Transcriptome pathways unique to dehydration tolerant relatives of modern wheat

Neslihan Z. Ergen · Jyothi Thimmapuram · Hans J. Bohnert · Hikmet Budak

Abstract Among abiotic stressors, drought is a major factor responsible for dramatic yield loss in agriculture. In order to reveal differences in global expression profiles of drought tolerant and sensitive wild emmer wheat genotypes, a previously deployed shock-like dehydration process was utilized to compare transcriptomes at two time points in root and leaf tissues using the Affymetrix GeneChip® Wheat Genome Array hybridization. The comparison of transcriptomes reveal several unique genes or expression patterns such as differential usage of IP_3-dependent signal transduction pathways, ethylene- and abscisic acid (ABA)-dependent signaling, and preferential or faster induction of ABA-dependent transcription factors by the tolerant genotype that distinguish contrasting genotypes indicative of distinctive stress response pathways. The data also show that wild emmer wheat is capable of engaging known drought stress responsive mechanisms. The global comparison of transcriptomes in the absence of and after dehydration underlined the gene networks especially in root tissues that may have been lost in the selection processes generating modern bread wheats.

Keywords Wild emmer wheat · Shock dehydration · Affymetrix GeneChip® wheat genome array · Abiotic stress · Transcriptome comparison

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Introduction

During the next two decades, global warming is expected to increase yield loss in crop plants due to increasing temperatures and decreasing water availability (Lobell et al. 2008). The major cereal crops such as rice, maize, and wheat are important components of human diet, and therefore, development of crop varieties with improved adaptation capacity to drought environments is receiving much scientific attention (Guy et al. 2006; Vasil 2007; Collins et al. 2008). Securing food supplies and achieving food safety worldwide for a still increasing human population is an important objective for the future.

Plants, being sessile organisms, have well-developed survival and reproduction strategies under drought environment through changes at the molecular, cellular, and physiological levels depending on factors such as species and genotype, length and severity of water loss, developmental stage, and tissue type. The defense against drought stress starts with the perception of water loss, which triggers abscisic acid (ABA)-dependent and ABA-independent regulatory systems involving signal transduction cascades of protein kinases/phosphatases (such as Ca^{2+}-dependent protein kinases/phosphatases) leading to the activation of a

number of transcription factors/regulators. The outcome is a change of transcription programs for the synthesis of new proteins enabling defenses against osmotic stresses, activating the removal of toxic compounds and enhancing functional protection or degradation of existing proteins (Ramanjulu and Bartels 2002; Bartels and Sunkar 2005; Mahajan and Tuteja 2005; Langridge et al. 2006; Shinozaki and Yamaguchi-Shinozaki 2007; Barnabas et al. 2008). Newly synthesized proteins in response to water deficit include late embryogenesis abundant (LEA) proteins, dehydrins, sugar transporters, aquaporins, osmolytes, antioxidants, lipid metabolism related genes, heat shock proteins (HSPs), and proteasome components. Several sugars (such as sucrose, trehalose, or sorbitol), sugar alcohols (e.g., mannitol), amino acids (such as proline), and amines like glycine betaine and polyamides accumulate in plants subjected to water loss and can function as osmolytes regulating cell turgor and stabilizing proteins (Seki et al. 2007). LEA proteins like dehydrins are extremely hydrophilic and may function in protecting existing proteins from unfolding and subsequent degradation (Wang et al. 2003; Mahajan and Tuteja 2005). HSPs, molecular chaperones first detected as HSPs, prevent denaturation of proteins or assist in refolding of denatured ones (Wang et al. 2003). In addition, a major cause for drought-induced problems is the inhibition of photosynthesis because it leads to the accumulation of reactive oxygen species (ROS). A complex and diverse group of chemical antioxidants, such as ascorbic acid, glutathione, or flavonoids, and a large number of enzymes, such as peroxidases or catalases, generally accompany drought responses to combat the accumulation of ROS (Shinozaki and Yamaguchi-Shinozaki 2007).

The ability of plants to use a genetic program that allows for tolerance under less than optimal growth conditions depends on its natural adaptation to diverse environments. There are plants growing under extreme halophytic (salt tolerant) and xerophytic (drought tolerant) conditions, which are commonly used as models to understand and engineer stress tolerance of glycophytes (Vinocur and Altman 2005; Bohnert et al. 2006). Ten thousand years of domestication and adaptation to optimal environments for maximum yield have resulted in the loss of genetic diversity in hexaploid modern wheat (*Triticum aestivum* L.) that enabled the wheat progenitors to cope with suboptimal growth environments. In contrast, tetraploid wild emmer wheat (*Tricitum turgidum* ssp. *diccocoides* (Korn.) Thell.) has retained its evolutionary adaptive characteristics and is therefore a promising candidate for abiotic stress tolerance genes or alleles that could be exploited for crop improvement (Araus et al. 2007; Dubcovsky and Dvorak 2007).

To identify new abiotic stress genetic determinants, a microarray platform was designed for genome-wide analysis of transcripts in wheat species for monitoring changes in the transcriptome of wild emmer wheat upon quickly imposed dehydration. The microarray analysis was used to test the hypothesis that some transcriptome pathways are unique to wild emmer wheats and correlated with their ability to withstand drought. Initially, approximately 200 wild emmer wheat genotypes were screened followed by the selection of 26 lines. The 26 wild emmer wheat genotypes native to Turkey, near the center of domestication (Dubcovsky and Dvorak 2007), were screened again for their tolerance to slow drought stress under greenhouse conditions (Ergen and Budak 2009). The most promising tolerant and sensitive genotypes from previous and present screens were selected based on physiological and agronomical responses to the drought environment in comparison to cultivated durum wheat (*Triticum turgidum* L. ssp. *durum*). The tolerant (TR; TR39477) and sensitive (TS; TTD-22) genotypes were then used for genome-wide comparison of transcript changes upon shock-like dehydration using the Affymetrix GeneChip® Wheat Genome Array.

Materials and methods

Plant material and shock-like dehydration

Selection of the most promising tolerant and sensitive wild emmer wheat (*T. turgidum* ssp. *diccocoides* (Korn.) Thell.) genotypes was performed by an experiment where the physiological and agronomical differences were monitored and compared to modern genotypes. The contrasting genotypes in the current study had characteristics that were consistent with their responses in a slow drought stress (Ergen and Budak 2009). For the shock-like dehydration treatment, seeds of TR39477 (tolerant) and TTD-22 (sensitive) genotypes were surface sterilized in 1% sodium hypochloride and pre-germinated in Petri dishes for 21 days at 4°C in the dark. Seedlings of a similar developmental stage were transferred to continuously aerated Hoagland's solution renewed every 3 days and grown under controlled conditions (16 h photoperiod, temperature 24/22°C, relative humidity 60%, and photon flux density of 600–700 μmol m^{-2} s^{-1}). At the age of 3 weeks after transfer, plants were stressed by removing them from tanks and leaving on paper towels under the same lighting conditions, while control plants were kept in fresh hydroponic solution. Root and leaf tissue samples from both stress and control plants were collected at the fourth and eighth hour of stress. Leaf tissues were directly frozen and stored at −80°C, whereas root tissues were kept in RNALater (Ambion) at 4°C overnight before freezing.

Physiological characterization and free proline determination

Leaf relative water content (LWC) was measured using third youngest leaf and calculated as LWC=[(fresh weight−dry weight)/(turgid weight−dry weight)] (Jones 2007). To determine free proline concentration (Li et al. 2004), 150 mg of leaf powder was incubated with 75% ethanol overnight with shaking, and 100 µl of aliquot was incubated with 900 µl of freshly prepared ninhydrin reagent. Samples were incubated at room temperature for 24 h after addition of 3 ml toluene (Sigma), and absorbance was measured at 520 nm. Free proline concentration was determined through a standard curve established using L-proline (Sigma). In both analysis, samples were collected from three independent individuals and measurements were performed as triplicates.

RNA isolation, labeling, and GeneChip hybridization

Three hundred milligrams of frozen tissue was used for total RNA isolation using the Trizol reagents (Invitrogen) according to the manufacturer's instructions. Isolated total RNA was quantified spectrophotometrically and after the integrity was confirmed by denaturing agarose gel electrophoresis. Five micrograms of total RNA were used to generate labeled cRNA using the One-Cycle Target Labeling and Control Reagents (Affymetrix) according to the manufacturer's protocol. Biotin-labeled cRNA (15 µg) were then fragmented and hybridized to Affymetrix GeneChip® Wheat Genome Array for 16 h at 45°C and 60 rpm using Hybridization Wash and Stain Kit (Affymetrix) in Fluidics Station 450 following the manufacturer's protocol and scanned using GeneChip® Scanner 3000 with GeneChip® Operating Software.

Data analysis

Hybridization experiments were carried out as biological triplicates of control and shock stress treated samples (separate total RNA isolation and cRNA labeling for each hybridization) of two genotypes, two tissues, and two time points for a total of 48 Affymetrix GeneChip® Wheat Genome Arrays. Cell intensity files were first analyzed with Partek® Genomics Suite version 6.3 Beta (Partek Incorporated) using robust multichip average normalization. The quality of data was then confirmed by principal component analysis and box–whiskers plots. Log-transformed expression values were further analyzed by analysis of variance (ANOVA) where filters were set to $p<0.01$ and differential expression (DE) either less than −3 or greater than +3. Raw data are available in the ArrayExpress database (http://www.ebi.ac.uk) under the submission number E-MEXP-1488. Differentially expressed probes in both genotypes, in all tissues, and at both time points were subjected to cluster analysis using Cluster/Treeview programme (Eisen et al. 1998).

Quantitative real-time RT-PCR

Two micrograms of total RNA were used for first-strand cDNA synthesis with the Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions, quantified spectrophotometrically and diluted to 500 ng/µl. One microliter of this cDNA was amplified with 0.8 µM of specific primers in a total of 20 µl volume using SYBR green polymerase chain reaction (PCR) master mix (Applied Biosystems) with Icycler Multicolor Real-Time PCR Detection Systems (Bio-Rad Laboratories) (Ergen et al. 2007; Cebeci et al. 2008; Ergen and Budak 2009). The quantification was performed according to Muller et al. (2002) using actin (GenBank accession AY663392; forward, GGATCTCACGGACTCCCTCAT/reverse, CGGC TGAGGTTGTGAAGGA) as an internal reference, and three independent PCR results with acceptable efficiency (1.8–2.2) were averaged. Quantitative real-time reverse transcription PCR (qRT-PCR) analyses were performed for four probes to confirm differential regulation between genotypes in different tissues. Selected probes and primer sequences designed to amplify a region of 100 to 150 bp are as follows: (1) linalool synthase (Ta.426.1.A1_at, forward, CACATGGTGGAGATGATTGC; reverse, TAGT GATGCCGTGATGAAGC); (2) nicotianamine synthase 7 (TaAffx.61928.1.S1_at; forward, CATCGCGCAGAAGTC CAG; reverse, CCTCTCTCTTCTG GCTCACG); (3) allene oxide synthase (Ta.27217.1.S1_at, forward, GGCAGCA GCTCTCTCATAGC; reverse, CACAAGGGCACCATGC AG); and (4) LEA protein, group 3 (Ta.23797.1.S1_x_at, forward, GCGCAGTACACCAAGGAGTC; reverse, TGG TGGTGGTGGTGTCCT).

Results

Stress phenotype, relative LWC and proline in contrasting genotypes

A previously deployed shock-like dehydration process (Talame et al. 2007) was utilized to establish the experiments reported in the present study using the 26 genotypes. One set of genotypes was used for the shock-like drought stress (4 and 8 h). Based on the morphological and physiological screen, where we obtain few seeds in the tolerant genotype (TR39477) compared to controls not exposed to the stress, the sensitive genotype (TTD-22) gave no seeds. Microarray study were carried out on these two

contrasting genotypes using root and leaf tissue samples from both stress and control plants.

