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Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley

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Abstract

Responses to drought and salinity in barley (*Hordeum vulgare* L. cv. Tokak) were monitored by microarray hybridization of 1463 DNA elements derived from cDNA libraries of 6 and 10 h drought-stressed plants. Functional identities indicated that many cDNAs in these libraries were associated with drought stress. About 38% of the transcripts were novel and functionally unknown. Hybridization experiments were analyzed for drought- and salinity-regulated sequences, with significant changes defined as a deviation from the control exceeding 2.5-fold. Responses of transcripts showed stress-dependent expression patterns and time courses. Nearly 15% of all transcripts were either up- or down-regulated under drought stress, while NaCl led to a change in 5% of the transcripts (24 h, 150 mM NaCl). Transcripts that showed significant up-regulation under drought stress are exemplified by jasmonate-responsive, metallothionein-like, late-embryogenesis-abundant (LEA) and ABA-responsive proteins. Most drastic down-regulation in a category was observed for photosynthesis-related functions. Up-regulation under both drought and salt stress was restricted to ESTs for metallothionein-like and LEA proteins, while increases in ubiquitin-related transcripts characterized salt stress. A number of functionally unknown transcripts from cDNA libraries of drought-stressed plants showed up-regulation by drought but down-regulation by salt stress, documenting how precisely transcript profiles report different growth conditions and environments.

Introduction

Abiotic stresses can severely impair plant growth and performance. Environmental factors, such as drought, extreme temperatures or high and fluctuating salinity, are also responsible for significant yield reductions in cultivated areas world-wide. Thus, the responses of plants to various stresses have for decades been the focus of physiological studies (Levitt, 1980) and, more recently, of molecular and reverse genetics studies and transgenic experimentation (Grover, 1999; Bajaj *et al.*, 2000; Forster *et al.,* 2000; Hasegawa *et al.*, 2000; Zhang *et al.*, 2000). A large and increasing number of genes, transcripts and proteins have been correlatively implicated in stress response pathways, while their precise functions in either tolerance or sensitivity often remain unclear (Bray, 1997). Rather than being indicators of stress resistance, many of these response components may be ancillary and irrelevant or may even be indicators of degenerative processes.

By necessity, the work followed a gene-by-gene or protein-after-protein process, which may now, as molecular genetic tools have improved, be replaced by global approaches to study gene and protein expression. We have begun to assemble transcript pop-

ulations to probe for abiotic (drought or salinity) stress-related transcript changes in microarray hybridizations. Microarray technology allows for the determination of transcript abundance for many or all transcripts in a genome by comparing control and experimental states. The RNAs from different treatments are distinguished by the incorporation of different fluorescent labels (Richmond and Somerville, 2000; Schuchardt *et al.*, 2000; Deyholos and Galbraith, 2001). With appropriate controls and repeat experiments, significant data are obtained. Statistical analysis of such microarray data has already established insights into a number of plant processes, such as seed development (Girke *et al.*, 2000), wounding responses (Reymond *et al.*, 2000), pathogen signaling (Maleck *et al.*, 2000; Schenk *et al.*, 2000), nutrient-dependent changes in expression profiles (Wang *et al.*, 2000), or salinity stress responses (Kawasaki *et al.*, 2001).

Barley (*Hordeum vulgare* L.) was chosen for monitoring the changes induced by water and salt stress in transcript profiles in comparison with control plants. Barley is a moderately drought- and salt-tolerant crop of great significance in countries around the Mediterranean basin. Several morpho-physiological and biochemical traits have been investigated in the attempt to improve barley yield under water-limited conditions (Avecedo, 1987; Grumet *et al.*, 1987; Blum, 1988; Matin, 1989; Sanguineti *et al.*, 1994; Ceccarelli and Grando, 1996; Borel *et al.*, 1997; Teulat *et al.*, 1997; Ceccarelli *et al.*, 1998). Among the most notable biochemical traits, accumulation of proline has received considerable attention, though contrasting conclusions have been reached concerning its role in the adaptive response to drought (Blum, 1988; Delauney and Verma, 1993). Extensive efforts have also been devoted to the characterization of genes induced or upregulated by drought, such as dehydrins (Close *et al.*, 1989, 1993). Correlative evidence suggests a possible role for a number of these drought-induced proteins in protecting cells from the harmful effects of dehydration (Bray, 1997; Close, 1997). For example, the up-regulation of a drought-induced barley gene (*HVA1*) improved tolerance to drought and salinity in rice grown under controlled conditions (Xu *et al.*, 1996). Encouraging as these results are, there is widespread scepticism that up-regulation of one or more genes encoding structural proteins may lead to meaningful results in terms of field tolerance to drought (Bajaj *et al.*, 2000; Bohnert and Bressan, 2001). A more promising strategy relies on genes encoding transcription factors (e.g., DREB-1A; Yamaguchi-Shinozaki and Shinozaki, 1994; Jaglo-Ottosen *et al.*, 1998; Smirnoff and Bryant, 1999), which lead to the induced expression of a suite of genes in response to drought. When the expression of *Arabidopsis DREB-1A* was regulated by a drought-inducible promoter in rice, resistance to drought under controlled conditions was significantly enhanced (Kasuga *et al.*, 1999). The presence of *DREB-1A* homologues in barley (e.g., *CBF1*, accession number AF298230; T.J. Close, personal communication) and maize (van Buuren *et al.*, 2002) has recently been reported. It will be important to resolve to what extent, if any, allelic variation in *DREB1* homologues affects drought tolerance in barley and other crops.

Genetic differences in tolerance to saline conditions have been reported among accessions of cultivated barley (Greenway, 1962; Richards *et al.*, 1987; Slavich *et al.*, 1990). A number of genes seem to affect Na^+ influx and concentration in cells (Schachtman and Liu, 1999). Although a Na^+ (net) uptake transporter has yet to be identified in higher plants, it seems that Na^+ enters cells through high-affinity K^+ carriers or low-affinity K^+ channels which are insufficiently selective for K^+ (Amtmann and Sanders, 1999). Recent evidence indicates that HKT-type transporters may be one of those default influx systems (Rus *et al.*, 2001). In contrast to Na^+ influx, more is known about genes responsible for the compartmentalization of Na^+ to the vacuole. This is accomplished by a tonoplast Na^+/H^+ antiporter, whose over-expression has been shown to increase salinity tolerance in *Arabidopsis thaliana* (Apse *et al.*, 1999). In all plants, tolerance to high salt concentrations relies on maintaining continued meristematic growth and sustained photosynthesis, which is made possible by control over the rate of salt accumulation and sequestering in leaves so that cytoplasmic concentrations remain below toxic levels (Greenway and Munns, 1980) while the supply of carbon to sinks is maintained (Munns, 1993). It has been argued that salt tolerance of barley may be a consequence of high growth rate associated with fast progression of phenological development and early flowering (Munns *et al.*, 2000).

Plant stress tolerance is governed by complex traits whose expression is subject to 'genotype × environment' interactions (G × E; Powell *et al.*, 1986; Conti *et al.*, 1994; Tuberosa *et al.*, 1998). This, in turn, suggests that monitoring changes in transcript profiles by microarray hybridizations may allow for the identification of networks of transcripts accounting for G × E

Table 1. Transcript profiles of drought-stressed barley (*H. vulgare* L. cv. Tokak). The category 'no hit' includes ESTs for which no homology in the databases was detected in BLAST searches. Unclassified proteins denote ESTs with homology to unknown, hypothetical or putative ESTs with unknown functions in other organisms.

Major functional categories	Leaf		Root		Total	
	n	%	*n*	%	*n*	%
Cell growth and division	14	2.8	49	5.1	63	4.3
Cell rescue, defense and aging	61	12.0	74	7.7	135	9.2
Cellular biogenesis	0	0.0	3	0.3	3	0.2
Cellular organization	8	1.6	3	0.3	11	0.8
Energy	20	3.9	38	4.0	58	4.0
Intracellular transport	3	0.6	14	1.5	17	1.2
Ion homeostasis	18	3.6	13	1.4	31	2.1
Metabolism	86	17.0	123	12.9	209	14.3
'No hit'	38	7.5	101	10.6	139	9.5
Photosynthesis	14	2.8	0	0.0	14	1.0
Protein destination	46	9.1	75	7.8	121	8.3
Protein synthesis	17	3.4	37	3.9	54	3.7
Signal transduction	18	3.6	59	6.2	77	5.3
Transcription	7	1.4	23	2.4	30	2.1
Transport facilitation	41	8.1	58	6.1	99	6.8
Unclassified proteins	116	22.9	286	29.9	402	27.5
Total	507	100	956	100	1463	100

interactions in different genotyes. We used a relatively drought-tolerant line of barley (*Hordeum vulgare* L. cv. Tokak; Altinkut *et al.*, 2001) to generate cDNA libraries from roots and leaves after drought stress for 6 and 10 h. The stress was applied in the form of a shock treatment because we wished to obtain transcripts which showed strong regulation, arguing that such a set might be informative to follow up in later experiments based on a more natural and slow drying of plants. ESTs were sequenced and ca. 1500 DNA elements were arrayed on glass slides. Results from the hybridization experiments confirmed the up-regulation of many transcripts which had previously been reported as responsive to stress; in addition, many more novel, functionally unknown transcripts were found. It is also obvious that the responses to drought and salt shock include only partially overlapping sets of transcripts.

Materials and methods

Plant materials and growth conditions

Hordeum vulgare L. cv. Tokak seeds were provided by TUBITAK, MRC, RIGEB (Kocaeli, Turkey). Seeds were surface-sterilized in 10% sodium hypochloride (30 min) and rinsed 15 times (1 min/each) in double-distilled H_2O. For drought experiments, plants were grown in sand and watered daily with one-third-strength Hoagland's solution containing double iron. For salt stress experiments, the plants were grown in aerated, hydroponic tanks in the same solution. Plants were grown under controlled conditions (28 °C day/25 °C night, 12 h photoperiod, $\sim$500 μmol m^{-2} s^{-1} photon flux density, 83% relative humidity) and stressed at the age of three weeks after germination.

Drought stress was applied by removing plants from sand and leaving them on paper towels under the conditions indicated above. Root and leaf tissues were harvested after 6 and 10 h of stress, frozen in liquid nitrogen and stored at -80 °C. Control plants were well-watered and harvested at the same time as the stressed plants. Salt stress was applied to plants in hydroponic tanks containing one-third Hoagland's

Table 2. Up-regulated transcripts in barley hybridized separately with 6 and 10 h drought-stressed leaf and root tissues and fold-time regulation values. For each of the indicated tissue and time point, the transcripts are listed according to their decreasing values of regulation (numbers in **bold**).

