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ESTs analysis in maize developing kernels exposed to single and combined water and heat stresses

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ABSTRACT: Molecular and metabolic response of plants to a combination of two abiotic stresses is unique and cannot be directly extrapolated from the response of plants to each of the stresses individually. cDNA macroarray has become a useful tool to analyze expression profiles and compare the similarities and differences of various expression patterns. A macroarray of approximately 2,500 maize (*Zea mays* L.) cDNAs was used for transcriptome profiling in response to single and simultaneous application of water and high temperature stress of maize developing kernels at 15 days after pollination. All stress treatments (water stress-WS, heat stress-HS and their combined application-CS) induced changes in expression of 106 transcripts with 54 up-regulated and 52 down-regulated. There were 11 up-regulated and 15 down-regulated transcripts in common for all three stresses. Although these common transcripts showed existence of a mutual mechanism in stress response, the 23 transcripts induced only in CS indicate that plants responded in a different manner when exposed to simultaneous effects of both stresses. A glimpse of functions regulated under WS, HS and CS is provided, and also the common and different responses between individual and simultaneous stresses.
Key words: abiotic stress, down-regulation, macroarray, up-regulation

Análise de ESTs de espigas de milho em desenvolvimento expostas a estresse simples e combinado de água e calor

RESUMO: A resposta molecular e metabólica de plantas a uma combinação de dois estresses abióticos é singular, e não pode ser diretamente extrapolada da resposta das plantas a cada um dos estresses individualmente. O macroarranjo do cDNA, tornou-se uma ferramenta útil para analisar os perfís de expressão e comparar as similaridades e diferenças de vários padrões de expressão. Um macroarranjo de 2.500 cDNAs de milho (*Zea mays* L.) foi usado para traçar um perfil de transcriptoma em resposta ao stress ocasionado por uma única e simultânea aplicação de água e alta temperatura em espigas em desenvolvimento, 15 dias após a polinização. Todos os tratamentos de stress (stress de água – SA, stress de calor – SC e sua aplicação combinada – AC) induziram modificações na expressão de 106 transcritos com 54 regulados acima e 52 regulados abaixo. Houve 11 transcritos regulados acima e 15 regulados abaixo em comum para os três estresses. Embora esses transcritos em comum mostrassem a existência de um mecanismo mútuo na resposta do estresse, os 23 transcritos induzidos somente em AC indicam que as plantas respondem de maneira diferente quando expostos aos efeitos simultâneos de ambos os estresses. Vislumbram-se funções reguladas por SA, SC e AC e também efeitos comuns e diferentes entre estresses individuais e simultâneos.
Palavras-chave: stress abiótico, regulação negativa, macroarranjo, regulação positiva

Introduction

Environmental abiotic stresses have detrimental impact on crop-yield worldwide and among the most important in temperate areas are drought and high temperatures. Drought at flowering and grain-filling induces high grain yield reductions in maize (Praba et al., 2009). Molecular and metabolic responses of plants to a combination of two different abiotic stresses are unique and cannot be directly extrapolated from the stresses individually (Mittler, 2006). Different stresses might require conflicting or antagonistic responses and a combination of drought and heat stress was found to alter plant metabolism in a novel manner compared with single stresses separately (Rizhsky et al., 2004). The stress combination should be regarded as a new state of abiotic stress in plants that requires a new defense or acclimation response (Mittler, 2006).

The complexity and polygenic nature induced by abiotic stress disabled conventional breeding to succeed in efficient and stable improvement of maize drought and heat tolerance. Recently, development of cDNA macroarray and microarray technologies enabled systematic analysis of the expression levels for thousand of genes simultaneously (Shi et al., 2005; Andjelkovic and Thompson, 2006; Zhang et al., 2009), thus contributing to the understanding of basic mechanisms underlying stress tolerance.

We performed comparative expressed sequence tags (ESTs) analysis in response to single and simultaneous application of drought and high temperature for identification of gene expression and affected pathways in developing maize kernels, using macroarray analysis. The aim

of this profiling was to indicate different and mutual responses in maize kernels to single and combined stresses during grain-filling, as molecular mechanisms of responses to drought at this stage are not fully understood, although maize is frequently exposed to severe stress in field with significant yield loss.

