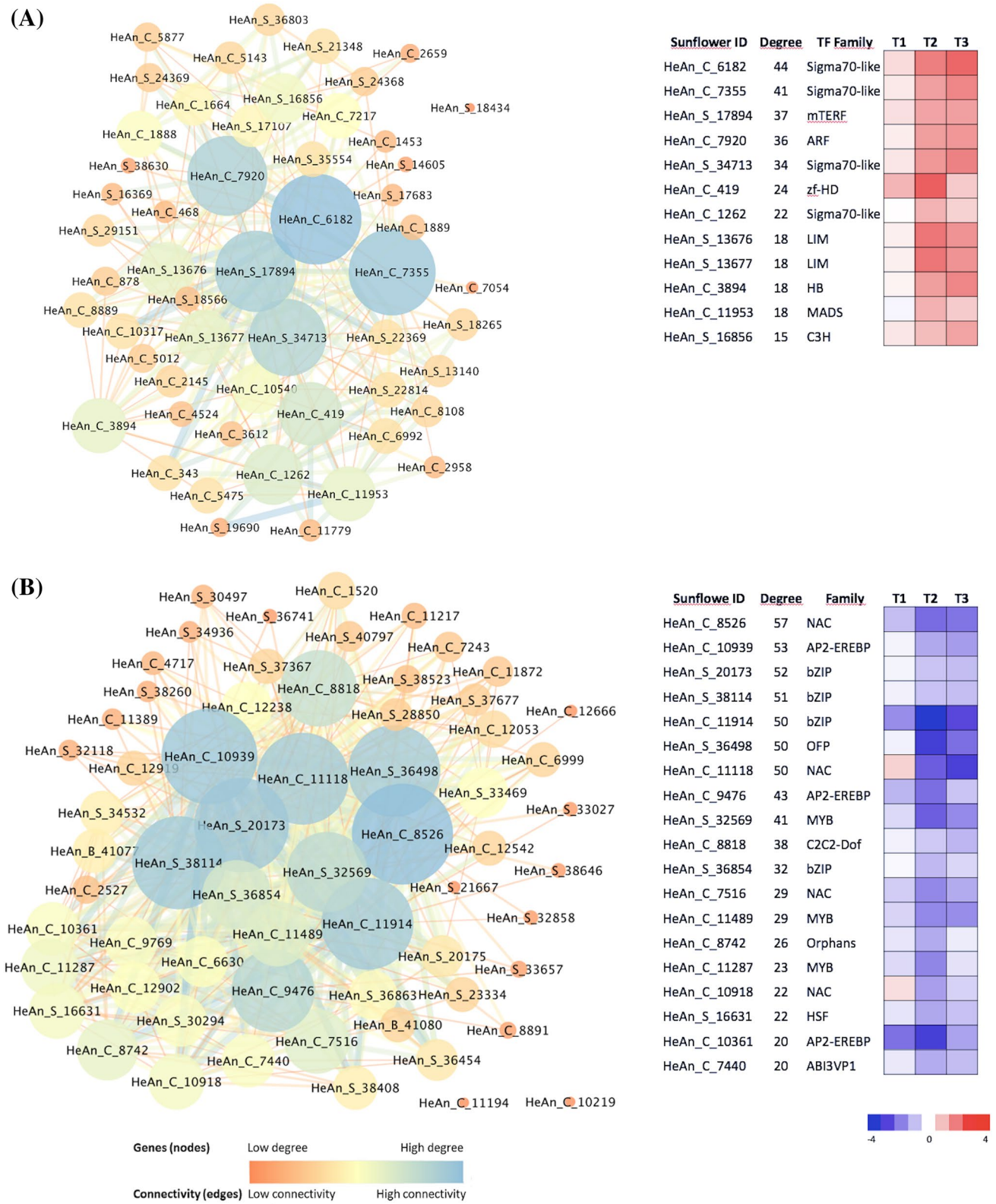

Fig. 8 Gene co-expression networks. **a** *Brown* and *blue* modules showing highly connected upregulated TFs and **b** Turquoise module showing highly connected downregulated TFs. The nodes represent TFs and the edges represent connections between them. The node size and colour are related to the number of connections, *large blue nodes* represent highly connected *hub* genes, *small orange nodes* represent genes with few connections. Strong connections are visualized as *wider lines*. The *tables* show the expression profile of the *hub* TFs at the different sampling points (T1, T2 and T3). Colour intensity corresponds to the expression ratio at logarithmic scale (*red* upregulated, *blue* downregulated)

Meanwhile, sugar content displayed high levels under drought conditions (Fig. 1c). Sugar accumulation is an important mechanism of drought tolerance, by preventing water loss and protecting membranes, enzymes and other cellular structures (Sperdouli and Moustakas 2012).

Recently, transcriptomics and functional genomics analysis have been extensively used to understand the molecular basis of drought responsive mechanisms in crop plants.

