Funct Integr Genomics (2005) 5: 201–207
DOI 10.1007/s10142-005-0141-6

ORIGINAL PAPER

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Gene expression profiling of potato responses to cold, heat, and salt stress

Received: 9 February 2005 / Revised: 25 March 2005 / Accepted: 26 March 2005 / Published online: 22 April 2005

Abstract In order to identify genes involved in abiotic stress responses in potato, seedlings were grown under controlled conditions and subjected to cold (4°C), heat (35°C), or salt (100 mM NaCl) stress for up to 27 h. Using an ~12,000 clone potato cDNA microarray, expression profiles were captured at three time points following initiation of the stress (3, 9, and 27 h) from two different tissues, roots and leaves. A total of 3,314 clones could be identified as significantly up- or down-regulated in response to at least one stress condition. The genes represented by these clones encode transcription factors, signal transduction factors, and heat-shock proteins which have been associated with abiotic stress responses in Arabidopsis and rice, suggesting similar response pathways function in potato. These stress-regulated clones could be separated into either stress-specific or shared-response clones, suggesting the existence of general response pathways as well as more stress-specific pathways. In addition, we identified expression profiles which are indicative for the type of stress applied to the plants.

Keywords Potato · Gene expression profiling · Abiotic stress

Introduction

Temperature, both high and low, and salinity are common stress conditions to plants. These stresses affect both plant growth and crop production. Understanding how plants perceive and respond to these conditions is of fundamental interest to biology. Plants have the ability to adapt to changes in their environment, and accelerated by the increased availability of genomic data, progress has been made in understanding the abiotic stress responses in a number of species, although primary efforts have been focused on Arabidopsis and rice (for review, see Thomashow 2001; Zhu 2001a,b; Iba 2002; Zhu 2002; Shinozaki et al. 2003). The sensing of these changes and the subsequent acclimation to the environment follow a general signal transduction pathway (Xiong et al. 2002). The signaling pathway is initiated by sensors which detect the stress and then relay the signal through secondary signaling molecules thereby initiating a phosphorylation cascade and activating transcription factors. Activated transcription factors, in turn, regulate gene expression forming the primary response of the plant that results in the protection and repair of the cell. If successful, these transcriptional changes result in the adaptation and tolerance to the abiotic stress condition.

Understanding the plant's response to abiotic stresses requires monitoring the transcriptome of the plant in a temporal and spatial manner. Therefore, expression profiling is the preferred method to identify genes involved in abiotic stress responses. Indeed, microarrays have been used extensively in abiotic stress research in various species (Kawasaki et al. 2001; Fowler and Thomashow 2002; Kreps et al. 2002; Seki et al. 2002; Rabbani et al. 2003). However, the diversity of microarray platforms used, coupled with the different types of stresses and the various plant species studied, makes it difficult to perform a global comparison of stress-related genes in flowering plants. Another issue with a majority of published abiotic stress gene expression profiling studies is the absence of biological replicates thereby preventing application of robust statistical analysis methods. Recently, several statistical methods have been developed to make use of biological replicates and identify genes that are both biologically and statistically significant (Smyth et al. 2003; Cui and Churchill 2003).

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s10142-005-0141-6.

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In this study, responses to cold, heat, or salt stress were monitored in potato plants using microarrays. Not only is potato an important crop worldwide, but it also serves as a model for other members of the Solanaceae family that includes tomato, tobacco, pepper, and petunia as their genomes are highly similar (Zamir and Tanksley 1988). Freezing tolerance and cold acclimation have been studied in potato, but only for a limited number of genes (Stone et al. 1993; van Berkel et al. 1994; Schneider et al. 1997). With the advent of genomics projects, a large dataset of expressed sequence tags (ESTs; ~193,322; dbEST Release 030405, March 4th 2005) is available for potato.

