IDENTIFICATION OF OSSPX1 INTERACTING PROTEINS AND THEIR FUNCTION IN LOW TEMPERATURE TOLERANCE IN ARABIDOPSIS THALIANA

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Identification of OsSPX1 Interacting Proteins and Their Function in Low
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Esta tesis se la dedico a mis padres, Edgar y Margarita, mi hermana Takhinsha, y a todas las demás personas que incondicionalmente apoyaron mis decisiones académicas durante mis estudios de bachillerato y posgrado. Sus palabras de inspiración y apoyo fueron lo que me hicieron seguir adelante. Sin ustedes no hubiese podido realizar este viaje. Gracias, nos vemos en la cima.

DEDICATION

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ABSTRACT OF THE THESIS

Identification of OsSPX1 Interacting Proteins and Their Function in Low Temperature Tolerance in Arabidopsis thaliana
by
Edgar Igor Campos
Master of Science in Biology with a Concentration in Microbiology
San Diego State University, 2013

Climate fluctuation represents a major threat to crops that are sensitive to low temperatures. The importance of developing crops with the ability to tolerate low temperatures can potentially save millions from famine. Understanding the mechanisms of in planta signaling pathways in response to low temperature stress can lead to the improvement of crop traits. I propose to study a recently discovered gene from rice, OsSPX1. This gene, when over-expressed, leads to enhanced low temperature tolerance in tobacco and Arabidopsis thaliana. However, the molecular mechanisms by which OsSPX1 functions during cold stress response remains elusive. Especially, at the protein level, it is not known which proteins are interacting with OsSPX1. Protein interactions are essential components in signal transduction. Therefore, the main goal of this project was to understand the molecular and physiological roles of OsSPX1 in the cold signaling pathway, and to identify potential OsSPX1 interacting proteins using in vivo and in vitro approaches.
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CHAPTER 1

INTRODUCTION

SIGNIFICANCE

Worldwide climate fluctuations pose detrimental effects on our food supply (Rosenzweig and Parry, 1994; Tilman, 1999). The understanding of the plant cold stress signaling pathway is of great importance for the development of low temperature resistant crops such as corn, wheat, and rice (Sanghera et al., 2011). Gene manipulation has been proven to increase low temperature tolerance. A recently discovered rice gene, OsSPX1, was found to enhance cold tolerance when over-expressed in tobacco and Arabidopsis thaliana (Zhao et al., 2009). Due to its potential in crop trait improvement, I chose to study OsSPX1 in order to further understand the function of OsSPX1 during low temperature stress tolerance.

LOW TEMPERATURE STRESS

There are different types of abiotic stresses that affect plants. Some examples include heat or freezing temperatures, drought, and salinity (Verslues et al., 2006). The ability of plants to cope with a given abiotic stress is crucial for its adaptation and survival (Fedoroff, 2010). The plant abiotic stress response is composed of complex signaling pathways and networks that ultimately induce the expression of specific stress-related genes (Seki et al., 2003). Low temperature stress response is species specific and can be defined as chilling (0°C to <15°C), or freezing (<0°C) (Sharma et al., 2005). During cold stress, the signaling cascade is proposed to start within the plasma membrane (Xiong, Schumaker, et al., 2002), followed by an initial surge in cytosolic calcium levels (Knight, 2000), increase of reactive oxygen species (ROS) (Okane et al., 1996) and possibly the activation of membrane proteins such as GPCRs (G-Protein Coupled Receptors) (Tuteja, 2009; Yadav et al., 2012). The transcriptional activation of COld-Responsive (COR) genes, leading to the plant cold acclimation and tolerance (Lee et al., 2002; Teige et al., 2004) is mediated through an extensive protein-protein interaction network. For instance, the initial surge of calcium can activate calcium-responding or binding proteins including Calcium-Dependent Protein
Kinases (CDPKs), Calcineurin B-like proteins (CBL), CBL-Interacting Protein Kinases (CIPK), Calmodulins (CaMs), and Calmodulin-like (CMLs) (Li and Komatsu, 2000; Cheong et al., 2003; Reddy et al., 2011; Xiang et al., 2007). Activation of GPCRs and other membrane receptors can turn on the activities of proteins such as phospholipid C (PLC), phospholipid D (PLD), that are involved in phospholipid signaling (Dixit and Jayabaskaran, 2012). The transcriptional activation of COR (COld-Related) genes has been shown to be regulated by a number of pathways. The two most extensively studied pathways are the Abscisic acid (ABA)-dependent pathway, and the ABA-independent pathway. The ABA-dependent pathway results in a direct activation of ABA-Transcription Factors that can bind to the ABRE elements (ACGT-containing ABA response elements), and subsequently activate the COR gene expression (Xiong, Lee, et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). The ABA-independent pathway is quite complex too, and involves protein phosphorylation, sumoylation, and the regulation of CBF transcription factor genes. The CBF transcription factors bind to the CRT/DRE cis-regulatory elements, leading to the activation of COR genes (Zarka et al., 2003; Agarwal et al., 2006; Chinnusamy et al., 2010; Lata and Prasad, 2011).

