

# TRANSCRIPT PROFILING OF SALINITY STRESS RESPONSES BY LARGE-SCALE ANALYSIS OF EST IN *Mesembryanthemum crystallinum*.

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## ABSTRACT

We have conducted large-scale expressed sequence tag (EST) analysis of randomly selected cDNAs derived from leaf tissues of well-watered and salinity-stressed (0.4 M NaCl for 30 hr and 48 hr) *Mesembryanthemum crystallinum*, a facultative Crassulacean acid metabolism (CAM) plant. At present, 9,733 ESTs have been obtained from leaf tissues representing 3,676 non-redundant sequences. The same number (2,782) of EST sequences were picked randomly from each library (total = 8,346) and analyzed further to compare expression profiles between the control and stressed leaf tissues. Abundance of transcripts encoding light harvesting and photosystem complexes and C3 photosynthetic enzymes decreased dramatically following salinity stress. In contrast, salt stress brought about pronounced increases in transcripts involved in CAM, disease and defense responses, abiotic stress adaptation, proteolysis and ion homeostasis. Moreover, stressed plants contained a higher percentage of ESTs encoding novel and/or functionally unknown proteins. The rapid discovery of new and unknown genes related to stress adaptation in *M. crystallinum* demonstrates the great utility of EST analysis in unraveling the complex set of adaptive mechanisms contributing to salinity and drought tolerance.

## INTRODUCTION

Crassulacean acid metabolism (CAM) is a biochemical and physiological adaptation of higher plants to environmental stress that limits evaporative water loss and increases water use efficiency (Osmond, 1978). The common ice plant, *Mesembryanthemum crystallinum*, has emerged as a useful model for understanding CAM and plant abiotic stress responses (Cushman and Bohnert, 1999; Bohnert and Cushman, 2001). This extremely stress tolerant, halophytic, inducible CAM species has a relatively short life cycle, a small genome (390 Mb or 2.5 times larger than *Arabidopsis thaliana*), and a growing collection of morphological and biochemical mutants available. In spite of these advantages, relatively few genes have been characterized from this species compared with other higher plant models.

Analysis of transcripts from expressed sequence tag (EST) data provide a rapid and powerful approach for gene discovery and invaluable insights for roles of transcriptionally regulated genes which respond to various environmental stimuli (Pih et al., 1997; Bockel et al., 1998; Machuka et al., 1999; Wood et al., 1999; Bohnert et al., 2001). Here we report on the development of an EST database for *M. crystallinum*. The EST collections generated by this effort are being used to construct cDNA microarrays for large-scale gene expression analyses (Desprez et al., 1998; Ruan et al., 1998; Seki et al., 2001).

