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**GENE EXPRESSION PROFILING IN RESPONSE TO HEAT AND
WATER STRESS IN MAIZE KERNEL**

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New high-throughput techniques, together with advances in
analytical and computational technologies are enabling systemic investi-
gations of molecular processes of biological systems. Clustering is widely
applied by biologists in genome sequencing projects and phylogenetic
studies. In the microarray expression analysis, the aim of cluster is to
better organize, but not to alter primary data: genes are visually organized
according to the statistical transformation and calculation, due to the ex-
pression pattern in maize kernel during heat and water stress. Besides
grouping together genes with similar patterns of expression, genes are
represented by increased intensity of red, e.g. green color, according to
their up- or down-regulation in stress condition, compared to control.

Key words: maize, cluster analysis, microarray, stress

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INTRODUCTION

In maize (*Zea mays* L.) drought stress that occurs two weeks before and after flowering makes large losses in final grain yield. Besides yield reductions as result of lower photosynthesis rate, significant decrease in seed number and harvest index in response to drought has been showed, too (EDMEADES *et al.*, 1999). Therefore, it is essential for maize drought tolerance improvement to understand the most affected pathways and process in the plants.

Recent advances in molecular biology and bioinformatics are enabling systematic investigation of biological systems. DNA array technology allows investigation the expression level for thousand of genes in different conditions at the same time. Obtained data could be used for multifunctional study instead of study the expression of genes one by one, that provided low biological insight.

First step in the analysis of large amount of data is to use specific software for determination genes with significant expression level compared to control conditions. Beyond that is important to classify and group genes involved in the same regulatory process.

Clustering enables extracting groups of co-expressed genes over different experiments. However, different clustering methods allows very different results and it is not yet clear which clustering method is most useful for gene expression analysis.

MATERIAL AND METHODS

Plant material, cultivation conditions and stress treatment. - *Zea mays* L. cv A 188 plants were grown under optimal conditions in a greenhouse prior to flowering. Five days after self-pollination different stresses were applied:

Heat stress (HS) – plants were transferred to the growth chamber and 10 days were exposed to 35°C / 25°C day / night cycle of 14 / 10 hours and 60% relative air humidity.

Water stress (WS) – plants were kept under the same growing conditions in a greenhouse, but without watering for 10 days.

Combine stress (CS) – maize plants were exposed to heat and water stress (as describe for a) and b)) simultaneously from 5-15 DAP (days after pollination).

Control – plants were grown under the same in the greenhouse and were harvested at the same time with stressed plants, e.g. 15 DAP.

Kernels from stressed and control plants were collected and frozen in the liquid nitrogen and stored at –80°C for further analysis.

Total RNA extraction and probe preparation. - Total RNA was isolated from 3-5gr of kernels using Perfect RNAtm kit according to manual instructions (Eppendorf Scientific, Inc.Hamburg. Germany).

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

Reverse transcriptions were performed at 43°C for 1 hour by adding 6µl of Superscript buffer (GibcoBRL), 3µl of 0.1M DTT, 3µl of 10mM [dATP, dGTP, dTTP], 3µl of 50µM d CTP, 3µl [$\alpha^{33}\text{P}$]dCTP 30µCi and 1µl of Superscript II RT (200U/µl, GibcoBRL). By adding 1µl of 1%SDS, 1V of 0.5M EDTA and 3µl 3M NaOH and incubation at 65°C for 30min, hydrolysis of RNA was started, followed by 15min at room temperature. In order to neutralize the reaction 10µl of Tris-HCl (pH8) and 3µl of 2N HCl were added. cDNAs were precipitated after addition of 5µl of 3M Na[CH₂COO](pH5.3), 5µl of yeast tRNA (10mg/ml) and 60µl of isopropanol at -20°C for 30min. After measuring of the incorporation by scintillation counter, dried pellets were resuspended in 100µl of sterile water.

cDNA preparation and analysis. - A library of ~2500 maize ESTs was PCR amplified. cDNA human desmin and nebulin were spotted on each filter in 4 different concentrations in multiple locations on the filter and were used as an internal control.

Arrays were prepared on 22.2cm² (6 field areas 8x12cm²) nylon membranes (Hybond N⁺, Amersham), which were pre-wetted in denaturing conditions (1.5M NaCl; 0.5M NaOH). The BioGrid robot (Biorobotics, UK) produced DNA spots in duplicates in a pattern 4x4. After spotting, filters were neutralized (1M Tris pH 7.6; 1.5M NaCl) and DNA was fixed to the membrane by UV radiation at 120.000 µJ/cm² for 30min using Stratalink (Stratagene, Netherlands).