The expression profiles from root and aerial tissue (referred to as leaf from hereon) were captured at three time points using three different abiotic stressors. Each time course was repeated independently (n=3). Across the three abiotic stresses, a total of 3,314 clones could be detected as up- or down-regulated following stress initiation. Based on pair-wise comparisons, 1,032 clones were significantly differentially regulated by cold, heat, or salt stress. Several transcription factors, DNA binding proteins, transporter proteins, phosphatases, and heat-shock proteins were identified as significantly up- or down-regulated in response to abiotic stress. In addition, a suite of genes with unknown function were also identified, providing new avenues for investigation of abiotic stress responses in plants.

Materials and methods

Plant growth and sample isolation

Commercially available true potato seeds (Variety Gilroy; Potato Products International Ltd, Gilroy, CA) were germinated on rafts floating on hydroponic medium in Magenta boxes (Sigma-Aldrich, St. Louis, MO), three plants per box. Plants were grown for 5 weeks prior to stress application under long day conditions (16 h light and 8 h dark) at 25°C with gentle agitation. Medium was provided as 0.5× Murashige and Skoog salts including vitamins (Sigma-Aldrich), 0.5 g l^{-1} MES, pH 5.7, and 0.5% (w/v) sucrose. To initiate stress, the medium was replaced with fresh medium prechilled to 4°C (cold stress), preheated to 35°C (heat stress), or supplemented with 100 mM NaCl (salt stress). Cold and heat stress were maintained for the duration of the experiment by placing the Magenta boxes on ice or in a water bath at 35°C. For each individual sample, two boxes of plants were used pooling a total of six plants per sample. Stress was initiated at the time the daylight period commenced. For each time point, a single control sample was used by changing the medium in a similar way as for the stress induction. A total of six boxes were combined for the pooled reference samples.

Plants were harvested at the appropriate time and snap-frozen in liquid nitrogen. Roots and leaf tissue were separated prior to freezing. The tissue was stored at −80°C until isolation. Total RNA was isolated using RNeasy isolation kit (Qiagen, Valencia, CA). RNA integrity was verified on agarose gel, and the concentration was adjusted to 3 μg/μl by ethanol precipitation and resuspension.

Fabrication, validation, and hybridization of potato microarrays

From the cDNA libraries used for expressed sequence tag (EST) sequencing (Ronning et al. 2003), 15,264 clones were selected for microarray fabrication. We successfully amplified and validated the clones through sequencing and gel electrophoresis; 74% (11,243 clones) of the original clone set could be validated, and the remaining 26% (4,021 clones) were excluded from all subsequent analysis. After amplification, clones were purified using Millipore (Millipore Corporation, Billerica, MA) filter plates and resuspended in 50% DMSO. A subset of clones from each 96-well plate was sequenced following purification to detect any tracking errors and validate spot identity on the array. Clones were spotted using 48 pins resulting in a 4×12 grid in duplicate, nonadjacent positions onto Ultra-GAP slides (Corning, Corning, NY) using an IAS arrayer (Brooks Automation Inc., Chelmsford, MA).

For annotation, we utilized the TIGR *Solanum tuberosum* Gene Index (StGI) in which ESTs and expressed transcripts (ETs) are clustered and assembled into a set of unique sequences referred to as tentative consensus sequences (TC) (Quackenbush et al. 2001), with sequences not in a cluster referred to as singleton ESTs or ETs. As the assembly of the sequences into TCs is a continuous and dynamic process, we refer to clones rather than genes in our analysis. The same TC can be represented by multiple clones on the array as with the availability of new sequence data, sequences collapse resulting in the merging of TCs and singleton ESTs into a single TC. Based on the latest build of the gene index (version 9), the clones on the microarray represent 9,774 genes (TCs, singleton ESTs and ETs). The gene index process uses ≥96% sequencing identity for clustering and assembling sequences into TCs (Quackenbush et al. 2001) and thus cannot merge gene family members that are not highly similar. Consequently, there will be clones on the array that represent closely related gene family members which will not be in the same TC and might cross-hybridize with RNA from other family members.

