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A snapshot of the low temperature stress transcriptome of developing rice seedlings (*Oryza sativa* L.) via ESTs from subtracted cDNA library

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Abstract Rice (*Oryza sativa* L.) is sensitive to chilling particularly during early seedling development. Given the biochemical complexity of tolerance mechanisms, genetic potential for this trait depends on highly coordinated expression of many genes. We used a simple cDNA subtraction strategy to develop Expressed Sequence Tags (ESTs) that represent an important subset of cold stress-upregulated genes. The 3,084 subtracted cDNA clones represent a total of 1,967 unigenes from 1,354 singletons and 613 contigs. As expected in the developing seedlings, genes involved in basic cellular processes, i.e., metabolism, growth and development, protein synthesis, folding and destination, cellular transport, cell division and DNA replication were widely represented. Genes with stress-related and regulatory functions comprised 23.17% of the total ESTs. These categories included proteins with known function in cellular defenses against abiotic (drought, cold and salinity) and biotic (pathogen) stresses, and proteins involved in developmental and stress response signalling and transcription. Based on the types of genes represented, tolerance mechanisms rely on precise integration of developmental processes with stress-related responses. A large fraction of the ESTs (38.7%) represents unknown proteins. This EST library is a rich source of cold stress-related genes, and supplements for other publicly available libraries for comprehensive analysis of the stress-response transcriptome.

Keywords cDNA subtraction · Cold tolerance · EST · Transcriptome · Upregulated genes

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Introduction

With the completion of the full genomic sequences of the model plant species *Arabidopsis thaliana* (L.) Heynh. (The *Arabidopsis* Genome Initiative 2000) and *Oryza sativa* L. (Goff et al. 2002; Yu et al. 2002), the next major goal of plant biology is to understand gene functions in the context of cellular biochemistry and physiology. Genes are initially identified based on the nucleotide sequence, and biological functions are first deduced from the temporal and spatial expression patterns. The basic tool for this type of analysis is Expressed Sequence Tags (EST), generated via large-scale sequencing of cDNA (Richmond and Somerville 2000).

Because of its many uses in genomics, ESTs have become an integral component of both small- and large-scale genome projects. As for the writing of this manuscript, at least 177,000 *A. thaliana* and 122,000 *O. sativa* ESTs have been deposited in the public databases (http:\\www.ncbi.nlm.nih.gov/dbest). This resource contains a vast amount of information that serves as a window for the array of genes expressed in higher plants at different developmental stages and under a variety of environmental conditions. Thus, EST development is a systematic means to catalog the transcriptome, and transcript profiling with gene arrays allows detailed studies of complex cellular processes including metabolism (Girke et al. 2000; White et al. 2000), development

(Fernandez et al. 2002) and mechanisms of plant adaptation to a variety of biotic and abiotic factors (Kawasaki et al. 2001; Chen et al. 2002). For instance, recent studies in *A. thaliana* showed that the mechanism of tolerance to low-temperature stress involves a highly coordinated expression of hundreds of genes. Gene expression is intricately regulated by a family of stress-specific transcription factors in response to changes in environmental temperatures. The products of these genes are presumed to play major roles in complex networks of adaptive biochemical processes (Seki et al. 2001; Fowler and Thomashow 2002). This mechanism defines the cellular and molecular bases of the cold-acclimation response, the process that mediates freezing-tolerance in temperate plants.

Unlike *Arabidopsis* and other temperate cereals such as wheat, barley and rye, rice is sensitive to chilling and does not acclimate to freezing. The optimum temperature range for germination and early seedling growth of rice is between 25 and 35 °C. In cooler regions, rice is normally direct-seeded during late spring or early summer. Low temperatures (10 °C to15 °C) during these periods can cause severe injury to seedlings at early stages of establishment, and often result in poor seedling vigor (De los Reyes and McGrath 2003). Genetic variation in early seedling-stage cold-tolerance has been reported in some rice cultivars particularly among the subspecies japonica (Sthapit and Witcombe 1998). However, our understanding of the molecular basis of this variation is limited, and little is known about the similarities between chilling-insensitive and sensitive species in terms of the low temperature-induced early gene expression. Better understanding of the molecular events that lead to enhanced-seedling cold tolerance can be achieved by global gene-expression analysis.

The degree by which the current publicly available rice EST collections represent the total transcriptome depends on the diversity of developmental stages and environmental conditions used in the cDNA library construction. This is an important issue that should be addressed when designing the composition of cDNA microarrays in order to ensure that most if not all of the critical genes are represented in a given experiment. To our knowledge cold stress-related gene expression during the early seedling developmental stages is poorly represented in the major rice EST libraries (Sasaki et al. 1994; Wu et al. 2002). More recently, ESTs derived from drought and salt-stressed rice have become available (Kawazaki et al. 2001; Reddy et al. 2002). Although salt, drought and low-temperature stresses share a common physiological effect, the former two EST libraries may be covering slightly different sets of genes relative to the overall cold-induced gene expression, and thus may exclude some of the most important genes specific to the responses of rice seedlings to low temperatures. To address this issue, we developed a new set of ESTs as a catalog for a specific subset of genes important in the cold-stress responses at the early seedling developmental stages. In this study, we used a simple cDNA enrichment strategy to specifically target the clones with high probability for being upregulated under cold conditions. Our objective was to supplement the publicly available stress-related EST libraries for future use in assembling cDNA microarrays that broadly represent the cold-stress transcriptome. We discuss in this report the biological implications of the findings from these ESTs in relation to known mechanisms of low-temperature stress tolerance in higher plants.

Materials and methods

Plant materials and cold-stress treatments

The rice cultivar CT6748-8-CA-17 (PI560247) obtained from the Arkansas Rice Research and Extension Center breeding nursery was used to develop the cDNA library. Seeds were surface-sterilized in 3% Chlorox. Control seedlings were prepared by growing the seeds in moist filter paper at 30 °C. Seedlings were harvested at coleoptile (S_1 stage, 2 to 3 days post-imbibition), radicle (S_2 stage, 4 to 5 days post-imbibition) and prophyll (S_3 stage, 6 to 7 days post-imbibition) emergence stages. Cold-stressed seedlings were prepared by first allowing the seeds to imbibe at 30 °C for 24 h and then allowing them to germinate and grow in moist filter paper at 13 °C/10 °C in a growth chamber. Seedlings were harvested at S_1 (4 to 6 days post-imbibition), S_2 (7 to 9 days post-imbibition) and S_3 (10 to 14 days post-imbibition) stages.