Both control and stress-treated plants were visually inspected and indicated that the control plants remaining in hydroponics showed turgescence throughout the experiment with LWC of both genotypes to be around or above 93%, while plants removed from hydroponics began to show wilting within 2 h. The first tissue collection was performed for 4 h where strong wilting in the sensitive genotype TTD-22 was observed. At this time point, the relative LWC of the tolerant genotype TR39477 was measured as 81% and that of the sensitive genotype TTD-22 as 77%. The second time point for tissue collection was selected at 8 h where complete leaf rolling was observed in both genotypes. The relative LWC of drought stressed TR39477 was measured as 78% and that of TTD-22 as 71%.

The initial proline content of leaf samples were approximately 0.07±0.01 µg/mg fresh weight in both genotypes. After 4 h of dehydration, no proline accumulation was detected in plants of the TR39477 genotype, while the proline concentration in TTD-22 increased slightly (0.09±0.01 µg/mg fresh weight). The accumulation of proline was detected in leaf tissues of both genotypes after 8 h of stress (TR39477, 0.11±0.03 and TTD-22, 0.27±0.02 µg/mg fresh weight).

Functional categorization of transcript profiles

The Affymetrix GeneChip® Wheat Genome Array contains 61,127 probe sets designed to target 55,052 wheat transcripts from *T. aestivum* (59,356 probe sets and 53,474 transcripts), *T. turgidum* (147 probe sets and 136 transcripts), *T. turgidum* ssp. *durum* (392 probe sets and 350 transcripts), *T. monococcum* (1,215 probe sets and 1085 transcripts), and *Aegilops tauschii* (five probe sets and four transcripts). Out of these more than 60,000 probes, 9,587 (15.7%) were found to be differentially expressed at least under one experimental condition ($p<0.01$ and DE<−3 or >3, Fig. 1, Supplementary Table S1). The Venn diagrams show the number of overlapping probes between genotypes (Fig. 2a), in leaf and root tissues (Fig. 2b), and at corresponding time points after the onset of shock-like dehydration (Fig. 2c). The results indicated that, although fewer than half of the differentially expressed probes are common in all cases, the transcriptome profiles were fairly specific to genotype, tissue, or time. Annotations of the stress responsive portion of the probes were performed in order to increase the number of meaningfully annotated transcripts (Supplementary Table S2).

Differentially expressed probes were functionally categorized into 33 groups according to the GO database (The Gene Ontology Consortium 2000). These included not only functionally well-defined categories, such as amino acid metabolism, cell growth and division, cellular organization, chaperone, defense, energy, hormone related, intracellular transport, lipid, general metabolism, nodulin, nucleotide, photosynthesis, protein kinase/phosphatase, protein synthesis, proteolysis, secondary metabolite, signal transduction, transcription factor/regulator and transporter, but also transcripts for proteins identified by shared structural domains [e.g., calcium binding, DNA/RNA binding domain, LEA related, leucine rich repeat protein, membrane binding and proline-rich], and by deduced functions like aquaporin, miRNA pathway, oxidoreductase, and retrotransposon. Probes lacking significant homology to any known genes or proteins with a cutoff *e* value less than 0.00001 were kept as "no hits", and those transcript probes with homologies to hypothetical or unknown proteins were grouped in the category "unclassified". The functional classifications of the stress responsive probe sets with significant differences between the two contrasting genotypes are given in Fig. 3. The results further supported transcript profiles and differences between the lines that showed genotype-specific, tissue-centered, and time-dependent changes in the response to drought.

Global comparison of transcriptome profiles upon quick dehydration

Detailed analyses of differentially expressed probes were performed to identify similarities and differences between genotypes with phenotypically known contrasting responses to drought conditions (TR39477, tolerant; TTD-22, sensitive).

Probes that were found to be differentially expressed in the two contrasting genotypes at both time points (394 probes from leaf and 926 probes from root; Supplementary Table S3) were subjected to average linkage clustering (Eisen et al. 1998), and the treeview results are given in Fig. 4. The linkage of the differentially expressed probes from the leaves of two contrasting genotypes showed that the regulations of transcriptomes with respect to the time the plants were subjected to shock-like dehydration were clustered together. In contrast, linkage of responses in root tissues was dependent on the genotype (Fig. 4). Accordingly, transcriptome changes in root tissues of the tolerant genotype subjected to water deficit clustered separately from those of the sensitive genotype, irrespective of the time of treatment.

The treeview analysis of the clustering (Eisen et al. 1998) for the top ten most highly up- and down-regulated probes is given in Fig. 5. It is noted that different probes designed for hybridization to the same gene or its isoforms (such as cold-regulated protein homologs and putative lipases) changed in an identical or very similar manner in magnitude upon quickly imposed dehydration (Fig. 5, Supplementary Table S1). However, a few probes designed to hybridize to the same transcript, such as germin-like

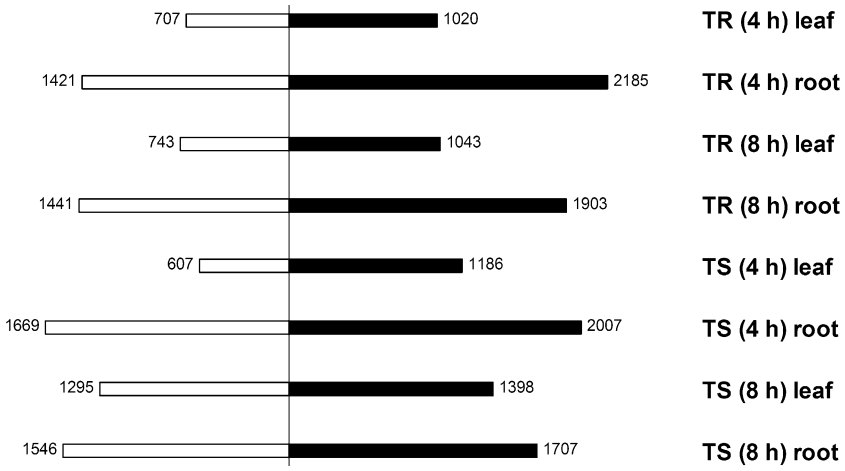

Fig. 1 Number of shock-like dehydration responsive genes in TR39477 and TTD-22. Listed are the number of probes differentially regulated in leaf and root tissues after 4 and 8 h of stress treatment. Up-regulated probes are represented in *black boxes*, whereas down-regulated probes in *white boxes*. *TR* tolerant genotype TR39477, *TS* sensitive genotype TTD-22

protein 1 precursors, showed significant differences in their hybridization pattern (Fig. 5, Supplementary Table S1). While technical difficulties cannot be ruled out, it appears more likely that polymorphisms in different part of a transcript sequence or alternative splicing were the cause of the discrepancy in hybridization intensities, considering that the target RNA derived from wild emmer wheat, whereas the Affymetrix GeneChip® Wheat Genome Array predominantly includes probes designed for transcripts from hexaploid wheat. However, although hybridization intensities may change, the trend of up- or down-regulation was generally constant for probes designed for the same transcript.

A 9-cis-epoxycarotenoid dioxygenase homolog involved in carotenoid biosynthesis, a putative ATP-binding protein, several Bowman–Birk type proteinase inhibitors, glutamine-dependent asparagine synthetase, homeodomain transcription factor Hox22 homolog, well-known drought stress accumulated LEA group 3 proteins, and proline-rich protein precursor were among the probes with highest up-regulation in leaf tissues of both genotypes at both time points (Fig. 5). A number of probes, such as basic region leucine zipper (bZIP) transcription factor family protein homologs, cysteine proteinase precursors responsible from protein degradation, germin-like protein precursors, lesion-

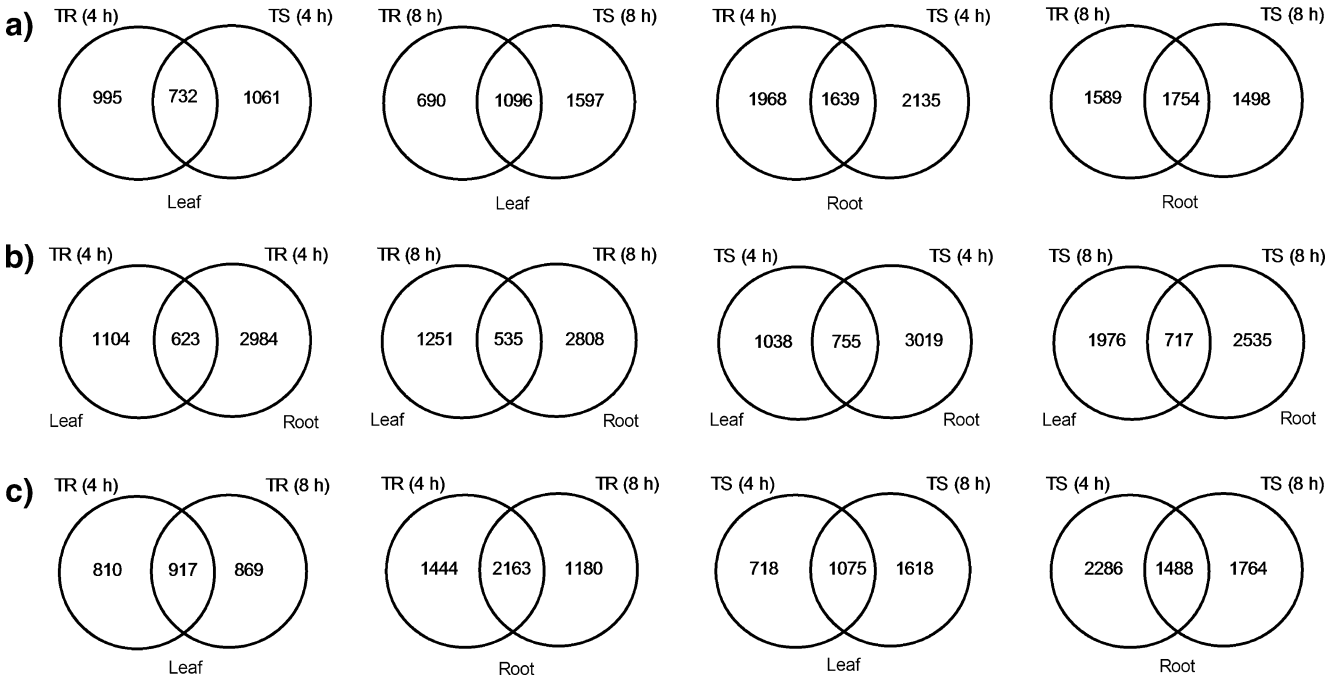

Fig. 2 Comparison of differentially expressed probes with respect to genotype, tissue and time. Venn diagrams showing the number of overlapping and specific genes with significant ($p<0.01$) expressional regulation (DE <−3 or >3). Comparisons are performed specifically between **a** genotypes, **b** tissues, and **c** time of shock treatment. *TR* tolerant genotype TR39477, *TS* sensitive genotype TTD-22