## METHODS AND MATERIALS

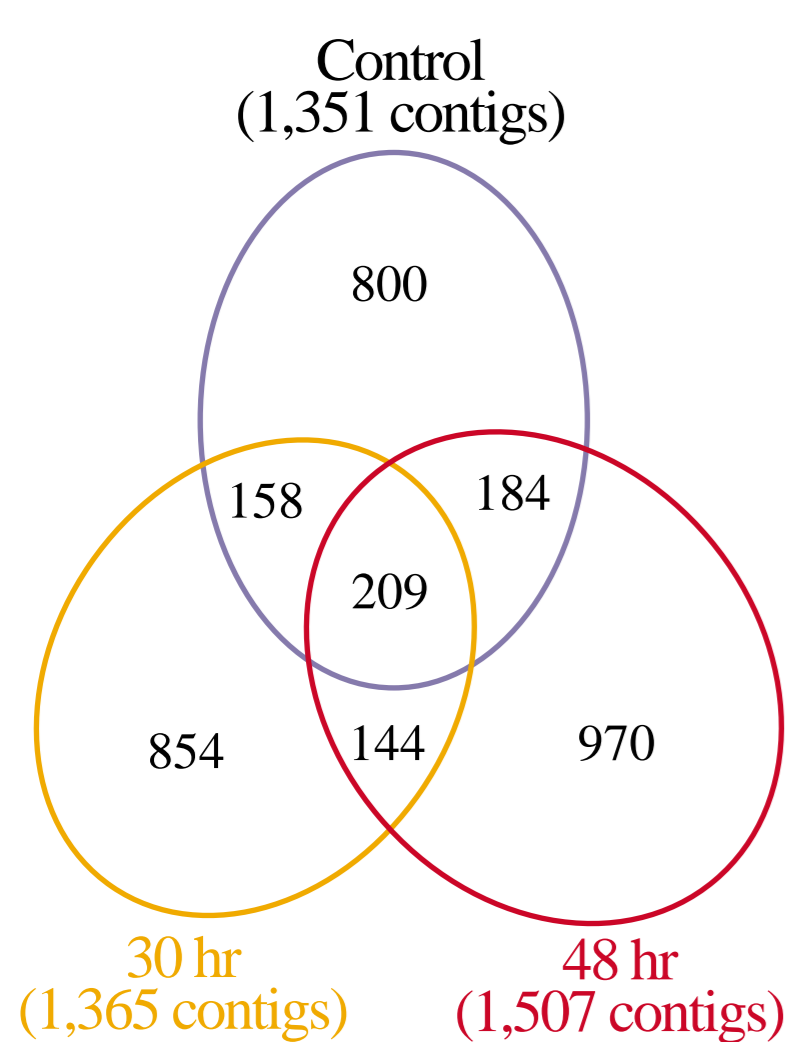
*Mesembryanthemum crystallinum* were grown in hydroponic culture with 1 x Hoagland's solution in a growth chamber on a 12-hr light (26°C)/12-hr dark (18°C) cycle. Fluorescence and incandescent lighting provided a photon flux density of 450 to 500  $\mu\text{E M}^{-2}\text{s}^{-1}$ . Six-week-old plants were stressed by adding 0.4 M NaCl (final) to the nutrient solution at beginning of the light period. Leaves were sampled just before adding NaCl (control) and 30 hr and 48 hr after starting the stress. Three kinds of directionally cloned (*EcoRI/XhoI*) cDNA libraries were constructed in Lambda Uni-Zap-XR according to manufacturer's instructions (Stratagene, Inc., La Jolla, CA) with mRNA purified by using oligo dT cellulose (Sigma) from total RNA isolated by Trizol<sup>®</sup> (Gibco BRL). The cDNA clones were converted to plasmids by the mass excision method described in the manufacturer's instructions and DNA sequencing reactions were conducted using DyeDeoxy<sup>™</sup> Terminator PRISM<sup>™</sup> mix (Perkin-Elmer-ABI, Inc.).

## CONCLUSIONS

- > The redundancy of transcripts in ice plant leaf tissue decreases during CAM induction.
- > The salinity-stressed (for 48 hr) leaf tissue contains a large number of unique transcripts that are not present in the other (control and 30 hr) leaf tissues.
- > The abundance of transcripts related to C3 photosynthesis (e.g., RuBisCO, photosystems and light harvesting complexes) decreased dramatically following salinity stress.
- > The abundance of transcripts related to CAM including CAM-specific C4 enzymes as well as glycolytic enzymes and vacuolar H<sup>+</sup>-ATPase increased dramatically following salinity stress.
- > Stress induces a prominent increase in the abundance of various transcripts encoding stress-related proteins, detoxifying enzymes, proteolytic functions, and ion homeostasis (especially Na<sup>+</sup>/H<sup>+</sup> antiporters).