Accession number	GenBank match	Annotation	Leaf		Root	
			6 h^{I}	10 h^{I}	6 h^{I}	10 h^{I}
HC106E12	Q00531	Jasmonate-induced protein 60KD	4.0	4.1	−2.0	3.1
HB108B06	AF127241	Arginine decarboxylase 2	**3.7**	3.5	−1.8	2.6
HB107F12	T05291	Arginine decarboxylase SPE2	**3.5**	3.0	−1.5	3.8
HB103A03	AAC16325.1	Asparagine synthetase	**3.2**	1.4	−0.4	1.2
HB105C09	S53503	Aldehyde dehydrogenase	**3.2**	2.0	0.1	3.6
HC106E11	P32024	Jasmonate-induced protein 23KD	**3.0**	0.5	−3.4	2.3
HB105H11	CAB86384.1	Allene oxide synthase	**3.0**	1.6	−0.8	1.7
HB107D06	AAF64042.1	Fatty acid alpha-oxidase	**2.9**	−0.2	1.2	1.2
HB106A11	P32024	Jasmonate-induced protein 23KD	**2.8**	0.9	−3.6	2.1
HB102C02	T05934	Jasmonate-induced protein 1	**2.8**	2.0	−3.0	1.8
HB107D05	AJ250864	Allene oxide synthase	**2.7**	0.9	0.3	1.8
HB108B04	P94029	Metallothionein-like protein type 2	**2.7**	1.8	−1.9	0.9
HC106E06	AAB70865	Lipoxygenase 2 (methyl jasmonate-inducible)	**2.7**	1.6	−2.0	1.5
HB108C02	T04374	Jakalin homolog	**2.5**	1.2	−0.5	1.3
HC102B08	O04226	Δ1-pyrroline-5-carboxylate synthetase	**2.4**	2.3	−1.7	1.2
HB108E04	AF043094	Dehydrin 9	**2.3**	0.4	0.1	4.3
HB102C07	P43283	Trypthophan synthase beta chain 1	**2.3**	0.9	0.9	3.6
HC106E10	X66376	Jasmonate-induced protein 6/thionin	**2.3**	3.1	−4.5	−1.3
HB104B09	P94029	Metallothionein-like protein type 2	**2.2**	1.7	−1.2	0.6
HC109F09	P46518	Late embryogenesis abundant protein LEA14-A	**2.2**	1.7	1.4	3.4
HB102G10	S33631	Tritin	**2.0**	0.0	−0.2	0.1
HB106F04	T05876	Cytochrome P450 homolog (*Arabidopsis thaliana*)	**1.9**	1.2	1.7	3.6
HB108B12	X95909	Receptor-like protein kinase LRK1	**1.9**	0.8	−0.2	0.8
HB108E05	BE438063	Unknown protein (*Hordeum vulgare*)	**1.9**	3.5	0.1	0.9
HC106E12	Q00531	Jasmonate-induced protein 60KD	4.0	**4.1**	−2.0	3.1
HB108B10	AB016087	RNA-binding protein	1.3	**4.1**	0.2	−0.7
HC109C08	JQ2361	Wheat aluminum-induced protein wali5	1.3	**3.9**	1.4	1.0
HC108A02	P22220	Arginine decarboxylase	4.2	**3.5**	−1.6	4.2
HB108B06	AF127241	Arginine decarboxylase 2	3.7	**3.5**	−1.8	2.6
HB108E05	BE438063	Unknown protein (*Hordeum vulgare*)	1.9	**3.5**	0.1	0.9
HC112B03	S71555	Proteinase inhibitor-related protein bsi1 precursor	0.5	**3.4**	0.9	0.1
HC106E10	X66376	Jasmonate-induced protein 6/thionin	2.3	**3.1**	−4.5	−1.3
HB107F12	P22220	Arginine decarboxylase SPE2	3.5	**3.0**	−1.5	3.8
HC114C12	AF323586	Aldehyde dehydrogenase	1.2	**2.9**	0.1	1.6
HB102B10	P42762	ERD1 protein precursor	0.6	**2.8**	−0.8	1.5
HB108E07	AJ010423	Glyoxalase 1	1.0	**2.7**	−0.7	1.5
HB107C10	AF014055	Asparagine synthetase	3.0	**2.7**	−0.5	0.6
HC108A08	JQ2361	Wheat aluminum-induced protein wali5	1.4	**2.7**	1.0	0.2
HC103G04	P46518	Late embryogenesis abundant protein LEA14-A	1.6	**2.6**	1.3	3.2
HC105D02	S66524	Ferritin 1 precursor	1.4	**2.5**	1.1	4.3
HC113B04	BG300231	Unknown protein (*Hordeum vulgare*)	1.5	**2.4**	0.1	2.7
SKFAVH12	BE040455	Unknown (*Oryza sativa*)	−1.5	**2.4**	F	−1.7
HC113G10	AF026538	Abscisic acid responsive protein	0.7	**2.4**	1.3	4.9
HC102B08	O04226	Δ1-pyrroline-5-carboxylate synthetase	2.4	**2.3**	−1.7	1.2
HB102B09	S22515	Thionin precursor	1.4	**2.3**	−2.8	−0.5
HC104F03	AC006300	Non-LTR retroelement reverse transcriptase	0.2	**2.3**	0.6	−6.9
HC102C08	P12940	Trypsin inhibitor 1 (Bowman-Birk type)	1.5	**2.3**	0.7	1.8

Table 2. Continued

Accession number	GenBank match	Annotation	Leaf		Root	
			6 h[1]	10 h[1]	6 h[1]	10 h[1]
HC101A03	P94029	Metallothionein-like protein	2.2	**2.3**	−1.0	2.9
HB107E04	U77345	Cell death supressor protein L1s1	1.2	**1.8**	−1.2	1.6
HC105B12	AF218627	Abscisic acid-induced plasma membrane protein	0.9	1.1	**3.9**	4.9
HC101C08	CAA09192.1	Glutathione transferase	0.1	0.8	**2.6**	3.3
HC109H11	T01696	Omega-3 fatty acid desaturase	−0.4	−0.9	**2.6**	3.1
HC103D05	T02191	Cytochrome P450 homolog (*Arabidopsis thaliana*)	1.0	1.1	**2.5**	3.3
HC102A07	T06986	Wheat aluminum-induced protein wali6	0.3	1.3	**2.5**	3.3
HC102D09	CAC11562	Hypothetical membrane protein (*Thermolasma acidophilum)*	0.3	0.0	**2.5**	0.5
HC108B09	Q10327	Unknown (*Schizosaccharomyces pombe*)	0.0	−0.4	**2.5**	−0.4
HC109C04	–	No hit	0.4	1.4	**2.5**	1.2
HC105D07	T17012	Protein phosphatase 2A	1.1	−1.2	**2.4**	1.3
HC108D06	P49075	Exoglucanase 3 precursor	0.8	−0.4	**2.3**	0.2
HC101A05	Q41001	Blue copper-binding protein homolog	1.3	0.3	**2.3**	2.5
HC101G12	S66342	Ribonuclease 2 (wound-induced)	0.5	1.5	**2.3**	2.3
HC104B09	P43279	Malic enzyme (NAPD-dependent, chloroplast)	0.0	−0.8	**2.2**	2.9
HC105G01	AF031195	Blue copper-binding protein homolog	−0.2	0.6	**2.1**	3.0
HC102E09	U60764	Pathogenesis-related protein	0.1	−0.3	**2.1**	5.0
HC101E01	AF123608	Cytochrome P450	−0.6	1.3	**2.0**	2.1
HC101B07	L37358	Lipoxygenase 2	1.2	0.4	**2.0**	2.4
HC105A09	AAD21417	Unknown (*Arabidopsis thaliana)*	−0.4	−0.8	**1.9**	2.3
HC107D02	AF250935	Germin protein F	−0.3	1.6	**1.9**	2.6
HC110G12	S71361	Actin-binding protein WCOR719	0.5	0.6	**1.9**	4.0
HC110A02	P40372	Ribosomal protein 60S L3-A	−0.6	−0.1	**1.9**	0.1
HC107G10	T05956	Germin-like protein	−0.3	0.4	**1.8**	2.5
HC110G02	BAB02761	Cytochrome c oxidoreductase-like protein	0.9	−0.3	**1.8**	2.4
HC113F12	T11580	12-oxophytodienoate reductase	−0.4	−0.8	**1.8**	3.5
HC101A07	Q01291	Ribosomal protein, s0, 40S	−0.3	0.5	**1.8**	0.4
HC109H08	T07107	Calcium-binding protein	0.3	1.6	**1.7**	3.0
HC110C03	AAF08575	Unknown (*Arabidopsis thaliana*)	1.3	0.2	**1.7**	1.6
HC103C11	P07987	Exoglucanase 2 precursor	−1.6	−3.3	**1.6**	F
HC112E03	AF026538	Abscisic acid responsive protein	0.6	1.5	**1.6**	2.8
HC107H07	AAF64042	Fatty acid alpha-oxidase	2.5	−0.8	F	**6.5**
HC114F01	T17012	Probable phosphoprotein phosphatase	−0.6	−2.2	F	**5.4**
HC104E01	T02763	Malate dehydrogenase	0.1	−4.9	0.3	**5.4**
HC114D12	AL365234	Unknown (*Arabidopsis thaliana*)	0.7	0.5	−0.6	**5.2**
HC102E09	T14817	Pathogenesis-related protein	0.1	−0.3	2.1	**5.0**
HC105B12	AF218627	Abscisic acid-induced plasma membrane protein	0.9	1.1	3.9	**4.9**
HB107A11	AF127241	Arginine decarboxylase 2	3.4	2.9	−1.3	**4.7**
HC107C01	D85764	Monodehydroascorbate reductase (cytosolic)	0.7	0.1	0.7	**4.6**
HC109B06	Q03460	Glutamate synthase (NADH dependent)	−0.2	0.4	1.1	**4.5**
HC105C11	AC010926	Protein phosphatase 2C	1.1	1.3	0.3	**4.5**
HC107A05	AF324993	Unknown *(Arabidopsis thaliana)*	0.6	1.3	1.3	**4.3**
HC105D02	S66524	Ferritin 1 precursor	1.4	2.5	1.1	**4.3**
HB108E04	AF043094	Dehydrin 9	2.3	0.4	0.1	**4.3**
HC109E10	BF256912	Unknown protein (*Hordeum vulgare*)	0.6	0.4	0.7	**4.2**
HC110G12	S71361	Actin binding protein WCOR719	0.5	0.6	1.9	**4.0**
HC114E06	AC010718	Unknown (*Arabidopsis thaliana*)	0.4	1.0	0.5	**4.0**

Table 2. Continued

Accession number	GenBank match	Annotation	Leaf		Root	
			6 h[1]	10 h[1]	6 h[1]	10 h[1]
HB106D02	AJ010829	Geminivirus Rep A-binding protein (GRAB1)	1.4	1.7	1.2	**4.0**
HC105D06	BG355459	Unknown protein (*Hordeum vulgare*)	0.3	0.2	−0.6	**3.8**
HB107F12	P22220	Arginine decarboxylase	3.5	3.0	−1.5	**3.8**
HC103E02	T01558	Auxin-induced protein	0.3	−2.0	−0.6	**3.8**
SKFAVG11	BAB09839	Water channel protein (WCP-IV)	0.6	−1.3	−0.3	**3.7**
HC112E09	AJ250864	Allene oxide synthase	2.3	0.9	0.7	**3.6**
HB106F04	T05876	Cytochrome P450 homolog (*Arabidopsis thaliana*)	1.9	1.2	1.7	**3.6**
HB105C09	S53503	Aldehyde dehydrogenase	3.2	2.0	0.1	**3.6**
HC107E10	AL161496	Protein phosphatase regulatory subunit	−0.6	0.0	0.6	**3.6**
HB102C07	P43283	Trypthophan synthase beta chain 1	2.3	0.9	0.9	**3.6**

[1] Values represent log2 ratios of stressed *vs*. control tissue. Changes in regulation were considered significant at 2.5-fold deviation from the control value (corresponding to log2 = 1.5). ESTs labeled 'F' were flagged because of high variability of signal intensity in repeat experiments.

solution supplied with double iron and 150 mM NaCl. Root and leaf tissues were collected from control and stressed plants after 24 h of stress treatment.