Total RNA samples were labeled essentially as described (Hegde et al. 2000). All control samples were labeled with the Cy5 dye and the query sample with Cy3. Hybridization and washing were performed essentially as described (Hegde et al. 2000). After the final washing step and spin-drying of the slide, slides were scanned using an Axon scanner at maximum laser power (Axon Instruments, Union City, CA) at both 532 and 635 nm. The PMT values for both wavelengths were adjusted to capture a similar number of normalized counts for each channel.

For validation of the arrays, we assessed technical reproducibility by calculating the Pearson's correlation coefficient between different technical replicates. For self–self hybridizations using the same RNA sample (Cy3, Cy5), an

average correlation of 0.93 (n=8, two slides, comparisons of all on-slide replicates) was observed. The average correlation between the normalized intensities of on-slide replicates (Cy3 and Cy5) was 0.94 (n=144, 72 slides, two channels). For 603 TCs that were represented by two clones on the array and found to be significantly differentially expressed, the correlation between the two clones among the 72 hybridizations was calculated; for 61% of the clones, the correlation is at least 0.80. Some variability is expected as clones represent different parts of the gene, and misassembly of clones into TCs may occur. These high correlation coefficients indicate that the potato cDNA microarray used resulted in reproducible hybridizations with a high confidence in spot identity.

Data processing and analysis

The TIFF images were quantified using Genepix 5.0 (Axon Instruments, Union City, CA). The software automatically flags spots that cannot be found in one of the channels; these are flagged and excluded from further analysis. Spots containing >30% saturated pixels in either channel or a diameter <70 μm were flagged and not used for subsequent analysis. Local background was subtracted from the signal value (mean pixel intensity). The data were normalized using the print-tip-loess method in the limma package (Smyth 2004) of Bioconductor (http://www.bioconductor.org; Dudoit et al. 2003). Flagged spots were given a weight of 0 using the weight function within the package which excludes these spots from affecting the normalization. Except for the limma analysis, all analyses used the average of the two on-slide replicates. If one of the two replicates was flagged, the remaining value was used for analysis.

Differentially expressed (DE) clones were detected using the limma package (Smyth 2004). A top clone list was generated using a false discovery rate to correct for multiple testing. The results were filtered on p<0.01 or <0.05 as indicated in the text. A clone was only termed DE when both on-slide replicates passed the indicated p value. All clones were filtered to have at least a signal of 250 in both channels (scale 0–65,535 units). The Pearson's correlation was calculated using the 'cor' function in the R-package (Ihaka and Gentleman 1996). Further statistical analysis was performed using GeneSpring (Silicon Genetics, Redwood City, CA), and R Welch t-test analyses used a family-wise error rate of 0.01 and using Bonferroni step-down multiple testing corrections. The consensus trees in Figs. 2 and 3 were made using complete linkage clustering with the Euclidean distance measure followed by bootstrapping the tree (Kerr and Churchill 2001) using the R-package (Ihaka and Gentleman 1996). For the classification of the samples, the pamr package within Bioconductor (Dudoit et al. 2003) was used (Tibshirani et al. 2002). A clone list was generated as an output with a threshold to contain the least number of clones and the expected least number of misclassified clones.

Results and discussion

Identification of all of up- and down-regulated clones after initiation of cold, heat, and salt stress

The potato plants used in this study were grown from true seeds for 5 weeks on rafts in hypertonic medium. A similar experimental setup was used for gene expression profiling of response of Arabidopsis to abiotic stress (Kreps et al. 2002). Due to this growth method, the roots of the potato plants were exposed to light, and some greening of the roots was observed. Cold (exposure to 4°C), heat (exposure to 35°C), or salt (exposure to 100 mM NaCl) stresses were initiated by exchanging with medium at the stress temperature or containing elevated salt concentration. Root and leaf tissue were harvested 3, 9, and 27 h after stress initiation.

Heat and salt treatment resulted in no gross visible effect on plant morphology (data not shown). However, 3 h after cold treatment, the plants collapsed presumably due to cold shock but recovered after 27 h (data not shown). For every condition, a time-matched reference sample was used; reference plants were not subjected to a stress condition, yet the medium (at 25°C, the normal growth temperature) was exchanged. To assess the effect of medium exchange alone, a set of control experiments was performed by comparing no medium exchange to medium exchange at 25°C (normal growth temperature) at each time point.