Material and methods

Maize (*Zea mays* L.) plants were grown in 10-L pots in greenhouse, under standard conditions with 16-h light period (300 $\mu E^{-2} s^{-1}$, 21-25ºC) and 8-h dark period (15ºC). Plants were grown on soil mix (soil : vermiculite = 3 : 2), irrigated daily and fertilized once a week. Self-pollinated ears were harvested at 15 days after pollination (DAP).

Stresses treatments were performed on plants at 5 DAP. Plants were subjected to water stress (WS) by completely withdrawing water until harvesting. Heat stress (HS) was applied by exposing the plants to 35ºC/25ºC day/night cycle for 16/8-h, with relative humidity maintained at 60%. Combination of heat and water stress (CS) was performed by simultaneously subjecting plants to HS and WS, under conditions already described. Control plants were grown under defined standard greenhouse conditions (21-25ºC/15ºC day/night cycle for 16/8-h). Leaf water status was determined by relative water content (RWC), measured on the control and stressed plants. After three days of withholding water (8 DAP) visible signs of stress such as leaf rolling and leaf blade coloration appeared on the lower part of the plants. Leaf samples were colleted between 12 a.m. and 1 p.m. at 0, 3 and 10 days after stress application and RWC was calculated as follows: RWC= fresh weight – dry weight) / (turgid weight – dry weight)*100 (Barr and Weatherley, 1962). The kernels of all plants, i.e. stressed and controlled, were collected at 15 DAP. All experiments were performed in triplicates and repeated three times. Pooled samples for each treatment were frozen in liquid nitrogen and stored at –80ºC. Total RNA was extracted using Perfect RNA^{tm} kit (Eppendorf Scientific, Inc. Hamburg, Germany) and prepared for macroarray probes.

Filter arrays were produced with ~2500 ESTs from unstressed maize tissues (mostly leaf and endosperm). Inserts were PCR amplified and the PCR products were separated by electrophoresis 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 four 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, expression was invariant. Normalization of each filter was performed by using the mean signal intensity of nebulin as a non-plant control (Desprez et al., 1998). Arrays were prepared on 22.2 cm^2 (six field areas of 8 $cm^2 \times 12$ cm^2) nylon membranes (Hybond N+, Amersham), which were pre-wetted under denaturing conditions (1.5 mol L^{-1} NaCl; 0.5 mol L^{-1} NaOH). The BioGrid robot (Biorobotics, UK) produced DNA spots in duplicates in a 4 × 4 pattern. After spotting, filters were neutralized (1 mol L^{-1} Tris pH 7.6; 1.5 mol L^{-1} NaCl) and DNA was fixed to the membrane by UV radiation at 120,000 μJ cm^{-2} for 30 min using Stratalinker (Stratagene, Netherlands).

Total RNA prepared from kernels of stressed and control samples were reverse-transcribed and used as probes for expression profile analysis. The reverse transcription reaction was performed at 43ºC for 1h, in a 30 µL reaction volume containing 5 × Superscript buffer (GibcoBRL), 0.01 mol L^{-1} M DTT, 1 mmol L^{-1} dNTP mix [dATP, dGTP, dTTP], 5 µmol L^{-1} dCTP, [α^{33}P] dCTP 3 µCi and 200 U of Superscript RT. Membranes were prehybridized in 20 mL of Church buffer (0.5 mol L^{-1} Na_2HPO_4 pH 7.2, 7% SDS, 1 mmol L^{-1} EDTA), including 200 µL of denaturated salmon sperm DNA (10 mg mL^{-1}), at 65ºC for 2h. The probes were denaturated by boiling for 5 min, followed by 5 min on ice. After prehybridization the denaturated probes were added to the buffer and hybridization was carried on overnight (at least 10 h) at 65ºC. Following hybridization the membranes were washed twice for 30 min at 65ºC in washing buffer (40 mmol L^{-1} Na2HPO4, pH 7.2 and 0.1% SDS). Subsequently, the filters were exposed to a phosphor screen overnight and scanned using ImageQuant software and Storm PhosphorImager (Molecular Dynamics).

The image analysis, quantification of signal intensities and first normalization by the average signal of all spots were done 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): i) we chose 'Automatic model selection for independent conditions' with minimum two (of four) valid observations required. It performed offset corrections by normalization across replicates. Outliers were detected and removed automatically prior to further analysis. ii) normalization across conditions was performed by 'Mean of reference values' (human nebulin). Reported output colored in blue data from genes exhibited different expression levels. Genes with changes in expression compared to control were identified as up- or down-regulated. Threshold of 2 is widely applied in distinguishing affected genes, even though it can still lead to some misclassification of genes (Deyholos and Galbraith, 2000). Herein, only genes with more than 2-fold change in expression are presented. BLASTN and BLASTX similarity searches against the non-reduntant NCBI protein database were performed and annotations were assigned to each query. Annotation and tentative contigs (TCs) were identified according to The Gene Index Database (http://compbio.dfci.harvard.edu/).

