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Transcript profiling of salinity stress responses by large-scale expressed sequence tag analysis in *Mesembryanthemum crystallinum*

Key words: Crassulacean acid metabolism, salinity stress, common ice plant

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Abbreviations: ABP, auxin-binding proteins; EST, expressed sequence tag; CAM, Crassulacean acid metabolism; GLP, germin-like protein; LEA, late embryogenesis abundant; NCBI, National Center for Biotechnology Information; nr, non-redundant; PR, pathogenesis-related; RuBisCO, Ribulose bisphosphate carboxylase/oxygenase; GA3P, glyceraldehyde-3-phosphate; HSP, heat shock protein.

Abstract

The common ice plant, *Mesembryanthemum crystallinum*, is a halophytic (salt-loving) member of the Aizoaceae, which switches from C_3 photosynthesis to Crassulacean acid metabolism (CAM) when exposed to salinity or water-deficit stress. CAM is a metabolic adaptation of photosynthetic carbon fixation that improves water use efficiency by shifting net CO_2 uptake to the night, thereby reducing transpirational water loss. To improve our understanding of the molecular genetic underpinnings and control mechanisms for Crassulacean acid metabolism (CAM) and other salinity stress response adaptations, a total of 9,733 expressed sequence tags (ESTs) from cDNAs derived from leaf tissues of well-watered and salinity-stressed (0.5 M NaCl for 30 h and 48 h) were characterized. Clustering and assembly of these ESTs resulted in the identification of a total of 3,676 tentative unique gene sequences (1,249 tentative consensus sequences and 2,427 singleton ESTs) expressed in leaves of ice plant under unstressed and salinity stressed conditions. The same number (2,782) of ESTs from each library (total = 8,346 ESTs) were randomly selected and analyzed to compare expression profiles among the control and salt stressed leaf tissues. EST frequencies for transcripts encoding CAM-related enzymes, pathogenesis-related, senescence-associated, cell death-related, and stress-related proteins such as heat shock proteins, chaperones, early light-inducible proteins, ion homeostasis, antioxidative stress, detoxification, and biosynthetic enzymes for osmoprotectants increased 2-12-fold in cDNA libraries constructed from salt stressed plants. In contrast, the frequency of ESTs encoding light-harvesting and photosystem complexes and C_3 photosynthetic enzymes decreased four-fold overall following salinity stress with transcripts for Ribulose bisphosphate carboxylase/oxygenase subunits decreasing seven-fold. Moreover, stressed plants contained a higher percentage of ESTs encoding novel and/or functionally unknown proteins. The rapid discovery of both known and unknown genes related to stress responses in *M. crystallinum* demonstrates the great utility of EST analysis in unraveling the complex set of adaptive mechanisms contributing to water use efficiency (CAM) and salinity tolerance.

1. Introduction

The common or crystalline ice plant, *M. crystallinum*, has been used extensively to investigate plant responses to salinity and drought stresses (Adams et al., 1998; Bohnert and Cushman, 2001). Interest in this plant arose initially from the discovery of its ability to shift from C_3 photosynthesis to CAM when exposed to water deficit or high salinity stress (Cushman 2001; Cushman and Borland, 2002). CAM is a plastic, metabolic adaptation of photosynthetic carbon fixation found in about 6% of angiosperm species that limits evaporative water loss and photorespiration, and improves water use efficiency under stress conditions (Dodd et al., 2002). Many aspects of CAM research including signaling events (Taybi et al., 1999; Bakrim et al. 2001; Taybi and Cushman, 2002) and circadian regulation (Taybi et al., 2000; Dodd et al., 2003) have been investigated using the ice plant model.

The ice plant has been used extensively to investigate halophytic responses to salinity stress involving sodium accumulation in shoot tissues and partitioning to the vacuole (Barkla et al., 1999, 2002; Golldack and Dietz, 2001; Yang and Yen, 2002; Kluge et al., 2003; Epimashko et al., 2004). In contrast to glycophytes, ice plant utilizes a long-distance *myo*-inositol-dependent Na^+ transport system of vacuolar sodium/inositol symporters to reduce sodium amounts in root cells and vascular tissues and sodium/proton antiporters to partition Na^+ in the vacuoles of leaf cells (Nelson et al., 1998, 1999; Chauhan et al., 2000). The ice plant also accumulates compatible solutes as osmoprotectants, including pinitol and precursor methylated inositols, following salinity stress (Vernon and Bohnert, 1992; Ishitani et al., 1996). Ice plant has also been used as a model for investigating potassium uptake (Su et al., 2001), water channels (Kirch et al., 2000), carbon, nitrogen, and amino acid metabolism and transport (Popova et al., 2002, 2003), and abiotic stress related responses such as reactive oxygen scavenging mechanisms (Miszalski et al., 1998; Niewiadomska et al., 1999), and the biosynthesis of betalains, a class of pigments that replace anthocyanins in most members of the Caryophyllales (Vogt et al., 1999; Ibdah et al., 2002, 2003).

Despite their apparent potential utility as a source of unique genetic determinants for salinity tolerance, gene discovery efforts in halophytes have been limited (Cushman 2003). Except for the halophytic models *Suaeda maritima* subsp. Salsa, (Zhang et al., 2001; 1,000 expressed sequence tags (ESTs) dbEST release Oct. 10, 2003), and *Thellungiella halophila*, a salt tolerant relative of *A. thaliana* (Bressan et al., 2001; Zhu, 2001; 1,739 ESTs dbEST release Oct. 10, 2003), no other EST collections from halophytic plants exposed to salinity stress have been reported. To overcome this deficiency, we have undertaken an EST sequencing project from leaves of unstressed and salinity-stressed *M. crystallinum.* Comparisons of gene representation in ESTs isolated from a cDNA library generated from leaves of unstressed plants and with those derived from two cDNA libraries from leaves of salinity-stressed plants for 30 and 48 h revealed that salinity stress resulted in pronounced differences in gene expression profiles.

2. Materials and methods

2.1 Plant Material

Common ice plant (*Mesembryanthemum crystallinum*) seeds were germinated in Metromix 200 (Scotts Sierra Horticultural Products, Marysville, OH) in a growth chamber on a 12-h light (26°C)/12-h dark (18°C) cycle. Fluorescent and incandescent lighting provided a photon flux density of 450-500 μmol m^{-2} sec^{-1}. Ten-day-old seedlings were then grown in hydroponic culture in 30 L buckets containing 0.5 X Hoagland's solution under constant aeration. Six-week-old plants were stressed by adding 0.5 M NaCl [final] to the nutrient solution at beginning of light period. Leaves were sampled just before adding NaCl (control) and 30 h and 48 h after imposition of salt stress.