Physiological analyses

Water loss of plants under drought conditions was determined by drying leaf samples taken from stressed and control plants at 80 °C for 6 h. Free amino acids from both drought-stressed (10 h) and control samples were isolated according to Thomas *et al.* (1992). The amino acid composition was determined in a Beckman 7300 amino acid analyzer (Beckman, Fullerton, CA).

RNA isolation and cDNA library construction

Total RNA was isolated by using TRIzol Reagent (Life Technologies, Rockville, MD) and converted to mRNA by the PolyAT-tract mRNA Isolation System IV (Promega, Madison, WI) according to established protocols. Two cDNA libraries were constructed from drought-stressed *H. vulgare* L. cv. Tokak. The library HB includes transcripts from 6 and 10 h drought-stressed leaves combined; library HC includes RNA from 6 and 10 h drought-stressed roots. Library construction utilized a cDNA Synthesis Kit (Stratagene, La Jolla, CA). The cDNAs were ligated into pBluescript II SK(+) (Life Technologies) and transformed into *Escherichia coli* XL10-Gold (Stratagene). After DNA isolation, inserts were sequenced from the 5′ ends and annotated on the basis of their homologies with sequences published in GenBank.

Library amplification and preparation of DNA microarrays

cDNA libraries were amplified in a 96-well PCR format using pBluescript T3 (5′-GAAATTAACCCTCACTAAAGG-3′) and T7 (5′-TGTAATACGACTCACTATAGGGC-3′) primers with amino-modified 5′ ends in 50 μl reaction volumes at an annealing temperature of 55 °C for 40 cycles. The products were separated in agarose gels to confirm amplification quality and quantity. PCR products were diluted by adding 100 μl binding solution (150 mM potassium acetate pH 4.8, 7 M guanidine hydrochloride), purified (Mafnob 96-well PCR Cleanup Kit; Millipore, Bedford, MA), freeeze-dried and re-suspended in 7 μl of 2× SSC. Products at a DNA concentration >400 ng/μl and of a size >400 bp were printed (Deyholos and Galbraith, 2001) on Sigma Screen glass slides (Sigma, St. Louis, MO) using the Omnigrid Spotter (GeneMachines, San Carlos, CA). Each DNA element was spotted in four replicates. In addition, five different human ESTs were spotted in four replicates in multiple locations on the slides. Each hybridization included Cy3/Cy5-labeled human probes of these ESTs each at a different concentration to control for low and high signal intensities and to adjust for equal Cy3 and Cy5 signal strength.

Preparation of fluorescent probes

mRNA samples were labeled with Cy3- or Cy5-labeled dUTP (Amersham Pharmacia Biotech, Dubendorf, Switzerland) by first-strand reverse transcription. Labeling was performed in a 50 μl reaction volume containing 1× reaction buffer, 2 μg oligo T(18)

Table 3. Down-regulated transcripts in barley hybridized separately with 6 and 10 h drought-stressed leaf and root tissues and fold-time regulation values. For each of the indicated tissue and time point, the transcripts are listed according to their decreasing values of regulation (numbers in **bold**).

Accession number	GenBank match	Annotation	Leaf		Root	
			6 h	10 h	6 h	10 h
SKFAVB08	–	No hit	**−4.8**	−1.1	−0.6	−1.9
HB108E01	P31251	Ubiquitin activating enzyme E1	**−4.3**	0.9	−3.1	−2.5
SKFAVA03	Q03664	Glutathione S-transferase (auxin-induced)	**−3.1**	0.0	F	F
HC103A06	AJ009695	Wall associated kinase 4	**−3.0**	−2.5	−1.8	−1.3
HB106B03	T06176	Ribulose-bisphosphate carboxylase activase A2	**−2.9**	−4.2	−2.2	−4.5
SKFAVH04	BAB10223	Glycosylation enzyme	**−2.8**	−0.5	−0.9	−0.3
HB102E09	68090	Rubisco small chain precursor	**−2.7**	−1.4	−4.3	−1.4
HB107E08	S21386	Chlorophyll a/b-binding protein CP29 precursor	**−2.7**	−3.1	−1.4	0.4
HC110D02	AF024589	Glycine decarboxylase P subunit	**−2.7**	−2.4	−1.7	−0.1
HC108B10	P48631	Omega-6 fatty acid desaturase	**−2.6**	−1.3	0.5	0.7
HB105B06	AF024589	Glycine decarboxylase P subunit	**−2.6**	−1.7	−1.6	−0.8
HC104A02	S53012	Root-specific protein RCc3	**−2.5**	2.1	−1.5	−1.6
SKFAVB04	T09555	Fibrillarin	**−2.5**	−0.4	−0.9	−0.9
HB108A05	T03962	r40g3 protein	**−2.5**	−1.4	−3.5	F
HB102H05	CAA10497	Unknown (*Secale cereale*)	**−2.5**	−2.4	−2.9	−1.6
HB108A08	AF009959	Metallothionein-like protein	**−2.4**	−4.6	−2.8	2.9
HC102H11	BAB17689	Heat shock protein DnaJ homolog Pfj4	**−2.4**	0.4	0.1	−2.3
HC110A04	T03438	Metallothionein-like protein	**−2.3**	−1.4	−1.4	−0.4
HC105H01	Z28347	Plastocyanin (*Hordeum vulgare*)	**−2.3**	0.7	−0.8	−0.9
SKFAVB01	–	No hit	**−2.2**	−1.4	−0.3	−2.4
SKFAVB12	S18991	High mobility group protein HMGd1	**−2.2**	−1.5	−0.9	−0.1
HC105G10	BAA94238	Lipase	**−2.2**	0.7	−0.4	−1.2
HC107H11	Y07752	Pherophorin-S	**−2.2**	0.4	0.1	−0.4
HC110C06	U69155	Prohibitin	**−2.2**	−1.9	−1.0	−0.3
HC104E01	T02763	Malate dehydrogenase	0.1	**−4.9**	0.3	5.4
HB108A08	AF009959	Metallothionein-like protein	−2.4	**−4.6**	−2.8	2.9
HC102A03	CAC14890	Nucleotide-sugar dehydratase (putative)	−0.8	**−4.4**	0.0	0.0
HB106B03	T06176	Ribulose-bisphosphate carboxylase activase A	−2.9	**−4.2**	−2.2	−4.5
HC106C09	Y11277	Alpha-amylase	−1.5	**−4.1**	−2.2	−0.1
HC106E05	CAA47251	Ethylene forming enzyme	0.0	**−3.4**	−1.4	−0.2
HB108G11	AAF61950.1	Phosphoethanolamine N-methyltransferase	−0.3	**−3.4**	−1.0	1.8
HC103C11	P07987	Exoglucanase 2 precursor	−1.6	**−3.3**	1.6	F
SKFAVB07	AAF36731	Ribosomal protein 60S L6 (YL16 like)	−1.9	**−3.2**	−0.8	−2.1
HB107E08	S21386	Chlorophyll a/b-binding protein CP29 precursor	−2.7	**−3.1**	−1.4	0.4
HB103B02	AAB67843.1	Carbamoyl phosphate synthetase large chain	−0.8	**−3.1**	−0.6	−2.6
HB108C03	Q40004	Rubisco small subunit	−2.9	**−3.0**	−2.6	−1.2
HC114E01	T09338	DnaJ-like protein MsJ1	0.1	**−2.9**	−1.4	0.6
HC102F01	T03263	Calcium dependent protein kinase 7	−0.1	**−2.7**	0.9	−0.9
HB105H01	T04121	S-2-hydroxy-acid oxidase (peroxisomal)	−1.9	**−2.6**	−1.1	−0.3
HC106C07	AF025292	HAK62 (High affinity potassium transporter)	−0.9	**−2.6**	−0.4	−0.2
HC105B07	AF200531	Cellulose synthase 7	−1.5	**−2.5**	0.1	0.2
HB105B06	AF024589	Glycine decarboxylase P subunit	−2.7	**−2.4**	−1.7	−0.1
HC106E03	AAK55421	Rieske protein	−1.2	**−2.4**	−1.4	−1.1
HB105C10	T48186	Light inducible protein ATLS1	−1.2	**−1.9**	−1.5	−0.4

