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BIOTIC AND ABIOTIC STRESS

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# Changes in gene expression in maize kernel in response to water and salt stress

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**Abstract** Increasing pressure on limited water resources for agriculture, together with the global temperature increase, highlight the importance of breeding for drought-tolerant cultivars. A better understanding of the molecular nature of drought stress can be expected through the use of genomics approaches. Here, a macroarray of ≈2500 maize cDNAs was used for determining transcript changes during water- and salt-stress treatments of developing kernels at 15 days after pollination. Normalization of relative transcript abundances was carried out using a human nebulin control sequence. The proportions of transcripts that changed significantly in abundance upon treatment (>2-fold compared to the control) were determined; 1.5% of the sequences examined were up-regulated by high salinity and 1% by water stress. Both stresses induced 0.8% of the sequences. These include genes involved in various stress responses: abiotic, wounding and pathogen attack (abscisic acid response binding factor, glycine and proline-rich proteins, pathogenesis-related proteins, etc.). The proportion of down-regulated genes was higher than that for up-regulated genes for water stress (3.2%) and lower for salt stress (0.7%), although only eight genes, predominantly involved in energy generation, were down-regulated in both stress conditions. Co-expression of genes of unknown function under defined conditions may help in elucidating their roles in coordinating stress responses.

**Keywords** Expression analysis · Macroarray · Salt and water stress · Zea mays L

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## Introduction

Environmental abiotic stresses, such as drought, extreme temperatures, or high salinity, severely compromise plant growth and development. Water availability is one of the major factors affecting crop-yield worldwide. Many irrigated areas are poorly drained so that additionally, serious problems of waterlogging, alkalinization and soil salinity are frequent.

Research on the two major abiotic stresses, drought and salinity, have much in common. Salinity reduces the ability of plants to take up water which quickly causes reductions in growth rate along with metabolic changes identical to those caused by water stress. Unfortunately, the complexity and polygenic nature of drought and salt stress tolerance make it difficult to select these characters in conventional breeding programs.

Abiotic stresses induce morphological, biochemical and physiological changes in plants during the acquisition of stress tolerance. At the cellular level, water deficit may cause cellular damage or initiate adaptive responses (Cellier et al. 1998). The products of stress-inducible genes can be classified into two groups (Bray 1997; Hasegawa et al. 2000).

– Genes that directly protect against stress.
– Genes that regulate gene expression and signal transduction in the stress response.

A large number of genes have been linked to stress response pathways although their precise functions often

remain unclear (Zhu 2000). Many salt-responsive genes do not increase tolerance, but induce stress damage and genes important for salt tolerance may not be expressed during salt stress. However, the genomic drought and salt stress responses both reflect the necessity for cellular protection by free-radical scavengers, chaperonins, and regulators of redox and osmotic potential (Hasegawa et al. 2000). A more complete understanding of the genes that promote cellular and whole plant tolerance to stresses is necessary for targeting loci for genetic improvement. New powerful tools, such as DNA microarray/macroarray analysis, provide high-throughput simultaneous analysis of thousands of genes. Arrays can be "microarrays" (spot size smaller than 200 μm in diameter; fabricated on glass slides or chips; Hauser et al. 1998) or "macroarrays" (spot size over 300 μm in diameter; on polypropylene membranes; Hauser et al. 1998). Most microarrays used today fall into two groups based on the characteristics of the probes: (a) cDNA probes that are products of polymerase chain reactions (PCR) and are <200 nucleotides in length; (b) oligonucleotides of maximum 80 nucleotides, which have greater specificity of gene family.

Massive expression data correlating with changes in plant physiological processes can be used as a clue to gene function. There are several potential applications of expression data:

1. Identification and mapping a large number of ESTs that provide candidate genes for QTL analysis (Davies et al. 1999). Expression levels could be analyzed one by one separately as any quantitative trait (Schadt et al. 2002; Kirst et al. 2003).
2. Construction of functional maps of ESTs sharing similarity in function or pathway (Schneider et al. 1999).
3. Detection of a pattern of expression that is connected with a particular trait, with some data reduction. Peréz-Encisco et al. (2003) pointed out that microarray data are more useful for analyzing monogenic traits, while polygenic traits are difficult to interpret due to the numbers of transcripts affected. Candidate genes for yield and other traits for crops growing under different stresses have been reported (Seki et al. 2001; Ozturk et al. 2002). Talamé et al. (2003) showed that the expression of a large number of transcripts was statistically significantly different under water-shock and water-stress treatments. This suggested that changes in expression level obtained with rapid, shock stress give information about changes occurring under semi-arid and arid field conditions. Using more gradual and thus more authentic water stress treatments, we have shown changes in expression of fewer transcripts and with a lower intensity ratio between stress and control treatments (data not published).