Expression profiles of stress-inducible cDNAs were also analyzed by the hierarchical clustering in the Gene Cluster software (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/ software / cluster).

Isolated RNA from stressed and control plants was also used for RNA gel-blot analysis. Total RNA (10 μg) was separated by electrophoresis in denaturating formaldehyde 1% agarose gel and then capillary transferred to Hybond N^+ membrane. Selected probes were labeled with $[^{32}P]$ dCTP using RediPrime DNA labeling system (Amersham). Hybridization was performed in hybridization buffer containing 5x SSC, 5x Denhardts, 50% formamide, 1% SDS and 100 μg μL^{-1} salmon sperm DNA, overnight at 42ºC. Filters were then washed twice, in 2x SSC, 0.1% SDS and in 0.2x SSC, 0.1% SDS, for 5 min at room temperature. Another washing was done in 0.2x SSC, 0.1% SDS for 15 min at 42ºC. Scanning was performed as described above. Each filter was stripped and re-hybridized with RNA probe pTA71 as a loading control.

Results and Discussion

To ensure that plants were grown under the required stress conditions RWC was monitored through the experiment. The reduction in RWC occurred at 3 day and at 10 day it was decreased by 13% in heat stress, 31% in water stress and 45% in combined stress in comparison with control plants. The greatest decrease in RWC was observed with the combined stress (Figure 1). Similar results were presented in maize (Hui-Yong et al., 2007) and wheat (Sharma and Kaur, 2009).

All three stress treatments (combined stress - CS, water stress - WS and heat stress - HS) induced changes in expression of 106 (4.3% of all analyzed) transcripts and 54 (2.2%) were up-regulated. There were 11 up-regulated transcripts in common for HS, WS and CS, seven for WS and CS, seven for HS and CS, one for WS and HS. Considering significantly up-regulated transcripts for each distinct stress treatment, there were 23 transcripts identified for CS, one for WS and four for HS (Figure 2). Detailed assessments on the expression ratios of all up-regulated maize ESTs are given in Table 1. Up-regulated transcripts include only ESTs with homology to previously identified or putative genes in *Z. mays* (24), *O. sativa* (18), *H. vulgare* (four), *A. thaliana* (five) and three share homology to other plants. A total of 54 up-regulated genes during the stress treatments were grouped into functional categories based on the categorization developed by the Gene Ontology (GO) Consortium (www.geneontology.org). With regard to biological process (Table 1), transcripts were assigned to nine groups (Table 1), including photosynthesis (three), transport (six), stress (19), cytoskeleton (three), metabolism (11), cell cycle (three), translation (two) and protein formation (one). The other six ESTs were assigned to the unclassified or unknown proteins category. Molecular function of the up-regulated ESTs was identified for 42 transcripts, while for 13 (23.6%) transcripts it could not be determined (Figure 3). Most of the transcripts are involved in protein binding (13 transcripts). For seven transcripts (grouped as *other*) following molecular functions were identified: lipid binding, oxygen binding, peptide binding, rRNA binding and translation initiation factor activity.

Here we focus on a set of up-regulated transcripts as less attention has been paid to down-regulation in expression studies. However, for an insight into a global answer to stress response, we analyzed down-regulated ESTs grouped together by cluster analysis (Figure 4). Detailed assessments on the expression ratios of down-regulated maize ESTs are given in Table 2. Down-regulated transcripts include only ESTs with homology to previously identified or putative genes. Ten out of 17 identified down-regulated transcripts are with homology to previously identified or putative genes in *Z. mays*, four to *O. sativa*, two to *A. thaliana* and one to *H. vulgare.* With regard to biological process, transcripts were assigned to six groups (Table 2), including photosynthesis

