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Water-stressed sunflower transcriptome analysis revealed important molecular markers involved in drought stress response and tolerance

Vivien Sarazin[a,b], Jérôme Duclercq[a], Xavier Guillot[b], Brigitte Sangwan[a], Rajbir S. Sangwan[a,*]

[a] EDYSAN FRE 3498 CNRS, UPJV, Amiens, France
[b] Laboulet Semences, Airaines, France

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ABSTRACT

Water stress is considered one of the most important factors limiting worldwide agricultural productivity and efficiency. Although sunflower (*Helianthus annuus* L.) is a deep-rooted crop, water stress strongly reduces its productivity. A holistic approach that integrates physiological, biochemical and molecular genetic tools could provide opportunities for breeding novel genotypes with stable yield under water-deficit conditions.

In this study, we have studied the responses of several *Helianthus annuus* genotypes, in water stress conditions, using genomics (RNA-seq and quantitative RT-PCR), physiological (growth, water statute, stomatal conduction evaluations, and transpiration rate) and biochemical analyses (LC–MS).

Our physiological analyses indicated a sunflower genotype-dependent water stress response. This variability in the water stress response could be observed transcriptomically with the identification of water stress core genes, as well as genes characteristic of tolerance and/or sensitivity to a water deficit. From these genes, we identified several components involved in abscisic acid synthesis and signaling *(NCED3*, *NCED5*, *ABI1* and *PYL4*), which must be involved in drought tolerance. Under well-watered conditions, we subsequently detected higher abscisic acid content in leaves of the sensitive genotypes.

We propose that sunflower water stress tolerance is correlated with a transcriptome fine-tuning leading to an efficient activation of ABA-dependent genes and not by ABA overproduction.

1. Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops in the world, and after five centuries of culturing, it became the fourth oil plant representing 7% of the world's total oil production in 2015 (41 Mt of grains; FAOSTAT). Due to its relatively short growing season, it represents one of the major cash crops (Seiler and Gulya, 2016). Sunflower is grown mainly in warm to moderate semi-arid climatic regions. Although sunflower can develop on a wide variety of soils, its yield will be optimum on soils suitable for maize or wheat production. In addition, because of its deep rooting system, sunflower is able to use nitrogen from soil layers that are inaccessible to wheat, corn or other field crops (Doré and Varoquaux, 2006). Apart from its drought tolerance and, more generally, its low need for inputs, the main advantage of this species is its high grain oil content (42–50% of seed weight on average). The characteristics of this plant are perfectly in line with the agricultural issue related to the current societal demand: promote crops requiring less fertilizers, pesticides and water.

Over time, farmers sought to value inhospitable environments while hoping to keep good yields and seed quality for many industrial/non-industrial applications (Chevalier et al., 2014). As *Helianthus annuus* is considered to be tolerant to drought (compared to other summer crops like corn and soybeans; (Merrien and Grandin, 1990)), sunflower is very often cultivated in water scarcity zones. Paradoxically, the main limitation of sunflower production is drought (Blanchet et al., 1981). Therefore, despite its strong drought tolerance, there remain significant annual fluctuations in its yield and oil content. For example, in France, yield losses of sunflowers were up to 20% in some areas in 2003, which was a particularly dry year (Amigues et al., 2006). This loss of yield reached 40% in 1976, when sunflower cultivations were overwhelmingly without irrigation and with low-performing varieties (Debaeke and Bertrand, 2008). Moreover, water stress has an impact not only on grain yields but also on the composition of fatty acids (Alberio et al., 2016; Howell et al., 2015). Consequently, it is necessary to manage this aspect in sunflower use, especially with actual climate change and global warming. Interestingly, current climate change

Abbreviations: ABA, abscisic acid; FTSW, fraction of transpirable soil water; LC–MS, high-performance liquid chromatography coupled with mass spectrometry; RWC, relative water content; Wd, dry weight; Wf, fresh weight; Wt, turgescent weight

* Corresponding author.
E-mail address: rajbir.sangwan@u-picardie.fr (R.S. Sangwan).

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models suggest selecting arable crop species such as sunflower (Boyer and McLaughlin, 2007; Moriondo et al., 2011; Cohn et al., 2016). Two to three waterings are currently recommended to optimize the performance of this crop, but due to a shortage of water, such irrigation use will be drastically reduced. An earlier study comparing different sunflower varieties with and without optimum irrigation showed that the lack of optimum irrigation has drastically reduced yield (up to ¼) (Cabelguenne et al., 1982). However, crops rarely have water supplies to maximize their potential seed production. Although total absence of rain is unlikely, these studies demonstrated the possible economic repercussions of severe water stress on sunflower farming (Andrianasolo et al., 2016). To address any future constraints related to sunflower culturing in areas with water limitations, it is necessary to characterize relationships between water stress and sunflower productivity. Through biochemical and/or molecular markers for tolerance/susceptibility to drought, it would be possible to enhance the varieties already selected, as well as to obtain new hybrids, that are adapted to changing climatic conditions for the future production of sunflower (Dhillon et al., 2012).