Table 3. Continued

Accession number	GenBank match	Annotation	Leaf		Root	
			6 h	10 h	6 h	10 h
HB102C11	D75542	Unknown (*Deinococcus radiodurans*)	−0.4	0.7	**−7.0**	0.9
HB104B12	S21150	Unknown (*Oryza sativa)*	−1.0	−0.3	**−4.7**	10.4
HC106E10	X66376	Jasmonate-induced protein 6/thionin	2.3	3.1	**−4.5**	−1.3
HB102E09	68090	Rubisco small chain precursor	−2.7	−1.4	**−4.3**	−1.4
HB106A11	P32024	Jasmonate-induced protein 23KD	2.8	0.9	**−3.6**	2.1
HB108A05	T03962	r40g3 protein	−2.5	−1.4	**−3.5**	F
HB105G03	P41067	Conjugal transfer protein TRAB	0.6	−0.7	**−3.2**	0.2
HB102B01	T15046	Arginine decarboxylase 1	1.6	−0.6	**−3.1**	0.0
HB108E01	P31251	Ubiquitin activating enzyme E1	−4.3	0.9	**−3.1**	−2.5
HB105B09	T02645	Unknown (*Arabidopsis thaliana*)	−0.9	−1.1	**−3.0**	−1.7
HB102C02	T05934	Jasmonate-induced protein 1	2.8	2.0	**−3.0**	1.8
HC109A05	P53990	Unknown (*Homo sapiens*)	0.8	0.3	**−3.0**	0.8
HB108A08	AF009959	Metallothionein-like protein	−2.4	−4.6	**−2.8**	2.9
HB106C03	AC025783	C-4 sterol methyl oxidase	1.7	1.8	**−2.8**	−0.2
HB102B09	S22515	Thionin precursor	1.4	2.3	**−2.8**	−0.5
HB105A10	CAA10497	Unknown (*Secale cereale*)	−0.4	−0.1	**−2.7**	0.9
HB107D07	S28871	Lipid transfer protein cw18	0.5	0.9	**−2.7**	−0.8
HB108A06	P13803	Electron transfering flavoprotein	−0.4	−1.9	**−2.6**	−1.1
HC102E01	BAB18760	Beta-cyanoalanine synthase	−1.2	F	**−2.6**	−1.0
HB106A08	AJ005840	Thioredoxin M	−1.9	−1.2	**−2.6**	0.0
HC104F03	AC006300	Non-LTR retroelement reverse transcriptase	0.2	2.3	0.6	**−6.9**
HB102F03	AJ243828	Phosphatase 2A regulatory A subunit	−1.0	0.0	−0.6	**−5.6**
HB106B03	T06176	Ribulose-bisphosphate carboxylase activase A	−2.9	−4.2	−2.2	**−4.5**
HC109H10	AAF69540	Unknown (*Arabidopsis thaliana*)	−0.2	1.2	0.0	**−4.4**
HC113A01	AC007060	Unknown (*Arabidopsis thaliana*)	−0.6	−1.4	F	**−4.1**
HB103A01	AK001301	Unknown (*Homo sapiens*)	−0.6	−0.3	−2.4	**−3.8**
HB108F01	–	No hit	−0.4	−0.2	−1.3	**−3.5**
SKFAVE05	CAA67362	Peroxidase ATP9a	−0.8	−0.5	−0.7	**−3.3**
HB108B05	AF003551	Lysine-ketoglutarate reductase/saccharopine dehydrogenase	0.1	0.4	−0.4	**−3.2**
HC113A12	AF250935	Germin protein F	−0.6	0.3	0.2	**−3.1**
HB102H11	P30110	Glutathione S-transferase 1	−0.6	0.6	−0.4	**−3.1**
HC114A01	AL509452	Unknown protein (*Hordeum vulgare*)	−0.9	−0.5	−0.5	**−3.0**
HC102B01	AJ006358	Ascorbate peroxidase	0.0	0.3	−0.4	**−3.0**
SKFAVE03	P12940	Trypsin inhibitor 1 (Bowman-Birk type)	−0.1	1.6	−0.6	**−3.0**
HC113B01	Q43082	Hydroxymethylbilane synthase	−0.4	0.5	F	**−3.0**
HC108B01	AL512082	Unknown protein (*Hordeum vulgare*)	−0.8	0.6	−0.3	**−2.9**
HB102H12	AB017042	Glyoxalase I	−0.1	−0.9	−0.4	**−2.9**
HC112F01	P49043	Vacuolar processing enzyme precursor	−0.4	0.9	−0.4	**−2.9**
HB102F01	T03727	Metallothionein-like protein type 2	1.0	1.2	F	**−2.7**
HC106F05	BAB11029	Beta-galactosidase	1.1	−0.9	−0.7	**−2.7**
HB103B02	U40341	Carbamoyl phosphate synthetase large chain	−0.8	−3.1	−0.6	**−2.6**
HC109E03	AL511500	Unknown protein (*Hordeum vulgare*)	−1.1	−1.1	−1.2	**−2.6**
HB105B03	X83413	Unknown (herpesvirus-6)	−1.7	0.0	−0.5	**−2.6**
HC108B03	AC069273	Unknown (*Arabidopsis thaliana*)	−0.4	1.4	−0.1	**−2.6**

Table 3. Continued

Accession number	GenBank match	Annotation	Leaf		Root	
			6 h	10 h	6 h	10 h
SKFAVC10	AC027662	Unknown (*Oryza sativa*)	−0.4	−2.1	−0.3	**−2.5**
HB108E01	P31251	Ubiquitin activating enzyme E1	−4.3	0.9	−3.1	**−2.5**
SKFAVG12	BE607400	Unknown (*Oryza sativa*)	−2.3	0.8	−0.9	**−2.5**

[1]Values represent log2 ratios of stressed *vs*. control tissue. Changes in regulation were considered significant at 2.5-fold deviation from the control value (corresponding to log2 = 1.5). ESTs labeled 'F' were flagged because of high variability of signal intensity in repeat experiments.

primer, 0.5 mM dATP, dCTP and dGTP, 0.2 mM dTTP, 2 nmol of either Cy3- or Cy5-labeled dUTP and 0.5 units of reverse transcriptase (SuperScript II, Life Technologies). After incubation at 42 °C for 1.5 h, RNA was degraded by adding RNase H. Fluorescently labeled target cDNAs were cleaned (Concert Rapid PCR Purification System, Life Technologies) and precipitated overnight at −20 °C after the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and one volume of isopropanol. The precipitate was collected by centrifugation, washed with 70% ethanol, dried and re-suspended in 15 μl of hybridization buffer containing 0.25% non-fat dry milk, 5× SSC, 0.1% SDS and 50% formamide.

Hybridization and data analysis

Microarrays were sealed with cover slips after the application of denatured target cDNAs from both control and treated plant tissues, which had been labeled by the incorporation of Cy3 or Cy5 nucleotides, respectively. Hybridization was carried out at 42 °C for 14–16 h in chambers containing 2× SSC solution. After hybridization, the slides were incubated in 2× SSC and 0.5% SDS for 5 min followed by two incubations of 5 min each in 0.5× SSC. Slides were dried by centrifugation.

Microarrays were scanned using a ScanArray 3000 (GSI Lumonics, Watertown, MA) and analyzed by ImaGene III Software (BioDiscovery, Los Angeles, CA). Local background was subtracted from the value of each spot on the array. Spots showing low signal values, covered with dust particles or having irregular shapes, as well as spots in high background areas were flagged as candidates for exclusion and, after further analysis, excluded from the data analysis. Normalization of the signal intensities was carried out according to Deyholos and Galbraith (2001). Changes in signal intensity between stress and control experiments exceeding a 2.5-fold or higher difference in repeat experiments were considered significant. An initial normalization between the Cy3 and Cy5 emission channels was achieved by adjusting the signal intensity of exogenously added non-plant control genes. Transcript regulation is expressed as the ratio of intensities between stress and control.

Real-time quantitative PCR

Real-time PCR amplifications were carried out with two genes that showed up- and down-regulation, respectively. The α-tubulin 2 gene (forward primer, 5′-AGTGTCCTGTCCACCCACTC-3′; reverse primer, 5′-AGCATGAAGTGGATCCTTGG-3′) was used as a control in the PCR. The transcript for the up-regulated gene was the cell death suppressor protein L1s1 (forward primer, 5′-GCCAGAAGCATTTCGTGTTT-3′; reverse primer, 5′-TGGTTTTCAACCCGACTTTT-3′), and for the down-regulated gene was the transcript for the light-inducible protein ATLS1 (forward primer, 5′-GGCTGAACCCTGATGTGAAC-3′; reverse primer, 5′-TGGCCATTAAACTGACACGA-3′). *L1s1* and *ATLS1* were amplified by Smart-Cycler Real-Time PCR (Cepheid, Sunnyvale, CA) in a 25 μl reaction volume containing 2.5 mM dNTP solution, 1× reaction buffer for reverse transcriptase, 10 mM of each primer, 250 ng total RNA, 2.5 mM $MgCl_2$, 10× SYBR Green I (Sigma), 0.2 units of *Taq* DNA Polymerase (Sigma) and 0.5 units of StrataScript Reverse Transcriptase (Stratagene). Reverse transcription was carried out at 55 °C for 10 min followed by annealing at 55 °C for 45 cycles. Results were analyzed according to the threshold value for RNA from stressed and control tissues.

Table 4. Up-regulated transcripts in salt-stressed leaf and root tissues of barley after 24 h of 150 mm NaCl stress and fold-time regulation values. For each of the indicated tissue, the transcripts are listed according to their decreasing values of regulation (numbers in **bold**).

Accession number	GenBank match	Annotation	Leaf [1]	Root[1]
HB107B12	P94029	Metallothionein-like protein type 2	**5.4**	−0.2
HC114F10	AC060755	Unknown (*Oryza sativa*)	**5.0**	0.1
HC102H11	BAB17689	Heat shock protein DnaJ homolog Pfj4	**5.0**	0.6
HB107D02	AAB27289	Protein-basic proline-rich proteins PRB1L precursor	**3.8**	−0.6
HB106D10	AJ250864	Allene oxide synthase	**3.7**	0.2
HB108D01	O57809	1-aminocyclopropane-1-carboxylate deaminase	**3.1**	0.1
HC114B10	T00745	Unknown (*Arabidopsis thaliana*)	**2.9**	0.0
SKFAVD12	P41095	Ribosomal protein acidic 60S PO	**2.8**	1.1
HB106D12	S28871	Lipid transfer protein cw18	**2.6**	−0.3
HB104B10	T06000	Aspartic proteinase	**2.5**	−0.3
SKFAVE09	T09657	Beta-glucosidase	**2.4**	0.3
SKFAVA03	Q03664	Glutathione S-transferase (auxin-induced)	**2.3**	−1.0
HC109B02	NP044447	Replicase-associated polyprotein	**2.3**	−1.1
HB106H12	T04177	Photosystem II 10K protein	**2.2**	−0.4
HB106D06	P31365	Transcription factor POU3A	**2.1**	−0.4
HC113H06	AF071894	Late embryogenesis abundant like protein	**2.0**	−0.2
HB106D08	BAB19328	Unknown (*Oryza sativa*)	**1.9**	−1.0
HC114B12	AC010795	Unknown (*Arabidopsis thaliana*)	**1.9**	−0.4
HC107B12	AC079887	UDP-glucose 6-dehydrogenase	**1.9**	−1.6
HC104E11	AL109787	Unknown (*Arabidopsis thaliana*)	**1.9**	−0.4
HB102H07	S37166	Pathogenesis-related protein 1a	**1.9**	−0.7
HC104D12	P43400	Metallothionein-like protein 1	**1.8**	0.5
HC102B08	O04226	Delta-1-pyrroline-5-carboxylate synthetase	**1.7**	−0.1
HB105A01	Q42829	S-adenosylmethionine decarboxylase precursor	**1.6**	0.4
HB102B10	P42762	ERD1 protein precursor	**1.6**	−0.8
HC107D06	T40637	Ribosomal protein 40S	**1.6**	−0.9
HC106E04	S37166	Pathogenesis-related protein	**1.6**	0.0
HC107H12	P17784	Fructose-bisphosphate aldolase	−0.4	**2.8**
HC107C04	1QDMA	Vacuolar aspartic proteinase	0.4	**2.7**
HC107C07	Q03033	Elongation factor 1-alpha	−0.2	**1.9**
HC109C11	X69422	Ubiquitin 4	−0.1	**1.8**
HC101A02	S28426	Ubiquitin 4	−0.1	**1.8**
HC107G10	T05956	Germin-like protein	0.1	**1.7**
HC103C02	U84969	Ubiquitin	1.5	**1.7**
HC113B10	CAA54603	Ubiquitin 10	0.9	**1.7**
HB105G07	CAA10497	Unknown (*Secale cereale*)	−0.8	**1.7**
HC101C05	AJ275318	dTDP-glucose 4-6-dehydratase	−0.8	**1.5**

[1] Values represent log2 ratios of stressed *vs.* control tissue. Changes in regulation were considered significant at 2.5-fold deviation from the control value (corresponding to log2 = 1.5). ESTs labeled 'F' were flagged because of high variability of signal intensity in repeat experiments.