2.2 cDNA Library Construction and DNA Sequencing

Total RNA was extracted from leaf tissue of the control and stressed plants using Trizol® reagent (Invitrogen, Carlsbad, CA). Poly(A+) RNA was purified from the total RNA using oligo(dT) cellulose (Sigma, St. Louis, MO). Directionally cloned (*Eco*RI/*Xho*I) cDNA libraries were generated from poly(A+) RNA using a Lambda Uni-Zap-XR cDNA synthesis kit according to manufacturer's instructions (Stratagene, La Jolla, CA). The cDNA libraries were then mass-excised *in vivo* and the resulting plasmids (pBluescript II KS-) propagated in the *E. coli* SOLR host strain. For all libraries, individual white colonies were transferred to 96-well plates and preserved at –80°C. Double-stranded plasmid DNA from individual cDNA clones was isolated with a Qiagen 96-well plasmid DNA preparation kit on a Qiagen BioRobot 3000 (Qiagen, Valencia, CA) and sequenced using dideoxy chain-termination method on an Applied Biosystems 373A-XL Stretch or 3700 automated DNA sequencing system using the Prism™ Ready Reaction Dyedeoxy™ Terminator Cycle Sequencing kit (Applied Biosystems Division, Perkin-Elmer, Foster City, CA).

2.3 DNA sequence data processing and analysis

Raw single-pass sequence data were analyzed using PHRED (Ewing et al., 1998; Ewing and Green, 1998) to call bases, CROSS-MATCH to remove vector sequences, and clustered with PHRAP using the PipeOnline 2.0 automated EST processing and functional data sorting program (Ayoubi et al., 2002). Following processing, EST sequences were polished manually as required to remove low quality and/or vector-linker sequences. Following removal of vector and low quality sequences, all sequences ≤ 50 bp in length were discarded. Tentative clusters (TCs) forming contigs and singlets were compared against the nr GenBank database using BLASTX (Altschul et al., 1997) with a cutoff Expect value of 10^{-3}. Tentative gene ontologies were assigned based on top BLASTX hit alignments.

3. Results and discussion

3.1 Properties of cDNA libraries and sequencing of EST clones

Three cDNA libraries from control (unstressed) leaf tissues of *M. crystallinum* plants were constructed: one from well-watered plants and two libraries from 30- and 48-h salinity-stressed plants. These time points were selected initially to monitor induction of transcripts associated

with CAM, which begin to show increased transcript abundance only after 24-48 h salt stress treatment of soil grown plants. After conversion of the lambda phage libraries to plasmid libraries, plasmid DNA was isolated from 196 clones from each library and subjected to *Eco*RI and *Xho*I restriction endonuclease digestion. Analysis of the digestion products by agarose gel electrophoresis showed that each cDNA library had a mean insert size of about 1.1 kb (data not shown). Of the 10,500 EST attempted by sequencing from the predicted 5' end of the cDNA clones, a total of 9,733 EST sequences were acquired that passed the cleansing process. The average length of cleansed EST sequences derived from control, 30 h-stressed and 48 h-stressed leaf libraries were 538, 490, and 604 nucleotides, respectively. The sequence accession numbers and results of database homology search results using BLASTX can be viewed as web supplement 1 at (http://www.ag.unr.edu/cam/iceplant.htm). The complete *M. crystallinum* EST database can also be viewed and downloaded at the TIGR Ice plant Gene Index (McGI Release 4.0, http://www.tigr.org/tdb/mcgi/). After cleansing, all ESTs were clustered. Within each cluster, from which a consensus sequence was derived, individual ESTs were identified as belonging to either a TC or a singlet. TCs and singlets were used to conduct BLASTX searches to obtain tentative functional assignments.

3.2 Assembly into non-redundant contigs

The 9,733 ESTs in total were combined and analyzed together by PHRAP. They were assembled into 1,249 contigs and 2,427 singlets. Therefore, the total estimated number of unique genes (transcripts) identified by this EST sequencing project as being expressed in ice plant leaves was 3,676. In order to compare the frequency of ESTs present within each of the three cDNA libraries, an identical number of randomly selected EST from each library (2,782) were sampled for further analysis. A total of 8,346 EST were reassembled into contigs and unique ESTs. Roughly the same number of unique transcripts appeared in the 0 h- (control) and 30 h-stressed leaf cDNA libraries (1,351 and 1,374, respectively), but the 48 h-stressed leaf cDNA library had a larger number of unique transcripts (1,507) (Fig. 1). Furthermore, the 48 h-stressed leaf cDNA library contained a larger number of transcripts that were present only in this library (970 transcripts) than the number of library-specific transcripts found in the 0 h- (800) or 30 h-stressed leaf (863) cDNA libraries. These results suggest that stressed leaves possessed a greater diversity of transcripts, a likely result of the increased abundance of various transcripts encoding stress adaptive determinants. The overlap of transcripts between the 30 h- and 48 h-stressed libraries (353 transcripts) was slightly smaller than that between other combinations (367 between 0 h and 30 h or 393 between 0 h and 48 h) (Fig. 1). In summary, we found that only 36% to 41% of contigs from each library also appeared in the other libraries whether derived from well watered or salinity stressed leaf tissue. This observation suggests that the number of ESTs isolated from each library was not saturating. Additional EST sequencing from normalized cDNA libraries is necessary to overcome this deficiency.

3.3 Highly redundant ESTs

Analysis of transcripts from EST data provides a rapid and powerful approach for gene discovery and can provide invaluable insights into the roles of transcriptionally regulated genes that respond to various environmental stimuli (Bohnert et al., 2001). If a large number of ESTs are isolated randomly from an unbiased cDNA library, the number of ESTs fitting a particular gene should reflect the abundance of the corresponding transcript in the tissue from which the library was derived (Ewing et al., 1999; Ohlrogge and Benning, 2000; Beisson et al., 2003). As an

initial assessment of the diversity of transcripts present in leaves transitioning from an unstressed condition to salinity stress, we analyzed exactly the same number (2,782) of cleansed ESTs from each library. The total number of ESTs included in the top 25 most redundant EST derived from the 0 h (control) cDNA library was 722 (28%) of the total number of ESTs counted. However, this total was only 480 (17%) and 528 (19%) in the 30 h- and 48 h-salinity stress libraries, respectively, suggesting that redundancy among abundant transcripts was significantly reduced as a result of salinity stress. A plot of the redundancy levels of the 25 most redundant ESTs also clearly demonstrates that the cDNA libraries from 30 h- and 48 h-salinity stressed plants displayed a marked reduction in transcript redundancy for highly abundant transcripts (Fig. 2). These results demonstrate that the 0 h library contained a higher frequency of redundant transcripts, most of which encoded structural components and enzymes associated with C_3 photosynthesis (see Table 1).

By tracking the most abundant ESTs represented in each library, we observed pronounced changes in the abundance of individual transcripts following salinity stress treatment (Table 1). The putative cellular roles of gene products encoded by transcripts were predicted by BLASTX amino acid sequence comparisons. The top five most abundant ESTs that showed increased relative abundance following salt stress accounted for only 1.4% of ESTs in the 0 h cDNA library, but these same five transcripts accounted for only 2.9% and 7.3% of ESTs in the 30 h and 48 h cDNA libraries, respectively (Table 1). The vast majority of ESTs with increased abundant in either the 30 and 48 h cDNA libraries encoding gene products with assigned functions in stress response and adaptation.