When applying a stress condition, the response in the plant may be variable due to the nature of the treatment, variation in the response by the plants, or natural variation between the plants. Consistency across biological replicates indicates a robust, reproducible response. Thus, rather than using average fold change and selecting the more extreme fold changes, we employed linear models (Smyth 2004) as a statistic to rank the clones in order of evidence (i.e., p value) of DE, thereby addressing any variability between biological replicates. After ranking the clones based on p value, a p value cutoff was chosen to construct a clone list of significantly DE clones. A more stringent cutoff results in identification of fewer clones but also fewer false positives. Only clones with a p<0.01 (for cold and salt stress) or p<0.05 (for heat stress) in at least one condition were selected. Due to the higher variability in the heat stress, a higher cutoff was needed to identify DE clones.

Overall, very few clones could be detected as significantly DE in the control samples (76 clones, p<0.05, media exchange only), demonstrating that the medium change alone does not induce large, consistent changes in expression and that the expression changes identified in stress-treated samples are due to the stress conditions themselves. The cold stress has the most DE clones (2,584 clones, p<0.01), followed by the salt stress (1,149 clones, p<0.01), and lastly the heat stress (998 clones, p<0.05). In total, the number of clones determined to be DE in at least one condition was 3,314. A full list of all the clones is available in supplemental Table 1. This set of clones was used for all

further analysis as it reflects the stress transcriptome of the potato plants. The number of clones that could be identified as DE in each stress treatment was compared using a Venn diagram (see Fig. 1a). Overall, the three stresses share numerous DE clones. Salt and cold stress responses share a larger number of DE clones in comparison to heat vs salt stress response and heat vs cold stress response.

Identification of shared- and stress-specific responses

The Venn diagram in Fig. 1a represents simple comparisons of clone lists, and *t*-tests ($p<0.01$) between each stress were applied to detect significantly differentially expressed clones between stresses. Combined root and leaf samples from all three time points were used as the analysis of leaf and root samples or different time points separately did not identify additional clones (data not shown). The number of clones that could be identified as significantly differentially regulated between the stresses is shown in Fig. 1b. A total of 1,032 clones were identified as significant in at least one comparison leaving 2,281 clones that are expected to be regulated in a somewhat similar way between the different stress treatments. Compared to the simple comparison (Fig. 1a), this more stringent statistical approach identified fewer clones that are expected to be truly DE between the three different stress treatments.

To compare the overall similarity of the expression profiles, the clones (3,314) that were identified as significantly DE in at least one condition were used in hierarchical clustering (Fig. 2). Only nodes in the tree with >60% confidence were used to generate the consensus tree. Three of the cold stress samples form outliers in the tree (Fig. 2; node E). The cold stress leaf late time points (9 and 27 h) cluster together with the late time point salt stress leaf samples (Fig. 2; node D), indicating a similar stress response. This supports the Venn diagram comparison; salt and cold stress responses share a larger number of DE clones in comparison to heat (Fig. 1a, b). Overall, the cold root 3-h sample (Fig. 2; node A) forms an outlier indicating a more distinct transcriptional response. All heat stress samples cluster together (Fig. 2; node B) with the 27-h root sample being an outlier within the cluster. Within the remaining salt samples (Fig. 2; node C), the 3-h root sample forms the outlier of the late time-point roots and the 3-h leaf sample in the middle.