RNA isolation and construction of the primary EST library

PolyA$^+$ RNA was isolated from about 1,200 cold-stressed seedlings at S_1, S_2 and S_3 stages with the PolyATract mRNA isolation system (Promega). Equal amounts (3 μg) of polyA$^+$ RNA from each developmental stage were pooled, and a 5-μg aliquot was used to synthesize double-stranded cDNA using the *Eco*RI/*Xho*I adapter cDNA synthesis kit (Stratagene). The adapter-ligated, double-stranded cDNA pool was enriched with fragments longer than 500 bp by sepharose gel-filtration chromatography. The double-stranded cDNAs were directionally cloned relative to the T3 promoter of Lambda Uni-Zap XR and packaged with Gigapack III Gold extracts. The primary library had a titer of $>2.7 \times 10^6$ pfu in XL1-Blue MRF′. An aliquot of the library containing 1×10^5 pfu was in vivo excised as pBluescript phagemid with ExAssist helper phage and used as the primary EST library.

Preparation and labeling of subtracted cDNA probes

A composite probe enriched with cDNA fragments corresponding to cold-induced transcripts was prepared with the PCR-Select cDNA subtraction kit (BD Biosciences Clontech). Subtraction was performed according to the manufacturer's protocol with the mRNA from control and cold-stress seedlings as driver and tester, respectively. Briefly, double-stranded cDNAs were synthesized from equal amounts (2 μg) of driver and tester polyA$^+$ RNA with AMV reverse transcriptase and an enzyme cocktail composed of RNase H, DNA polymerase I and *Escherichia coli* DNA ligase. Double-stranded cDNA was digested with *Rsa*I for 2 h at 37 °C. The digested tester cDNA was diluted to a concentration of 0.2× of the original sample, and two 2-μl aliquots were ligated to the adaptors (adaptors 1 and 2R) provided in the kit. The first subtraction step was performed by denaturing the two types of adaptor-ligated tester cDNAs, followed by hybridization with an excess amount (10×) of denatured driver cDNA without adaptors at 68 °C for 8 h. The second enrichment step was performed by further hybridizing the two primary hybridization products with each other and with an additional amount of driver cDNA without an adaptor at 68 °C overnight. The upregulated cDNA fragments

were selectively amplified from the subtraction hybridization products by nested PCR. The primary PCR was performed with the outer primer 1 provided in the kit. Amplification was performed for 27 cycles in the PTC-200 thermal cycler (MJ Research) by the following parameters: initial denaturation for 30 s at 95 °C, step-denaturation at 94 °C for 15 s, 30 s annealing at 65 °C for 30 s and extension at 72 °C for 2 min. The secondary PCR was performed with nested primers 1 and 2R provided in the kit, for 15 cycles by the following parameters: initial denaturation for 30 s at 95 °C, step-denaturation at 94 °C for 15 s, annealing at 68 °C for 30 s and extension at 72 °C for 2 min. The amplified subtracted cDNA was purified by phenol-chloroform precipitation and then labeled with ^{32}P-dCTP using the RediPrime random priming kit (Amersham Biosciences).

Selection of cDNA clones for EST development

The excised primary library was plated at a density of about 5,000 cfu per 150-mm culture dish containing LB-agar + ampicillin (50 μg/ml) + IPTG + X-Gal. The library was grown at 37 °C for 16 h. More than 76,000 white colonies were picked from the master plate and arrayed on 384-well microtiter plates containing LB freezing medium + ampicillin by the Flexys Colony Picker robot (Genome Solutions). The colony plates were incubated at 37 °C overnight and then colony lifted on nylon membranes with a manual 384-well pin replicator/spotter. The filter-bound colonies were grown over LB-agar + ampicillin at 37 °C for 14 h and processed according to standard procedures.

The filters were pre-hybridized for 4 h at 65 °C in Denhardt's solution + 5× SSPE + 0.1% SDS and denatured salmon-sperm DNA. Hybridization with the labeled subtracted cDNA probe was performed overnight at 65 °C on a platform-shaking hybridization incubator. Stringency washes were performed at 65 °C three times with 2× SSC + 0.1% SDS and twice with 0.1× SSC + 0.1% SDS. The hybridized filters were autoradiographed for 3 to 5 days at –80 °C. The specific clones that hybridized to the subtracted probe were identified from the master plates by visual inspection with the colony lift autoradiograms as a reference. Selected clones were grown overnight at 37 °C in 96-well plates containing LB freezing medium + ampicillin.

cDNA sequencing and EST analysis

Plasmid DNA templates were prepared with the QIAwell 96 Ultra plasmid kit (Qiagen). The cDNAs were sequenced from the 5′ end by the Michigan State University Genomics Technology Support Facility, East Lansing, Mich., through the ABI Prism 3,700 DNA Analyzer (Applied BioSystems). The ESTs were clustered with StackPack 2.1 (Burke et al. 1999) and identified by comparing with the *Arabidoposis*, nr and dBEST databases by BlastN and BlastX algorithms (Altschul et al. 1997) at the threshold e-value of 10^{-4} or better. The ESTs were grouped into functional categories either manually or with the aid of the Kyoto Encyclopedia of Genes and Genomes (KEGG) graphical pathway maps (Kanehisa and Goto 2000).

Northern-blot and dot-blot analyses

The Northern blots were prepared by electrophoresis of polyA$^+$ RNA (1 μg) from control and cold-stress germinated seedlings (pooled from $S_1 + S_2 + S_3$) in a 1.2% formaldehyde-agarose gel and by blotting on Hybond N$^+$ nylon membrane (Amersham Biosciences) with the turbo blotter device. The cDNA probes were labeled with ^{32}P-dCTP by the RediPrime random priming kit (Amersham Biosciences). The RNA filters were hybridized overnight at 42 °C in Ultrahyb buffer (Ambion). Post-hybridization washes were performed according to standard procedures. Filters were autoradiographed for 24 to 48 h at –80 °C.

The cDNA samples used for the dot blot were PCR-excised from the recombinant plasmids with the T3 and T7 primers. Aliquots of the excised insert DNA (200 ng/μl) were arrayed on a 96-well plate and used to print six identical dot blots on Hybond N$^+$ nylon membrane with a 96-well manual pin spotter/replicator device. The cDNA probes for the dot blots were prepared by reverse transcription of equal amounts (0.5 μg) of polyA$^+$ RNA from the control and low temperature-grown seedlings ($S_1 + S_2 + S_3$). The cDNA probes were synthesized with the ReversePrime cDNA labeling kit (GenHunter Corp.) with the T_{20} primer, MMLV reverse transcriptase and ^{32}P-dCTP. One set of triplicate dot blots (filters 1A, 2A and 3A) was hybridized with the control probe and the second set (filters 1B, 2B and 3B) with the stress probe. Hybridization was performed overnight at 42 °C in Ultrahyb buffer. Dot blots were washed and autoradiographed according to standard procedures. The filters were stripped and re-hybridized in an inverse fashion (i.e., filter set A for the stress probe and filter set B for the control probe) to insure reproducibility. Two sets of filters with the most consistent results were used for data collection.