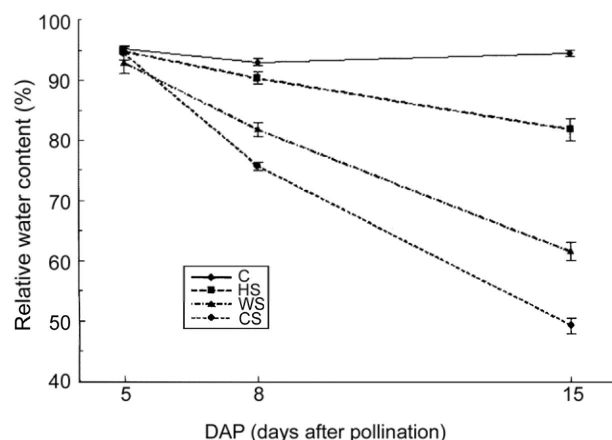

Figure 1 – Changes of leaf relative water content (RWC) in maize leaves under stress treatments at days 5, 8 and 15 DAP. C-control, HS-heat stress, WS-water stress and CS-combined stress. Data shown are means ± SE of three replicates.

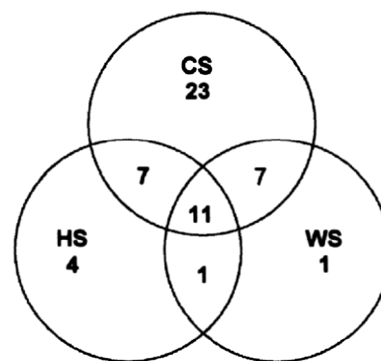

Figure 2 – Venn diagram of up-regulated transcripts (expression ratio stressed/control >2 fold) in maize developing kernels in response to the applied stresses: heat stress (HS), water stress (WS) and combination of heat and water stress (CS). Numbers in the circle overlap indicate the number of transcripts common to the compared stresses and numbers outside the overlap indicate the number of transcripts exclusive to the particular stress.

Table 1 – List of significantly up-regulated ESTs in response to water (WS), heat (HS) and/or combined (CS) stress treatments in developing maize kernels.

Tentative contig (TC)	Annotation	Expression ratio			Biological function
		WS/Control	HS/Control	CS/Control	
In HS and CS					
TC506584	auxin-induced protein (*O. sativa)*		12.7	7.2	other
TC468236	ATP synthase (*Z .mays*)		2.3	4.3	transport
TC464003	vacuolar ATPase B subunit (*H .vulgare*)		3.9	3.8	transport
TC487563	catalase isozyme 3 (*Z. mays*)		2.5	3.2	stress
TC467312	heat shock protein 81 (*O. sativa*)		3.4	2.8	stress
TC463096	pathogenesis-related protein (*Z. mays*)		2.2	2.9	stress
TC458346	ABA-responsive element- (ABRE) (*Z. mays*)		2.7	2.9	stress
In CS only					
TC542593	male sterility protein 2 (*A. thaliana*)			4.5	unknown
TC481964	alpha-6 tubulin (*Z. mays*)			4.5	cytoskeleton
TC467824	alpha-3 tubulin (*Z .mays*)			4.5	cytoskeleton
TC505652	fatty acyl coA reductase (*O. sativa*)			4.5	metabolism
TC289703	cytochrome P450 monooxygenase (*O. sativa*)			3.6	transport
TC479478	cytosolic glyceroldehyde-3-phosphate dehydrogenase (*Z. mays*)			3.4	stress
TC525318	alpha-1 tubulin (*Z. mays*)			3.3	cytoskeleton
TC462753	cell division protein (*Z. mays*)			3.3	cell cycle
TC4460	chlorophyll a /b-binding protein (*H. vulgare*)			3.1	photosynthesis
TC458293	glucose-6-phosphate (*O. sativa*)			3.1	metabolism
TC464688	eucaryotic translation initiation factor 5 (*Z. mays*)			2.9	translation
TC458802	translation initiation factor (*Z. mays*)			2.9	translation
TC286854	phospholipid cytidylyltransferase (*O. sativa*)			2.8	metabolism
TC483484	heat shock protein 81(*O. sativa*)			2.7	stress
TC474466	cycloartenol synthase (*O. sativa)*			2.6	metabolism
TC302534	cell wall glycoprotein (*A. thaliana*)			2.5	other
TC458415	phytoene synthase (*Z. mays*)			2.4	metabolism
TC546852	polyubiquitin (*T. caerulescens*)			2.3	metabolism
TC441536	cyclophilin (*O. sativa*)			2.3	protein formation
TC458487	superoxide dismutase (*Z. mays*)			2.2	stress
TC290568	ADP-glucose pyrophosphorylase small subunit (*O. sativa*)			2.1	metabolism
TC487801	glucose-1-phosphate adenylyltransferase (*Z. mays*)			2.0	metabolism
TC468456	d-UDP-glucose dehydratase (*O. sativa*)			2.0	metabolism
In WS and HS					
TC467940	non-specific lipid-transfer protein (*Z. mays*)	3.5	6.5		transport
In WS only					
TC494687	60S ribosomal protein (*O. sativa*)	2.1			cell cycle
In HS only					
TC458178	disease resistance protein (*Z. mays*)		2.3		stress
TC300734	chilling-inducible protein (*O. sativa*)		2.2		stress
TC499474	glycine-rich RNA-binding, ABA inducible protein (*Z. mays*)		2.2		stress
TC464194	actin-depolymerizing factor 3 (*Z. mays*)		2.0		stress

