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Semi-global Analysis of the Early Cold Stress Response Transcriptome of Developing Seedlings of Rice (Oryzasativa L.,japonica)

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SEMI-GLOBAL ANALYSIS OF THE EARLY COLD STRESS RESPONSE TRANSCRIPTOME OF DEVELOPING SEEDLINGS OF RICE (*Oryza sativa* L., japonica)

By

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An Abstract of the Thesis Presented
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Plants are either sensitive or insensitive to low temperatures. Cool-season species acclimate to chilling and develop tolerance to freezing. Warm-season species vary in the degree of sensitivity to chilling and are not capable of acclimation. Both freezing and chilling tolerance involve the activity of an intricately coordinated network of genes. The regulatory network that defines cold acclimation and freezing tolerance is well understood in *Arabidopsis*. The centerpiece of this network is a group of transcriptional activators that coordinate a battery of downstream defense-related genes. In contrast, little is known about the corresponding regulon in non-acclimating species.

This study utilized the available tools of functional genomics and genome sequence resources of rice (*Oryza sativa* L.), a chilling-sensitive species, to examine the cold stress transcriptome of young seedlings of a relatively tolerant japonica cultivar (CT6748-8-CA-17). The goal was to use rice as a model towards understanding the dynamics of the early response regulatory network through a survey of gene expression changes during the critical first 24 hours of stress. The expression of 1,550 unique genes represented as spotted cDNA probes on a microarray was profiled by interrogation with a pair of control (28°C) and stressed (10°C) RNA isolated after 0.5, 2, 6, 12 and 24 hours. The expression data showed that early response involves two

waves of induction and repression. The first wave started within the first 2 hours of stress, hence the genes were collectively designated as 'rapidly induced/repressed early response genes' (Group-I). The second wave did not start until after 2 hours of stress, hence the genes were collectively designated as 'delayed induced/repressed early response genes' (Group-II).

The functional categories of genes overlap between Groups-I and II. In general, balanced upregulation and downregulation of genes involved in signal transduction, growth and development, metabolism, transport, protein synthesis, modification and degradation reflects the 'physiologically stressed' status of the plant, consistent with the initial reduction in growth rate. Early gene expression responses also suggest an active mechanism by which cellular resources are temporarily being redirected from growth related processes to overall physiological adjustments and early defenses.

Potential regulators of early responses were also identified in the survey. The first is a novel cold stress-related bZIP transcription factor (*OsbZIP*) induced rapidly but transiently within the first 2 to 6 hours. Other potential early regulators are transiently expressed after 6 hours. These include a bHLH transcription (*OsICE*) factor similar to the ICE1 of *Arabidopsis*, a Myb protein similar to the previously identified cold stress response regulator Osmyb4, and a C3HC4 zinc-finger protein related to HOS1 of *Arabidopsis*. The layered fashions by which these transcription factors are expressed suggest a mechanism for fine-tuned regulation of the early response genetic network. The transcriptome survey also suggests that ABA is not essential in the early responses and other molecules such as H_2O_2 are probably involved. The results of this study set the stage for future investigation of the entire pathway by a combinatorial approach that includes genome-wide transcript profiling, promoter-reporter assay, protein-DNA interaction analysis and reverse genetics.

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Chapter 1

INTRODUCTION

Many agriculturally important plants are highly prone to stress that is commonly caused by abiotic factors such as drought, salinity and temperature extremes. In the temperate regions of the world, stress due to low temperature is a major environmental factor that impacts the ecological distribution of plants and limits their productivity even in managed agricultural ecosystems. Plant species of temperate origins are generally less vulnerable to low temperature-induced injuries because they have evolved more efficient mechanisms to physiologically acclimate or adjust to the fluctuations in temperature that occur at virtually all stages of growth. However, the same is not true with plants of tropical or subtropical origins, which exhibit higher levels of low temperature sensitivity because of less efficient or lack of effective physiological mechanisms to adapt to the extreme fluctuations in environmental temperatures.

With the fast growth in global population and rapidly shrinking area of prime land devoted to agriculture, it has been predicted that more and more marginal lands will be utilized in the coming years in order to insure sustainable and environmentally friendly systems of food production. Both uplands and lowlands that are marginal between cool-season and warm-season zones are increasingly being utilized for large-scale cultivation of low-temperature sensitive crops for human and animal consumption and, more recently, for energy production. Three of the most important cereal species (rice, maize and sorghum) that are grown in large acreage in temperate or sub-tropical countries of North and South America are highly sensitive to chilling temperature. For instance, rice (*Oryza sativa* L.) is cultivated as a major crop for both local consumption and export in many southern states of the U.S. including Arkansas, California, Louisiana, Mississippi, Missouri and Texas. Rice crops in these states are normally direct-seeded in the field

(i.e., broadcast from small airplanes) in late spring or early summer when the soil and water temperatures can fluctuate depending on the weather and time of day from ambient to chilling, at lethal or sub-lethal range. Chilling injury at the early stages of rice seedling development has severe manifestations on the agronomic potential of the crop. For example, chilling temperatures can cause severe injuries or kill rice seedlings at the early stages of germination and vegetative growth. These injuries are often manifested in terms of reduced seedling emergence and vigor, which then result in poor stands and decreased crop competitiveness against weeds, insect herbivores and pathogens, resulting in negative impacts on plant survival and yield potential. Additionally, cold stress slows down early seedling developmental processes resulting in the delay in flowering and further exposure of developing microspores to cold snap during late summer or early fall (Li and Rutger, 1980).

The mechanism that allows temperate plant species to survive freezing during winter is a very well studied biological phenomenon (Pearce, 1999; Thomashow, 1999; Thomashow, 2001). However, the genetic potential that defines chilling tolerance in some genotypes of warm-season plants, especially cereals has not been studied to the same extent as freezing tolerance in temperate plants have been studied. Elucidation of the precise biochemical pathways and genetic regulatory mechanisms involved in chilling tolerance of warm-season crops will be important for future development of genetic strategies for crop improvements. Accomplishing this goal has continued to be a major challenge because of the complex genetics governing the mechanisms of adaptation to low-temperature and other abiotic stress. Tolerance mechanisms involve the synergistic functions and cumulative effects of many genes that are inherited as quantitative trait loci or QTL (Redona and MacKill, 1996). For example, five QTL for cold tolerance have been genetically mapped across the 12 chromosomes of rice (Kim et al., 2000). The total number of genes contained in these QTL is not known.

A number of important features have made rice an ideal model plant for studying complex quantitatively inherited traits such as tolerance to environmental stresses. Relative to most crop species, rice has a very small genome consisting of ~390 megabase pairs encoding about 40,000 genes (Goff et al., 2002). The genome of rice, which is divided into 12 pairs of chromosomes (2n = 2x = 24) has been well mapped both genetically and physically, including extensive high-density genetic linkage maps and comprehensive transcript maps (Sasaki and Burr, 2000; Harushima et al., 1998; Wu et al., 2002, Chen et al., 2002). Furthermore, rice is relatively easy to manipulate genetically by *Agrobacterium*-mediated transgene expression and T-DNA insertion mutagenesis, making it an excellent model for detailed analysis of gene function (Sasaki and Burr, 2000). In addition, rice exhibits a high level of synteny with the other members of the cereal family. As the smallest cereal genome in the monocot-dicot divide, genes identified in rice can be used as fundamental basis for comparative genomics and identification of homologous stress-related genes in the more complex monocot genomes such as maize and sorghum (Gale and Devos, 1998; Messing and Llaca, 1998).

Using rice as a model and taking advantage of the available tools of structural and functional genomics that are currently available in this species, this study was aimed at establishing the foundation for future reverse genetic analysis by further investigating the genomic basis of an important aspect of the responses of warm-season plants to chilling stress. The emphasis of this research is the analysis of the transcriptome of developing rice seedlings during the initial phase of cold stress on a semi-global scale. The scope of this study was defined based on the genetic and biochemical network hypothesis and genomic resources such as abiotic stress EST libraries and candidate cold-stress-associated genes developed earlier by this group (De los Reyes et al., 2003; Morsy et al., 2005; Antoine et al., 2005). Thus, the centerpiece of this study is the

quantitative survey and parallel analysis of the expression of candidate cold-stress related genes based on the hypothesis proposed from the snapshot genomic data reported by De los Reyes et al. (2003).

Chapter 2

REVIEW OF LITERATURE

Physiology of plant responses to low temperature

Changes in temperature (low or high) have very profound effects on all aspects of growth, development and reproduction of plants. Therefore, temperature is a major environmental factor that limits their ecological distribution. Plants can be categorized either as 'chilling-sensitive' or 'chilling-insensitive' depending on the limits and/or magnitude of their susceptibility to low temperature stress (Pearce, 1999). Insensitive species, which thrive in the temperate zones, are damaged only by temperatures below freezing (0°C) and many can withstand or acclimate to freezing temperatures with prior exposure to chilling temperatures (Levitt, 1980; Ingram and Bartels, 1996). On the other hand, many important crop and horticultural plants of tropical and sub-tropical origins are considered chilling-sensitive because they are easily injured or killed by exposure to low but non-freezing temperatures, usually between the 2°C to 20°C range depending on genotype and developmental stage.

Extensive physiological and biochemical studies conducted during the last few decades have shown that the cell membrane system is the primary site of cellular injury during both freezing and chilling stress (Steponkus, 1984; Uemura and Steponkus, 1994). The first apparent physical effect of low temperature on plant cells is the change in membrane dynamics from a flexible liquid-crystalline state to a solid gel-like phase (Kratsch and Wise, 2000; Raison and Chapman, 1976). This phase transition often leads to alteration of the otherwise semi-permeable and biologically functional cell membrane further resulting in cellular leakiness and ion imbalance. The physiological consequences of this perturbation affect plant cells in many ways, including abnormal metabolism, and accumulation of toxic metabolites and reactive oxygen species (Nishida

and Murata, 1996; Lyons, 1973; McKersie and Bowley, 1997). The mechanism of freezing tolerance of temperate plants is not a constitutive process but an acquired adaptive response that results from prior exposure to a continuous period of low, but non-freezing temperatures (chilling). This process is called cold acclimation (CA) or hardening (Hughes and Dunn, 1996; Thomashow, 1999). When plants growing during the warmer months are exposed to artificial freezing stress in the laboratory during the vegetative growth phase, these plants would have very limited tolerance to freezing. However, gradual exposure to low but nonfreezing temperatures serves as a stimulus that mimic the signals associated with an approaching winter. This signal is an essential process that triggers a series of intricate molecular events that determine adaptation and survival under freezing conditions. For instance, non-acclimated rye (*Secale cereale*) is killed when exposed to about -5°C, but can survive freezing down to about -30°C with prior exposure to low but non-freezing temperature, i.e., 4 weeks of cold acclimation (Webb et al., 1994; Thomashow, 1999).

Many studies have contributed to our current knowledge of the important mechanisms that define the molecular and cellular differences between plants that had been cold acclimated and plants that have not been acclimated (Uemura et al., 1995; Webb et al., 1994). In general, membrane lesions caused by freeze-induced destabilization is prevented or minimized with a change in overall lipid composition of the membrane. CA increases the phospholipid (PL) content of the plasma membrane of both herbaceous and woody species (Yoshida and Uemura, 1984; Lynch and Steponkus, 1987; Uemura and Steponkus, 1994; Ishikawa and Yoshida, 1985; Yoshida, 1984). In addition, an increase in the proportion of PL was also shown to be associated with varied capacity for CA in two *Solanum* species. For instance, membrane PL increased in *S. commersonii*, which increased tolerance with CA, but not in *S. tuberosum*, which did not show an increase in freezing tolerance (Palta et al., 1993).

Changes in lipid composition during CA affect the cryostability of the plasma membrane and this is manifested by the occurrence of specific freeze-induced lesions. These changes profoundly influence freezing tolerance. Without CA, isolated leaf protoplasts of rye (*Secale cereale*) are injured when frozen at a temperature range of 0°C to -5°C, a type of freezing injury referred to as '*expansion-induced lysis*' (Uemura and Steponkus, 1989). As a consequence of osmotic excursions during freezing, endocytotic vesiculation of the plasma membrane occurs, which leads to a significant reduction in membrane surface area and cell volume (Dowgert and Steponkus, 1984; Gordon-Kamm and Steponkus, 1984). Since the severe plasma membrane loss and cellular reduction are irreversible, protoplasts burst upon thawing when water reenters the cell.

Deleterious membrane lesions are also formed when non-acclimated protoplasts are frozen to much lower temperatures (e.g., -10°C). The severe dehydration and associated alteration of the plasma membrane ultrastructure leads to increased permeability to water and solute, hence, there is a complete loss of osmotic responsiveness (LOR) during re-warming (Dowgert and Steponkus, 1984; Crowe et al., 1983; Steponkus, 1984). Loss of osmotic responsiveness is characterized by several membrane morphological changes, including lateral separation of the plasma membrane, formation of large aparticulate lamellae (domains) around the membrane, and phase transition from lamellar-to-hexagonal$_{II}$ (Hex_{II}, inverted membrane hexagonal symmetry structure formed as a result of lipid aggregation) (Gordon-Kamm and Steponkus, 1984; Crowe et al., 1983). Moreover, the association of CA with these injuries is even more significant, given both expansion-induced lesion and freeze-induced formation of Hex_{II} phase are normally not observed in cold-acclimated protoplasts (Uemura et al., 1995; Webb et al., 1994).

Genes associated with low temperature stress response

Most of the known mechanisms of low temperature stress tolerance in higher plants have been discovered from studies on cold acclimation (CA). Questions about homologous mechanisms in chilling-sensitive species are just beginning to be addressed using the tools of functional genomics and reverse genetics. The acquisition of freezing tolerance after CA is associated with a plethora of cellular adaptations such as increased synthesis of sucrose (Strauss and Hauser, 1986), Late Embryogenesis Abundant (LEA) and cryoprotective proteins (Dure, 1993; Worrall et al., 1998), compatible osmolytes (Holmstrom et al., 2000), and proteins responsible for oxygen radical scavenging and cellular detoxification (Roxas et al., 1997; Dipiero and Leonardis, 1997). These responses are necessary to stabilize membranes against freeze-induced injuries mentioned earlier and other potential cellular damages, including production of reactive oxygen species (oxidative stress) (McKersie and Bowley, 1997) and cell rupture caused by intracellular ice adhesion with cell walls and membranes (Olien and Smith, 1977).

The occurrence of massive alteration in gene expression patterns during CA was first established from the experiments of Guy et al. (1985) in spinach (*Spinacea oleracea*). Since then many studies have focused on the identification of cold-inducible genes and determining the roles of their products on tolerance mechanisms. Initially, many of the genes that were identified as induced during CA encode proteins with known enzymatic functions, providing important clues on some of the critical biochemical or physiological defenses contributing to overall freezing tolerance. For instance, the *FAD8* gene of *Arabidopsis thaliana,* which encodes a fatty acid desaturase, was implicated in freezing tolerance mechanisms by virtue of its role in altering the lipid composition of cell membranes during CA (Gibson et al., 1994). A number of genes encoding molecular chaperones including *hsp70* from spinach have also been implicated in the CA process (Anderson et al., 1994). It was established that molecular chaperones

have important roles in stabilizing proteins during the process of freeze-induced denaturation, thereby preventing irreversible aggregation (Antoine et al., 2005).

The other class of cold-inducible genes encodes proteins that are not directly involved in biochemical or physiological defenses. The products of these genes are known to contribute to freezing tolerance mechanisms by controlling the expression of defense-associated genes or by regulating the activities of associated proteins through a very complex signal transduction pathway. This group of genes includes the calcium (Ca^{2+})-binding and calmodulin-binding proteins (Polisensky and Braam, 1996), mitogen-activated protein kinase family, i.e., MAPK, MAPKK, MAPKKK (Mizoguchi et al., 1993) and 14-3-3 proteins (Jarillo et al., 1994; DeLille et al., 2001).

Cytoplasmic Ca^{2+} levels increase rapidly in response to low temperature, as shown in both *Arabidopsis* and alfalfa (Knight et al., 1996; Polisensky and Braam, 1996; Monroy and Dhindsa, 1995), indicating the important role of Ca^{2+} in low temperature signal transduction. Indeed, Ca^{2+} is one of the many molecules that function as second messengers, which eukaryotic cells use to deliver external stimuli caused by environmental stressors to specific downstream cellular targets leading to responses. These responses include changes in enzyme activity and gene expression, and thus have a profound role in many aspects of plant cell signaling (Bouche et al., 2005; Hetherington and Brownlee, 2004; White and Broadley, 2003). Calmodulin (CaM) is the most studied Ca^{2+}-sensing protein which, upon binding Ca^{2+}, activates numerous proteins involved in various cellular processes, including low temperature response and acclimation. For instance, cold stress induces several CaM and CaM-like genes, which are presumably responsible for translating stress signals (Braam and Davis, 1999; van der Luit et al., 1999). A plasma mernbrane Ca^{2+}/H^{+} transporter from *Arabidopsis* (CAX1) has been shown to affect freezing tolerance following CA (Catala et al., 2003). In addition, Ca^{2+} and CaM are likely involved in protein stabilization because *HSP* gene

expression and protein accumulation are significantly upregulated by Ca^{2+} addition and repressed by Ca^{2+} chelator or antagonists (Liu et al., 2003; Bouche et al., 2005).

Mitogen-activated protein (MAP) kinases are known to be activated by various growth factors and they are essential for integrating a plethora of cellular signals delivered by secondary messages such as Ca^{2+} flux (Bouche et al., 2005). This process has been associated with various abiotic stress responses. For instance, an alfalfa gene $p44^{MMK4}$ was shown to be induced shortly (within 10 minutes) of low temperature treatment and appeared to exhibit a cold-responsive pattern. In contrast, two other alfalfa MAP kinases, *MMK2* and *MMK3*, have been identified as not responsive to cold stress (Jonak et al., 1996). *MAKKK*, another member of the large MAP kinase class of proteins has been shown to be activated simultaneously by touch, cold and water stress (Mizoguchi et al., 1996). In addition to protein kinases, another class of signaling proteins, the 14-3-3-type proteins, play an important role in the regulation of the stress response signal transduction. These proteins are known to play important roles in various pathways by regulating a variety of target proteins through phosphorylation-mediated protein-protein interactions (DeLille et al., 2001; Roberts et al., 2002).

Molecular genetic analysis of the CA process in the model species *Arabidopsis* has also resulted in the identification of many novel cold-inducible genes. These are referred to as cold-regulated (*COR*) (Thomashow, 1999; Gilmour et al., 1992), low temperature-induced (*LTI*) (Nordin et al., 1993; Nordin et al., 1991), responsive to desiccation (*RD*) (Shinozaki and Yamaguchi-Shinozaki, 2000; Yamaguchi-Shinozaki and Shinozaki, 1993), cold inducible *(KIN*) (Tahtiharju et al., 1997), and early dehydration-inducible (*ERD*) (Thomashow, 1999). The nomenclature for these genes also reflects the fact that they are induced in response to conditions associated with water and osmotic stress including drought, high salinity and increased intracellular levels of the hormone, abscisic acid (ABA). Freeze-induced injuries to plant cells are essentially due to osmotic

effects resulting from the movement of water molecules into the intercellular space, where ice crystallization occurs more rapidly than in intracellular compartments. Hence, freezing essentially mimics the physiological effect of dehydration (Steponkus, 1984; Steponkus et al., 1993).

Results of some recent studies established that the products of many novel cold-inducible genes are indeed essential components of the freezing tolerance mechanism (Thomashow, 1999; Monroy et al., 1993; Hong et al., 1992; Houde et al., 1992). One example was shown by the study of *COR15a* expression in transgenic *Arabidopsis*. Constitutive expression of *COR15a*, which encodes a chloroplast-associated polypeptide resulted in a significant increase in freezing tolerance of the transgenic plants at the cellular level. It was established from this study that the COR15a protein contributes to freezing tolerance of cells by stabilizing the chloroplast membrane during freeze-induced dehydration (Artus et al, 1996). This study also demonstrated that overexpression of the *COR15a* transgene minimized the harmful biochemical changes and structural alterations of the chloroplast membrane, which often take place during freeze-induced dehydration of non-acclimated *Arabidopsis* cells (Steponkus et al., 1998).

Regulation of genes associated with low temperature stress response

Expression of eukaryotic genes is mediated by transcription factors that specifically recognize and bind to highly conserved sequence motifs in the target gene promoters (*cis*-elements, i.e., enhancers, repressors, insulators) (van Driel et al., 2003). Detailed analysis of the promoter regions of many members of the *COR/RD* gene family resulted in the identification of a conserved DNA regulatory element, termed C-repeat (CRT) or Dehydration Responsive Element (DRE). This element contains a conserved core sequence of CCGAC (Yamaguchi-Shinozaki and Shinozaki, 1994). Transcription of *COR/RD* genes is activated through the interaction (binding) of the transcriptional

activator with the CRT/DRE. This transcriptional activator belongs to the AP2/EREBP (Apetala2/Ethylene Response Element Binding Protein) family of plant-specific DNA-binding regulatory proteins (Riechmann and Meyerowitz, 1998) called CBF (C-repeat binding factor) or DREB (Drought-responsive element binding protein). Three members of the CRT/DRE family of regulatory proteins have been identified in *Arabidopsis* and designated *CBF1/DREB1b*, *CBF2/DREB1c* and *CBF3/DREB1a* (Gilmour et al., 1998; Stockinger et al., 1997; Liu et al., 1998). The importance of these regulatory proteins as mediators of low temperature and drought stress adaptive responses have been demonstrated in *Arabidopsis* or *Brassica napus*. For instance, overexpression of *CBF1/DREB1b* and *CBF3/DREB1a* in *Arabidopsis* led to strong activation of their target CRT/DRE-containing *COR/RD* genes and subsequently enhanced tolerance of transgenic plants to freezing and dehydration without prior exposure to CA conditions (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Liu et al., 1998; Jaglo et al., 2001; Gilmour et al., 2000). Collectively, all of these genes constitute a genetic regulatory network called the *CBF*-regulon (Thomashow, 2001). This network defines the genetic mechanism involved in the induction of CRT/BRE-containing genes by the CBF/DREB1 transcription factors.