Results

A new seedling EST library from subtracted cDNA clones

As an initial step towards the analysis of the global gene expression during the early seedling development of rice under sub-optimal temperature conditions, we developed a catalog of candidate cold stress-related genes by selective, single-pass sequencing of potentially upregulated cDNAs. We selected the japonica cultivar CT6748-8-CA-17 (PI560247) as the model genotype for this study. In laboratory experiments, this cultivar exhibited a high level of cold tolerance based on a higher percentage and shorter duration of germination at 13 °C/10 °C, as well as higher seedling survival following germination at chilling temperatures (data not shown).

We constructed a primary cDNA library with average insert size of 1.0 kb from cold-stressed seedlings at coleoptile (S_1), radicle (S_2) and prophyll (S_3) emergence (Counce et al. 2000), in an attempt to gather a meaningful subset of genes that are induced either transiently or for longer terms during the early stages of seedling development. To select for such cDNAs, we screened 76,800 clones from the primary unamplified library with a composite cDNA probe derived from PCR-select cDNA subtraction. This probe consisted of short cDNA fragments that had been enriched for upregulated transcripts by the removal of those that were expressed at equal levels in the control seedlings. The resulting subtracted library was composed of 4,519 clones that hybridized strongly to the subtracted cDNA probes. Sequencing of the 5′ ends of 3,760 clones yielded a total of 3,084 high quality tags with an average length of 437 bp.

One of the possible effects of the gene enrichment strategy used in this study was the increased level of EST redundancy as a consequence of multiple sampling of cDNAs corresponding to a single highly upregulated gene. In order to assess the level of redundancy and to estimate the number of unique genes represented in the subtracted set, we assembled the 5′ ESTs into contigs through their overlapping sequences. A total of 1,730

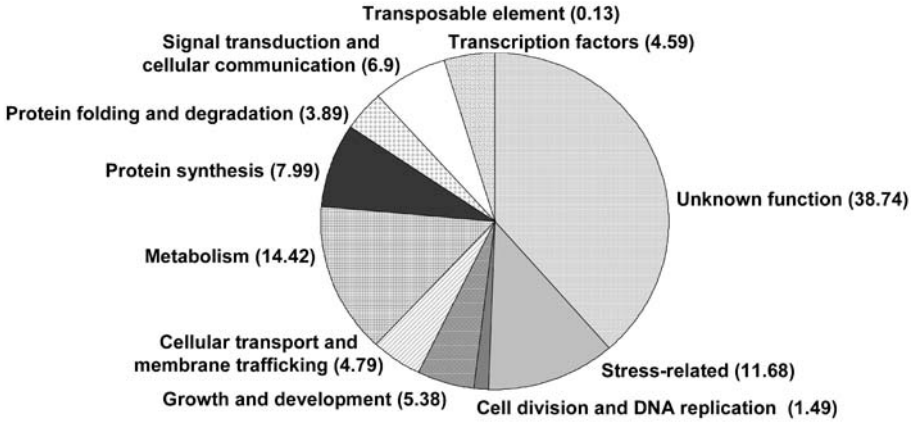

Fig. 1 Percentage distribution of the redundant ESTs according to functional categories

ESTs (56.1% of the total EST) assembled into 613 contigs, composed of 2 to 48 ESTs. The other 1,354 ESTs (43.9% of the total EST) remained as singletons. Overall, the current subtracted set consists of 1,967 unique cDNAs, presumably enriched with potential cold stress-upregulated genes.

A glimpse of cellular physiology during cold stress

Since we used a selective cDNA sequencing approach as a means to increase our probability of sampling the upregulated genes, we would expect a good representation of the cellular processes involved in the cold stress-response mechanisms. To test this supposition, we used two different approaches to extract meaningful information from the redundant EST data.

For the first approach, we used all EST singletons and the longest sequence from each contig to assign a putative function to each tag by comparison (BlastX) with the non-redundant nucleotide and protein databases of the GenBank. The ESTs were manually annotated using the information from the highest scoring matches (10^{-4} or better) returned by BlastX. The EST groupings according to functional categories are summarized in Fig. 1. Of the 3,084 total redundant ESTs, 61.3% can be identified by significant similarities with known genes or proteins. The other 38.7% were classified as "unknown functions" or do not have significant similarities with known proteins or genes at the threshold e-value. Members of this group include those that are similar only to other unknown plant ESTs (expressed proteins), or those that are similar only to hypothetical proteins predicted from genomic sequences (putative proteins) or both. Given the assumption that the library is enriched with cold stress-upregulated genes, this large number of unknown ESTs could be a rich source of yet to be discovered gene functions specific to seedlings developing at low temperatures.

As expected in a developing seedling, the gene classes that are involved in basic cellular housekeeping are widely represented in the subtracted set. Despite the sub-optimal growth conditions due to chilling, active resumption of cellular activities as a result of the transition of the seed from quiescent to growing state was indicated by the occurrence of transcripts involved in metabolism (e.g., starch hydrolysis, protein catabolism, glycolysis, TCA cycle and ATP synthesis), growth and development (e.g., cell division and elongation, modification or synthesis of new cell-wall materials, cell structure and cytoskeleton), DNA replication and repair, de novo protein synthesis, protein folding and destination (e.g., chaperones, transporters), and cellular transport and trafficking. Whether the transcription of some of these housekeeping genes are integrated with the overall developmental and stress-response mechanisms is a question that will be addressed in the future by in-depth analysis of the gene regulatory circuits.

The functional categories that can be directly associated with the cold-induced changes in gene expression patterns are those that encode for known or putative stress-related proteins and those with putative roles in the regulation of developmental or stress-related responses (transcription factors, signal transduction and cellular communication). When combined, these categories constitute 23.17% of the total redundant EST-set indicating the high activity of these cellular processes at the specific developmental and environmental status of the seedlings used in this experiment.