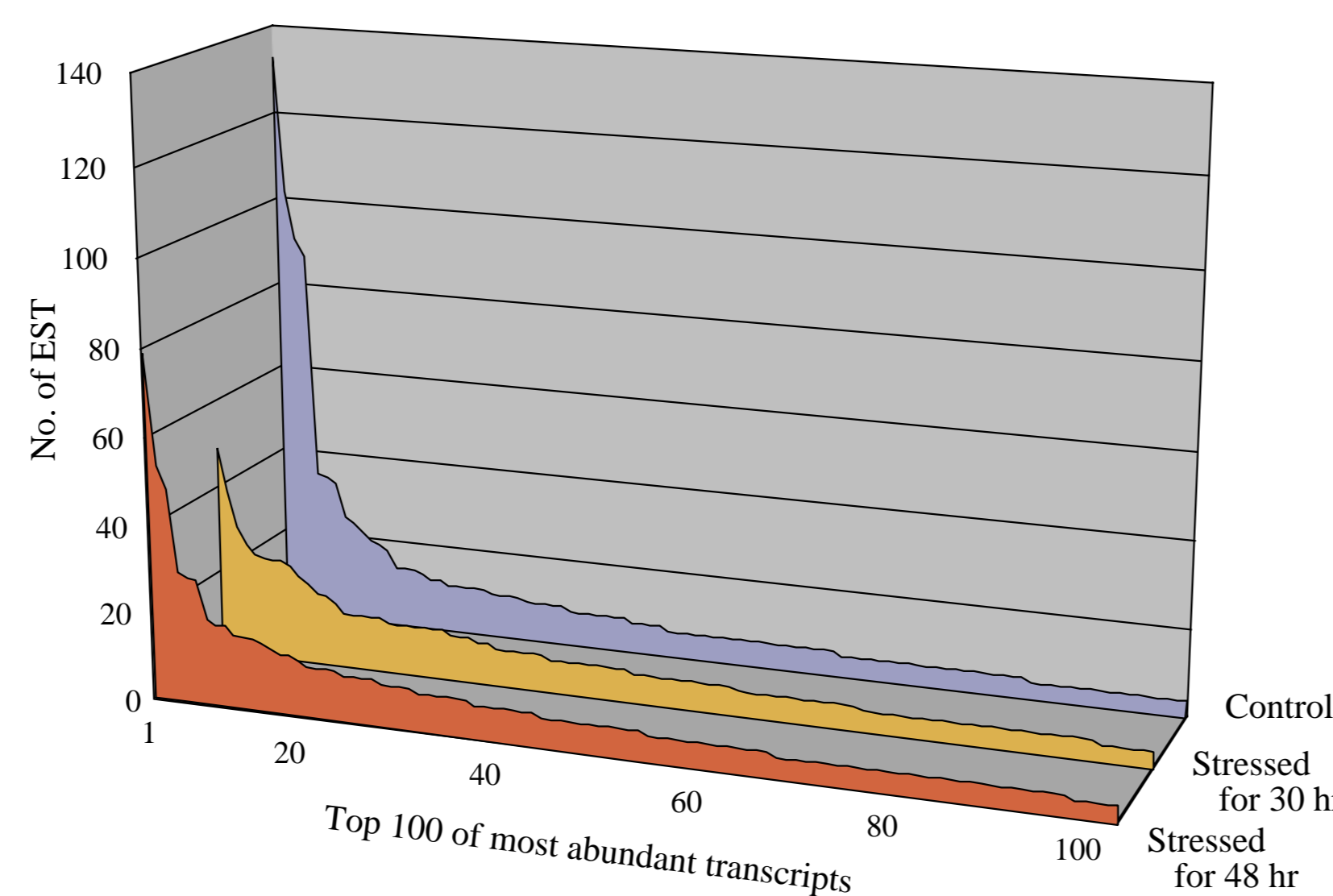
Table 1. Top 30 of most abundant transcripts from unstressed (control) leaves.		Table 2. Top 30 of most abundant transcripts from stressed (30 hr) leaves.		Table 3. Top 30 of most abundant transcripts from stressed (48 hr) leaves.	
1 RuBisCO, small subunit*	8.34%	1 RuBisCO small subunit*	2.98%	1 Ferredoxin-NADP <sup>+</sup> reductase	2.84%
2 Chlorophyll a/b-binding protein, LHC I & II*	3.99%	2 RuBisCO large subunit*	1.76%	2 <i>myo</i> -Inositol-1-phosphate synthase	1.94%
3 Ferredoxin-NADP <sup>+</sup> reductase (FNR)	3.67%	3 Chlorophyll a/b-binding protein, LHC I & II*	1.15%	3 Early light-inducible protein I	1.76%
4 RuBisCO, large subunit*	3.31%	4 Ferredoxin-NADP <sup>+</sup> reductase	1.11%	4 Carboxyvinyl-carboxyP phosphorimutase	1.08%
5 Unclear classification** (protein1)	1.26%	5 Nonspecific lipid-transfer protein1	0.97%	5 Pathogenesis-related protein 1a	1.04%
6 ATP synthase subunit 9 (mitochondrial)	1.19%	6 Phosphoenolpyruvate carboxylase 1	0.90%	6 60S Ribosomal protein L34	1.04%
7 RuBisCO activase	0.90%	7 GA3P dehydrogenase (cytosolic)	0.86%	7 RuBisCO small subunit*	0.97%
8 Photosystem II, K subunit	0.86%	8 ATP synthase, subunit 9 (mitochondrial)	0.86%	8 Heat shock protein (HSP18.2)	0.72%
10 Na <sup>+</sup> /solute symporter	0.72%	9 Antimicrobial peptide 1	0.83%	9 Cold inducible protein 1	0.68%
11 Polyubiquitin	0.68%	10 Na <sup>+</sup> /solute symporter	0.75%	10 S-adenosylmethionine decarboxylase	0.68%
12 Photosystem II, X subunit	0.64%	11 Unclear classification** (protein2)	0.61%	11 Chlorophyll a/b-binding protein, LHC I and II*	0.65%
13 Auxin-binding protein 1	0.54%	12 Cysteine proteinase	0.61%	12 H <sup>+</sup> -ATPase, subunit G (vacuolar)	0.61%
14 Auxin-binding protein2	0.50%	13 Cystatin	0.54%	13 Polyubiquitin	0.61%
15 Carbonic anhydrase	0.50%	14 Unclear classification** (protein3)	0.47%	14 Unclear classification** (protein1)	0.61%
16 S-Adenosylmethionine decarboxylase	0.50%	15 TMV-induced protein I	0.47%	15 Nonspecific lipid-transfer protein	0.58%