EST clusters encoding transcripts for non-specific lipid-transfer proteins were the most abundant of all transcripts that showed increased abundance in the salt-stressed cDNA libraries. Although the precise roles of non-specific lipid transfer proteins remains unclear, they have been implicated to function in cuticle formation, in pathogen defense as efficient antifungal agents, and in long-distance systemic resistance signaling (Blein et al., 2002). Other ESTs that increased greatly in abundance following salt stress with anti-fungal functions related to microbial signaling and defense included those encoding pathogenesis-related protein 1a and a thaumatin-like protein. All of these pathogen-related proteins are also widely regarded as important food allergens.

The second most abundant EST TC that showed increased abundance in the salt-stressed cDNA libraries encoded *myo*-inositol 1-phosphate synthase, a pivotal enzyme responsible for the accumulation of methylated inositols, D-ononitol and D-pinitol, which serve as osmoprotectants (Ishitani et al., 1996). Transcripts for this enzyme increase approximately 10-fold during salinity stress consistent with the observed EST frequency increase in the 48 h-salinity stress library where *myo*-inositol 1-phosphate synthase transcripts accounted for 1.9% of all transcripts (Table 1). The relatively high EST frequency (0.50%) of *myo*-inositol 1-phosphate synthase transcripts in unstressed leaf libraries suggests that this transcript was being well expressed in 6 week-old plants even under unstressed conditions. ESTs encoding *myo*-inositol 4-O-methyltransferase, the enzyme that catalyzes the first step in the biosynthesis of D-pinitol also showed increased frequency in the 30 h and 48 h cDNA libraries, consistent with the previously reported salinity-induced expression of this gene in ice plant (Vernon et al., 1992). ESTs representing transcripts encoding galactinol synthase, which catalyses the first step in the biosynthesis of raffinose family oligosaccharides from UDP-galactose, were well represented (0.5%) in the 48 h-stressed cDNA library, but were not found in the other libraries (Table 1). Galactinol and raffinose accumulate in response to water deficit, high salinity, and cold stress in

Arabidopsis and are likely to function as osmoprotectants (Taji et al., 2002).

The third most abundant EST TC class encoded a early light-inducible protein (ELIP), a chlorophyll a- and lutein-binding protein, which plays a protective role against photooxidative stress, by acting as either a chlorophyll pigment carrier or as a sink for excess excitation energy. ELIP transcripts are induced by UV-B irradiation, high light, cold, high salinity, and wounding stress (Savenstrand et al., 2004). Four of the most abundant stress-induced EST TCs encode various small heat shock proteins, which function as ATP-independent chaperones that prevent irreversible protein aggregation and facilitate subsequence protein renaturation in cooperation with ATP-dependent chaperones (Basha et al., 2004). These sHSPs are likely to protect a large set of proteins with diverse cellular functions against damage resulting from salinity stress. Cysteine proteinase transcripts increased in abundance in both cDNA libraries from salt-stressed plants, whereas no ESTs for this TC were present in the 0 h control cDNA library (Table 1). This salt-stress-induced cysteine proteinase gene has been described previously in ice plant (Forsthoefel et al., 1998). Two TCs that increased in abundance in the salt stressed cDNA libraries encoded vacuolar H^+-ATPase subunits B and G consistent with previous reports that these two subunits exhibited salt-stress-induced transcript accumulation in ice plant (Kluge et al., 2003). Interestingly, three highly abundant EST TCs encoding proteins with similarity to previously characterized genes whose functions remain unclear, designated as unclear classification proteins 1-3, and one EST TC encoding a novel proteins with no similarity to other known sequences, were found to increase in abundance following salt stress. Based on their relative abundance, these unknown and novel TCs are predicted to encode proteins with functions related to stress-adaptation.

In contrast, the vast majority of ESTs that were most abundant in the 0 h cDNA library and decreased in abundance following salt stress, as illustrated by a decrease in relative frequency in the 30 h- or 48 h-stress cDNA libraries, represented transcripts encoding gene products with assigned functions in C_3 photosynthesis or its regulation (Table 1). The top five most abundant ESTs in the 0 h cDNA library accounted for nearly 20% of transcripts in terms of their relative abundance (Table 1). However, in the 30 h and 48 h cDNA libraries, these same five transcripts accounted for only 7.1% and 4.8% of ESTs, respectively. Specifically, EST abundance for the large and small subunits of RuBisCO decreased seven-fold. ESTs encoding chlorophyll a/b binding proteins of the photosystem I and II light harvesting complexes decreased six-fold. ESTs encoding RuBisCO activase, carbonic anhydrase, photosystem I and II reaction center subunits, oxygen evolving complexes, and mitochondrial ATP synthase and plastidic photosynthetic electron transport chain complex components also showed marked declines in abundance (Table 1). Two TCs encoding proteins homologous to the germin-like proteins (GLPs) were highly abundant in the 0 h cDNA library (0.47% and 0.4%), whereas only five such ESTs in total were present in the 30 h cDNA library and no such ESTs were found in the 48 h cDNA libraries. Michalowski and Bohnert (1992) isolated the cDNA of another member of GLP gene family from *M. crystallinum* root tissue (GenBank accession No. M93041) and observed that mRNA level of this GLP declined during salt stress in the root tissue. GLPs belong to the 'cupin superfamily', which encodes other proteins with a wide variety of functions, such as oxalate oxidases, several types of dioxygenases and auxin-binding proteins (ABP) (Dunwell et al., 2000). In fact, both of the GLP transcripts described here are highly similar to ABP19/20 from peach (Ohmiya et al., 1998). Further investigations are required to elucidate the function of these GLPs in leaf and root tissues.

In contrast to ESTs associated with C_3 photosynthesis, a transient increase in the abundance

of several CAM-related ESTs was observed in the 30 h stress cDNA libraries. For example, ESTs for both NAD-glyceraldehyde 3-phosphate dehydrogenase (*GapC1*) and a CAM-specific isozyme of phospho*enol*pyruvate carboxylase (*Ppc1)*, displayed a transient increase in frequency in the 30 h cDNA library (Table 2).. This decline in EST abundance after 48 h was unexpected, however, since transcript abundance for both *GapC1* and *Ppc1* transcripts increase steadily over a period of five days during CAM induction (Ostrem et al., 1990; Cushman et al., 1989). The observed differences in *GapC1* and *Ppc1* EST abundance between the 30 h and 48 h libraries are likely to reflect circadian changes in the abundance of these transcripts, because the control and 48 h stressed leaf cDNA were made from tissues harvested just before the beginning of light period, whereas 30 h-stressed leaves were harvested at 6 h after the beginning of light period. Detailed time-course studies of transcript abundance have confirmed that both of these genes display circadian expression patterns with increased abundance during the light period (Boxall et al., 2001). Other ESTs that display a transient increase in EST frequency in the 30 h library may also be subjected to circadian regulation (Table 2). Similarly, control of genes under different phase outputs of the circadian clock may be represented by ESTs with transient decreases in frequency in the 30 h cDNA library (Table 2). However, detailed mRNA expression profiling of these genes is required to confirm this hypothesis. A more detailed summary of the abundance distribution of all ESTs within each cDNA library is available as web supplement 2 at http://www.ag.unr.edu/cam/iceplant.htm.