TCs and annotations according to The Gene Index Database (www.compbio.dfci.harvard.edu). Functional categories based on the categorization developed by the Gene Ontology (GO) Consortium (www.geneontology.org)

(three), transport (three), stress (three), metabolism (four), cell cycle (one) and translation (one). The other two ESTs were assigned to the unknown proteins category. No down-regulated transcripts were identified only in CS (data not presented). This could probably be due the fact that ESTs used in our profiling were obtained from unstressed maize tissues. Thus, it could be presumed that the overall transcripts' expression would probably be higher if macroarray was performed using library constructed from stressed tissues.

Eleven up-regulated transcripts were in common for all three stress treatments. (F-test, *p*-value 0.05, and Step-down Bonferroni as a multiple test correction method).These transcripts had the highest expression ratios among all the increased ESTs. Two of these transcripts (thaumatin-like protein and glycine-rich protein) were by far the most induced transcripts, highly above three times the threshold in all three stresses. Thaumatin-like protein belongs to pathogenesis-related proteins. Similar to other pathogenesis-related proteins, thaumatin-like protein synthesis can occur under general stress conditions and may be constitutively present in response to environmental or physiological stimuli (Zamani et al., 2004). Gene expression of glycine-rich proteins (GRP) can be modulated by various environmental stimuli, including wounding, pathogens, osmotic stresses, cold, light, hormones and circadian rhythm (Sachetto-Martins et al., 2000). The diverse response of GRPs to various environmental and developmental signals suggests that these proteins may play different but important roles in the maintenance of plant function and in adaptation to stress. Some plant glycine-rich proteins possess nucleic acid binding ability and are involved in gene expression regulation (Kim et al., 2007; Cai et al., 2008).

All the other transcripts belonging to this group were more expressed in CS compared to WS and HS. This difference is mostly pronounced for metallothionein and proline-rich protein. Metallothioneins function in plant protection and up-regulation in response to different stresses were confirmed in the other macroarray studies of Reymond et al. (2000) and Kawasaki et al. (2001). They play important roles in metal homeostasis and detoxification because of their ability to bind different heavy metal ions (Vasak and Hasler, 2000). Although there has been no report to indicate DNA-binding activity of a metallothionein protein, their nuclear localization in various cellular events has been reported, which has led to the hypothesis that the metallothionein mediates gene expression by donating zinc, directly or indirectly, to transcription factors (Butcher et al., 2004; Cai et al., 2008). Regulatory studies indicate that proline-rich proteins (PRP), have a structural role in the cell wall, as well as a storage or defense function (Jose-Estanyol et al., 1992). In addition, wounding, endogenous elicitors, funga1 elicitor, ethylene, cell culturing, and light can affect PRP gene expression (Sheng et al., 1991). Proline-rich proteins were shown to be up-regulated during water-deficit in *A. thaliana* (Bray, 2002).

Two of three up-regulated transcripts encoding components of the photosynthesis pathways were identified in all three stress treatments. Transcripts involved in photosynthe-

Table 2 – List of significantly down-regulated ESTs in response to water (WS), heat (HS) and combined (CS) stress treatments in developing maize kernels.