Given the large number of genes involved in the *CBF/DREB* regulon, it is clear that the induction of a single *COR* or *RD* gene would have little effect on stress tolerance at the whole plant level. However, overexpression of a cold–inducible transcription factor such as the *CBF/DREB* has been shown to cause dramatic increases in freezing tolerance of transgenic plants due to its ability to coordinate the expression of individual genes involved in the network (Steponkus et al., 1998; Artus et al., 1996; Gilmour et al., 2000). More recently, global gene expression profiling experiments with microarrays or GeneChip arrays showed more evidence of the importance of the *CBF/DREB* network and other similar gene regulons to cold and perhaps dehydration and high salinity

tolerance mechanisms in higher plants (Seki et al., 2001; Fowler and Thomashow, 2002).

In a recent study that surveyed the transcript accumulation patterns for more than 8,000 *Arabidopsis* genes, it was found that more than 300 genes are responsive to cold, i.e., either upregulated or downregulated (Fowler and Thomashow, 2002). Many of these genes are either known or putative transcription factors (48 of the 306). Two of these transcription factors (*RAP2.1* and *RAP2.6*) were activated by constitutive overexpression of the *CBF* gene in plants that are not subjected to CA condition. These findings indicate the existence of sub-regulons or branched pathways.

Fowler and Thomashow (2002) also found that not all cold responsive genes in *Arabidopsis* are members of the *CBF* regulon, as many exhibited expression patterns that were apparently independent of *CBF* expression. Thus, many different types of transcription factors such as *ZAT12*-like and *RAV1*-like, presumably activate multiple regulatory pathways in addition to the *CBF* pathway. Furthermore, gene repression also appeared to have an integral role in the CA process, since nearly half (42 out of 88) of the genes were downregulated by low temperature in a long-term manner, and eight of those were also shown to be repressed when the activator *CBF* gene was expressed at warmer temperatures. These observations were further supported by a similar survey of 1,300 *Arabidopsis* genes (Seki et al., 2001). The precise number and type of genes under each sub-regulon and other parallel pathways are the specific emphases of the current studies. Nevertheless, it was proposed that *CBF/DREB* together with the putative *ZAT12*-like and *RAV1*-like regulons constitute a *'super-regulon'* that governs the overall plant response to low temperature in a complex network-like manner.

Comparative genomics of the *CBF/DREB* regulatory networks in different plant species showed that many of the genes involved in the *Arabidopsis* network have putative homologs in other temperate and tropical plants including *Atriplex*, barley,

canola, tomato, soybean, wheat, rye and rice (Shen et al., 2003; Choi et al., 2002; Jaglo et al., 2001; Shen et al., 2003; Dubouzet et al., 2003). These findings indicate that the *CBF/DREB* network of genes are highly conserved among both cool-season and warm-season species and that similar mechanisms that operate in CA plants may also be operational to a certain extent in warm-season plants that are not capable of CA. This paradigm presents an interesting hypothesis by which experiments on chilling tolerance mechanisms in warm-season cereals are currently being pursued.

Role of abscisic acid in plant responses to abiotic stress

The plant hormone abscisic acid (ABA) has long been implicated as the major regulatory molecule that affects seed development, germination and adaptation to various abiotic stresses (Leung and Giraudat, 1998). ABA accumulates endogenously during seed development and accumulation normally persists during the first two thirds of the seedling developmental process. Accumulation tapers down to a much reduced rate as the seed gets closer to the maturation and desiccation phase of development (Rock and Quatrano, 1995). Exogenous ABA was essential for the inhibition of precocious germination and promotion of dormancy of immature seeds in embryos from an ABA-biosynthetic mutant of maize (*Zea mays*) which were found to germinate while still attached to the mother plant (McCarty, 1995). Seeds of ABA-biosynthesis mutants from *Arabidopsis* and wild tobacco (*Nicotiana plumbaginifolia*) do not become dormant (Koornneef et al., 1982; Koornneef et al., 1984; Marin et al., 1996).

ABA is also a major regulator of stress response and it is an essential mediator for the initiation of the signaling pathway involved in responses to various environmental stimuli (Zeevaart and Creelman, 1988). The important role of ABA in the stress signaling mechanism of plants has been established from the numerous studies involving ABA-deficient mutants. Mutation in these genes appeared to have major impacts on the

regulation of many other genes involved in dehydration, salinity and cold stress (Giraudat et al., 1994; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996). However, expression of many of the osmotic stress-responsive (OR) genes were shown to be independent of ABA and this conclusion was based on analysis of the expression of downstream target genes in ABA-insensitive (defective in ABA perception) and ABA-deficit (impaired ABA-biosynthesis) mutants (Gilmour and Thomashow, 1991; Nordin et al., 1991; Gosti et al., 1995).

Many of the *COR/RD* genes that are induced during CA and drought are also induced by exogenous ABA in *Arabidopsis.* A conserved regulatory *cis*-element distinct from the CRT/DRE has been found in the promoters of many of the ABA-responsive *COR/RD* genes indicating another regulatory pathway apart from the CBF/DREB-mediated pathway (Busk and Pages, 1998; Rock, 2000; Yazaki et al., 2004). The conserved promoter motif critical to ABA-induced gene expression is called ABRE (ABA response element). The ABRE core sequence of ACGTG has been shown to be the specific binding target of a basic leucine-zipper (bZIP)-type transcription factor (Chandler and Robertson, 1994; Giraudat et al., 1994). Based on these findings, it was proposed that two signaling pathways, ABA-dependent and ABA-independent, occur in parallel in the stress response signal transduction pathway. Based on current data, it is believed that cold stress-regulated gene expression is generally independent of ABA (Shinozaki and Yamaguchi-Shinozaki, 2000). However, many cold-related genes suggest the opposite because many of the newly identified cold regulated genes are also responsive to exogenous addition of ABA (Baker et al., 1994). This is suggestive of a possible cross-talk between the cold stress branch of the signaling pathway and other secondary pathways that involves ABA. Examples of such ABA-responsive genes include the CA-associated *LEA-like* and *KIN1*, both encoding proteins with roles in protecting against freezing injury (Thomashow, 1999; Wang and Cutler, 1995). The manner by which

genes like *KIN1* is regulated is a strong indication of the potential interaction between ABA responses and direct responses to low temperature. In addition, in a genetic study involving various signaling mutants of *Arabidopsis*, Ishitani et al. (1997) created mutant lines that exhibited bioluminescence in response to low temperature. Based on the results of these experiments, an alternative scheme of cold and osmotic stress signal transduction has been proposed in which ABA-independent and ABA-dependent pathways function in an overlapping fashion, eventually converging and leading to activation of the downstream osmotic stress responsive gene transcription.

Sensitivity of rice to chilling temperatures

Rice is chilling sensitive with an optimal temperature for growth and development between 25°C to 35°C. Specific stages of germination and early seedling growth are particularly sensitive to fluctuations in environmental temperature below the optimum range (Adair, 1968). Low temperature stress causes either dramatic reduction or failure of germination, depending on genotype. Furthermore, cellular injuries caused by low temperature stress often result in poor seedling vigor, which leads to negative effects on seedling survival, development and growth (Peterson et al., 1978). Cold tolerance is associated with genetic variation in seedling cold hardiness. In general, japonica cultivars are more tolerant than most indica types. Cold tolerance of japonica rice is characterized by the ability to sustain normal germination and close to normal growth at sub-optimal temperatures of 10°C or below (Sthapit and Witcombe, 1998; MacKill and Lei, 1997; Lyons, 1973). In addition, a more quantitative measure of this genotypic variation for seedling cold tolerance was provided by a number of parameters measured under laboratory conditions, in which highly tolerant genotypes such as the model used in this study (CT6748-8-CA-17), were able to germinate at a higher rate at 10°C, and displayed a lower degree of membrane leakiness and higher level of seedling re-growth

after periods of chilling stress compared to many of the less tolerant indica cultivars (Morsy et al., 2005).

Genetics of seedling cold tolerance in rice

Unlike the well-studied cold acclimation (CA) process in chilling-insensitive, overwintering plants the molecular basis of adaptive responses in chilling-sensitive, warm-season species is largely unknown. The CA process is biochemically and genetically very complex as observed in the mechanisms elucidated using *Arabidopsis* as a model. This mechanism involves a very intricate coordination and integration of multiple signaling pathways. Although rice is incapable of CA, more cold-tolerant japonica cultivars are expected to respond to cold stress through the coordination of hundreds of defense related genes. During the last five years, a number of laboratories have established the groundwork required to elucidate the genetic regulatory network that defines tolerance mechanisms. For instance, a library of Expressed Sequence Tags (ESTs) was recently developed from the cold tolerant model CT6748-8-CA-17 to establish a subset of candidate cold stress-regulated genes for functional genomic analysis (De los Reyes et al., 2003). The strategy employed in this study involved the use of a composite pool of PCR-subtracted cDNA probes enriched with upregulated transcripts to screen for genes that are upregulated at by low temperature from a primary cDNA library constructed from cold stressed (10°C) rice seedlings. A total of 1,967 unique cDNAs were identified from the subtracted ESTs library.

Several of the genes represented in the EST collection are similar to the CA and dehydration associated *COR/RD* genes previously identified in *Arabidopsis* and other cold-acclimating plants. Among the highly expressed tags are putative orthologs of the *Arabidopsis* rare cold-inducible, *RCI2* (Capel et al., 1997; Medina et al., 2001; Morsy et al., 2005), barley low-temperature inducible, *blti101* (Goddard et al., 1993), wheat cold-

acclimation gene, *WCOR413* (AY057118), and Δ-1-Pyrroline 5-carboxylase synthetase (*P5CS*) (de los Reyes et al., 2003). In addition, many ESTs in the library appear to be similar to GenBank entries that are annotated to be responsive to ABA, dehydration, salinity stress [including *osr40g2* (CAA10714)] (Kawasaki et al., 2001), water stress-induced (*PWSI18*) (Joshee et al., 1998), the *Arabidopsis* early responsive to dehydration *ERD15* (Kiyosue et al., 1994), and *salT* (Claes et al., 1990). Although most of these genes do not have well understood biochemical functions, *P5CS* is a known target of CBF/DREB transcription factor in *Arabidopsis* (Gilmour et al., 2000), suggesting the possibility of a *CBF/DREB*-like regulatory pathway involved in the rice cold tolerance mechanism.

Analysis of the promoters of the putative *COR/RD* homologs of rice showed the presence of the CRT/DRE and ABRE-related motifs, which are commonly shared by many known *COR/RD* genes (Morsy et al., 2005). Other conserved sequence signatures characteristic of other stress-inducible promoters such as the as1/ocs-like, GCC-box, G-box and Myb-like elements (Chen et al., 2002; Shinozaki and Yamaguchi-Shinozaki, 2000; Schindler et al., 1992) have also been found among the putative rice homologs of *COR/RD* genes, indicating the involvement of multiple pathways in the regulation of adaptive responses. Among the many candidate cold-stress ESTs, are genes of regulatory function, including transcription factors and proteins involved in cellular signal transduction (De los Reyes et al., 2003). Full-length sequence analysis of an AP2-EREBP-type and bHLH-type of transcription factors in the EST library indicated that they are highly similar to the *Arabidopsis CBF3* and *ICE1* genes, respectively (Chinnusamy et al., 2003). Since ICE1 is the upstream regulator of CBF/DREB-mediated gene expression, it was hypothesized that the *CBF3*-like transcription factor might be the regulator that targets those *COR/RD*-like genes found in the same EST collection (Morsy et al., 2005), hence the possibility of a *CBF/DREB*-like regulon in rice.

The first line of evidence of the functionality of a *CBF/DREB*-like pathway in rice was shown in the study of two closely related *COR/RD* homologs discovered from the EST library developed by De los Reyes et al. (2003). These ESTs represent transcripts of the *OsLti6a* and *OsLti6b* genes, which are similar to *Arabidopsis RCI2* and barley *blti101*, respectively. The cold induced expression patterns of these genes were correlated with the expression of a *CBF3*-like regulator and are genotype-specific. The temporal expression patterns of the CRT/DRE containing target genes, *OsLti6a*, *OsLti6b* and *P5CS* appeared to be synchronized with the expression of the putative *CBF3*-like regulator gene (*'regulator-target'* relationship). Similar expression profiles were observed in both tolerant and intolerant genotypes in terms of the order of induction, i.e., regulator and then targets, but the intolerant line showed a much delayed expression (Morsy et al., 2005). Furthermore, given the fact that *OsLti6a* and *OsLti6b* encode plasma membrane-associated peptides, the physiological differences between rice genotypes with respect to the level of cold-induced membrane injury is presumably due at least in part to the synthesis of such protective proteins. Activation of these protective genes and other downstream defense-related genes is dependent upon the activation of the CBF/DREB regulators.

Functional genomics of abiotic stress tolerance mechanisms in rice

While *Arabidopsis,* the smallest-genome higher plant species is the genetic model for dicot, rice is considered the genetic model for monocot species (Goff et al., 2002; Sasaki et al., 2002; Feng et al., 2002). The rice genome consists of 389 megabase pairs (Mb) of DNA, about three times the size of the *Arabidopsis* genome (120Mb) and it is the smallest genome among the members of the cereal family (Sakaki and Burr, 2000). There are an estimated 40,000 to 60,000 genes encoded in the genome depending on the *ab initio* gene prediction method used for analysis. For

instance, analysis of the map-based rice genome sequence predicted a total of 37,544 genes (International Rice Genome Sequencing Project, 2005). It appeared that 71% of those genes have a putative homolog in the *Arabidopsis* genome, and in reciprocal 90% of the *Arabidopsis* genes have a putative counterpart in rice. Overall, a total of 2,859 genes seem to be unique to rice and other cereals, many of which are presumably the genes that define the fundamental differences among plant lineages, e.g., monocot versus dicot. In addition, a large number (29%) of rice genes appear to occur as members of clustered gene families. While some of the duplicated genes may be functionally redundant, differential expression patterns exhibited by the individual members of gene families also add a layer of complexity to genetic traits in rice.

The availability of the complete rice genome sequence and subsequent *in silico* analysis of the sequence have enabled researchers to begin to look at the complete set of genes expressed in the cells at a given time and condition and to identify the proteins responsible for every cellular process. The current emphasis of molecular genetic research in rice and other plants is on the integration of the genome structure with function by global gene expression analysis and genetic manipulation using available functional genomic tools such as gene microarray, gene knockouts and overexpression lines and naturally occurring allelic variation. For instance, in order to understand the molecular basis of genotypic variation for cold tolerance in rice, it is necessary to distinguish the most critical physiological and biochemical differences among these genotypes. A starting point to address this goal would be to identify genes that encode the major proteins in the pathways through gene expression profile analysis. Therefore, this study took advantage of one of the available functional genomic tools, microarray technology (also known as a 'gene chip' in a slightly different format) to address the project's goals. Microarray technology is a robust technique that allows parallel analysis of the expression of thousands of genes in a single experiment (Brown and Botstein,

1999; Richmond and Somerville, 2000). Gene-specific DNA sequences are spotted or synthesized onto a chemically treated glass matrix by computer-controlled robotic tin pins. Millions of copies of each gene (as DNA molecules) in solution are fixed on a specific spot on the slide. Because each spot is only about 100μm or less in diameter, this allows tens of thousands of unique genes (clones) to be included in a single microarray matrix. By measuring fluorescence intensity that reflects the relative abundance of transcripts, activities of each of the thousands of genes are captured, providing a quantitative measure of changes in gene expression that occur at a given time and condition.

Chapter 3

OBJECTIVES OF THE STUDY

Rice (*Oryza sativa* L.), the most important staple food for more than half of the world's population, is sensitive to chilling particularly during the early seedling development and flowering stages. Like many other tropical and subtropical crops such as maize, sorghum, tomato, and soybean, rice is usually planted in late spring to early summer when field temperature sometimes drops below normal. Therefore, for many years, one of the greatest challenges for crop breeding has been the improvement of cold tolerance of warm-season plants in order to reduce the risks of chilling-induced crop failure and to hopefully expand their cultivation to more temperate zones. However, cold tolerance is a complex trait defined by many quantitative trait loci (QTL) (Redona and MacKill, 1996; Peterson at al., 1978). The functions of these QTL are manifested in a very complex manner and are cumulative in nature. Evidence from works published during the last five years (De los Reyes et al., 2003, Yazaki et al., 2004; Morsy et al., 2005; Antoine et al., 2005) support the hypothesis that the mechanisms of tolerance to chilling involve a genetic regulatory network similar to the CA regulatory network. Thus, it is likely that the mechanisms of cold tolerance exhibited by japonica rice seedlings depend on highly coordinated expression of hundreds of genes.

Earlier studies have shown that seedlings of cold stress intolerant indica rice (cv. INIAP12) exhibit a delayed response in the expression of the *CBF3*-like regulon compared to the tolerant japonica cultivar (CT6748-8-CA-17) (Morsy et al., 2005). This differential expression was observed as early as 2 hours after the initiation of cold stress, when *CBF3*-like expression in CT6748 peaked, and followed by the induction of its target genes 8 to16 hours after initiation of the cold stress. On the other hand, the peak expression of *CBF3*-like and target genes in the intolerant INIAP12 did not occur until 8

hours after imposition of cold, with a consequent delay in the induction of downstream target genes (after 12 to 24 hours) (Morsy et al., 2005). Therefore, it was suggested that the first 24 hours following a cold snap is a critical time window for the expression of genes that provide a robust activation of a complex defense response mechanism.

The overall goal of this study was to use the currently available functional genomic resources to strengthen the basis and fine-tune the current gene regulon hypothesis of chilling tolerance mechanism in warm-season cereals using rice as a model. The foundation of this study was built from the hypothesis and genomic resources established from pervious works published in this lab during the last five years (De los Reyes et al., 2003; Morsy et al., 2005; Antoine et al., 2005). The goal is to establish a more quantitative survey of gene expression changes that occur during the early phase of cold stress in developing rice seedlings. The experimental strategy involved a semi-global study of the temporal expression patterns using available transcriptome analysis platforms, i.e., a stress cDNA microarray and the whole genome sequence of japonica rice. The specific objectives of this study are the following:

1) To define the early cold-induced changes in the transcriptome of *Oryza sativa* L. by interrogating a >6,000 cDNA microarray with transcripts from seedlings exposed to varying durations of cold stress (10°C).
2) To validate the gene expression data obtained from microarrays by RNA gel-blot analysis of representative candidate genes.
3) To identify candidate transcriptional regulators involved in early responses to cold stress.
4) To study the effect of abscisic acid (ABA) on the expression and regulation of candidate early cold-stress responsive genes.

Chapter 4

MATERIALS AND METHODS

Plant materials, growth conditions, and stress treatments

Rice varieties belonging to the japonica ecotype are generally more cold tolerant than most indica varieties. All experiments in this study were performed using the japonica cultivar CT6748-8-CA-17 (PI560247) as a model. This cultivar represents the higher end of the total genetic variation for cold tolerance (chilling) based on previous laboratory and field-based phenotypic screening. The genotypic differences in cold tolerance were determined at a critical temperature of 10°C during early seedling developmental stages, i.e., from S_3 (prophyll) to V_3 (three-leaf) stage (Morsy et al., 2005).

Seed germination. Mature, non-dormant seeds were first surface-sterilized in 30% ethanol for about 5 minutes and then rinsed thoroughly with sterile deionized water. After washing-off excess ethanol, the seeds were soaked in water for about 10 minutes to initiate imbibition and then germinate on 1.5% agar plates at 28-30°C. Successful germination was reached at the point of 80% emergence of the coleoptile and radicle, defined as the prophyll (S_3) stage (Counce et al., 2000). Completion of normal germination in a growth chamber (28-30°C) typically takes between 4-5 days after imbibition. To limit experimental variation, all germinated seedlings were subjected to chilling stress treatments 6 days after imbibition.

Low temperature stress. S_3-stage seedlings on agar plates were subjected to chilling stress in a growth chamber (Percival, Model E-30BHO) maintained at a constant temperature of 10°C. The chamber was also programmed to run a 12-hour daylength

and 50-60% optimal relative humidity throughout the duration of the stress treatment. Tissue samples, i.e., coleoptile and radicle (~3g) were collected from cold-stressed seedlings at 0.5, 2, 6, 12, and 24 hours after the initiation of the stress treatment. Control (unstressed) plants were sampled independently for each time point for use in microarray experiments. A single sample of control tissues representing 0 hour treatment was used for the RNA gel-blot analysis. Tissue samples were frozen at -80°C until RNA isolation.