Table 5. Down-regulated transcripts in salt-stressed leaf and root tissues of barley after 24 h of 150 mm NaCl stress and fold-time regulation values. The transcripts are listed according to their decreasing values of regulation (numbers in **bold**).

Accession number	GenBank match	Annotation	Leaf[1]	Root[1]
HB102D03	AAB71969	Auxin-induced protein	**−8.3**	−0.5
HC109H01	AL162508	Unknown (*Arabidopsis thaliana*)	**−6.8**	0.2
HC107F09	AC007858	Unknown (*Oryza sativa*)	**−6.8**	−0.8
HC112D03	T09657	Beta-glucosidase	**−6.6**	0.1
HC109H03	JQ2361	Wheat aluminum-induced protein wali5	**−6.1**	0.3
HC105F01	Y08501	ORF107a (*Arabidopsis thaliana*)	**−4.8**	0.2
HC109F09	P46518	Late embryogenesis abundant protein LEA14-A	**−4.8**	−0.3
HC112B09	AAG52429	Aminopeptidase N	**−4.7**	−0.9
HC109H10	AAF69540	Unknown (*Arabidopsis thaliana)*	**−4.7**	−0.4
HC101B09	O49203	Nucleoside diphosphate kinase III precursor	**−4.4**	−0.8
HC109D01	T14189	Unknown (*Arabidopsis thaliana*)	**−4.3**	−0.1
HC107D01	S34285	Ubiquitin	**−4.2**	−0.2
HC103B10	S30569	Glutamine synthetase	**−4.1**	1.2
HB105D02	AF113541	Ethylene response sensor (putative)	**−4.0**	−0.4
HB108C11	AAF07846	Aminotransferase (putative)	**−3.8**	F
HC107H01	P40392	Ras-related protein RIC 1	**−3.7**	−0.3
SKFAVA11	CAA75793	Sucrose synthase 2	**−3.6**	0.2
HC110E01	AAK20858	Fasciclin-like arabinodalactan-protein 2	**−3.5**	−1.0
HC107H03	AAG09228	Coatomer alpha subunit	**−3.5**	0.5
HC105B05	T36632	Oxidoreductase	**−3.4**	−0.1
HC105D06	BG355459	Unknown protein (*Hordeum vulgare*)	**−3.4**	0.5
HB107A01	AJ275310	Unknown (*Cicer arietinum*)	**−3.4**	−0.5
HC101D02	T02667	Proteinase inhibitor	**−3.3**	−0.6
HC101F12	T05276	Unknown (*Arabidopsis thaliana*)	**−3.3**	0.4
SKFAVG12	BE607400	Unknown (*Oryza sativa*)	**−3.3**	−0.5
HC108B05	–	No hit	**−3.3**	−0.5
HC108E01	AE003723	Unknown (*Drosophila melanogaster*)	**−3.3**	−0.2
SKFAVH12	BE040455	Unknown (*Oryza sativa*)	F	**−4.3**
HB108G10	S28871	Lipid transfer protein cw18	0.3	**−2.1**
SKFAVA01	AAD50376	Ripening related protein	−0.3	**−1.7**
HB105C02	AL078637	Unknown (*Arabidopsis thaliana*)	0.5	**−1.7**
HC106C05	L12707	Photosystem 1	−0.7	**−1.7**
HB108C01	AAG00249	Unknown (*Arabidopsis thaliana*)	−0.4	**−1.7**
HC110D04	P49043	Vacuolar processing enzyme precursor	0.2	**−1.7**
HC107B12	AC079887	UDP-glucose 6-dehydrogenase	1.9	**−1.6**
HC106C09	Y11277	Alpha-amylase	F	**−1.6**

[1] Values represent log2 ratios of stressed *vs.* control tissue. Changes in regulation were considered significant at 2.5-fold deviation from the control value (corresponding to log2 = 1.5). ESTs labeled 'F' were flagged because of high variability of signal intensity in repeat experiments.

Results and discussion

Physiological parameters and drought-specific transcript profiles

For the drought-shock experiment, samples from stressed and control tissues were taken at the same time points to eliminate variation due to diurnal changes in gene expression. During that time period, relative water content of the plants declined to ca. 90% within the initial 4 h, and then more rapidly to ca. 70% (6 h) and 64% (10 h). Replanting after 10 h of drought indicated that the plants were able to survive and re-

Table 6. Transcripts with up- or down-regulation under drought and salt stress experiments.

Accession number	GenBank match	Annotation	Drought				Salt	
			6 h leaf	10 h leaf	6 h root	10 h root	24 h leaf	24 h root
With up-regulation								
HB107D05	AJ250864	allene oxide synthase 1	**2.7**	0.9	0.3	**1.8**	**3.7**	0.2
HB108B04	P94029	metallothionein-like protein	**2.7**	**1.8**	−1.9	0.9	**5.4**	−0.2
HB102B10	P42762	ERD1 protein precursor	0.6	**2.8**	−0.8	**1.5**	**1.6**	−0.8
HC102B08	O04226	Δ1-pyrroline-5-carboxylate synthetase	**2.4**	**2.3**	−1.7	1.2	**1.7**	−0.1
HC107G10	T05956	germin-like protein	−0.3	0.4	**1.8**	**2.5**	0.1	**1.7**
With down-regulation								
HC106C09	Y11277	α-amylase	−1.5	**−4.1**	**−2.2**	−0.1	F	**−1.6**
HB107D07	S28871	lipid transfer protein cw18	0.5	0.9	**−2.7**	−0.8	0.3	**−2.1**
HC109H10	AC06922	ABC transporter	−0.2	1.2	0.0	**−4.4**	**−4.7**	−0.4
HC112F01	P49043	vacuolar processing enzyme precursor	−0.4	0.9	−0.4	**−2.9**	0.2	**−1.7**
SKFAVG12	BE607400	unknown (*Oryza sativa*)	**−2.3**	0.8	−0.9	**−2.5**	−3.3	−0.5

Data are log2 ratios of stressed *vs.* control tissue. Changes in regulation were considered significant at 2.5-fold deviation from the control value (corresponding to log2 = 1.5). ESTs labeled 'F' were flagged because of high variability of signal intensity in repeat experiments.

sume growth but leaves that had already developed did not recover (not shown). As an additional physiological parameter, the composition of free amino acids was determined in whole plants. Within 10 h, the total amount of amino acids had increased more than 2.5-fold, from 8008 to 22 979 pmol per 50 μl total amino acids. A substantial proportion of the increase was in proline (from 28 to 5711 pmol per 50 μl) which, after 10 h of drought, amounted to 25% of all amino acids. Similar stress-dependent increases in proline have been observed in many plants (Yoshiba *et al.*, 1997).

Two cDNA libraries, from roots and leaves, respectively, were generated and the DNA of ca. 2200 clones was sequenced. Redundant clones were eliminated before printing which resulted in 1463 DNA elements from drought-stressed leaf and root tissues included on the slides (Table 1). The transcript profile sampled here is small, includes mainly abundant transcripts, and is characterized by the large number of ESTs related to cell defense functions, which amounted to almost 10% of all transcripts. The low number of transcripts in the photosynthesis category (2.8% of ESTs in leaves) supports that these clones were indeed derived from stressed plants (Table 1). In unstressed plants, about 15% of all leaf transcripts can typically be categorized as photosynthesis-related, but in drought-stressed leaves this percentage is much lower. This value (Table 1) includes transcripts for functions of light reactions only. When other chloroplast functions are counted in addition, the value increases to ca. 5% of the leaf transcripts. At least 38% of all transcripts are functionally unknown. They are grouped into two categories, 'unknown' and 'no hit', the latter indicating that transcripts in this class have not previously been described from other organisms. In reality, however, the number of functionally unknown transcripts is larger because many transcripts are assigned to categories based on domain homologies with other deduced protein sequences while their actual function has not been documented.

Microarray hybridization analysis

The result of a hybridization to ESTs derived from the root and leaf cDNA libraries is shown in Figure 1. DNA elements were deposited in quadruplicate: the average signal intensities were determined after subtraction of the local background intensity. The figure reveals the variability that is typically obtained which may lead to difficulties in interpreting hybridization results when spots are either too small or not well defined. Spots with aberrant size, washed-out spots, or unevenly hybridized spots were flagged and excluded from the analyses. Such variability depends on the purity of the DNA samples (e.g., ion content), slide surface properties, solubilization of the target probe, and quality of the pins used to deposit the DNA on the glass surface. The Cy3/Cy5 signal intensities were

Table 7. Quantitative determination of transcript abundance. The change in transcript abundance in leaf tissue during drought stress observed by microarray analysis was confirmed by duplicate real time PCR. The threshold values were used for quantitative expression of PCR results. α-tubulin 2 gene (specific primers *Tub2*) was used as control, cell death suppressor protein L1s1 (specific primers *lls1*) for confirming up-regulation and light-inducible protein ATLS1 (specific primers *Atls1*) for down-regulation. A. Threshold values of replicate real time PCR. B. Quantitative comparison of microarray and PCR results.

A

Tissue	Primer	Exp. 1 threshold value	Exp. 2 threshold value
6 h drought-stressed leaf	*Tub2*	13.8	14.2
Control leaf for 6 h drought stress	*Tub2*	13.5	13.9
10 h drought-stressed leaf	*Tub2*	14.0	14.5
Control leaf for 10 h drought stress	*Tub2*	13.3	13.8
6 h drought-stressed leaf	*lls1*	11.6	11.8
Control leaf for 6 h drought stress	*lls1*	14.9	15.3
10 h drought-stressed leaf	*lls1*	10.8	11.1
Control leaf for 10 h drought stress	*lls1*	15.8	16.4
6 h drought-stressed leaf	*Atls1*	16.1	15.6
Control leaf for 6 h drought stress	*Atls1*	14.7	15.0
10 h drought-stressed leaf	*Atls1*	16.6	16.5
Control leaf for 10 h drought stress	*Atls1*	15.0	15.1

B

Tissue	Primer	PCR	Microarray
6 h drought-stressed leaf vs. control	*Tub2*	0.8	1.0
10 h drought-stressed leaf vs. control	*Tub2*	0.6	0.8
6 h drought-stressed leaf vs. control	*lls1*	10.8	2.3
10 h drought-stressed leaf vs. control	*lls1*	36.2	3.5
6 h drought-stressed leaf vs. control	*Atls1*	−1.9	−2.3
10 h drought-stressed leaf vs. control	*Atls1*	−2.8	−1.9

adjusted with the help of exogenously added control genes which had been placed in different sections of the microarray slides to compensate for variable background levels (see Materials and methods). A comparison of hybridization intensities by using labeled root (Cy3) and leaf (Cy5) targets is presented in Figure 2. The distribution of log2 signal intensities indicated that most of the transcripts on the array are not specific to either leaf or root tissue. More than 90% of all transcripts are equally expressed in both tissues. Examples for leaf-specific transcripts are an unknown protein (HB104B12; S21150), a metallothionein-like protein (HB108A08; AF009959), a membrane protein (HB108C09; AAF18512) and an ADP/ATP translocase (HC107E02; S33630). Root-specific transcripts are glyoxalase-I (HB102H12; AB107042), a non-LTR retroelement reverse transcriptase (HC104F03; AC006300), a phosphatase-2A regulatory subunit (HB102F03; AJ243828) and an unknown protein (HC109H10; AAF69540).