In Arabidopsis, it was shown that many pathways involved in cold stress response are also involved in plant responses to other abiotic stresses (Chen, 2012). In addition, cross talk among different signal-transduction networks is also common, and reflects the complexity of molecular mechanisms during abiotic stress responses. The understanding of the signaling networks during low temperature stress is crucial for the development of cold and freezing resistant crops in the future.

**OsSPX1: The SPX Domain, Phosphate Homeostasis, and Low Temperature Stress**

Phosphorous, in the form of inorganic phosphate (Pi), is an essential nutrient for plant growth and development (Schachtman et al., 1998). The sensing, and signaling of inorganic phosphate by specific proteins, as well as phosphate homeostasis, is crucial for the plant survival.

Studies of the SPX domain-containing proteins of the yeast PHO regulon (Secco, Wang, Shou, et al., 2012) revealed that these proteins were involved in inorganic phosphate homeostasis (Barabote et al., 2006). In plants, proteins containing the highly conserved SPX
domain have been found to be involved in inorganic phosphate signaling (Rouached et al., 2010). The SPX domain, named after the discovery of the yeast Syg1: Suppressor of yeast gpa1; Pho81: Phosphatase 81; and the human Xpr1: Xenotropic and Polytropic Retrovirus receptor 1, is about 180-residue long. The plant SPX-domain containing proteins are categorized into 4 distinct families: class 1 contains a single SPX domain only, class 2 contains an SPX-EXS domain, class 3 contains an SPX-MFS domain, and class 4 contains an SPX-RING domain (Secco, Wang, Arpat, et al., 2012). In Arabidopsis, the class one family contains genes such as AtSPX1 (At5g20150), AtSPX2 (At2g26660), AtSPX3 (At2g45130), and AtSPX4 (At5g15330). The AtSPX1 and AtSPX3 genes were found to be involved in the positive regulation of inorganic phosphate signaling under the phosphate limiting conditions (Duan et al., 2008). In rice, the class 1 family is composed of OsSPX1 (Os06g40120), OsSPX2 (Os02g10780), OsSPX3 (Os10g25310), OsSPX4 (Os03g61200), OsSPX5 (Os03g29250), and OsSPX6 (Os07g42330). The OsSPX1 gene has been found to be involved in phosphate signaling under inorganic phosphate limiting conditions, as well as in the tolerance to cold stress (Z. Y. Wang et al., 2009; Zhao et al., 2009); OsSPX3 has also been shown to be involved in inorganic phosphate starvation. However, its involvement in cold tolerance has not been reported (Z. Y. Wang et al., 2009). The class 2 (SPX-EXS), class 3 (SPX-MFS), and class 4 (SPX-RING) families are also involved in the homeostasis of inorganic phosphate. For instance, the rice OsPHO1;2 (Os02g56510) gene from class 2 is involved in root to shoot inorganic phosphate transfer (Secco et al., 2010); the rice OsSPX-MFS1 (Os04g48390) gene from class 3 is involved in inorganic phosphate homeostasis in the leaves (C. Wang et al., 2012), and the Arabidopsis AtNLA (At1g02860) gene from class 4, has been shown to regulate inorganic phosphate under limiting nitrogen conditions (Kant et al., 2011).