17 <i>myo</i> -Inositol-1-phosphate synthase	0.50%	16 Anti-fungal protein 1	0.47%	16 Galactinol synthase	0.50%
18 Photosystem I reaction centre, N subunit	0.40%	17 Clp protease proteolytic subunit (ClpP)	0.47%	17 Antimicrobial peptide 1	0.50%
19 Oxygen-evolving enhancer protein 2	0.40%	18 Nonspecific lipid-transfer protein2	0.47%	18 Heat shock protein (HSP83)	0.47%
20 Unclassified (Novel) protein1***	0.40%	19 Unclear classification** (protein4)	0.43%	19 Cytochrome P450 monooxygenase	0.43%
21 Early light-inducible protein 1	0.40%	20 40S Ribosomal protein S4	0.43%	20 Histone H3	0.43%
22 ATP synthase, epsilon subunit (chloroplastic)	0.40%	21 <i>myo</i> -Inositol 4-O-methyltransferase	0.43%	21 H <sup>+</sup> -ATPase, subunit c (vacuolar)	0.43%
23 Photosystem II reaction centre, W subunit	0.36%	22 Photosystem II, 10 kD polypeptide	0.43%	22 <i>FisH</i> -like protein	0.40%
24 Oxygen-evolving enhancer, 33 kD subunit	0.36%	23 Low-temperature-regulated gene1	0.43%	23 Unclear classification** (protein5)	0.40%
25 Antimicrobial peptide 1	0.36%	24 Cold inducible protein 1	0.43%	24 Unclassified (Novel) protein1***	0.40%
26 Photosystem I reaction centre, subunit II	0.32%	25 Unclear classification** (protein1)	0.43%	25 RuBisCO large subunit*	0.40%
27 Unclassified (Novel) protein2***	0.32%	26 Unclassified (Novel) protein3***	0.40%	26 Cysteine proteinase	0.36%
28 Cytochrome <i>b<sub>6</sub>-f</i> complex, Fe-S subunit	0.32%	27 Elongation factor 1-alpha	0.40%	27 Elongation factor 1-alpha	0.36%
29 <i>ycf4</i> (predicted ORF in the plastid genome)	0.32%	28 Photosystem II, K subunit	0.40%	28 Clp protease proteolytic subunit (ClpP)	0.36%
30 Low-temperature-regulated gene 1	0.32%	29 Peptidylprolyl isomerase	0.36%	29 Photosystem II, K subunit	0.36%
		30 H <sup>+</sup> -ATPase, subunit B (vacuolar)	0.36%	30 Abscisic stress ripening protein 1	0.32%
				30 Heat shock protein (HSP17.7)	0.32%
				30 Glycolate oxidase	0.32%
				30 Thaumatin-like protein (RP-5)	0.32%
				30 Na <sup>+</sup> /solute symporter	0.32%