Prehybridisation was done for 0.5-2h at 65°C in 20ml of Church buffer (0.5M Na₂HPO₄, pH 7.2; 7% SDS; 1mM EDTA) including 200 µl of denatured salmon sperm DNA (10mg/ml).

The probe solution was boiled for 5min and then rapidly applied to the filters and hybridization was carried out overnight (at least 10h) at 65°C. Washing step was done by briefly rinsing at room temperature and by incubations 2x30min at 65°C in a washing solution (40mM Na₂HPO₄, 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 Storm PhosphorImager (Molecular Dynamics).

The images 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).

Cluster and tree view software were used to group and display genes with similar expression profiles (EISEN *et al.*, 1998; <http://rana.lbl.gov> updated by DE HOON, 2003; <http://bonsai.ims.u-tokyo.ac.jp/~mdhoon/software/cluster>). We used the default options of the centroid linkage hierarchical clustering with city-block distance similarity metric. The method used for gene's color was on the basis of their log₂ (ratio) in each experiment: log₂ (ratio) values close to zero colored black, with log₂ (ratio) values greater than zero colored red and those with negative values colored green. Each column in the cluster represents single experiment and each row represents the expression level of particular gene.

RESULTS

In order to compare expression data in more details, the similarities between transcript profiles were measured using clustering and visualized by tree view software (see Material and methods). Relationships among genes are represented by a tree whose branch length (node values) reflects the degree of similarity between the objects.

Grouping of genes that are regulated by specific stress and/or co-regulated by combinations of different treatments was made within six groups (Fig.1 and 2), according to the cluster analysis.

Group I consists of genes preferentially induced by heat stress. Among them are: a) members of HSP (heat shock protein) family (HSP 22 and HSP 82). Their induction and possible protective function in response to high temperatures has been reported in many studies (MOHR and SCHOPFER, 1995; SCHALTER *et al.*, 2002); b) genes induced in response to stress and with detoxification function: LEA proteins, jasmonic acid and stress inducible proteins, thaumatin-like, zinc and disease resistance, monooxygenase (BAJAJ *et al.*, 1999; BRAY 2002); c) tubulins and Ca-binding proteins with a basic role in cytoskeleton formation have a protective function in abiotic stresses, too (ANTHONY *et al.*, 1999; FISCHER *et al.*, 2002).

Group II consists of co-expressed genes in heat and water stress, together with eight genes induced by combine stress. Besides some genes already defined as stress responsive, the main portion belongs to genes involved in protein synthesis. For different ribosomal proteins up-regulation is confirmed in response to UV-B light (CASATI and WALBOT, 2003) and salt stress in barley (OZTURK *et al.*, 2001), but in expression study in yeast, a group of 112 ribosomal proteins was down-regulated in response to heat stress (EISEN *et al.*, 1998). Within a group of eight genes co-expressed in all treatments, four of them are related to protein biosynthesis.

Genes from group III are preferentially up-regulated in combine stress, although they were slightly down-regulated in response to single heat and/or water treatment. Common characteristics for most of them is their function in plant protection: disease resistance, bacterial blight resistance, basal layer antifungal protein, glycin and praline rich protein, as well as genes active in their biosynthesis (KAWASAKI *et al.*, 2001). Genes involved in glycolysis and TCA cycle (EISEN *et al.*, 1998) and cell transport.

Groups IV and VI consist of genes induced in response to more severe stresses e.g. heat and combine stress, while during water stress they were down-regulated or even without expression. Stronger but similar influence of those two stresses is showed in cluster for conditions: they are grouped together in the same cluster. Up-regulated genes in these groups are responsible for plant protection and defence, like glycine rich protein, wound and herbicide inducible protein, ethylene-responsive coactivator. Protective function is confirmed for histones in Arabidopsis and tomato (ASCENZI and GRANT, 1999; SCIPA *et al.*, 2000). Besides, important