Ion homeostasis is crucial for the survival of plants during low temperature stress. Evidence for the correlation between phosphate levels and low temperature tolerance was demonstrated in a study of the Arabidopsis mutant pho1-2, which contains low shoot phosphate level, and the mutant pho2-1, which contains high shoot phosphate (Hurry et al., 2000). This study showed that low phosphate levels (pho1-2) resulted in the metabolic and enzymatic changes that correlates with an enhanced cold acclimation. Interestingly, over-expression of OsSPX1 was shown to enhance plant’s low temperature tolerance and resulted
in decreased level of total leaf inorganic phosphate in tobacco leaves (Zhao et al., 2009). This further suggests that the enhanced cold stress tolerance might be mediated by the SPX domain-containing proteins through reducing the inorganic phosphate level. However, it is yet to be discovered if the interaction of both, the inorganic phosphate molecule and the SPX-containing protein, is the result of a direct interaction between the SPX domain and inorganic phosphate; or by indirect interaction, were a mediator or multiple proteins are required for inorganic phosphate homeostasis.

**REGULATION OF SUCROSE AND OTHER OSMOLYTES PRODUCTION DURING LOW TEMPERATURE STRESS**

Upon abiotic stress, plants accumulate osmolytes such as sugars (e.g., sucrose), sugar-derived alcohols, amino acids (e., free proline), and quaternary ammonium compounds (e.g., glycine betaine) (Hare et al., 1998). Regulation of osmolyte production during water stress ensures proper macromolecular structure and function inside the cell (Yancey et al., 1982). Cold stress can cause major damage to the plant cell, if not acclimated. The plant cell membrane is vulnerable to freezing stress due to possible phospholipid bilayer destabilization (Steponkus et al., 1988), which can result in water leakage and osmotic stress. In order to restore osmolyte balance, it is essential for the plant cell to increase osmolyte production (Yamazaki et al., 2009). In addition, plant cells also make changes in lipid composition (Steponkus et al., 1988) in order to reduce freezing damage.

There is evidence suggesting that the SPX-domain containing proteins also play a role in osmolyte production. Besides the reduced accumulation of inorganic phosphate in leaves, the OsSPX1 over-expressing transgenic tobacco plants also displayed significantly higher levels of sucrose and free proline than those found in wild-type plants after cold stress (Zhao et al., 2009). Interestingly, under the limiting inorganic phosphate conditions, it was found that plants accumulate sugars and other carbon sources in their leaves (Hammond and White, 2011). Thus, SPX-domain containing proteins, such as OsSPX1, might be important signal transduction molecules involved in inorganic phosphate sensing, signaling and homeostasis. Although it is clear that OsSPX1 over-expression affects osmolyte and inorganic phosphate levels, which correlates with enhanced cold tolerance, the molecular mechanisms are not yet completely understood.
**PROTEIN IDENTIFICATION**

To understand the role of OsSPX1 in the cold signaling pathway, it is critical to identify proteins that potentially interact with OsSPX1. In order to ensure the success of identifying OsSPX1 interacting proteins, we employed two approaches in detecting protein-protein interaction in this study. The first approach was a yeast two-hybrid system. The advantage of this system is that it allows for a high-throughput screening of a large number of colonies from a cDNA library (Mohr and Koegl, 2012). The cDNA library is generated from a reverse transcription of all the messenger RNAs expressed at a specific time point, and/or under a specific condition in the cell. In a normal cell, there are mechanisms that prevent or select transcripts from being translated and becoming proteins (Carey and Smale, 2000). However, generating a cDNA library in a yeast two-hybrid system allows for selective expression of all transcripts under the proper induction system (refer to Clontech “Mate & Plate™” library system manual for review). Since the individual cDNA transcripts expressed (in vivo) in the yeast cell will be translated into proteins, the use of a bait protein can help identify potential interacting proteins. These protein interactions can be identified when the physical interaction between an activation domain (AD) protein-fusion (e.g., cDNA transcript from library) and a binding domain (BD) protein-fusion (e.g., OsSPX1), are close enough to bring both AD and BD domains into proximity, resulting in reporter gene activation (e.g., LacZ and LEU2).