In fact, Mir et al. (2012) have reported different genes involved in cellular protection under drought conditions (e.g. proteins involved in osmotic adjustment, degradation, repairs, detoxification and structural adaptations) and/or in regulation mechanisms (protein kinases and transcription factors). Drought could affect photosynthesis by the diffusion limitations through the stomata and the mesophyll or by alterations in photosynthetic metabolism (Chaves et al. 2009).

Sunflower is a crop with low resistance to water transport through the plant and low stomatal resistance (Andrade and Gardiol 1994). Genes related to photosynthesis and photorespiration, sugar synthesis and starch degradation are upregulated under drought compared to control conditions, whereas genes related to sucrose degradation are downregulated (Fig. 7). Indeed, this leads to higher levels in sugar contents, as observed by metabolic analysis (Fig. 5). Nevertheless, in addition to the photosynthesis maintenance, sugar accumulation could be explained by growth reduction under drought stress. Sugar accumulation, which produces osmotic adjustment, is a key adaptation mechanism for drought tolerance in plants. This mechanism avoids cellular dehydration, by maintaining leaf turgor to improve stomatal conductance (Kiani et al. 2007a, b) and promoting water uptake in roots (Chimenti et al. 2006; Farooq et al. 2012). These results suggest a mechanism of drought tolerance in sunflower involving an increase of photosynthesis related genes and higher sugar levels during droughts.

Furthermore, the higher levels of different amino acids and derivatives (proline, tyramine, glycine, malonate and γ-aminobutyrate) underline the osmotic adjustment hypothesis. Glycinebetaine and proline are two major organic osmolytes that accumulate in some plant species in response to environmental stresses such as drought, salinity, among others (Ashraf and Foolad 2007). Glycine betaine is synthesized in chloroplast from serine or direct *N*-methylation of glycine (Hanson and Scott 1980) which showed an accumulation under drought conditions (Fig. 5). We also detected accumulation of proline under drought conditions. Several researchers reported that proline contributes to the cytoplasmic osmotic adjustment in drought or salinity stress responses (Yamada et al. 2005; Planchet et al. 2014; Yaish 2015; Ben Rejeb et al. 2015; Jahantigh et al. 2016).In addition, Ashraf and Foolad (2007) demonstrated that proline accumulation correlates with stress tolerance in several species and that its concentration is generally higher in stress tolerant plants.

Carbon accumulated under drought conditions promotes the synthesis of secondary metabolites. Genes related to flavonoids and terpenes metabolism pathways also showed upregulation during drought conditions. Flavonoids are a major component of specialized/secondary metabolites in plants and display several physiological functions in response to biotic and abiotic stress (Saito et al. 2013; Nakabayashi et al. 2014a). In Arabidopsis, Nakabayashi et al. (2014b) demonstrated that flavonoid accumulation was key to enhance tolerance to both oxidative and drought stresses. Moreover, terpene accumulation is involved in many functions in growth, development and resistance to environmental stresses, in a number of plant species (Gershenzon and Dudareva 2007; Palmer-Young et al. 2015). Terpenes are involved in at least two roles during droughts. They enhance membrane stability during transient heat stress (Sharkey 2001; Sharkey et al. 2008) and have direct antioxidant effects (Vickers et al. 2009). Vickers et al. 2009 sugeted that that unsaturated terpene hydrocarbons react with ROS and therefore quenched these oxidants.

Environmental factors such as drought may cause nutrient deficiencies, even in fertilized fields, by reducing the mobility and absorbance of individual nutrients (Amtmann and Blatt 2009). The participation of each ionic nutrient during the drought stress response is ambiguous and can differ between different plant species (Ciríaco da Silva et al. 2011).

Among the ionic nutrients detected, we found higher levels of chloride and lower levels of nitrate during drought (Fig. 6). The nitrogen status of plant is highly influenced by its water relation. Nitrogen mobility is severely affected by a prolonged drought (Ciríaco da Silva et al. 2011). Drought conditions cause decreased leaf nitrogen concentration in *Coffea canephora* (DaMatta et al. 2002) and *Lactuca sativa* (Ruiz-Lozano and Azcón 1996) grown with low soil nitrogen supply.

Chloride and all other cationic nutrients accumulated with drought (Fig. 6) and this could be due to an osmotic adjustment. Mahouachi et al. (2006) reported accumulation of chloride, potassium and sodium in leaves and roots of *Carica papaya* and Utrillas et al. (1995) detected an increase in calcium content in *Cynodon dactylon* subjected to drought. Ammonium assimilation enzymes were differently affected under drought condition in *Lotus* (Borsani et al. 1999). Glutamate dehydrogenase activity is important for ammonium assimilation (Masclaux-Daubresse et al. 2002; Skopelitis et al. 2006) specially during senescence and abiotic stresses in which ammonium concentration increases. Under these conditions, ammonium assimilated by glutamate dehydrogenase that acts as a detoxification system contributes to maintain

glutamate and glutamine levels, which are precursors of several osmoprotectant compounds (Díaz et al. 2014).