Class prediction of expression profiles allows for identification of the smallest set of clones which can accurately classify a sample and the identification of clones that differentiate the treatments. The class prediction method (Tibshirani et al. 2002) was used on the samples grouped by tissue in order to identify clones with a tissue-specific response or alternatively by stress to identify stress-spe-

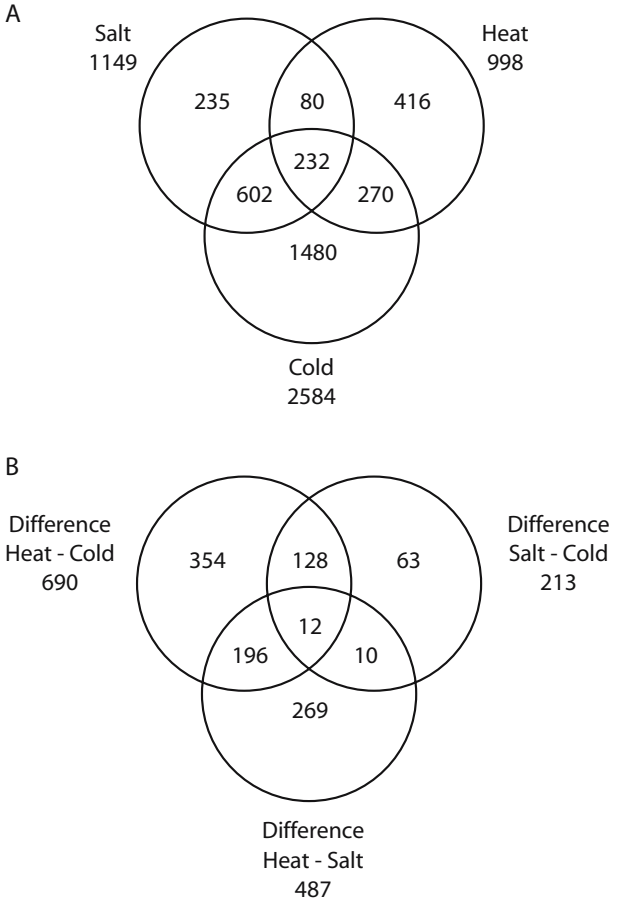

Fig. 1 Identification of shared- and stress-specific responses. **a** Venn diagram comparing the number of clones identified as DE in each stress treatment. **b** Venn diagram comparing clones that were identified as significantly different between each treatment using a *t*-test between each stress treatment

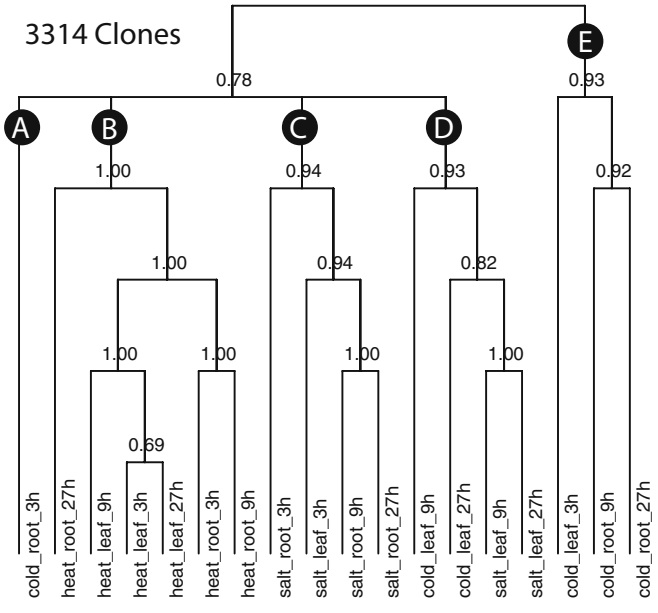

Fig. 2 Global analysis of expression data responses. Hierarchical clustering of expression profiles of clones that were found to be significantly up- or down-regulated in at least one treatment (3,314 clones total). Shown is the consensus tree after bootstrapping; only those nodes with a confidence of 60% or higher were drawn (see Materials and methods for details)

cific responses. The stress-based class prediction identified 305 clones, whereas tissue-based classification identified 280 clones (Fig. 3). Identification of specific genes using the classification method is further supported by hierarchical clustering followed by bootstrapping. Figure 3a shows the result for the stress grouping; all three stresses are clustered in three different branches. The heat and salt stress are separated from the cold stress. These profiles can be considered signature expression profiles for each stress, capable of identifying the type of stress. This knowledge can be used in comparison with other stress samples to identify the nature of the stress. In a field situation, plants may experience several abiotic stresses simultaneously, and knowledge about a stress-specific expression profile may enable establishment of the type and level of different stresses. Figure 3b shows the result for the tissue classes. Although the confidence of the clustering is 62%, the samples can be grouped into different branches based on roots or leaves. These results suggest that the clones identified by the class predictions are the best candidates for further analysis to lead to a better understanding of the shared and specific pathways in the adaptation response to various abiotic stresses.