The majority of the genes under the "stress-related" category encode for proteins with known or putative roles in the prevention and/or repair of cellular injuries caused by low temperatures, dehydration, salinity, ion toxicity, light stress and hypoxia (Table 1). The cellular processes included in this group are osmotic adjustment and ion homeostasis, water channeling, oxygen radical scavenging and modification of membrane lipid compositions. Many of these genes are also known to be regulated by stress-response signalling hormones such as abscisic acid (ABA) and ethylene (Shinozaki and Yamaguchi-Shinozaki 2000). The occurrence of genes involved in different stress factors is consistent with the common physiological effects of low temperature and dehydration to plant cells. Additionally, 18.32% of the ESTs classified as being "stress-related" are known to have major roles in plant responses to pathogen invasion (Table 1). The expression of these genes reflects a secondary stress response, as

Table 1 Selected examples of ESTs for known or putative stress-related genes

Putative function/Homology	Representative dBEST Acc.	Type of stress
Alcohol dehydrogenase	CB000919, CA999554, CB000462	Abiotic
Aquaporin/water channel protein	CB000222, CA998222	Abiotic
A. thaliana Avr9 elicitor response protein-like	CA998031	Biotic
A. thaliana AvrRpt2-induced protein 2	CA998891	Biotic
A. thaliana drought-induced protein Di19-like	CB000279	Abiotic
A. thaliana early responsive to desiccation *ERD15*-like	CA997987, CA998049, CA998070	Abiotic
A. thaliana rare cold-inducible *RCI2*-like	CB000413, CA999649	Abiotic
A. thaliana salt-inducible protein AB006698	BU099179	Abiotic
β-13-Glucanase-like protein	CA999814	Abiotic/biotic
Barley low-temperature induced *blti101*-like	CA998755	Abiotic
Catalase	CA999044, CA998957	Abiotic/biotic
Chitinase-like proteins	CB000861	Abiotic/biotic
Chloride channel protein-like	CA998936	Abiotic
C. arietinum water stress-regulated protein	CB000083	Abiotic
C. junos iron stress-related protein	CA998229	Abiotic
Cold acclimation protein WCOR413-like	CA999631	Abiotic
D. antarctica cold acclimation associated protein	CA999448	Abiotic
Fatty acid desaturase	CA998805	Abiotic
Ferritin	CA998472	Abiotic
Germin-like protein	CA998978	Abiotic
Glutathione peroxidase	CB000231	Abiotic/biotic
Glutathione reductase	CB000921	Abiotic/biotic
Glutathione S-transferase	CA998124	Abiotic/biotic
HMG-CoA reductase	CA999526	Abiotic
H. vulgare Cf2/Cf5 disease resistance protein homolog	CA999238	Biotic
LEA protein	CB000198	Abiotic
Metallothionein-like proteins	CB001014, CA999942, CA998871	Abiotic
$Na^+/K^+/Cl^-$ cotransporter-like protein	CA998487	Abiotic
N. tabacum dehydration-induced protein	CA999835	Abiotic
N. tabacum ion homeostasis protein SLT1	CA998042	Abiotic
O. sativa ABA and salt stress-related protein osr4g3	CA999365	Abiotic
O. sativa anaerobically induced early gene 2	CA998038	Abiotic
O. sativa chilling tolerance-related protein	CA998398	Abiotic
O. sativa cold-inducible protein	CB001070	Abiotic
O. sativa early drought/high-molecular-weight group	CB000760	Abiotic
O. sativa elicitor-induced GDP dissociation inhibitor	CA999763	Biotic
O. sativa ethylene-inducible protein	CA997945	Abiotic/Biotic
O. sativa hypersensitive response protein RHIR1	CA999179, CA998776	Biotic
O. sativa pathogenesis-related protein PR10a/b	CA999048, CA998218, CA999843	Biotic
O. sativa pathogen-related protein Rir1b	CB000862	Biotic
O. sativa salt and ABA-inducible protein osr40g2	CA999657	Abiotic
O. sativa salT gene	CA999358, CB000748	Abiotic
O. sativa salt-induced mannose-binding lectin	CB000699	Abiotic
O. sativa Sgt1	CB001052	Biotic
O. sativa unknown elicitor inducible protein	CB000230	Biotic
O. sativa water-stress induced protein WSI18	CA998010	Abiotic
O. sativa18 kDa cold-induced protein	CB000948	Abiotic
Peroxidases	CA999326, CA997920, CA999786	Abiotic/biotic
Porin-like	CA998440	Abiotic
Potassium transporter	CB000477	Abiotic
Prolamin	CA999406, CA999291, CA998150	Abiotic
Proteinase inhibitor	CB000816	Biotic
Putative ion homeostasis/stress-related protein	CB000247	Abiotic
Pyrroline 5-carboxylase synthetase	CA998314	Abiotic
S-Adenosylmethionine decarboxylase	CA997853	Abiotic
Shaggy-related protein	CA999756	Abiotic/biotic
S. tuberosum respiratory burst oxidase protein	CA999224	Abiotic/biotic
S. bicolor water-stress protein WS1	BU099177	Abiotic
S. polyrhiza ABA and stress-induced protein PDR5-like	CA999520	Abiotic
Superoxide dismutase	CA999904	Abiotic/biotic
Thaumatin-like protein	CB035351	Abiotic/biotic
Thionin	CB001058, CB000929	Biotic
T. aestivum aluminum stress-induced protein wali6	CB001053	Abiotic
T. aestivum aluminum tolerance protein S22	CA999272	Abiotic

plants that are already injured via abiotic stress effects are more vulnerable to opportunistic pathogens (Kneebone 1976).

Cellular signalling is the primary event that leads to transcriptional activation of inducible genes. Hence, these two processes are the principal regulators of the low-temperature stress-response pathway. Processes related to cellular signalling in the cold-stressed seedlings can be deduced from the ESTs encoding for proteins with known or predicted functions as membrane-bound receptors, Ca^{2+} homeostasis proteins, various Ca^{2+}/calmodulin-dependent protein kinases, protein phosphatases and mediators of protein-protein interactions (Table 2). The integration of developmental and stress-related transcriptional machineries can also be inferred from the proteins with known or predicted functions as transcriptional regulators for both pathways (Table 2). These ESTs were identified by similarities to proteins containing signature DNA-binding motifs including AP2, EREBP, bZIP, zinc finger, Myb-like and Myc-like domains, and MADS box proteins. Because many of the known stress-related transcriptional regulators in plants contain some of these signature domains (Stockinger et al. 1997; Liu et al. 1998; Chen et al. 2002), the current ESTs provide some insight on the possible range of regulators that function in the cold-stress response during the early seedling development.