Different methods of co-expression networks analysis have been used to unravel gene function, especially in agricultural crops, thus providing a powerful tool to understand biological processes (Schaefer et al. 2016). Transcription factors are key proteins in the regulation of gene expression and signal transduction networks that regulate different biological processes, and represent promising candidate genes for drought tolerance improvement in crops.

In this study, we identified 12-candidate *hub* TFs with high expression levels under drought conditions (Fig. 8a). These TFs were contained in gene network modules with a positive correlation with some sugar metabolites that have been proposed to act as osmotic regulators. Sigma70-like TFs were highly connected in the gene network, highlighting them as potential regulators of sunflower drought responses. Plastid genes, such as sigma-like factors, are expressed in response to developmental and environmental signals (Allison 2000). In fact, members of this family are induced under various stress conditions, such as high light, low temperature, high salt and high osmotic conditions (Nagashima et al. 2004). However, the regulation mechanism for these plastid sigma factors in response to distinct stress signals is still unknown (Chi et al. 2015).

HeAn_C_419, a sunflower zf-HD TF, showed high transcription levels at a very early stage, at pre-anthesis sampling (T1 and T2) (Fig. 8a). Members of the zf-HD TF family play an important role in plant developmental and stress responses. For instance, Arabidopsis members of zf-HD were induced under dehydration, salt stress and abscisic acid (ABA) treatment. Specifically, ZHD1 TF interacts with some NAC TFs and the resulting simultaneous overexpression of ZHD1 and NAC genes enhanced drought tolerance in Arabidopsis (Tran et al. 2006). Recently, a study of the zf-HD family in Chinese cabbage reported several members being significantly induced under photoperiod or vernalization conditions, as well as under abiotic stresses (Wang et al. 2016). Interestingly, our results highlight this TF as a promising candidate gene for drought response in plants.

Members of HD-Zip TF family, a closely related TFs family with homeodomain (HD), has been studied in plants as regulators of organ and vascular development, meristem maintenance, mediating the action of hormones or involved in responses to different environmental conditions (Ariel et al. 2007). *HeAn_C_3894* contig ID, which codes for sunflower HAHB1 TF, was highly expressed and co-expressed in our analysis. HaHB1 and the Arabidopsis homologous AtHB13 have been studied in response to cold tolerance, which confers cell membrane stabilization and inhibit the recrystallization of ice (Cabello et al. 2012).

HaHB4, another member of the HD-Zip TF family, has also been extensively studied in response to different biotic and abiotic stresses, (Gago et al. 2002; Dezar et al. 2005a, b; Manavella et al. 2006, 2008a, b). Ectopic expression of this gene in Arabidopsis showed strong tolerance to water stress and a reduction in the rate of growth and development (Dezar et al. 2005b). In our study, *HaHB4* (HeAn_C_11893 contig Id) showed an upregulation during drought conditions with a strong increase of transcript levels toward the post-anthesis time (T3) (Table S3).

Most of the downregulated *hub* TFs (Fig. 8b) correspond to members of NAC, AP2-EREBP, bZIP and MYB TFs families and showed negative correlation with sugar accumulation. These TFs families are involved in the senescence process and nutrient recycling by decreasing photosynthetic activity (Moschen et al. 2016a). The sugar accumulation and the downregulation of these TFs evidence photosynthesis maintenance mechanisms helping to keep an osmotic adjustment as drought tolerance mechanism.

In a previous network analysis, we assessed the leaf senescence process under natural growing conditions and detected several TFs candidate genes that could activate this recycling process, specially NAC, AP2-EREBP and MYB TF families (Moschen et al. 2016a, b). In this study, we compared the expression profiles of these TF families associated to leaf development under drought condition (Table S5). Most of these candidate genes showed lower expression levels in drought conditions compared to natural leaf development. This result suggests a delay in the senescence process activation under drought conditions, thus highlighting this event as a tolerance strategy in sunflower.

In sunflower, in addition to its highly explorative root system, the osmotic adjustment mechanism seems to play a very important role in drought tolerance. In this study, we detected an increase in the expression level of photosynthesis related genes, which lead to an accumulation of sugars, different osmoprotectant amino acids, an increase of ionic nutrients and a delay in senescence process under drought condition. This mechanism of osmoprotectant accumulation may act by preventing water loss and protecting membranes, enzymes and other cellular structures. Moreover, we detected promising TFs candidate genes as *hubs* in network correlations, which could be involved in drought response regulation, some of them belonging to families with members previously reported in drought response.

In summary, the results of this study emphasised the importance of the integration of transcriptomic and metabolomic data as a useful tool to identify molecular markers and/or candidate genes that could provide a better understanding of the molecular basis of drought tolerance. They also represent interesting molecular tools for applications in molecular and/or biotechnological breeding to improve stress tolerance and increase crop yield.

