

Genetic variability for physiological traits under drought conditions and differential expression of water stress-associated genes in sunflower (*Helianthus annuus* L.)

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Abstract Genotypic variation for water status and gas exchange parameters under different water treatments (well-watered and water-stressed plants before and after rehydration) were investigated in a population of recombinant inbred lines (RILs) of sunflower (*Helianthus annuus* L.). Afterwards, four RILs and parental lines presenting contrasting responses to dehydration and rehydration were selected to determine the differential expression of four water-stress associated genes: aquaporin, dehydrin, leafy cotyledon1-like protein and fructose-1,6 biphosphatase. Water stress revealed a high genetic variability for water status and gas exchange parameters when compared with well-watered genotypes. Genetic gain when selected RILs were compared with the best parent was significant for most traits due to transgressive segregation. QTL mapping and graphical genotyping showed that RILs carrying different genomic regions for some QTLs presented also physiological different

characteristics as well as gene expression patterns. The expression level of aquaporin genes in leaves of four RILs and their parents was down regulated by water stress and was associated with relative water content (RWC). Down-regulation was also associated with genomic regions having alleles with negative effects on plant water status. The level of dehydrin transcripts increased in leaves of all studied RILs in response to water stress. Transcript accumulations of dehydrin and leafy cotyledon1-like genes, likely involved in protective tolerance processes, were not correlated directly with plant water status or QTL effects. Down-regulation of fructose-1,6 biphosphatase was observed under water stress. Net photosynthesis rate (P_n) and the fructose-1,6 biphosphatase gene expression levels were associated mainly after rehydration. This phenomenon indicates an association between physiological response to water stress and differential expression of water-stress related genes.

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Introduction

Water stress, the most limiting factor of plant growth and crop production, induces various biochemical and physiological responses in plants. Water stress decreases plant growth by slowing the rate of cell division and expansion, mainly due to loss of turgor, which results in a decline of the water status components of the plant cells. Relative water content (RWC) and water potential (Ψ_w) and its components, turgor potential (Ψ_t) and osmotic potential (Ψ_s), are parameters most commonly used to assess plant water status. Turgor maintenance by means of increases in cell

solute concentration, osmotic adjustment (OA), is probably the most important mechanism for maintaining physiological activity as water potential (Ψ_w) falls (Morgan 1984; Ober et al. 2005). Gas exchange parameters, transpiration (Tr) and net photosynthesis (P_n), regulated by stomatal conductance (g_s), decrease as water stress increases in sunflower (Tezara et al. 2002). Stomata are mainly sensitive to leaf water status, tending to close with decreasing leaf water potential. Moreover, water stress can also limit net photosynthesis by metabolic impairment of CO_2 fixation, leading to an increase of the intercellular CO_2 concentration (C_i) (Flexas and Medrano 2002). In sunflower, different water stress treatments decrease relative water content (RWC), leaf water potential (Ψ_w) and osmotic potential (Ψ_s), resulting in progressive and significant decline in stomatal conductance (g_s) and net photosynthesis (P_n) (Tezara et al. 2002; Maury et al. 1996). Genetic variation and QTL mapping has been reported for photosynthesis and water status traits under well-watered conditions in sunflower (Hervé et al. 2001). Variation was also observed for osmotic adjustment in sunflower genotypes under water stress (Jamaux et al. 1997). As far as we know, the genetic bases of physiological traits associated with drought tolerance in sunflower have not yet been reported.

Genetic variability in the stress response has been suggested to be mainly due to the differential expression of stress-responsive genes (Krishnan et al. 1989; Joshi et al. 1997). The interface between the expression of stress responsive genes and plant physiological response to drought stress is critical for translating molecular genetics into advances in crop production under stress conditions (Bruce et al. 2002). A large number of genes have been described that respond to drought at the transcriptional level and the mechanisms of the molecular response to water stress in higher plants have been analyzed by studying the expression of genes responding to drought and other abiotic stresses (Bohnert et al. 1995; Ingram and Bartels 1996; Bray 2002, 2004; Shinozaki and Yamaguchi-Shinozaki 1997, 1999, 2000; Seki et al. 2001, 2002; Watkinson et al. 2003; Oono et al. 2003; Boominathan et al. 2004). Stress-inducible genes have been also used to improve the stress tolerance of plants by gene transfer (Holmberg and Bülow 1998; Bajaj et al. 1999). It is thus critical to study the functions of stress-induced genes to understand the mechanisms involved in stress tolerance in plants. Correlating phenotypic adaptations with molecular responses should enable us to evaluate the role of drought-induced changes in gene expression during adaptation. Indeed, linking the

expression of a gene to a high degree of tolerance suggests a possible role for this gene in adaptation (Ouvard et al. 1996).

In the present study, among the different ways in which sunflower can maintain physiological activity as water stress increases, relative water content, turgor maintenance and protective tolerance processes were investigated in 38 recombinant inbred lines (RILs). Based on the genetic characteristics of the RILs, four RILs and parental lines presenting contrasting responses to dehydration and rehydration were selected to determine the differential expression of four water-stress associated genes. Plant water status characters (relative water content, leaf water potential and turgor potential) and their effects on gas exchange (photosynthesis and stomatal conductance), as the water stress phenotypic adaptation parameters, were evaluated in the selected RILs and their parents. The putative correlations between these phenotypic adaptations and the expression of four selected genes (aquaporin, fructose-1,6 bisphosphatase, dehydrin and leafy cotyledon1-like protein) were also evaluated in order to determine their putative role in adaptation. Among these four genes, an aquaporin gene (*suntip7*), which is expressed in guard cells of sunflower during the phase of stomatal closure, (Sarda et al. 1997) has been chosen because of its direct involvement in plant water status (Sarda et al. 1999; Luu and Maurel 2005; Jang et al. 2004). Fructose-1,6 bisphosphatase (*cFB-Pase*) is a key enzyme of the photosynthetic carbon reduction cycle and extremely sensitive to H_2O_2 produced under stress (Charles and Halliwell 1981). Reduced fructose-1,6 bisphosphatase leads to a reduction in photosynthesis activity (Zrenner et al. 1996). Therefore a part of photosynthesis reduction under water stress may be due to change in fructose-1,6 bisphosphatase gene expression. The dehydrin gene used in our experiment (*HaDhn1*) is a gene of the *D-11* subgroup of late-embryogenesis-abundant (LEA) proteins (Dure et al. 1989; Close et al. 1993), which is correlated with drought tolerance in sunflower (Ouvard et al. 1996; Cellier et al. 1998). The fourth gene (*HaLIL*) used in the present study is a sunflower gene encoding leafy cotyledon1-like protein (Fambrini et al. 2006). Leafy cotyledon is a regulator of late-embryogenesis-abundant proteins (LEA), which are involved in drought and salt tolerance (Parcy et al. 1997; Reid and Walker-Simmons 1993; Moons et al. 1995). Therefore they might be involved directly in drought tolerance or via changes in LEAs (dehydrins) during vegetative growth stages. The putative association of these four genes with phenotypic adaptation (plant water status and photosynthesis) was evaluated.

Finally, for each selected genotype (RILs and parents), the possible relationship between genomic regions with positive or negative allele effects and transcript abundance was investigated.

Materials and methods

Plant material

The 38 recombinant inbred lines (RILs) used in this experiment are F9 pure lines coming from a population of 150 RILs which were developed through single seed descent from F₂ plants derived from a cross between ‘PAC-2’ and ‘RHA-266’. RILs and their parents were grown in the greenhouse under controlled conditions. Plants were individually grown in plastic pots (4.0l) containing a mixture of 40% soil, 40% compost and 20% sand. Temperature was maintained at 25/18 ± 2°C (day/night) and relative humidity was about 65–85 ± 5%. Supplementary light giving an approximately 16 h light period and 8 h dark period was maintained during experiment. The experiment consisted of three blocks, and each block was split into two main plots (well-watered and water-stressed). Three plants per genotype were cultivated in each main plot and randomly allocated at the beginning of the experiment.