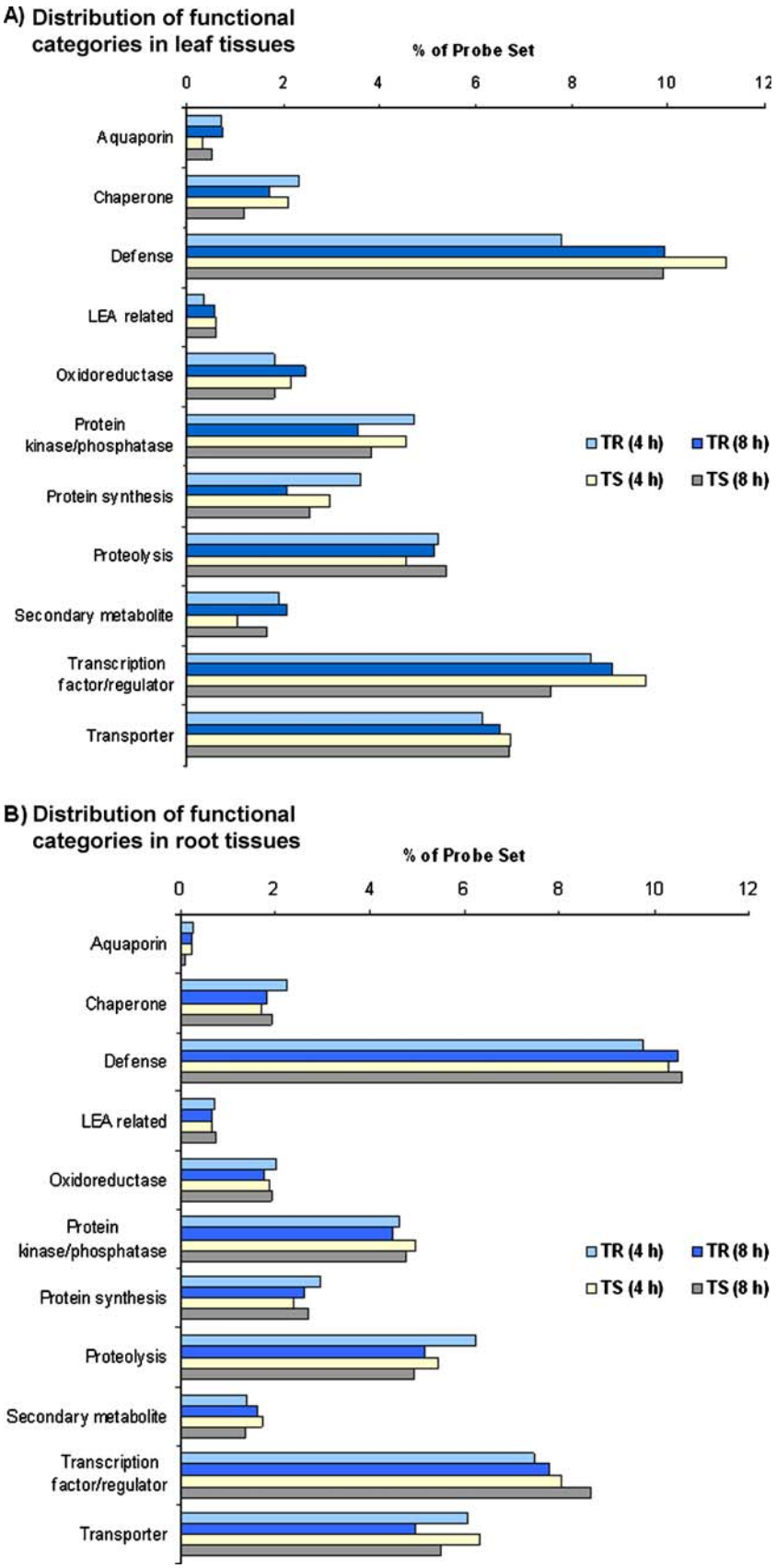

Fig. 3 Percentages of functional categories of shock-like dehydration responsive probes in leaf (**a**) and root (**b**) tissues. The categories "no hit" and "unclassified" and a number of categories with no significant differences between the two genotypes at both time points were not included to the graphs. *TR* tolerant genotype TR39477, *TS* sensitive genotype TTD-22

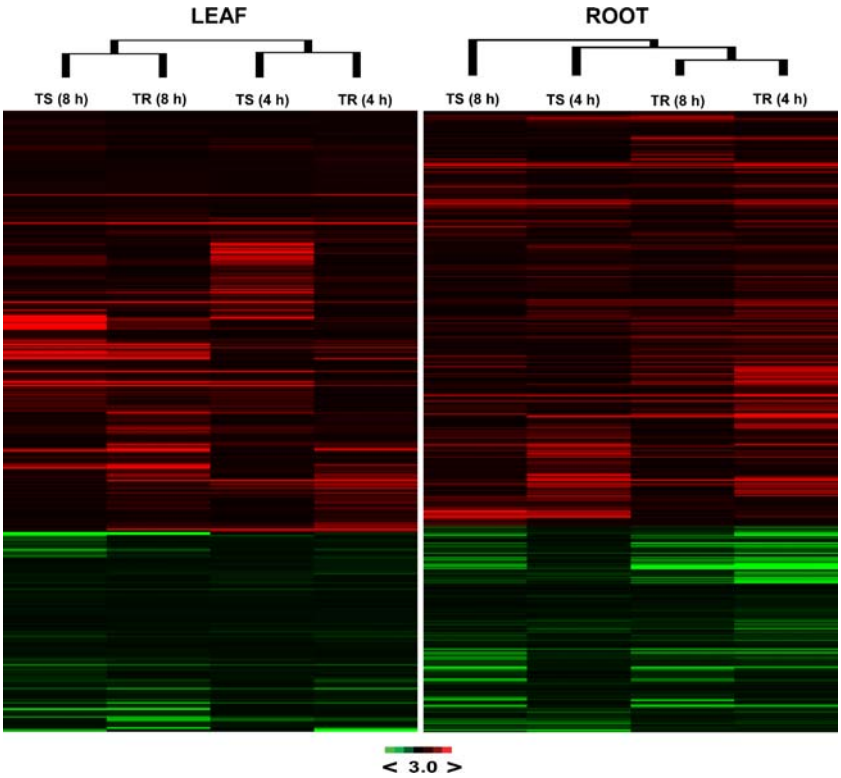

Fig. 4 Cluster analysis of probes with differential regulation at both time points in the contrasting genotypes. List of probes used in cluster analysis are given in Supplementary Table S3. The color saturation reflects the fold change, where *green* is for more than threefold down-regulated and *red* is for more than threefold up-regulated probes with $p<0.01$. *TR* tolerant genotype TR39477, *TS* sensitive genotype TTD-22

stimulating disease one like possible zing finger binding protein (LOL1), a two-component system signal receiver domain bearing rice type-A response regulator OsRR9 homolog, a putative cytochrome P450, and G/C rich motif binding Myb-like transcription factor RADIALIS, were among the significantly down-regulated probes in leaf tissues of the two contrasting phenotypes (Fig. 5).

In root tissues, flavin-dependent 12-oxophytodienoic acid reductase, a number of cold-regulated proteins, cold shock protein A-2 and ABA-inducible cold responsive LEA/RAB-related COR protein WRAB1, several dehydrins, a glutathione transferase functioning in ROS scavenging, pathogen-related protein homologs, putative lipases, a Rab GTPase homolog, an actin depolymerization factor WCOR719, and Myb-domain transcription factor Hv1 were among the probes with highest up-regulation in the two contrasting genotypes (Fig. 5). Several HSPs, plant-specific B12D proteins with unknown function, RmlC-type cupin domain containing proteins, two putative early nodulin 93 proteins, GTP-binding EF hand family protein, RING-H2 finger protein ATL2B responsible for protein–protein interaction and wound-responsive family proteins were strongly down-regulated in root tissues of genotypes with distinct responses to water deficit (Fig. 5).

The comparison of probes with similar up- or down-regulation independent of genotype, tissue, or time of treatment showed that only a very small number of transcripts were inversely regulated. A linalool synthase and terpene synthase 6, enzymes involved in terpene synthesis, were inversely regulated in leaf tissues of the two contrasting genotypes (Supplementary Table S4a, b). A photosynthesis-related gene and two transcriptional regulators (a CCH-type zing finger protein and an EREBP-like protein) were down-regulated in leaf tissues of the tolerant genotype whereas up-regulated in that of the sensitive (Supplementary Table S4a, b). Inverse differential regulation was much more pronounced in root tissues, especially 4 h after drought stress (Supplementary Table S4c). Several major intrinsic proteins, putative, or known aquaporins were up-regulated after 4 h of drought in the tolerant genotype TR39477. Four nicotianamine synthase isoforms, an enzyme responsible for the production of nicotianamine necessary for the synthesis of iron-chelating mugineic acid family generating protein family (phytosiderophore synthesis), were inversely regulated in the two contrasting genotypes. Expression of two cytochrome P450 homologs, a myosin-like protein XIG homolog and a phoshatidylglycerol specific phospholipase C, all of which belong to a large family of often observed proteins with differential responses to stress conditions, showed opposite differential expression between the two genotypes. Cell wall modification-related enzymes, an ethylene-induced esterase and a xylanase inhibitor precursor, were specifically induced in drought stress-sensitive genotype. Interestingly, a calmodulin-binding mildew resistance gene Mlo3 homolog and two putative nematode-resistance proteins were up-

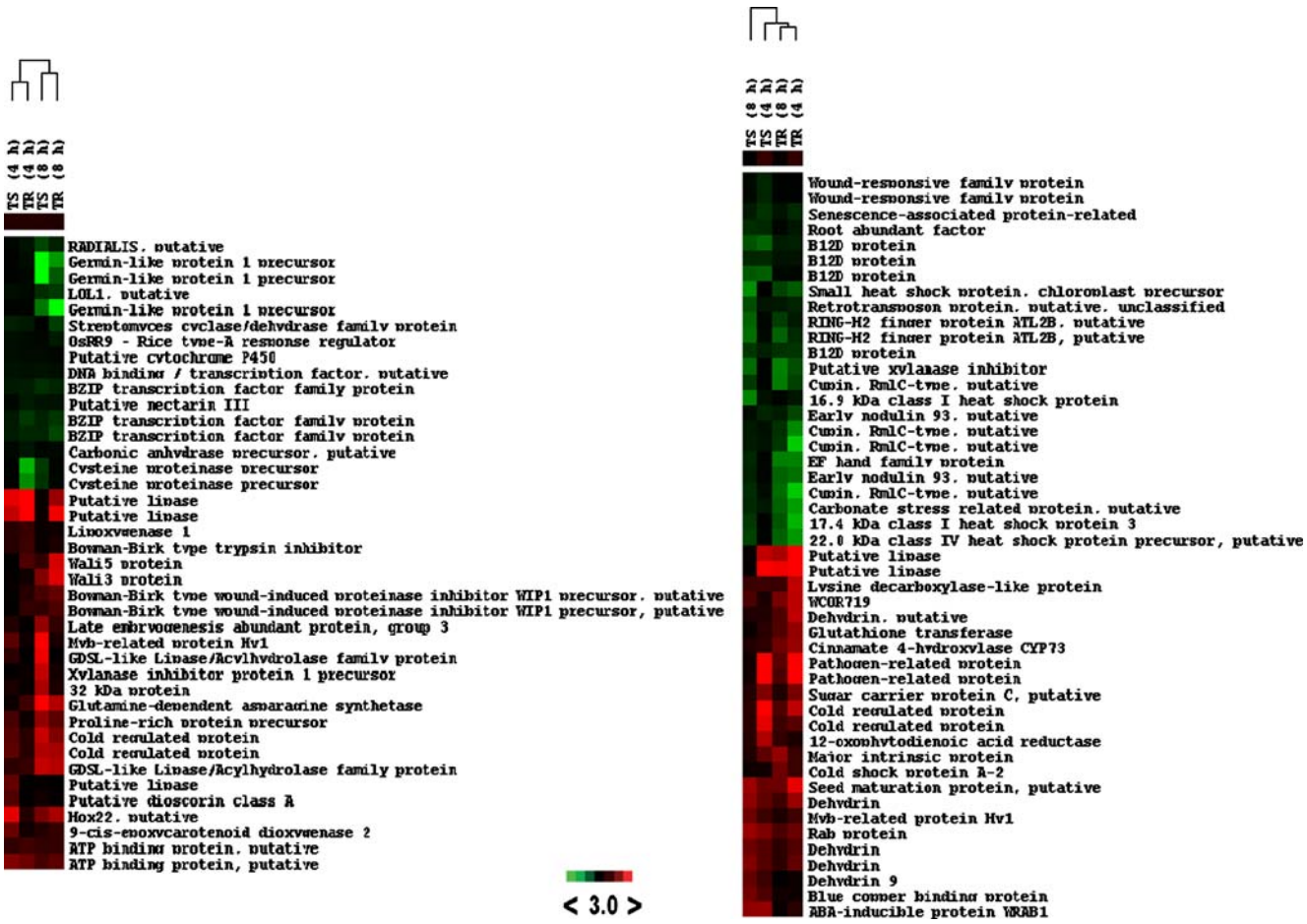

Fig. 5 Cluster analysis of highly up- and down-regulated probes at both time points in leaf (*left panel*) and root (*right panel*) tissues. Most highly up- and down-regulated 30 probes were used and unknown or hypothetical proteins were excluded from cluster analysis. The color saturation reflects the fold change, where *green* is for more than threefold down-regulated and *red* is for more than threefold up-regulated probes with $p<0.01$. *TR* tolerant genotype TR39477, *TS* sensitive genotype TTD-22

regulated after 4 h of drought stress in the sensitive genotype TTD-22 while strongly repressed in the tolerant TR39477. Only a few probes were oppositely regulated in root tissues after 8 h of water deficit (Supplementary Table S4d), a putative nematode resistance protein, an iron-phytosiderophore transporter, glutathione transferase and an enzyme involved in thiamine biosynthesis were up-regulated only in the tolerant, while a single probe, a putative bacterial-induced peroxidase, was induced in the sensitive genotype.