The second identification system we used was the Tandem Affinity Purification (TAP) method, coupled with mass spectrometry (LC-MS/MS). This system relies on the use of epitope tags for single or sequential purification of protein complexes from whole cell extracts (Van Leene et al., 2008). Under appropriate conditions, the proteins present in the cell extract form a protein-complex (in vitro) with the protein of interest. The protein complex can then be purified from a cell extract by the use of specific antibodies against the epitope tags (e.g., Flag-, HA-, GST-tag, etc.), which is fused with the protein of interest. After the purification of the protein complex, liquid chromatography, coupled with mass spectrometry (LC-MS/MS) is used to identify the components in the protein complex.
CHAPTER 2

EXPERIMENTAL METHODS

This chapter contains a description of the experimental methods I used to conduct my research. This includes methods for characterization of rice OsSPX1 in Arabidopsis thaliana, protein-interaction and identification studies in both in vivo and in vitro assays.

CONSTRUCT DESIGN AND CLONING

The rice OsSPX1 gene, in the pCOU binary vector, was kindly provided by Dr. Zhen Su laboratory (State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China). The OsSPX1 gene was fused to an N-terminus TAG (3XFlag-2XStrep) and was mobilized into the pCHF3 plant transformation vector. Supporting constructs were also generated using similar cloning methods such as the one listed above. The constructs generated were pCFH3::Nter-TAG (vector only), pCHF3::Nter-OsSPX1, and pCFH3::Nter-AtSPX1. These constructs were transformed into DH5α heat shock competent cells. DNA plasmid minipreps were conducted using Fermentas GeneJet™ Plasmid Miniprep kit (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturers’ instructions. Before plasmid DNA transformation into agrobacterium competent cells, all samples were digested to check for presence of fragment, and then sequenced using forward and reverse gene primers (Appendix A). Confirmed DNA plasmid miniprep constructs were transformed into electro-competent Agrobacterium GV3101 strain using a Bio-Rad GenePulser II Electroporator™ (Hercules, CA, USA) (9 msec at 2.47 kV, 400 Ω, and 25 μF). Transformation reactions were spread in LB + Spec [100 μg/μl] + Gent [25 μg/μl] and incubated at 30°C for 2-3 days.

AGROBACTERIUM MINIPREPS

Colonies from LB +Spec +Gent plates were tested for the presence of the pCHF3 plant transformation vector, insert (OsSPX1 or AtSPX1), and agrobacterium (GV3101). A total of 3 colonies per pCHF3 construct were inoculated overnight on 3 mls of LB +Spec + Gent medium at 30°C. Next day, inoculums were pelleted twice and their supernatant
removed each time. Pellets were resuspended in 100 µl of MPS1 solution (40mM Glucose, 10 mM EDTA, 25 mM Tris pH=8.0), and incubated at room temperature for 5 minutes. Samples were digested on 20 µl of 20 mg/ml lysozyme solution at 37°C for 15 minutes, and then resuspended in 200 µl of fresh MPS2 solution (5N NaOH, 10% SDS). Right after addition of MPS2, solutions were neutralized with 150 µl of MPS3 solution (5M Potassium acetate, 11.5% glacial acetic acid) and incubated on ice for 5 minutes. Each solution was treated with 400 µl of 25:24:1 Phenol:Chloroform:Isoamyl alcohol, and then after phase separation, the aqueous layer was decanted. This step was repeated again with Chloroform only, and then followed by DNA precipitation with 100% isopropanol. After 70% ethanol wash, pellets were resuspended in a total of 50 µl TE Buffer + RNase.

Agrobacterium minipreps were tested for the presence of pCHF3 binary vector and gene of interest. Polymerase Chain Reactions (PCR) were assembled in 20 µl reactions (2 µl Biopioneer 10X Buffer Mix, 2 µl 25 mM MgSO₄, 2 µl 25 mM dNTP mix, 0.4 µl forward and reverse primers, 0.2 µl Biopioneer Taq pol™, and 11.4 µl H₂O) containing 2 µl of 1/10 dilution of agrobacterium minipreps. The primers used were 5'-35s-Upstream and 3'-rbcs-downstream (Appendix A). The PCR program was set to standard conditions (Denaturation 95°C for 5 minutes, Activation 95°C for 30 seconds, Annealing 55°C for 30 seconds, Extension 72°C at 1 minute/kilobase) for 35 cycles.