Four genotypes contrasting in their response to water stress and their two parental lines were used for gene expression analysis by real-time RT-PCR in order to determine putative relationships between plant physiological response and genomic regions with positive or negative allele effects as well as with water stress-induced gene expression.

Water stress treatments

Water deficit was induced in 45-day-old plants near the stage R1 (Schneiter and Miller 1981), with the 14th true leaf fully expanded, for a period of 12 days. Both well-watered and water-stressed plants were weighed and water loss replaced carefully. Well-watered (control) plants received sufficient water to maintain soil water content close to pot capacity. Water-stressed plants were subjected to a progressive water stress and irrigated with a water volume of 60, 50, 40 and 30% of pot capacity during 12 days with weighing and replacing of the lost water volume. After 12 days of differential watering, plants were not watered the following morning and the physiological measurements were made that day. When measurements were finished, a young fully expanded leaf was harvested from well-watered and water-stressed plants, frozen in liquid

nitrogen, and stored at –80°C for RNA extraction and gene expression analysis. Both well-watered and water-stressed plants were rehydrated close to pot capacity after physiological measurements. Thirty-six hours after rehydration, photosynthesis and related traits (gas exchange parameters) were measured. A young fully expanded leaf was again harvested from each plant for RNA extraction.

Physiological traits

Leaf water potential (Ψ_w) was measured with a pressure chamber (Model 3000, Soil Moisture Equipment Crop, Santa Barbara, CA, USA; Scholander et al. 1965) on the last fully expanded leaf (around leaf number 15) for each individual plants. After the measurement of Ψ_w , relative water content (RWC) was determined ($RWC = \frac{F_w - D_w}{T_w - D_w}$), where F_w is fresh weight, T_w is turgid weight after 24 h rehydration at 4°C in a dark room by placing the petioles in a container with distilled water, and D_w is dry weight after oven drying for 24 h at 80°C. In all cases half the lamina of the sampled leaf was used for RWC determination (lamina with midrib vein) and the remaining half (without the midrib vein) was used for measurement of leaf osmotic potential (Ψ_s). Leaf turgor potential was also determined ($\Psi_t = \Psi_w - \Psi_s$). Both leaf osmotic potential (Ψ_s) and osmotic potential at full turgor (Ψ_{sFT}) were measured on expressed sap of frozen and thawed leaves using 10 µl aliquots placed in an osmometer (Wescor Model 5520, Logan, UT, USA) calibrated with manufacturer solutions. Osmotic potentials (Ψ_s) were determined by converting the osmometer reading (in mmol kg⁻¹) using the Van’t Hoff relation: $\Psi_s = -RTdc$, where R is the gas constant, T the temperature in Kelvin, d the density of water at temperature T , and c the concentration of osmotically-active solutes, given by the osmometer. Osmotic adjustment (OA) is the accumulation of solutes induced by water stress, which was calculated using the rehydration method (Blum 1989), with the following equation: $OA = \Psi_{sFT(WW)} - \Psi_{sFT(WS)}$, where OA is osmotic adjustment, $\Psi_{sFT(WW)}$ is the osmotic potential at full turgor of well-watered plants and $\Psi_{sFT(WS)}$ is the osmotic potential at full turgor of water-stressed plants.

Measurements of gas exchange were done with a portable Li-6400 photosynthesis system (Li-Cor, Lincoln, NE, USA) between 10 and 14 h. Leaves at the same position as that sampled for RWC were used for gas exchange measurements. Measurements of net photosynthetic rate (P_n), stomatal conductance (g_s) and intercellular CO₂ concentration were made at light-saturation of 1,500 µmol m⁻² s⁻¹ photosynthetic

photon flux density (PPFD), ambient CO₂ concentration (approximately 400 μmol mol⁻¹), leaf temperature controlled at 25 ± 2°C and with relative humidity of 60 ± 5%.

Statistical analysis

Physiological trait data were analyzed by ANOVA and GLM with SAS v.8.0 for PC (SAS Institute Inc, NC, USA). Comparison of mean treatments was made with a GLM and the LSD test for a priori pair wise comparisons (all treatment means versus control) and SNK test for comparison of the means of other parameters.

QTL mapping

An AFLP and SSR genetic linkage map developed by our department (Al-Chaarani et al. 2004) was used to map QTLs involved in the expression of water status traits (RWC, Ψ_w, Ψ_s, Ψ_t and OA) as well as net photosynthesis rate (P_n) under water stress before rehydration. The QTL mapping of the studied traits was performed by composite interval mapping (CIM), using QTL Cartographer, version 1.16 (Basten et al. 2002). GGT software (Van Berloo 1999) was then used to create a “graphical genotype” for each offspring, to show how chromosome segments at each QTL locations were inherited from each parent. Graphical genotypes were used to show the differences among four selected RILs for RT-PCR in term of chromosomal segments at QTL positions.

RNA extraction and cDNA synthesis

Young fully expanded leaves, harvested from each treatment combination (well-watered and water-stressed plants before and after rehydration) were frozen in liquid nitrogen, and stored at -80°C. For each treatment combination, the leaves collected from three plants were pooled in one sample prior to RNA preparation. The pooled leaf samples were used to

extract total RNA as described by Verwoerd et al. (1989). Three biological repeats were performed. Single-stranded cDNA was synthesized from DNase-treated RNA using the Advantage RT-for-PCR Kit (Ozyme). The reaction mixture containing 5 μg of total RNA and 40 pMol oligo (dT15) was heated at 70°C for 10 min. Then 8 μl of 5× reaction buffer, 2 μl of dNTP mix (0.625 mM each), 1 μl (1 Unit) of RNase inhibitor and 2 μl (400 Units) of MMLV reverse transcriptase were added. The reaction was incubated at 42°C for 1 h. An additional 200 Units of MMLV reverse transcriptase was added and the mixture was incubated for another 60 min at 42°C followed by heating at 70°C for 15 min to stop the synthesis reaction.

Quantitative real-time RT-PCR

The expression levels of four selected genes were tested in the four above-mentioned RILs and two parental lines (‘PAC-2’ and ‘RHA-266’), which present contrasting responses to water stress and different genomic regions. Gene expression analysis was performed using different treatment combinations (well-watered and water-stressed plants before and after rehydration) in three replications. Gene-specific primers were designed using the Primer Express software, version 2.0 (Applied Biosystems, Courtaboeuf, France). Oligonucleotide primer sequences are shown in Table 1. Quantitative real-time RT-PCR was performed using the method described by Hewezi et al. (2006). Quantification of the relative changes in gene expression was performed using the 2^{-ΔΔCT} method as described by Livak and Schmittgen (2001).