Abscisic acid (ABA) treatment. Seedlings were germinated essentially as described in the agar germination method but with slight modification. Instead of agar plates, seeds were germinated in sterilized and water-soaked Whattman No.3 filter paper to allow post-germination treatment with ABA. After the completion of the S_3 (prophyll) stage, the seedlings were transferred to fresh petri dishes containing Whattman No.3 filter papers soaked in 100μM solution of (+)-cis, trans-abscisic acid (S-5-(1-Hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-(2Z,4E)-pentadienoic acid (GIBCO-Invitrogen, Carlsbad, CA). The seedlings were maintained at the control temperature (28-30°C) throughout the whole duration of the ABA experiment. Tissue samples (~3g) were collected from the plants after 2 and 24 hr of ABA treatment. Control tissues were collected from seedlings grown in petri dishes containing water-soaked (deionized water) Whattman No.3 filter paper. Excised tissue samples were frozen at -80°C until RNA isolation.

Total RNA isolation

Coleoptile and radicle tissues were excised from the endosperm and immediately frozen in liquid nitrogen and stored at -80°C until used for RNA isolation. Total RNA was isolated by the TRIzol method (Invitrogen) according to the manufacturer's protocol.

Briefly, tissue samples were pulverized with liquid nitrogen in an RNase-free mortar and pestle (baked at 120°C overnight). The powered tissue was homogenized in the TRIzol reagent (1ml/ 100mg of tissue). Aliquots (1ml) of the tissue slurry were transferred in diethylpyrocarbonate (DEPC)-treated microcentrifuge tubes and incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes in the buffer solution. The tissue homogenate in TRIzol buffer was mixed thoroughly with chloroform (0.2 volume chloroform: 1 volume TRIzol) and then centrifuged for 15 minutes at 13,000 rpm to separate the organic and aqueous phases. The RNA-containing aqueous phase was transferred into fresh DEPC-treated microcentrifuge tubes and then precipitated for 10 minutes by the addition of isopropyl alcohol (0.5 volumes isopropyl alcohol: 1 volume TRIzol homogenate). The precipitated RNA formed a white gel-like pellet, which was then washed with 75% ethanol to remove residual contaminants. The RNA pellet was dissolved in DEPC-treated water (15 µl/ mg tissue).

RNA concentration was determined by measuring absorbance at 260nm (A260) using the SmartSpec Plus spectrophotometer (Bio-Rad Laboratories). An RNA-specific conversion factor of 40µg/ml was used to estimate the concentration of total RNA, which was calculated by multiplying the A260 reading of the sample with the conversion and dilution factors. Quantity and quality of total RNA was also assessed by running a small amount of sample in denaturing formaldehyde-agarose gel. The relative concentration of total RNA was normalized across samples through the signal intensity of the ethidium bromide (EtBr)-stained ribosomal RNA bands under UV light. Two separate RNA isolations were performed from independent stress and control experiments for use as biological replicates in the microarray studies.

Microarray experiments

Spotted cDNA microarrays consisting of >6,000 non-redundant cDNA clones from cold (De los Reyes et al., 2003) and drought stress (J. Bennett and R. Bruskiewich, International Rice Research Institute, unpublished; Kawazaki et al., 2001) EST libraries were assembled by the Michigan State University Genomic Technology Support Facility (www.gtsf.msu.edu). Annotations of all EST accessions are available at dBEST (www.ncbi.nih.nlm.gov). The rationale for the construction of these cDNA microarrays was to develop an expression profiling platform that is enriched with candidate abiotic stress-related cDNAs and therefore, genes that are likely to change in expression in response to cold stress.

Inserts from the >6,000 EST clones were excised from the pBluescript-SK plasmids (Stratagene, La Jolla, CA) by PCR with T3 and T7 primers and then extracted and purified from 1% TBE-agarose gels. Samples of the purified cDNA inserts were spotted robotically on amine-coated glass microarray slides (Telechem International). The >6,000 cDNA clones were printed onto the amine-coated glass slides in duplicate panels to assemble a >12,000-spot microarray matrix by the Michigan State University Genomic Technology Support Facility (www.gtsf.msu.edu). The duplicate spots served as internal replicates for use in assessing the quality and technical reproducibility of microarray hybridization. The gene-array list (GAL-file) was annotated according to the most recent information available from dBEST. The GAL-file (EST spreadsheet) was constructed in a format that is compatible with the GenePix Pro 4200 microarray data collection and analysis software (Axon Instruments, Sunnyvale, CA)

Preparation of target cDNA and hybridization with microarray probes. Equal amounts of total RNA (40-50 µg) were reverse transcribed and labeled with Cy3 and Cy5 fluorescent dyes by the post-cDNA synthesis method using the CyScribe cDNA Post

labeling kit (Amersham-GE Healthcare, Piscataway, NJ). Synthesis and labeling were performed in a two-step procedure according to the manufacturer's protocol. First, the cDNA synthesis reaction was primed by anchored oligo-dT and catalyzed by CyScript reverse transcriptase (a modified MMLV reverse transcriptase, 100 units). Aminoallyl-dUTPs were incorporated into first-strand cDNA with an optimized nucleotide mix supplied in the labeling kit. In the second step, control and cold stress cDNA samples with aminoallyl modifications were chemically labeled with Cy5 and Cy3 fluorescent dyes, respectively. After the post-synthesis fluorescent labeling reaction was completed, the control and cold stress (treatment) cDNA samples from the same time point were combined in equal quantities in a single solution and then dried by speed-vacuum centrifugation.

The dried cDNA sample was dissolved in 1X microarray hybridization buffer (Amersham-GE Healthcare) with formamide (50%). Microarray hybridization was performed by applying the mixture of labeled control and treatment target cDNAs onto the area of the microarray slide corresponding to the matrix of spotted probe cDNAs. The spotted area of the slide was covered with a thin glass cover slip (Corning Life Sciences, Acton, MA) to prevent evaporation of the hybridization solution during prolonged exposure to warm temperatures. After assembling the hybridization set-up, the microarray slides were mounted in a tightly sealed portable hybridization cassette (TeleChem International). A humidified environment was created inside the hybridization cassette by adding a small drop of water (~15 µL) inside the tightly sealed mini-chamber. Humid condition is necessary to prevent irreversible binding of the labeled target cDNA on the surface of the glass slide when the buffer evaporates and also to allow competitive hybridization of control and stress target cDNA molecules with the probes immobilized on the surface of the glass slides. The hybridization chambers were wrapped with aluminum foil to prevent exposure of the fluorescently labeled target cDNA

to light. The hybridization reaction was carried out in a 42°C constant temperature water bath for 16-18 hours.

To comply with the basic requirements for microarray expression profiling experiments as outlined in the regulations of MIAME (Minimum Information about a Microarray Experiment; Brazma et al., 2001), two parallel hybridization experiments using RNA targets derived from two independent control and stress treatments were performed as biological replicates. Following hybridization, the cover slips were gently peeled-off and the microarray slides were washed sequentially in three stringency buffer solutions (TeleChem International): Buffer 1 - 2X SSC (3M sodium chloride, 0.3M sodium citrate) plus 1% sarcosyl; Buffer 2 - 2X SSC; and Buffer 3 - 0.2X SSC. All three wash steps were carried out at room temperature for 5 minutes with moderate agitation. At the end of the wash procedure, the microarray slides were centrifuged for 5 minutes at low speed (500 *g*) to remove residual droplets of the wash buffer on the surface of the fluorescent cDNA spots.

Microarray image scanning and data analysis. Gene expression data was acquired by measuring the fluorescence intensity of each spot at both 532nm (Cy3, green) and 635nm (Cy5, red) with the GenePix® Pro 4200 Microarray scanner (Axon Instruments). The individual spot corresponding to a unique gene or feature in the GAL-file was located through the spot-finding function of the scanner software. The GAL-file contains specific locations and identifiers for each feature in the microarray matrix (block, column and row), EST accession numbers and/or contigs for each feature and annotation based on the information obtained from the GenBank nr, PDB and dBEST databases. Each microarray feature was functionally identified using available annotation in public databases. The signal intensity of each spot was represented as medians of all the pixels based on background subtraction [signal minus background; F635 (or F532)

Median - B635 (or B532)] as described in the GenePix® Pro User's Guide and Tutorial (http://www.moleculardevices.com/).

A complex quality control query was used to identify and select high quality spots (features) from the entire microarray matrix. This query was formulated to exclude features that belong to any of the following filter criteria: 1) The feature intensity is near background level; 2) The feature is irregular or not uniform (spotting irregularity); and 3) The background around the feature is not uniform (hybridization irregularity). Only the features that passed these stringent filter criteria were included in subsequent bioinformatics analysis of the gene expression data. Gene expression data in each image was normalized globally (i.e., ratio of medians of all the features is equal to 1) prior to high level bioinformatics analysis.

The gene expression values were expressed as base-two logarithm of the ratio of medians [$\log_2$ (F635median-B635)/ (F532median-B532)]. This type of log transformation allows a more robust comparison of the levels of over- and under-activities through a linear-type relationship. For instance, a log base 2 ratio of 1 (i.e., a ratio value of 2) would represent a 2-fold increase in gene expression (GenePix® Pro User's Guide and Tutorial; http://www.moleculardevices.com/). Thus, change in gene activity was expressed as a fold-change difference between control and stress (chilling) values. The gene expression values from two technical replicates (duplicate panels of spotted cDNAs in the microarray matrix) were averaged. In cases where only one of the duplicate spots passed the quality control threshold, the one spot that exhibited the high quality value was used as valid data. Genes with at least one good quality data (feature) from the technical duplicates in both biological replicates were included for subsequent analysis. Gene expression values from two biological replicates were averaged.

Only the microarray features with data available from all five time points were included in cluster analysis. Hierarchical clustering of gene expression data was

performed using the Acuity Bioinformatics Suite (Axon Instruments; Eisen et al., 1998). The decision on which clustering models or assumptions to consider was based both on the size and composition of the resulting groups with critical consideration of the potential biological significance of the resultant groupings within the context of stress response biochemistry and physiology. For example, an initial attempt to cluster all the genes that passed the quality control criteria without any assumption was tried. The resulting clusters were too large and consisted of genes of very random functional categories. By adjusting the hierarchical clustering parameters, two selected subsets of genes with significant changes in transcript abundance ($\geq$ 2-fold induction or repression) at one or more time points during the 24 hours were used for hierarchical clustering. This approach resulted in identification of two biologically meaningful groupings of cold-responsive genes. Group-I includes genes that exhibit $\geq$1.8-fold induction in expression within the first 2 hours; and Group-II includes genes that exhibit $\geq$ 2-fold induction in expression at one or more time points between 6 and 24 hours after stress. Note that 1.8-fold increase might not have been considered significant in the conventional sense. However, given the small number of genes that clustered in this group (total of 15 genes) at the default 2-fold threshold, a slightly lower threshold appeared more logical to increase the chance of capturing a few more genes (especially those with regulatory functions) that often exhibit relatively lower, but a statistically and biologically acceptable, change in expression level.

Validation of the microarray expression data

The gene expression data obtained from the microarray experiments were validated by the analysis of expression of representative clones from each cluster or group. Genes known to be regulated by low temperature and related stresses (drought and salinity) based on published information were used as positive controls. Candidate

genes for validation were selected from each expression cluster with consideration of the likelihood that such genes are indeed regulated by low temperature based on putative functions and related information from the literature. Preference was given to genes with homology or similarity to previously characterized or candidate stress-regulated genes based on information available from gene annotation and published research.

Cloning of cDNA probes by RT-PCR. The ESTs of the candidate genes for validation were obtained from the dBEST (www.ncbi.nih.nlm.gov). In cases where there were multiple hits to a single array feature, the matching sequences were aligned to identify the highly conserved region. A set of three EST-specific nested primers were designed for each candidate gene to obtain a cDNA fragment for use as a probe in gel-blot analysis (Table 1). The first EST-specific primer (R2) was designed in the reverse or antisense orientation to prime the synthesis of the first strand cDNA from the RNA template. The second (R1) and third (F1) primers constituted a pair of reverse and forward primers, respectively. These primers were used to synthesize and amplify double stranded cDNA from the first strand cDNA template. Sequence alignment, primer design and analysis were performed using the BioEdit software (Hall, Ibis Therapeutics) and OligoAnalyzer 3.0 (Integrated DNA Technologies; www.idtdna.com).

The cDNA fragment for each candidate gene was amplified from total RNA (0.5 µg) obtained from cold-stressed rice seedlings. The cDNAs were synthesized with an optimal amount (recommended by the manufacturer) of ImProm-II™ reverse transcriptase (Promega, Madison, WI) with the EST-specific R2 cDNA synthesis primer (0.1 µg), $MgCl_2$ (3 mM), nucleotide mix (0.5 mM), and 1X ImProm-II RT-reaction buffer. The reverse transcription reaction was performed at 42°C in a thermal cycler for 60 minutes. The resulting first strand cDNA was amplified by *Taq* DNA polymerase (0.625 units) with F1 and R1 primers (0.1 µg each). All amplification reactions were performed

using the BioRad iCycler dual block thermal cycler with the following cycling parameters: a) 94°C for 3 minutes to denature the mRNA/cDNA hybrid; b) step-denaturation at 94°C for 30 seconds; c) primer annealing for 30 seconds at primer-specific temperature based on the melting temperature (T_m) of individual EST-specific primers (1-5°C below the T_m); d) extension reaction at 72°C for 45 seconds. The 3-step amplification cycle (b to d) was repeated 35 times. A 5 minute final extension was performed at the end of the 35-cycle PCR to seal the ends and gaps of the synthesized products. The PCR products were analyzed in a 1% TBE-agarose gel using standard procedures (Sambrook et al., 2001)

The amplified cDNA fragments were ligated with the TA-cloning vector pCR2.1® (Invitrogen) according to the manufacturer's protocol. This cloning system takes advantage of the fact that *Taq* DNA polymerase has a non-template-dependent activity that adds a terminal deoxyadenosine (A) to the 3' ends of newly synthesized DNA fragment. This feature allows AT-mediated ligation of the PCR product with the linearized pCR 2.1 vector, which contains a single 3' deoxythymidine (T) overhang. The ligation reaction was carried out by T4 ligase (4 Weiss units) overnight at 14°C.

The recombinant pCR2.1 containing the cDNA insert was transformed into chemically competent TOP10 *E. coli* One Shot® cells (Invitrogen) by the heat shock method (42°C) according to the manufacturer's protocol. Selection and screening of recombinant *E. coli* was performed in Luria-Bertani (LB) medium-agar plates containing ampicillin (Amp, 100 mg/ml) and 5'-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 40 mg/ml) for blue-white colony differentiation. Plates of recombinant *E. coli* cells were incubated overnight at 37°C and transferred to 4°C to allow efficient blue-white color development of the transformed cells.

Representative Amp-resistant, white colonies were picked from the plate with sterile toothpicks and grown at 37°C in overnight (16-18 hr) suspension culture in 5 ml LB-Amp liquid medium with continuous shaking. Recombinant plasmids were extracted

and purified from cell suspension by the standard alkaline lysate method using the Qiagen plasmid miniprep kit (Qiagen, Valencia, CA). The purified plasmid DNA was evaluated for the presence of the cDNA insert by *Eco*RI digestion and agarose gel electrophoresis. One representative clone with the correct insert size was further analyzed by sequencing to verify the correct identity of the cloned PCR product.

Table 1. List of primers used for cloning cDNA probes.

Primer Name	Sequence (5' to 3')	Target Gene/cDNA (Annotation)
EREBP1-R2	CTAACGGGGCATGTCATCAAAGCT	AP2-domain, EREBP-type transcription factor
EREBP1-R1	GGTTCTAAATCAGGCAGATCAACA	
EREBP1-F1	CGCAAGGGTGTTCGTGTCTGGCTT	
LRR-R2	AGCCTCACCTTCTCCAAACTCAGC	putative ATP sulfurylase
LRR-R1	AACTCAAGCGCTTATTGATATGGG	
LRR-F1	GTAATGATGTTTTGGTGTAATCCA	
AU069042-R2	CGTCTCCCTCGACCTCTACAACCA	ESTs Acc. No. AU069042; unknown function
AU069042-R1	GTCACTGCTTCGCATGCATTTGTC	
AU069042-F1	GGATGCGGCAGCCTTAAAGCTCTC	
PK-like-R2	TTGTGAAGGTTCGACATCTGGATG	Similar to *Arabidopsis* putative protein kinase
PK-like-R1	TATTCACTTCAGTCTTGGTTGCCC	
PK-like-F1	CCCTGGCTCGCTCATCTGACAAAC	
putative TF-R2	GCTGACCTTGCTGCGTCGTCTGCG	putative bZIP-type transcription factor
putative TF-R1	ACCGTCAGTTATCTCGCCAGATGC	
putative TF-F1	GTCTGCGGCAAGGTCAAAGGAGCG	
Vacuo-sort-R2	CCCAGATCCTGTTGATTCCCGCCG	Vacuolar sorting protein
Vacuo-sort-R1	CCAACTTCCATCTTGATGCTATTG	
Vacuo-sort-F1	AGGTAAAAGGGTGGAGCACCAAGG	
NOD26-R2	GATCGATGAACCCGGCGAGGACGC	MIP family of channel proteins; Similar to a
NOD26-R1	GTCTAATCGCCCTGACGCCATGGA	putative NOD26-like membrane integral protein
NOD26-F1	TCGCCAGCTGATTATACCAGCGAG	

Table 1. (cont'd).

Primer Name	Sequence (5' to 3')	Target Gene/cDNA (Annotation)
CA759231-R2	AAGGGTGTTGCTCCTCTTGCTGTG	Transmembrane protein similar to a putative anion transporter
CA759231-R1	GAGCAGGCTAGAAGAGCTCAGTT	
CA759231-F1	AGCCTGCTACATCTGCATCCTTCT	
CA759247-R2	AGCCTGCTTCTCAGCTCAATGGTG	Protein belonging to the phosholipase family
CA759247-R1	AATCTGCTTTGGTCACCATGAGGC	
CA759247-F1	CTCCAGCTTATCCAGAGTAGGATC	
CB096630-R2	CCCCTGCCAAGCCCAGACTATGTC	Putative Serine/Threonine protein kinase
CB096630-R1	AAGATGAAAGTTTGGGGTAACCTC	
CB096630-F1	GGGTCTCGATGCAAAGCTGCTCAG	
CA767317-R2	CATTCTTTGTTCATGCTGCCTGGG	Putative leucine-rich repeat (LRR) protein
CA767317-R1	CTTGCAGCTCTGGGCCGTTGAGCC	
CA767317-F1	CCTCCAATACCTGGAGCTCTACAG	
ferritin-R2	AGCTTCTTCCTCAAGCAGCTTCTG	Ferritin 1
ferritin-R1	TCACTCTCAACGAAGTCGGTCAGC	
ferritin-F1	GACCGTGACAACGTTGCTCTCAAG	
GDPdiss-R2	GGACTGAGTGAGAGTCATTTGTGC	Rab GDP dissociation inhibitor-alpha
GDPdiss-R1	TCAAATTCAACCTTGCAGTCCGGC	
GDPdiss-F1	GTGACGATACCGTGGATTTCATCG	

Table 1. (cont'd).

Primer Name	Sequence (5' to 3')	Target Gene/cDNA (Annotation)
enolase-R2	TGCAGTCTTCAAGAGCTCAAGACC	Putative enolase
enolase-R1	AATGAAGCGGCCCCAGTAGGAAGG	
enolase-F1	GTGAACTCGGTTATTGCCCCAGCT	
CA760356-R2	TGCCCCTCCAAGCTATGCAATTCC	Ligand-gated ion channel family protein
CA760356-R1	TGGAGGTCGCGAAGAACGTAACTC	
CA760356-F1	GGCCTTCCTGCATTAATTTCTACA	
Germin-R2	ACGTAATCAATTTCCCGGGCCTCA	Germin-like protein subfamily 1
Germin-R1	AAGGGTGATGTGTTCGTGTTCCCT	
Germin-F1	TAACGCACAAGGGAACAATCAAAA	
salT-R2	ATCGCCAACTTTATTGTACTACGG	Salt-stress-related salT protein
salT-R1	GGAATCAAGGGTGGACGTAGATGC	
salT-F1	GGAGATTTCTGGAACCCATGGCCC	

Sequencing and analysis of cDNA. The cloned partial cDNAs corresponding to the candidate cold-stress-regulated genes were sequenced from the 5' end using M13 forward primer flanking the cloning sites of the pCR2.1 plasmid. Sequencing was performed by the University of Maine DNA Sequencing Facility through the ABI 3730 Capillary DNA Analyzer (Applied Biosystems). Vector sequence was trimmed manually by identifying the landmark sequences (*Eco*RI sites) flanking the insert sequence. The sequenced cDNAs were identified by alignment with the databases of The Institute of Genomic Research (TIGR) Rice Genome Annotation Project (http://www.tigr.org/tdb/e2k1/osa1/index.shtml) through WU-BLAST 2.0 (Washington University in St. Louis; http://blast.wustl.edu/), a software based on blastX (Altschul et al., 1990).