The goals of this work were to identify genes whose expression in maize kernel was affected by salt and water–shock stresses and to indicate those promising for marker-assisted selection and breeding for drought tolerance, by analysing their expression profiles.

## Material and methods

### Plant material and stress treatments

Maize (*Zea mays* L. A188, provided by the Istituto Sperimentale de la Cerealicoltura, Bergamo, origin Maize Genetics Cooperative, provenance Charles Burnham, University of Minnesota) plants were grown under green-house conditions with 16-h light period (300 μE $m^{-2}$ $s^{-1}$, 21–25°C) and 8-h dark period (15°C). Plants were irrigated daily and fertilized once a week. Self-pollinated ears were harvested at 12 DAP. Stalks of maize plants were cut 10 cm below and above the ear, leaving the ear leaf. Progressive drought stress was initiated by dehydration treatment: stalks, together with ears were weighed and placed on filter paper under the same greenhouse conditions. The relative weight of 90% of initial weight was reached after 3 days of dehydration treatment.

For salt stress at 12DAP, cobs were removed from the plants in the same way as for the dehydration treatment and partially submerged in sterile liquid medium. The medium was modified according to Donovan and Lee (1977) and Cully et al. (1984). The composition for 1 l of medium was 220 mg $CaCl_2 \cdot 2H_20$, 340 mg $KH_2PO_4$, 370 mg $MgSO_4 \cdot 7H_2O$, 28 mg $FeSO_4 \cdot 7H_2O$, 37.3 mg $Na_2EDTA \cdot 2H_2O$, 6.2 mg $H_3BO_3$, 16.9 mg $MnSO_4 \cdot 2H_2O$, 8.6 mg $ZnSO_4 \cdot 7H_2O$, 0.83 mg kJ, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$, 0.025 mg $CuSO_4 \cdot 5H_2O$, 0.025 mg $CoCl_2 \cdot 6H_2O$, 0.4 mg thiamine HCl, 100 mg myo-inositol and 30 g sucrose; amino acids L-alanin (1 g; 11.2 mM), L-aspartic acid (0.5 g; 3.7 mM), L-asparagin (1.5 g; 11.4 mM), L-glutamic acid (0.9 g; 6.1 mM), L-glutamine (2.5 g; 17.1 mM), L-leucine (1.0 g; 7.6 mM), L-proline (0.5 g; 4.3 mM). For salt stress 150 mM NaCl was added to the medium and sampled cobs were incubated for 3 days under greenhouse conditions. The pH of both media was adjusted to 5.8.

For the control samples, cobs with 10 cm of stalk, were removed from the plants and immediately transferred to the fresh culture medium without 150 mM NaCl for 3 days. The kernels from control and stressed treatments were sampled at the same time (e.g. 15DAP) for RNA extraction. Pooled samples for every treatment were frozen in liquid nitrogen and stored at −80°C for further analysis.

### Total RNA isolation and probe preparation

Total RNA for array and RNA blot was isolated from 3–5 g of kernels from 15 DAP using Perfect RNA$^{tm}$ kit according to instructions in the manual (Eppendorf Scientific, Inc. Hamburg, Germany). An additional re-precipitation at the end of the extraction procedure was carried out.

A mixture of maize total RNA (25–30 μg), human nebulin RNA (~2 ng) as a spiking control and oligo $(dT)_{15}$ (500 ng) was denaturated at 70°C for 10 min, chilled on ice and equilibrated at 43°C for 5 min.

Reverse transcriptions were performed at 43°C for 1 h by adding 6 µl of Superscript buffer (GibcoBRL), 3 µl of 0.1 M DTT, 3 µl of 10 mM [dATP, dGTP, dTTP], 3 µl of 50 µM dCTP, 3 µl [$\alpha^{33}$P] dCTP 30µ Ci and 1 µl of Superscript II RT (200 U/µl, GibcoBRL). Hydrolysis of the RNA was started by adding 1 µl of 1% SDS, 1 µl of 0.5 M EDTA and 3 µl of 3 M NaOH and incubation at 65°C for 30 min, followed by 15 min at room temperature. In order to neutralize the reaction 10 µl of Tris-HCl (pH 8) and 3 µl of 2 N HCl were added. cDNAs were precipitated after addition of 5 µl of 3 M sodium acetate (pH 5.3), 5 µl of yeast tRNA (10 mg/ml) and 60 µl of iso-propanol at −20°C for 30 min. After centrifugation and determination of the incorporation by scintillation counter, dried pellets of labeled cDNA were resuspended in 100 µl of sterile water.