Results

Genetic variation for physiological traits

Analysis of variance for 38 RILs and their parents ‘PAC-2’ and ‘RHA-266’ summarized in Table 2,

Table 1 Primers used for quantitative real-time RT-PCR expression analysis

Sequence name	Putative function	Accession	Sequence of primers (5′-3′)
X95950	Aquaporin	X95950	F: TCCACCTTGCTCTTCGTCTTC R: TCTGCTGTCAATTTGGCGTAA
X92647	Dehydrin	X92647	F: TGAAGGAATTGTGTGAGGCGTAT R: GGAGCCATGCAAACCTTTATTCTT
AJ863116	Putative leafy cotyledon1-like protein	AJ863116	F: CGCAAAAGAAACGATCCAAGA R: TCATTTCGTTACCTGTCCACA
DH0AL23ZC07ZM1	Fructose-1,6 biphosphatase	CD850867	F: TGGCACGTGTCGTTCTCGTA R: CTCCTTGCCATGCCTTGAG

Table 2 Analysis of variance for physiological traits on well-watered and water-stressed genotypes of sunflower before and after rehydration

Trait	Before rehydration						After rehydration					
	Well-watered			Water-stressed			Well-watered			Water-stressed		
	MSG	Range	Mean	MSG	Range	Mean	MSG	Range	Mean	MSG	Range	Mean
P_n	32.14 ^{NS}	11.8/25.0	19.7	7.94 ^{**}	0.2/6.7	3.3	33.05 ^{NS}	13.4/26.7	18.9	41.43 [*]	6.5/20.6	12.4
g_s	0.09 ^{NS}	0.37/1.14	0.72	0.0007 ^{NS}	0.00/0.08	0.03	0.09 ^{NS}	0.20/1.04	0.59	0.03 ^{NS}	0.06/0.45	0.22
Ci	884.18 ^{NS}	270/359	300	7911.5 ^{**}	139/361	215	855.4 ^{NS}	244/313	288	1583.45 ^{NS}	190/280	244
Tr	2.97 ^{NS}	3.96/8.56	7.03	0.34 ^{NS}	0.13/1.37	0.77	3.40 ^{NS}	4.94/8.68	6.50	3.44 ^{NS}	1.40/5.81	3.48
RWC	23.12 ^{NS}	80.4/91.7	86.0	92.19 ^{***}	59.5/80.7	71.3						
Ψ_w	0.027 ^{NS}	-0.97/-0.58	-0.78	0.21 ^{**}	-1.3/-2.54	-1.8						
Ψ_s	0.028 [*]	-1.05/-1.43	-1.21	0.25 ^{**}	-1.31/-2.69	-1.84						
Ψ_t	0.02 ^{NS}	0.31/0.75	0.43	0.03 ^{**}	-0.24/0.39	0.04						
OA	-	-	-	0.06 ^{***}	-0.1/0.58	0.20						

MSG genotype mean square, g_s stomatal conductance (mol H₂O m⁻² s⁻¹), Ci intercellular CO₂ concentration (μmol mol⁻¹), Tr transpiration (mmol H₂O m⁻² s⁻¹), RWC relative water content (%), Ψ_w leaf water potential (MPa), Ψ_s osmotic potential (MPa), Ψ_t turgor potential (MPa), OA osmotic adjustment (MPa)
 *, **, *** and NS significant at 0.05, 0.01, 0.001 levels and non-significant, respectively

showed highly significant differences among genotypes (MS_G) for some of traits studied under the four treatment combinations (well-watered and water-stressed plants; before and after rehydration). After rehydration, variation among RILs was significant only for net photosynthesis (P_n). Gas exchange trait variation summarized in Table 2 shows that net photosynthesis (P_n), stomatal conductance (g_s), transpiration (Tr) and intercellular CO₂ concentration (Ci) of RILs were decreased due to water stress. For example, leaf net photosynthesis (P_n) of well-watered RILs ranged from 11.8 to 25.0 μmol CO₂ m⁻² s⁻¹. This was reduced in water-stressed RILs from 0.2 to 6.7 μmol CO₂ m⁻² s⁻¹. All values of gas exchange parameters were increased after rehydration (Table 2) and a wide range of net photosynthesis values (6.5–20.6 μmol CO₂ m⁻² s⁻¹) in rehydrated water-stressed plants was observed. However, rehydrated water-stressed plants presented a lower value for gas exchange parameters when they are compared with well-watered plants, suggesting that some RILs had not recovered 36 h after relief of water stress.

Plant water status traits presented in Table 2 show that relative water content (RWC) of all RILs declined with water stress. RWC of well-watered plants ranged from 80.4 to 91.7% while in water-stressed plants it ranged from 59.5 to 80.7%. Water stress also decreased leaf water potential (Ψ_w) and its components, osmotic potential (Ψ_s) and turgor potential (Ψ_t), in RILs. Osmotic adjustment (OA) was observed in RILs subjected to water stress with the mean value of 0.20. OA ranged from -0.1 to 0.58 MPa showing high genetic variation in RILs.

Genetic variability for all physiological traits studied is presented in Table 3. No significant difference was detected between the two parental lines for all traits studied under water stress before rehydration. The differences between the mean of 38 RILs (\bar{X}_{RIL}) and the mean of their parents (\bar{X}_P) in the four treatment combinations (well-watered and water-stressed plants before and after rehydration) were not significant for all traits studied except for osmotic potential (Ψ_s). The comparison between the best parent and the best RIL showed a significant difference for most of physiological studied traits among water-stressed plants. Significant differences were also observed when the mean parent was compared with the mean of 10% selected RILs for some physiological traits (Table 4).

Correlations between different physiological traits are presented in Table 5. Highly significant positive correlations were shown between leaf water potential (Ψ_w), relative water content (RWC), turgor potential (Ψ_t) and net photosynthesis (P_n). Significant positive

Table 3 Genetic gain for gas exchange parameters on well-watered (WW) and water-stressed (WS) genotypes of sunflower before and after rehydration

Parameter	Before rehydration						After rehydration									
	P _n		g _s		Ci		Tr		P _n		g _s		Ci		Tr	
	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS
PAC-2 (P1)	16.3	3.2	0.54	0.03	276	222	6.35	0.78	22.8	15.8	0.64	0.32	293	252	7.40	4.62
RHA-266 (P2)	20.8	3.5	0.81	0.03	304	177	7.34	0.68	22.8	7.4	0.75	0.18	293	261.3	8.03	3.14
P1-P2	4.5 ^{NS}	0.2 ^{NS}	0.27 ^{NS}	0.00 ^{NS}	28 ^{NS}	45 ^{NS}	0.99 ^{NS}	0.10 ^{NS}	0.0	8.4 ^{**}	0.09 ^{NS}	0.14 ^{NS}	0.0 ^{NS}	9.3 ^{NS}	0.63 ^{NS}	1.48 ^{NS}
$\bar{X}_{P=(P1+P2)/2}$	18.5	3.3	0.67	0.03	291	200	6.84	0.73	22.8	11.6	0.69	0.25	293	256.7	7.71	3.88
\bar{X}_{RIL}	19.7	3.3	0.72	0.03	300	215	7.03	0.77	18.9	12.4	0.59	0.21	289	244.4	6.50	3.48
$\bar{X}_{RIL} - \bar{X}_P$	1.2 ^{NS}	0.0 ^{NS}	0.05 ^{NS}	0.00 ^{NS}	9.0 ^{NS}	16 ^{NS}	0.18 ^{NS}	0.04 ^{NS}	3.9 ^{NS}	0.8 ^{NS}	0.10 ^{NS}	0.04 ^{NS}	4.0 ^{NS}	12.3 ^{NS}	1.21 ^{NS}	0.40 ^{NS}
B RIL	25.0	6.7	1.14	0.08	360	319	8.56	1.74	26.8	20.6	1.04	0.45	313	280.7	8.69	5.81
GGB= BRIL - BP	4.2 ^{NS}	3.3 ^{**}	0.33 ^{NS}	0.05 ^{**}	56 ^{NS}	97 ^{NS}	1.22 ^{NS}	0.96 ^{**}	4.0 ^{NS}	4.8 ^{NS}	0.29 ^{NS}	0.12 ^{NS}	20 ^{NS}	19.4 ^{NS}	0.66 ^{NS}	1.187 ^{NS}
10%SRIL	24.5	6.3	1.02	0.06	330	286	8.45	1.46	23.3	19.9	0.91	0.41	312	275.5	8.39	5.48
GG10% = 10%SRIL - \bar{X}_P	5.9 ^{NS}	2.6 ^{NS}	0.35 ^{NS}	0.03 ^{NS}	39 ^{NS}	86 ^{NS}	1.61 ^{NS}	0.73 ^{NS}	0.5 ^{NS}	8.3 ^{**}	0.22 ^{NS}	0.16 ^{NS}	19 ^{NS}	18.8 ^{NS}	0.68 ^{NS}	1.59 ^{NS}
LSD _{0.05}	9.44	3.25	0.63	0.04	67.25	111.27	3.36	0.81	8.53	7.97	0.44	0.28	49.38	65.52	2.88	2.93