RNA gel-blot analysis. Equal amounts of total RNA samples (5-10 µg) from control (28-30°C), cold stressed (10°C) or ABA-treated (100 µM) rice seedlings were fractionated by electrophoresis in a standard denaturing agarose gel containing formaldehyde buffer (Ambion, Austin, TX). After electrophoresis, the agarose gel was rinsed with DEPC-treated water to remove traces of formaldehyde, which can reduce the efficiency of RNA transfer. The RNA gel was blotted onto Hybond N^+ nylon membrane (Amersham-GE Healthcare) with 20X SSC buffer for 3 hours using the turbo blotter downward blotting device (Schleicher and Schuell Bioscience). The RNA filter was rinsed twice for 10 min in fresh 20X SSC buffer and then was covalently cross-linked by brief (15 seconds) exposure to UV light.

The cDNA probes were excised from the pCR2.1 backbone either by *Eco*RI single digestion or *Eco*RI/*Xho*I double digestion at 37°C. The excised cDNA inserts were separated by electrophoresis in 1X TBE-agarose gel. The cDNA fragment was extracted and purified from the agarose gel with QIAquick Gel Extraction Kit according to the manufacturer's (Qiagen) protocol. The gel-purified cDNA (~50 ng) was labeled with ^{32}P-dCTP (40 µCi) through an overnight random priming reaction with Klenow DNA polymerase-

l at room temperature using the Rediprime kit labeling cocktail (Amersham-GE Healthcare). Unincorporated ^{32}P-dCTP was removed from the probe by the QIAquick Nucleotide Removal kit (Qiagen) according to the manufacturer's instructions. Purified labeled probes were denatured by boiling in a water bath for 10 minute before adding to the prehybridized RNA filters.

RNA filters were prehybridized in the Ultrahyb buffer (Ambion). Prehybridization was performed overnight in a rotisserie hybridization incubator maintained at 42°C before the addition of radiolabeled probe. Hybridization was carried out at 42°C for 24 hours. Following hybridization, the filters were washed twice in low stringency buffer of 2X SSC + 0.1% SDS (sodium dodecyl sulfate) at ambient temperature, and then twice in high stringency buffer of 0.2X SSC + 0.1% SDS at 42°C. The hybridized RNA filters were autoradiographed at -20°C for 12 to 96 hours depending on the radioactive signal intensity of the blot. EtBr-stained rRNA bands were used to monitor equal loading across lanes in the formaldehyde-agarose gels. Filters were stripped for rehybridization by boiling in a 1% SDS solution.

Chapter 5

RESULTS

Generation of microarray data set

The cDNA microarray used in this study was composed of a total of 5,855 unigenes assembled from cold and drought stress EST libraries and printed in duplicated panels (De los Reyes et al., 2003; J. Bennett and R. Bruskiewich, International Rice Research Institute, unpublished; Kawasaki et al., 2001). This microarray matrix was interrogated with two independent RNA sample pairs collected from duplicated experiments (biological replicates) on S_3-stage rice seedlings at 0.5, 2, 6, 12, and 24 hours after the imposition of low temperature stress (10°C). Thus, a total of 11,710 data points representing the technical replication were displayed in each channel per biological replicate. The fluorescence intensity values of the individual microarray features were measured for the control (Cy5, red) and stress (Cy3, green) samples in each of the duplicated features for both biological replicates, gathering a set of four data points for each time window. Sample scans of the duplicate panels in each biological replicate are shown in Fig. 1.

A flagging query was used to select for the '*good quality*' features in each panel within a microarray matrix. A feature was flagged as a '*bad quality spot*' if such feature had either a low intensity signal in either channel (low hybridization efficiency or near background intensity level), irregular in shape or with high or irregular background levels. These types of features were excluded in higher level data analysis. The stringent filtering criteria used in the data quality control yielded between 47% to 75% high-quality features per microarray matrix (Table 2). A lower filter passing rate was observed in one of the biological replicates for the 2-hour hybridization (21% and 28% for the two panels respectively) indicating the relatively lower quality of the data derived from this part of

the experiment. In all cases, the data from the two duplicated panels were highly consistent with each other indicating a high level of technical reproducibility of the microarray data (Table 2, Fig. 1). The fluorescent intensity values (expressed as base-two logarithm of the ratio of background subtracted medians) from the technical replicates were averaged if the feature from both panels passed the filtering criteria. When only one of the two features from the duplicate panels passed the filtering criteria, such feature was used as the valid data and was eventually used for higher level analysis. The average values from the two biological replicates were used to evaluate the differences in transcript levels for the individual genes in the set.

A two-fold change in gene expression is commonly used as a threshold level of significance in standard cDNA microarray-based gene expression profiling experiments. However, because this study is focused on identifying candidate genes during the first 24 hours of stress when overall gene expression may not be very high, a preliminary analysis of the different proportions of differentially expressed genes at three threshold levels seemed logical during the early phase of the study. This analysis showed a general trend of a decreasing number of differentially expressed genes (both upregulated and downregulated) at threshold levels of 2-fold, 2.5-fold and 3-fold (Fig. 2). However, these data indicated that a good number of candidate genes can reasonably be captured at a slightly permissive threshold level of 2-fold. For instance, at 24-hour, as many as 95 genes can be identified as differentially expressed at a 2-fold threshold, but the number of qualified genes is drastically reduced to only 5 when the cut-off is increased to 3-fold. Part of the assumption in deciding to initially use a slightly permissive 2-fold cut-off was that microarray data will be confirmed by 'gene-by-gene' expression analysis by RNA gel-blot. Based on this analysis, it was concluded that the cut-off level of around 2-fold was inclusive enough to allow efficient identification of biologically meaningful candidate early cold stress responsive genes (Fig. 3).

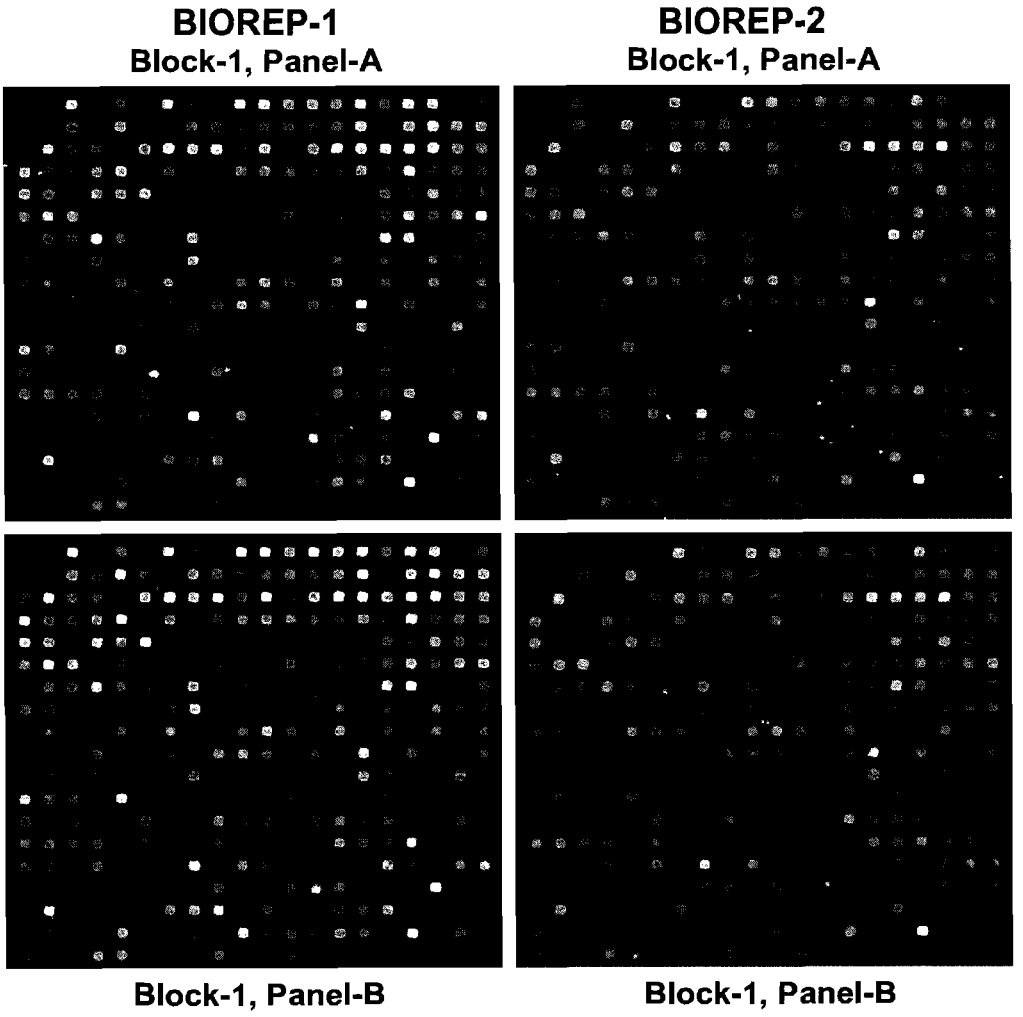

Figure 1. Sample scan of the cDNA microarray assembled from the cold stress and drought stress EST libraries of rice. Microarray matrix is an amine-coated glass slide. Equal amount of control (28°C) and cold stress (10°C) cDNA samples with aminoallyl modifications were labeled with Cy5 (red) and Cy3 (green) fluorescent dyes, respectively. Shown here is a section of a scan image of differential expression at 6 hr after low temperature stress at 10°C. Red signal = downregulated by cold stress; Green signal = upregulated by cold stress; Yellow signal = expression not affected by cold stress. Biological replicates (BIOREP) and technical replicate (Panel) are shown.

Table 2. Summary of microarray data quality control showing the proportion of the total 5,855 microarray features that passed printing and hybridization quality standards for high level data analysis.

Replicate*	0.5 hr Total	(Percent)	2 hr Total	(Percent)	6 hr Total	(Percent)	12 hr Total	(Percent)	24 hr Total	(Percent)
Rep1-Panel1	4,267	(73)	3,766	(65)	3,739	(64)	2,805	(48)	3,009	(51)
Rep1-Panel2	4,080	(70)	3,674	(63)	4,363	(75)	2,758	(47)	3,204	(55)
Rep2-Panel1	3,077	(53)	1,643	(28)	3,650	(62)	3,682	(63)	4,089	(70)
Rep2-Panel2	3,260	(56)	1,230	(21)	3,472	(59)	2,989	(51)	3,581	(61)
Total features included in analysis	**3,595**	**(61)**	**1,890**	**(32)**	**4,024**	**(69)**	**2,985**	**(51)**	**3,382**	**(58)**

*Rep = Independent biological replication of control and cold stress experiments; Panel = Duplicate prints of all microarray features printed on the glass slide.

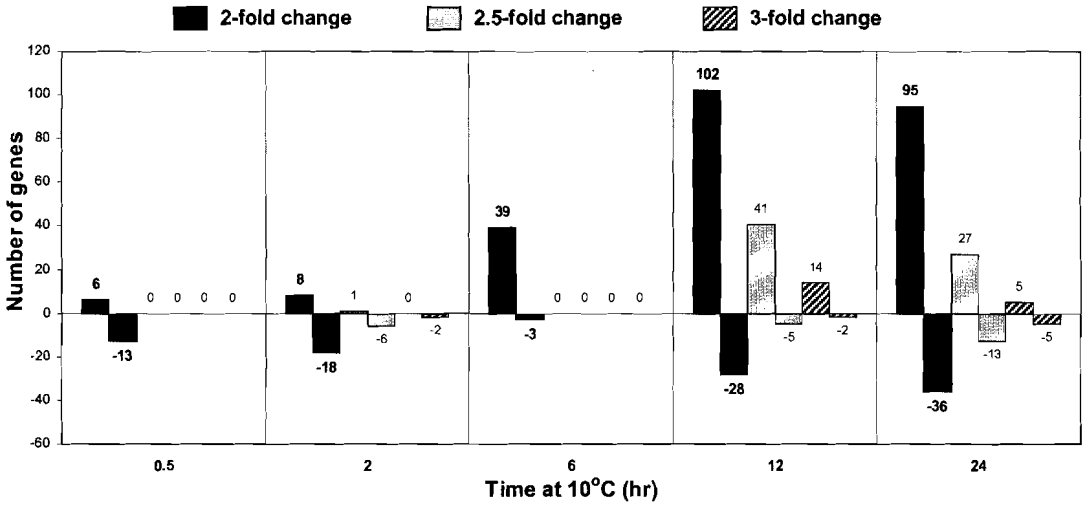

Figure 2. Graph showing the proportion of differentially expressed genes at different fold-change cut-off levels (2-fold, 2.5-fold and 3-fold). Positive and negative values indicate the proportion of genes that are upregulated and downregulated by cold stress (10°C), respectively.

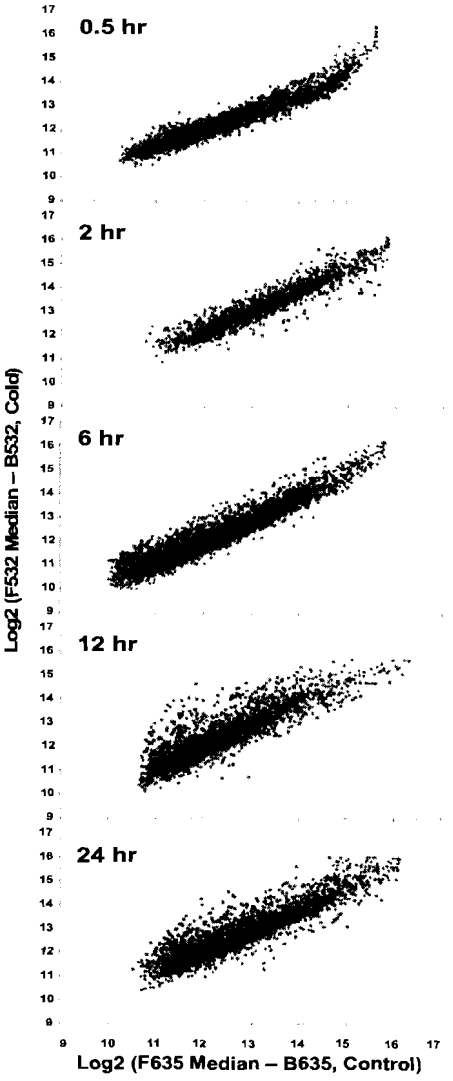

Figure 3. Scatter plots showing the distribution of significant changes in gene expression across time point. The gene expression data points were plotted using base-two logarithm of the background-corrected medians of each channel. In this scatter plot, significant changes in gene expression were determined using a cut-off level of two-fold increase or decrease in transcript abundance. Green dots = Upregulated genes; Red dots = Downregulated genes; Blue dots = Genes that did not show significant change in transcript abundance.

Early cold stress response transcriptome of rice seedlings

The filtered microarray data from the various time points of low temperature treatments were combined into a composite transcriptome profile data series in order to assemble a temporal analysis of gene expression during the early phase of cold stress. Data across all five time points were combined for pattern searches by hierarchical clustering. The basic requirement established for this analysis is that no time window in the temporal series should have a missing data points. Thus, data sets with one or more missing data point along the series were further removed to create a database of complete gene expression profiles during the first 24 hours of cold stress. After combining the data from all five time points, the number of data points that are inclusive of the whole series was further reduced to 1,550, representing about 26% of the total features in the microarray (Table 2). Separate hierarchical clustering was performed for the upregulated and downregulated components of the transcriptome.

Initial attempts to perform hierarchical clustering on all 1,550 genes without any regard to whether these genes are within the threshold level of differential expression as established in Fig. 2, resulted in very large clusters with no apparent biologically meaningful trends (data not shown; Cheng et al., 2005; http://abstracts.aspb.org/pb2005/public/P79/8279). To further refine the query for biologically meaningful trends in gene expression, the subset of genes that displayed significant increases or decreases in expression levels at one or more time points during the 24 hours cold stress based on a 2-fold cut-off was selected and used for hierarchical clustering (Fig. 4 and 8, respectively). The clusters that resulted from the analysis of the genes with significant increase in expression are shown in Fig. 4. The groupings of these 88 upregulated genes based on broad functional categories are summarized in Fig. 7 and their expression levels further illustrated in Table 3. The majority of upregulated genes are associated with cellular metabolism (28.5%), protein synthesis, modification

and degradation (18.6%), and signal transduction and cellular communication (13.7%). A closer look at the hierarchical clusters in Fig. 4 revealed that this group of 88 genes can be subdivided into three major groups (A, B and C) according to temporal expression pattern. Groups A and B combined include the majority of genes that are induced within 6 to 12 hours after cold stress. Many are short acting (group B), while others remained until 24 hours and maybe beyond (group A). Analysis of the possible correlation between the clusters based on temporal expression patterns and broad functional categories did not reveal any meaningful trends that can be associated with critical biological or biochemical pathways as all functional categories are more or less randomly distributed across temporal groups A, B and C.

Since the number of upregulated genes is not that large (88 in total) and the number of genes included in groups A, B and C are even smaller to be used for a meaningful prediction of critically altered biological process, it was thought that a simplified clustering scheme would be more applicable for this type of data. Manual inspection of the hierarchical clustering in Fig. 4 suggests that this group of 88 genes can roughly be divided into two major groups that are either rapidly induced (labeled in red) or less rapidly induced (labeled in black). By manual inspection, at least the less rapidly induced group appeared to have a much larger number of genes than the earlier temporal clustering of A, B and C. To establish this scheme, the clustering query was further refined to allow grouping of the 88 upregulated genes into two major clusters (Figs. 5 and 6). This clustering scheme revealed a simpler and more meaningful grouping of the 88 upregulated genes. The first of the two major clusters (Group-I) includes the genes that started getting induced by cold stress within the first 2 hours, hence they were referred to as the '*rapidly induced early response*' genes. Initially, the number of genes that belonged to this group was small (15 genes). It was hypothesized that perhaps the great majority of genes that are rapidly induced by cold stress would be

those with regulatory functions and thus, although they are induced, their expression levels may not be that high to be picked-up by a 2-fold threshold. To address this issue, the clustering query for this group was adjusted to include a slightly lower threshold of 1.8-fold. This was done initially as a trial and error but the data turned out to be useful in biological interpretation (more detail in the discussion). As a result of the slightly lower cut-off level, five more genes (in addition to the 15 genes identified initially at the 2-fold cut-off) that would have otherwise been overlooked in a 2-fold threshold, were identified. These genes are involved in cellular transport and trafficking, and metabolism, i.e., vacuolar sorting protein and S-adenosylmethionine synthetase represented by probes: S345A_E06 and S234D1_E07, respectively (Table 3). In addition, this lower cut-off level appeared to capture a potentially very important regulatory gene, for instance, at the 2-fold-cut-off-based grouping, a potential basic region leucine zipper-type transcription factor (*OsbZIP*) that has not been reported previously to be cold stress-related, would not have been included in the list of candidate genes under group-I. However, according to the results from gel-blot analysis, its expression reached peak level within 2 hours of cold stress (Fig.12). Another example of a gene that is not so highly expressed, but is certainly induced rapidly by cold stress, is the gene encoding GDP-dissociation inhibitor. This gene (represented by probes S345V_A11; Table 3) showed only a 1.82-fold increase at 2 hr. It appeared to exhibit two waves of induction first after 2 hours and then second after 24 hours (Fig. 12). The 1.8-fold cut-off reflected this temporal expression well, and the first induction would have been overlooked by a 2-fold cut-off. A total of 20 genes were included in Group-I at a 1.8-fold cut-off.

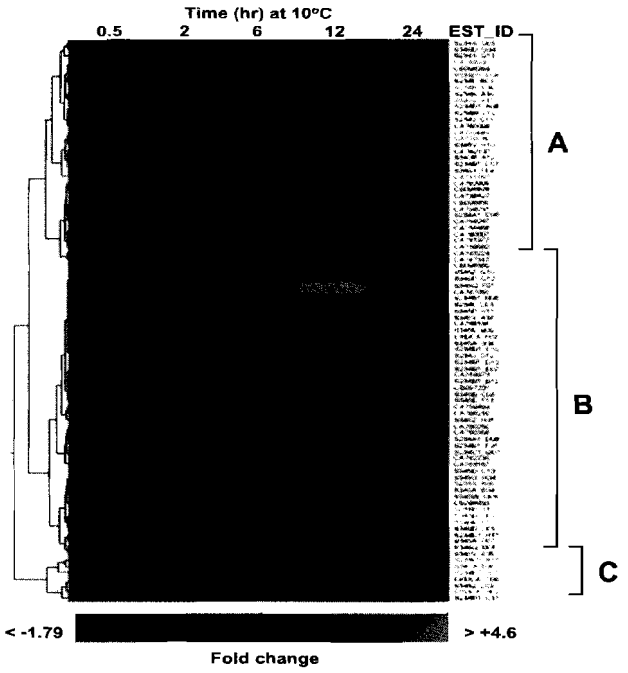

Figure 4. Grouping of rice genes that exhibited significant increase in expression level. Genes (88 in total) that exhibited at least a 2-fold increase in expression at one or more time points during the first 24 hours of cold stress (10°C) were grouped by hierarchical clustering. Positive fold-change values for genes indicate upregulation and negative values represent downregulation. Base on these hierarchical clusters, the upregulated components of the early cold stress response transcriptome can be divided into three major groups: A, B and C. Genes that were upregulated within the first 2 hours of cold stress are labeled in red text (EST_ID).