\* All transcripts combined  
\*\* Similar to known sequences whose functions are unclear.  
\*\*\* No similarity to other known sequences.



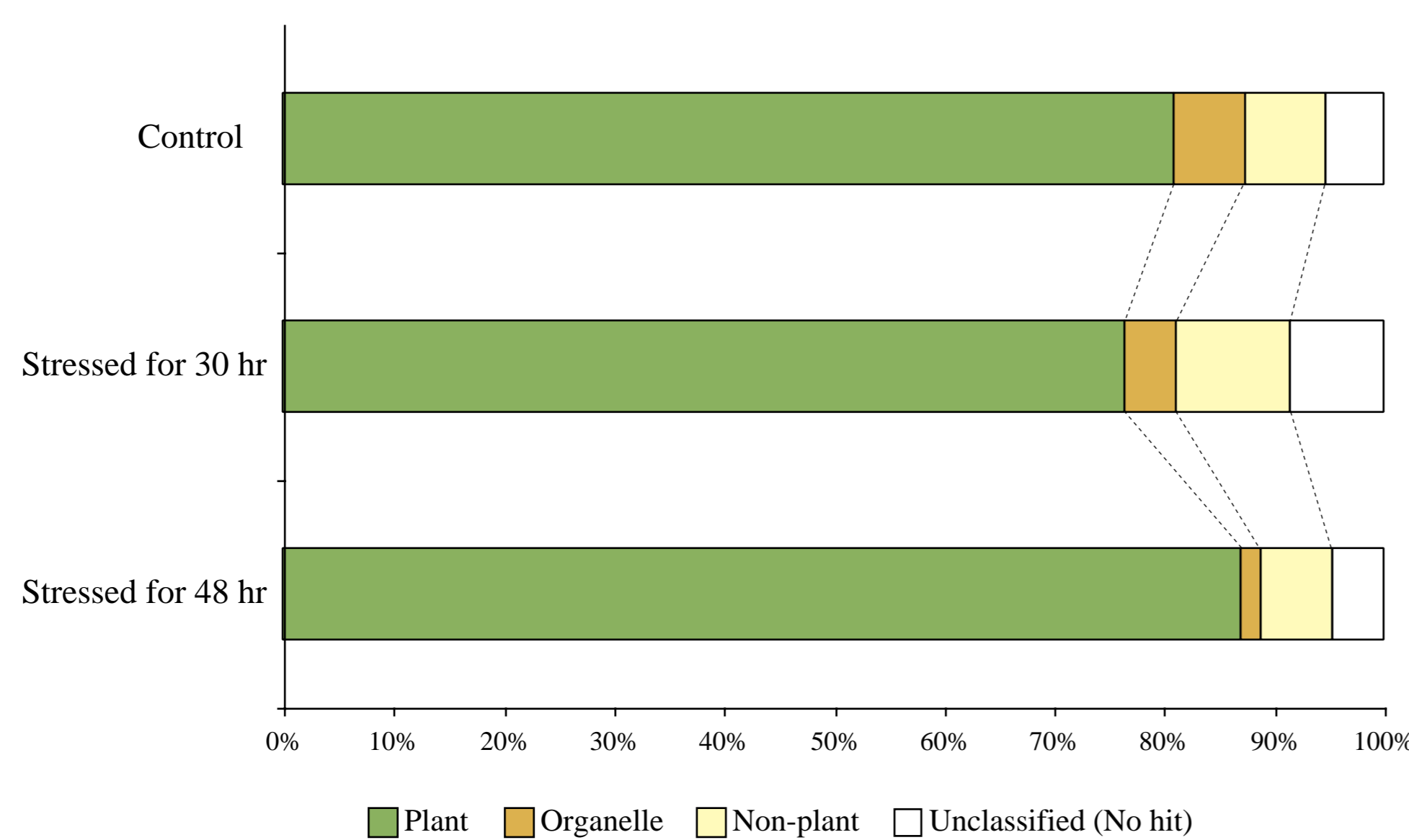
**Figure 1. Overlap among ESTs in cDNA libraries from unstressed (control) and stressed (for 30 hr and 48 hr) leaves.**

The same number (2,782) of EST sequences from each library (Total: 8,346 ESTs) were combined and analyzed together by PHRAP. They were assembled into 3,328 unique transcripts (contigs). 1,351, 1,365 and 1,507 contigs were found in the unstressed and stressed (30 hr and 48 hr) leaf libraries, respectively. This figure shows the number of contigs appearing only in each of the three libraries and commonly found in two or all three of them. More numbers of unique contigs were present in the stressed leaf libraries than in the control library presumably reflecting a higher diversity of transcripts required for stress adaptation.