P_n net photosynthesis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), g_s stomatal conductance ($\mu\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), Ci intercellular CO₂ concentration ($\mu\text{mol mol}^{-1}$), Tr transpiration ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), PAC-2 (P1) and RHA-266 (P2) parental lines, \bar{X}_P mean of two parental lines, \bar{X}_{RIL} mean of recombinant inbred lines (RILs), BRIL the best RIL, BP the best parent, 10%SRIL 10% selected RILs, GGB genetic gain when the best RIL is compared with the best parent, GG10% genetic gain when 10% selected RILs are compared with mean of parents, LSD_{0.05} least significant differences calculated using $t_{0.05}$ and error mean square of each trait

*** and NS significant at 0.01 level and non-significant

correlations were also shown between relative water content (RWC), leaf water potential (Ψ_w) and stomatal conductance (g_s) indicating the response of stomata to water supply.

High genetic variability was observed in four selected RILs and their parents mainly under water stress conditions (Table 6); these treatments and lines were used for RT-PCR gene expression analysis.

QTL mapping

The QTL analysis was performed in plants grown under water-stressed conditions before rehydration (Fig. 1). QTL mapping showed the presence of several QTLs involved in the expression of water status traits (RWC, Ψ_w , Ψ_s , Ψ_t and OA) as well as net photosynthesis rate (P_n). Co-localization was observed for some traits on different linkage groups. The genomic regions with alleles increasing these traits come from both parental lines (Fig. 1). Graphical genotyping for the selected RILs (C100, C133, C147 and LR54) used for RT-PCR showed how chromosome segments having the alleles with positive or negative effects were inherited from parental lines (Fig. 1), which partially explains the differences among RILs in term of physiological traits as well as transcript abundance.

Quantitative real-Time RT-PCR analysis of gene expression

The results of analysis by real-time RT-PCR of aquaporin expression presented in Fig. 2a show that expression of the aquaporin gene in the leaves of all studied lines is down-regulated by water stress. A large variation was observed in the relative expression of aquaporin among our studied genotypes (Fig. 2a). Aquaporin transcript level decreased from five-fold control in 'PAC-2' to about 100-fold control in LR54 under water stress. The transcript level of aquaporin greatly increased by rehydration in all RILs when compared with its level of expression before rehydration (Fig. 2b). However, transcript level differed among RILs and both up and down-regulation was observed in the studied genotypes (Fig. 2b). The level of dehydrin transcript was increased in leaves of all studied RILs in response to water stress (Fig. 2c). Major differences were observed among RILs for the accumulation of dehydrin transcript. Dehydrin transcript accumulated about 90-fold in C100 compared to well-watered control plants of this genotype. The transcript level of dehydrin strongly decreased in all RILs after rehydration but differed among RILs (Fig. 2d). 'RHA-266' showed the highest transcript

Table 4 Genetic gain for plant water relation parameters on well-watered (WW) and water-stressed (WS) genotypes of sunflower

Parameter	RWC		Ψ_w		Ψ_s		Ψ_t		OA
	WW	WS	WW	WS	WW	WS	WW	WS	WS
PAC-2 (P1)	88.2	71.5	-0.79	-1.62	-1.15	-1.61	0.36	0.00	0.06
RHA-266 (P2)	86.2	69.4	-0.75	-1.65	-1.12	-1.68	0.46	0.03	0.03
P1-P2	4.0 ^{NS}	2.1 ^{NS}	0.04 ^{NS}	0.03 ^{NS}	0.03 ^{NS}	0.07 ^{NS}	0.10 ^{NS}	0.03 ^{NS}	0.03 ^{NS}
$\bar{X}_{P=(P1+P2)/2}$	87.2	70.4	-0.77	-1.63	-1.13	-1.64	0.41	0.01	0.04
\bar{X}_{RIL}	86	71.3	-0.78	-1.80	-1.21	-1.84	0.43	0.04	0.20
$\bar{X}_{RIL} - \bar{X}_P$	1.2 ^{NS}	0.90 ^{NS}	0.01 ^{NS}	0.17 ^{NS}	0.08 ^{NS}	0.20 ^{**}	0.02 ^{NS}	0.03 ^{NS}	0.16 ^{NS}
B RIL	91.7	80.7	-0.58	-1.30	-1.05	-1.30	0.75	0.39	0.58
GGB= BRIL - BP	5.5 ^{NS}	9.2 ^{**}	0.17 ^{NS}	0.32 ^{**}	0.07 ^{NS}	0.31 ^{**}	0.29 ^{NS}	0.36 ^{**}	0.52 ^{**}
10%SRIL	90.9	80	-0.61	-1.43	-1.06	-1.49	0.58	0.26	0.48
GG10% = 10%SRIL - \bar{X}_P	3.7 ^{NS}	9.6 ^{**}	0.16 ^{NS}	0.20 ^{NS}	0.07 ^{NS}	0.17 ^{**}	0.17 ^{NS}	0.25 ^{**}	0.44 ^{**}
LSD _{0.05}	8.62	6.92	0.54	0.23	0.54	0.16	0.65	0.23	0.23

PAC-2 (P1) and RHA-266 (P2) parental lines, BRIL the best RIL, BP the best parent, 10%SRIL 10% Selected RILs, GGB genetic gain when the best RIL is compared with the best parent, GG10% genetic gain when 10% selected RILs are compared with mean of parents, LSD_{0.05} least significant differences calculated using $t_{0.05}$ and error mean square of each trait, RWC relative water content (%), Ψ_w leaf water potential (MPa), Ψ_s osmotic potential (MPa), Ψ_t turgor potential (MPa), OA osmotic adjustment (MPa)

** and NS significant at 0.01 and non-significant

Table 5 Simple correlation coefficients between relative water content (RWC), leaf water potential (Ψ_w), turgor potential (Ψ_t), net photosynthesis (P_n), stomatal conductance (g_s), transpiration (Tr) and osmotic adjustment (OA) in Recombinant Inbred Lines (RILs) and two parents ('PAC-2' and 'RHA-266') of sunflower

	RWC	Ψ_w	Ψ_t	P_n	g_s	Tr
Ψ_w	0.86***					
Ψ_t	0.56***	0.75***				
P_n	0.72***	0.77***	0.67***			
g_s	0.63***	0.70***	0.63***	0.88***		
Tr	0.81***	0.80***	0.72***	0.93***	0.95***	
OA	-0.42***	-0.51***	0.24**	0.12 ^{NS}	0.03 ^{NS}	0.005 ^{NS}

RWC relative water content (%), Ψ_w leaf water potential (MPa), Ψ_t turgor potential (MPa), P_n net photosynthesis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), g_s stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), Tr transpiration ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), OA osmotic adjustment (MPa),

, * and NS, significant at 0.01, 0.001 probability levels and non-significant

level after rehydration. 'RHA-266' could not recover from drought stress after rehydration as indicated by its net photosynthesis after rehydration (Table 6). The expression level of putative leafy cotyledon1-like protein gene decreased under water stress in all genotypes, suggesting down-regulation of this gene under water stress (Fig. 3a). Differences in expression patterns were found in the putative leafy cotyledon1-like protein gene among RILs. The expression level of leafy cotyledon1-like protein in LR54, which displayed lower RWC and photosynthesis under water stress (Table 6), decreased to 5.5-fold control (Fig. 3a). The expression patterns of fructose-1,6 bisphosphatase under water stress and rehydration conditions are shown in Fig. 3c and d. The transcript level of fructose-1,6 bisphosphatase was decreased by water stress in all genotypes except in C100, which showed a slight transcript increase under water stress, close to control levels.