For the second approach, we used a sample of 46 ESTs classified either as stress-related, growth-related or housekeeping proteins, and another 46 randomly selected "unknown protein" ESTs to estimate the proportion of the subtracted set that are actually upregulated by chilling. We used the standard dot-blot technique as a preliminary means to compare the relative transcript abundance between the control and cold-germinated seedlings (Fig. 2, Table 3). The gene-expression patterns obtained from this analysis are reasonably consistent among the triplicate blots. Based on conventional knowledge of stress-gene expression in plants, many of the genes that were expected to respond positively to the cold treatments exhibited such patterns in the dot blots. A total of 20 of the 46 randomly selected "unknown protein" tags in the dot blot showed some indication of induction in response to cold. Because this technique is not as sensitive and robust as the standard Northern blot or RT-PCR, the results from this analysis may not reflect the actual proportionof stress-inducible genes in the subtracted EST-set. Nevertheless, this strategy provided a good and easy way to validate the efficacy of the cDNA enrichment procedures.

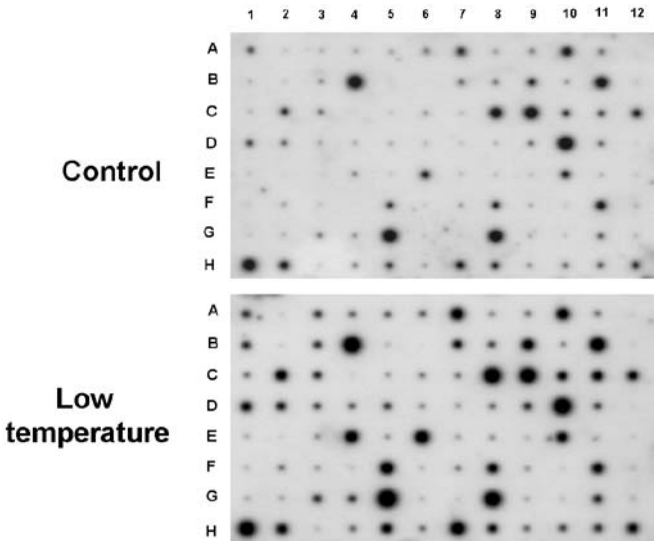

Fig. 2 Analysis of transcript abundance for some candidate stress-related ESTs. Identities of the ESTs used in the dot blot are listed in Table 3. Control: 30 °C. Low temperature: 13 °C/10 °C

Profiles of highly expressed genes

The number of ESTs assembled in each contig allowed the identification of the most highly expressed genes in rice seedlings under cold conditions. Figure 3 shows a summary of the contigs composed of more than six overlapping or redundant ESTs. The majority of these clusters correspond to housekeeping genes. Some encode for proteins with no known function and others for proteins with known or putative roles in the abiotic stress response. The most abundant cDNA species were assembled into three separate contigs and are similar to some members of the metallothionein-like (MT) protein family. The cold-induced expression of this gene is further confirmed in the Northern-blot data (Fig. 4A). In similar transcript profiling studies using SAGE, MT proteins were reported to be the second most highly expressed and induced genes in rice seedlings grown under anaerobic stress (Matsumura et al. 1999). Similarly, high expression of MT was also reported in drought (Reddy et al. 2002) and salt-stressed (Kawazaki et al. 2001) seedlings.

Other genes with some roles in plant responses to cold, drought or both, such as catalase, aquaporin, alcohol dehydrogenase, prolamin and 14-3-3 protein, were also among the most-abundant tags in this subtracted set (Jarillo et al. 1993, 1994; Matsumura et al. 1999; Kawazaki et al. 2001). In northern blots, these genes were clearly induced by cold (Fig. 4A). Except for the 14-3-3 protein, gene-expression profiles were characterized by high or medium activity in the control and an even further increase in transcript levels in the stressed seedlings. This result implies that these genes are important for the integration of developmental and stress-related responses.

COR gene orthologs in rice

In over-wintering plants, the development of freezing tolerance requires prior exposure to low but non-freezing temperatures, a process known as cold acclimation (CA). CA is accompanied by massive changes in gene expression that include the induction of a family of cold-

Table 2 Selected examples of ESTs for genes with known or putative regulatory functions

Putative function/Homology	Representative dBEST Acc.	Cellular process
14-3-3 protein	CA999400, CA998433, CB000845	Signal transduction
Ankyrin kinase	CB000654	Signal transduction
Ankyrin repeat protein	CA999110, CA997979	Signal transduction
A. thaliana bHLH transcription factor	CA998014	Transcription
A. thaliana C3HC4 zinc-finger protein	CA999534	Transcription
A. thaliana CDK5 activator protein-like	CA999616	Signal transduction
A. thaliana CONSTANS B-box zinc-finger transcription factor	CA999812	Transcription
A. thaliana ETTIN auxin response-like	CA999254	Transcription
A. thaliana protein kinase ATN1	CA999665	Signal transduction
A. thaliana protein phosphatase PEPINO	CB035352	Signal transduction
A. thaliana RING3 protein	CA999052	Transcription
A. thaliana signal recognition particle 72kDa protein-like	CA998275, CA999301	Signal transduction
A. thaliana trigger factor protein-like	CB000035	Signal transduction
A. thaliana two-component response regulator protein-like	CA998945	Signal transduction
A. thaliana VP1/ABI3 regulatory protein	CA999533	Signal transduction
Auxin response transcription factor	CA999797	Transcription
bZIP transcription factor	CB000843	Transcription
Calcineurin-like protein	CA998498, CB000355	Signal transduction
Calmodulin	CA999506	Signal transduction
Calmodulin-binding protein	CA999267	Signal transduction
Calreticulin	CA999388	Signal transduction
Cyclic nucleotide and calmodulin-regulated ion channel	CB000792	Signal transduction
Cyclin-dependent kinase F	CB001061	Signal transduction
D. carrota phytosulfokine/LRR-receptor protein	CA998409	Signal transduction
EF-hand calcium-binding protein	CB000272	Signal transduction
AP2-type transcription factors	CB000708	Transcription
EREBP1transcription factors	CA997903	Transcription
Ethylene-responsive GTP-binding protein	CA999035, CB000847	Signal transduction
F-box protein	CB035353	Transcription
G. hirsutum leucine-rich repeat protein kinase	CB000707	Signal transduction
Histone acetyltransferase	CB000679	Transcription
Histone deacetylase	CA999490	Transcription
Homeobox protein	CA998391	Transcription
Jun-activation domain binding protein-like	CA999423	Transcription
Knotted1-like	CA998293	Transcription
Leucine zipper domain protein	CB035354	Signal transduction
L. esculentum leucine-rich repeat protein LRP	CA999809	Signal transduction
MADS box protein	CA997982	Transcription
Mitogen activated protein kinase	CA998105	Signal transduction
Myb-related transcription factor	CA999492	Transcription
Myc-like protein	CA999311	Transcription
O. sativa APF1 transcription factor	CB000217	Transcription
O. sativa apospory protein	CA998036	Transcription
O. sativa auxin response factor	CB000143	Transcription
O. sativa calcium-dependent protein kinase OsCDPK7/OSK1	CB000044, CA999524	Signal transduction
O. sativa Bax cell-death protein inhibitor BI-1	CB000435	Signal transduction
O. sativa chilling-induced leucine zipper protein glip19	CA998766	Transcription
O. sativa protein kinase APC1/SNF1/SPK3	CA998860, CA999884, CA998915	Signal transduction
O. sativa putative transcription factor Riaa1	CA999980	Transcription
O. sativa S-locus receptor protein kinase RLK13-like	CB000018	Signal transduction
Phosphoinositide-specific phospholipase C	CB001002	Signal transduction
P. sativum circadian rhythm protein GDA2	CA997985	Transcription
Protein phosphatase 2c	CA998003	Signal transduction
Ring H2 finger protein	CA997885	Transcription
Ser/Thr protein kinase	CA999745	Signal transduction
S. bicolor far red response protein	CA999958	Transcription
TIR-NBS-LRR type protein	CA997921, CA998443	Signal transduction
WD-repeat protein	CA999721	Signal transduction
Whitefly-induced gp91 calcium-binding EF-hand protein	CA999848	Signal transduction
Xa-21-like receptor kinase	CA998023	Signal transduction
zf-C3HC4/zf-CCCH domain protein	CB001046	Signal transduction
zfwd2/zinc-finger protein	CB000299	Signal transduction