**PLANT GROWTH**

All *Arabidopsis thaliana* genotypes’ seeds were vernalized at 4°C for 3 days. Seeds were sown in 2 ¼” or 3 ½” pots containing Metro-Mix® 360 soil (Sungro™, Seba Beach, Canada) and grown for 2 to 3 weeks in a Percival LT-36VL (Geneva Scientific, Fontana, Wisconsin) growth chamber at 22°C under a 16/8 photoperiod with an average light intensity of ~100 microeinsteins (m²·sec⁻¹). These conditions are defined hereafter as “regular growth conditions.” Plants were watered every other day, and pots were randomized to prevent slow germination or growth retardation.

**PLANT TRANSFORMATION**

All pCHF3 constructs were confirmed via PCR and used to generate plant transformants on either Wild-Type (WT) or mutant *Arabidopsis thaliana* backgrounds (atspx1/039445c) using the Agrobacterium-mediated transformation method (Clough and
Bent, 1998). Plant transformation was performed as described in (Toki et al., 2006) with minor modifications.

**FREEZING EXPERIMENT**

This protocol represents a modified version of Tsutsui et al. (2009) freezing experiment. Two-week-old seedlings were grown in regular growth conditions in ½ MS plates containing 2% sucrose plates. Plates were transferred to a plastic tray and covered with crush ice. The growth chamber was stabilized for 10 to 20 minutes at -1°C with the lights set to off. After the growth chamber temperature was stabilized at -1°C, the tray with ice-covered plates was moved into the growth chamber and left 1 hr until temperature was normalized across all plates. After 1 hour at -1°C, the growth chamber’s temperature was programed (96 step) to decrease by 2.4°C per hour until -8°C. The growth chamber temperature was set to manual (-8°C) after 3 to 4 hours to prevent cycling of the 96-step program. Seedlings were treated at -8°C for 48 hours to 72 hours, depending on the experiment. After the freezing treatment period, the tray with ice-covered plates was thawed in the dark at 4°C for 12 hours. The growth chamber was set back to regular growth conditions, and seedlings were left to recover for 24 to 48 hours or until complete recovery.

**DROUGHT EXPERIMENT**

Four to five week-old plants were grown at regular growth chamber conditions in pots with Metro-Mix® 360 soil (Sungro™, Seba Beach, Canada). Plants were randomized every other day and watered once per week. Two days before the experiment, pots were visually examined for soil humidity. Plants were then deprived from water for two weeks. During the course of the experiment, pots were randomized to prevent positional effects. After 1.5 weeks, pots and plants were assessed for complete dryness of the soil and plant tissue. Plants were recovered by watering (approximately 50 ml H₂O/pot); and assessed thereafter for drought stress recovery after 6 hours and 24 hours.

**PHOSPHATE ASSAY**

This phosphate assay protocol was optimized for *Arabidopsis thaliana* plant tissue according to Mercan and Bennett (2010) phosphate determination methods. Plants were grown in 3 ½” pots with a mesh-covered soil dome. After 2.5 to 3 weeks of growth in regular
growth chamber conditions, plants were cold treated at 4°C for 48 hours. Leaf tissue was harvested (approximately 6 leaves/plant) and flash frozen in liquid nitrogen at 0-hour and 48-hour time points. Frozen samples were homogenized in liquid nitrogen for approximately 5 minutes using a pestle and mortar. Aliquots of 50 mg (+7 mg, -2 mg) of tissue were made and quickly stored at -80°C in 1.7 ml eppendorf tubes.

Plant extracts were prepared from homogenized tissue aliquots. A total of 8-10 samples were taken out from the -80°C freezer to start the first round of extract preparation. Samples were mixed with 1 ml of 0.083 M H$_2$SO$_4$ (sulfuric acid), and then incubated at room temperature for 10 minutes. A timer was started after addition of sulfuric acid to the last sample of each extract round. Reaction was stopped after 10 minutes, by the immediate addition of 33.2 µl of 5 N NaOH (sodium hydroxide) followed by quick vortexing. Neutralized samples were centrifuged at 12000 x g for 10 minutes at 4°C. Clear supernatants were carefully removed without disturbing the plant tissue pellet. A 750 µl aliquot from the clear supernate was transferred to a clean eppendorf tube, and clarified by centrifugation at 12000 x g for 10 minutes at 4°C. A final 500 µl aliquot of clear supernatant was transferred to a clean eppendorf tube, and stored at room temperature (12 hours max). The same above process was repeated multiple times in rounds of 8-10 samples max.