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References

Aguirrezábal L, Orioli G, Hernández LF, Pereyra V, Miravé J (1996) Girasol: Aspectos fisiológicos que determinan el rendimiento. Balcarce, Argentina

Allison LA (2000) The role of sigma factors in plastid transcription. Biochimie 82:537–548

Alonso R, Salavert F, Garcia-Garcia F, Carbonell-Caballero J, Bleda M, Garcia-Alonso L, Sanchis-Juan A, Perez-Gil D, Marin-Garcia P, Sanchez R, Cubuk C, Hidalgo MR, Amadoz A, Hernansaiz-Ballesteros RD, Alemán A, Tarraga J, Montaner D, Medina I, Dopazo J (2015) Babelomics 5.0: functional interpretation for new generations of genomic data. Nucleic Acids Res 43:W117–W121. doi:10.1093/nar/gkv384

Alpert P, Simms EL (2002) The relative advantages of plasticity and fixity in different environments: when is it good for a plant to adjust? Evol Ecol 16:285–297. doi:10.1023/A:1019684612767

Amtmann A, Blatt MR (2009) Regulation of macronutrient transport. New Phytol 181:35–52. doi:10.1111/j.1469-8137.2008.02666.x

Andrade FH, Gardiol JM (1994) Sequía y producción de los cultivos de maíz, girasol y soja. Boletín técnico 132. EEA INTA Balcarce

Andrianasolo F, Casadebaig P, Langlade N, Debaeke P, Maury P (2016) Effects of plant growth stage and leaf aging on the response of transpiration and photosynthesis to water deficit in sunflower. Funct Plant Biol 43:797. doi:10.1071/FP15235

Ariel FD, Manavella P a, Dezar C a, Chan RL (2007) The true story of the HD-Zip family. Trends Plant Sci 12:419–426. doi:10.1016/j.tplants.2007.08.003

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25:25–29. doi:10.1038/75556

Ashraf M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59:206–216. doi:10.1016/j.envexpbot.2005.12.006

Ben Rejeb K, Lefebvre-De Vos D, Le Disquet I, Leprince A-S, Bordenave M, Maldiney R, Jdey A, Abdelly C, Savouré A (2015) Hydrogen peroxide produced by NADPH oxidases increases proline accumulation during salt or mannitol stress in *Arabidopsis thaliana*. New Phytol 208:1138–1148. doi:10.1111/nph.13550

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B 57:289–300. doi:10.2307/2346101

Borsani O, Díaz P, Monza J (1999) Proline is involved in water stress responses of lotus corniculatus nitrogen fixing and nitrate fed plants. J Plant Physiol 155:269–273. doi:10.1016/S0176-1617(99)80018-2

Cabello JV, Arce AL, Chan RL (2012) The homologous HD-Zip I transcription factors HaHB1 and AtHB13 confer cold tolerance via the induction of pathogenesis-related and glucanase proteins. Plant J 69:141–153. doi:10.1111/j.1365-313X.2011.04778.x

Cabello JV, Giacomelli JI, Piattoni CV, Iglesias AA, Chan RL (2016) The sunflower transcription factor HaHB11 improves yield, biomass and tolerance to flooding in transgenic Arabidopsis plants. J Biotechnol 222:73–83. doi:10.1016/j.jbiotec.2016.02.015

Cattivelli L, Rizza F, Badeck F-W, Mazzucotelli E, Mastrangelo AM, Francia E, Marè C, Tondelli A, Stanca AM (2008) Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. Field Crop Res 105:1–14. doi:10.1016/j.fcr.2007.07.004

Cellier F, Conejero G, Breitler JC, Casse F (1998) Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower. Accumulation of dehydrin transcripts correlates with tolerance. Plant Physiol 116:319–328

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103:551–560. doi:10.1093/aob/mcn125

Chi W, He B, Mao J, Jiang J, Zhang L (2015) Plastid sigma factors: their individual functions and regulation in transcription. Biochim Biophys Acta 1847:770–778

Chimenti CA, Marcantonio M, Hall AJ (2006) Divergent selection for osmotic adjustment results in improved drought tolerance in maize (*Zea mays* L.) in both early growth and flowering phases. F Crop Res 95:305–315. doi:10.1016/j.fcr.2005.04.003

Ciríaco da Silva E, Mansur Custódio Nogueira RJ, Almeida da Silva M, Bandeira de Albuquerque M (2011) Drought stress and plant nutrition. Plant Stress 5:32–41

Connor DJ, Jones TR (1985) Response of sunflower to strategies of irrigation II. Morphological and physiological responses to water stress. Field Crop Res 12:91–103

Connor DJ, Palta JA, Jones TR (1985) Response of sunflower to strategies of irrigation. III. Crop photosynthesis and transpiration. Field Crop Res 12:281–283

Corti Monzón G, Pinedo M, Di Rienzo J, Novo-Uzal E, Pomar F, Lamattina L, de la Canal L (2014) Nitric oxide is required for determining root architecture and lignin composition in sunflower. Supporting evidence from microarray analyses. Nitric Oxide 39:20–28. doi:10.1016/j.niox.2014.04.004