Table 3 List of ESTs used in the dot blot (refer to Fig. 2)

Clone identification	Expression*	Clone identification	Expression*
A1 *O. sativa* salT gene	I	E1 *A. thaliana* hypothetical protein AB007649	NI
A2 *O. sativa* ethylene-inducible AC079632	UD	E2 No significant hit (CA998030)[a]	UD
A3 *A. thailana* ERD15	I	E3 *O. sativa* hypothetical protein AJ243961	I
A4 *S. bicolor* water-stress inducible (WS1)	I	E4 *A. thaliana* unknown protein AY086551	I
A5 *A. thaliana* salt-inducible AB006698	I	E5 *A. thaliana* unknown protein AY088117	I
A6 *O. sativa* anaerobically induced early gene	NI	E6 *P. sativum* hypothetical protein U60592	I
A7 *O. sativa* water stress inducible WSI18	I	E7 *O. sativa* unknown protein AC105730	NI
A8 MAPK-like protein[a]	NI	E8 *O. sativa* hypothetical protein AL66027	NI
A9 *A. thaliana* ethylene-responsive[a]	NI	E9 *O. sativa* hypothetical protein AL606998[a]	NI
A10 *O. sativa* disease resistance protein AC084295	I	E10 Catalase	I
A11 *A. thaliana* ethylene-responsive[a]	NI	E11 *O. sativa* hypothetical protein AL606998[a]	NI
A12 *A. thaliana* disease resistance protein AL161533	UD	E12 *O. sativa* hypothetical protein AL606632	UD
B1 *A. thaliana* Avr9 response protein-like	I	F1 *O. sativa* hypothetical protein AP003433	UD
B2 TIR-NBS-LRR type disease resistance protein	UD	F2 *O. sativa* hypothetical protein AC091734	I
B3 *O. sativa* pathogenesis-related PR-10b AF274851	I	F3 *A. thaliana* hypothetical protein AC011661	I
B4 Peroxidase	I	F4 *O. sativa* hypothetical protein AL606632	UD
B5 *O. sativa* unknown protein AC087182	NI	F5 *A. thaliana* hypothetical protein AY075661	I
B6 *O. sativa* unknown protein C97584	UD	F6 No significant hit (CA997952)	UD
B7 *A. thaliana* Ca^{2+}-dependent protein kinase	I	F7 *O. sativa* hypothetical protein AP003516	NI
B8 *Xa-21*-like receptor protein kinase	I	F8 *O. sativa* hypothetical protein AP003235	I
B9 Protein phosphatase 2C	I	F9 No significant hit (CA998019)	NI
B10 MAPK-like protein[a]	NI	F10 No significant hit (CA998030)[a]	UD
B11 40S ribosomal protein S9-type	I	F11 No significant hit (BU099188)	NI
B12 Serine/threonine-specific protein kinase	UD	F12 No significant hit (CA998080)	UD
C1 *O. sativa* S-locus receptor-like protein kinase	I	G1 No significant hit (CA998028)	NI
C2 *Z. mays* HMG-like DNA-binding protein X66077	I	G2 No significant hit (CA998057)	NI
C3 *A. thaliana* MADS-box protein	I	G3 No significant hit (BU099201)	I
C4 *P. sativum* circadian rhythm protein GDA2	UD	G4 *O. sativa* unknown protein AU108260	I
C5 Transcription factor EREBP1	I	G5 Glutathione S-transferase	I
C6 *O. sativa* apospory protein AF358770	NI	G6 *O. sativa* endosperm protein H144B12	UD
C7 Putative tumor suppressor protein U55048	I	G7 *O. sativa* shoot protein AU222993	UD
C8 *A. thaliana* regulatory protein	I	G8 *O. sativa* callus protein C97731	I
C9 Heat shock protein	I	G9 *O. sativa* leaf protein AU173890	NI
C10 *A. thaliana* harpin-induced protein AT2G4420.1	I	G10 *O. sativa* cytosolic protein E60301	UD
C11 Gibberellin C-20 oxidase	I	G11 No significant hit (BU099203)	I
C12 Nitrogen fixation protein (nifS)	NI	G12 No significant hit (BU099205)	UD
D1 *O. sativa* hypothetical protein AP002525	I	H1 Fructose bisphosphate aldolase	NI
D2 No significant hit (BU099173)	I	H2 Pyruvate decarboxylase	NI
D3 *A. thaliana* unknown protein E1366	I	H3 Transaldolase	UD
D4 *A. thaliana* unknown protein AP002897	I	H4 Glyceraldehyde 3-PO_4 dehydrogenase	NI
D5 *A. thaliana* putative protein NM_121938	I	H5 Pyruvate kinase	I
D6 *A. thaliana* unknown protein AC007454	NI	H6 Enolase	NI
D7 *A. thaliana* unknown protein AB018116	NI	H7 Malate dehydrogenase	I
D8 *O. sativa* unknown protein AL606615	I	H8 Ribosomal protein L3	I
D9 *A. thaliana* unknown protein NM_120126	I	H9 Elongation factor 2	NI
D10 *A. thaliana* hypothetical protein AC005170	I	H10 40S ribosomal protein	I
D11 *A. thaliana* unknown protein AY079375	NI	H11 Elongation factor-1α	NI
D12 *A. thaliana* unknown protein AP001304	UD	H12 Ubiquitin	I

* Expression pattern based on visual inspection of dot-blot signal intensity. I = induced, NI = not induced, UD = undetected
[a] ESTs that are represented twice in the dot-blot panel

regulated or *COR* genes (Thomashow 1999). Since rice is incapable of CA, we then raised the question of whether similar *COR* genes are expressed as an early response of developing seedlings to cold. If not, are there other non-CA-related stress proteins that are expressed during the early stages of seedling development under low-temperature conditions? To address these questions, we searched our annotated stress-related ESTs for some genes with similarities to known *COR* genes from *Arabidopsis thaliana* and other cold-acclimating plants.