Discussion

Genetic variation for physiological traits

Water stress revealed genotypic variability (MS_G) for the physiological traits studied as more significant variances were observed among water-stressed RILs compared with well-watered ones (Table 2). Burke (2001) and Srikanthbabu et al. (2002) have also shown that genetic variability for the stress response could only be seen upon exposure to an induction stress. Some RILs maintained their RWC under water stress. Drought resistance of a plant is related to its ability to maintain higher relative water content in the leaves under water stress (Suprunova et al. 2004). Net photosynthesis (P_n) in rehydrated water-stressed plants differed substantially between the two parents, indicating differential abilities for recovery from water stress (Table 3). This suggests that parental lines carry

Table 6 Physiological characteristics of four RILs and their parents ('PAC-2' and 'RHA-266') selected for gene expression analysis by quantitative real-time RT-PCR

Genotype	P_n		g_s		Ci		Tr		RWC		Ψ_w		Ψ_s		Ψ_t		OA
	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WS
Before rehydration																	
C100	18.7 ^a	3.9 ^{ab}	0.51 ^a	0.03 ^b	314 ^a	183 ^{ab}	6.36 ^a	0.69 ^b	86.7 ^a	79.0 ^a	-0.76 ^a	-1.50 ^a	-1.11 ^a	-1.56 ^a	0.35 ^a	0.05 ^b	0.25 ^b
C133	20.5 ^a	6.7 ^a	0.74 ^a	0.06 ^a	287 ^a	203 ^{ab}	7.39 ^a	1.36 ^a	87.4 ^a	73.0 ^{ab}	-0.79 ^a	-1.82 ^{ab}	-1.25 ^a	-1.79 ^{bc}	0.46 ^a	0.03 ^b	0.06 ^d
C147	19.3 ^a	4.4 ^{ab}	0.65 ^a	0.03 ^b	257 ^a	143 ^b	5.55 ^a	0.79 ^b	85.4 ^a	78.8 ^a	-0.97 ^a	-1.60 ^{ab}	-1.33 ^a	-1.62 ^a	0.35 ^a	0.03 ^b	0.11 ^c
LR54	20.9 ^a	1.6 ^b	0.70 ^a	0.02 ^b	302 ^a	264 ^a	6.98 ^a	0.51 ^b	85.5 ^a	59.5 ^d	-0.81 ^a	-2.00 ^c	-1.15 ^a	-2.33 ^d	0.34 ^a	0.33 ^a	0.49 ^a
PAC-2	16.3 ^a	3.2 ^{ab}	0.54 ^a	0.03 ^b	277 ^a	222 ^{ab}	6.35 ^a	0.78 ^b	86.7 ^a	71.5 ^{ab}	0.79 ^a	-1.62 ^{ab}	-1.15 ^a	-1.62 ^a	0.36 ^a	0.00 ^c	0.06 ^d
RHA-266	20.6 ^a	3.4 ^{ab}	0.76 ^a	0.02 ^b	298 ^a	177 ^{ab}	6.86 ^a	0.68 ^b	85.7 ^a	69.4 ^c	0.87 ^a	-1.65 ^{ab}	-1.27 ^a	-1.68 ^{ab}	0.40 ^a	-0.03 ^c	0.03 ^d
After rehydration																	
C100	14.8 ^a	20.3 ^a	0.57 ^a	0.45 ^a	290 ^a	264 ^a	6.28 ^a	5.77 ^a									
C133	23.7 ^a	20.3 ^a	0.88 ^a	0.38 ^{ab}	302 ^a	259 ^a	8.20 ^a	5.08 ^a									
C147	21.9 ^a	16.9 ^{ab}	0.54 ^a	0.33 ^{ab}	271 ^a	262 ^a	6.10 ^a	4.37 ^a									
LR54	14.1 ^a	13.7 ^{ab}	0.30 ^a	0.19 ^b	269 ^a	246 ^a	4.94 ^a	3.62 ^a									
PAC-2	22.8 ^a	15.8 ^{ab}	0.64 ^a	0.32 ^{ab}	293 ^a	252 ^a	7.40 ^a	4.62 ^a									
RHA-266	22.8 ^a	7.4 ^b	0.75 ^a	0.18 ^b	293 ^a	261 ^a	8.02 ^a	3.14 ^a									

In a column, means followed by common letter(s) are not significantly different at 0.05 level by SNK

WW well-watered plants, WS water-stressed plants, P_n net photosynthesis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), g_s stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), Ci intercellular CO_2 concentration ($\mu\text{mol mol}^{-1}$), Tr transpiration ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), RWC relative water content (%), Ψ_w leaf water potential (MPa), Ψ_s osmotic potential (MPa), Ψ_t turgor potential (MPa), OA osmotic adjustment (MPa)

different genes for recovery, and these are inherited in RILs via transgressive segregation. This results in significant differences among RILs after rehydration for net photosynthesis (Table 2). Otherwise, the two parents did not differ significantly for net photosynthesis before rehydration. According to these results, it is probable that the genes responsible for drought resistance could be different from those responsible for recovery from water stress during rehydration. Significant differences between the best parent and the best RIL and/or between 10% selected RILs and the mean of parents (Table 3) might be due to transgressive segregation resulting from the accumulation of favorable alleles in some RILs.

The significant correlation between osmotic adjustment (OA) and turgor potential (Ψ_t) in our study showed that the plants subjected to water stress maintained turgor through osmotic adjustment, which consequently resulted in maintenance of photosynthesis under water stress (Table 5). OA is considered to be important for the maintenance of photosynthetic activity during dehydration (Conroy et al. 1988), through its role in turgor maintenance (Turner and Jones 1980; Maury et al. 2000; Ober et al. 2005). The correlation between net photosynthesis (P_n) and stomatal conductance (g_s) was also highly significant. Stomatal limitation is considered to decrease both net photosynthesis (P_n) and CO_2 concentration in the intercellular space of the leaf (Ci) (Cornic 2000). In our experiment water stress increased intercellular CO_2 concentration (Ci) in some RILs and decreased it in