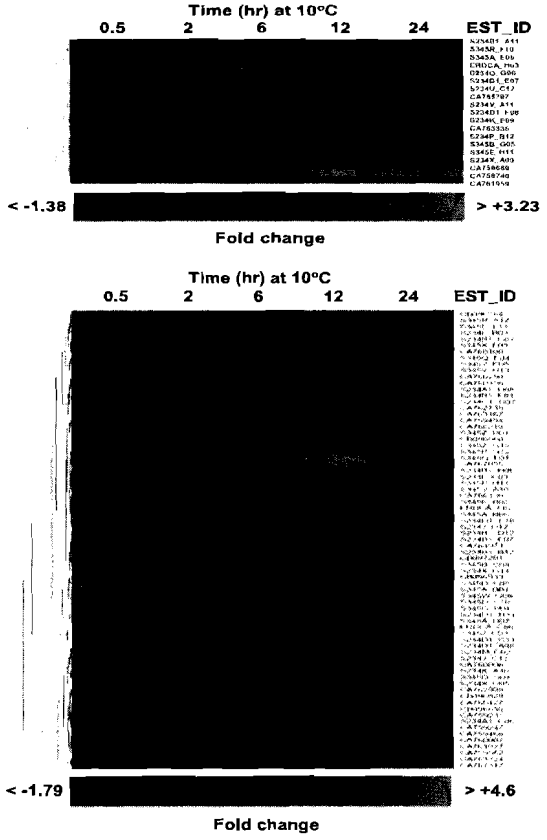

Figure 5. Temporal grouping of cold stress-upregulated rice genes. Group-I genes correspond to the microarray features labeled in red text in the hierarchical clustering presented in Fig. 4. This group consists of the '***rapidly induced early response***' genes. These genes were identified with a cut-off level of at least a 1.8-fold increase in expression within the first 2 hours of cold stress. Group-II consists of the '***delayed induced early response***' genes. These genes were identified with a cut-off level of at least a 2-fold increase in expression 2 hours after the initiation of cold stress.

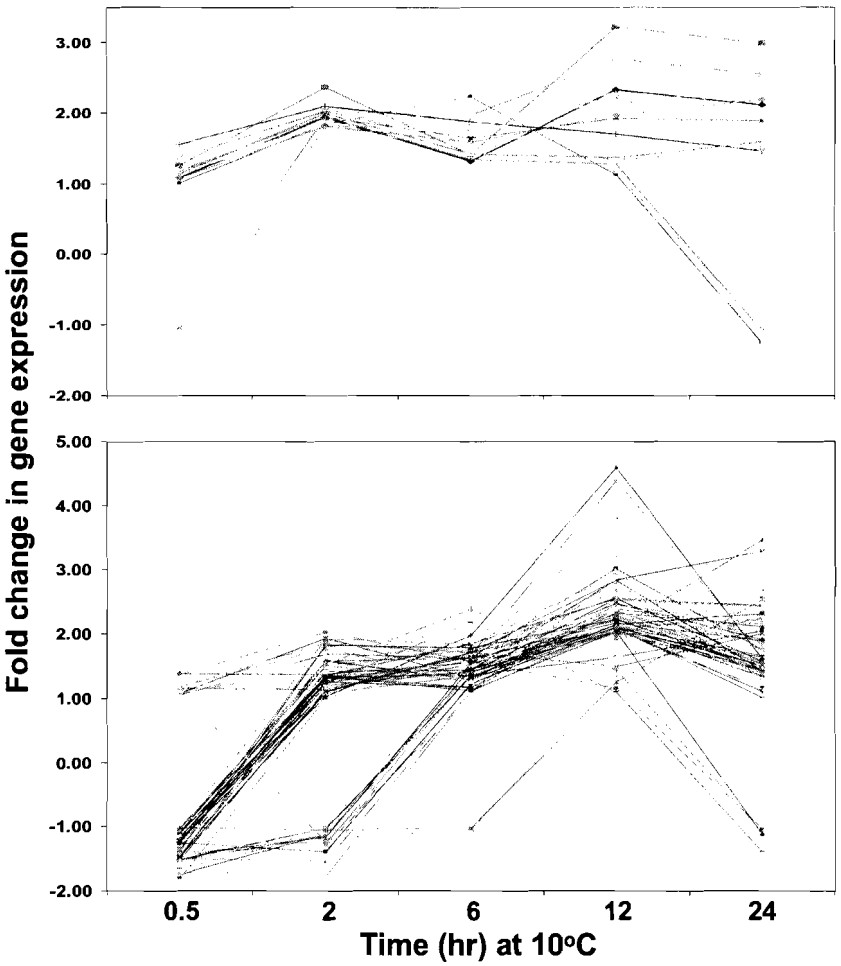

Figure 6. Time series expression profiles of the 20 genes that belong to Group-I or '***rapidly induced early response***' genes (upper panel, also shown in Fig.5) and 73 genes that belong to Group-II or '***delayed induced early response***' genes (Lower panel, also shown in Fig. 5). Positive fold-change values for genes indicate upregulation and negative values represent downregulation.

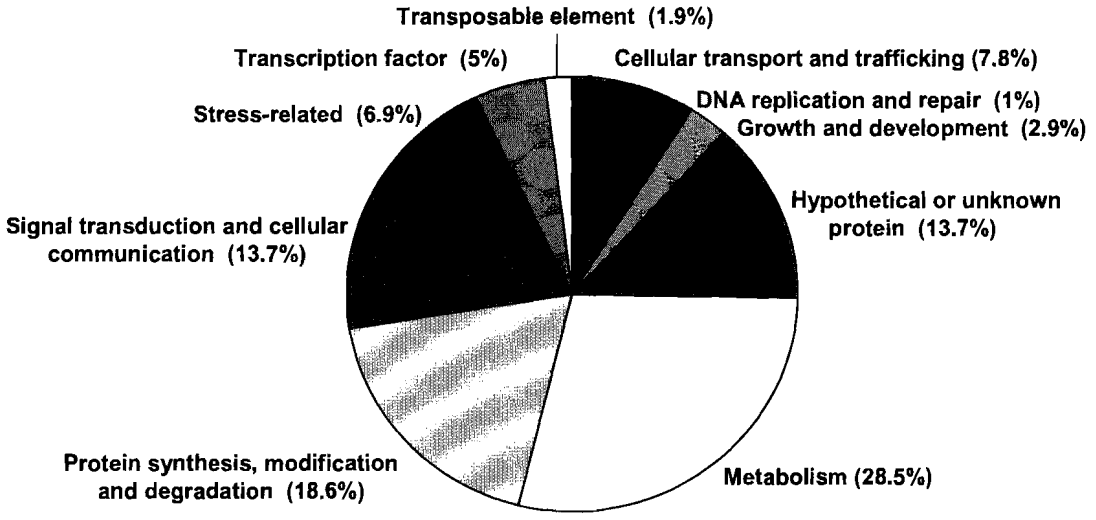

Figure 7. Functional distribution of rice genes that exhibit a significant increase in expression level in at least one time point during the first 24 hours of cold stress.

The second cluster includes the genes that did not start getting induced by cold until after the first 2 hours, hence they were referred to as the '*delayed induced early response*' genes. A survey of the microarray raw data indicated that the signal intensities of the features that clustered in Group-II appeared higher than the signal intensities of the features that belong to Group-I. Therefore, it was thought that, in general genes exhibit much higher expression levels during this phase of cold stress, and thus a 2-fold threshold would be sufficiently high to capture the critical genes in this stage.

The functional distribution of genes appeared to overlap between Group-I and Group-II since no single functional category was found to be specifically overrepresented in one group versus the other. A survey of the functional distribution of the genes that were upregulated during the initial phase of cold stress revealed some potential biochemical characteristics of the relatively cold tolerant japonica rice that are probably critical in the early response (Fig. 7). First, consistent with what is known about freezing and drought stress response mechanisms, the onset of chilling stress might initially cause perturbations in protein and enzyme functions. At the specific developmental stage examined in this study, the seedlings were in a very active growth state and proteins and enzymes that are synthesized shortly before or immediately after the initiation of the stress are negatively affected by the sudden change in environmental temperature. Thus, a mechanism to minimize the negative effects of low temperature on protein-mediated processes is expected to be part of the early response mechanism. This is indicated in the current transcriptome data by the induction of expression of genes involved in protein synthesis, modification, turn-over and replacement, which represents 18.6% of the total number of early cold stress upregulated genes.

Second, genes associated with signal transduction are upregulated at different time points during the first 24 hours, including a phospholipase and phosphatidylinositol transfer protein, protein kinases (ser/thr and casein kinases), two different GDP

dissociation inhibitors, pathogen induced proteins (avrRpt2- and harpin- induced), a leucine-rich repeat (LRR) protein, an impaired sucrose induction protein, nodulin and ankyrin. The induction of these genes is consistent with the fact that signal transduction is one of the critical early events that trigger subsequent gene expression. Third, a total of five transcription factors were found to be significantly induced during the 24-hour time window. These genes include a basic region leucine zipper (*OsbZIP*), a basic helix-loop-helix (bHLH, LOC_Os01g70310), a Myb protein (*OsMyb*), and a zinc finger-like protein (LOC_Os02g36740). Alteration of the overall transcriptional machinery is one of the most critical early events in abiotic stress and transcription factors are known to be the major components that coordinate these responses. The individual analysis of these transcription factors are described in a separate section of the results. Fourth, a significant proportion of genes that are upregulated during the first 24 hours of cold stress are involved in growth and development (2.9%) and metabolism (28.5%). These results suggest the potential involvement in the general (and/or initial) adjustment of metabolic processes, which are probably necessary to either maintain or prevent excessive perturbation to basic biochemical processes. Lastly, some stress-related genes, either known (e.g., ferritin) or novel (e.g., drought inducible high mobility group protein or HMG) were also found to be induced at different time points during the 24 hour time window.

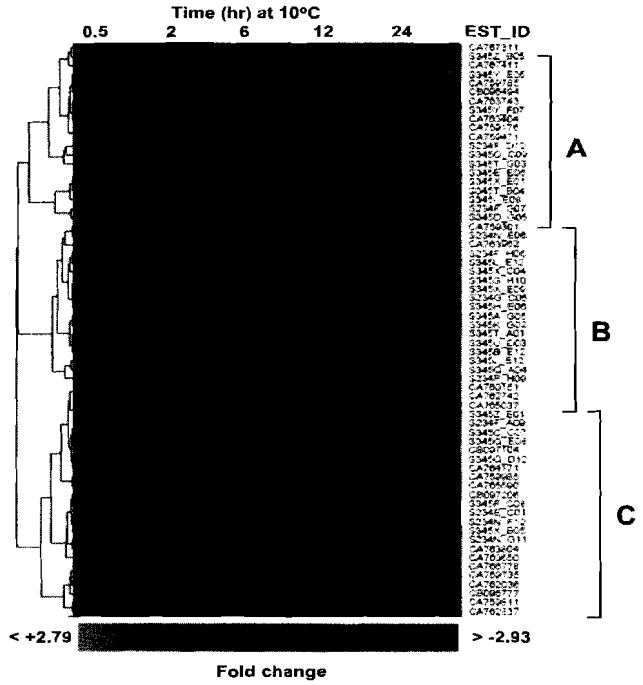

Figure 8. Grouping of rice genes that exhibited significant decrease in expression level. Genes (64 in total) that exhibited at least 2-fold decrease in expression at one or more time points during the first 24 hours of cold stress (10°C) were grouped by hierarchical clustering. Positive fold-change values for genes indicate upregulation and negative values represent downregulation. Base on these hierarchical clusters, the downregulated component of the early cold stress response transcriptome can be divided into three major groups: A, B and C.

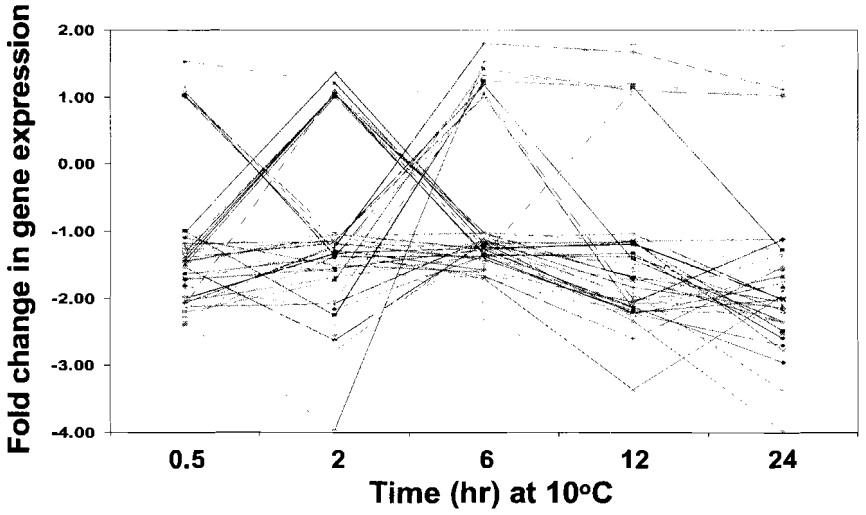

Figure 9. Time series expression profiles of the 64 genes that exhibit significant downregulated of expression in at least one time point (also shown in Fig. 8). Positive fold-change values for genes indicate upregulation and negative values represent downregulation.

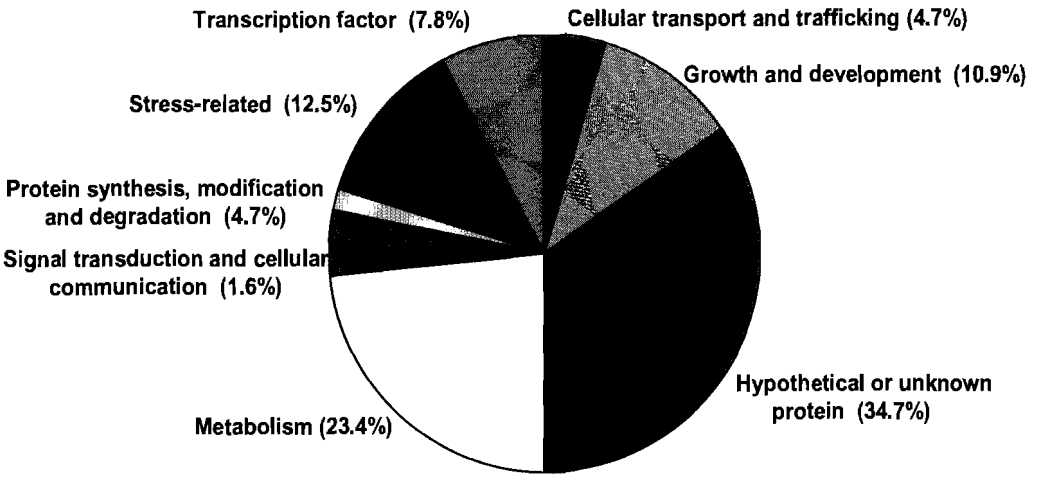

Figure 10. Functional distribution of rice genes that exhibit significant decrease in expression level in at least one time point during the fist 24 hours of cold stress.

A similar clustering strategy was employed for the downregulated group, in which the genes that displayed a 2-fold decrease in expression at one or more time points during the first 24 hours were selected. A total of 64 genes were included in this class. The profiles of these genes showed three distinct waves of downregulation (Figs. 8 and 9). Like the pattern exhibited by the upregulated genes, the downregulated groups were repressed within the first 2 hours of cold stress (Fig. 8, Group A). However, these responses all seem to be transient and did not continue past the first 2 hours. The second group of downregulated genes was not repressed until after the first 2 hours. Most of these genes continued to exhibit downregulated patterns throughout the whole 24 hours of cold stress (Fig. 8, Group B). The rest of the genes in the downregulated component appeared to respond late (Fig. 8, Group C). Overall, downregulation of gene expression in response to chilling stress appears to be transient and occurs at multiple waves during the 24-hour period. Many genes that belong to this group are housekeeping proteins and growth-related (Fig. 10), suggesting that downregulated expression is an important indication of a state of physiological stress. One of the more obvious morphological features of the cold-stressed seedlings is their slower growth rate compared to those grown at optimum temperature. A significant proportion of genes that are downregulated by cold stress were found to fall under metabolism- and growth and development-associated functions, i.e., 23.4% and 10.9% respectively (Fig. 10). This observation is consistent with the possibility that growth-related processes are the first to be affected by significant decreases in metabolic rate caused by downregulation of gene expression.

The reduction in growth rate observed in cold-stressed rice seedlings might also be a result of the repression of development-related transcription factors, which was detected in the transcript profile (e.g., Myb-type proteins). In addition, within the repressed group are many stress-related genes (12.5%). As mentioned earlier, most of them are

transient. Therefore, the repression of these stress-related genes could be the result of cold-shock. Finally, despite these characteristics, a large portion of the repressed genes encode putative proteins with unknown function (34%). This is most likely due to the fact that very limited efforts have so far been devoted to functional characterization of genes that are repressed by different forms of stress in any organism.

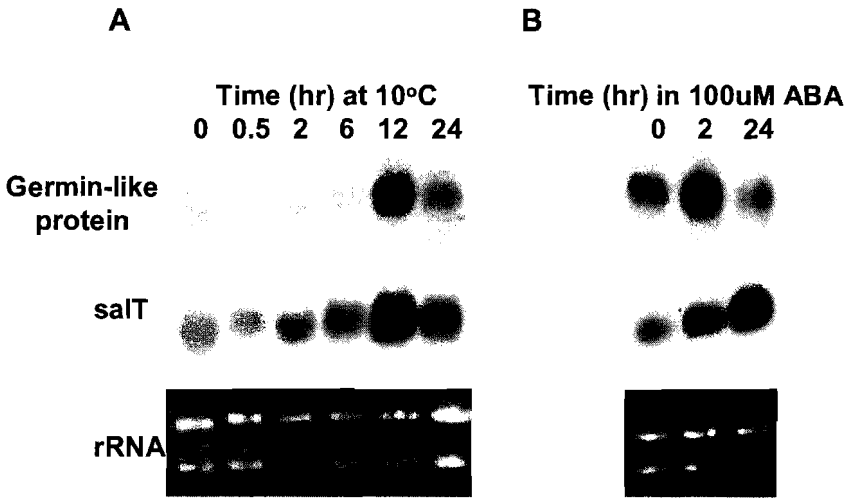

Figure 11. RNA gel-blot analysis of known cold- (A) and ABA-inducible (B) genes of rice. The expression of these genes was used as positive control of gene expression analysis. EtBr stained rRNA bands are presented as RNA loading controls.

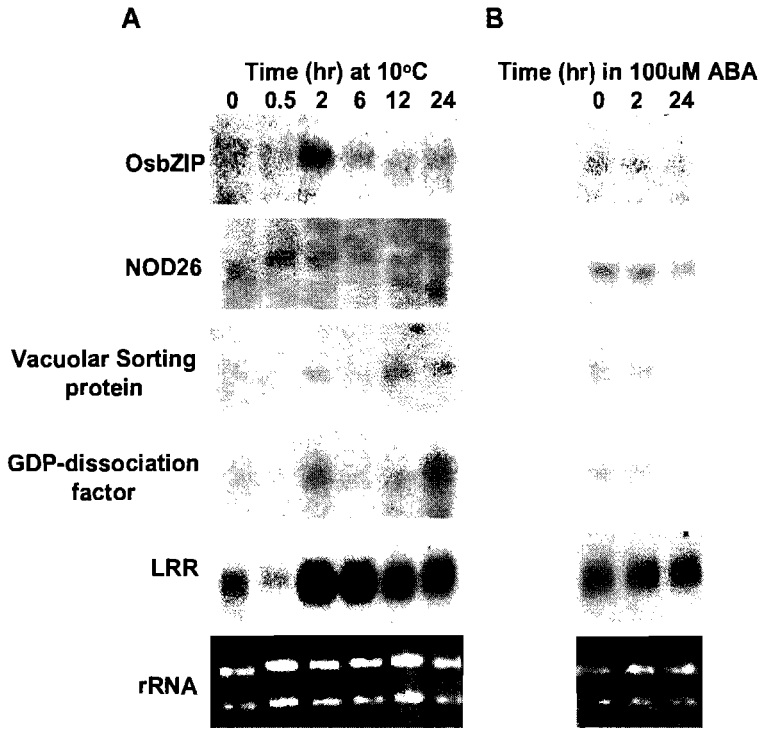

Figure 12. RNA gel-blot analysis of the '***rapidly induced early response***' genes (Group-I based on hierarchical clustering of microarray data). (A) Cold-induced expressions of candidate genes representing major functional groups, including transcription factor (OsbZIP), cellular transport and trafficking (NOD26 and vacuolar sorting protein), and signal transduction and communication [GDP-dissociation factor and leucine-rich-repeat (LRR)]. (B) Expression of the candidate genes with exogenous addition of ABA. EtBr stained rRNA bands are presented as RNA loading controls.