3.4 Origins of homologous sequences based on the nr database

The sequences of non-redundant clusters (contigs and singlets) were compared with the 'nr' protein database using BLASTX and classified according to the origin (*M. crystallinum*, *A. thaliana*, other plants, non-plant, organelle, or unclassified) (Fig. 3). The vast majority (70% to 80%) of ice plant ESTs sequences showed significant matches to ice plant itself or other plant gene accessions in the nr database. The proportion of organellar matches declined with increasing duration of salt stress, largely due to the decline in abundance of ESTs for the large subunit of RuBisCO. Homologous sequence matches from non-plant accessions were less than 2% in every library. Even with a default threshold E-value of 10e-3, a small percentage of transcripts did not have any similarities with other known sequences in the nr database (Fig. 3). Such unclassified (no similarity to other known sequences) transcripts comprise 16.1% (422 ESTs) of ESTs from the 0 h (control) cDNA library, whereas 25% (695 ESTs) were from the 30 h-stressed leaf cDNA library. The percentage of unclassified ESTs from the 48 h-stressed cDNA library was 15.2% (449 ESTs).

3.5 Functional categorization of ESTs

ESTs were given a putative functional assignment according to the classification developed for plant genes by Bevan et al. (2000) dividing functional assignments into categories and subcategories of, for example, Photosynthesis (02.30), Ion transporters (07.01), Transport ATPases (07.22) and Stress responses (11.05) (Table 3). Functional category assignments were inferred based entirely on significant similarity scores from BLASTX reports. The most striking difference in EST abundance observed between control and salt-stressed leaf cDNA libraries was the reduction of transcripts related to C_3-photosynthesis during salinity stress, excluding genes encoding chloroplastic H^+-ATPase subunits. A major proportion of ESTs (27%) from the control leaf cDNA libraries represented transcripts involved in C_3-photosynthesis (02.3001) mainly due to the high expression of RuBisCO subunits, light harvesting complex

components, and cytochrome b-559 genes. The abundance of C_3-photosynthesis-related transcripts was reduced by approximately one-fourth in the 48 h salinity stress cDNA library relative to the control library largely as a result of declines in almost all ESTs in this category.

The relative proportion of ESTs related to Sugar/polysaccharide metabolism (01.05) and organic acid metabolism (01.08), TCA pathway (02.10), and Fermentation (02.15) increased more than two-fold in the 48 h cDNA library. CAM induction in *M. crystallinum* is accompanied by substantial increases (10-20-fold) in the activities of several phosphorylytic and amylolytic starch degrading enzymes, including alpha and beta-amylases, starch phosphorylase and glucanotransferase (Paul et al. 1993; Häusler et al. 2000; Dodd et al. 2003). The subcategory Sugar/polysaccharide metabolism (01.05) includes starch-synthesizing/degrading enzymes such as alpha and beta-amylases, starch-branching enzymes and starch synthases. EST redundancies in this category increase more than two-fold after 48 h of salt stress consistent with the known increases in transcript abundance for many genes encoding such enzymes.

Slightly increased redundancy for ESTs representing photorespiration-related functions might suggest that transcripts for such genes increase in abundance after 30 h and 48 h of salt stress. Although CAM is thought to be a CO_2 concentration mechanism, high rates of photorespiration are suspected to occur when CAM plants are assimilating atmospheric CO_2 with open stomata at the end of light period (Maxwell et al., 1999; Niewiadomska et al., 1999). Among enzymes related to photorespiration, a pronounced increase was observed in EST abundance of glycolate oxidase (0.04% ESTs in the control versus 0.32% ESTs in the 48 h cDNA library; see Table 1), whereas the overall proportion of ESTs in the Photorespiration subcategory (02.3003) increased only slightly in cDNA libraries constructed from salt stressed plants relative to control plants. This observation is largely consistent with direct measurements of photorespiratory enzyme activities, including glycolate oxidase activity, which differed very little between control and salt-stressed ice plants (Whitehouse et al., 1991). It is necessary to further investigate the regulation of glycolate oxidase gene expression during CAM induction in order to elucidate the possible significance of this predicted increase transcript abundance.

EST redundancy sampling was also able to confirm induction of transcripts known to exhibit increased abundance following salt stress such as those encoding vacuolar H^+-ATPase subunits B and c (Low et al., 1996; Tsiantis et al., 1996; Kluge et al., 2003). In contrast, EST sampling indicated a decline in mitochondrial and chloroplastic H^+-ATPase transcript abundance (Table 3). Transcript abundance increases for *myo*-inositol 4-O-methyltransferase (Vernon and Bohnert, 1992), *myo*-inositol 1-phosphate synthase (Ishitani et al., 1996), and galactinol synthase (Taji et al, 2002) are reflected in the more than 3.5-fold increase in EST frequencies for these mRNAs in leaf libraries from salt stressed plants relative to unstressed plants (Table 1). EST sampling also predicts increases in Sodium transporter (07.0101) mRNA abundance consistent with the fact that *M. crystallinum* is a leaf succulent that accumulates and sequesters high concentrations of salt within its leaf tissues (Adams et al., 1998). Finally, almost all subcategories in the Disease/defense category (11) displayed dramatic increases in EST abundance following salt stress. This category includes defense-related proteins such as pathogenesis-related (PR) proteins, senescence-associated proteins, cell death-related proteins, and stress proteins such as heat shock proteins (HSPs), chaperones, early light-inducible proteins, and late-embryogenesis abundant proteins (data not shown). Overall, ESTs of HSPs and chaperones (11.0501) were induced four-fold at 48 h following a transient decline at 30 h. Many of the genes encoding these types of proteins are well known to be induced during salt, osmotic and/or water deficit stress in other higher plants (Ozturk et al., 2002; Kreps et al., 2002; Seki et al., 2002a,b). Antioxidative

enzymes, especially glutathione S-transferases and L-ascorbate peroxidase (see Table 2), also greatly increased together with proteins related to detoxification (11.06) such as cytochrome P450s and metallothioneins. These observations are consistent with recent observations that mRNA abundance for some antioxidative enzymes in *M. crystallinum* increases following salt stress (Slesak et al., 2002). Within the category Transporters (07), EST abundance within each subcategory differed greatly probably dependent on specific requirements of stress adaptation and/or CAM, whereas overall EST abundance in this category was unchanged during stress. In some other subcategories, such complex patterns of changes in EST abundance were also observed. In addition to C_4-Photosynthesis/CAM (02.3002), the abundance of ESTs related to Glycolysis (02.01) and Phenylpropanoids (20.01) increased transiently at 30 h, but returned to almost the same percentages as in the control at 48 h.