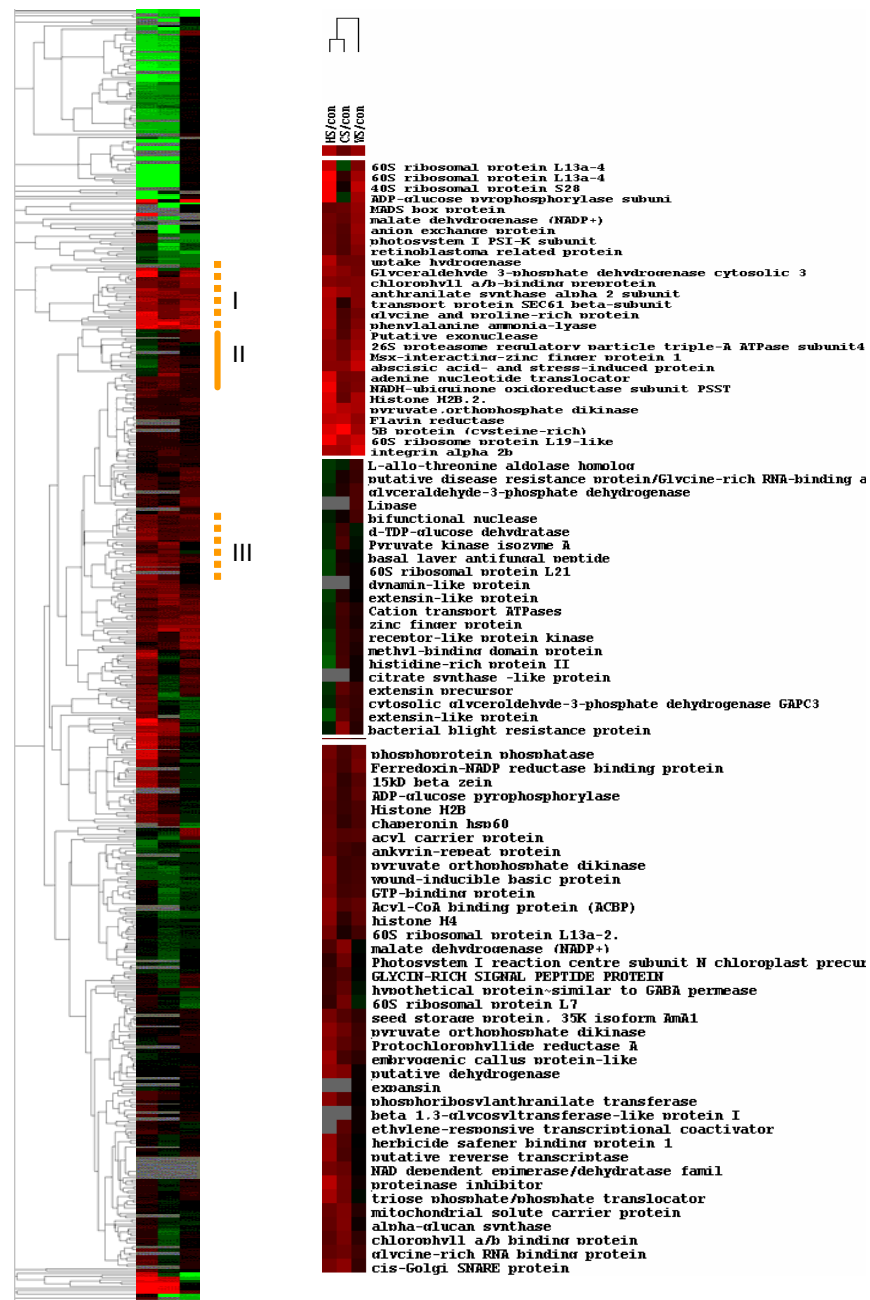


Fig 1. Expression of transcription factor genes during heat, water and combined stress (Group I-III)