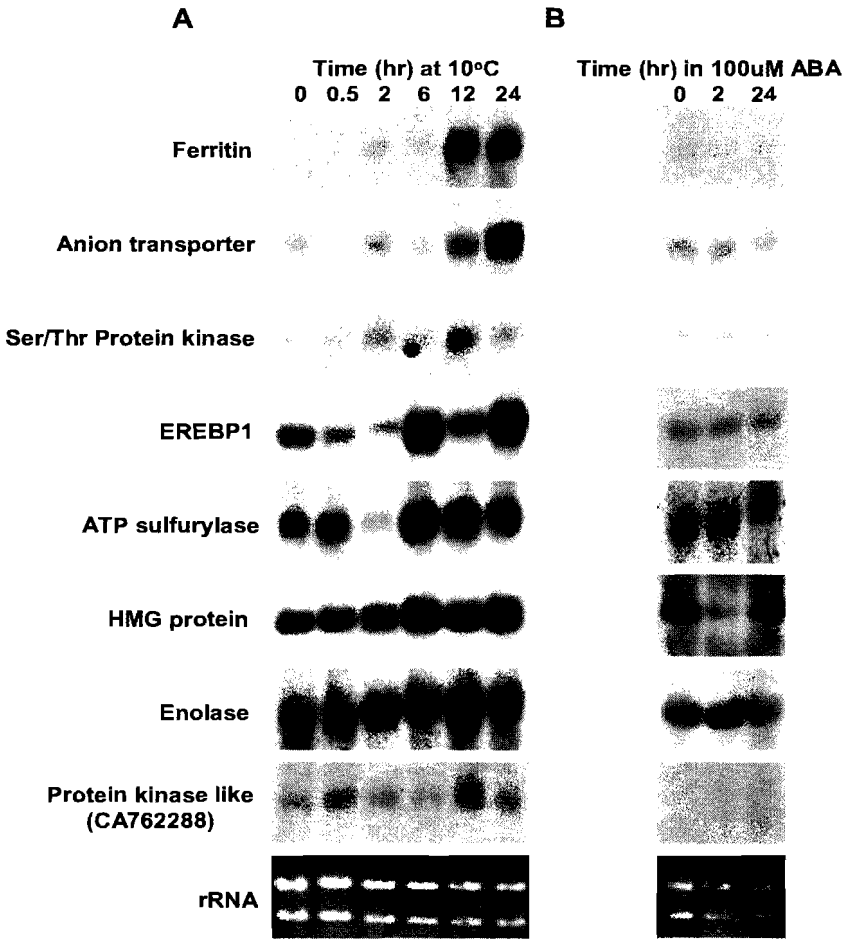

Figure 13. RNA gel-blot analysis of the '***delayed induced early response***' genes (Group-II based on hierarchical clustering of microarray data). (A) Cold-induced expressions of candidate genes representing major functional groups, including transcription factor (EREBP), stress-related [Ferritin and high mobility group (HMG) protein], cellular transport and trafficking (Anion transporter), metabolism (ATP sulfurylase and enolase), and signal transduction and communication (protein kinases). (B) Expression of the candidate genes with exogenous addition of ABA. Note that the protein kinase-like protein gene (CA762288) is not included in the cluster due to missing microarray data point at 2-hour (missing in one of the two biological replicates). This gene was selected for validation prior to finalizing clustering criteria. It is clear that the gene is upregulated after 12 hour of cold stress, which is consistent with microarray data showing strong upregulation (more than 2-fold) at this time point. EtBr stained rRNA bands are presented as RNA loading control.

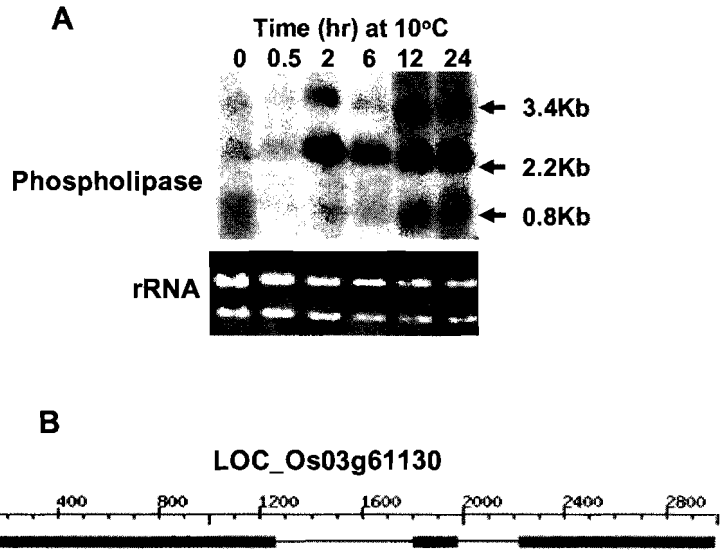

Figure 14. Analysis of the cold-induced transcription pattern of a gene encoding a putative phospholipase (LOC_Os03g61130). This gene belongs to the '***delayed induced early response***' group (Group-II) based on microarray data. (A) Gel-blot of the gene appeared to contain putative splice variants. The estimated sizes of each variant are as indicated. (B) The *ab initio* predicted gene structure of the encoding gene (LOC_Os03g61130) according to TIGR rice genome annotation is shown consisting of three exons and two introns. The 3.4-kb transcript appears to correspond to the pre-mRNA (unspliced version). The 2.2-kb transcript is consistent with the splice sites as shown in the splice annotation by TIGR. The 0.8-kb transcript appears to correspond to the last exon.

Gel-blot analysis of candidate early cold-induced genes

To minimize the false positive rate associated with technical and experimental variations inherent to microarray data, gene expression data obtained from such experiments were validated by individual analysis of the expression of representative genes. RNA gel-blot was used to further study the temporal expression of representative cold stress-upregulated genes from Group-I and Group-II. The expression of two known stress-inducible genes was first monitored to establish the quality of RNA gel-blots and efficacy of the stress treatments. The expression of a germin-like protein and *SalT* genes both have been previously shown to be cold-inducible (De los Reyes et al., 2003; De los Reyes and McGrath, 2003). As expected, RNA gel blot analysis of both genes showed that they are indeed induced by the stress conditions used in all experiments (Fig. 11). The same RNA gel-blot preparation and stress treatments were then used to analyze selected genes from each of the two temporal groups of upregulated genes (Group-I and Group-II).

Four genes, including the bZIP transcription factor (ERDCA_H03) discussed in the previous section, a GDP-dissociation factor (S345V_A11), and two proteins involved in cellular transport and trafficking (NOD26-like membrane protein, CA761959; vacuolar sorting protein, S345A_E06), were selected to represent the small group of *'rapidly induced early response'* genes (Group-I). All four genes were first induced within 2 hours of cold stress (Fig.12). The expression of the bZIP gene (*OsbZIP*) remained slightly upregulated at 6 hours and then faded slowly to the control level. In the microarray array data, this gene was induced within the first two hours and the expression increased at 6 hours (Table 3). Nonetheless, its temporal expression was consistent with the grouping criteria discussed in the previous section. In addition, both genes encoding GDP-dissociation factor and vacuolar sorting protein showed a second level of induction, which started at 12-hour and increased through 24-hour. This second wave of induction

was observed in microarray data for the GDP dissociation inhibitor but not for the vacuolar sorting protein. Collectively, the results of the gel-blot analysis supported the clustering of the Group-I genes based on the analysis of microarray data.

Among the genes that belong to the *'delayed induced early response'* group, 9 representative genes were validated from each of the major functional categories (Figs. 13 and 14). These include some stress-related genes (ferritin, S234J_C11; high mobility group protein, S234D1_A08), cellular transport and trafficking gene (anion transporter, CA759231), signal transduction and cellular communication genes (Ser/Thr kinase, CB096630; kinase-like, CA762288; phospholipase, CA759247), a transcription factor (EREBP1, CB096828), and genes involved in metabolism (ATP sulfurylase, S345Z-_H01; enolase, S234C1_G07) (Figs. 13 and 14). Gel-blots results were virtually consistent with the microarray data in terms of the temporal patterns (Table 3). The only exception is a leucine-rich repeat protein (LRR, CA767317). According to microarray data, this gene is not induced until 24 hours after the imposition of cold stress. However, gel-blot analysis showed a clearly sustained induction from 2 to 24 hours of cold stress (Fig. 12).

Note that the protein kinase-like protein (CA762288) is shown here, but was not included in the cluster due to missing microarray data at 2 hours (missing in one of the biological replicates). This gene was selected for validation prior to the final clustering. Nonetheless, it is clear that the gene is upregulated at 12 hours, which is consistent with the microarray data showing strong upregulation at 12 hours.

As shown in Fig. 14, the transcript profile of a phospholipase gene that belongs to Group-II showed multiple hybridizing bands in the RNA gel-blot. This result suggests the possibility of stress-induced splice variants. Analysis of the genomic sequence of this gene (LOC_Os03g61130) showed that it contains three exons and two introns. The predicted total length of the gene is 2,197-bp (exons 1 to 3 are 1256bp, 716bp, and

765bp, respectively), which is consistent with the 2.2-kb transcript that was induced within 2 hours and lasted throughout the 24 hours period. The other putative splice variants occurred after 12 hours and are 3.4-kb and 0.8-kb in size, perhaps representing the unspliced pre-mRNA (~3-kb) and the third exon (765bp), respectively. These observations suggest a possible involvement of alternative splicing as a mean of regulating the expression of stress-related genes. This hypothesis is based purely on a very limited and preliminary set of data hence, it requires further study.

Role of abscisic acid in the early events of cold stress signaling in rice seedlings

Genetic studies in *Arabidopsis* are consistent with the theory that the signaling mechanisms involved in cold acclimation are largely independent of ABA-regulation (Shinozaki and Yamaguchi-Shinozaki, 2000). Insights on the possible involvement of ABA in regulating the early gene expression events associated with cold stress responses of chilling-sensitive rice was established in this study by monitoring the effect of exogenously applied ABA on the expression of candidate genes in the absence of a low temperature signal. It has been reported earlier by this laboratory that 100 μM ABA is sufficient to induce the expression of a number of cold regulated genes at ambient temperature (Morsy et al., 2005; Antoine et al., 2005). The expression of two previously characterized ABA-responsive genes was used as a positive control in this study. RNA gel-blot analysis revealed the transcript level of a germin-like protein and the novel salT protein were both induced at 100uM ABA (De los Reyes and McGrath, 2003; De los Reyes et al., 2003). The former showed a very high level of induction 2 hours after treatment with ABA, while the latter was highly induced after 24 hours of treatment with ABA (Fig. 11). These results showed that the ABA concentration used in this study is sufficient to cause a significant induction of known ABA-regulated genes. In contrast to the positive control, none of the experimental genes from either Group-I or Group-II were

induced by exogenously supplied ABA. These results suggest that ABA is probably not an important factor involved in the regulation of the early cold stress responsive genes of rice, at least during the early phase of cold stress.

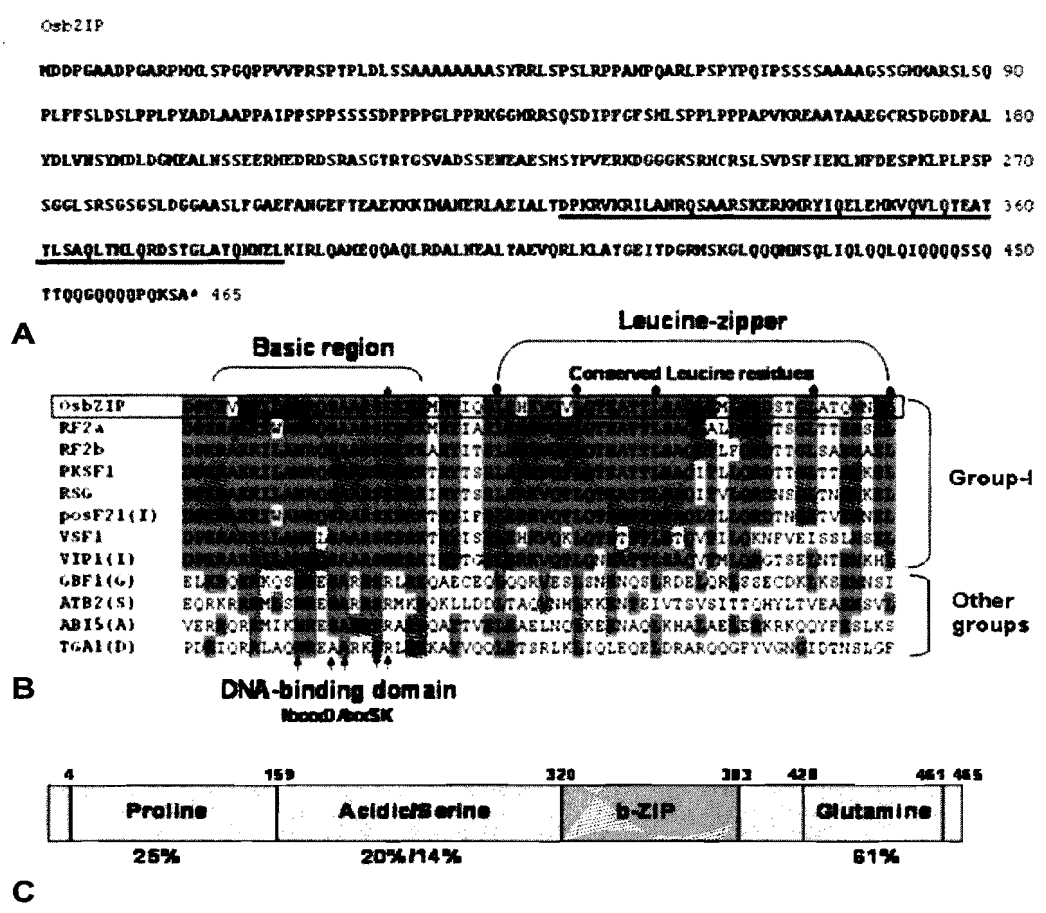

Figure 15. Structure of the cold-induced OsbZIP transcription factor of rice (LOC_Os08g43090). This gene belongs to the '***rapidly induced early response***' group (Group-I). The analysis of the conserved bZIP domain of this protein is shown. (A) Full-length amino acid sequence of the protein encoded by *OsbZIP*. (B) Multiple sequence alignment of the bZIP domains of OsbZIP and other group-I bZIP homologs, including RF2a (rice), RF2b (rice), PKSF1 (*Paulownia kawakamii*), RSG (tobacco), posF21 (*Arabidopsis*), VSF1 (tomato), and VIP1 (*Arabidopsis*). Alignments with representative genes from the other major subgroups of bZIP proteins *(Arabidopsis* GBF1, ATB2, ABI5, and TGA1) are also shown. Residues identical among sequences are highlighted. Solid circles indicate the positions of conserved leucine residues in the bZIP proteins. Solid diamond shows the -10 position in the bZIP domain related to the first conserved leucine residue. Arrow heads indicate amino acids important for DNA-binding specificity. Note that a DNA-binding signature, NxxxDAxxSK, is highly conserved among the members of the Group-I type of bZIP proteins. (C) Schematic diagram showing the domain structure of OsbZIP. Three possible functional domains in addition to the bZIP domain and their characteristic amino acid compositions are indicated.

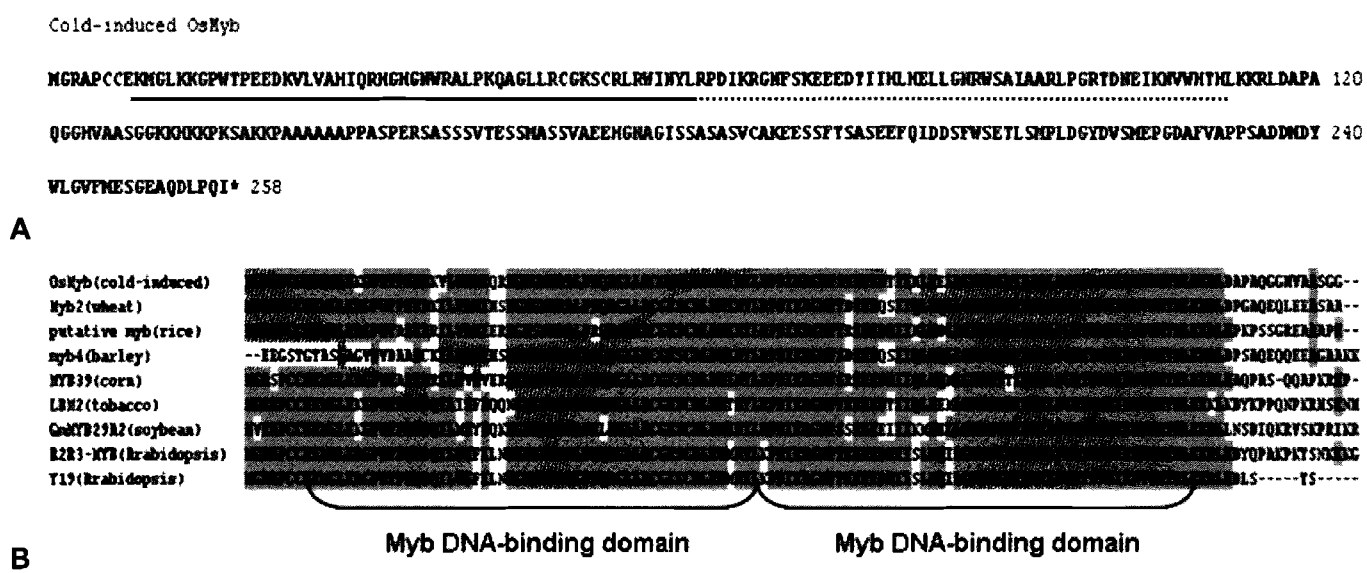

Figure 16. Structure of the cold-induced Myb homolog of rice. *OsMyb* (LOC_Os04g43680). This gene belongs to the '***delayed induced early response***' group of rice genes (Group-II). (A) Deduced amino acid sequence of cold induced OsMyb. The Myb DNA-binding domains (two) are underlined (solid and dashed lines). (B) Sequence alignment of the conserved Myb DNA-binding regions of OsMyb and other homologous Myb proteins from other plants. GenBank accession numbers are Myb2 (wheat), AAT37168; putative myb (rice), XP466825; MYB39 (corn), CAJ42203; LAM2 (tabacco), BAA88222; GmMYB29A2 (soybean), BAA81732; R2R3-MYB (*Arabidopsis*), CAA74603; Y19 (*Arabidopsis*), CAA62032.

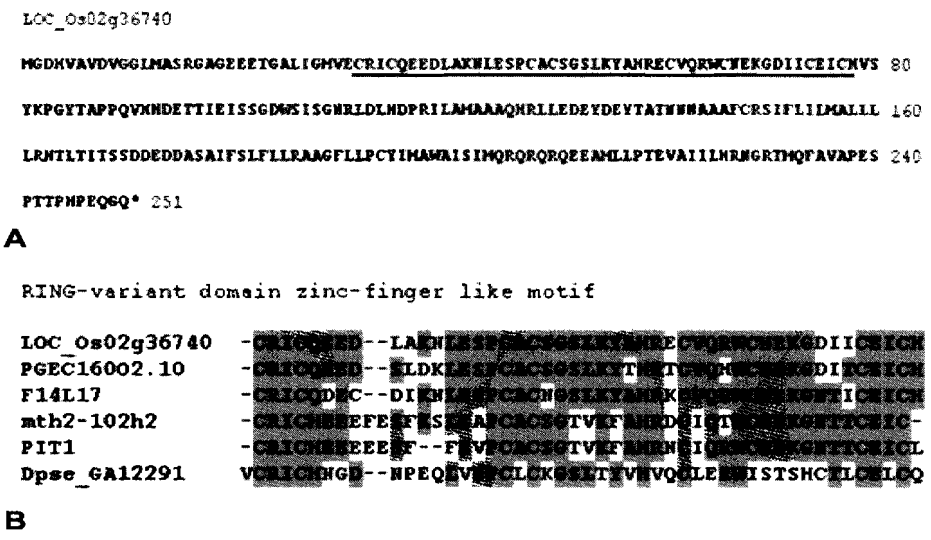

Figure 17. Structure of the cold-induced zinc finger-like protein (LOC_Os02g36740) of rice. This gene belongs to the '***delayed induced early response***' group of rice genes (Group-II). (A) Amino acid sequence of the region containing a RING-variant (RINGv) domain, a C3HC4-type zinc finger. The RINGv-domain (residue 32-78) is indicated by solid line. (B) Sequence alignment of the RINGv domain of cold-induced zinc-finger protein of rice and other similar RINGv containing proteins from other species. Identical sequence is highlighted in blue. The origin of the proteins, with GenBank accession numbers and amino acid numbers in parentheses are: PGEC160o2.10, *Solanum demissum* (AAT66770, 62-108); F14L17, *Arabidopsis thaliana* (AAF43925, 57-103); mth2-102h2, *Medicago truncatula* (ABE78108, 20-67); PIT1, *Arabidopsis thaliana* (NP567222, 20-65); DPSE_GA12291, *Drosophila pseudoobscura* (EAL25558, 195-242).