Hybridizations with RNA extracted from leaf and root tissues of either drought-stressed (6 and 10 h) or salt-stressed (24 h, 150 mM NaCl) plants are shown in Figures 3–5, presenting the normalized log-

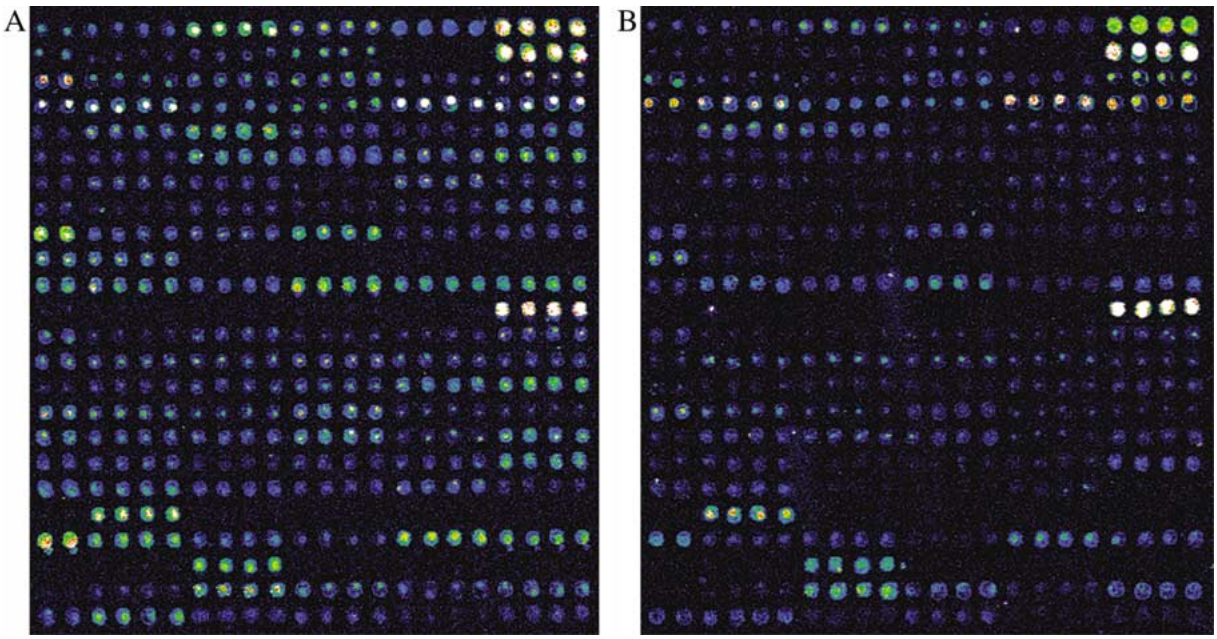

Figure 1. Microarray hybridization to cDNAs immobilized on glass slides. A segment of a microarray slide is shown after hybridization with Cy5-labeled-dUTP target from drought-stressed roots (10 h) (A) and Cy3-labeled-dUTP target from untreated roots (B). The saturation of signals with Cy3- and Cy5-labeled targets is represented by colors (blue, green, red, yellow to white), where blue is for low signal intensity and white for high intensity.

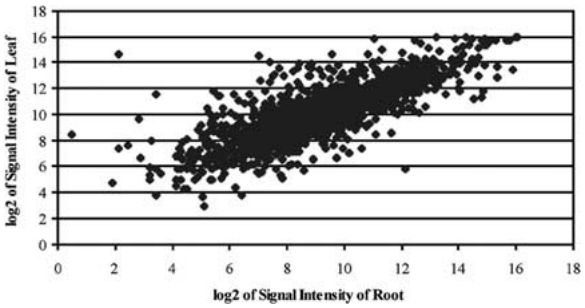

Figure 2. Tissue specificity of transcript expression. Drought stress (10 h) hybridization intensities on log2 basis of root samples compared to leaf samples. The distribution of signal ratios indicates that most transcripts used in these experiments are not specific for either tissue.

10 hybridization intensity ratios of stressed vs. control tissues plotted against the EST number. We considered a 2.5-fold change (corresponding to 0.6 log10 or 1.5 log2) in the stressed tissue as significant for up- and down-regulation. Transcripts above or below the boxed area (Figures 3–5) represent those exhibiting significant changes in expression following stress. In order to ascertain the reproducibility of the changes in transcripts of a particular sample, mRNAs from the same isolation and labeling reaction were compared in repeated hybridizations (Figures 3B and C; Figures 5B and C). The conformity of up- and down-regulation in repeat experiments was c.a. 50% for all transcripts showing significant changes in regulation, i.e., about half of the transcripts that we identified as significantly altered in response to drought or salinity showed the same regulation in repeat experiments. This percentage was the same irrespective of whether the mRNA derived from the same or different experiments. However, a relaxation of the significance level to a 1.6-fold change (Kawasaki *et al.*, 2001) indicated that more than 90% of the transcripts which showed either up- or down-regulation in one hybridization, did likewise in repeat experiments. A similar degree of variability has been observed for rice salinity-stress-regulated transcripts (Kawasaki *et al.*, 2001).

Up-regulated transcripts in drought-stressed leaves

Examples of significantly drought-affected transcripts in barley are given in Tables 2 and 3. Highly up-regulated at the 6 h time point of the drought treatment in leaves (Table 2) are transcripts for the biosynthesis of jasmonate (e.g., several allene oxide synthases), which is well-known as a signal in pathogen defense and under drought conditions (Reymond and Farmer, 1999; Wierstra and Kloppstech, 2000). Strongly

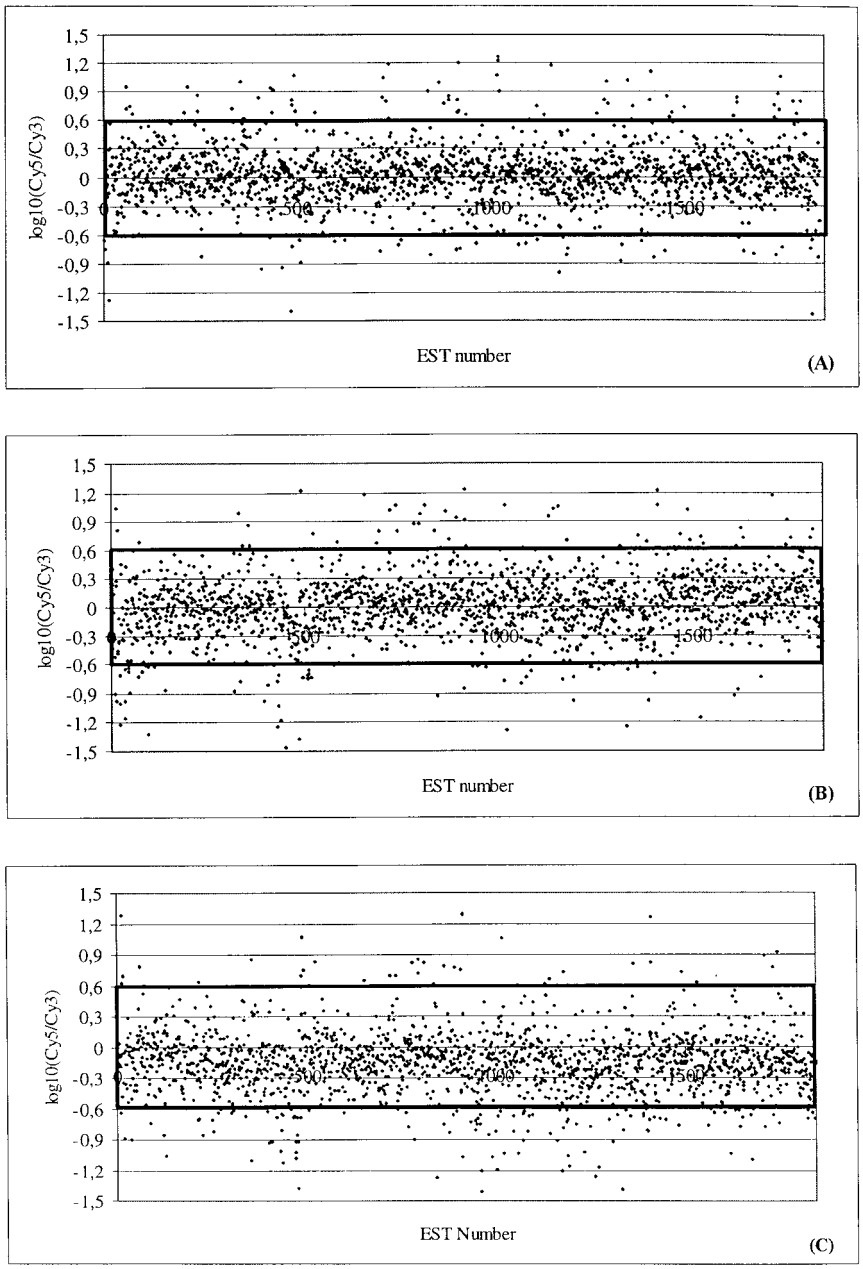

Figure 3. Hybridization ratios (log10) for drought-stressed leaf tissues. A. 6 h drought-stressed leaf tissue *vs.* control leaf target after normalization. B. 10 h drought-stressed leaf tissue *vs.* control after normalization. C. A repeat experiment for 10 h drought-stressed leaf tissue *vs.* control with mRNA from the same sample and labeling is shown. Boxed areas indicate the interval (−2.5- to 2.5-fold regulation) in which changes in expression were considered not significant.