Couso LL, Fernández RJ (2012) Phenotypic plasticity as an index of drought tolerance in three Patagonian steppe grasses. Ann Bot 110:849–857. doi:10.1093/aob/mcs147

Cramer GR, Ergül A, Grimplet J, Tillett RL, Tattersall EAR, Bohlman MC, Vincent D, Sonderegger J, Evans J, Osborne C, Quilici D, Schlauch KA, Schooley DA, Cushman JC (2007) Water and salinity stress in grapevines: early and late changes in transcript and metabolite profiles. Funct Integr Genom 7:111–134. doi:10.1007/s10142-006-0039-y

DaMatta FM, Loos RA, Silva EA, Loureiro ME, Ducatti C (2002) Effects of soil water deficit and nitrogen nutrition on water relations and photosynthesis of pot-grown Coffea canephora Pierre. Trees 16:555–558. doi:10.1007/s00468-002-0205-3

De Witt T, Sih A, Wilson D (1998) Costs and limits of phenotypic plasticity. Trends Ecol Evol 13:77–81

Dezar CA, Fedrigo GV, Chan RL (2005a) The promoter of the sunflower HD-Zip protein gene Hahb4 directs tissue-specific expression and is inducible by water stress, high salt concentrations and ABA. Plant Sci 169:447–456

Dezar CA, Gago GM, Gonzalez DH, Chan RL (2005b) Hahb-4, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to *Arabidopsis thaliana* plants. Transgenic Res 14:429–440

Díaz P, Betti M, García-Calderón M, Pérez-Delgado CM, Signorelli S, Borsani O, Márquez AJ, Monza J (2014) Amino acids and drought stress in lotus: use of transcriptomics and plastidic glutamine synthetase mutants for new insights in proline metabolism. In: Anjum NA, Gill SS, Gill R (eds) Plant adaptation to environmental change: significance of amino acids and their derivatives. CABI International, Boston

Dubois M, Gilles K, Hamilton J, Rebus P, Smith F (1956) Colorimetric method for the determination of sugars and related substances. Anal Chem 28:350–356

Dumas A (1826) Annales de chimie 33:342

El-Maarouf-Bouteau H, Sajjad Y, Bazin J, Langlade N, Cristescu SM, Balzergue S, Baudouin E, Bailly C (2015) Reactive oxygen species, abscisic acid and ethylene interact to regulate sunflower seed germination. Plant Cell Environ 38:364–374. doi:10.1111/pce.12371

Farooq M, Hussain M, Wahid A, Siddique KHM (2012) Drought Stress in plants: an overview. In: Aroca R (ed) Plant responses to drought stress—from morphological to molecular features. Springer, Berlin, pp 1–33

Fernandez P, Rienzo J Di, Fernandez L, Hopp HE, Paniego N, Heinz RA (2008) Transcriptomic identification of candidate genes involved in sunflower responses to chilling and salt stresses based on cDNA microarray analysis. BMC Plant Biol 8:1–18. doi:10.1186/1471-2229-8-11

Fernandez P, Di Rienzo JA, Moschen S, Dosio GA, Aguirrezabal LA, Hopp HE, Paniego N, Heinz RA (2011) Comparison of predictive methods and biological validation for qPCR reference genes in sunflower leaf senescence transcript analysis. Plant Cell Rep 30:63–74. doi:10.1007/s00299-010-0944-3

Fernandez P, Soria M, Blesa D, DiRienzo J, Moschen S, Rivarola M, Clavijo BJ, Gonzalez S, Peluffo L, Príncipi D, Dosio G, Aguirrezabal L, García-García F, Conesa A, Hopp E, Dopazo J, Heinz RA, Paniego N (2012) Development, characterization and experimental validation of a cultivated sunflower (*Helianthus annuus* L.) gene expression oligonucleotide microarray. PLoS ONE 7:1–11. doi:10.1371/journal.pone.0045899

Gago GM, Almoguera C, Jordano J, Gonzalez DH, Chan RL (2002) Hahb-4, a homeobox-leucine zipper gene potentially involved in abscisic acid-dependent responses to water stress in sunflower. Plant Cell Environ 25:633–640. doi:10.1046/j.1365-3040.2002.00853.x

Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. Nat Chem Biol 3:408–414. doi:10.1038/nchembio.2007.5

Giordani T, Natali L, D'Ercole A, Pugliesi C, Fambrini M, Vernieri P, Vitagliano C, Cavallini A (1999) Expression of a dehydrin gene during embryo development and drought stress in ABA-deficient mutants of sunflower (*Helianthus annuus* L.). Plant Mol Biol 39:739–748

Hanson AD, Scott NA (1980) Betaine synthesis from radioactive precursors in attached, water-stressed barley leaves. Plant Physiol 66:342–348

Huang L, Ye Z, Bell RW, Dell B (2005) Boron nutrition and chilling tolerance of warm climate crop species. Ann Bot 96:755–767