## Library amplification and preparation of DNA macroarrays

### *Maize Full Length cDNA Macroarray*

Clones for the production of filter arrays were obtained from Michael McMullen, Theresa Musket and Ed Coe (University of Missouri), Riccardo Velasco, Antonio Serna and Heinz-Albert Becker (Max-Planck Institute, Cologne, Germany, http://www.mpiz-koeln.mpg.de/~riehl/ArrayDB/MzArrayDB.htm). The library was constructed from different unstressed maize tissues. In total, about 2500 maize ESTs with known or putative transcript-coding capacity were PCR amplified in a 96-well format in 100 µl reaction volumes by using primers complementary to the vector sequences flanking both sides of the cDNA insert. The PCR products were electrophoresed on agarose gels to confirm amplification quality and quantity, and spotted onto the membrane using a robotic spotting device (BioGrid robot Biorobotics, UK). Reproducibility of the experiment was achieved by arraying each cDNA clone twice per array and by repeating the same experiment three times. To check the sensitivity of the detection system, genes uidA and a ribosomal cistron (pTA71) were used as internal controls. pBluescript plasmid probe was used to assess hybridization to the cloning vector; and cDNA clones encoding human nebulin and desmin, which have no homology in maize, were employed as negative controls. For some of the so-called housekeeping genes, expression levels varied with different experimental treatments, but for the gene pTA71, as confirmed by Northern blot (Figs. 1b, 2b and 3b), expression was invariant. A human nebulin cDNA was spotted in four different concentrations on the filter. After hybridization with synthetic nebulin cDNA, we chose a concentration which has a coefficient of linearity ~1 ($R^2$=0.998) to the increased percentage of nebulin (data not shown). About 2 ng of spiking control was incorporated into sample RNA and therefore the ratio (w/w) was 1:15000. Normalization of each filter was performed by using the mean signal intensity of nebulin as a non-plant control.


**Fig 1.** **a** Comparison of cDNA macroarray and RNA gel blot analysis for clone 5C04E06 in dehydration and salt stress conditions: line 1—control; line 2—water stress (macroarray value stress/control = 17.7); line 3—salt stress (macroarray value stress/control = 15.9); **b** Loading control pTA71. Clones are listed according to *F*-value

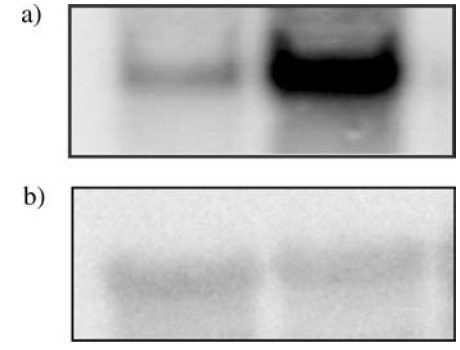


**Fig 2.** **a** Comparison of cDNA macroarray and RNA gel blot analysis for clone 1785 in salt stress condition: line 1—control; line 2—salt stress (macroarray value stress/control = 9.7); **b** Loading control pTA71. Clones are listed according to *z*-value

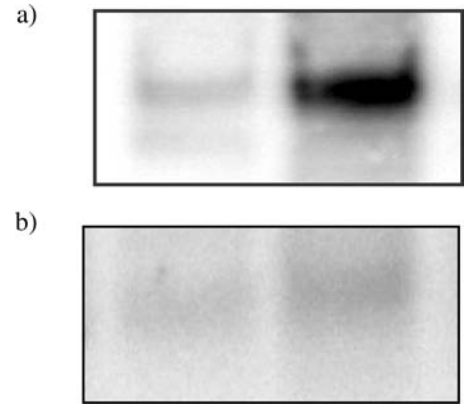


**Fig 3.** **a** Comparison of cDNA macroarray and RNA gel blot analysis for clone 1387 in dehydration stress condition: line 1—control; line 2—water stress (macroarray value stress/control = 5.7); **b** Loading control pTA71. Clones are listed according to *z*-value

As controls, DNA fragments of pBluescript, uidA, pTA71genes as well as cDNA human desmin and nebulin were spotted at four different concentrations, and in multiple locations on the filter.