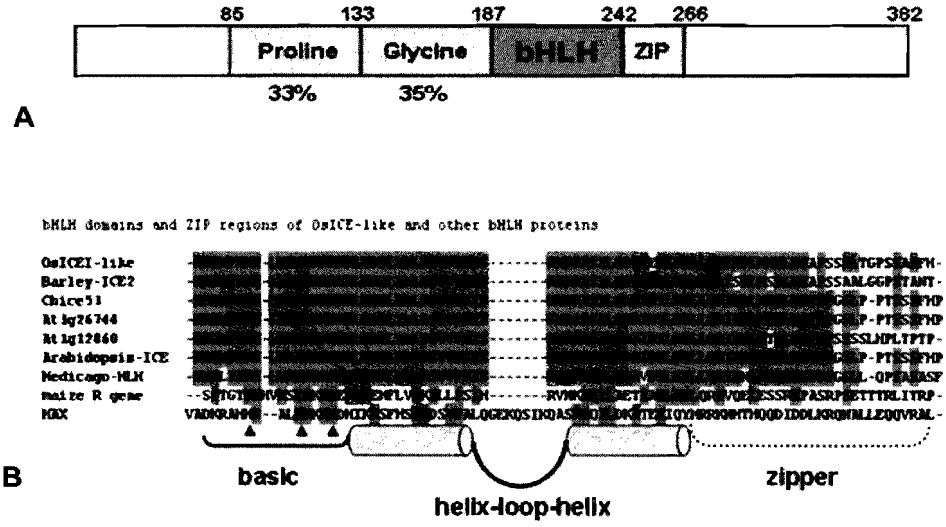

Figure 18. Structure of the cold-induced ICE1-like transcription factor of rice. *OsICE1* (LOC_Os01g70310) encodes a bHLH-type (basic helix-loop-helix) protein. This gene belongs to the '***delayed induced early response***' group of genes (Group-II). (A) Overall domain structure of the OsICE1 protein based on predicted sequence, including a proline-rich region, glycine-rich region, bHLH domain, and putative zipper region. (B) Alignment of the sequence of the conserved bHLH domains and possible ZIP regions of OsICE1 with homologs from other plants and animal (*Danio rerio*, MAX). Species origin of each homologous gene is as indicated (Cbice53 is from *Capsella bursa-pastoris*). Identical residues are highlighted in blue. Solid triangle indicates the amino acid residues that are known to interact with DNA based on the study conducted in MAX (Grandori et al., 2000; Chinnusamy et al., 2003)

Candidate cold stress-related transcription factors

Five transcription factors were found to be significantly upregulated by low temperature stress during the first 24 hours. One of these genes (EREBP1, CB096828) encodes an AP2 domain-type transcription factor that is known to be induced by various types of abiotic stress including low temperature (Fig.13 and Table 3; Fowler and Thomashow). The successful detection of the induction of this gene is further evidence to the efficacy of the low temperature experiments and validity of the microarray data. Two other candidate cold-induced transcription factor genes, which encode a basic region leucine zipper protein (*OsbZIP*, ERDCA_H03) and a zinc finger-type protein (S345D_G04) are potentially novel because their induction by low temperature has not yet been reported in the literature. The other two transcription factor genes encode a Myb-type protein (S345J_C03) and a basic helix-loop-helix (bHLH, S234D1_H11). Homologs of these genes have been reported earlier to play important roles in cold stress signaling in *Arabidopsis* and rice.

The initial identification of the functional homology for these transcription factors was based on limited EST data. To gain further insights on the function of these genes, their structural features and homology were studied by sequence analysis. The open reading frame for the OsbZIP protein, which was induced within 2-hour, was found in locus LOC_Os08g43090 based on the rice genome sequence annotation of The Institute of Genomic Research (TIGR). The homology and structural features of this protein are shown in Fig. 15. The predicted amino acid sequence is 465 residues in length and includes a bZIP domain (residues 320-383) (Fig.15A). The full-length sequence of OsbZIP is most similar to tomato bZIP (VSF-1) that controls vascular gene expression (Ringli and Keller, 1998). Multiple sequence comparison showed that the bZIP domain is highly similar but not identical to two rice bZIP-type transcription factors, RF2a and RF2b, which have been implicated in the mechanism of resistance to disease caused by

the rice tungro virus (Dai et al., 2004). In addition, a number of characteristics suggested that OsbZIP most likely belongs to the subgroup-I of plant bZIP according to the classification scheme established from the analysis of all bZIP-type proteins of *Arabidopsis* (Jackoby et al., 2002). For instance, the bZIP domain of OsbZIP has very high identity (83-89%) to other Group-I bZIP-type proteins, but is less similar (52-59%) to the bZIP domains from other sub-groups (Fig.15B). Group-I bZIP domains share a conserved leucine-zipper and a DNA-binding signature in the basic region. The OsbZIP has up to three putative functional domains in addition to the DNA-binding bZIP domain (Fig. 15C). A proline-rich domain was located near the N-terminal end (aa-4 to aa-159, 25%). Between the proline-rich and bZIP domains is a 162-aa-long stretch rich in acidic and serine residues (20% and 14%, respectively). A glutamine-rich (61%) region is found near the C-terminal end from aa-428 to aa-461. Based on this analysis the OsbZIP appears to be a member of the Group-I type of plant bZIP proteins.

The predicted amino sequence of the cold-induced Myb homolog (*OsMyb*, LOC_Os01g70310, Fig. 16A) is virtually identical to the product of the gene *Osmyb4*, which has been shown to cause an increase in chilling and freezing tolerance when overexpressed in *Arabidopsis* (Vannini et al., 2004). Based on conserved domain search (Marchler-Bauer and Bryant, 2004) this protein is characterized by its two Myb DNA-binding domains present in tandem near the N-terminus of the 258-aa-long protein (Fig. 16A). While the rest of the protein sequence showed little similarity among homologous Myb proteins (28-67%), the tandem Myb-domain structure is conserved across plant species (Fig. 16B).

The ORF of the zinc-finger protein is encoded in LOC_Os02g36740. The predicted sequence of the 251-aa-residue protein is characterized by a conserved RING-variant domain (RINGv domain, from aa-32 to aa-78) (Fig. 17). The RINGv is a C3HC4-type zinc finger (i.e., consensus sequence Cys-X_2-Cys-X_{9-39}-Cys-X_{1-3}-His-X_{2-3}-

Cys-X_2-Cys-X_{4-48}-Cys-X_2-Cys, where X is any amino acid residue) that coordinates two zinc ions (Saurin et al., 1996; Borden and Freemont, 1996). So far, not much is known about the function of this orthologous group in relation to stress response.

The fourth transcription factor is a bHLH-type (basic helix-loop-helix) protein similar to the recently characterized ICE1 of *Arabidopsis*. The rice homolog (OsICE-like) appears to be different from the *Arabidopsis* gene by virtue of the fact that the latter is constitutively expressed while the former is apparently induced by low temperature. The ORF of *OsICE* is encoded in LOC_Os01g70310 in the rice genome sequence, with a predicted 382-aa protein product. The bHLH is located halfway in the central part of the polypeptide (aa-187 to aa-242), followed by the 24-aa-long probable ZIP region. Proline-rich (33%) and glycine-rich (35%) regions were located at the N-terminus (aa-85 to aa-133, and aa-134 to aa-187) (Fig. 18A).The bHLH domain is highly conserved between OsICE and ICE1 as well as other plant bHLH proteins (Fig.18B). A conserved glutamate residue in fish bHLH (MAX) facilitates contact with nucleotides of MYC-elements (Grandori et al., 2000). This glutamate residue is conserved in all bHLH domains including OsICE (Fig.18B).

In silico promoter analysis in representative early cold stress response genes

The occurrence of two putative novel and two known stress-related transcription factors among the candidate early response genes provides a rationale for the analysis of the promoter structures of other non-transcription factor genes that belong to Group-II. It is assumed that such a correlative analysis may provide some insights on how the transcriptional changes involved in the early response are regulated. To address this hypothesis, the promoter sequences of four genes that belong to the '*delayed induced early response*' group (Group-II) were investigated. The upstream 1,000-bp region

(-1,000) was extracted from ferritin (LOC_Os11g01530), high mobility group protein (LOC_Os06g51220), anion transporter (LOC_Os03g05390), and ATP sulfurylase (LOC_Os03g53230) and analyzed by *ab initio* survey of conserved stress-related *cis*-elements of plant genes. This analysis showed a number of candidate conserved motifs corresponding to the binding sites of a number of transcription factors specifically associated with abiotic stress (Fig. 19). For instance, G-box, as1/ocs, and ABRE, which are known to be recognized by a bZIP-type transcription factor, were found in all four genes. Despite the limited number of genes analyzed, overrepresentation of the as1/ocs-like elements, which is also known to be involved in H_2O_2-mediated signaling, appeared to be the dominant trend.

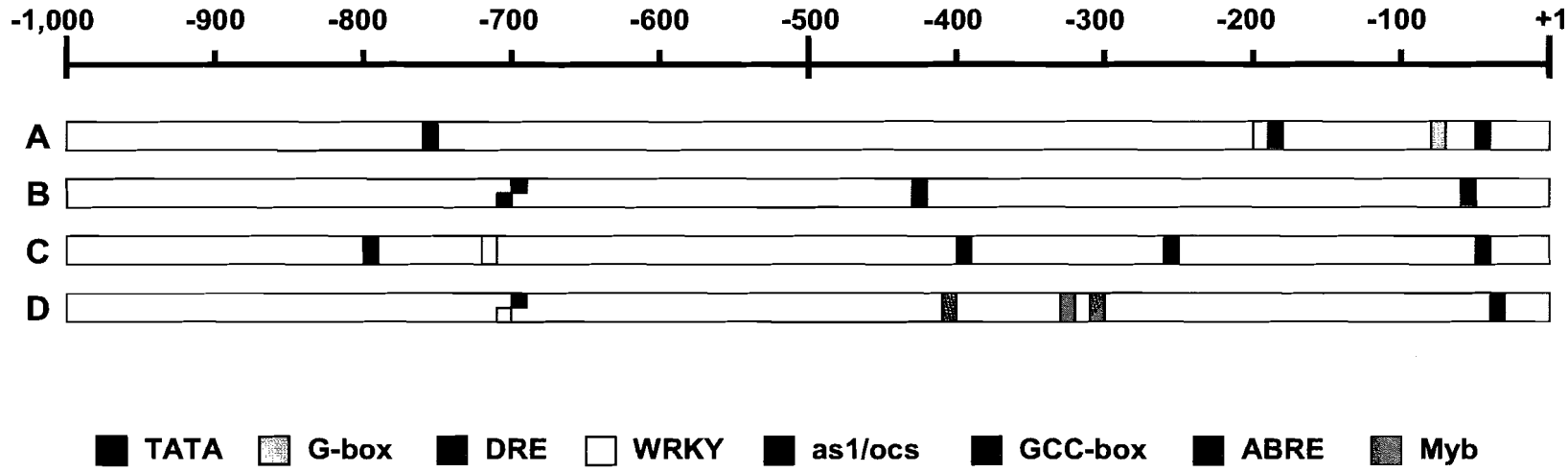

Figure 19. Graphical representation of the promoters of cold-inducible rice genes that belong to the '***delayed induced early response***' group (Group-II). A = Ferritin (LOC_Os11g01530), B = High mobility group protein (LOC_Os06g51220), C = Anion transporter (LOC_Os03g05390), D = ATP sulfurylase (LOC_Os03g53230). G-box (CACGTG), DRE-Drought response element (core sequence, CCGAC), WRKY (TTGACC), as1/ocs (TGACG), GCC-box (GCCGCC), ABRE-ABA response element (core sequence, ACGTG).

Table 3. List of candidate rice '***early response***' cold stress genes identified from microarray experiments.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-I									
CA759689	1.08	1.94	1.33	2.33	2.12	β-galactosidase complementation protein	4e-10	-	-
CA759740	1.27	2.36	1.41	3.22	3.00	Hypothetical protein XP_237701 (*Rattus norvegicus*)	9e-08	-	-
CA761959	1.39	2.11	1.47	2.07	2.48	NOD26-like integral membrane protein ZmNIP2-3 (*Zea mays*)	5e-24	P0425F05	2.0e-121
CA763335	1.38	2.03	1.96	2.77	2.56	Expressed protein (*Arabidopsis thaliana*)	3e-26	OJ1060_G11	8.0e-65
CA765797	-1.02	1.95	1.65	1.93	1.90	Unknown protein (*Arabidopsis thaliana*)	2e-35	OSJNBa0014E22	1.8e-108
ERDCA_H03	1.02	1.85	2.23	1.14	-1.25	bZIP protein (*Oryza sativa*)*	8e-32	P0623F08	8.1e-63
S234D1_A11	1.56	2.09	1.88	1.71	1.47	Hypothetical protein (*Oryza sativa*)	4e-78	OJ1126_D01	6.9e-105
S234D1_E07	1.19	2.02	1.58	1.35	1.51	S-adenosylmethionine synthetase-1 (*Oryza sativa*)	3e-81	P0519E07	0
S234D1_E08	1.22	1.81	1.78	2.25	2.04	Putative malate dehydrogenase (*Oryza sativa*)	9e-72	OJ1316_H05	8.2e-67

* Annotation in GAL was updated. These genes were previously annotated to be similar to a region of particular BAC clone or to a putative function that was less specific.

Table 3 cont'd.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-I									
S234K_E09	1.18	1.87	1.71	2.21	2.04	Putative mitochondrial processing peptidase alpha-II chain precursor (*Oryza sativa*)	1e-38	P0710E05	2.6e-29
S234L_F12	1.14	1.85	1.44	1.32	1.23	Putative transposase (*Oryza sativa*)	1e-159	P0010D0	6.5e-186
S234O_G06	1.19	1.84	-1.02	1.04	-1.26	Similar to *avrRpt2*-induced protein-2 (*Arabidopsis thaliana*)	2e-33	OJ1679_B08	4.4e-106
S234P_B12	-1.26	1.85	1.72	2.27	1.55	α-Amylase isozyme-3E precursor	2e-76	P0013B04	1.6e-122
S234X_A09	1.12	1.91	1.98	2.83	1.80	Putative ubiquitin-conjugating enzyme (*Oryza sativa*)	6e-93	OSJNBb0038H12	1.0e-80
S345B_G05	-1.37	1.83	1.79	2.23	1.50	Impaired sucrose induction 1-like protein (*Oryza sativa*)*	4e-28	P0538C01	0
S345E_H11	-1.02	1.92	1.84	2.29	1.58	Unknown protein (*Oryza sativa*)	6e-25	OSJNBb0004M10	5.6e-115
S345R_F10	1.18	1.95	1.35	1.28	-1.06	Harpin induced gene-1 homolog (*Oryza sativa*)*	3e-88	OSJN00092	9.7e-66
S345U_C12	1.14	1.99	1.42	1.37	1.59	Putative casein kinase-II beta subunit (*Oryza sativa*)	4e-72	OSJNBa0027P10	3.8e-116

* Annotation in GAL was updated. These genes were previously annotated to be similar to a region of particular BAC clone or to a putative function that was less specific.

Table 3 cont'd.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-I									
S345V_A11	1.08	1.82	1.59	1.97	2.19	GDP dissociation inhibitor protein (*Oryza sativa*)	1e-93	P0579A05	7.0e-54
S345A_E06	1.37	2.04	1.57	1.17	-1.03	Similar to vacuolar sorting protein (*Arabidopsis thaliana*)	1e-107	P0038B07	7.4e-86
Group-II									
CA759231	-1.25	-1.38	1.22	2.16	2.33	Putative anion transporter (*Oryza sativa*)	4e-53	OSJNBa0067N01	7.9e-118
CA759247	1.08	-1.12	1.41	2.39	2.87	Putative phospholipase (*Oryza sativa*)	1e-45	OJ1111_B11	1.6e-136
CA759466	-1.18	-1.19	-1.04	1.36	2.33	phosphatidylinositol transfer-like protein III (*Lotus japonicus*)	2e-27	P0030G11	3.60e-138
CA759494	-1.06	1.05	1.76	2.94	2.08	Putative cytidine deaminase (*Zea mays*)	1e-23	OJ1058_C08	1.4e-70
CA759562	1.12	1.30	1.30	2.01	2.59	40S Ribosomal protein S3A (*Oryza sativa*)	6e-52	OSJNBa0009F05	8.6e-127
CA760006	-1.11	1.35	1.86	2.55	2.45	Putative glutamate dehydrogenase*	4.8e-220	OSJNBb0012J10	2.3e-34

* Annotation in GAL was updated. These genes were previously annotated to be similar to a region of particular BAC clone or to a putative function that was less specific.

Table 3 cont'd.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-II									
CA760007	1.16	1.15	1.53	2.08	3.47	NADH-glutamate synthase (*Medicago sativa*)	1e-35	P0019E03	1.3e-129
CA760100	-1.09	1.59	1.29	2.46	2.16	ORF_OSJNBa0047D12.20 (*Oryza sativa*)	3e-45	OSJNBa0062A24	0
CA760250	-1.70	-1.79	1.12	2.90	1.39	SF21-like protein (*Oryza sativa*)*	0	P0695H10	3.3e-79
CA760356	-1.14	-1.30	1.41	3.36	1.64	Probable ligand-gated ion channel protein (*Arabidopsis thaliana*)	3e-05	SJNBa0090D06	2.1e-142
CA761977	-1.30	-1.11	1.19	1.45	2.33	Aldehyde dehydrogenase (*Arabidopsis thaliana*)	2e-33	OSJNBa0052O08	8.5e-32
CA762735	1.10	1.19	1.51	2.90	2.49	Putative transposon protein, mutator sub-class (*Oryza sativa*)*	2e-15	P0463G11	1.4e-117
CA762909	-1.02	-1.02	1.56	1.71	2.37	Putative ADP-ribosylation factor (*Oryza sativa*)	9e-14	P0425G02	3.8e-29
CA763167	1.17	1.34	1.19	3.20	1.85	Putative 50S ribosomal protein L13 (*Oryza sativa*)	1e-74	OSJNBa0004B23	7.4e-74
CA763224	-1.00	-1.05	-1.02	1.26	2.55	Similar to OsENOD93a gene for early nodulin (*Oryza sativa*)	6e-35	P0535G04	7.3e-118

* Annotation in GAL was updated. These genes were previously annotated to be similar to a region of particular BAC clone or to a putative function that was less specific.

Table 3 cont'd.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-II									
CA764973	-1.22	1.18	1.43	2.12	1.54	Putative cullin (*Oryza sativa*)	2e-87	P0560B06	1e-159
CA766136	-1.26	1.14	2.38	3.54	1.48	Ankyrin-like protein (*Oryza sativa*)*	4e-116	P0506B12	3.5e-169
CA766210	-1.32	-1.41	1.52	2.14	1.74	Putative drought-induced protein (*Oryza sativa*)*	3e-48	P0518C01	1.4e-105
CA766427	-1.39	-1.30	1.10	2.11	2.25	Putative ripening regulated protein (*Oryza sativa*)*	2e-43	OSJNBa0069D17	0
CA767055	-1.50	1.09	1.67	4.39	1.69	Polyubiquitin (*Sporobolus stapfianus*)	1e-118	OSJNOa0048I04	0
CA767317	1.10	-1.23	1.08	1.15	2.34	Putative leucine-rich repeat protein (*Oryza sativa*)	4e-72	OSJNBa0051C19	1.2e-87
CB096284	-1.15	1.35	1.45	2.33	1.89	Unnamed protein product (*Oryza sativa*)	1e-98	P0453A06	6.1e-119
CB096560	-1.08	1.11	1.61	2.23	1.46	GDP dissociation inhibitor protein (*Oryza sativa*)	2e-41	B1023E01	6.8e-117
CB096630	-1.49	-1.54	1.12	2.53	2.44	Putative serine/threonine protein kinase (*Oryza sativa*)	1e-68	OJ1409_C08	8.1e-167
CB096828	-1.22	-1.26	1.39	1.92	2.24	EREBP1 (*Oryza sativa*)	1e-104	OJ1311_D08	1.9e-179

* Annotation in GAL was updated. These genes were previously annotated to be similar to a region of particular BAC clone or to a putative function that was less specific.