Many morphological, physiological, molecular and phenological traits/mechanisms have been associated with drought stress adaptation. For example, drought triggers many plant responses such as the production of abscisic acid (ABA), stomatal closure and the expression of drought-inducible genes (Shinozaki and Yamaguchi-Shinozaki, 2007; Lim et al., 2012). ABA is involved in the response to many biotic and abiotic stresses and is mostly known to play a primary role in the ability of plants to cope with water stress (Pandey et al., 2013). At the root level in sunflower, ABA's role has been known for a long time (Robertson et al., 1985). During water stress, ABA synthesized at the root level goes up to the leaves by transpiration stream (in crude sap), where it will activate the guard cells' receptors PYR/PYL, which will generate a change in ion concentrations (K^+ and anions) leading to the stomatal cells' intracellular plasmolysis and the stomata closing to minimize water loss (Osakabe et al., 2014). The plant's response to water stress, even if it is necessary, limits many physiological activities of the plant, especially photosynthesis. Although stomatal conductance is a major factor influencing photosynthetic performance under drought stress, non-stomatal limitations may considerably reduce CO_2 assimilation, mostly in severe drought conditions (Vassileva et al., 2011). Studies have shown that for sunflower, stomatal parameters do not seem to be prevailing in photosynthesis regulation (Steduto et al., 2000; Noreen and Ashraf, 2008; Noreen et al., 2012).

Phenotyping and global transcriptome analysis are major sources of information used to unravel gene networks involved in the water stress response (Rengel et al., 2012), so we chose these approaches to advance our understanding of sunflower drought responses. In this study, using physiological markers, we screened sunflower lines under water-deficit conditions to find sensitive and tolerant genotypes in order to compare their transcriptome. In addition, as ABA is a key factor to cope with drought, we measured this plant hormone in the selected lines and hybrids.

2. Materials and methods

2.1. Plant materials

The experiment was conducted in a controlled growth chamber with seven genotypes: L001, L002, L003, L004, L005, L006 and H001. L001 and L002 are fertility restorer lines. L003, L004, L005 and L006 are male sterile lines. H001 is a hybrid from a L005xL001 crossing.

All lines are oleic sunflowers from the Laboulet Semences selection program.

2.2. Growth conditions

Seeds were sowed in fully watered pots containing 6.0 kg of peat with 10% sand. Water was provided by dripping (0.3 l d^{-1}). Sunflowers were placed in a controlled growth chamber for 31 days (22 °C +/− 1 °C; photoperiod, 16 h:8 h), then separated in two groups: Well-watered (with irrigation) and Water Stress (without irrigation) for nine days. The water stress was intense enough to trigger a physiological response without making all the leaves unusable for analysis (especially for the most sensitive genotypes), and it corresponded to a critical period for sunflower crops (Göksoy et al., 2004). Then, plant heights were measured and leaves 7–8 (fully extended) were collected in dry ice. Six repetitions/pots for each genotype and condition were performed.

Relative water content (RWC) was calculated as follows:

$$RWC = 100 \times (Wf - Wd)/(Wt - Wd)$$

Fresh weight (*Wf*) was measured from two leaves of the last fully expanded leaves (7–8) used for gas exchange measurements (Hervé et al., 2001). Turgescent weight (*Wt*) was determined from the same leaf incubated for 4 h at 4 °C in a water bath with a saturated humidity atmosphere. Dry weight (*Wd*) was determined after 24 h at 80 °C in a stove.

In field conditions, seeds were sowed in a clay-loam soil next to Lavaur (France, 43° 41′ 59″ north, 1° 49′ 11″ east) in May 2015.

2.3. RNA isolation, RNA sequencing and quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. A DNase treatment was carried out for 15 min at 25 °C using the RNase-free DNase Set (Qiagen, Germany). RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer. All RNA samples were rejected if they did not reach a minimum concentration of 100 ng μl^{-1}, a 260 nm/280 nm ratio between 1.8 and 2.0 and an RNA integrity number superior to 7.5, measured with an Agilent 2100 Bioanalyzer (Agilent, USA). RNA sequencing was performed at the VIB Nucleomics Core (www.nucleomics.be). Library preparation was performed using the Illumina TruSeq Sample Preparation v2 kit (Illumina, USA), followed by 1 × 50 bp sequencing on two lane of a 300 Gb flowcell of the Illumina HiSeq 2000 instrument (Illumina, USA). FastQ files containing sequence reads were trimmed to remove low quality ends (< Q20) using the FASTX-toolkit (v0.0.13 from Assaf Gordon Hannon lab), adapters (at least 10 bp overlap and 90% match) with cutadapt 1.2.1 (Martin, 2001), small reads (length < 50 bp), polyA-reads (more than 90% of the bases equal A), ambiguous reads (containing N) and low quality reads (more than 50% of the bases < Q25). In addition, we removed reads that align to phix-illumina (technical spike-in), HA383-chloroplast (chloroplast genome of sunflower), GRCh37.71 (human hg19) using Bowtie 2.1.0 (http://bowtie-bio.sourceforge.net). Reads were then assembled into contigs (candidate transcripts) with the Trinity package (version trinityrnaseq-r2013-02-25) (Grabherr et al., 2011) and concatenated with reference contigs from Dryad (http://datadryad.org/resource/http://dx.doi.org/10.5061/dryad.rs4k0). All contigs were then clustered together and assembled into longer contigs with the TGICL package v2.1 (Pertea et al., 2003). At this step, we considered both singleton sequences and representatives of the contig-clusters as approximates of exon chains. The reads were aligned to the reference with Tophat v2.0.8b (Trapnell et al., 2009). SAMtools v0.1.19-44428cd (Li et al., 2009) was used to remove reads from the alignment that are non-primary mappings or have a mapping quality ≤20, to sort the reads from the alignment according to the chromosomes and to index the resulting bam-files. Per sample, the transcripts were identified from the mappings with Cufflinks v2.1.1 (Trapnell et al., 2010). With Cuffmerge from the Cufflinks toolkit, we merge all per-sample transcript lists into one file. A list of gene-level coordinates was constructed by merging the exon chains of transcripts that belong to the same gene, using mergeBed from the Bedtools v2.17.0 toolkit (Quinlan and Hall, 2010). For each gene, we computed the

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