The Malachite Green (MG) Assay was used to measure soluble inorganic phosphate from plant extracts. The Malachite Green Reagent (MGR) was made fresh by preparing a 1:1:2 solution of MG1 (0.135 % malachite green oxalate salt in water), MG2 (4.2 % w/v ammonium molybdate in 4 N HCl), and 10 % Tween r 20. The MGR solution was ready to use after incubating at room temperature for 5 minutes followed by another 5 minutes on ice. Phosphate standards were prepared by making 7 different phosphate concentrations in nanomoles of phosphate in increments of two (1, 2...10, 12) using a stock solution of 100 µM of K$_2$HPO$_4$.

Plant extracts to be measured for soluble inorganic phosphate were prepared on disposable plastic cuvettes. Sample duplicates (2 cuvette/sample) were prepared by adding a 2/15 dilution of plant extract (20 µl) and ultrapure water (130 µl) to each cuvette. A timer was started (set to 10 minutes) after adding 850 µl of MGR solution to each cuvette. Absorbance was measured after 10 minutes at 650 nm using a spectrophotometer (Unico S1200™, New Jersey, USA).
**Sucrose Assay**

This sucrose assay was optimized for *Arabidopsis thaliana* tissue and performed as described by Vanhande (1968) with minor modifications. Plants were grown in 3½” pots with a mesh-covered soil dome. Plants were grown for 2.5 to 3 weeks at regular growth chamber conditions, and subsequently cold treated at 4°C for 48 hr. Leaf tissue was harvested (approximately 6 leaves/plant) and flash frozen in liquid nitrogen at 0-hour and 48-hour time points. Frozen samples were homogenized in liquid nitrogen for approximately 5 minutes using a pestle and mortar. Aliquots of 100 mg (+5 mg, -5 mg) of tissue were made and quickly stored at -80°C in 2.5 ml eppendorf tubes.

Arabidopsis tissue extracts were prepared by adding 1 ml of 80% ethanol per 100 mg of tissue powder. Samples were incubated for 2 hours at 80°C with brief vortexing every 15 minutes. After incubation, all samples were clarified by centrifugation at 14000 rpm or max speed. Supernatants were decanted into new tubes and vacuum dried to evaporate ethanol solution. Dried tissue extracts were resuspended in 500 µl of ultrapure water, and saved at room temperature or 4°C until soluble sugar determination.

Tissue extracts were hydrolyzed for sucrose determination. Aliquots of 100 µl were made for each stock extract (500 µl resuspension), and then combined with 100 µl of 30% KOH (potassium hydroxide) solution. Samples were hydrolyzed by incubation at 100°C for 10 minutes followed by cooling at room temperature. Extracts were kept at room temperature until addition of anthrone solution. A stock solution of 1% w/v sucrose in water was used to make a standard curve with 5 standards. Each standard corresponded to an amount of micrograms of known sucrose present (20 µg, 40 µg...100 µg) in a final volume of 100 µl of water.

A solution of 0.2% anthrone in 98% v/v sulfuric acid [20.4 N] was prepared on ice to increase solubility. Aliquots of 100 µl of hydrolyzed extract were decanted from the original 200 µl hydrolyzation reaction (100 µl stock + 100 µl 30% KOH), and combined with 1.5 ml of anthrone solution per sample. The 1.6 ml anthrone reaction samples were incubated for 15 minutes at 40°C. A total of 1 ml of each reaction mixtures was transferred to disposable plastic cuvettes, after incubation. Samples, including sucrose standard curve, were measured at 620 nm using a spectrophotometer (Unico S1200™, New Jersey, USA).
HEAT MAP CONSTRUCTION