Comparisons of cellular metabolism through glycolysis, the pentose phosphate cycle, and lignin biosynthesis are given in Fig. 6. The results showed that, although some of the enzymes in glycolysis (such as phosphofructokinase, fructose-6-phosphate-2-kinase, and cytosolic triose phosphate) were regulated similarly in both genotypes, in all tissues, and at all time points, others like hexokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase revealed both genotype and tissue-specificity and time-dependent regulation of biochemical pathways in response to drought stress (Fig. 6a). The unique metabolic regulation was obvious in enzymes with differential regulation in pentose phosphate cycle (Fig. 6b), where 6-phosphogluconate deyhdrogenase expression was induced in root tissues at all times, transaldolase was up-regulated only in leaf tissues at both time points, and D-ribulose-5-phosphate 3-epimerase was down-regulated in leaf tissues after 8 h exposure to drought. All differentially expressed components of a drought responsive pathway, lignin biosynthesis, were up-regulated in contrasting phenotypes (Fig. 6c), implying that wild wheat is capable of inducing known drought stress responsive mechanisms similar to other plants.

Comparison of differentially expressed probes in the absence of stress

The comparison of transcriptomes of drought tolerant and sensitive wild emmer wheat genotypes revealed a number of genotype-specific and stress-responsive probe sets expressed in the absence of stress. Out of over 60,000 probe

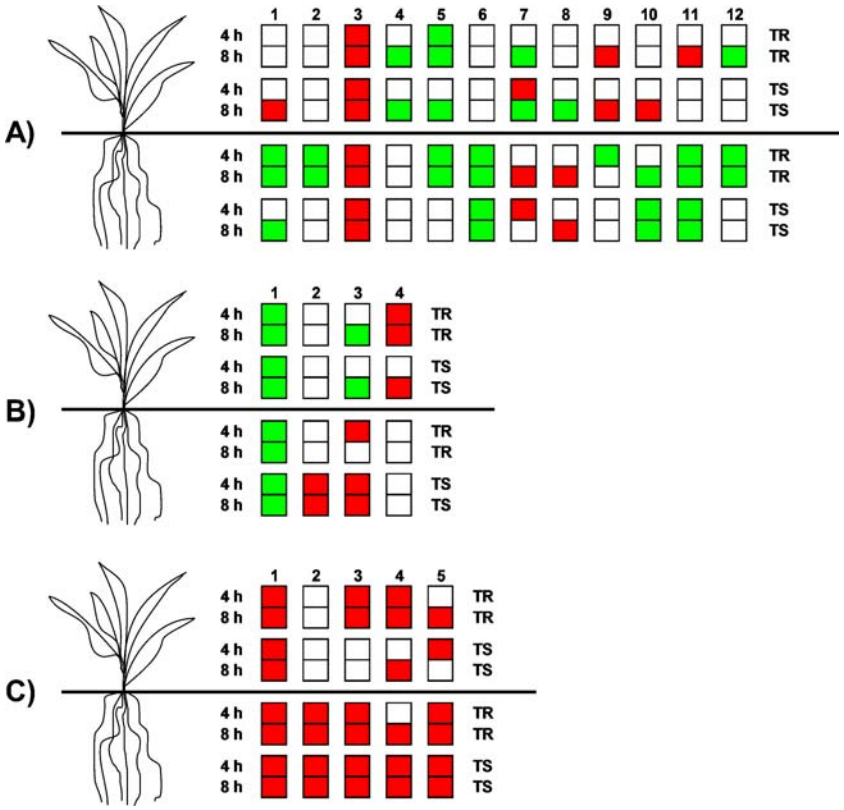

Fig. 6 Expressional changes in enzymes involved in glycolysis (**a**), pentose phosphate (**b**), and lignin biosynthesis (**c**). For each metabolic pathway, only enzymes found in differentially expressed probe lists are included. Regulation changes represent common behavior of probes coding for the same enzyme. Up-regulation of an enzyme is highlighted with a *red box* and down-regulation with *green box*. Empty cells indicate no significant differential regulation. *TR* tolerant genotype TR39477, *TS* sensitive genotype TTD-22. **a** *1* Hexokinase, *2* glucose-6-phosphate isomerase, *3* phosphofructokinase, *4* fructose-6-phosphate-2-kinase, *5* fructose 1,6-biphosphate aldolase, *6* cytosolic triosephosphate isomerase, *7* glyceraldehyde-3-phosphate dehydrogenase, *8* phosphoglycerate kinase, *9* phosphoglycerate mutase, *10* enolase, *11* pyruvate kinase, *12* phosphoenolpyruvate carboxylase. **b** *1* Phosphogluconolactonase, *2* 6-phosphogluconate dehydrogenase, *3* D-ribulose-5-phosphate 3-epimerase, *4* transaldolase. **c** 1, 1-phenylalanine ammonia lyase, *2* cinnamate 4-hydroxylase, *3* 4-coumarate CoA ligase, *4* caffeoyl-CoA *O*-methyltransferase, *5* cinnamoyl CoA reductase

sets present at the Affymetrix GeneChip® Wheat Genome Array, a very small number (261 probes for TR and 672 for TS leaf tissues, 330 for TR and 755 for TS root tissues) were found to be differentially expressed both at the control and dehydrated tissues (Supplementary Table S4). Distribution of functional categories indicated that 10% of the differentially expressed probe sets in the presence or absence of stress belongs to defense-related functions (Fig. 7).

The detailed analysis of the differential expression of the defense-related probe sets in the leaf tissues revealed that the drought- tolerant genotype TR39477 specifically expressed several cold acclimation proteins (such WCOR80, WCOR518, and WCOR825) and a universal stress protein family homolog even in the absence of stress (Supplementary Table S5a). In the case of the sensitive genotype TTD-22, a number of pathogenesis-related proteins (WIR1B, pathogenesis-related protein 1a, pathogen-related protein, rust-resistance protein Lr21, GRAB1, and GRAB2) were found to be expressed under control conditions (Supplementary Table S5a).

The expressions of stress-related proteins in the non-treated root tissues were much more diverse than that of the leaf tissues (Supplementary Table S5b). The homologs of several cold acclimation proteins and pathogenesis-related proteins were observed in root tissues of the tolerant genotype TR39477. The genotype-specific expression of defense-related proteins in the drought-sensitive genotype TTD-22 revealed the higher transcription of probes homologous to wound-induced, pathogenesis- and cold-regulated proteins. Interestingly, stress-responsive and ABA-induced proteins, such as RD22 and HVA22, were found to be specifically expressed in the root tissues of this genotype, along with a high osmolarity sensitivity protein 3 (Supplementary Table S5b).

Quantitative reverse transcription polymerase chain reaction

Quantitative real-time PCR was performed on selected probes (linalool synthase, nicotianamine synthase 7, allene oxide synthase, and LEA protein, group 3) to confirm

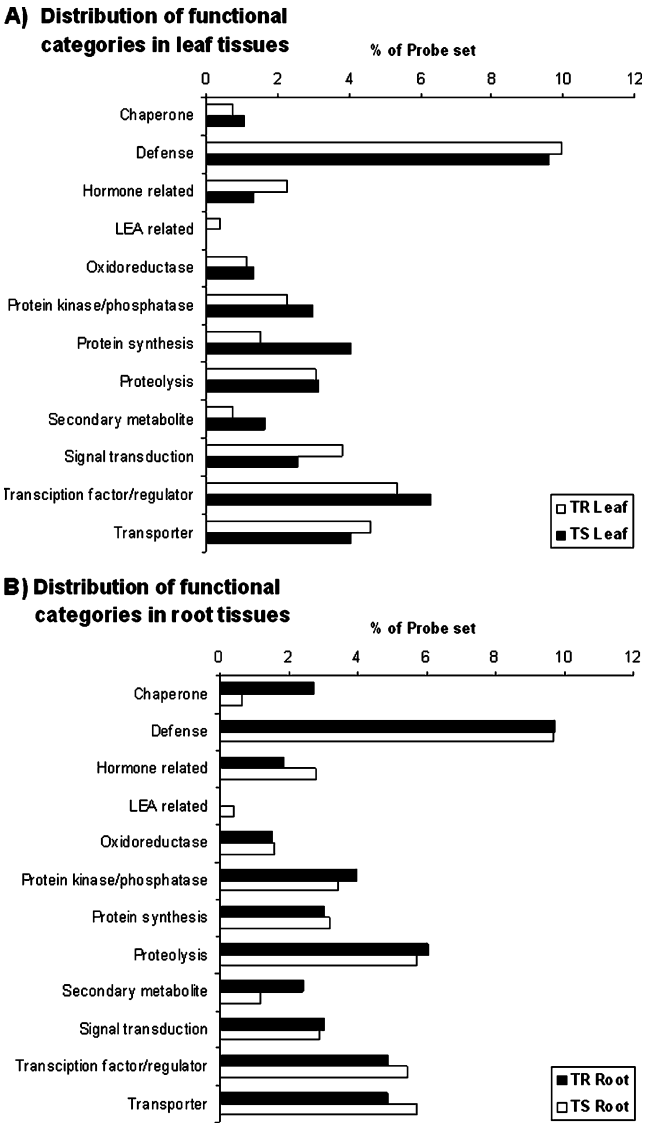

Fig. 7 Percentages of functional categories of stress-responsive probes expressed in the absence of stress in leaf (**a**) and root (**b**) tissues. The categories "no hit" and "unclassified" and a number of categories with no significant differences between the two genotypes were not included in the graphs. *TR* tolerant genotype TR39477, *TS* sensitive genotype TTD-22

changes in the expression levels determined by the Affymetrix GeneChip® Wheat Genome Array hybridization. The quantification using actin (GenBank accession AY663392) as internal reference (Muller et al. 2002) showed that microarray and qRT-PCR results were in good agreement with respect to trends of regulation (Fig. 8).