Arrays were prepared on 22.2 $cm^2$ (six field areas of 8 $cm^2$ × 12 $cm^2$) nylon membranes (Hybond $N^+$, Amersham), which were pre-wetted under denaturing conditions (1.5 M

NaCl; 0.5 M NaOH). The BioGrid robot (Biorobotics, UK) produced DNA spots in duplicates in a 4×4 pattern. After spotting, filters were neutralized (1 M Tris pH 7.6; 1.5 M NaCl) and DNA was fixed to the membrane by UV radiation at 120,000 μJ/cm$^2$ for 30 min using Stratalinker (Stratagene, Netherlands).

### Hybridization and data analysis

Prehybridisation was done for 0.5–2 h at 65°C in 20 ml of Church buffer (0.5 M $Na_2HPO_4$, pH 7.2; 7% SDS; 1 Mm EDTA) including 200 μl of denatured salmon sperm DNA (10 mg/ml).

The probe solution was boiled for 5 min and then rapidly applied to the filters and hybridization was carried out overnight (at least 10 h) at 65°C. Washing was done by briefly rinsing at room temperature and by incubations of 2×30 min at 65°C in a washing solution (40 mM $Na_2HPO_4$, pH 7.2 and 0.1% SDS). Subsequently the filters were wrapped in Saran Wrap, exposed to a phosphor screen overnight and scanned using Image Quant software and a Storm PhosphorImager (Molecular Dynamics).

The image analysis, quantification of signal intensities and first normalization by the average signal of all spots, were done by using the Array Vision 5.0 software (Imaging Research, Canada).

Statistical analysis of the data obtained was performed in two steps by Array Stat software (Imaging Research, Canada):

1. We chose 'Automatic model selection for independent conditions' with minimum two (of four) valid observations required. It performed offset corrections by normalization across replicates. According to the relationship of the means to their standard deviations, the 'Pooled: Curve fit' or 'Pooled: Common error' estimation method was performed. Outliers were detected and removed automatically prior to further analysis .The first step yielded a table of cleaned-up data without outliers.
2. Normalization across conditions was performed by 'Mean of reference values' (human nebulin). For 'Independent data with two conditions' the z-test was applied with a *p*-value 0.05 (5% probability of making false positive error) and 'Bonferroni method' for multiple test correction.' For tree independent conditions an *F**-test was performed with a *p*-value 0.05 and 'Step-down Bonferroni' as a multiple test correction method. Reported output colored in blue data from genes exhibited significantly different expression levels.

### RNA gel-blot analysis

Total RNA (10 μg) from control and stressed kernels was electrophoresed through 1.0% agarose/formaldehyde gels and capillary-transferred to Hybond N$^+$ membrane according to the manufacturer's protocol (Amersham Pharmacia Biotech). The filters were hybridized overnight at 42°C with radiolabled probes synthesized using RediPrime DNA labeling system (Amersham) and [$^{32}$P] dCTP in hybridization buffer (5× SSC, 5× Denhardts, 50% formamide, 1% SDS and 100 μg/μl of salmon sperm DNA). After hybridization, filters were washed twice in 2× SSC, 0.1% SDS and in 0.2× SSC, 0.1% SDS for 5 min at room temperature and once in 0.2× SSC, 0.1% SDS for 15 min at 42°C. Detection was carried out using a phosphorimager and data analysis by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Each filter was stripped and re-hybridized with RNA probe pTA71 as a loading control.

## Results

Identification of water- and salt-inducible genes with the cDNA macroarrays cDNA macroarrays were hybridized with probes from water and salt-stressed maize kernels and from unstressed samples, prepared as described in Material and Methods. Identification and quantification of each signal was performed using commercial software. An average signal of duplicate spots was used for the analysis. Adjustment of signal intensities with respect to that of the exogenously added control gene (normalisation) was carried out using Arraystat software. A first criterion for selection of genes of interest was according to statistically significant difference in expression level between stress and control conditions. For the chosen genes we calculated the ratio of signal intensity from the experimental conditions.

Two independent experiments were carried out, with induction by both stresses, and a ratio stress/control >2 was identified for 20 genes (Table 1).

These genes encode proteins with a protective function (e.g. HSP and chaperonins, proline and glycine-rich proteins); proteins responsible for plant defense (pathogenesis-related protein, thaumatin) and detoxification (metallothioinen and peroxidase). For some genes, induction by stress has been previously confirmed, although not always with a clear function, such as for a cytochrome P450 monooxygenase, light-harvesting chlorophyll a/b binding protein, alpha-keto glutarate dehydrogenase, auxin-induced protein, and ABA response-binding factor (Seki et al. 2002). Partial overlapping of transcripts in response to drought and salt stress could be due to overlap in early responses to these stresses (Munns 2002).