Finally, many genes with homologues in other organisms that have not been assigned a function (designated as "unclear classification" in Table 3) and have no recognizable homologue in any other organisms (designated as "unclassified" in Table 3) were highly represented as ESTs in leaves of both well watered and salinity stressed plants. It will be important to determine the potential functions of such unknown genes, as they may be responsible for making a unique contribution to, for example, the halophytic character of *M. crystallinum*. The increased transcript abundance of functionally unknown or novel, particularly in salinity-stressed ice plant, may also aid in the functional assignment of orthologous genes in other plant species. Both *S. maritima* and *M. crystallinum* are leaf succulents that accumulate and sequester high concentrations of salt within foliar tissues. A detailed comparison of EST derived genes from these two species relative to glycophytic species, may reveal unique coding sequences that are essential for the survival of euhalophytes under extremely high concentrations of salt. Such genes may provide the foundation for novel genetic engineering strategies for improving salinity tolerance in crop plants.

4. Conclusions

In summary, we have characterized 9,733 ESTs representing 3,676 tentative unique genes from both unstressed and salinity stressed *M. crystallinum* cDNA libraries. cDNA libraries derived from unstressed leaves contained more redundant transcripts from a fewer number of highly expressed genes encoding enzymes and structural component of the C_3-photosynthetic apparatus. These highly redundant ESTs undergo a four-fold reduction in relative expression following salinity stress. ESTs derived from the 30 h- or 48 h-stressed leaf libraries, however, displayed a greater range of functional diversity with EST having stress response and adaptation functions increasing in relative abundance 2-12-fold. Thus, the use of stressed leaf libraries can facilitate the isolation of less abundant, but more functionally diverse sets of transcripts within the same EST sample size. Furthermore, a higher proportion of ESTs with novel and unknown functions were recovered from cDNA libraries constructed from 30 h and 48 h salinity stressed leaves, respectively, suggesting that salinity stress-related genes are less likely to be represented or functionally characterized in cDNA libraries derived from plants grown under unstressed conditions.

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Figure Legends

Figure 1. Distribution of EST contigs among three cDNA libraries from unstressed (control) and salt-stressed (for 30 h and 48 h) leaves. Numbers in two or more rings indicate the overlap of EST-derived contigs present within each individual library.

Figure 2. Comparison of redundancy among the top 25 most abundant ESTs among the three cDNA libraries from unstressed (0 h, control) and salt-stressed (30 h and 48 h) leaves. The number of ESTs within each of the most abundant contigs among three cDNA libraries from unstressed (0 h, control) and salt-stressed (for 30 h and 48 h) leaves is plotted against the gene number.

Figure 3. Distribution of ice plant ESTs based on gene origins. The relative percentage of ESTs, not contigs, within each category of origin was plotted. Sequence homology-based BLASTX searches were performed for corresponding contigs and singlets against the nr protein database.