increased leaf transcripts encode known jasmonate-induced proteins (JIPs) and a methyl-jasmonate inducible lipoxygenase reported earlier (Voros *et al.*, 1998). Two arginine decarboxylases are induced (at 6 and 10 h in leaves and also at 10 h in roots), possibly reflecting the altered synthesis of polyamines that are observed in many stressed plants (Masgrau *et al.*, 1997; Soyka and Heyer, 1999). Lipoxygenase and an equally strongly induced fatty acid α-oxidase may be in the signal transduction pathway that is regulated by jasmonates. Finally, LEA/dehydrin protein and metallothionein encoding transcripts, as

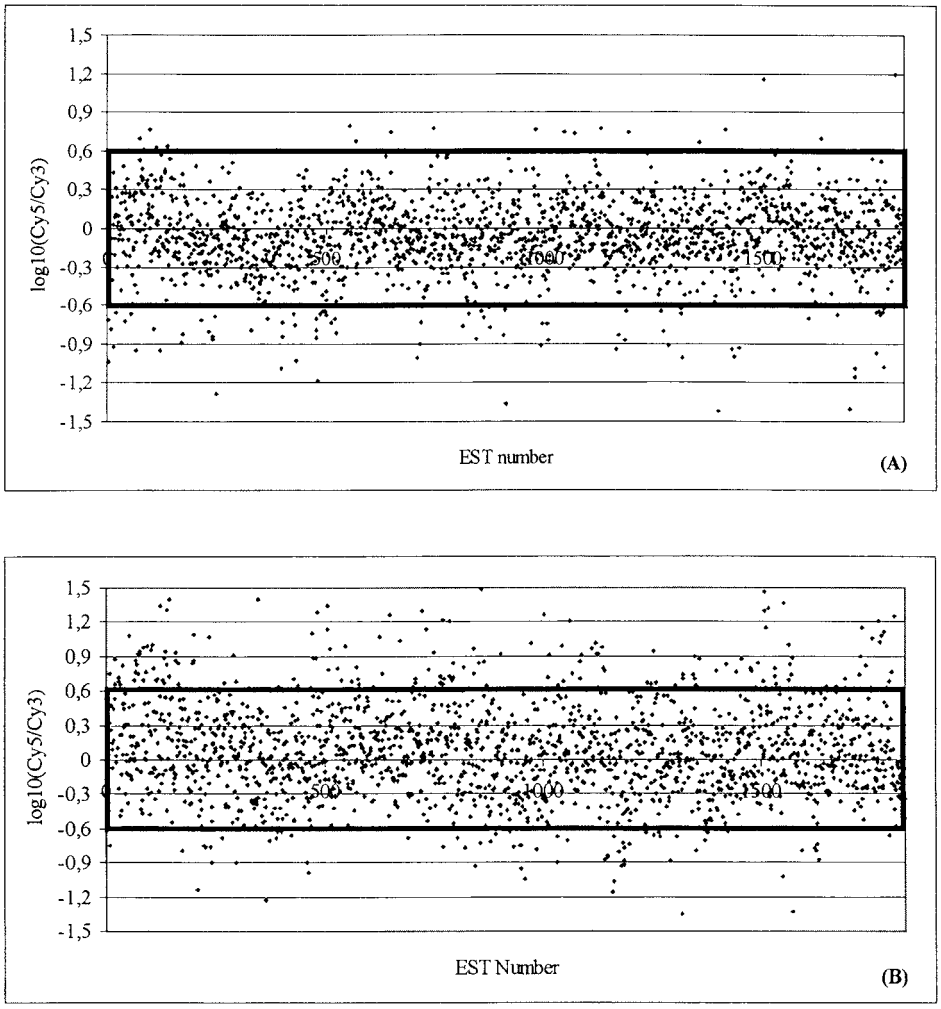

Figure 4. Hybridization ratios (log10) for drought-stressed root tissues. A. 6 h drought-stressed root tissue *vs.* control after normalization. B. 10 h drought-stressed root tissue *vs.* control after normalization. Boxed areas indicate the interval (−2.5- to 2.5-fold regulation) in which changes in expression were considered not significant.

well as △1-pyrroline-5-carboxylate synthetase are up-regulated, the latter encoding a rate-limiting enzyme in proline biosynthesis. Free proline does indeed accumulate rapidly (see above). For a number of other 6 h-induced transcripts, no function can be ascertained although functions in stress responses, for example, for aldehyde dehydrogenase, asparagine synthetase, a cytochrome P450 enzyme or a receptor-like protein kinase can be postulated. Only a more detailed analysis, which was not the purpose of this study, will show their precise functions. We point out, however, that the library for the 6 h time point of drought stress includes, among the less strongly up-regulated transcripts, other cDNAs that could potentially have a large effect on the response and defense capacity of the plants. Future studies should attempt to isolate additional and less-abundant transcripts from this library.

The leaf 10 h stress time point shows a different profile in terms of up-regulated transcripts (Table 2). Transcripts for jasmonate-responsive proteins are less prominent, although several JIPs are still among the most highly up-regulated transcripts; this also applies to LEA/dehydrins and metallothionein-like transcripts, and for proline or polyamine biosynthesis functions. At this time point, the first ABA-induced protein transcript is found to be up-regulated, presenting good indications for jasmonates and ABA serving in separate signal transduction pathways. Also, at the 10 h time point, transcripts emerge that encode diverse functions, whose mechanisms and possible

significance in plant stress tolerance are unknown. The unknown putative functions are indicated by descriptive labels, such as putative RNA-binding protein, proteinase inhibitor-related protein BSi1, aluminum-induced protein or thionin precursor.

Up-regulated transcripts in drought-stressed roots

In leaves, about half of the up-regulated transcripts at the 6 h time point are also among the most highly induced transcripts after 10 h, but in roots a very different pattern is observed. First, the root and leaf expression profiles are fundamentally different (Table 2). Second, the 6 h profile of up-regulated transcripts includes many ESTs that are also strongly up-regulated at 10 h, yet at 10 h an additional set of transcripts is up-regulated; those transcripts are at background level at 6 h. Also, analogous to what has been reported for salt stress-dependent transcript regulation in rice (Kawasaki *et al.*, 2001), up-regulated transcripts for which no function is known or which are placed into the 'no hit' category are more often encountered in roots than in leaves, possibly reflecting the relative paucity of studies with plant roots. Several highly up-regulated transcripts constitute putative signal transduction intermediates or producers of secondary messengers (other than the jasmonate pathway). Others are known for their defense (including pathogen defense) functions, in scavenging of oxygen radical species or in redox control, and in metabolic adjustments to stress (e.g., nitrogen metabolism [glutamate synthase] and mitochondrial respiration [malate dehydrogenase]). The functions of yet other proteins, should the up-regulated transcripts be translated, remain obscure. This is, for example, the case for the transcript of a water channel protein (similar to BAB09839) because the increased synthesis of a channel that facilitates water movement in drought-stressed roots could be considered counterproductive unless the real function of such channels is different from water transport.

Down-regulation of transcripts upon drought shock

Significantly down-regulated transcripts are compiled in Table 3. From hybridization with RNA from drought-stressed leaves, down-regulation was obvious for a number of basic biosynthetic functions, including photosynthesis and photorespiration, and amino acid and carbohydrate metabolism. Also, a high-affinity potassium transporter, several protein kinases and DnaJ-like transcripts were down-regulated.

Although Tables 2 and 3 provide a glimpse of the many different functions regulated under stress conditions, their role and importance in tolerance or sensitivity is impossible to judge from the limited selection of ESTs that are contained on the microarray slides. With approximately 1400 ESTs, our hybridizations covered about 10% or less of all transcripts expressed in the leaf or root tissues. This number is based on experiments on renaturation kinetics, in which it had been estimated that roots and leaves may express around 8000 transcripts, each with about one third of the transcripts expressed similarly in both tissues (Kamalay and Goldberg, 1980).

The response to salt shock in comparison to drought stress

The results of the hybridization of transcripts from 24 h, 150 mM NaCl-stressed leaf and root tissues showed the fundamentally different stress response programs of these tissues to the two stress factors considered in our study (Tables 4 and 5). There was very little overlap among both the most highly up- or down-regulated transcripts in the two tissues when comparing high salinity and lack of water. Transcripts which are equivalently regulated in drought and salinity conditions are compiled in Table 6.

An enzyme in the biosynthesic pathway leading to jasmonate (allene oxide synthase 1) is among the most significantly up-regulated transcript under both conditions. This may indicate similar pathways related to water deficiency reported through a jasmonate-based signal. A Δ1-pyrroline-5-carboxylate synthetase which largely controls proline *de novo* synthesis is also up-regulated in response to both stresses; in fact, barley accumulates proline during drought and salinity episodes (Stewart and Voetberg, 1985; Stewart *et al.*, 1986). Several other transcripts, and in most cases also the encoded proteins, have been shown to accumulate under abiotic stress conditions (metallothionein-like protein, ERD1, germin-like protein; Lane *et al.*, 1992; Hsieh *et al.*, 1995; Hsieh *et al.*, 1996; Nakashima *et al.*, 1997) and it should not be surprising that they are also reported by our microarray hybridizations. What exactly their functions are, with their up-regulation most likely related to water deficit, is unknown. Comparably down-regulated transcripts under both stress conditions include transport proteins such as a lipid transfer protein and an ABC transporter. Others include an unknown protein for which a rice homologue has been found and