It can be assumed that the stress is initially perceived in the roots, as this is where the stress is applied, and then progresses to the leaves. The data showed highly similar responses in roots and leaves, and therefore stress responses progressed rapidly through the entire plant. The stress response may be delayed such that clones which are not significant at a certain time point may be so at later time points or at least have a similar trend. This is illustrated in Fig. 3b in which the heat stress samples cluster with the leaf or root samples of the salt and cold stress indicating that in response to the heat stress, similar expression changes occur, although overall stress levels are probably lower. These results raise the question whether different stresses should be compared at the same time point. Although different abiotic stresses share pathways, the stress itself may not be apparent at an identical time point. Different stresses, such as a higher temperature or a higher salinity, may also induce more severe stress to the plant at an earlier time point. This can be illustrated by the salt and cold stress treatments; the response appeared to be quite different at the 3-h time point, but at the 9- and 27-h time points, the response is quite similar (see Fig. 2), suggesting that an initial difference in stress perception is followed by more similar responses. Various abiotic stressors could be better compared based on the level of stress perceived rather than time point.

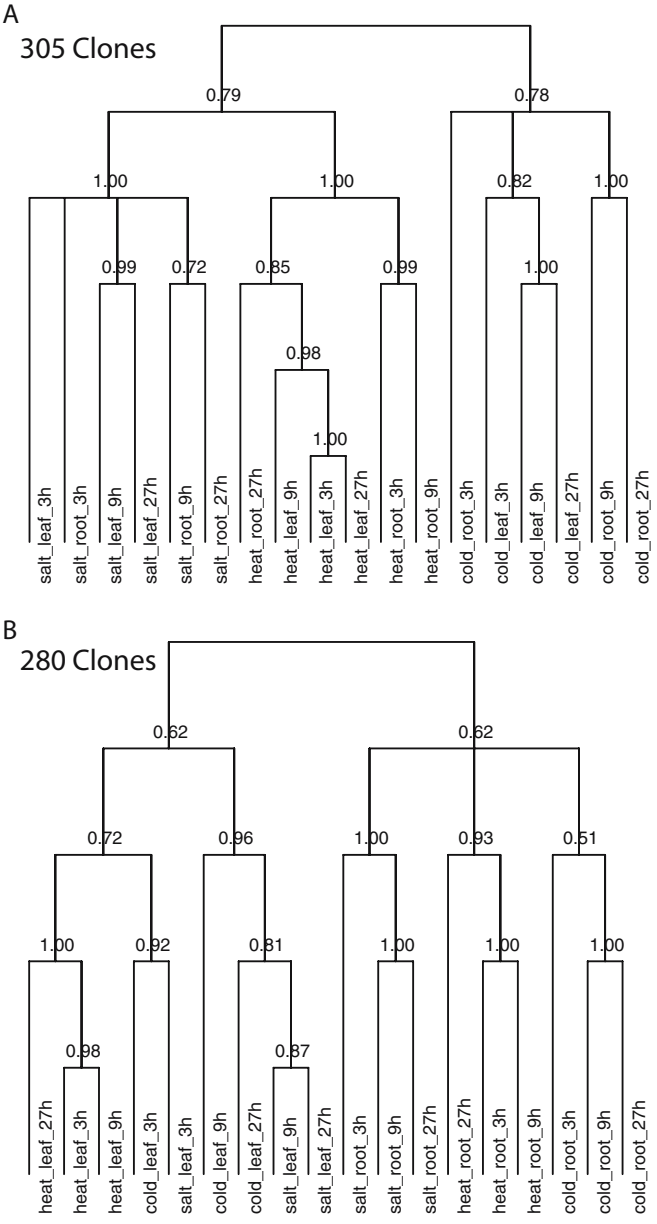

Fig. 3 Class prediction based on stress or tissue grouping. **a** Consensus tree of stress-specific clones; 305 clones were identified as classifiers for the abiotic stress treatments. **b** Consensus tree for tissue-specific clones which are shared between abiotic stresses; 280 clones were identified that could classify the samples based on tissue origin