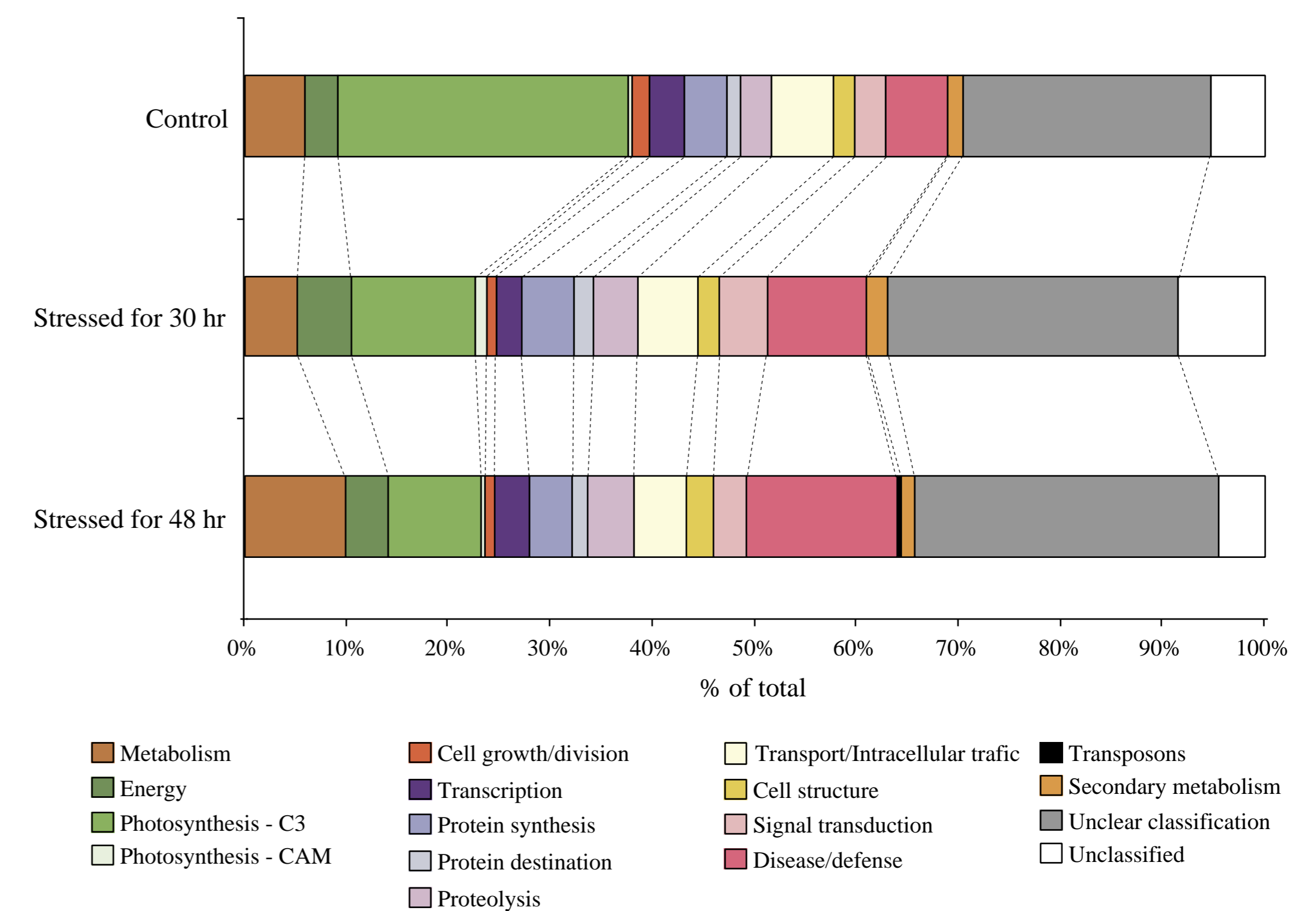
**Figure 2. Comparison of redundancy among unstressed (control) and stressed (30 hr and 48 hr) leaf libraries.**