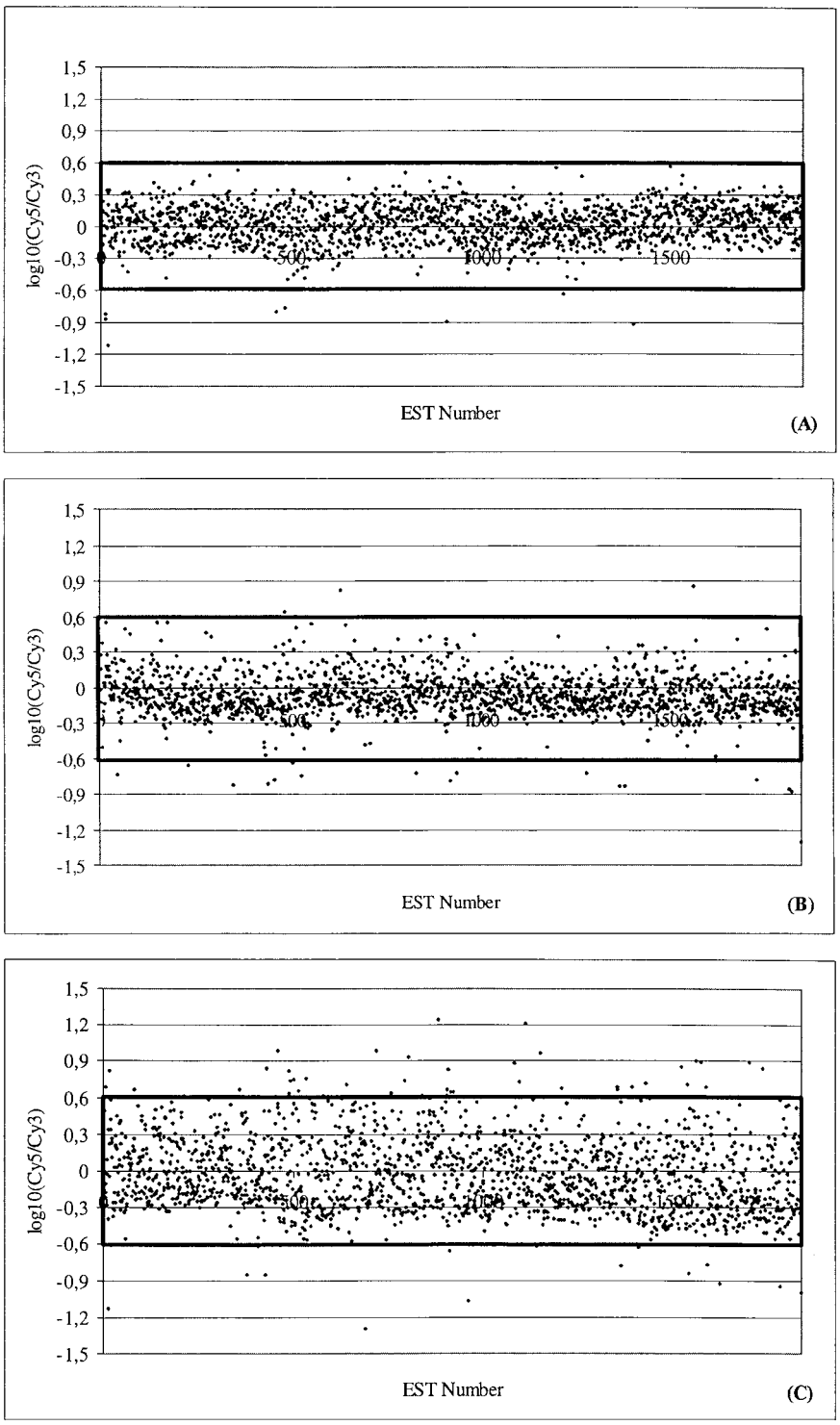

Figure 5. Hybridization ratios on a log10 basis for 24 h 150 mM salt-stressed tissues. A. Leaf tissue *vs.* its control after normalization. B. Root tissue *vs.* its control after normalization. C. Repeat experiment for root tissue *vs.* its control in which mRNA from different extractions was used. Boxed areas indicate the interval (−2.5- to 2.5-fold regulation) in which changes in expression were considered not significant.

this transcript is up-regulated in both rice and barley (Kawasaki *et al.*, 2001). In fact, for transcripts whose sequences are conserved by more than ca. 87% between different grasses, hybridization of barley target first-strand cDNA to rice printed microarray elements produced the same signal as hybridization between homologous probes and targets. Also, a vacuolar processing enzyme of unknown specific function is strongly induced.

Apart from the similarly regulated transcripts listed in Table 6, drought and salinity affect very different transcripts. Regulation differences of genes under different stress conditions indicate the difference of plant stress response to changes in environmental conditions. This difference is not just related to the stress type and conditions but also the species and degree of tolerance of the plant. For this experiment we used a relatively drought-tolerant barley species which also showed high tolerance to elevated salinity. The conditions (24 h, 150 mM NaCl) used here did not severely affect the plants, which had already recovered after 24 h, as also indicated by the microarray analysis. It may be that the pronounced natural salt tolerance of this line is responsible for the relatively small number of regulated transcripts and also for the fact that we did not observe much overlap between drought- and salinity-regulated transcripts.

How closely do microarrays report the induction or repression of transcripts?

As a control for the validity of microarray results, PCR amplifications were carried out for genes that showed up- and down-regulation, respectively (Table 7). Several studies have already shown that a tight correlation exists between the changes reported by RNA-blot hybridization analyses and microarray data (e.g., Ruan *et al.*, 1998; Reymond *et al.*, 2000; Kawasaki *et al.*, 2001; Yale and Bohnert, 2001). One potential problem of microarrays is a relatively narrow dynamic range which is due to the low level of DNA deposited. Thus, abundant transcripts or transcripts that increase dramatically under the experimental conditions chosen may not report true amounts or changes in the amounts, as was seen for several of the highly abundant transcripts shown in Figure 2. Likewise, for rare transcripts, the ratio of change may not reflect reality because signal intensities close to background levels cannot be recorded accurately with the microarrays.

Real-time PCR is a novel technique suitable for simultaneous amplification and detection (Livak *et al.*, 1995). The basic PCR amplification method is used in combination with intercalating dyes, which provide a measure of the amount of double-stranded DNA obtained after each amplification cycle. The data output consists of a baseline, representing an unbound probe that may form double-stranded segments or autofluorescing components during early amplification steps, and a log-linear phase of increasing signal representing the exponential amplification of the desired product. A 'threshold value' at which amplification products of a reaction become detectable provides the basis of quantification. This value depends on the initial amount of target present in the reaction that can thus be calculated. For a robust and reliable quantification of amounts, repeat experiments and a standard curve based on a known amount of target are necessary. Several studies in the laboratory indicated that microarray hybridizations generate data, with respect to regulatory changes, comparable to data obtained by qualitative PCR and RNA-blot analyses (Kawasaki *et al.*, 2001; Yale and Bohnert, 2001; M. Deyholos, C.B. Michalowski, D.W. Galbraith and H.J. Bohnert, in preparation). Microarray analyses tend to report lower differences than the other techniques and in very few instances (e.g., Yale and Bohnert, 2001) results diverge. In those cases, divergence is due to very low transcript abundance.

A tubulin EST was chosen as an example of a transcript that did not change in abundance according to the microarray data during drought stress. For this transcript, *tub2*, both techniques indicate no changes or only non-significant changes (down-regulation) during stress. Differences in repeat experiments, not exceeding a factor of 2, seem to reflect differences in RNA quality and possible systematic errors. In the case of the *tub2* transcript, the differences between PCR and microarray results are not significant and both techniques confirm that transcript amounts for *tub2* do not change under our experimental conditions. The up-regulated *IIs1* (average increase 3-fold by microarray analysis), encoding a cell death suppressor protein, showed much higher up-regulation by quantitative PCR (more than 10-fold) than by microarray hybridization. Since microarray analyses can overestimate differences for transcripts whose signal intensities are close to background values, it is important to consider absolute signal strength. Miscalculation seems not to have been a problem because the signal for *IIs1* in the unstressed state was about 10 times above background and increased during stress, indicating that a real increase was measured.

Finally, *ATLS1*, coding for a light-inducible, down-regulated protein, showed closely matching transcript amounts with both techniques; in both cases down-regulation was observed. These genes were selected because they were unique transcripts based on our EST analysis and all three transcripts belonged to approximately the same abundance class. Likely, other members of presumptive gene families, we argued, would not distort the results. In general, the results obtained with real-time PCR amplification (assuming identical amplification efficiency) agreed with the trend observed in microarray hybridizations. The real-time quantitative PCR amplification technique could thus replace RNA-blot analyses and, once sufficiently miniaturized, might be able to replace microarray hybridizations.

Conclusions

This first microarray analysis of barley transcripts, which are putatively involved in drought tolerance responses, is merely a beginning. We focused on a set of transcripts, which were obtained from cDNA libraries of shock-stressed young barley plants to determine the feasibility of the approach and to obtain data which, we hope, could facilitate future work in this direction. Admittedly, the shock treatment administered here is not comparable to a field situation, and, thus, the results might be of only limited value for crop physiologists and breeders. However, having identified transcripts responsive to shock treatment should provide clues about severe stress situations with a database available for comparison with data from stress conditions as they develop in the field naturally. Even though the collection of transcripts used here was small, we can draw several conclusions from the results.

Drought and salinity stresses affect largely different sets of transcripts. Yet the differences are often in isoforms of transcripts for similarly encoded functions, i.e., the same function seems to be required by the plant to adapt to more than one abiotic stress. The differences then should lie in different activation circuits either through alternative signal transduction, separate transcription factors, and/or altered promoter structures. Disregarding several ESTs in the 'functionally unknown' or 'no hit' categories and known regulated proteins with a general, not well-defined, defense function, the similarities are in a few signal transduction intermediates, among which are members of the *PP2A* and *PP2C* gene families. The *PP2C* induced in our study is homologous to the *Arabidopsis ABI1/2* genes.

One result deserves discussion: the utility of a barley EST microarray for monitoring expression changes in other grass species. For example, in both barley and rice, the transcript for a metabolite facilitator protein (WCP-IV, a putative water channel protein) showed up-regulation under salt-stress conditions. This up-regulation was observed in rice after an adaptation period of several days (Kawasaki *et al.*, 2001) but up-regulation was observed in barley within 24 h after stress imposition indicating, presumably, the relatively higher stress tolerance of the barley line.

Among repressed transcripts in barley and rice, transcripts for a glycosylation enzyme showed very similar behavior. A transcript encoding a metallothionein-like protein (barley EST), different from an up-regulated transcript encoding this function, was down-regulated; this distinct behavior is found in barley and rice (Kawasaki *et al.*, 2001). Also down-regulated in both grasses is the transcript encoding a high-mobility-group protein, HMGd1, whose signal intensity declined by more than a factor of four. Finally, a highly expressed transcript encoding a peroxidase, ATP9a, was equally down-regulated. For all the outlined examples, the transcripts showed signal intensities that were significantly above background such that the systematic error could be considered negligible.

Even more pronounced is the salinity stress-dependent concomitant up- and down-regulation in barley and rice. The most highly regulated transcripts in both species are a metallothionein-like protein, β-glucosidase, glutathione *S*-transferase (auxin-induced), *S*-adenosylmethionine decarboxylase, and a ribosomal protein 40S and elongation factor 1-α. Coincident regulation was also observed for significantly down-regulated transcripts in both grass species: a nucleoside diphosphate kinase III precursor, aminotransferase, and sucrose synthase 2.

When focusing on the drought-induced transcripts in barley roots and leaves, we list the roughly 100 most strongly up-regulated ones, although more transcripts are included in our complete analysis. Among those, about half are functionally unknown (and a number of these transcripts have not been reported as drought-induced). In a global sense, ca. 20% of the transcripts included in our microarray are either up- or down-regulated significantly in this population of relatively abundant transcripts from non-subtracted

cDNA libraries. Is such a small array sufficient for providing meaningful information from a breeding standpoint? Clearly, we have cloned a large number of the barley transcripts that had already been identified as drought-stress induced. Those and the additional drought-regulated transcripts will now be analyzed with target RNAs from barley plants and breeding lines that experienced natural drought conditions. It may be that we will need additional ESTs that are selected from the transcript profiles from different developmental stages, and that we will need to add transcripts for selected stress sensing, signaling, and/or biochemical pathways to better understand drought through microarray analysis and the correlative quantitative transcript profiles (CQTPs) which, coupled with an appropriate QTL analysis, could possibly lead to the identification of candidate genes for agronomically valuable traits.

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