Inskeep WP, Bloom PR (1985) Extinction coefficients of chlorophyll a and b in N, N-dimethylformamide and 80% acetone. Plant Physiol 77:483–485

Jahantigh O, Najafi F, Badi HN, Khavari-Nejad RA, Sanjarian F (2016) Changes in antioxidant enzymes activities and proline, total phenol and anthocyanine contents in *Hyssopus officinalis* L. plants under salt stress. Acta Biol Hung 67:195–204. doi:10.1556/018.67.2016.2.7

Kiani SP, Talia P, Maury P, Grieu P, Heinz R, Perrault a., Nishinakamasu V, Hopp E, Gentzbittel L, Paniego N, Sarrafi a (2007a) Genetic analysis of plant water status and osmotic adjustment in recombinant inbred lines of sunflower under two water treatments. Plant Sci 172:773–787. doi:10.1016/j.plantsci.2006.12.007

Kiani SP, Grieu P, Maury P, Hewezi T, Gentzbittel L, Sarrafi A, Kiani PS (2007b) Genetic variability for physiological traits under drought conditions and differential expression of water stress-associated genes in sunflower (*Helianthus annuus* L.). Theor Appl Genet 114:193–207. doi:10.1007/s00122-006-0419-7

Kiniry JR, Blanchet R, Williams JR, Texier V, Jones K, Cabelguenne M (1992) Sunflower simulation using the EPIC and ALMANAC models. Field Crop Res 30:403–423

Kratsch HA, Wise RR (2000) The ultrastructure of chilling stress. Plant Cell Environ 23:337–350

Langfelder P, Horvath S (2008) WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 9:559. doi:10.1186/1471-2105-9-559

Lohse M, Nagel A, Herter T, May P, Schroda M, Zrenner R, Tohge T, Fernie AR, Stitt M, Usadel B (2014) Mercator: a fast and simple web server for genome scale functional annotation of plant sequence data. Plant Cell Environ 37:1250–1258. doi:10.1111/pce.12231

Luedemann A, Strassburg K, Erban A, Kopka J (2008) TagFinder for the quantitative analysis of gas chromatography–mass spectrometry (GC-MS)-based metabolite profiling experiments. Bioinformatics 24:732–737. doi:10.1093/bioinformatics/btn023

Maas EV, Hoffman GJ (1977) Crop salt tolerance, current assessment. J Irrig Drain Div ASCE 103:115–134

Mahouachi J, Socorro AR, Talon M (2006) Responses of papaya seedlings (*Carica papaya* L.) to water stress and re-hydration: growth, photosynthesis and mineral nutrient imbalance. Plant Soil 281:137–146. doi:10.1007/s11104-005-3935-3

Manavella PA, Arce AL, Dezar CA, Bitton F, Renou J-PP, Crespi M, Chan RL (2006) Cross-talk between ethylene and drought signalling pathways is mediated by the sunflower Hahb-4 transcription factor. Plant J 48:125–137

Manavella PA, Dezar CA, Bonaventure G, Baldwin IT, Chan RL (2008a) HAHB4, a sunflower HD-Zip protein, integrates signals from the jasmonic acid and ethylene pathways during wounding and biotic stress responses. Plant J 56:376–388. doi:10.1111/j.1365-313X.2008.03604.x

Manavella PA, Dezar C, Ariel FD, Drincovich MF, Chan RL (2008b) The sunflower HD-Zip transcription factor HAHB4 is up-regulated in darkness, reducing the transcription of photosynthesis-related genes. J Exp Bot 59:3143–3155

Masclaux-Daubresse C, Valadier M-H, Carrayol E, Reisdorf-Cren M, Hirel B (2002) Diurnal changes in the expressionof glutamate dehydrogenase and nitrate reductase are involved in the C/N balance of tobacco source leaves. Plant Cell Environ 25:1451–1462. doi:10.1046/j.1365-3040.2002.00925.x

Mir RR, Zaman-Allah M, Sreenivasulu N, Trethowan R, Varshney RK (2012) Integrated genomics, physiology and breeding approaches for improving drought tolerance in crops. Theor Appl Genet 125:625–645. doi:10.1007/s00122-012-1904-9

Montaner D, Dopazo J (2010) Multidimensional gene set analysis of genomic data. PLoS ONE 5:e10348. doi:10.1371/journal.pone.0010348

Moschen S, Bengoa Luoni S, Paniego NB, Hopp HE, Dosio GAA, Fernandez P, Heinz RA (2014a) Identification of candidate genes associated with leaf senescence in cultivated sunflower (*Helianthus annuus* L.). PLoS ONE 9:e104379. doi:10.1371/journal.pone.0104379

Moschen S, Radonic LM, Ehrenbolger GF, Fernández P, Lía V, Paniego NB, López Bilbao M, Heinz RA, Hopp HE (2014b) Functional genomics and transgenesis applied to sunflower breeding. In: Arribas JI (ed) Sunflowers: growth and development, environmental influences and pests/diseases. Nova Science Publishers, Hauppauge, pp 131–164