Salt stress induced 37 genes identified by the cDNA macroarray analysis (Table 2). Up-regulation of transcripts NaCl-inducible, salt-tolerant, ABA and stress inducible, ABC transporter was reported in expression study of salt stress in rice (Kawasaki et al. 2001). A group of salt-induced transcripts has a putative protective function (jasmonic-inducible, monooxygenase, zinc-finger, superoxide dismutase); a number of salt up-regulated transcripts is important for plant growth and morphogenesis such as extensin-like proteins, auxin-induced genes, and those related to the tricarboxylic acid cycle which contribute to ATP production (Umeda et al. 1994). Members of the tubulin family, heat shock-related proteins, and translation initiation factors are also induced in our study, but were reported to be down-regulated in rice.

**Table 1** Up-regulated transcripts during dehydration and salt stress

| Genbank accession | Clone no. | Homology | Salt/control | Dehydr/control | *F* value |
|---|---|---|---|---|---|
| Q9SBM1 | 1813 | Hydroxyproline-rich glycoprotein | 16.7 | 20.7 | 52.7 |
| T15294 | 5C05E10 | Metallothionein | 15.7 | 2.1 | 25.5 |
| Q7Y1Y5 | H39 | Auxin-induced protein | 7.0 | 11.9 | 15.4 |
| T14723 | 5C04E06 | Glycine rich protein | 16.0 | 17.6 | 15.0 |
| BI993305 | px60 | Proline-rich protein | 10.7 | 2.4 | 12.3 |
| T18326 | 5C06H12 | Chloroplast ATP-binding chaperonin | 8.2 | 4.5 | 9.9 |
| T18416 | 6C02E07 | Vacuolar ATPase B subunit | 3.9 | 3.6 | 5.6 |
| T18391 | 6C02B05 | Cytosolic glyceraldehydes 3 phosphate | 5.2 | 4.1 | 4.9 |
| T18823 | csu257 | Plastocyanin | 6.2 | 3.1 | 4.7 |
| U64437 | csu48 | Novel protein/elicitor-responsive gene | 3.9 | 3.1 | 4.6 |
| T14680 | 5C04B08 | Glyceraldehyd proteophosphoglycan | 3.3 | 2.6 | 4.1 |
| T14732 | 5C04F07 | Pathogenesis-related protein | 3.0 | 2.1 | 4.1 |
| T18386 | 6C02A09 | Alpha-ketoglutarate dehydrogenase | 3.6 | 3.0 | 3.9 |
| T20359 | 6C01G02 | Cytochrome P450 monooxygenase | 4.6 | 2.7 | 3.7 |
| T18833 | csu274 | Heat shock protein 90 | 2.7 | 3.2 | 3.2 |
| BT018008 | 1891 | Cyclin-dependent protein kinase | 2.6 | 3.1 | 3.0 |
| T15328 | 6C06C07 | Ccatalase isozyme 3 | 3.1 | 2.3 | 2.7 |
| M18976 | csu104 | Light-harvesting chlorophyll a /b protein | 2.6 | 2.2 | 2.6 |
| AJ867403 | 1895 | Glycosyltransferase peroxidase | 3.0 | 3.2 | 2.4 |

A list of significantly drought-induced transcripts in maize kernels is given in Table 3. The DRE (*dehydration-responsive element*) is a conserved sequence important for induction of gene expression under drought conditions (Yamaguchi-Shinozaki and Shinozaki 1994), and has been identified as up-regulated under water stress in this study. Expression of transcripts RAB 8 (*related to ABA*), and many abscisic acid and water stress-inducible proteins are regulated via ABRE (*ABA-responsible elements*) (Bray 1997; Ingram and Bartels 1996). The identification of a group of transcripts with putative protective functions in the response to dehydration stress confirms the validity of our approach.

## Genes down-regulated by salt and water stress

Whilst numerous salt and water-stress-induced genes have been analysed, currently less attention has been paid to down-regulated genes. Transcripts significantly down-regulated in dehydration and drought stress include those responsible for plant protection from oxidative stress and for energy production in cells (Table 4). In a comparative study of salt and water stress, photosynthesis decreases in both stresses with the time of treatment (Munns 2002). Osmotic stress predominantly limits plant growth, but in salt-sensitive species there is also a gradual loss of capacity to produce photoassimilate. Mosst of the 16 transcripts down-regulated during salt-stress are responsible for assimilate production and plant growth, with a few encoding enzymes involved in detoxification and cell defense (Table 5). The number of transcripts down-regulated in response to dehydration was less than that seen during salt stress, with most of them having basic biosynthetic functions, mainly in carbohydrate metabolism (Table 6). These results are consistent with previous reports that water stress inhibits photosynthesis (Tezara et al. 1999; Ozturk et al. 2002).