Tentative contig (TC)	Annotation	Expression ratio			Biological function
		WS/Control	HS/Control	CS/Control	
TC470272	abscisic acid- and stress-induced protein (*O. sativa*)	-19.3	-5.5	-5.2	stress
TC349303	malate dehydrogenase (NADP+) (*Z. mays*)	-13.5	-6.5	-4.4	metabolism
TC458526	interacting-zinc finger protein 1 (*Z. mays*)	-11.1	-5.8	-3.4	transport
TC495400	photosystem I PSI-K subunit (*H. vulgare*)	-10.4	-5.3	-3.0	photosynthesis
TC526421	phosphatidylinositol transfer protein(*O. sativa*)	-10.3	-1.0	-7.6	transport
TC475504	legumin-like protein (*Z. mays*)	- 6.9	-2.7	-6.9	unknown
TC468236	ATP synthase gamma chain chloroplast (*Z. mays*)	-5.9	-3.5	-5.3	photosynthesis
TC506023	BETL2 protein (*Z. mays*)	-6.3	-3.0	-4.9	stress
TC464507	CCAAT-binding transcription factor subunit A (*O. sativa*)	-4.7	-4.2	-4.4	cell cycle
TC345125	phosphoprotein phosphatase (*O. sativa*)	-4.6	-5.1	-2.3	metabolism
TC468023	early nodulin (*A. thaliana*)	-4.0	-4.7	-3.2	transport
TC503833	19 kD zein protein (*Z. mays*)	-4.1	-3.0	-4.3	metabolism
TC333458	nitrilase-associated protein (*A. thaliana*)	-4.0	-5.9	-2.7	unknown
TC564432	chloroplast membrane (*Z. mays*)	-3.5	-3.8	-2.7	stress
TC522429	chlorophyll a/b-binding preprotein (*Z. mays*)	-2.7	-2.1	-5.3	photosynthesis
TC470704	60S acidic ribosomal protein(*Z. mays*)	-1.6	-7.4	-1.0	translation
TC481030	starch branching enzyme IIb(*Z. mays*)	-1.7	-2.8	-2.0	metabolism

TCs and annotations according to The Gene Index Database (www.compbio.dfci.harvard.edu). Functional categories based on the categorization developed by the Gene Ontology (GO) Consortium (www.geneontology.org).

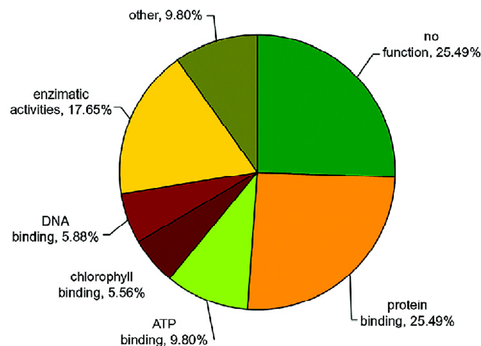

Figure 3 – Molecular function of stress induced transcripts. A total of 54 up-regulated genes during stress treatment were grouped based on the categorization developed by the Gene Ontology (GO) Consortium (www.geneontology.org). Each category was given a percentage of all induced genes.

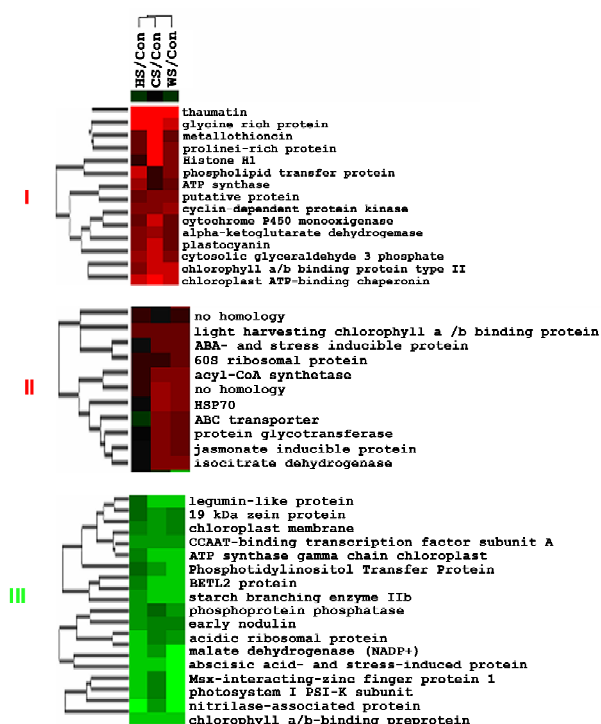

Figure 4 – Expression profiles of maize kernel transcripts subjected to heat stress (HS), combined stress (CS) and water stress (WS). Ratio treatment/control was $\log_2$ transformed and subjected to hierarchical clustering. Up-regulation (or suppression) ranges from black (no expression changes) to saturated red (up-regulation) and green (down-regulation). Three groups with distinct expression patterns were created - group I with 15 genes remarkably expressed at least under two stress treatments; group II with 11 genes with expression ratios stress/control slightly above 2 and group III with 17 genes down-regulated in response to all applied stresses.