Moschen S, Bengoa Luoni S, Di Rienzo J, Caro M, Tohge T, Watanabe M, Hollmann J, González S, Rivarola M, García-García F, Dopazo J, Hopp HE, Hoefgen R, Fernie A, Paniego N, Fernández P, Heinz R (2016a) Integrating transcriptomic and metabolomic analysis to understand natural leaf senescence in sunflower. Plant Biotechnol J 14:719–734. doi:10.1111/pbi.12422

Moschen S, Higgins J, Di Rienzo JA, Heinz RA, Paniego N, Fernandez P (2016b) Network and biosignature analysis for the integration of transcriptomic and metabolomic data to characterize leaf senescence process in sunflower. BMC Bioinformatics 17:174. doi:10.1186/s12859-016-1045-2

Nagashima A, Hanaoka M, Shikanai T, Fujiwara M, Kanamaru K, Takahashi H, Tanaka K (2004) The multiple-stress responsive plastid sigma factor, SIG5, directs activation of the psbD blue light-responsive promoter (BLRP) in *Arabidopsis thaliana*. Plant Cell Physiol 45:357–368. doi:10.1093/PCP/PCH050

Nakabayashi R, Mori T, Saito K (2014a) Alternation of flavonoid accumulation under drought stress in *Arabidopsis thaliana*. Plant Signal Behav 9:e29518

Nakabayashi R, Yonekura-Sakakibara K, Urano K, Suzuki M, Yamada Y, Nishizawa T, Matsuda F, Kojima M, Sakakibara H, Shinozaki K, Michael AJ, Tohge T, Yamazaki M, Saito K (2014b) Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. Plant J 77:367–379. doi:10.1111/tpj.12388

Ouvrard O, Cellier F, Ferrare K, Tousch D, Lamaze T, Dupuis JM, Casse-Delbart F (1996) Identification and expression of water stress- and abscisic acid-regulated genes in a drought-tolerant sunflower genotype. Plant Mol Biol 31:819–829

Palmer-Young EC, Veit D, Gershenzon J, Schuman MC (2015) The sesquiterpenes(E)-ß-farnesene and (E)-α-bergamotene quench ozone but fail to protect the wild tobacco *Nicotiana attenuata* from ozone, UVB, and drought stresses. PLoS ONE 10:e0127296. doi:10.1371/journal.pone.0127296

Peluffo L, Lia V, Troglia C, Maringolo C, Norma P, Escande A, Esteban Hopp H, Lytovchenko A, Fernie AR, Heinz R, Carrari F (2010) Metabolic profiles of sunflower genotypes with contrasting response to Sclerotinia sclerotiorum infection. Phytochemistry 71:70–80. doi:10.1016/j.phytochem.2009.09.018

Pereyra-Irujo GA, Velázquez L, Granier C, Aguirrezábal LAN (2007) A method for drought tolerance screening in sunflower. Plant Breed 126:445–448. doi:10.1111/j.1439-0523.2007.01375.x

Pérez-Rodríguez P, Riaño-Pachón DM, Corrêa LGG, Rensing SA, Kersten B, Mueller-Roeber B (2010) PlnTFDB: updated content and new features of the plant transcription factor database. Nucleic Acids Res 38:D822–D827. doi:10.1093/nar/gkp805

Pinheiro J, Bates D, DebRoy S, Sarkar D (2012) nlme: linear and nonlinear mixed effects models. R package

Planchet E, Verdu I, Delahaie J, Cukier C, Girard C, Morère-Le Paven M-C, Limami AM (2014) Abscisic acid-induced nitric oxide and proline accumulation in independent pathways under water-deficit stress during seedling establishment in *Medicago truncatula*. J Exp Bot 65:2161–2170. doi:10.1093/jxb/eru088

R Core Team (2012) R: a language and environment for statistical computing. R Foundation for Statistical Computing. ISBN 3-900051-07-0

Raineri J, Ribichich KF, Chan RL (2015) The sunflower transcription factor HaWRKY76 confers drought and flood tolerance to *Arabidopsis thaliana* plants without yield penalty. Plant Cell Rep 34:2065–2080. doi:10.1007/s00299-015-1852-3

Roche J, Hewezi T, Bouniols A, Gentzbittel L (2007) Transcriptional profiles of primary metabolism and signal transduction-related genes in response to water stress in field-grown sunflower genotypes using a thematic cDNA microarray. Planta 226:601–617. doi:10.1007/s00425-007-0508-0

Roessner-Tunali U, Hegemann B, Lytovchenko A, Carrari F, Bruedigam C, Granot D, Fernie AR (2003) Metabolic profiling of transgenic tomato plants overexpressing hexokinase reveals that the influence of hexose phosphorylation diminishes during fruit development. Plant Physiol 133:84–99. doi:10.1104/pp.103.023572

Rousseaux MC. C, Hall AJ, Sanchez RA (1996) Far-red enrichment and photosynthetically active radiation level influence leaf senescence in field-grown sunflower. Physiol Plant 96:217–224