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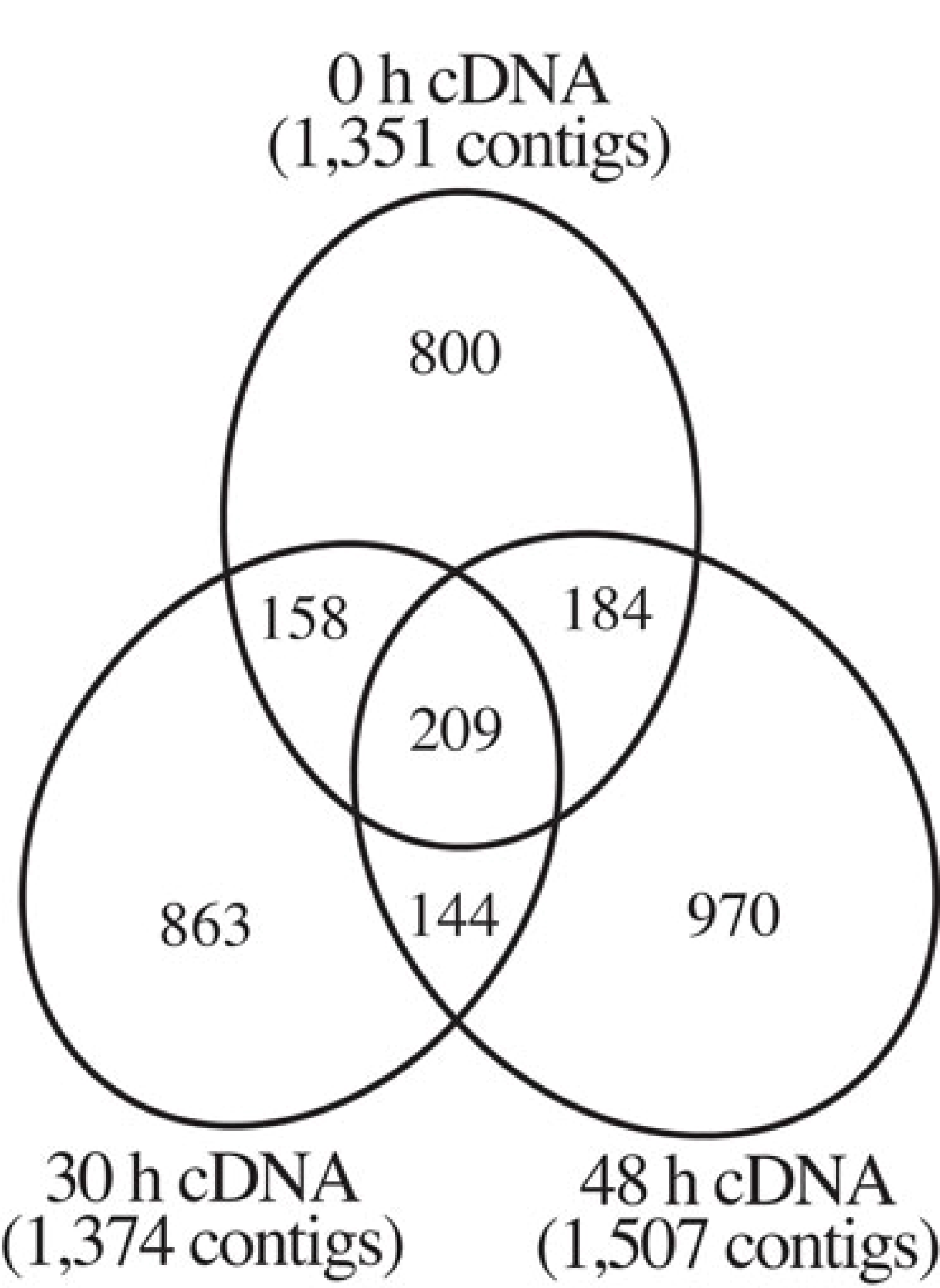
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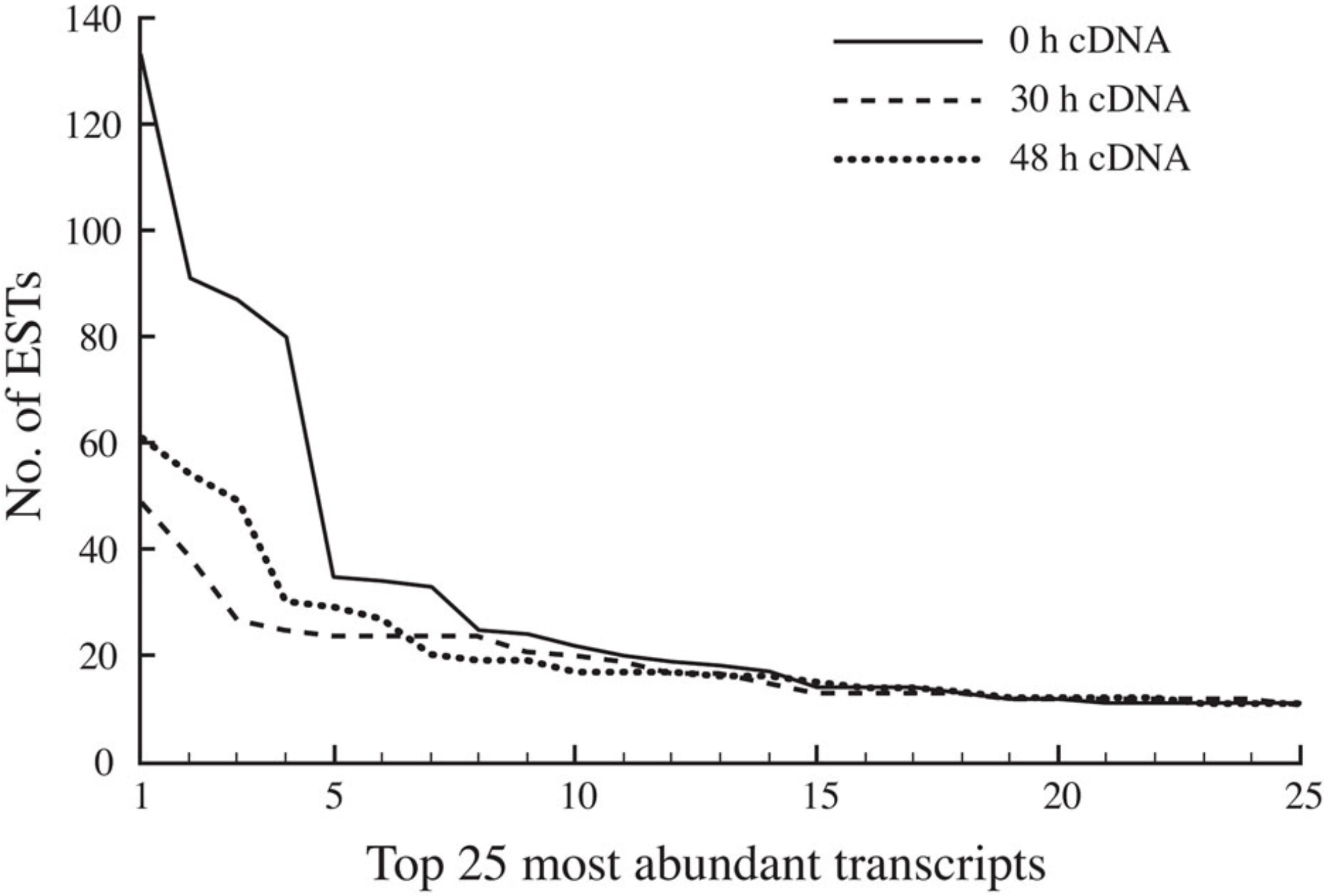
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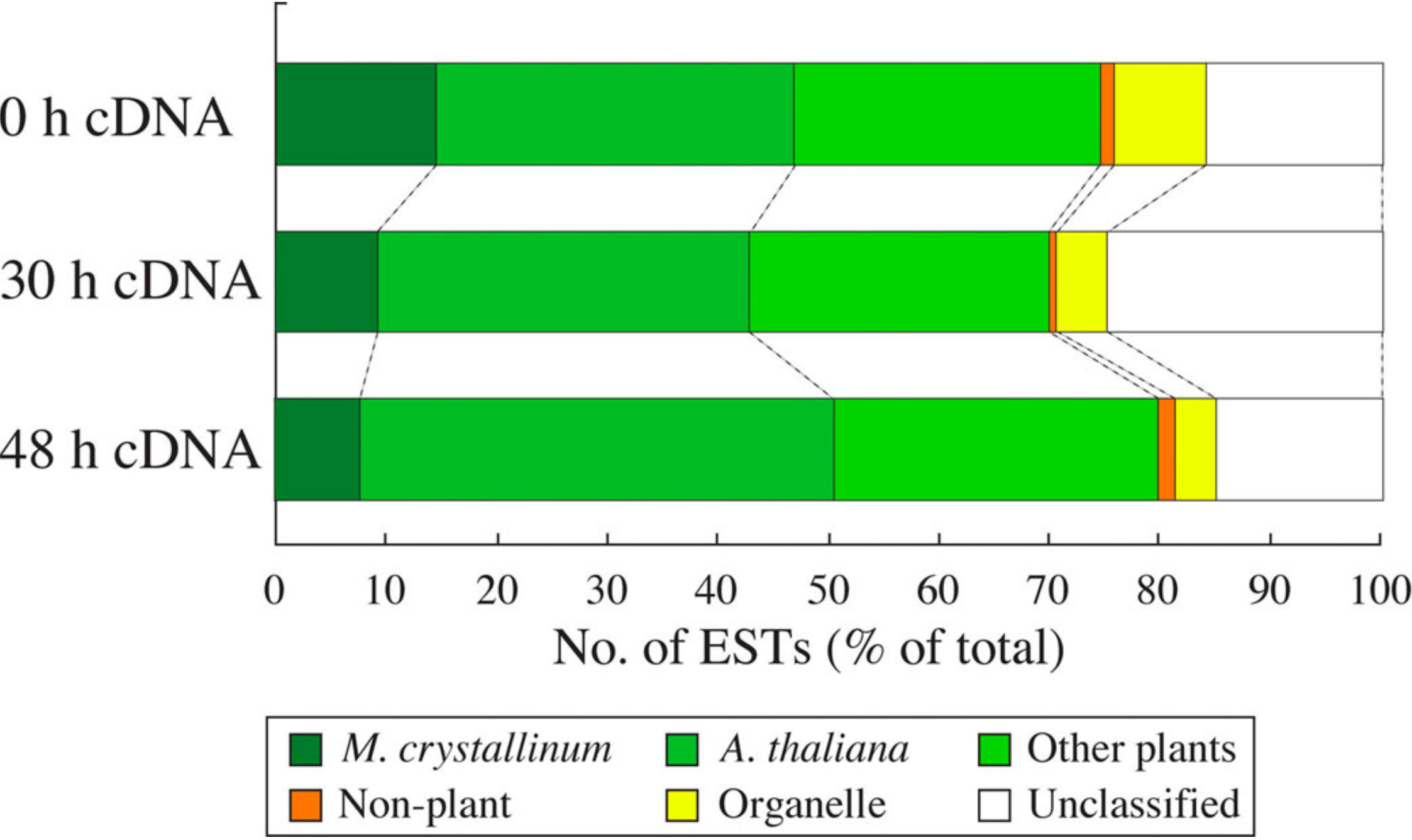
No. of ESTs
0 h cDNA
30 h cDNA
48 h cDNA
1
5
10
15
20
25
Top 25 most abundant transcripts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48 h cDNA
0
10
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30
40
50
60
70
80
90
100
No. of ESTs (% of total)
M. crystallinum
A. thaliana
Other plants
Non-plant
Organelle
Unclassified

Table 1. ESTs of most redundant transcripts with increased or decreased frequency in control and salt-stressed (30 h and 48 h) leaf cDNA libraries.