sis were up-regulated in nonphotosynthetic organs in response to stress (Wang et al., 2003; Zhuang et al., 2008). This was explained by the hypothesis that they were involved in the control of redox state under water stress (Grossman et al., 2001). Cytochrome P_{450} monooxygenase and plastocyanin are known to be involved in electron transport processes and have a role in the control of redox state. They were approximately two fold increased in CS compared to WS and HS. Cytochrome P_{450} monooxygenase mediates the biosynthesis of lignins, terpenes, alkaloids and the variety of secondary metabolites, which act in plant defense and oxidative detoxification (Persans et al., 2001). Up-regulation of cytochrome P_{450} monooxygenase was detected in drought stress in barley (Ozturk et al., 2002) and aluminium stress in *A. thaliana* (Goodwin and Sutter, 2009). Plastocyanin up-regulation was identified under water and salt stress of developing maize kernels (Andjelkovic and Thompson, 2006).

Cytosolic glyceraldehyde 3 phosphate dehydrogenase, which is up-regulated under all three stress treatments, was also up-regulated in leaves and roots of maize seedlings in response to polyethylenglicol stress (Zheng et al., 2004). Suppression of genes encoding glyceraldehyde-3-phosphate dehydrogenase and other enzymes related to glucose-phosphate metabolism has been noted in response to dehydration shock treatment in barley (Talame et al., 2007) and drought stress in tolerant maize landraces (Hayano-Kanashiro et al., 2009).

Alpha-ketoglutarate dehydrogenase and protein kinase activity transcripts were the least induced in CS among all eleven transcripts. The function of alpha-ketoglutarate dehydrogenase could not be established according to the categorization developed by the Gene Ontology (GO) Consortium. Rhizky et al. (2004) showed that transcripts encoding signal transduction, including protein kinases, were elevated during a combination of drought and heat stress in *A. thaliana* and Yu and Setter (2003) showed that a calcium dependent protein kinase was up-regulated in both maize endosperm and placenta.

Apart from the common ESTs for all three stress treatments, 24 transcripts were up-regulated only in CS. These specifically induced transcripts are involved in stress response, metabolism, photosynthesis, translation, as well as protein and cytoskeleton formation. The expression ratios were in the range from 2.0 to 4.5, in general considerably lower than expression ratios of common transcripts induced in all three stresses. These results confirm that stress combination should be regarded as a new state of abiotic stress in plants.

As the same ESTs library was used in the previous experiments (Andjelkovic and Thompson, 2006), in which developing kernels were analyzed under water and salinity stress treatments, the results from both studies were compared. In the previous study the stress was applied in the form of a shock treatment - stalks of maize plants were cut 10 cm below and above the ear and placed on filter paper (drought stress), i.e. submerged in liquid medium (salt stress) for three days, until sampling. Four common up-regulated transcripts with high expression ratios were identified in both studies for all stresses applied. These are glycine rich protein, metallothionein, plastocyanin and cytosolic glyceraldehyde 3 phosphate. Another six common up-regulated transcripts were

identified in the salt and CS (alpha-6 tubulin, alpha-3 tubulin, cycloartenol synthase, phytoene synthase, male sterility protein, eukaryotic translation initiation factor 5*)*. However, the shock treatment is not comparable to field situation, and for this reason in the present study we performed HS, WS and CS with stress conditions more similar to natural conditions.

Drought triggers the production of abscisic acid (ABA) which induces expression of stress-related genes. Some genes are up-regulated and others down-regulated resulting in overall synthesis of genomic products which may play a role in plant survival under different environmental conditions. In our study the highest suppression was detected for abscisic acid (ABA)/stress-induced protein in all stress treatments, while Guo et al. (2009) detected its up-regulation in response to 3-days of drought stress in barley. ABA also causes stomatal closure and consequently the inhibition of photosynthesis. In this respect, transcript level of photosynthesis-associated genes for photosystem I PSI-K subunit, ATP synthase gamma chain chloroplast and chlorophyll a/b-binding preprotein decreased during applied WS, HS and CS treatments. Sugar metabolism is closely related to photosynthesis and is also affected by stress stimuli (Hayano-Kanashiro et al., 2009). Although three transcripts involved in sugar metabolism were slightly induced only in CS, their expression ratios were at the threshold level (Table 1). Also, suppression of malate dehydrogenase, an enzyme involved in gluconeogenesis, was detected under all three stress treatments, coinciding with results obtained for barley (Ozturk et al., 2002). Inhibition of genes associated with starch biosynthetic pathway was detected during shade stress in maize (Zinselmeier et al., 2002) and in our experiments starch branching enzyme IIb transcript level was significantly decreased only under heat strees.