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. Bioinform Methods Protoc 132:365–386

Ruiz-Lozano JM, Azcón R (1996) Mycorrhizal colonization and drought stress as factors affecting nitrate reductase activity in lettuce plants. Agric Ecosyst Environ 60:175–181. doi:10.1016/S0167-8809(96)01074-2

Sadras VO, Whitfi eld DM, Connor DJ (1991) Regulation of evapotranspiration and its partitioning between transpiration and soil evaporation by sunflower crops. A comparison between hybrids of different stature. Field Crop Res 28:17–37

Saito K, Yonekura-Sakakibara K, Nakabayashi R, Higashi Y, Yamazaki M, Tohge T, Fernie AR (2013) The flavonoid biosynthetic pathway in Arabidopsis: structural and genetic diversity. Plant Physiol Biochem 72:21–34. doi:10.1016/j.plaphy.2013.02.001

Sartor MA, Leikauf GD, Medvedovic M (2009) LRpath: a logistic regression approach for identifying enriched biological groups in gene expression data. Bioinformatics 25:211–217. doi:10.1093/bioinformatics/btn592

Schaefer RJ, Michno J-M, Myers CL (2016) Unraveling gene function in agricultural species using gene co-expression networks. Biochim Biophys Acta-Gene Regul Mech. doi:10.1016/j.bbagrm.2016.07.016

Schmidhuber J, Tubiello FN (2007) Global food security under climate change. Proc Natl Acad Sci USA 104:19703–19708. doi:10.1073/pnas.0701976104

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 13:2498–2504. doi:10.1101/gr.1239303

Sharkey TD (2001) Isoprene increases thermotolerance of fosmidomycin-fed leaves. Plant Physiol 125:2001–2006. doi:10.1104/pp.125.4.2001

Sharkey TD, Wiberley AE, Donohue AR (2008) Isoprene emission from plants: why and how. Ann Bot 101:5–18. doi:10.1093/aob/mcm240

Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED, Delis ID, Yakoumakis DI, Kouvarakis A, Papadakis AK, Stephanou EG, Roubelakis-Angelakis KA (2006) Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in

tobacco and grapevine. Plant Cell 18:2767–2781. doi:10.1105/tpc.105.038323

Smyth G (2005) Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W (eds) Bioinformatics and computational biology solutions using R and bioconductor. Springer, New York, pp 397–420

Sperdouli I, Moustakas M (2012) Interaction of proline, sugars, and anthocyanins during photosynthetic acclimation of *Arabidopsis thaliana* to drought stress. J Plant Physiol 169:577–585. doi:10.1016/j.jplph.2011.12.015

Tardieu F, Tuberosa R (2010) Dissection and modelling of abiotic stress tolerance in plants. Curr Opin Plant Biol 13:206–212. doi:10.1016/j.pbi.2009.12.012

Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY, Stitt M, Bläsing O, Krüger P, Müller L a (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J 37:914–939. doi:10.1111/j.1365-313X.2004.02016.x

Thomas WTB (2015) Drought-resistant cereals: impact on water sustainability and nutritional quality. Proc Nutr Soc 74:191–197. doi:10.1017/S0029665115000026

Tran L-SP, Nakashima K, Sakuma Y, Osakabe Y, Qin F, Simpson SD, Maruyama K, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K (2006) Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the ERD1 gene in Arabidopsis. Plant J 49:46–63. doi:10.1111/j.1365-313X.2006.02932.x

Utrillas MJ, Alegre L, Simon E (1995) Seasonal changes in production and nutrient content of *Cynodon dactylon* (L.) Pers. subjected to water deficits. Plant Soil 175:153–157. doi:10.1007/BF02413021

van Kleunen M, Fischer M (2005) Constraints on the evolution of adaptive phenotypic plasticity in plants. New Phytol 166:49–60. doi:10.1111/j.1469-8137.2004.01296.x

Vickers CE, Gershenzon J, Lerdau MT, Loreto F (2009) A unified mechanism of action for volatile isoprenoids in plant abiotic stress. Nat Chem Biol 5:283–291. doi:10.1038/nchembio.158

Wang W, Wu P, Li Y, Hou X (2016) Genome-wide analysis and expression patterns of ZF-HD transcription factors under different developmental tissues and abiotic stresses in Chinese cabbage. Mol Genet Genom 291:1451–1464. doi:10.1007/s00438-015-1136-1

Yaish MW (2015) Proline accumulation is a general response to abiotic stress in the date palm tree (*Phoenix dactylifera* L.). Genet Mol Res 14:9943–9950. doi:10.4238/2015.August.19.30

Yamada M, Morishita H, Urano K, Shiozaki N, Yamaguchi-Shinozaki K, Shinozaki K, Yoshiba Y (2005) Effects of free proline accumulation in petunias under drought stress. J Exp Bot 56:1975–1981. doi:10.1093/jxb/eri195