	Relative percentage[a]			
	Control	30 h	48 h	Total
Increased following salt stress				
Non specific lipid-transfer protein[b]	0.36	1.65	1.72	0.91
myo-Inositol-1-phosphate synthase	0.50	0.04	1.94	0.82
Early light-inducible protein	0.40	0.22	1.76	0.79
Heat shock protein, 18.2 kDa[b]	0.14	0.00	1.54	0.56
Cysteine proteinase[b]	0.00	1.00	0.39	0.47
Carbokyvinyl-carboxyphosphonate phosphorylmutase	0.22	0.04	1.08	0.44
Pathogenesis-related protein 1a	0.18	0.04	1.04	0.42
Cold or osmotic stress inducible protein	0.04	0.43	0.68	0.38
Vacuolar H^+-ATPase, G subunit	0.22	0.29	0.61	0.37
Heat shock protein, 18 kDa	0.11	0.00	0.97	0.36
ClpP ATP-dependent protease, proteolytic subunit	0.22	0.47	0.36	0.34
Metallothionein[b]	0.18	0.36	0.39	0.33
Cystatin	0.14	0.54	0.25	0.31
Vacuolar H^+-ATPase, c subunit	0.18	0.18	0.43	0.26
myo-Inositol 4-O-methyltransferase	0.07	0.43	0.25	0.25
Heat shock protein, 18.2 kDa	0.14	0.00	1.54	0.56
Unclassified[d] (protein 1)	0.04	0.61	0.11	0.25
Histone H3	0.22	0.07	0.43	0.24
Senescence-associated protein	0.04	0.25	0.43	0.24
Unclear classification[c] (protein 1)	0.07	0.32	0.25	0.22
Unclear classification[c] (protein 2)	0.11	0.11	0.39	0.2
Vacuolar H+-ATPase, B subunit	0.07	0.36	0.18	0.2
Peptidylprolyl isomerase	0.04	0.36	0.18	0.19
Formate dehydrogenase (NAD-dependent), mitochondrial	0.04	0.25	0.25	0.18
Heat shock protein, 83 kDa	0.04	0.00	0.47	0.17
Cellulase	0.04	0.22	0.25	0.17
Galactinol synthase	0.00	0.00	0.50	0.17
Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	0.07	0.18	0.22	0.16
Glycolate oxidase	0.04	0.11	0.32	0.16
Thaumatin-like protein	0.04	0.11	0.32	0.16
Heat shock protein, 17.8 kDa	0.11	0.00	0.32	0.14
Unclear classification[c] (protein 3)	0.11	0.04	0.28	0.14
FtsH-like protein	0.04	0.00	0.39	0.14
Decreased following salt stress				
RuBisCO, small subunit[b]	8.34	2.98	0.97	4.1
Cytochrome *b559*, alpha and beta subunits	2.88	0.86	2.19	1.97
Chl *a*/*b*-binding protein, LHC I & II[b]	3.90	1.11	0.65	1.89
RuBisCO, large subunit	3.31	1.76	0.39	1.82
Unclassified[d] (protein 2)	1.26	0.43	0.61	0.76
ATP synthase, subunit 9, mitochondrial	1.19	0.86	0.14	0.73
Unclear classification[c] (protein 3)	0.72	0.75	0.32	0.6
Photosystem II, K subunit	0.86	0.40	0.36	0.54
RuBisCO activase	0.90	0.18	0.07	0.38
Ultraviolet-B-repressible protein	0.65	0.18	0.18	0.33
Oxygen-evolving enhancer protein 2	0.40	0.32	0.11	0.28
Photosystem I reaction center, N subunit	0.40	0.32	0.00	0.24
Oxygen-evolving enhancer protein 1	0.40	0.32	0.11	0.24
Photosystem II reaction center W protein precursor	0.36	0.18	0.11	0.22
ycf4	0.32	0.05	0.18	0.22
Carbonate dehydratase (Carbonic anhydrase)	0.50	0.07	0.00	0.19
Cytochrome B6-F complex Iron-sulfur subunit precursor	0.32	0.11	0.14	0.19
Germin-like (protein 1)	0.47	0.07	0.00	0.18
Unclassified[d] (protein 3)	0.32	0.14	0.07	0.18
Germin-like (protein 2)	0.40	0.11	0.00	0.17
Photosystem I reaction center subunit II	0.32	0.11	0.04	0.15
ATP synthase B' subunit (subunit II), chloroplastic	0.29	0.07	0.11	0.15
Photosystem I reaction center subunit IV precursor	0.21	0.11	0.11	0.14
Phosphopyruvate hydratase	0.21	0.14	0.07	0.14
Pheromone response factor 1	0.21	0.21	0.00	0.14

[a] Percentage of ESTs from each library.

[b] All transcripts combined

[c] Similar to known sequences whose functions are unclear.

[d] No similarity to other known sequences.

Table 2. ESTs of most redundant transcripts with transiently increased or decreased frequency in control (0 h) and salt-stressed (30 h and 48 h) leaf cDNA libraries.

	Relative percentage[a]			
	Control	30 h	48 h	Total
Increased transientlyfollowing salt stress				
Antimicrobial peptide 1 precursor	0.36	0.72	0.50	0.52
Glyceraldehyde 3-phosphate dehydrogenase (cytosolic)	0.29	0.86	0.29	0.48
Phosphoenolpyruvate carboxylase 1	0.07	0.90	0.14	0.37
Photosystem II 10 KD polypeptide precursor (light inducible)	0.21	0.43	0.18	0.27
Unclassified[d] (protein 4)	0.04	0.61	0.11	0.25
40S Ribosomal protein S4	0.14	0.43	0.04	0.2
Elicitor-inducible protein	0.07	0.46	0.00	0.18
Macrophage migration inhibitory factor family protein	0.04	0.43	0.04	0.17
Phase-change related protein	0.14	0.32	0.00	0.15
Unclassified[d] (protein 5)	0.00	0.15	0.00	0.15
Glycine dehydrogenase (decarboxylating) mitochondrial precursor	0.07	0.29	0.07	0.14
Progesterone receptor	0.11	0.29	0.00	0.13
Apocytochrome F precursor (cytochrome f)	0.07	0.29	0.07	0.13
Dormancy-associated protein	0.07	0.32	0.00	0.13
Unclassified[d] (protein 6)	0.00	0.39	0.00	0.13
L-ascorbate peroxidase	0.07	0.25	0.04	0.12
Photosystem I reaction center subunit VI precursor	0.07	0.21	0.04	0.11
Latex profilin	0.04	0.25	0.04	0.11
Unclear classification[c] (protein 4)	0.04	0.29	0.00	0.11
Decreased transiently following salt stress				
Polyubiquitin	0.68	0.18	0.61	0.49
Fructose-bisphosphate aldolase (plastidic)	0.61	0.18	0.57	0.45
S-adenosylmethionine decarboxylase proenzyme	0.50	0.18	0.68	0.45
Plastocyanin	0.29	0.14	0.18	0.02
Jasmonate-induced protein, 23 kDa	0.18	0.07	0.25	0.17
Unclassified[d] (protein 7)	0.21	0.04	0.18	0.14
Tonoplast intrinsic protein	0.18	0.00	0.18	0.12
Unclear classification[c] (protein 5)	0.14	0.07	0.18	0.12
Unclear classification[c] (protein 6)	0.18	0.00	0.14	0.11

[a] Percentage of ESTs from each library.