This figure shows EST number belonging to the most abundant (top 100) transcripts (contigs) from each libraries. The blue area represents 44% of ESTs from the control leaf library, whereas the orange and red areas represent 35% and 36% of ESTs from the stressed (30 hr and 48 hr) leaf libraries. Note the higher frequency of redundant transcripts in the unstressed library.



**Figure 3. Origins of sequences most homologous to ice plant transcripts.**

The sequences of transcripts were analyzed by BLASTX using the "nr" peptide sequence database. Primary homologues are grouped by their origins.



**Figure 4. Overview of expression profiles of transcripts from unstressed (control) and stressed (30 hr and 48 hr) leaf tissues.**

Each EST sequence was classified into a category shown in Table 4 according to their putative functions. Unclear classifications are sequences similar to known sequences whose functions are unclear. Unclassified sequences are those that have no similarity to other known protein sequences.

**Table 4. Abundance of transcripts with various roles in the control and stressed (30 hr and 48 hr) leaf of ice plant**

Functional category	% Control	% 30 hr*	% 48 hr*	Functional category	% Control	% 30 hr*	% 48 hr*
01 Metabolism	5.90%	5.14%	9.78%	07 Transporters	5.54%	5.61%	4.82%
01.01 Amino acid	1.19%	1.33%	1.69%	07.0101 Sodium transporter	0.04%	0.11%	0.11%
01.02 Nitrogen/sulfur	0.50%	0.11%	0.40%	07.0109 Other ion transporters	0.25%	0.58%	0.36%
01.03 Nucleotide	0.68%	0.32%	0.47%	07.07 Sugar transporter	0.00%	0.04%	0.07%
01.04 Phosphate	0.11%	0.14%	0.22%	07.10 Amino acid transporter	0.22%	0.04%	0.14%
01.05 Sugars/polysaccharide	2.12%	1.58%	5.28%	07.16 Purine/pyrimidine transporter	0.04%	0.18%	0.11%
01.06 Lipid and sterol	1.01%	1.47%	1.47%	07.2201 Mitochondrial H <sup>+</sup> -ATPase	1.47%	1.26%	0.50%
01.07 Cofactor	0.29%	0.18%	0.25%	07.2202 Chloroplastic H <sup>+</sup> -ATPase	0.86%	0.14%	0.29%
				07.2203 Vacuolar H <sup>+</sup> -ATPase	0.93%	2.05%	1.83%
02 Energy	32.03%	18.58%	13.70%	07.2209 Other H <sup>+</sup> -ATPases	0.04%	0.07%	0.07%
02.01 Glycolysis	0.93%	1.83%	0.72%	07.25 ABC-type transporter	0.22%	0.11%	0.22%
02.02 Gluconeogenesis	0.18%	0.11%	0.00%	07.30 Water channel	0.25%	0.04%	0.07%
02.07 Pentose phosphate	0.18%	0.07%	0.22%	07.99 Others transporter	1.22%	1.01%	1.04%
02.10 TCA pathway	0.25%	0.47%	0.54%				
02.13 Respiration	0.83%	1.65%	1.40%	08 Intracellular traffic	0.54%	0.25%	0.40%
02.15 Fermentation	0.07%	0.22%	0.36%	08.02 Chloroplast	0.04%	0.00%	0.00%
02.20 E-transport	0.61%	0.40%	0.65%	08.04 Mitochondrial	0.00%	0.04%	0.04%
02.3001 C3-Photosynthesis	28.47%	12.15%	9.13%	08.07 Vesicular	0.40%	0.14%	0.29%
02.3002 C4-Photosynthesis/CAM	0.40%	1.22%	0.25%	08.13 Vacuolar	0.00%	0.04%	0.00%
02.3003 Photorespiration	0.11%	0.47%	0.43%	08.19 Import	0.11%	0.04%	0.07%