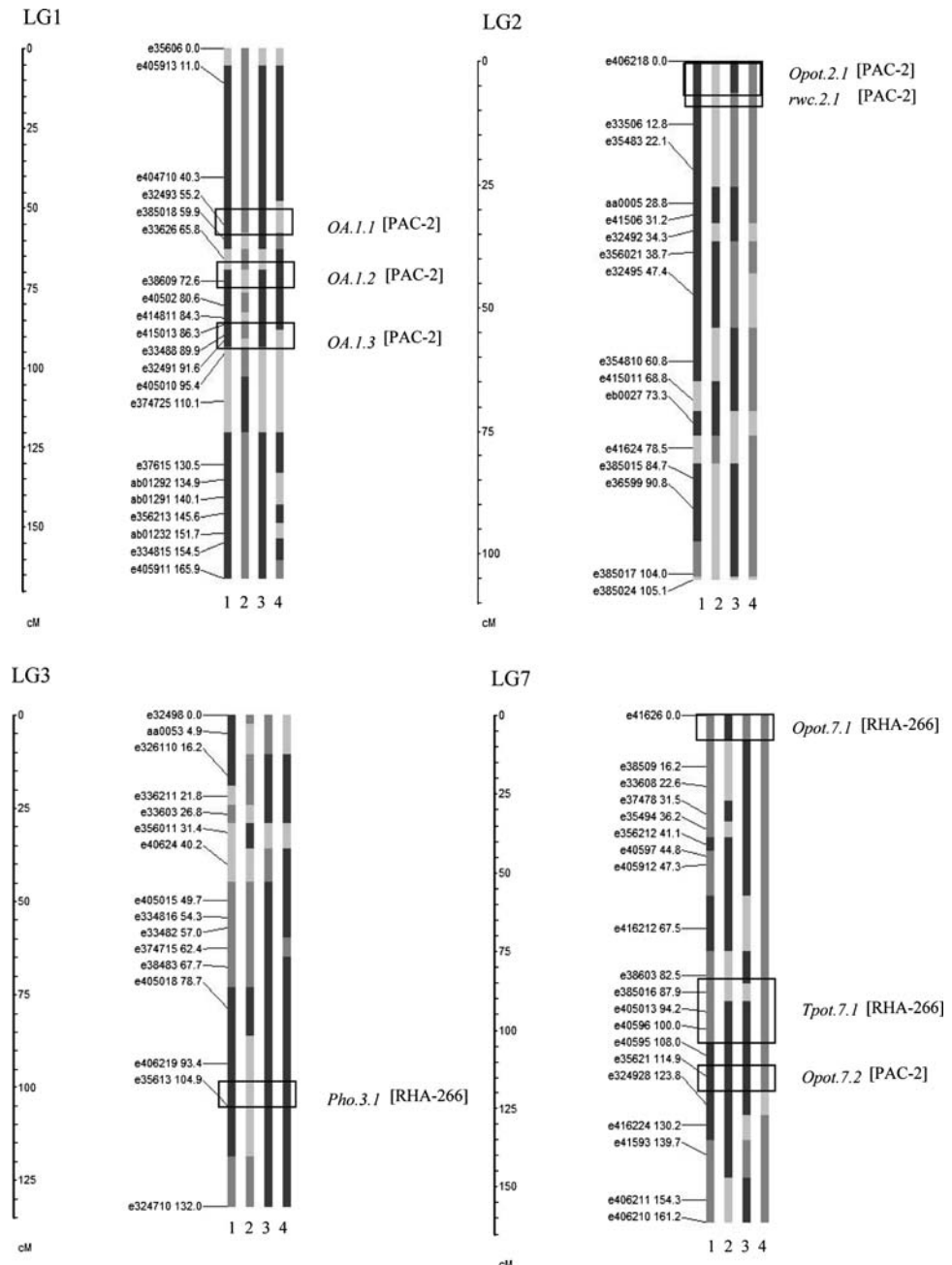
some others depending on genotypes (variation range; Table 2). These results indicate that while stomatal conductance (g_s) is decreased, intercellular CO_2 concentration (Ci) can be increased due to metabolic limitation of photosynthesis or by increased CO_2 production from respiration relative to photosynthesis, which has been also reported by Lawlor (1995).

Large differences were observed among genotypes selected for analysis of gene expression (Table 6). Net photosynthesis (P_n) of C100 and C133 genotypes may not be limited by metabolic factors because these RILs maintain high RWC under water stress. Therefore intercellular CO_2 concentration (Ci) decreased due to drawing down by photosynthesis under water stress, and was increased after rehydration via stomatal opening. In the LR54 genotype, limited photosynthesis by metabolic factors (non-stomatal limitation) resulting from low RWC, results in increase of intercellular CO_2 concentration (Ci), as has been previously described by Lawlor and Cornic (2002) and Lawlor (2002). We propose that intercellular CO_2 concentration (Ci) decreases in C100 and C133 genotypes due to a high rate of photosynthesis, whereas Ci is increased in LR54 because of respiration and metabolic limitations on photosynthesis.

Quantitative real-Time RT-PCR analysis of gene expression and QTL mapping

The expression level of aquaporin is closely associated with RWC, leaf water potential (Ψ_w) and osmotic

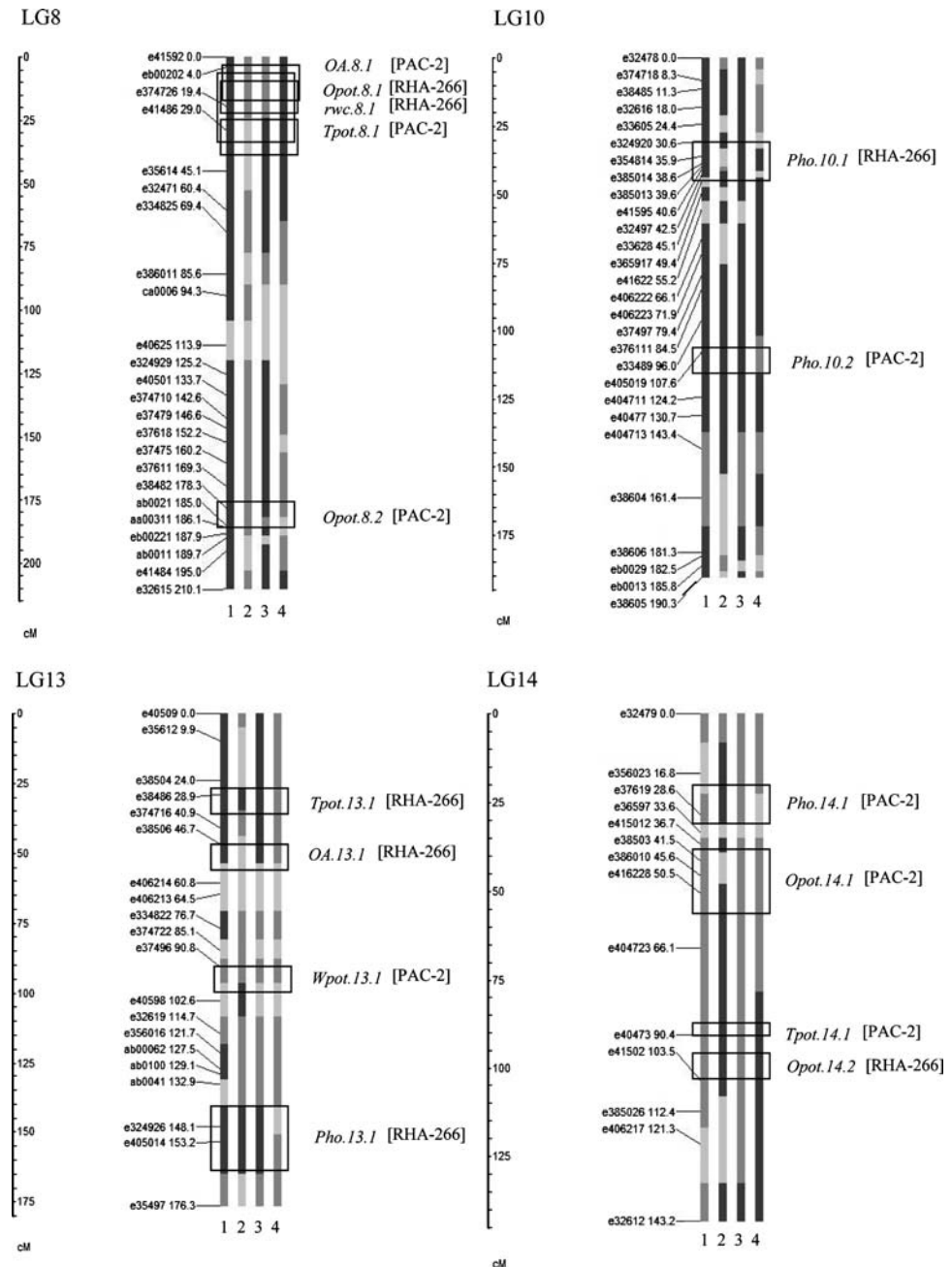
Fig. 1 Graphical genotyping to visualize the linkage groups (LG) and genomic regions of C100 (1), C133 (2), C147 (3) and LR54 (4) from PAC-2 (black) and RHA-266 (dark gray) and unknown genomic regions (light gray). The markers with their positions are presented in the left side and QTLs are presented in the right side of linkage groups. The QTLs were designated as osmotic adjustment (*OA*), relative water content (*rw*), water potential (*Wpot*), osmotic potential (*Opot*), turgor potential (*Tpot*) and photosynthesis (*Pho*) following linkage group and QTL number. For each QTL, [PAC-2] and [RHA-266] show that genomic region with positive alleles come from PAC-2 or RHA-266, respectively (determined by QTL cartographer V 1.6 as the sign of additive gene effect)