[b] All transcripts combined

[c] Similar to known sequences whose functions are unclear.

[d] No similarity to other known sequences.

Table 3. EST abundance in 0 h (control) and salt-stressed (30 h and 48 h) leaf cDNA libraries of *M. crystallinum*

Functional category		Control[a]	30 h[b]	48 h[c]
01	**Metabolism**	**5.50**	**4.67**	**9.63**
01.01	Amino acid	1.19	1.11	1.62
01.02	Nitrogen/sulfur	0.50	0.11	0.36
01.03	Nucleotide	0.58	0.22	0.36
01.04	Phosphate	0.11	0.11	0.22
01.05	Sugars/polysaccharide	1.94	1.40	5.03
01.06	Lipid and sterol	0.86	1.44	1.44
01.07	Cofactor	0.22	0.18	0.25
01.08	Organic acid	0.11	0.11	0.36
02	**Energy**	**30.7**	**17.7**	**11.5**
02.01	Glycolysis	0.93	1.87	0.79
02.02	Gluconeogenesis	0.18	0.11	0.00
02.07	Pentose phosphate	0.18	0.07	0.22
02.10	TCA pathway	0.25	0.43	0.54
02.13	Respiration	0.32	0.79	0.68
02.15	Fermentation	0.11	0.40	0.61
02.20	E-transport	0.61	0.40	0.58
02.3001	C_3-Photosynthesis	27.2	11.5	7.12
02.3002	C_4-Photosynthesis/CAM	0.40	1.22	0.29
02.3003	Photorespiration	0.50	0.93	0.72
03	**Cell growth/division**	**0.58**	**0.54**	**0.75**
03.01	Cell growth	0.07	0.07	0.00
03.13	Meiosis	0.04	0.00	0.07
03.16	DNA synthesis/replication	0.07	0.07	0.04
03.19	Recombination/repair	0.00	0.04	0.04
03.22	Cell cycle	0.04	0.04	0.11
03.26	Growth regulators	0.29	0.29	0.29
03.99	Others	0.07	0.04	0.22
04	**Transcription**	**2.59**	**1.94**	**2.88**
04.19	mRNA synthesis	0.83	0.65	1.15
04.1901	General transcription factors	0.07	0.07	0.04
04.1904	Specific transcription factors	0.61	0.47	0.79
04.1907	Chromatin modification	0.04	0.07	0.11
04.22	mRNA processing	0.50	0.29	0.36
04.31	RNA transport	0.04	0.00	0.00
04.99	Other RNA binding protein	0.50	0.40	0.43
05	**Protein synthesis**	**3.88**	**4.85**	**3.24**
05.01	Ribosomal proteins	2.77	3.06	1.65
05.04	Translation factors	1.01	1.44	1.37
05.07	Translation control	0.00	0.22	0.00
05.10	tRNA synthases	0.11	0.14	0.22
06	**Protein destination**	**4.10**	**5.68**	**5.64**
06.01	Folding and stability	0.29	0.72	0.50
06.04	Targeting	0.25	0.29	0.25
06.07	Modification	0.32	0.36	0.36
06.10	Complex assembly	0.36	0.25	0.22
06.13	Proteolysis	2.88	4.06	4.31
07	**Transporters**	**3.41**	**3.63**	**4.35**
07.0101	Sodium transporter	0.04	0.04	0.11
07.0109	Other ion transporters	0.43	0.79	0.65
07.07	Sugar transporter	0.00	0.04	0.07
07.10	Amino acid transporter	0.18	0.00	0.14
07.16	Purine/pyrimidine transporter	0.04	0.18	0.11
07.2201	Mitochondrial H^+-ATPase	0.29	0.40	0.36
07.2202	Chloroplastic H^+-ATPase	0.86	0.14	0.29
07.2203	Vacuolar H^+-ATPase	0.93	1.98	1.83
07.2209	Other H^+-ATPases	0.04	0.00	0.07
07.25	ABC-type transporter	0.14	0.04	0.22
07.30	Water channel	0.25	0.04	0.07
07.99	Others transporter	0.22	0.00	0.43
08	**Intracellular traffic**	**0.43**	**0.18**	**0.32**
08.04	Mitochondrial	0.00	0.04	0.04
08.07	Vesicular	0.43	0.14	0.29
09	**Cell structure**	**1.80**	**1.40**	**2.05**
09.01	Cell wall	0.18	0.11	0.29
09.04	Cytoskeleton	0.61	0.75	0.32
09.10	Nucleus	0.07	0.18	0.07
09.13	Chromosomes	0.50	0.14	0.75
09.25	Vacuole	0.18	0.00	0.22
09.28	Chloroplast	0.07	0.18	0.29
09.99	Others	0.18	0.04	0.11
10	**Signal transduction**	**2.37**	**3.38**	**2.73**
10.01	Receptors	0.04	0.07	0.14
10.04	Mediators	0.54	0.86	0.93
10.0404	Kinases	0.97	1.04	0.90
10.0407	Phosphatases	0.54	0.54	0.32
10.0410	G proteins	0.29	0.54	0.43
10.99	Others	0.00	0.32	0.00
11	**Disease/defense**	**6.61**	**8.48**	**15.1**
11.01	Resistance genes	0.29	0.14	0.11
11.02	Defense-related	1.37	2.41	3.49
11.03	Cell death	0.07	0.43	0.61
11.05	Stress responses	2.23	3.38	3.52
11.0501	HSP and chaperons	1.22	0.14	4.89
11.06	Detoxification	1.44	1.98	2.48
12	**Unclear classification[e]**	**20.3**	**20.7**	**24.9**
13	**Unclassified[f]**	**16.1**	**25.0**	**15.2**
14	**Transposons**	**0.11**	**0.04**	**0.29**
20	**Secondary metabolism**	**1.51**	**1.80**	**1.44**
20.01	Phenylpropanoids	0.32	0.72	0.36
20.02	Terpenoids	0.11	0.11	0.07
20.05	Amines	0.72	0.68	0.86
20.07	Tetrapyroles	0.25	0.22	0.07
20.99	Other secondary metabolism	0.11	0.07	0.07

[a]Percentage of ESTs in each category present in the 0 h (control) cDNA library from unstressed leaves.
[b]Percentage of ESTs in each category present in the cDNA library from plants salt-stressed for 30 h.
[c]Percentage of ESTs in each category present in the cDNA library from plants salt-stressed for 48 h.
[d]The percentages were calculated from the total number of ESTs present within each functional category from each library.
[e]Unclear classification are sequences with significant similarity to known sequences whose functions are unclear.