Four EST classes, with the highest scoring matches with *COR* genes, encode for probable orthologs of the *A. thaliana* rare cold-inducible, *RCI2* (Capel et al. 1997; Medina et al. 2001), barley low-temperature inducible, *blti101* (Goddard et al. 1993), wheat cold-acclimation gene, *WCOR413* (AY057118), and a one-helix protein that was annotated in the GenBank to be induced after 24 h of CA in *Deschampsia antarctica* (AY090544) (Table 1). The precise biochemical functions of these *COR* genes are not known, but they all share a common feature of being inducible early during the period of CA of chilling-insensitive species. Analysis of the *RCI2*-like and *blti101*-like full-length cDNAs showed that they encode for hydrophobic peptides (data not shown). The expression patterns of these putative *COR* gene orthologs in the northern blot showed that they were all induced by

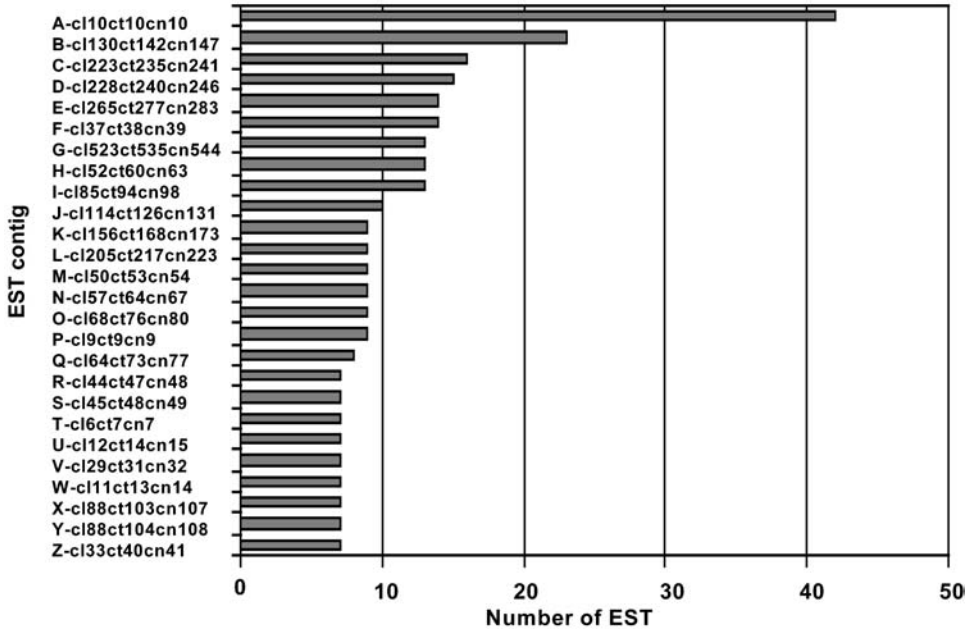

Fig. 3 Summary of the 26 largest contigs (>6 ESTs) assembled from the subtracted EST library. A, B, C = Metallothionein-like proteins; D = Elongation factor-1α; E = 60S Ribosomal protein L10; F = Glyceraldehyde 3-PO_4 dehydrogenase; G = No significant hit; H = Alcohol dehydrogenase; I = No significant hit; J = NADP-specific isocitrate dehydrogenase; K = Fructose bisphosphate aldolase; L = Heat-shock protein 82; M = 40S ribosomal protein; N = Triose phosphate isomerase; O = 14-3-3 protein; P = Aquaporin; Q = 40S ribosomal protein S3A; R = Prolamin; S = No significant hit; T = Ribosomal protein S7; U = Glucose 6-PO_4 isomerase cytosolic; V = Nascent polypeptide-associated complex alpha chain; W = Similar to *A. thaliana* unknown protein At1g10840.1; X = *O. sativa* unknown protein AF172282_26; Y = *O. sativa* reversibly glycosylated polypeptide; Z = Catalase

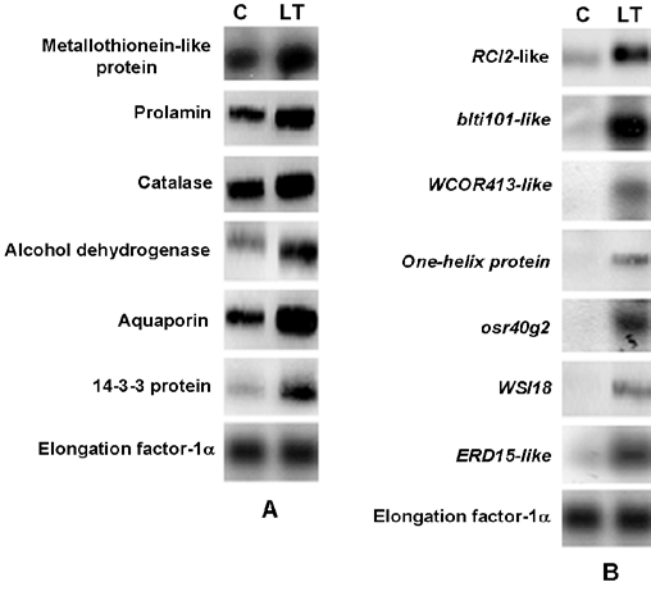

Fig. 4 *Panel A*: analysis of transcript levels for some of the most abundant ESTs. RNA gel-blots were probed with representative ESTs for each contig: metallothionein-like proteins (CA999942), alcohol dehydrogenase (CB000919), 14-3-3 protein (CA999400), aquaporin (CA998222), prolamin (CA999406) and catalase (CA998957). *Panel B*: Northern-blot analysis of putative *COR* gene orthologs and dehydration-responsive genes. RNA gel blots were probed with representative ESTs for *RCI2*-like (CB000413), *blti101*-like (CA998755), *WCOR413*-like (CA999631), one-helix protein from *D. antarctica*-like (CA999448), *osr40g2* (CA999657), *WSI18* (CA998010) and *ERD15*-like (CA998049). Elongation factor-1α (CA997978) was used as a loading-control probe. *C* = control; *LT* = low temperature

cold at the early seedling stage (Fig. 4B). The occurrence of these genes in rice suggests that the early events in the cold-stress response pathway have some overlaps between chilling-insensitive and sensitive species.