Discussion

As part of the current study, approximately 200 wild emmer wheat genotypes have been compared based on their physiological and agronomical responses to drought followed by re-watering. From this collection of local wild emmer wheat lines, additional screening has been carried out using a slow drought stress regime (Ergen and Budak 2009). Based on their physiological and morphological responses, one tolerant (TR39477) and one sensitive (TTD-22) genotype were selected for transcriptome analysis of changes in expression profiles upon shock-like dehydration using the Affymetrix GeneChip® Wheat Genome Array. The choice of shock-like stress was based on two previous studies, which indicated that the dynamics of drought stress affect the number of transcripts and the level of fold-changes, and validated the use of the shock treatment (Ozturk et al. 2002; Talame et al. 2007). It was suggested that despite certain differences, a shock-like dehydration treatment could provide an easier, fast, and reliable way to detect alleles involved in drought response (Talame et al. 2007). In physiological terms, a change in relative LWC of the contrasting genotypes indicated their different susceptibility to water scarcity, in which LWC of TR39477 decreased to 81% after 4 h and 78% after 8 h, whereas that of TTD-22 showed a decline from 77% to 71%. This change in LWC, in the tolerant wild emmer wheat genotype (TR39477), indicated moderate drought stress tolerance after 8 h of water deficit where the sensitive genotype (TTD-22) was more severely affected when compared with results from studies on hexaploid wheat (Xue et al. 2008).

Proline is not significantly accumulated in the tolerant wild emmer wheat

Proline is an amino acid, which has often been described to accumulate upon dehydration. It is considered to function in osmotic adjustment, stability of subcellular structures, free radical scavenging, and as an energy sink or a signaling molecule (Kishor et al. 2005). It is generally accepted that there is a direct correlation between proline accumulation and a plant's ability to tolerate abiotic stresses (Seki et al. 2007). However, data obtained in this experiment were not in agreement with using proline accumulation as a reliable indicator for tolerance to drought stress in wild emmer wheat. The tolerant genotype TR39477 did not accumulate significant amounts of proline, although its LWC decreased to 78%, indicating that the plants experienced moderate water deficit, whereas proline content in the sensitive genotype TTD-22 increased threefold after 8 h of shock drought with a LWC decrease to 71%. Two recent reports comparing proline accumulation in hexaploid wheat have also showed similar responses upon salt (Poustini et al. 2007) and drought (Xue et al. 2008) stress treatments. In both cases, sensitive genotypes accumulated higher amounts of proline than tolerant plants compared at early stages of stress treatment.

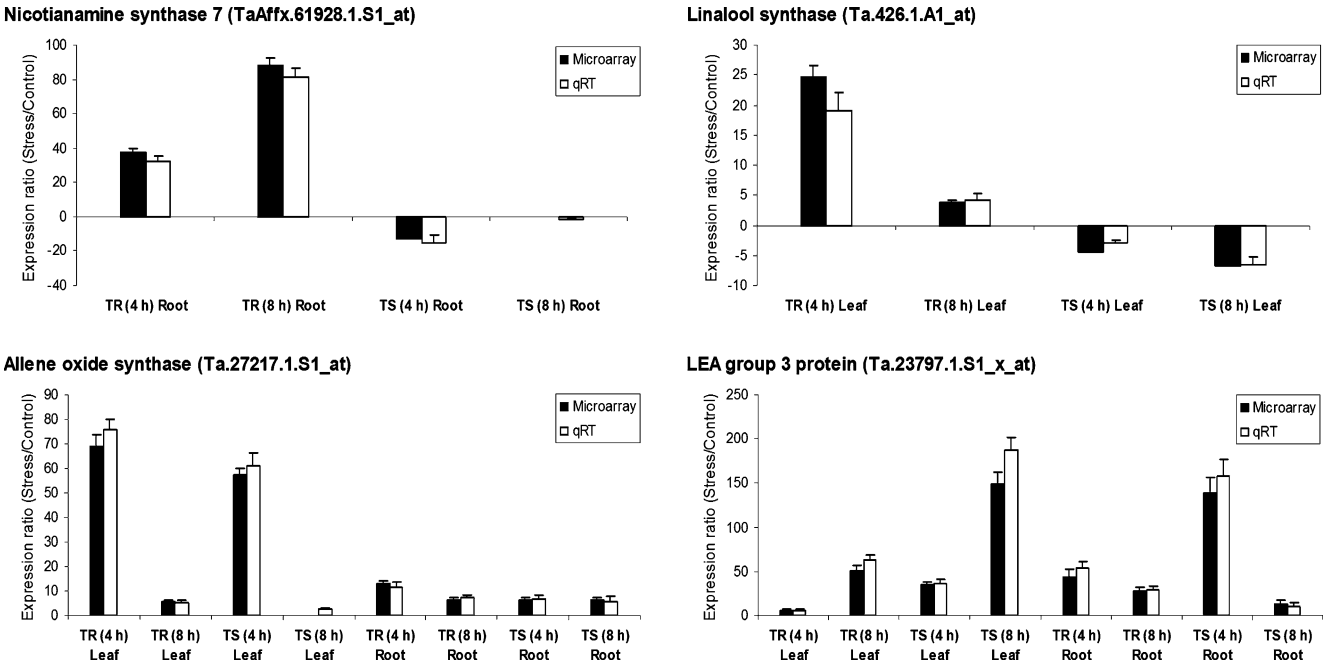

Fig. 8 Verification of differential expression by quantitative real-time PCR for nicotianamine synthase 7, linalool synthase, allene oxide synthase, and late embryogenesis abundant protein, group 3. *TR* tolerant genotype TR39477, *TS* sensitive genotype TTD-22

Proline is synthesized either from glutamate (delta 1-pyrroline-5-carboxylate synthetase and pyrroline-5-carboxylate reductase) or ornithine (ornithine aminotransferase), catabolized or oxidized by proline oxidase/dehydrogenase, and distributed throughout the plant by several proline transporters (Deuschle et al. 2001; Kishor et al. 2005). The comparison of fold changes of enzymes responsible from proline biosynthesis, degradation, or transport found to be significantly differentially expressed in response to shock dehydration stress showed similar regulation of enzymes related with proline synthesis between the two contrasting genotypes (Table 1). The differential up-regulation of a delta 1-pyrroline-5-carboxylate synthetase, a key enzyme in the synthesis of proline from glutamate, was approximately threefold higher in the leaf tissues of the tolerant genotype compared to that of the sensitive after 4 h of shock drought stress, whereas measurements did not detect any proline accumulation in the tolerant genotype at this time point. The ratio of fold changes of the same probe in leaf tissues after 8 h of drought stress showed approximately twofold increase in the sensitive genotype, which was relatively the ratio observed in free proline accumulation measured at this time point (2.5 times more proline in the sensitive genotype). The induction of putative ornithine aminotransferases in leaf tissues observed after 8 h of drought stress, with similar fold changes in both genotypes, and suggested that glutamate was the potential source for proline synthesis at the early stages of drought stress, and further proline accumulation was achieved by synthesis from ornithine.

Although the induction of enzymes in the proline synthesis pathway implied the potential of the tolerant genotype to accumulate this osmolyte in significantly higher than or comparable amounts with the sensitive genotype, the lack of accumulation could be related with oxidation of synthesized proline for utilization by mitochondrial energy production to compensate with decreased chlorophyll function, which could also be a reason for better adaptation of this genotype to lack of water. The probes available for proline oxidase/dehydrogenase homologs on the Affymetrix GeneChip® Wheat Genome Array were mostly down-regulated in leaf tissues while up-regulated in roots (Table 1); nevertheless, the homolog can be absent in the array to prove this hypothesis. The up-regulation of enzymes in proline synthesis and up-regulation of several proline transporters, with an emphasis on Ta.30603.1.S1_s_at, nicely coincide with the plant's response to drought stress (Table 1); therefore, the absence of proline accumulation or rather initiation of proline accumulation in later stages of stress response in the tolerant genotype could also be explained with prevention of accumulation of proline to high concentrations, which is known to be detrimental during recovery from stress (Deuschle et al. 2001).

Global comparison of differentially expressed probes indicates conserved responses

Cluster analyses of all differentially expressed probe sets were performed separately for leaf (Supplementary Fig. S1)

Table 1 Fold changes of enzymes in proline synthesis, degradation, or transport

		LEAF				ROOT			
Probeset ID	**Annotation**	**4 h TR**	**8 h TR**	**4 h TS**	**8 h TS**	**4 h TR**	**8 h TR**	**4 h TS**	**8 h TS**
Ta.7091.1.S1_at	Delta 1-pyrroline-5-carboxylate synthetase	**18.4**	**74.3**	**5.9**	**121.5**	**14.0**	**18.4**	**20.8**	**59.2**
Ta.591.1.S1_at	Pyrroline-5-carboxylate reductase		**3.5**		**4.5**				
Ta.12337.1.S1_at	Ornithine aminotransferase, putative	**3.8**	**10.8**		**8.7**	**4.0**			
Ta.21613.1.S1_at	Ornithine aminotransferase, putative		**3,7**		**4,6**				
Ta.28040.1.A1_at	Ornithine aminotransferase, putative		**3,1**		**3,6**				
Ta.3696.2.S1_at	Proline oxidase/dehydrogenase 2, putative				*-6,2*	**3,4**	**11,1**		
Ta.3696.2.S1_x_at	Proline oxidase/dehydrogenase 2, putative					**8,0**		**9,0**	
Ta.3696.3.S1_a_at	Proline dehydrogenase family protein		*-6,8*		*-6,3*	**15,2**	**4,5**	**3,7**	
Ta.7736.1.A1_at	Prolyl 4-hydroxylase alpha subunit-like protein							*-3,8*	
TaAffx.91995.1.A1_at	Prolyl 4-hydroxylase alpha subunit-like protein				*-3,6*			*-5,3*	
TaAffx.117566.1.S1_at	Putative prolyl 4-hydroxylase					*-12,0*	*-7,8*		
Ta.12049.1.A1_at	Prolyl 4-hydroxylase alpha subunit-like protein					*-3,0*		*-3,1*	
Ta.7736.1.A1_at	Prolyl 4-hydroxylase alpha subunit-like protein							*-3,8*	
Ta.12735.1.S1_at	Acetylglutamate kinase-like protein							**4,2**	
Ta.5321.1.S1_at	Putative acetylornithine aminotransferase							**3,7**	
TaAffx.82909.1.S1_at	Argininosuccinate synthase		**3,7**		**4,5**				
Ta.10058.1.A1_at	Proline transporter	**4.0**			**3.1**	**7.4**		**7.1**	**18.8**
TaAffx.93058.2.S1_at	Proline transporter					**27.1**	**10.2**	**7.0**	**11.9**
Ta.30603.1.S1_s_at	Proline transporter				**3.7**	**214.6**	**18.9**	**79.5**	**41.4**

The dataset contains probes with $p<0.01$ and DE<-3 or >3. Empty cells indicate no significant differential regulation. Given are fold changes calculated by ANOVA. All possible proline transporter isoforms are included in the table. No isoforms of other enzymes were found in shock-like dehydration responsive probes. Up-regulation, as fold change, is highlighted with bold characters, whereas down-regulation fold changes by italics. *TR* tolerant genotype TR39477, *TS* sensitive genotype TTD-22 (highlighted with red characters)

and root (Supplementary Fig. S2) tissues to compare the two wild emmer wheat genotypes with contrasting drought stress response. Treeview results indicated that transcriptome changes in leaf tissues resembled each other; however, the difference between the contrasting genotypes was clear on the root tissues (Fig. 4). The tolerant genotype showed similar differential changes at both time points (4 and 8 h), indicating that the stress response program in root tissues started earlier than the sensitive genotype and enhanced with the time after quick and severe stress treatment. The comparison of global expression profiling upon shock-like dehydration in wild emmer wheat genotypes with contrasting tolerance using the Affymetrix GeneChip® Wheat Genome Array strongly suggests that differences causing distinct stress response are more pronounced in root tissues.