Legumin-like proteins and zeins are seed storage proteins usually found in seed endosperm. In our study, legumin-like protein and 19 kD zein transcripts were suppressed in response to all applied stresses, but in roots of maize seedlings, legumin-like proteins were up-regulated in response to water stress (Zhu et al., 2007). Zeins are particularly sensitive to high temperature and it was found that their concentration in response to this stress was reduced during early developmental of maize kernel under heat stress (Monjardino et al., 2005).

Translation factor proteins, like ribosomal proteins, can be differentially expressed under abiotic stress conditions. In our study, 60S acidic ribosomal protein was suppressed only in response to heat stress. (z-test, $p < 0.05$). Ozturk et al. (2002) found that ribosomal proteins were induced in salt-stressed leaves of barley 24 h after stress application, but suppressed or without expression after shorter exposure (6 h and 10 h) to the stress.

Hayano-Kanashiro et al. (2009) found that a number of transcription factors were differentially expressed in response to drought stress and after recovery, depending upon maize landraces susceptibility. Members of zinc finger protein and CCAAT-binding transcription factor subunit A were among them, and these transcripts were strongly suppressed under all three stresses applied in our experiment.

To further evaluate these results, a hierarchical clustering analysis of the expression profiles of genes induced by HS, WS and CS was performed (Figure 4). Hierarchical clustering analysis was in agreement with the macroarray expression profiles. Tree groups with distinct expression patterns were created based on the results of cluster analysis. In group I there are 15 genes remarkably expressed at least under two stress treatments. Group II encompasses 11 genes with expression ratios stress/control slightly above 2. Clustering put together 17 genes in the group III with down-regulation in response to all applied stresses.

To evaluate the validity of the cDNA macroarray study RNA gel-blot analysis was performed. The RNA obtained from stress treatments was examined for a number of ESTs. In general, the results of RNA gel-blot were consistent with the expression data obtained by macroarray analysis. As an illustration, gel blot analysis for metallothionein clone 5C05E10 is presented in Figure 5. A ATP-dependent Clp protease ATP-binding subunit EST was chosen as an example of a transcript that did not change in abundance according to the macroarray data during the applied stress treatments (neutral control). For this transcript both techniques confirm no changes under our experimental conditions.

Conclusion

Combined effects of water and heat stresses, compared to single stress effects, alter plant response in a novel manner and can be regarded as a new state of abiotic stress that requires a new defense or acclimation response. The role and importance of both identified up and down regulated transcripts is hard to judge from the limited selection of ESTs (~2500).

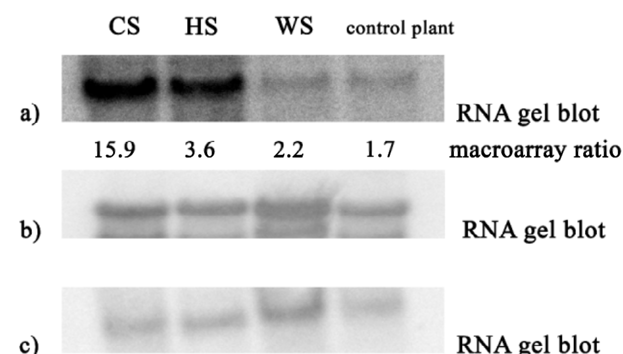

Figure 5 – RNA gel blot analysis and corresponding cDNA macroarray data. **a)** Verification of macroarray results by RNA gel blot analysis for clone 5C05E10 (metallothionein) in stress conditions: CS– combined stress, HS – heat stress WS – water stress. The gene expression ratios from the macroarray are shown under the corresponding RNA blot. **b)** PTA71 was used as loading control. **c)** Clone 5C02D08 (ATP-dependent Clp protease ATP-binding subunit) was used as neutral control - a transcript that did not change in abundance according to the macroarray data during the applied stress treatments.

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