Table 1 Annotation of the ten most up- and down-regulated clones for leaf and root samples

Functional category	Leaf samples	Root samples
Transcriptional regulation	9 (14%)	12 (9%)
Hormone related	6 (9%)	5 (4%)
Molecular chaperone/heat shock	11 (17%)	11 (8%)
Enzymatic activity	13 (20%)	24 (18%)
Signal transduction	5 (8%)	7 (5%)
Unknown	10 (16%)	31 (24%)
Transport	6 (9%)	2 (2%)
Other	4 (6%)	24 (18%)
Development/stress inducible	0	8 (6%)
Photosynthesis	0	7 (5%)

The top ten clones were selected for each condition (time point and stress) and compiled into a single list. The clones were grouped by functional category; shown is the number of clones and percentage for each functional category

Functional categorization of genes involved in the stress response

To survey the putative gene products of the clones present on the microarray, the ten most up- and down-regulated genes from each condition were identified and grouped by putative function (see Table 1). A full list of clones is shown in supplemental Table 2. The functional annotation analysis was separated for the root and leaf samples.

Overall, the gene products found in the leaf and root samples are similar, although a more diverse set of stress-related genes was found in the root samples (134 clones) compared to leaf samples (64 clones). In contrast to leaves, photosynthesis-related and development-related gene products were down-regulated in roots in response to the stress. As the roots displayed some greening, it is not surprising to find photosynthesis-related genes. The down-regulation of these clones could also be observed in the leaves (data not shown) but not listed in the top ten down-regulated genes in the leaf samples. Gene products observed in the set of ten up- and down-regulated genes further included genes previously implicated in stress adaptation, such as molecular chaperons, heat-shock proteins, late embryogenesis abundant proteins, and gene products with enzymatic activity. Several transcription factors, signal transduction proteins, and hormone-signaling related genes were identified. The identified transcription factors are of special interest as these may be the key regulators in the overall response to any of the stress treatments. Although this is the first study with potato, similar experiments have been performed with Arabidopsis, rice, maize, and barley (Hazen et al. 2003). All studies including this potato study were able to identify large numbers of genes that are DE upon abiotic stress indicating overall similar responses among different species. In contrast to Arabidopsis and rice, there is only limited genomic information available for potato, which complicates cross-species comparisons. A large number of clones represent genes with no known function; these genes could provide a basis for the discovery of novel stress-related proteins.

The annotation of clones with the largest fold change of the significantly DE clones strongly suggests that these genes have a role in the stress response and indicates that the experimental setup and downstream data analysis methods employed in this study are appropriate for the identification of stress-induced genes in potato. The findings in this study further illustrate the complexity of the abiotic stress response and the need for detailed comparisons at multiple time points. Expression profiling allowed for the global comparisons of the transcriptome as illustrated in this study; the availability of numerous characterized potato varieties with variability in stress tolerance will allow for a more detailed analysis of stress-induced genes.

Data availability

All expression data are available at the TIGR website (http://www.tigr.org/tdb/potato/) and through the Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov/geo/ under platform GPL1901. The list of significantly DE clones is available in supplemental Table 1, and the detailed annotation of the ten most up- and down-regulated clones is available in supplemental Table 2.

Acknowledgements The work on potato abiotic stress and fabrication of potato cDNA microarrays is funded by the National Science Foundation (DBI-9975866 and DBI-0218166). We thank Renae Malek for critical reading of the manuscript.

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