The most highly up- and down-regulated probes in both genotypes indicated that abiotic stress response mechanisms known from other plant species are also functional in wild emmer wheat (Fig. 5). Several marker genes for osmotic stress such as LEA protein group 3 homolog, proline-rich protein homolog, glutathione transferase, and a number of dehydrin homologs were strongly up-regulated in both genotypes. The transcriptome dynamics showed that most of the ubiquitously observed responses to abiotic stresses, such as cytochrome P450 and several HSPs, and proteins involved in cell wall modification (germin-like proteins, xylanase inhibitors, cupins), in cytoskeleton organization (WCOR719), and in proteolytic cleavage (cysteine protease and proteinase inhibitors). The conserved response in the contrasting genotypes indicated that, rather than associated with tolerance, these proteins are more likely to be related with demonstration of stress damage and/or damage control.

Downstream elements analyses indicated similar and conserved regulation in wild emmer wheat genotypes. For instance, aquaporins are important elements of water balance both during normal growth and upon water stress. Their role in the drought stress response is not clear, and the many different aquaporins are known to respond to dehydration by up- or down-regulation or show no response at all (Kaldenhoff et al. 2008). Similar behavior characterized aquaporin homologs in wild emmer wheat genotypes (Supplementary Table S6), where some members were

strongly up-regulated in both genotypes, in both tissues, and at all times, whereas others were mostly down-regulated.

As an important antioxidant for scavenging of ROS, differential expression of glutathione-related genes were reported to be dependent on both genotype and intensity of stress (Mohammadi et al. 2007). Most glutathione-related genes in the two contrasting wild emmer wheat genotypes were induced upon shock drought stress but, nevertheless, showed clear differences depending on the genotype, tissue, and time after stress treatment (Supplementary Table S6). A close look at glutathione transferases suggests involvement of this antioxidant generator predominantly in the tolerant wild emmer genotype.

Accumulation of LEA family proteins and compatible solutes (such as glycine betaine) are commonly observed results of dehydration, as both are important for osmotic adjustment and possibly in preventing protein degradation (Wang et al. 2003; Rampino et al. 2006). Induction of probes of LEA proteins, the subfamily of dehydrins, and betaine aldehyde dehydrogenase involved in glycine betaine synthesis indicated the conserved role of these proteins in shock-like dehydration responses of wild emmer wheat (Supplementary Table S6). In accumulation of LEA family proteins, there appeared to be some preferences depending on the genotype: TaAffx.46097.2.S1_at and Ta.13255.1.S1_at were highly induced in the tolerant genotype TR39477, whereas Ta.23797.1.S1_x_at and Ta.2704.1.S1_at accumulated to higher amounts in the sensitive TTD-22. It is not possible to deduce any effect on selective accumulation of these proteins in contrasting genotypes, as the roles of LEA proteins in dehydration responses are not yet understood (Wang et al. 2003; Rampino et al. 2006). Accordingly, the results clearly demonstrated that both wild emmer wheat genotypes induced genes commonly associated with general drought stress response mechanisms in plants.

Comparison of wild emmer wheat response to those of other plants

Comparison of differentially regulated probe sets revealed only a small number of probes inversely regulated in response to drought stress, several of which appears to be unique to wild emmer wheat (Supplementary Table S4). The most pronounced inverse regulation in leaf tissues was a putative linalool synthase and a terpene synthase 6 homolog, both responsible for isoprene and monoterpene synthesis, and both up-regulated to different extents in leaf tissues of the tolerant genotype TR39477 but down-regulated in the sensitive genotype TTD-22 (Supplementary Table S4a). Interestingly, several nicotianamine synthase homologs were up-regulated in root tissues of the tolerant wild emmer wheat genotype TR39477 while repressed in the sensitive TTD-22, upon 4 h of shock drought stress (Supplementary Table S1b). Nicotianamine synthase is the key enzyme in the biosynthesis of the mugineic acid family of phytosiderophores, which are natural chelators secreted from roots to solubilize iron for efficient uptake (Higuchi et al. 1999, 2001). Although all higher plants are capable of secreting mugineic acids, the level of nicotianamine synthase activity was found to be highly correlated with secretion for iron acquisition among graminaceous plants (Higuchi et al. 2001). In wheat and barley, to our best of knowledge, there is no report with respect to induction of this iron acquisition. As the potential function of this enzyme in response to abiotic stresses, other than iron deficiency, has not been studied at all, the possible involvement of nicotianamine synthase in the drought tolerance of wild emmer wheat genotype TR39477 remains largely conjectural.

Tables 2 and 3 provide a comparison of the expressed sequence responses to water deficit/drought and indicates that some responses in wild emmer wheat may be missing in modern bread wheats. The data were compared with the affymetrix microarray results of hexaploid wheat, in which data has been uploaded into PLEXdb (www.plexdb.org, TA2 Wheat Genechip experiment).

The selective up-regulation of linalool synthase (Ta426.1.A1_at, gb:BQ172153) and terpene synthase 6 (TaAffyx.38062.1.A1_at, gb:BJ252458) in leaf tissues of tolerant wild emmer wheat genotype suggest a potential role of terpenes in drought tolerance (Table 2). Terpenes are widely produced by plants and carry out essential functions as hormones and agents of polysaccharide synthesis, pigments, electron carriers, and membrane components, and when emitted, they can act as mediators of biotic interactions with other organisms, as toxic defense compounds and as anti-pollinators (Cseke et al. 1998). The up-regulation of several nicotianamine synthase homologs in response to dehydration has not been discussed in modern wheat varieties, emphasizing the unique responses of wild emmer wheats. In some instances, probes that up-regulated for 4 h but down-regulated for 8 h (or vice versa) in the present study are not yet sufficiently understood to postulate on their contribution the distinct drought stress responses in studied genotypes. It is evident however that the identification of these new drought stress genetic determinants needs further investigation in the wild emmer wheats.

The up-regulation of enzymes involved in isoprene and monoterpene synthesis (i.e., Ta.25053.1.S1_at, gb:AF442967.1 and gb:CK157996, Ta.6065.1.A1_x_at) in modern wheat varieties upon abiotic stresses has not been reported or discussed in the literature (NC, Table 2). Drought experiments measuring terpene emission in *Ros-*

Table 2 The regulation of genes detected in responses to water deficit/drought in emmer wheat

GeneBank	Probe ID	Annotation	This Study	PLEX Wheat Chip Data
gb:AF442967.1	Ta.25053.1.S1_at	Thaumatin-like protein TLP5	Up-regulated[a]	NC
gb:CK157996	Ta.6065.1.A1_x_at	CCCH-type zinc finger protein, putative	Down-regulated[a]	NC
gb:CA615555	Ta.24453.1.S1_at	Germin protein type 1, putative	Up-regulated[a]	NC
gb:CA643445	Ta.21042.1.S1_s_at	Tonoplast intrinsic protein 1	Up-regulated[a]	NC
gb:CD452716	Ta.19010.1.S1_at	Cytochrome P450, putative	Up-regulated[a]	NC
gb:CK207522	Ta.999.1.S1_at	3′-N-debenzoyltaxol N-benzoyltransferase, putative	Up-regulated[a]	NC
gb:CD915559	Ta.5805.1.S1_at	Cytosolic aldehyde dehydrogenase RF2D	Up-regulated[a]	NC
gb:CD37411	Ta.9143.1.S1_at	Aquaporin NIP5.1, putative	Up-regulated[a]	NC
gb:CA638492	Ta.18537.1.S1_at	ATP binding protein, putative	Up-regulated[a]	NC
gb:CA674419	Ta.2641.2.S1_a_at	NADH dependent glutamate synthase precursor	Down-regulated[a]	NC
gb:BE604247	Ta.516.1.S1_s_at	Nematode-resistance protein, putative	Down-regulated[a]	NC
gb:CA662086	Ta.21353.1.S1_a_at	Ethylene-induced esterase, putative	Down-regulated[a]	NC
gb:CA733223	TaAffx.78376.1.S1_at	Cytochrome P450, putative	down-regulated[a]	NC
gb:BJ284338	Ta.24121.1.S1_x_at	EF hand family protein	Down-regulated[a]	NC
gb:BE404653	Ta.116.1.S1_at	XIG	Down-regulated[a]	NC
gb:CA615901	TaAffx.113315.1.S1_at	Iron-phytosiderophore transporter	Up-regulated[a]	NC
gb:CK212980	Ta.27594.1.S1_at	Thiamine biosynthetic enzyme	Up-regulated[a]	NC
gb:CK194302	Ta.7091.1.S1_at	Delta 1-pyrroline-5-carboxylate synthetase	Up-regulated[a]	NC
gb:CA638052	Ta.12337.1.S1_at	Ornithine aminotransferase, putative	Down-regulated[a]	NC
gb:CA697121	Ta.21613.1.S1_at	Ornithine aminotransferase, putative	Down-regulated[a]	NC
gb:BJ234133	TaAffx.93058.2.S1_at	Proline transporter	Down-regulated[a]	NC
gb:AJ606017.1	Ta.4456.1.S1_at	Mitogen-activated protein kinase 8	Up-regulated[a]	NC
gb:BT008938.1	Ta.19020.1.S1_at	Mitogen-activated protein kinase kinase kinase 1, putative	Up-regulated[a]	NC
gb:CA646761	TaAffx.56682.1.S1_at	MAPKKK16, putative	Down-regulated[a]	NC
gb:CA742246	TaAffx.4427.1.S1_at	Basic helix-loop-helix (BHLH)-like protein	Up-regulated[a]	NC
gb:BQ166412	Ta.10723.1.A1_at	Putative bZIP transcription factor	Up-regulated[a]	NC
gb:BQ789086	Ta.14000.1.S1_at	Ethylene responsive element binding factor3	Up-regulated[a]	NC
gb:BJ286857	Ta.28539.1.A1_x_at	NAC domain transcription factor	Up-regulated[a]	NC
gb:BQ801563	TaAffx.122104.1.S1_at	NAC domain transcription factor	Down-regulated[a]	NC
gb:CA602588	Ta.5127.2.S1_a_at	Putative NAC domain protein NAC1	Up-regulated[a]	NC
gb:CA642180	Ta.961.2.S1_a_at	WRKY DNA binding domain containing protein	Down-regulated[a]	NC
gb:BI479196	Ta.2714.1.S1_x_at	OsWRKY16	Down-regulated[a]	NC
gb:BQ484139	Ta.25091.1.S1_at	Transcription factor OsWRKY99	Up-regulated[a]	NC
gb:BQ172153	Ta.426.1.A1_at	Linalool synthase, putative	Up-regulated[b]	Down-regulated
gb:AF442967.1	Ta.25053.1.S1_at	Thaumatin-like protein TLP5	Up-regulated[a]	NC
gb:CK157996	Ta.6065.1.A1_x_at	CCCH-type zinc finger protein, putative	Up-regulated[a]	NC
gb:BQ161967	Ta.23366.3.A1_at	Class III peroxidase 62 precursor	Down-regulated[b]	Up-regulated
gb:CK198845	Ta.10130.1.S1_at	EREBP-like protein, putative	Down-regulated[b]	Up-regulated
gb:BQ172153	Ta.426.1.A1_at	Linalool synthase, putative[b]	Up-regulated[b]	Down-regulated[b]

The data were compared with the affymetrix microarray results of hexaploid wheat that were uploaded into PLEXdb (www.plexdb.org, TA2 Wheat Genechip Experiment)

NC not changed or not reported.