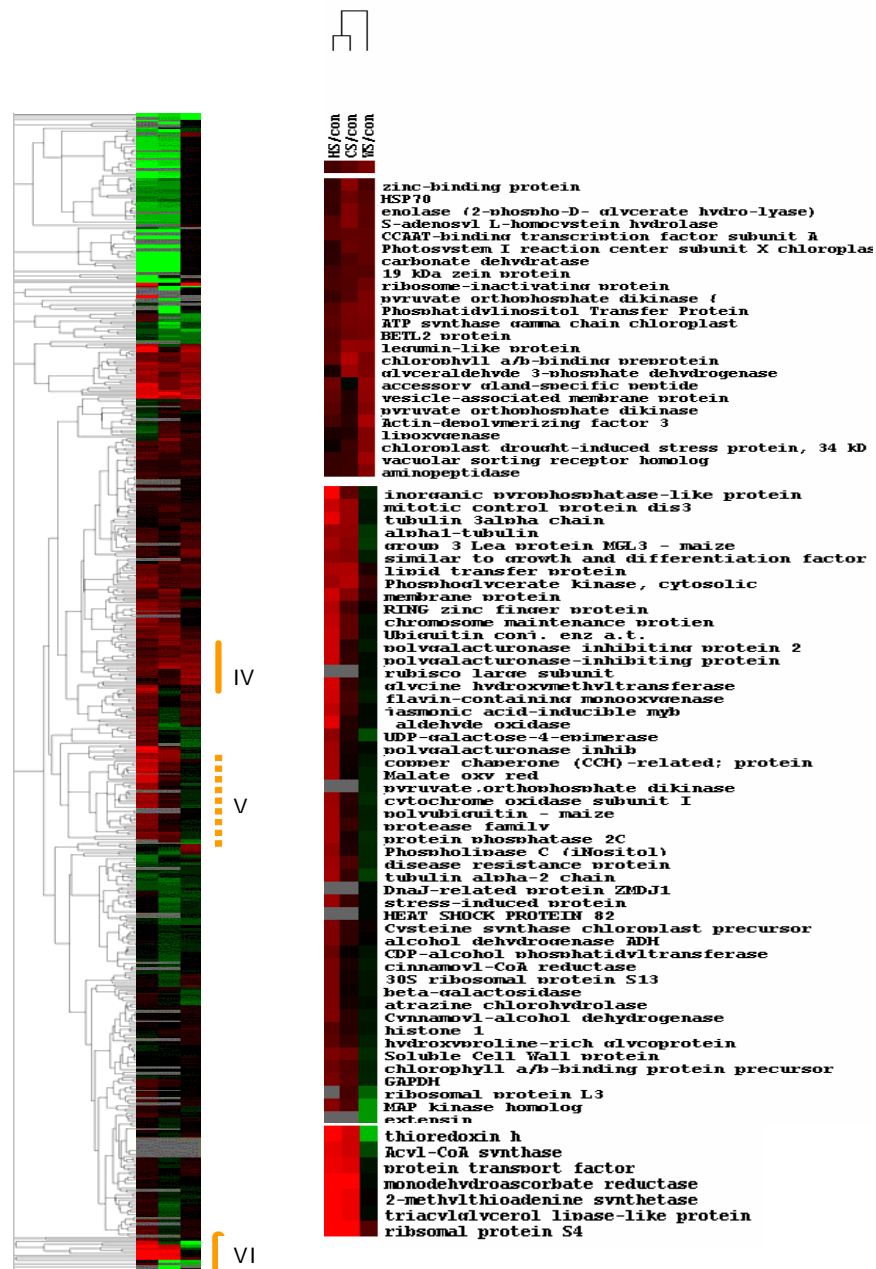


Fig 2. Expression of transcription factor genes during heat, water and combined stress (Group IV-VI)

role belongs to the genes involved in non-photosynthetic energy production within a cell. Group VI consists of seven genes only, but among them are genes responsible for already described function. They are expressed in response to high temperature and combine stress, as well, but the position is at the bottom of the cluster tree, distant from group IV.

Cluster analysis helped in grouping genes induced by water stress into a group V. However, according to computational analysis of described and the other experiments (unpublished data), withholding water from the pot was without significant difference in gene expression compared to control conditions. Genes already discussed are grouped together, but dark-red color indicates that their level of up-regulation was not very high (HSP 70, zinc binding and basal layer antifungal protein, lipoxigenase).

CONCLUSIONS AND OUTLOOK

Today biology is on the way of transition into information-driven science. This could be useful only by developing models with enough information about important biological processes and metabolic control in development, diseases and response to abiotic stresses (ZWEIGER, 1999).

Microarray-based genomic study and other high-throughput approaches are becoming increasingly important in biology. One of the difficulties is to “see” the information in the massive tables of data. Clustering represents relatively easy way to extract useful information, but it tells us only which genes are co-regulated. Results presented here shows that genes expressed and organized together share common function.

Of course, expression data alone can provide only partially useful information about essential genes. We have partially characterized transcriptoms associated with response to drought stress in maize. Analysis shows it is complex process involving induction of functionally different groups of genes. However, the role they play in development and in plant stress responses need to be verified using other approaches. In order to understand the mechanisms it is important to construct model of regulatory interactions between the genes. This requires inference of the casual relationships among genes, i.e. functional analysis and reverse genetics.

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EKSPRESIJA GENA U ZRNU KUKURUZA KAO ODGOVOR NA TOPLOTNI I VODNI STRES

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Izvod

Nove tehnike, zajedno sa dostignućima u analitičkoj i kompjuterskoj tehnologiji omogućile su sistematsko ispitivanje molekularnih procesa u biološkim sistemima. Klaster analiza se široko primenjuje od starne biologa, u programima sekvencioniranja genoma i filogenetskim proučavanjima. U proučavanju ekspresije gena korišćenjem «microarray» tehnike, cilj klaster analize je da se bolje organizuju, ali ne i zamene izvorni podaci: geni su vizuelno organizovani na osnovu statističkih transformacija i proračuna, a prema načinu ekspresije u uslovima toplotnog i vodnog stresa u zrnu kukuruza. Pored grupisanja gena sa sličnim načinom ekspresije, svaki gen je predstavljen različitim intenzitetom crvene, tj. zelene boje u zavisnosti od toga da li je utvrđeno povećanje ili smanjenje ekspresije gena u uslovima stresa u odnosu na kontrolu.

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