## RNA gel blot analysis

Analyses of gene expression are conventionally performed by RNA blot hybridizations, RNase protection assays and quantitative reverse transcription-polymerase chain reactions (QRT-PCR). DNA microarrays are superior to these methods in terms of the number of genes that can be simultanously analysed. However, the sensitivity of microarray transcript measurements is slightly inferior to Northern blot analyses and to QRT-PCR (Taniguchi et al. 2001). To confirm the validity of cDNA macroarray results, we performed Northern blot analyses on randomly selected clones. Among 20 up-regulated genes, only 5 had a ratio stress/unstressed in both conditions significantly high (>4). As an example of an up-regulated transcript macroarray results of clone 5C04E06, encoding a glycine-rich protein are confirmed and presented in Fig. 1.

Salt stress induced 37 genes, but most of them had a ratio stress/control of between 2 and 3. One exception, clone No 1785, encoding an NaCl-inducible $Ca^{2+}$ binding protein had a ratio stress RNA/control RNA of 9.7 (Fig. 2.).

Dehydration stress induced for the most part genes specifically expressed under this condition and almost all of them had high ratios of stress/unstressed transcript concentrations. As an exmple, Clone No 1387, encoding a *LEA* protein, was up-regulated in response to dehydration ( Fig. 3).

In general, the macroarray analysis and RNA gel blot data confirmed the same trends in gene expression. Weak

**Table 2** Up-regulated transcripts during salt stress

| Genbank accession | Clone no. | Homology | Salt/control | *z* Value |
|---|---|---|---|---|
| X57077 | 2389 | Histone H1 | 8.7 | 13.1 |
| X03658 | 1828 | HSP70, MADS-domain transcription factor | 4.2 | 10.2 |
| T15321 | 6C06B09 | Alpha-6 tubulin | 4.4 | 8.2 |
| T18407 | 6C02D05 | Alpha-3 tubulin | 4.4 | 7.3 |
| T15324 | 6C06C01 | Male sterility protein 2 | 4.4 | 6.7 |
| AB039929 | 1014 | Early-responsive to dehydration stress | 2.8 | 6.1 |
| X13499 | 1953 | Antifreeze glycopeptide AFGP | 2.6 | 5.7 |
| NM_196259 | 1886 | Glyceraldehyd cytidylyltransferase | 2.9 | 5.7 |
| W2166 | csu960 | ATP synthase delta chain mitochondrial precursor | 2.1 | 5.0 |
| CAA65502 | pxa2 | Isocitrate dehydrogenase | 3.0 | 4.9 |
| Y10814 | 1456 | Defense-related protein | 7.1 | 4.7 |
| T70662 | 7C02F04 | Dolichyl-di-phosphooligosaccharide-protein | 3.0 | 4.7 |
| X13499 | 2398 | Cell wall glycoprotein | 2.1 | 4.5 |
| O22514 | 1165 | Proline rich protein | 2.7 | 4.4 |
| T20361 | 6C01G06 | Putative senescence-associated protein | 3.7 | 4.3 |
| T25279 | 6C04E06 | Alpha-2 tubulin | 2.1 | 4.2 |
| X68682 | 1190 | Light-harvesting chlorophyll a /b-binding protein | 3.1 | 4.2 |
| Z54153 | 2379 | Acyl-CoA synthetase | 3.4 | 4.1 |
| T18784 | csu200 | Zinc finger protein 232 | 2.2 | 4.1 |
| Q8ZQY2 | 1847 | Monooxygenase | 2.5 | 4.0 |
| W49910 | csu399 | Alpha-1 tubulin | 3.3 | 3.6 |
| AAC2825 | px50 | Translation initiation factor 4B | 2.8 | 3.6 |
| T12739 | csu146 | FtsH protease, putative; cell division protein | 3.2 | 3.3 |
| X99517 | csu154 | Eukaryotic translation initiation factor 5 | 2.9 | 3.4 |
| CD435579 | 1671 | Maltose-binding protein | 2.1 | 3.3 |
| X16084 | csu375 | NADP-malate dehydrogenase chloroplast precursor | 2.5 | 3.3 |
| AY536122 | px57 | FLAVIN reductase /glycerol dehydrogenase | 3.3 | 2.8 |
| T70673 | 7C02H02 | Superoxide dismutase mitochondrial precursor | 2.2 | 2.6 |
| AA054808 | csu630 | ABA- and stress inducible-like protein | 2.2 | 2.4 |
| T18827 | csu265 | Cycloartenol synthase | 2.5 | 2.4 |
| AB060277 | 1306 | Putative glucose-6-phosphate | 3.0 | 2.3 |
| T18686 | 5C04E09 | Glyceroldehyde-3-phosphate dehydrogenase | 2.2 | 2.2 |
| NM_197152 | 1208 | Microtubule-severing protein subunit | 2.1 | 2.1 |
| AY300530 | csu572 | Phytoene synthase | 2.4 | 2.1 |
| T14750 | 5C01C04 | Cyclophilin 2 | 2.2 | 2.0 |
| T18321 | 5C06H09 | Exonuclease RRP41 | 2.1 | 1.9 |