[a] The gene expression regulation has been detected in this study but not detected in PLEXdb

[b] Gene expression regulation differs in this study and PLEXdb

Table 3 The comparison of differentially regulated genes detected in responses to water deficit/drought in emmer wheat to modern wheat

GenBank ID	Probe List	Annotation	This study	PLEX Wheat chip Data base
gb:CK163859	Ta.29307.1.A1sat	10-deacetylbaccatin III 10-O-acetyltransferase, putative	Up-regulated	Up-regulated
gb:BQ619994	TaAffx.122294.1.S1at	No apical meristem, putative	Up-regulated	Up-regulated
gb:CD884700	Ta.28750.1.S1_x_at	Photosystem II 10 kDa polypeptide, chloroplast precursor	Up-regulated	Up-regulated
gb:BJ252458	TaAffx.38062.1.A1at	Terpene synthase 6, putative	Up-regulated	Up-regulated
gb:CK157996	Ta.6065.1.A1xat	CCCH-type zinc finger protein, putative	Up-regulated	Up-regulated
gb:CA654024	TaAffx.61928.1.S1at	Nicotianamine synthase 7, putative	Up-regulated	Up-regulated
gb:CK211942	Ta.22831.1.S1at	Membrane protein, putative	Up-regulated	Up-regulated
gb:BJ286808	Ta.5145.3.S1xat	Nicotianamine synthase 2, putative	Up-regulated	Up-regulated
gb:BT009515.1	Ta.27142.1.S1at	MDR-like ABC transporter	Up-regulated	Up-regulated
gb:BJ281448	Ta.5549.2.A1at	Nicotianamine synthase 1	Up-regulated	Up-regulated
gb:CD454914	Ta.25293.1.A1at	Common plant regulatory factor 7, putative	Up-regulated	Up-regulated
gb:CD915560	Ta.28630.1.S1at	16.9 kDa class I heat shock protein	Up-regulated	Up-regulated
gb:CA695864	TaAffx.80586.1.S1sat	Nematode-resistance protein, putative	Down-regulated	Down-regulated
gb:CD453080	Ta.1897.1.S1xat	ZFP16-2	Down-regulated	Down-regulated
gb:BE430407	Ta.20434.2.S1xat	Xylanase inhibitor precursor	Up-regulated	Up-regulated
gb:CD373859	Ta.25774.1.S1at	Mlo3	Down-regulated	Down-regulated
gb:CA607753	Ta.20720.1.S1xat	Nematode-resistance protein, putative	Up-regulated	Up-regulated
gb:BQ804925	Ta.3418.2.S1xat	Glutathione transferase F5	Up-regulated	Up-regulated
gb:CA638066	Ta.18497.1.S1at	Bacterial-induced peroxidase, putative	Down-regulated	Down-regulated
gb:BF474041	Ta.28040.1.A1at	Ornithine aminotransferase, putative	Up-regulated	Up-regulated
gb:CA647018	Ta.3696.3.S1aat	Proline dehydrogenase family protein	Up-regulated	Up-regulated
gb:CA665054	TaAffx.82909.1.S1at	Argininosuccinate synthase	Down-regulated	Down-regulated
gb:CK213919	TaAffx.38461.1.A1at	Phospholipase C, putative	Up-regulated	Up-regulated
gb:BJ214989	Ta.13185.2.A1xat	Phospholipase C1, putative	Up-regulated	Up-regulated
gb:BQ169503	Ta.13277.1.A1at	BHLH protein-like	Up-regulated	Up-regulated
gb:BG906258	Ta.26797.1.A1at	Basic/helix-loop-helix 113, putative	Up-regulated	Up-regulated
gb:CK193816	Ta.5367.1.S1sat	NAC domain transcription factor	Up-regulated	Up-regulated
gb:CA707172	TaAffx.79863.1.S1sat	OsWRKY16	Down-regulated	Down-regulated

The data were compared with the affymetrix microarray results of hexaploid wheat that were uploaded into PLEXdb (www.plexdb.org, TA2 Wheat Genechip Experiment)

marinus officinalis, *Pinus halepensis*, *Citrus albidus*, and *Quercus cocifera* suggests the possible role of terpenes, especially monoterpenes, in the protection of leaves from harmful effects of drought, temperature, light, and ozone stresses; however, their exact function under drought stress conditions is largely unknown (Ormeno et al. 2007).

As indicated in Table 3, the expression profile of wheat in response to drought are similar to those of wild emmer wheat with respect to the induction of several transcription factor families such as basic/helix–loop–helix domain (bHLH), bZIP, and homeodomain-leucine zipper (HD-ZIP), carbohydrate metabolism, aquaporins, glutathione-related genes, and LEA family proteins reported earlier (Chao et al. 2006; Xue et al. 2008). These data indicate that, in the dehydration response explored in the present paper, wild emmer wheat is fully capable of engaging drought stress responsive pathways conserved among plants.

Signal transduction, signaling, and hormone-dependent regulation pathways are different in wild emmer wheat genotypes

Signal transduction pathways appeared to be engaged in the response to shock drought stress between the two contrasting wild emmer wheat genotypes: 1,4,5-triphosphate (IP_3) signaling and differentially regulated mitogen-activated protein kinase (MAPK) cascade elements (Table 4). The most obvious difference between the tolerant and sensitive genotypes was the regulation of phospholipase C, which is involved in IP_3 signaling, leading to the activation of tonoplast-bound calcium channels and changes in the

Table 4 Differentially regulated probes in phospholipid signaling and MAPK cascade

			LEAF				ROOT			
	Probeset ID	Annotation	4 h TR	8 h TR	4 h TS	8 h TS	4 h TR	8 h TR	4 h TS	8 h TS
Phospholipid										
	TaAffx.117121.1.S1_at	Phospholipase C, putative					3.0			
	TaAffx.38461.1.A1_at	Phospholipase C, putative		15.7		7.4				
	Ta.13185.2.A1_x_at	Phospholipase C1, putative					9.0	3.3		4.4
	Ta.8726.1.S1_at	Putative phosphatidylinositol-specific phospholipase C					3.1			
	TaAffx.105881.1.S1_at	Putative phosphatidylglycerol specific phospholipase C					9.1	3.3		
MAPK										
	Ta.29358.1.A1_at	Mitogen-activated protein kinase	3.2							
	Ta.6053.1.S1_at	Mitogen-activated protein kinase 12			3.3					
	TaAffx.3077.1.S1_at	Mitogen-activated protein kinase 7	6.0			6.1			6.1	
	Ta.4456.1.S1_at	Mitogen-activated protein kinase 8					12.3	3.9	3.2	3.3
	Ta.3238.1.A1_a_at	MAPKK-related protein kinase domain containing protein					8.5	5.4		
	Ta.19020.1.S1_at	Mitogen-activated protein kinase kinase kinase 1, putative		13.6	12.9	8.5			11.3	
	TaAffx.56682.1.S1_at	MAPKKK16, putative		5.7	7.0	10.9			5.4	

The dataset contains probes with $p<0.01$ and DE <−3 or >3. Empty cells indicate no significant differential regulation. Given are fold changes calculated by ANOVA

TR tolerant genotype TR39477, *TS* sensitive genotype TTD-22 (highlighted with red characters)

cytosolic concentration of calcium ions, which act as secondary messengers (Xiong et al. 2002; Kaur and Gupta 2005). Almost all possible probes for phospholipase C homologs appeared to be quickly up-regulated in root tissues of TR39477, the drought tolerant genotype, whereas induction in the sensitive genotype occurs only for two probes and at a much later time point, after 8 h of stress. In MAPK cascade elements, some probes were up-regulated only or in higher values in root or leaf tissues of the tolerant genotype after 4 h of dehydration. Even the comparison of two common components of signal transduction pathways in response to quick and severe water loss suggests that the tolerant wild emmer wheat TR39477 is able to sense the absence of water before losing too much of its water reservoir and quickly activates the signal transduction pathways for activation of downstream elements for survival and reproduction strategies.

Some transcriptional factors have unique responses to shock-like dehydration in the two contrasting genotypes, suggesting differences in hormone-dependent regulations. One of the main changes that occur after perception of water loss is the activation of proteins belonging to several transcription factor families for differential regulation of gene expression. bHLH (Toledo-Ortiz et al. 2003), bZIP (Rodriguez-Uribe and O'Connell 2006), ethylene responsive element binding (EREBP; Shen et al. 2003), HD-ZIP (Lee and Chun 1998), NAC (Olsen et al. 2005), and WRKY (Eulgem and Somssich 2007) proteins are examples of transcription factor families responsive to drought stress. Differential regulations of drought responsive elements belonging to these families are given in Table 5, where not only different families but homologs from the same family demonstrate unique expressional changes in response to water scarcity.

bHLH, NAC, and WRKY families of transcription factors are known to be involved in the induction of gene expression in response to several abiotic stressors (Ramanjulu and Bartels 2002; Olsen et al. 2005; Eulgem and Somssich 2007). Comparison of differential regulation of members of these families in the drought stress tolerant and sensitive wild emmer wheat genotypes did not show clear differences that can explain unique stress responses but indicated apparent induction of a number of homologs. The implication here is that their role in regulation of gene expression in wild emmer wheat genotypes in response to drought is similar to their known function in other species. Other families of transcription factors (bZIP, EREBP, and HD-ZIP), on the contrary, showed interesting differences between wild emmer wheat genotypes with distinct drought stress responses (Table 5). Members of the bZIP transcription factor family are known to be able to bind to ABA-responsive elements upstream of the transcription start site to induce gene expression (Ramanjulu and Bartels 2002; Rodriguez-Uribe and O'Connell 2006). In addition, both water stress and exogenous ABA treatment were shown to stimulate some HD-ZIP proteins (Lee and Chun 1998; Ramanjulu and Bartels 2002). bZIP and HD-ZIP family transcription factors were selectively induced in the tolerant

Table 5 Shock-like dehydration response of selected transcription factor families