In our search for putative *COR* gene orthologs, we also found a number of ESTs (Table 1) with homology to some entries that are annotated in the GenBank as responsive to water stress, ABA and/or salt stress. Examples include the *osr40g2* (CAA70174) and water stress-induced (*WSI18*, Joshee et al. 1998) genes from *O. sativa*, and early responsive to dehydration gene (*ERD15*, Kiyosue et al. 1994) from *A. thaliana*. To our knowledge, none of these genes have been previously reported to be cold inducible, and their precise biochemical functions are unknown. However, their cold-induced expressions (Fig. 4B) suggest that, as with freezing stress, chilling may also cause some reduction in cellular-hydration capacity. Furthermore, the cold-induced expressions of the known ABA-responsive genes also suggest that as in cold-acclimating species, signalling mechanisms in non-acclimating plants are also mediated by ABA.

Discussion

Direct-seeded tropical crops like rice rely on intrinsic mechanisms to survive sudden changes in environmental temperatures during the critical seedling-developmental stages. This response is suggested by the ability of some japonica cultivars to continue with the early seedling-

developmental processes even at temperatures that are below the optimum (Sthapit and Witcombe 1998). Given the presumed physiological and biochemical complexities associated with adaptation, the genetic potential for this trait is defined by the total set of genes that are expressed under such conditions. To our knowledge, large-scale analysis of gene expression related to the responses of rice to cold, particularly during the early seedling developmental stages, has not yet been reported. Hence, the expressed gene catalog presented here will provide the starting point for more in-depth study of the molecular genetic basis of early seedling cold-tolerance in rice by transcriptome profiling. In this small-scale EST project, we used a simple and more direct way to obtain a picture of the cellular processes that are critical in the expression of seedling cold-tolerance by targeting the genes that are upregulated by cold. Both the *in silico* and RNA gel-blot expression-data provided some evidence that this EST-set is indeed enriched with such genes, indicating the high efficiency of the cDNA subtraction strategy.

Germination and early seedling development are critical stages that determine the successful transition of the seed into an autotrophic young plant. A desiccated, quiescent seed is highly tolerant to low temperatures, but this status is immediately reversed upon imbibition. When hydrated, the seed is metabolically reactivated to initiate the processes of seedling development and growth. Transition from a quiescent to a germinating state is critical to the survival of the seedling and cannot be reversed without deleterious effects (Thomas 1993; Holdsworth et al. 1999). At this point, the once-hardy seed becomes highly sensitive to major environmental impediments such as low temperature and moisture conditions. As implied by the type of gene functions represented in this EST-set, more tolerant genotypes are able to complete with such difficult developmental transition by precise coordination of growth-related and stress-related gene expressions. One of the more obvious examples is the abundance of metallothionein-like (MT) proteins. The MTs have been reported previously as one of the most-highly expressed genes in germinating rice (Matsumura et al. 1999). Further increase in its expression in response to cold suggests that very high activity is probably required in order to perform both its essential roles in the normal cellular physiology of developing seedling and other functions critical to the overall stress-adaptive mechanism. In addition to its function in heavy metal detoxification (Hsieh et al. 1995), other important growth-related function of MTs includes a role as a major transporter of metal cofactors for biochemical reactions involved in cell elongation and cell-wall lignification (Omann et al. 1994; Yu et al. 1998). During chilling, the seed may exhibit a reduced hydration capacity. Under such conditions, increased MT functions may be required in order to maintain low concentrations of heavy metal ions in the cell, which at higher levels could lead to some toxicity by causing a rapid build-up of oxygen radicals. We speculate that MTs may be an integral component of the general defense mechanism against the negative physiological manifestations of chilling.

At the cellular level, responses to sub-optimal environmental conditions occur through a succession of distinct gene activities. For instance, global gene-expression data showed that the responses to salt stress in rice seedlings involves precise timing of gene expression defined by the genes involved in: (1) initial metabolic adjustment, (2) signalling and alteration of transcriptional machineries, (3) cellular defenses and damage repair, and (4) recovery (Kawazaki et al. 2001). The types of genes represented in the cold stress-related gene-enriched EST-set is consistent with this general scheme. Although this comparison needs to be substantiated with detailed temporal expression analysis, it appears that this EST-set is a good representative of the essential genes involved in the overall mechanism of adaptation to cold and, in combination with other stress-response EST libraries from public collections, it will provide powerful tools for precise mapping of the beginnings and endpoints of the cold-stress response pathway in developing seedlings.

The cold-acclimation (CA) response in chilling-insensitive species is controlled by cold-responsive transcriptional activators, which trigger the activation of a battery of target genes, i.e., the *CBF-COR* genes regulon (Stockinger et al. 1997; Gilmour et al. 1998; Jaglo-Ottosen et al. 1998). Through this mechanism, temperate plants are able to acquire the ability to survive subzero temperatures by allowing low but non-freezing temperatures to activate in advance an array of cellular-defense processes. Because rice is incapable of CA, we hypothesized that adaptation to cold stress probably involves genes that are different from the *COR* genes in freeze-tolerant species. With the current EST data, we identified and characterized a few *COR* gene orthologs (e.g., *RCI2*-like, *blti101*-like and *WCOR413*-like). Based on the current data, we can speculate that some of the components of the cold-stress response mechanism are probably conserved between cold-acclimating and non-acclimating species, and that the divergence of the chilling-sensitive from chilling-insensitive plants is accompanied by the loss of the major components of a functional CA pathway, or vice versa. Complete expression analysis of this EST-set should provide some support to this hypothesis. Additionally, the large number of potentially upregulated unknown proteins in this EST-set provide an opportunity to discover some genes that that may be specific to chilling-insensitive plants.

The current biological-research paradigm relies on the use of gene databases to develop hypotheses. We summarized in this report a new set of ESTs developed by using a simple cDNA subtraction strategy. Despite its small size, this EST-set appears to be substantial in content and, therefore, could contribute to the enrichment of the publicly available stress-related EST libraries. This resource will be useful to develop hypotheses on the mechanism of cold tolerance in rice seedlings. Because of the equally important role of down-regulation of gene expression to the cold-stress response pathway (Fowler

and Thomashow 2002), future expansion of this gene catalog should target the repressed genes.

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