Table 3 cont'd.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-II									
CB096933	-1.09	1.57	1.64	2.08	1.12	2-Oxoglutarate/malate translocator (*Panicum miliaceum*)	1e-106	OJ1430_B02	6.4e-119
CB097201	-1.34	1.24	1.64	2.37	1.54	Geranyl diphosphate synthase (*Arabidopsis thaliana*)	1e-56	OJ1651_G11	1.3e-166
ERDCA_C06	1.37	2.04	1.57	1.17	-1.03	Proteasome subunit alpha type 2 (*Oryza sativa*)	7e-99	OJ1643_A10	6.40e-74
ERDCA_F02	-1.74	-1.11	1.46	2.10	1.02	Polyubiquitin (*Pinus sylvestris*)	6e-33	P0547F09	1.3e-107
S234A1_D08	1.39	1.37	1.56	2.49	1.91	Amylogenin (*Oryza sativa*)	6e-68	OSJNBa0015K02	1.2e-148
S234B1_B12	-1.45	1.27	1.63	3.02	1.76	Sec61p (*Triticum aestivum*)	1e-84	P0512H04	1.5e-92
S234B1_C01	-1.03	1.34	1.18	2.40	2.11	Cytoplasmic malate dehydrogenase (*Oryza sativa*)	1e-125	OSJNBa0055P24	1.1e-104
S234B1_D12	-1.20	1.20	1.43	2.05	1.64	Putative 60S ribosomal protein L7 (*Oryza sativa*)*	3e-47	B1047A05	4.5e-61
S234B1_E07	-1.51	-1.03	1.35	2.10	1.52	EF-1 alpha (*Oryza sativa*)	7e-86	OSJNBa0061L19	5.5e-131
S234B1_F01	-1.35	-1.14	1.07	2.28	1.74	Cytosolic glyceraldehyde-3-phosphate dehydrogenase	1e-92	OJ1791_B03	5.6e-89

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Table 3 cont'd.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-II									
S234B1_H06	-1.44	-1.17	1.56	2.09	1.45	ORF P0501G01.5 (*Oryza sativa*)	5e-82	P0501G01	0
S234C1_G07	1.03	1.21	1.68	3.80	2.68	Enolase (*Oryza sativa*)	4e-84	OSJNBb0015J03	9.1e-68
S234D1_A08	-1.51	1.08	1.35	2.02	2.40	High mobility group protein (*Oryza sativa*)	3e-25	P0017G10	1.1e-79
S234D1_C11	-1.25	1.34	1.12	2.05	-1.11	phenylalanine ammonia-lyase (*Oryza sativa*)	6e-97	B1215B07	7e-120
S234D1_E10	-1.80	1.01	1.58	2.81	1.30	Polyubiquitin (*Euphorbia esula*)	1e-106	P0547F09	5.8e-130
S234D1_H11	1.18	1.77	1.28	2.44	1.38	Transcription factor ICE1-like (*Oryza sativa*)*	6e-94	OSJNBa0093F16	3.0e-126
S234J_C11	-1.46	1.42	1.45	2.84	3.30	Ferritin (*Oryza sativa*)	2e-69	OSJNBa0052H10	4.4e-87
S234J_D12	1.07	1.53	2.39	1.45	-1.08	OSJNBa0064G10.14 (*Oryza sativa*)	1e-37	OSJNBa0064G10	0
S234K_A10	-1.24	1.34	1.36	1.65	2.06	40S Ribosomal protein S3A	1e-109	OSJNBa0091J11	2.1e-81
S234L_B03	1.06	1.69	1.62	2.17	1.91	β-glucosidase (*Oryza sativa*) (*Oryza sativa*)	1e-128	OJ1212_C08	3.2e-106

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Table 3 cont'd.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-II									
S234L_C03	-1.52	-1.14	1.42	2.03	1.44	Putative 60S ribosomal protein (*Oryza sativa*)	2e-93	P0562A06	3.8e-145
S234M_C02	-1.52	-1.03	1.22	1.65	2.24	DnaJ-like protein (*Oryza sativa*)	7e-50	P0519E06	2.4e-52
S234X_G05	-1.15	1.12	1.72	1.50	2.01	Putative transaldolase (*Oryza sativa*)	1e-101	SJNBa0093F16	2.3e-81
S234X_G11	-1.60	1.18	2.06	2.06	1.72	Peroxisomal multifunctional protein (*Oryza sativa*)	1e-108	P0413A11	2.4e-107
S345A_B04	-1.14	1.38	1.78	2.87	1.32	Nt-iaa28 deduced protein (*Nicotiana tabacum*)	2e-64	OSJNBb0062H20	1.3e-124
S345D_C05	-1.31	1.61	1.82	2.05	1.34	Proteasome alpha subunit (*Oryza sativa*)	5e-55	OJ1112_E06	1.4e-106
S345D_C10	-1.20	1.51	1.76	2.58	1.34	Glycine-rich cell wall structural protein precursor (*Oryza sativa*)	5e-07	OSJNBa0030J04	4.3e-81
S345D_G04	-1.22	1.43	1.69	1.63	2.42	Zinc finger (C3HC4-type RING finger) protein (*Oryza sativa*)*	6e-111	B1342F01	4e-85

* Annotation in GAL was updated. These genes were previously annotated to be similar to a region of particular BAC clone or to a putative function that was less specific.

Table 3 cont'd.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-II									
S345E_F11	-1.23	1.31	1.40	2.55	1.58	Phosphoglycerate kinase, cytosolic (*Triticum aestivum*)	1e-64	P0669G09	1.2e-147
S345J_C03	-1.46	1.10	1.71	2.85	1.44	myb protein homolog (*Oryza sativa*)	8e-36	OSJNBa0073E02	5e-69
S345P_G12	-1.17	1.08	1.57	2.47	1.51	smr domain-containing protein (*Oryza sativa*)*	8e-130	OJ1147_D11	5e-168
S345P_H11	-1.36	1.13	1.72	2.68	1.76	Mannose-6-phosphate isomerase (*Oryza sativa*)	4e-16	OSJNBa0034P08	1.1e-94
S345Q_E04	-1.23	1.57	1.29	2.15	1.34	Putative cellulose synthase-8 (*Arabidopsis thaliana*)	0	P0669H03	1.4e-143
S345Q_F01	-1.20	1.03	1.98	4.60	1.63	Ribulose-bisphosphate carboxylase large chain precursor, rice chloroplast (*Oryza sativa*)	1e-25	BAC OJ1136_E8	8.1e-79
S345R_A12	-1.18	1.26	1.18	2.12	1.67	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	1e-125	B1148D12	2.0e-100
S345V_A10	-1.65	-1.11	1.47	2.25	1.43	Enolase (*Oryza sativa*)	5e-65	OSJNBb0015J03	1.9e-98
S345V_E05	-1.02	1.51	1.27	2.05	1.45	Probable inorganic diphosphatase (*Vigna radiata*)	6e-44	OSJNBa0032L17	3.1e-123

* Annotation in GAL was updated. These genes were previously annotated to be similar to a region of particular BAC clone or to a putative function that was less specific.

Table 3 cont'd.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-II									
S345V_H10	1.07	1.67	1.27	2.13	1.74	Putative ethylene-forming enzyme (*Oryza sativa*)	4e-06	OSJNBa0054H04	3.5e-31
S345X_F09	1.18	1.52	1.50	2.42	2.17	Putative disulfide isomerase precursor (*Oryza sativa*)	1e-137	P0431G05	2.4e-119
S345Z_G10	-1.43	-1.12	1.66	3.00	1.48	Unknown ORF OSJNBb0059K02.8 (*Oryza sativa*)	2e-50	OSJNBb0091I11	0
S345Z_H01	-1.73	-1.67	2.43	2.57	1.75	Putative ATP sulfurylase (*Oryza sativa*)	1e-128	OSJNBb0036F07	2.0e-166
S234A1_C06	1.05	-1.00	1.63	2.55	2.84	Glyceraldehyde 3-phosphate dehydrogenase, cytosolic	5e-92	OJ1499_A07	1.1e-84
S345A_B06	-1.28	1.11	1.84	2.41	1.28	Unknown ORF P0003E08.15 (*Oryza sativa*)	7e-40	P0003E08	5e-149
S345A_D07	-1.10	1.23	2.19	1.10	-1.38	OSJNBb0061C13.5 (Oryza sativa)	2e-42	OSJNBa0073E02	0
S345B_C08	-1.47	1.44	1.83	2.94	1.86	EF-1 alpha (*Oryza sativa*)	1e-107	OSJNBa0061L19	4.2e-127
S345E_B02	-1.47	-1.15	1.47	2.02	1.18	Ribosomal protein S19 (*Oryza sativa*)	1e-46	OSJNBa0061C08	6.3e-148

* Annotation in GAL was updated. These genes were previously annotated to be similar to a region of particular BAC clone or to a putative function that was less specific.

Table 3 cont'd.

Probe ID	Fold-change in expression 0.5hr	2hr	6hr	12hr	24hr	Functional Homology	Function e-value	BAC Clone	BAC e-value
Group-II									
S345G_H04	-1.35	1.26	1.40	2.10	1.14	Ribosomal protein S15 (*Oryza sativa*)	2e-51	OJ1559_F09	1.3e-111
S345W_G08	-1.05	1.56	1.89	2.75	1.32	Putative aux/IAA protein (*Oryza sativa*)*	1e-72	P0485D09	2.0e-132

* Annotation in GAL was updated. These genes were previously annotated to be similar to a region of particular BAC clone or to a putative function that was less specific.

Chapter 6

DISCUSSION

Molecular studies conducted during the last decade on the genetic model *Arabidopsis* indicated that plant acclimation to low temperature involves a highly coordinated expression of a battery of cold-responsive genes mediated by a specific family of transcriptional regulators called the CBF/DREB (Thomashow, 1999; Thomashow, 2001). This network of genes constitutes the first regulatory network to be directly associated with abiotic stress tolerance in plants (Shinozaki and Yamaguchi-Shinozaki, 2000). Unlike *Arabidopsis*, warm-season plants are sensitive to chilling stress. Rice for example, can easily be injured by low but non-freezing temperatures especially during the early stages of seedling development. Nevertheless, some traditional cultivars, especially those that belong to the japonica ecotype, exhibit a lesser degree of sensitivity than many other genotypes.

Like the cold acclimation process in *Arabidopsis*, the molecular genetic basis underlying the variation in cold sensitivity in rice is very complex. However, limited efforts have so far been devoted to identify candidate genes that participate in the genetic network defining cold tolerance mechanisms in rice, despite the fact that more appropriate functional genomic tools are already available in this monocot genetic model. A recent study based on the analysis of a very small number of genes indicated the possible activity of a *CBF*-like gene regulon in rice (Morsy et al., 2005).

The subject of this thesis is centered within the issue that little is currently known about the molecular genetic and/or functional genomic basis of low temperature adaptive responses in chilling sensitive plants and that further analysis of gene expression at least on a semi-global scale is necessary for a genetic model like rice. In an effort to address this issue, this study was focused on investigating the early events in the

complex genetic circuit, which are believed to comprise a very critical aspect of tolerance mechanisms. The central hypothesis is that the early responses are critical to tolerance because they trigger or initiate an intricate network of gene activity that results in a highly coordinated transcriptome. To address this hypothesis, a semi-global survey of gene expression during the critical first 24 hours of cold stress was conducted in the relatively chilling tolerant japonica cultivar (CT6748-8-CA-17). This study also aimed at utilizing the results of the transcriptome survey to either establish a new hypothesis or support previously established hypotheses on the molecular genetic aspects that differentiate the mechanism of low temperature responses between chilling-sensitive and chilling-insensitive plants by comparing available data from rice and *Arabidopsis*.

The microarray-based semi-global analysis of gene expression resulted in a number of interesting results. First, japonica rice appears to respond rapidly to a low temperature signal by massive induction and repression of gene expression. This is evident from the relatively large number of genes that are either upregulated (total of 88 genes or 5.7% of the total number of rice genes studied) or downregulated (total of 64 genes or 4.1% of the total number of rice genes studied) during the first 24 hours. Based on these results, 152 rice genes were classified as cold-regulated. The total number of genes in rice is estimated to be at least 37,500 based on the recent analysis of the japonica genome sequence (International Rice Genome Sequencing Project, 2005). Thus, the findings of this study suggest that a significant proportion of the total genes of rice are probably associated with cold stress response in either a direct or indirect manner or as part of a generalized response. Given the fact that only a small percentage of the rice genome was surveyed in this study (1,550 genes, which is only 4% of the coding capacity of the genome) and the survey was limited to a very narrow time window during the stress period (first 24 hours), it can be assumed that an even

larger set of genes could be identified as part of the responses to low temperature and to abiotic stress in general, if a genome-wide survey is be conducted.

The expression profiles of the cold stress response genes indicate that the initial transcriptional changes occur in an intricately coordinated temporal pattern even within a very narrow time window. For instance, the upregulated group of genes shows two distinct temporal groups, one being rapidly induced (rapidly induced early response genes or Group-I) and the other less rapidly induced (delayed induced early response genes or Group-II). The downregulated group follows very similar trends with some genes being repressed rapidly and some less rapidly.

The functional categories represented in the rapidly induced or repressed set of genes provide a good picture of the metabolic status of the cell. For instance, it appears that the cell is in a state of 'physiological stress' as early as 2 hours after the plants are subjected to sub-optimal temperature. Signal transduction genes associated with stress response are well represented indicating the initiation of a cascade of events possibly leading to defenses. Apparently, growth related processes are halted by the modulation of metabolic processes, which is reflected by the seemingly balanced upregulation and downregulation of various genes with possible roles in growth and development, metabolism, cellular transport, and protein synthesis, modification and degradation. A number of growth related transcription factors are also repressed early consistent with the overall slowing down of growth related cellular processes. These events are likely indications that intermediates and cellular resources that are otherwise devoted to growth and normal metabolic processes are being diverted towards the initial physiological adjustments and temporary defenses (Kawasaki et al., 2001). The not so rapidly induced and repressed groups are similarly comprised of genes involved in growth and development, metabolism, cellular transport, protein synthesis, modification and degradation and stress response, indicating that the cell is in an

advancing state of stress and that cellular adjustments established in the earlier phase are being continued or reinforced.

Another noteworthy observation from this study is the fact that within each temporal group, some genes are induced or repressed in a transient manner (short-lived induction or repression) and others are induced or repressed in a more sustained manner (long-lived induction or repression). It is quite noticeable that genes with regulatory functions tend to be expressed in a more transient fashion. For instance, *OsbZIP* is expressed very early (during the first 2 hours) but induction is very transient, perhaps lasting only within the first 4 to 5 hours of cold stress. Increased expression of *Myb* and *OsICE-like* transcription factors were observed at 12 hours but not 24 hours, also suggesting the transient fashion of expression. In addition, the induction of the zinc finger protein at 24-hour and beyond the time window of this study is likely also transient. This layered pattern suggests a complex interplay among the various transcriptional regulators that may be critical for the regulation of early responses. Furthermore, the fact that both the rapid and less rapid groups exhibit distinct temporal patterns despite their involvement in common or highly overlapping cellular processes, reinforces the paradigm that sequential gene expression is an evolutionary adaptation that ensures the precision and fine-tuning of regulation. It has been proposed that fine-tuned regulation is a major factor that determines genotypic differences in stress tolerance (Morsy et al., 2005).

The identification of candidate early response transcription factors (OsbZIP, Myb homolog, OsICE-like bHLH, and zinc finger proteins) is another potentially important result of this study. The early induction of these genes points to a possibility that they are involved in the initiation of a genetic regulatory network that links the initial signal to the downstream response. *OsbZIP*, which exhibits the earliest induction among the four genes, is a newly identified cold stress-related transcription factor. Results of a previous

genome-wide survey of the *Arabidopsis* cold acclimation transcriptome did not identify a homolog of this gene suggesting two possibilities. First, this gene could be part of a monocot-specific regulon and second, it may be part of an early branch of a regulatory network that occurs in chilling-sensitive but not in chilling-insensitive plants (Fowler and Thomashow, 2002; Seki et al., 2001; Seki et al., 2002; Chen et al., 2002). OsbZIP is unique among the four early response transcription factors because it is the only one that is rapidly induced (Group-I) and its expression is transient at least during the early phase (first 24 hours). Its predicted protein sequence is similar but not identical to other plant bZIPs including two from rice that have been associated with rice viral response (Dai et al., 2004). So far, no other known cold stress-related transcription factor exhibits the same temporal expression characteristics as *OsbZIP*. *In silico* analysis of four Group-II (delayed induced early response) genes showed the presence of multiple copies of putative target *cis*-element of bZIP-type transcription factors (as1/osc, G-box, and ABRE). These findings however do not necessarily mean on a functional context that OsbZIP regulates some of the group-II genes via these *cis*-elements. However, this circumstantial evidence suggests that a bZIP-mediated transcriptional regulation is probably one of the predominant pathways during the first 24 hours. Additionally, these findings are also useful in establishing hypotheses on the early response genetic regulatory network and set the stage for a number of potential studies for the future. By studying protein-DNA interactions, it will be possible to show: 1) Whether OsbZIP is the direct positive regulator of these Group-II genes, and if not; 2) What *cis*-element the OsbZIP interacts with, and; 3) What the transcription activator is for these group-II genes.

The second of the four early response transcription factors is a Myb-type protein. Based on sequence analysis, the predicted amino acid sequence of this Myb homolog is virtually identical to that of *Osmyb4*, which was previously isolated from a coleoptile

cDNA library of rice (cv. Arborio) (Solinas et al., 1997). *Osmyb4* was shown to be expressed at low level in warm grown (29°C) rice but strongly induced at 4°C. Earlier, this gene was proposed to be a potential master switch in a low temperature response transcriptome based on the observation that transgenic *Arabidopsis* overexpressing this gene showed significant increases in both chilling and freezing tolerance and altered expression of genes involved in different cold-induced pathways (Vannini et al., 2004). Given the fact that the induction of this Myb homolog occurs early and strongly (2.9-fold at 12-hour) in the tolerant genotype used in this study, it is likely that it might also act as a regulator of the early branches of the chilling tolerance genetic network of rice. This hypothesis must be confirmed by comparing the expression of this Myb homolog between tolerant and intolerant rice cultivar genotypes, and/or by transgenic approaches to assess the effects of overexpression and silencing to whole plant-level chilling tolerance.

A bHLH-type protein similar to the *Arabidopsis* ICE1 was also induced early when rice seedlings were subjected to cold stress. ICE1 is a positive regulator of the cold acclimation genetic regulatory network upstream of *CBF3* (Chinnusamy et al., 2003). Previously, rice *CBF* homologs were cloned and found to have multiple copies of the MYC-element (CATATG of consensus CANNTG) in its promoter (Almutairi, 2003). The identification of the early response *OsICE* is consistent with what has been proposed earlier by Morsy et al. (2005) that a *CBF*-like pathway occurs in rice. However, an intriguing aspect of *OsICE* is the fact that it is transcriptionally regulated in rice while in *Arabidopsis ICE1* is regulated post-translationally by phosphorylation (Chinnusamy et al., 2003). The reason for such a striking contrast cannot be explained from the current data.

Although not much biological data are available on the C3HC4-type zinc finger protein among the homologous group, a RING-finger domain containing protein (HOS1)

in *Arabidopsis* has been implicated as a negative regulator of plant cold responses (Dong et al., 2006). In this fairly recent study, Dong et al. present evidence that HOS1 acts as an E3 ligase that is required for the cold-induced degradation of ICE1. This HOS1 contains a RING domain that is very similar to the C3HC4-type RINGv domain identified in this study except the first Cys is a Leu (Lee et al., 2001). Despite the variant Cys residue, the RING domain of HOS1 was shown to be functional and is essential for HOS1 function as an E3 ligase.

Lastly, the results of this study support the current theory about the role of ABA in cold stress response signaling. Current results suggest that the early branch of the cold stress response pathway of rice is likely to be independent of ABA-mediated regulation or at least ABA is probably not absolutely required for early gene expression. Apart from the two positive control genes that are known to be ABA-inducible (germin-like protein and *salT*), none of the representative Group-I and Group-II genes examined by RNA gel-blot turned out to be induced by ABA at least during the first 24 hours. Interestingly, two of the four early response gene promoters (high mobility group protein, LOC_Os06g51220 and ATP sulfurylase, LOC_Os03g53230) that were analyzed *in silico* appeared to contain sequence motifs characteristic of an ABA-responsive element (ABRE). It is possible that the ABREs predicted *in silico* are false positives. Additionally, it is also possible that the ABA-mediated regulation of the Group-I and Group-II genes is dependent upon the interaction of an ABRE-binding factor induced by ABA and another transcription factor that is induced by cold but not ABA at a later stage of cold stress. Further experiments are required to address these hypotheses. It is also interesting to point out that copies of the as1/ocs-like *cis*-element were present in all but one of the group-II genes examined. This *cis*-element is a known enhancer of H_2O_2-mediated gene expression in plants. Among the genes that are downregulated early by cold stress, is a peroxidase (data not shown). It can therefore be hypothesized that H_2O_2 rather than

ABA could be involved in the regulation of the early cold stress response transcriptome of rice. H_2O_2 has been shown to be a potential secondary messenger of abiotic stress response signaling in plants (De los Reyes and McGrath, 2003; Kovtun et al., 2000).

While the *CBF* regulon is thought initially to be the centerpiece of the low temperature genetic networks, not only in plants that acclimate to freezing but also in chilling-sensitive plants like rice that do not cold-acclimate, increasing evidence based on global or semi-global transcript profiling experiments indicates that different regulatory pathways may also be operational in non-acclimating plants. Hence, the composition of the cold stress genetic network in rice may not be completely inclusive of the cold acclimation regulon in *Arabidopsis*. For example, at least one of the early response transcription factors identified in this study (OsbZIP) has not been identified as part of the cold acclimation circuit in *Arabidopsis*. However, understanding the exact similarities and differences between cold acclimation (*Arabidopsis*) and chilling stress adaptation (rice) will improve as whole genome transcriptome analysis becomes available in the near future. The interpretation of the transcriptome data in this study should provide a robust foundation for more detailed analysis of the critical components of the early cold stress response genetic network of rice. Future studies should be focused on validating the interpretations of the current transcriptome data using a multidisciplinary functional genomic approach that includes mutation and overexpression studies, promoter deletion-reporter assays, protein-DNA interaction and comparative genomics. Finally, the identification of a number of hypothetical or unknown proteins as cold-regulated should contribute to the annotation of these genes and should set the stage for functional characterization.

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