potential (Ψ_s) (Fig. 2a; Table 6). LR54 showed the greatest decrease in transcript level of aquaporin (100-fold control) (Fig. 2a). This genotype also had the lowest relative water content (RWC), leaf water potential (Ψ_w), osmotic potential (Ψ_s) and net photosynthesis (P_n) as well as the highest intercellular CO_2 concentration (C_i), probably due to chloroplastic impairments under water stress (Table 6). Other genotypes, which presented higher RWC (up to 79%), Ψ_w and Ψ_s under water stress, showed lower decreasing of aquaporin gene expression (Table 6). On the other

hand, when RWC and Ψ_w or Ψ_s is decreased, aquaporin gene expression is also decreased in order to conserve remaining cellular water against further stress. Smart et al. (2001) reported that down-regulation of aquaporin gene expression results in reduced membrane water permeability and may encourage cellular water conservation during periods of water stress. In contrast, there are examples of aquaporin genes, which are up-regulated by water stress resulting in greater osmotic water permeability and facility of water flux (Yamaguchi-Shinozaki et al. 1992; Yamada et al. 1997).

Fig. 1 continued



QTL mapping revealed that for QTLs of RWC (*rwc.2.1*) and Ψ_s (*Opot.7.2*), located respectively, on linkage groups 2 and 7, the favorable alleles come from ‘PAC-2’. Graphical genotyping (Fig. 1) showed that for the two mentioned QTLs, LR54 is the only genotype that carries a negative genomic region from ‘RHA-266’ (Fig. 1; LG2, and LG7). These genomic regions (*rwc.2.1* and *Opot.7.2*) may be associated with differences in aquaporin transcript abundance and also water status. Negative alleles of LR54 could decrease aquaporin gene expression under conditions of

low RWC and Ψ_s . However, LR54 carries genomic regions having alleles with positive effects for all QTLs of turgor potential (Ψ_t) and OA, which explains why LR54 showed the highest level of Ψ_t and OA compared with other genotypes (Fig. 1). This suggests that two mechanisms could be involved when the plants are subjected to water limited conditions: one that decreases aquaporin gene expression to conserve the cellular water, and another that increases turgor potential via osmotic adjustment. Comparison of aquaporin gene expression between LR54 and ‘RHA-266’

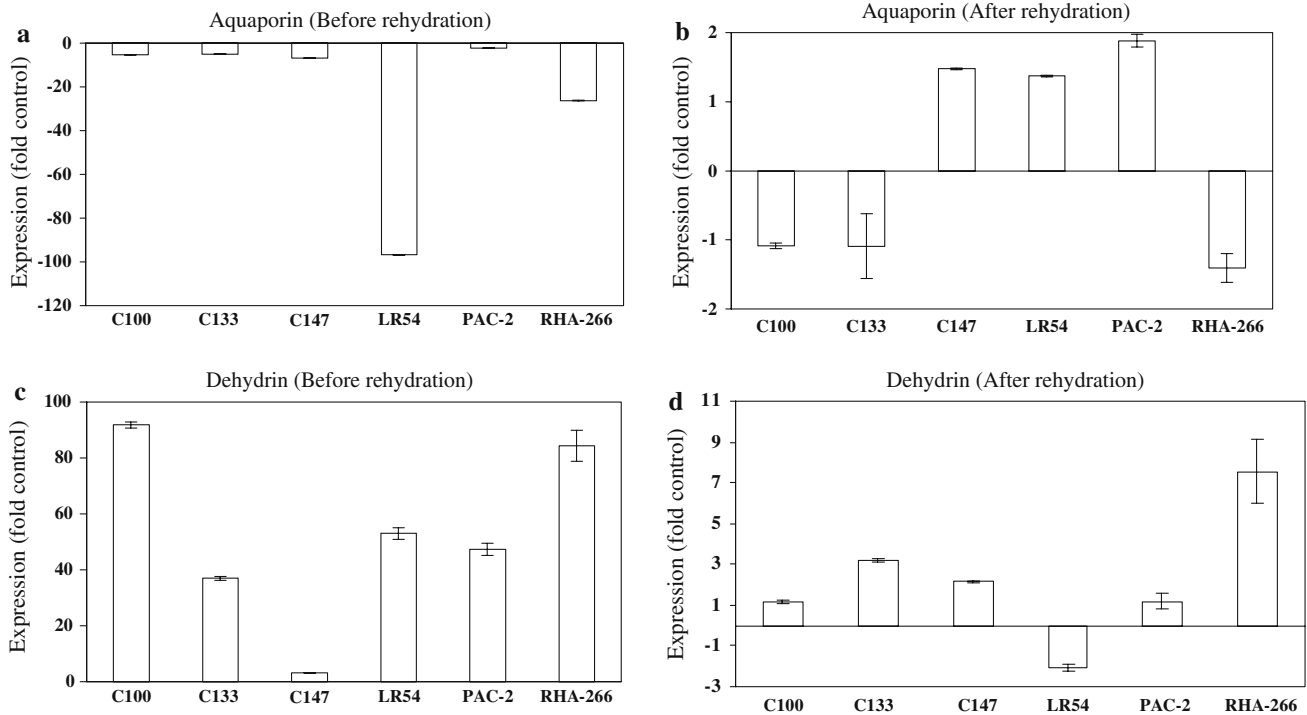


Fig. 2 Effect of water stress on the expression of the aquaporin and dehydrin genes in four sunflower RILs and parental lines (‘PAC-2’ and ‘RHA-266’) studied by quantitative real-time RT-PCR. The transcript levels of each gene in the leaf of water-stressed plants before rehydration (Fig. 2a, c) and water-stressed

plants after rehydration (Fig. 2b, d) were plotted as the relative expression (fold) of the non-stressed (well-watered) control plants exposed. The transcript level of actin was used as a reference. Values are mean ± SE of three independent replicates