expression or low specific activity of cDNA probes (Seki et al. 2001) could account for differences reported between macroarray results and RNA gel blots (Taniguchi et al. 2001) or QRT-PCR (Desprez et al. 1998; Kurth et al. 2002).

## Discussion

Low and high temperatures, salinity and water availability severely reduce grain yields in agricultural systems. In maize, drought at pollination induces yield reductions 2–3-fold higher compared to drought periods at other developmental stages (Grant et al. 1989). Early plant responses to environmental stresses include gens involved in perception of the environmental change and signal transduction to initiate biochemical and physiological responses, together with expression of the genes responsible for these responses. Several techniques for gene-expression analysis are available, such as Northern blotting, differential display, serial analysis of gene expression (SAGE), dot blot analysis. The main disadvantage for all of them is the limited number of genes that can be studied at the same time. Recently, an improvement in throughputs of expression studies has become available with cDNA microarray anal-

**Table 3** Up-regulated transcripts during dehydration stress

| Genbank accession | Clone no. | Homology | Dehydr/control | *z* Value |
|---|---|---|---|---|
| TC271114 | csu393 | Chloroplast drought-induced stress protein, 34 Kd | 40.6 | 24.0 |
| W49866 | csu336 | Abscisic acid- and stress-induced protein | 23.6 | 17.3 |
| AC000106 | 1317 | Water stress protein | 17.5 | 13.3 |
| T18666 | 5C02G05 | ABA—and H2O-inducible | 15.7 | 10.9 |
| U09276 | 1871 | ABA and ripening-inducible protein | 10.4 | 6.1 |
| Y11029 | 2059 | Glutathion deppendant formaldehyde dehydrogenase | 9.7 | 5.9 |
| AF033496 | 1112 | Herbicide safener binding protein | 9.0 | 5.4 |
| U41103 | 1519 | Ethylene-response protein | 8.8 | 5.7 |
| AY105489 | 1480 | Kinase-like protein | 8.7 | 4.2 |
| Z54153 | 2495 | Disease resistance protein | 8.7 | 5.6 |
| Q6H660 | pxa30 | Stress-induced protein sti1 –like protein | 8.6 | 5.6 |
| T18813 | csu234 | Ras-related protein RAB8-4 | 8.5 | 5.6 |
| U74296 | 1277 | Water stress inducible protein | 8.5 | 5.6 |
| W49439 | csu1140 | Calcium-binding protein; calreticulin | 8.1 | 5.5 |
| AF244682 | 2202 | Glutathione *s*-transferase | 7.7 | 5.3 |
| T18632 | 5C07B01 | Heat shock protein, 82 kDa | 7.6 | 5.2 |
| AF493800 | 1955 | Zea mays DRE binding factor 1 | 7.2 | 4.8 |
| T18793 | csu211 | Glycine-rich protein | 6.9 | 4.7 |
| T14700 | 5C04D01 | 70-kDa heat shock protein | 6.8 | 5.4 |
| AY111746 | 1475 | Copper chaperone -related protein | 6.6 | 4.9 |
| D88451 | 1430 | Aldehyde oxidase | 6.6 | 4.8 |
| AJ012301 | 1838 | Proline-rich protein | 6.5 | 4.6 |
| T18789 | csu205 | Glyceraldehyd transfer protein | 5.9 | 6.5 |
| D26552 | 1387 | Group 3 Lea protein MGL3 – maize | 5.8 | 4.5 |
| T27550 | csu352 | ATP synthase | 4.1 | 3.6 |
| T18440 | 6C02G12 | Putative disease resistance protein | 2.1 | 2.2 |

ysis. Recently, accurate and efficient methods for the large scale analysis of gene expression have been developed.