03 Cell growth/division	1.76%	1.01%	1.01%	09 Cell structure	2.05%	2.08%	2.70%
03.01 Cell growth	0.07%	0.07%	0.00%	09.01 Cell wall	0.32%	0.54%	0.65%
03.13 Meiosis	0.04%	0.00%	0.07%	09.04 Cytoskeleton	0.68%	0.83%	0.40%
03.16 DNA synthesis/replication	0.25%	0.22%	0.18%	09.10 Nucleus	0.11%	0.18%	0.14%
03.19 Recombination/repair	0.07%	0.07%	0.07%	09.13 Chromosomes	0.50%	0.14%	0.75%
03.22 Cell cycle	0.04%	0.04%	0.14%	09.25 Vacuole	0.18%	0.00%	0.29%
03.26 Growth regulators	1.22%	0.47%	0.29%	09.28 Chloroplast	0.07%	0.25%	0.29%
03.99 Others	0.07%	0.14%	0.25%	09.99 Others	0.18%	0.14%	0.18%
04 Transcription	3.31%	2.44%	3.34%	10 Signal transduction	3.09%	4.85%	3.06%
04.01 rRNA synthesis	0.04%	0.00%	0.00%	10.01 Receptors	0.25%	0.93%	0.32%
04.19 mRNA synthesis	1.51%	1.19%	1.69%	10.04 Mediators	0.86%	0.90%	1.04%
04.1901 General TFs	0.07%	0.07%	0.00%	10.0404 Kinases	1.01%	1.62%	0.86%
04.1904 Specific TFs	0.36%	0.29%	0.68%	10.0407 Phosphatases	0.58%	0.54%	0.36%
04.1907 Chromatin modification	0.11%	0.11%	0.11%	10.0410 G proteins	0.40%	0.54%	0.47%
04.22 mRNA processing	0.65%	0.32%	0.40%	10.99 Others	0.00%	0.32%	0.00%
04.31 RNA transport	0.07%	0.00%	0.00%				
04.99 Other RNA binding protein	0.50%	0.47%	0.47%				
05 Protein synthesis	4.28%	5.07%	4.28%	11 Disease/defense	5.97%	9.60%	14.92%
05.01 Ribosomal proteins	2.98%	3.06%	2.70%	11.01 Resistance genes	0.29%	0.22%	0.14%
05.04 Translation factors	1.19%	1.55%	1.37%	11.02 Defense-related	1.40%	2.52%	3.56%
05.07 Translation control	0.00%	0.22%	0.00%	11.03 Cell death	0.04%	0.18%	0.18%
05.10 tRNA synthases	0.11%	0.14%	0.22%	11.05 Stress responses	1.91%	4.39%	4.60%
05.99 Other	0.00%	0.11%	0.00%	11.0501 HSP and chaperons	0.93%	0.22%	3.77%
				11.06 Detoxification	1.40%	2.08%	2.66%
06 Protein destination	4.39%	6.22%	6.00%	14 Transposons	0.11%	0.14%	0.29%
06.01 Folding and stability	0.47%	0.72%	0.65%	14.01 LTR retroelements	0.04%	0.00%	0.11%
06.04 Targeting	0.29%	0.50%	0.32%	14.02 Non-LTR retroelements	0.00%	0.04%	0.04%
06.07 Modification	0.22%	0.43%	0.36%	14.99 Other	0.07%	0.11%	0.14%
06.10 Complex assembly	0.36%	0.25%	0.22%				
06.13 Proteolysis	3.06%	4.31%	4.42%	20 Secondary metabolism	1.37%	1.91%	1.40%
06.20 Storage proteins	0.00%	0.00%	0.04%	20.01 Phenylpropanoids	0.36%	0.75%	0.40%
				20.02 Terpenoids	0.11%	0.18%	0.07%
				20.05 Amines	0.72%	0.68%	0.86%
				20.99 Other secondary metabolism	0.18%	0.29%	0.07%
12 Unclear classification	24.44%	28.61%	29.76%				
13 Unclassified	5.25%	8.48%	4.57%				

a) % of ESTs in each category for the library of control leaf.  
b) % of ESTs in each category for the library of leaves from plants stressed for 30 hr.  
c) % of ESTs in each category for the library of leaves from plants stressed for 48 hr.

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