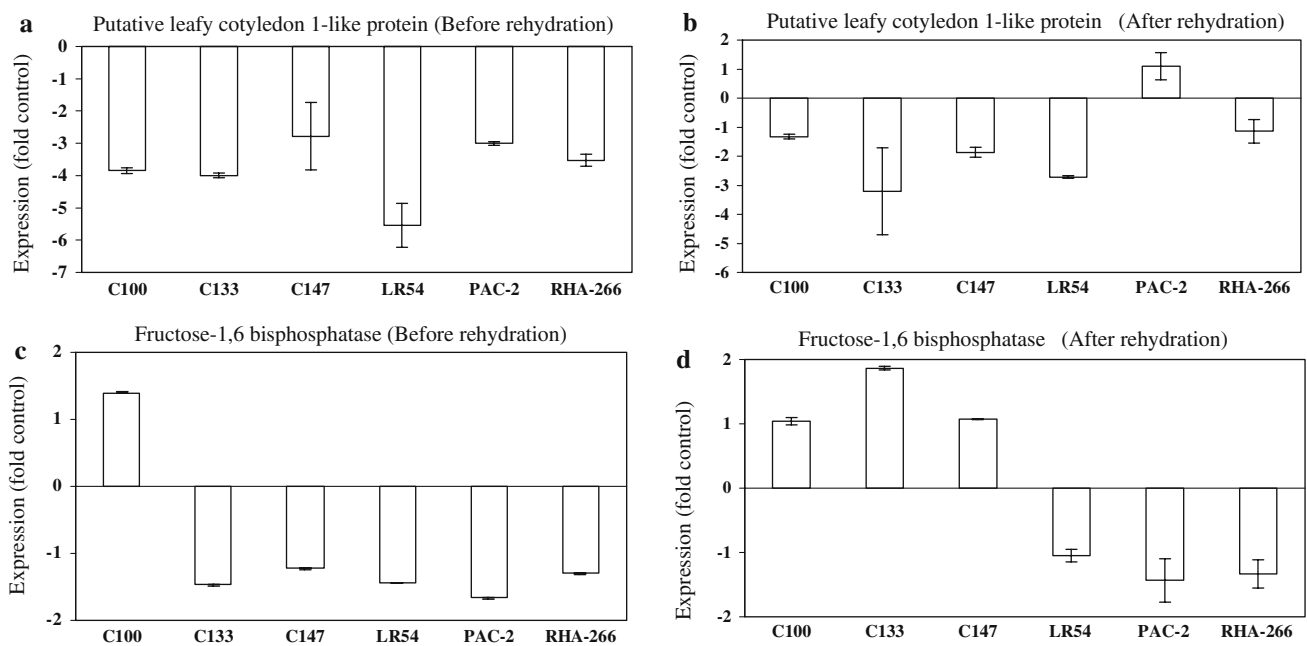


Fig. 3 Effect of water stress on the expression of Putative leafy cotyledon1-like protein and Fructose-1,6 biphosphatase genes in four sunflower RILs and parental lines (‘PAC-2’ and ‘RHA-266’) studied by quantitative real-time RT-PCR. The transcript levels of each gene in the leaf of water-stressed plants before

rehydration (Fig. 3a, c) and water-stressed plants after rehydration (Fig. 3b, d) were plotted as the relative expression (fold) of the non-stressed (well-watered) control plants exposed. The transcript level of actin was used as a reference. Values are mean ± SE of three independent replicates

before and after rehydration shows the probable role of aquaporin in recovery from water stress. Since both ‘RHA-266’ and LR54 showed the lowest aquaporin gene expression under water stress (before rehydration), the observed higher transcript level in LR54 compared with ‘RHA-266’ after rehydration may be associated with ability to recover from water stress (Table 6). Aquaporins play a significant role in recovery from water stress in *Arabidopsis* (Martre et al. 2002). Aquaporin gene expression in our experiment increased during leaf rehydration, which should increase membrane water permeability and facilitate water transport in and out of leaves as described by Vander Willigen et al. (2004). Sarda et al. (1997) showed that the transcripts of aquaporin (*suntip7*) accumulate when stomata are closed.

Despite a high accumulation of dehydrin (up to 90-fold control) under water stress, the transcript level of dehydrin is not associated simply with plant water status and the RILs with the same RWC and Ψ_w showed a great difference in dehydrin transcript accumulation under water stress (Table 6; Fig. 2). C100 and C147 genotypes, which showed the highest (90-fold control) and the lowest (five-fold control) dehydrin accumulation respectively, presented equivalent water status and gas exchange under water stress (Table 6; Fig. 2c). Our results are in agreement with those previously reported by Cellier et al. (1998) in which dehydrin transcripts generally accumulate to high levels in water-stressed sunflower lines, but transcripts did not accumulate as a function of leaf water potential. The results of QTL mapping and graphical genotyping confirmed that there is not a clear relationship between dehydrin transcript accumulation and positive alleles carried by RILs for different QTLs (Figs. 1, 2c). Otherwise, we could not find any QTL for which there is a difference between C147 (presenting the lowest transcript accumulation) and other genotypes in term of positive or negative allele effects. This is not surprising because dehydrin is reported to be involved in water stress tolerance via structural protection rather than changes in water status traits or photosynthesis (Ramanjulu and Bartels 2002). Our results suggest that the preferential accumulation of dehydrin transcripts in the leaves of some RILs such as C100 (Fig. 2c) is associated with the adaptive response occurring in this genotype during a period of drought. The high level of dehydrin in ‘RHA-266’ after rehydration suggests that dehydrin may be involved in structural protection, which has been reported previously (Dure et al. 1989; Baker et al. 1988). ‘RHA-266’ could not recover from water stress in terms of photosynthesis and dehydrin

gene expression remained high after rehydration (Table 6).

Reduction of putative leafy cotyledon1-like protein in our RILs under water stress (up to six-fold compared with control) showed for the first time its potential role in water stress response in sunflower. We showed that the expression level of putative leafy cotyledon1-like protein gene is decreased significantly by dehydration and increased by rehydration; it may play an essential role in drought tolerance. However, it is not accumulated as a function of plant water status; and QTLs were not associated with transcript abundance. Moreover, this regulator may not contribute in up-regulation of dehydrin gene. Future studies would dissect the role of putative leafy cotyledon1-like protein in water stress tolerance and in regulation of dehydrins as a member of LEA in sunflower.

In our study, photosynthesis was decreased by water stress in all RILs (Table 6) which may be partially due to down-regulation of fructose-1,6 biphosphatase under water stress (Fig. 3c). Two QTLs for photosynthesis (‘*Pho.10.2*’ and ‘*Pho.13.1*’) was closely associated with photosynthesis capacity of four RILs but not with transcript abundance of fructose-1,6 biphosphatase under water stress (Fig. 1). These two QTLs explain large differences observed between LR54 and other genotypes for P_n (Table 6). Another QTL, which may contribute to the greater photosynthesis capacity observed for C133 under water stress, is located on linkage group 14 (*Pho.14.1*). C133 carries positive alleles from ‘PAC-2’ for this QTL. Although the four RILs carry different genomic regions for three mentioned QTLs, they presented almost the same transcript abundance of fructose-1,6 biphosphatase (Fig. 3c). Therefore these three QTLs were not associated with transcript abundance, but were associated with phenotypic variation. Two other QTLs, for which all four genotypes received negative alleles from parental lines, are located on linkage group 3 (*Pho.3.1*) and 10 (*Pho.10.1*). Alleles increasing phenotypic responses associated with these two QTLs come from ‘RHA-266’ but all four RILs received these QTL regions from ‘PAC-2’ or an unknown segment (C133). These QTLs might be associated with down-regulation of fructose-1,6 biphosphatase because all RILs showed the same down-regulation of fructose-1,6 biphosphatase and also contain negative alleles for these QTLs. During rehydration after water stress, the RILs that showed a high net photosynthetic rate (P_n) (Table 6) displayed a transcript level of fructose-1,6 biphosphatase close to well-watered plants (Fig. 3d). This association between photosynthesis and fructose-1,6 biphosphatase is necessary as sucrose

synthesis must be balanced with the momentary rate of photosynthesis (Stitt 1990). These results show that down-regulation of fructose-1,6 biphosphatase could play a role in non-stomatal limitation of photosynthesis, resulting in decrease of photosynthesis under water stress.

We can conclude that sunflower genotypes subjected to progressive drought at the pre-flowering stage, as often happens in field conditions, presented a large variation and contrasted responses in terms of water status maintenance and photosynthetic potential. The four selected RILs and parents, which display contrasting response to water stress, present relationships between (1) water status and aquaporin gene expression and (2) net photosynthesis and fructose-1,6 biphosphatase gene expression mainly after rehydration. The transcript levels of aquaporin and fructose-1,6 biphosphatase are associated with QTLs having positive or negative alleles controlling water status and photosynthesis. In contrast, dehydrin and leafy cotyledon1-like genes expression are not correlated directly with plant water status, probably due to the slow drought establishment, which allowed different adaptive responses in our study. The large genetic variation among RILs studied here could be useful for selecting genotypes in terms of a given physiological characteristic for investigation of water stress tolerance.

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