			LEAF				ROOT			
	Probeset ID	Annotation	4 h TR	8 h TR	4 h TS	8 h TS	4 h TR	8 h TR	4 h TS	8 h TS
bHLH										
	Ta.13277.1.A1_at	BHLH protein-like		**13.4**		**6.5**	**8.2**	**6.3**	**8.9**	**10.1**
	Ta.24775.1.S1_s_at	BHLH protein family-like	**4.7**							
	Ta.14164.1.S1_s_at	BHLH family protein, putative				**5.5**				
	TaAffx.4427.1.S1_at	Basic helix-loop-helix (BHLH)-like protein		**6.7**	**66.9**	**47.0**	**14.1**	**8.3**	**13.4**	**8.1**
	Ta.25394.2.S1_a_at	BHLH transcription factor, putative	*-3.7*	*-3.9*						
	Ta.26797.1.A1_at	Basic/helix-loop-helix 113, putative	*-7.6*	*-12.8*	*-10.0*	*-17.5*				
bZIP										
	Ta.19597.1.S1_s_at	BZIP transcription factor					**3.7**			
	Ta.10723.1.A1_at	Putative bZIP transcription factor					**8.5**	**5.8**	**7.1**	**7.8**
	Ta.11513.1.S1_a_at	Putative bZIP transcription factor					**4.2**			**3.3**
	TaAffx.29225.1.S1_s_at	Putative bZIP transcription factor					**3.9**			
	Ta.29834.1.S1_at	Putative bZIP transcription factor ABI5			**4.7**		**3.6**			
EREBP										
	Ta.14000.1.S1_at	Ethylene responsive element binding factor3	**3.6**		**5.3**	**3.4**			**3.8**	
	Ta.5369.1.A1_s_at	Ethylene-responsive element binding factor							**3.2**	
	Ta.10130.1.S1_at	Putative EREBP-like protein	*-3.0*		**3.4**	**4.3**				
HD-ZIP										
	Ta.1808.1.A1_s_at	Class III HD-Zip protein 4					**4.3**			
	Ta.1940.2.S1_at	Class III HD-Zip protein 4					**3.8**			
NAC										
	Ta.28539.1.A1_x_at	NAC domain transcription factor		**5.4**	**5.8**	**3.9**			**21.9**	
	Ta.5367.1.S1_s_at	NAC domain transcription factor		**3.4**	**3.2**	**3.1**			**7.6**	
	TaAffx.122104.1.S1_at	NAC domain transcription factor		**3.0**	**4.8**	**7.7**			**9.5**	
	Ta.5127.2.S1_a_at	Putative NAC domain protein NAC1				**10.0**	**9.2**		**4.1**	
	Ta.18638.1.S1_at	Putative NAC domain protein NAC2				**5.8**			**4.2**	**4.3**
	Ta.8685.1.S1_at	Putative development regulation gene OsNAC4					**3.2**			**3.3**
	Ta.12286.1.A1_at	NAC domain protein NAC5, putative					**57.5**	**14.2**		
	Ta.16273.1.S1_at	OsNAC5 protein, putative	**4.4**	**3.2**					**26.4**	
	Ta.8928.1.S1_at	NAC domain-containing protein 18, putative					**5.8**			
	TaAffx.25555.1.S1_at	NAC domain-containing protein 68, putative					**4.5**			**3.5**
WRKY										
	Ta.4678.1.S1_at	WRKY transcription factor			**23.0**	**6.6**			**10.4**	
	Ta.29394.1.S1_at	Putative WRKY DNA-binding protein				**3.1**				
	Ta.961.2.S1_a_at	WRKY DNA binding domain containing protein		**3.6**	**3.3**	**5.8**			**4.1**	
	Ta.25475.1.S1_x_at	DNA-binding protein WRKY3	**4.5**						**3.9**	
	Ta.5641.1.A1_at	Putative WRKY transcription factor				**3.9**				
	Ta.26978.1.A1_at	OsWRKY16					**13.7**	**6.1**	**15.6**	**10.2**
	Ta.2714.1.S1_x_at	OsWRKY16					**4.9**	**6.1**	**12.7**	**5.5**
	TaAffx.79863.1.S1_s_at	OsWRKY16					**4.1**	**4.2**	**10.9**	**5.4**
	TaAffx.109191.1.S1_at	WRKY transcription factor 19		**5.0**			*-3.7*	**3.5**		
	TaAffx.99272.1.A1_at	WRKY21						**4.1**		**5.4**
	Ta.8482.1.A1_at	OsWRKY24			**3.5**	**3.6**			**5.3**	**10.4**
	TaAffx.23458.1.S1_at	WRKY transcription factor 28, putative			**10.0**				**10.8**	
	Ta.4944.1.S1_at	OsWRKY46							**3.2**	
	Ta.8614.1.S1_at	WRKY transcription factor 45			*-3.4*			**6.2**		
	TaAffx.64818.1.S1_at	WRKY transcription factor 48		**3.7**			**6.7**	**15.7**		
	TaAffx.54575.1.A1_at	WRKY transcription factor 51, putative	**4.6**							
	TaAffx.128663.1.S1_at	WRKY transcription factor 53		**4.8**						
	TaAffx.65806.1.A1_at	WRKY transcription factor 65		**5.2**						
	TaAffx.128870.1.S1_at	WRKY transcription factor 68	**4.5**							*-4.6*
	Ta.25091.1.S1_at	Transcription factor OsWRKY99	**3.7**		**3.0**		**7.9**	**8.3**	**4.9**	**7.8**
	Ta.5433.1.S1_a_at	Transcription factor OsWRKY99	**11.2**					**3.0**	**7.1**	**4.5**

The dataset contains probes with $p<0.01$ and DE <−3 or >3. Empty cells indicate no significant differential regulation. Given are fold changes calculated by ANOVA. Up-regulation, as fold change, is highlighted with bold characters, whereas down-regulation fold changes by italics

TR tolerant genotype TR39477, *TS* sensitive genotype TTD-22 (highlighted with red characters)

genotype TR39477, whereas induction of EREBP family members was confined almost exclusively to the sensitive genotype TTD-22 (Table 5). The observation suggested differences in hormone-regulated gene expression in wild emmer wheat genotypes.

Fold-changes of enzymes involved in ABA (9-cis-epoxycarotenoid dioxygenase and aldehyde oxidase), ethylene (ACC synthase and ACC oxidase), and jasmonate (allene oxide synthase, allene oxide cyclase, and 12-oxo-phytodienoic acid reductase) synthesis (Table 6) showed

Table 6 Regulation of hormone synthesis in response to quick and severe dehydration

			LEAF				ROOT			
	Probeset ID	Annotation	4 h TR	8 h TR	4 h TS	8 h TS	4 h TR	8 h TR	4 h TS	8 h TS
ABA										
	TaAffx.76007.1.S1_at	9-cis-epoxycarotenoid dioxygenase 2	**16.9**	**54.4**	**104.7**	**35.4**	**64.9**	**34.7**	**125.4**	**74.7**
	Ta.16216.1.S1_s_at	Aldehyde oxidase-2					**4.7**	**3.1**		
	Ta.21109.1.A1_at	Aldehyde oxidase-2					**10.7**	**10.8**		
	Ta.6172.3.A1_a_at	Putative aldehyde oxidase	**4.6**	**4.2**						
	TaAffx.97075.1.S1_at	Putative aldehyde oxidase-like protein								**3.5**
Ethylene										
	TaAffx.71241.1.A1_at	ACC synthase				**4.5**			**3.2**	**8.6**
	Ta.24076.1.S1_at	ACC synthase						**4.9**	**3.4**	**3.5**
	Ta.25755.1.A1_at	ACC synthase	**10.7**	**4.2**	**5.7**	**6.3**	**26.1**	**31.1**	**11.8**	**29.0**
	TaAffx.84150.1.S1_at	1-aminocyclopropane-1-carboxylate synthase 2b							**3.7**	
	Ta.20570.1.A1_at	1-aminocyclopropane-1-carboxylate oxidase					**4.7**	**3.7**		
	Ta.425.3.A1_at	1-aminocyclopropane-1-carboxylate oxidase				**7.9**			**16.9**	
	Ta.9107.2.S1_at	1-aminocyclopropane-1-carboxylate oxidase		**3.2**	**10.0**	**29.9**				
	TaAffx.100446.1.S1_at	1-aminocyclopropane-1-carboxylate oxidase	**4.5**	**3.8**	**21.6**	**42.4**				
Jasmonate										
	Ta.13362.1.S1_at	Allene oxide synthase	**606.3**	**14.7**	**104.7**		**98.8**	**7.3**	**52.0**	**20.1**
	Ta.27217.1.S1_at	Allene oxide synthase	**68.9**	**5.6**	**57.2**		**12.9**	**6.5**	**6.1**	**6.1**
	TaAffx.9452.1.S1_x_at	Allene oxide synthase, putative	**51.6**	**5.0**	**43.5**		**39.9**	**6.3**	**30.9**	**9.8**
	Ta.7703.1.S1_a_at	Allene oxide cyclase precursor	**11.1**	**3.8**	**7.4**		**8.5**	**3.2**	**6.2**	**3.4**
	Ta.30735.1.S1_at	12-oxo-phytodienoic acid reductase	**5.2**	**3.1**	**9.2**	**7.0**		**3.6**	**7.2**	
	Ta.5509.1.S1_at	12-oxo-phytodienoic acid reductase	**3.1**				**10.5**	**6.7**	**6.1**	**3.5**
	TaAffx.128684.1.S1_at	12-oxo-phytodienoic acid reductase	**10.0**	**3.7**	**4.8**	**3.3**	**16.9**	**69.1**	**56.1**	**18.9**
	Ta.1207.1.S1_at	12-oxophytodienoic acid reductase	**15.0**	**3.0**	**9.6**		**39.5**	**68.2**	**237.0**	**56.4**
	Ta.25473.1.S1_at	12-oxo-phytodienoic acid reductase							**25.1**	**6.4**

The dataset contains probes with $p<0.01$ and DE<−3 or >3. Empty cells indicate no significant differential regulation. Given are fold changes calculated by ANOVA

TR tolerant genotype TR39477, *TS* sensitive genotype TTD-22 (highlighted with red characters)

induction of these hormone biosynthesis pathways in both genotypes. While not expected, emphasis was on ABA production in root tissues of the tolerant genotype TR39477 and ethylene synthesis in roots of the sensitive genotype TTD-22, which explained differences observed in ABA- and ethylene-dependent transcription factors. Interestingly, a 9-cis-epoxycarotenoid dioxygenase probe (TaAffx.76007.1.S1_at) was significantly induced in both genotypes in all tissues at all times; however, aldehyde oxidase homologs, encoding a downstream component in ABA biosynthesis, were almost solely induced in TR39477, suggesting that the tolerant genotype was able to induce ABA synthesis enzymes faster than the sensitive genotype. This rapid synthesis of ABA, a long distance chemical signal in dehydration response, could give the tolerant genotype an advantage over the sensitive for stimulating growth under stress conditions and preservation of leaves, as ethylene synthesis, which was up-regulated in the sensitive genotype, is known to slow down or inhibit the growth (Munns et al. 2006).

Differential expression between the two contrasting genotypes in the control hybridizations, stress-responsive, and defense-related elements of the transcriptome were depicted in Fig. 7 (Supplementary Table S7). Specific induction of the leaf tissues of the tolerant genotype was characterized by the pre-stress transcription of several WCOR proteins (WCOR80, a dehydrin; WCOR518, a lipid-transport protein; and WCOR825, a hydrophilic, low-temperature-regulated protein) and that of the sensitive genotype TTD-22 was the up-regulation of a number of

pathogenesis-related probes. The comparison of genotype-specific induction of stress-related probes in the root tissues showed the transcription of several pathogenesis-related, wound-induced, and cold-responsive proteins in the absence of dehydration, indicating that both genotypes were prepared for abiotic and biotic stresses to certain extend. Surprisingly, the genotype TTD-22 significantly up-regulated homologs of dehydration-responsive RDD22-like protein in the absence of stress (Supplementary Table S5b), even though it was characterized by its less tolerance to water deficit in our previous slow drought experiments (Ergen and Budak 2009). This suggests that, apart from halophytes and xerophytes, which are naturally and metabolically adapted to extreme environmental conditions, genotypic differences leading to better tolerance to abiotic stress conditions might be more complex in plants usually growing in optimal or semi-optimal environment, but naturally armed or adapted to quickly respond to suboptimal growth conditions to ensure survival and reproduction.

In summary, mechanisms leading to developmental and physiological adjustments, defense, and possibly damage control, such as hormone-responsive transcription factors, aquaporins, ROS scavenging antioxidant generators, compatible solutes, and LEA proteins, were found to be similarly regulated in the two contrasting wild emmer wheat lines, resembling reports on the response to abiotic stresses in a wide range of plants from *Arabidopsis thaliana* (Bray 2004) to rice (Zhou et al. 2007) and grapevine (Cramer et al. 2007). The analysis of expression profiles of wheat in response to drought is mainly limited to *T. aestivum* and mostly to the high-throughput expressed sequence tag library sequencing or cDNA arrays; nevertheless, similar induction of several transcription factor families such as bHLH, bZIP, and HD-ZIP, carbohydrate metabolism, aquaporins, glutathione-related genes, and LEA family proteins were reported earlier (Chao et al. 2006; Mohammadi et al. 2007; Xue et al. 2008). The similar differential expression of several abiotic-stress-regulated elements indicate that wild emmer wheat is fully capable of engaging drought stress responsive pathways conserved among plants even in response to quickly imposed dehydration. In addition to this response, the identifying selective up-regulation of linalool synthase and terpene synthase 6 and several nicotianamine synthase homologs, differential usage of IP_3-dependent signal transduction pathways, and inverse differentially regulated transcripts regulated in opposite directions at the different time points indicate the unique responses of wild emmer wheats.

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