1. Sequence based (SAGE-serial analysis of gene expression, Velculescu et al. 1995; MPSS-massively parallel signature sequencing, Brenner et al. 2000)
2. Fragment based (cDNA-AFLP, Bachem et al. 1996; geneCalling, Bruce et al. 2000) or
3. Hybridization based (macro- and micoarray, Schena et al. 1995; Lockhart et al. 1996).

DNA-microarray technology permits the identification of a number of genes that are induced or suppressed by environmental stresses.

In this report, macroarray technology has been used to analyze expression profiles of 2500 clones in the maize kernel during water and salt stress. We have identified 20 dehydration and salt-inducible genes, and 37 induced solely by salt stress and 26 specifically dehydration-induced genes. Most of these genes and their expression patterns have been previously characterized as stress-inducible in *Arabidopsis*, rice or barley. The primary response to water and salt stress is essentially identical (Munns 2002) and in our study 0.8% of the transcripts were induced by both stresses.These shared expression patterns imply a common protective mechanism. Synergism in the action of ABA and osmotic stress on endogenous gene expression was previously reported by Xiong et al. (1999). Dehydration and salt stress also induced a number of stress-specific transcripts. Thirty-seven genes were induced only by salt and 26 only by water-stress in the present study.

Gene expression varies with the duration and intensity of stress. We applied salt-shock stress only to initiate a plant response. Among the transcripts up-regulated in salt stress, most of them are related to water stress, rather than specifically to salt stress (Tables 1 and 2). Initiation of water stress-related genes soon after salt-induced genes and the specific effect of salt application over a longer time have been reported by Munns (2002).

Two types of controls for data quality:

1. Biological—we used the same experimental conditions for all plants and each treatment; kernels were collected in parallel fashion from three cobs within salt, water-stress and control conditions.
2. Array—we applied relatively rigorous criteria for selecting differently expressed genes. (a) Usage of mean

value of duplicate spots from the filter; b) normalization by mean value of all signals to eliminate background effect; (c) normalization by mean value of spiking control human nebulin; d) statistical test enabling extraction of genes; (e) ratio stress/control was calculated only for transcripts that showed statistically differential expression. We presented and discussed transcripts with ratio stress/control >2 from triply replicated experiments. The expression pattern was confirmed by other reports for several stress-inducible genes (Kawasaki et al. 2001; Ozturk et al. 2002) and by Northern blotting in our experiments.

Our experiments (unpublished) on withholding water from maize plants from 5–15 DAP and results of Talamè et al. (2003) showed that water stress affected a lower number of transcripts compared to control conditions. Considering that, we applied intense and rapid dehydration to induce and predict changes that occur during drought.

Here we focused on a set of transcripts induced by stress treatment, approximating to, if not identical to, field conditions. The transcripts identified in response to shock treatment provide a clue about metabolic changes and reactions of plants during drought and salt stress. However, in field conditions one stress factor is rarely present alone: water shortage is generally accompanied by high temperature and increased soil salinity. It is necessary to examine the plant response to simultaneous stresses that will be more comparable to real conditions. The results presented here confirmed that expression analysis by macroarray could be used for identification of stress-inducible genes in maize.

Due to their size, large proportion of repetitive sequences and complexity, crop genomes present challenges for genome-wide analysis. Whereas a complete genome sequence is available for the model species *Arabidopsis*, for most crops gene discovery is based on sequencing of ESTs with its numerous limitations, such as cross-hybridization between related sequences and members of small gene families. The *in silico* identification of TUGs (*tentative unique genes*) in maize (Fernandes et al. 2002) and the use of synthetic oligonucleotide microarrays are two landmarks that will help in defining regulatory and metabolic pathways involved in response to environmental stresses, an effort that will require a complex systems analysis to finally reveal the biochemical and physiological basis of stress response in plants.

So far, expression data are mostly being used *per se*, but correlated with a complex trait could be used to improve QTL mapping (Peréz-Enciso et al. 2003). Usage of new tools for assigning function to genes and to improve adaptabilty of crops is an important challenge for breeding purposes.

Major progress in microarray analysis has provided made possibilities for more practical application of the results (Kawasaki et al. 2001; Ozturk et al. 2002) including the possibility of identifying TQL (transcript quantity loci) and to connect with QTL of interest or to associate clusters of genes with QTLs (Khavkin and Coe 1997; Tuberosa et al. 2002).

The rapid improvement in proteomics and metabolomics e.g. co-localisation between PQL (protein quantitative loci) and QTL (de Vienne et al. 1999) is another promising example for applications in breeding. Further studies combining genomics and genetics, proteomics and MAS (marker-assisted selection) are opening possibilities for crop improvement for better performance in stress environments.

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