Rice GeneChip® Microarray data generated by Dr. Zhen Su’s laboratory in China (State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China). The microarray data were obtained from wild type rice (*Oryza sativa japonica* (Nipponbare) and two independent *OsSPX1*-antisense rice lines treated at 4°C for 24 hours. Experimental signal data in the form of tab-delimited files (.txt) was median-based normalized and filtered against low intensity probesets (<50) using the “max” excel function. Data files were separated into three different analyses (7 comparisons total): WT-Cold/WT-Control, A1-Cold/A1-Control, and A2-Cold/A2-Control samples; A1-Control/WT-Control, and A2-Control/WT-Control samples; A1-Cold/WT-Cold, and A2-Cold/WT-Cold samples. Each comparison was split into two files, one for 2-fold up-regulation and another for 2-fold down-regulation. A total of 14 comparisons were generated.

A single database file (.accdb) was generated in Microsoft Access 2010 (version 14.0.6129.5000, 32-bit) using the “union” query to combine all 14 excel tables by removing redundant genes (57,308 total genes). The database file was exported as an excel file, and Affymetrix™ internal control genes were removed (24,566 total genes). The database was further filtered by first comparing the control antisense lines ratio (A1-Control/A2-Control) and keeping genes in the range of 1.9 fold up-regulation and 0.6 fold down-regulation. This generated a spreadsheet of 18,202 probesets. To this list, a comparison was made of the cold antisense lines ratio (A1-Cold/A2-Cold), and probesets with expression ratio higher than 1.9 fold and lower than 0.6 fold were kept. This generated a database of 13,260 genes.

The new data file (13,260 genes) was adjusted and log transformed using Cluster version 2.20 (Eisen et al., 1998). The settings “Eweight and Gweight” were set to 1, “log transform” was selected, “mean center” was selected (with “log transformed” deselected). The “self-organizing map” (SOM) was generated by reloading the new adjusted file into Cluster and by selection the option “YDIM” (software defined groups). A new text file was generated, and was further adjusted in Excel by setting GWEIGHT and EWEIGHT values equal to 1. A final CDT file was generated by re-loading the excel adjusted file into Cluster and selecting the option “average linking clustering.” The CDT file was loaded into TreeView 1.60 (Eisen et al., 1998) software to generate a hierarchical clustering analysis heat map of 13,260 genes.
**Rice Multiple-Time-Point Cold Experiment**

Rice was initially vernalized at 35°C for 48 hours in the dark, and then moved to a growth chamber set to short day 12 hour -12 hour photoperiod; 8 am day start with 28°C temperature and 80 % RH; 8 pm day end with 25°C temperature, lights off, and 70 % RH. Rice seedlings were grown for 5 days after germination (DAG). Day 1 after germination was right after 48 hours of vernalization.

At DAG #5, seedlings from two rice jars were randomly collected and labeled as 0-hour time point and flash frozen in liquid nitrogen. The growth chamber temperature was then set to 4°C diurnal (short day), and rice was treated for 5 days and collected at 6-hour, 24-hour, and 5-day time point. Both leaf and root tissue were harvested and flash frozen in liquid nitrogen. Approximately 50-100 mg of tissue aliquots was used for protein and RNA extraction.

**RNA Extraction and DNaseI Treatment for qPCR Experiments**

RNA extraction of rice tissue samples was prepared according to TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) manual specifications (Chomczynski, 1993). After RNA extraction, samples were treated with Ambion® recombinant DNaseI, rDNaseI, (Life Technologies, Carlsbad, CA, USA) to remove genomic DNA contamination according to manufacturers specifications.

RNA quantity was assessed with a GeneQuant II™ RNA/DNA (Pharmacia Biotech, Piscataway, NJ, USA) spectrophotometer. Visual confirmation of RNA integrity (quantity and quality) was assessed in a formaldehyde-based agarose denaturing gel with ethidium bromide (1.5 % agarose in 40 mls of 1X MOPS-EDTA containing 9 ml formaldehyde and 3 µl of ethidium bromide). The presence of sharp and equal intensity bands from 28s and 18s rRNAs across all samples was evidence for good RNA integrity.

**cDNA Preparation of Rice Cold Experiment Samples**

A reverse transcriptase reaction was used to prepare cDNA from rDNaseI-treated RNA (2 µgs) from rice samples. Reactions were assembled according to Thermo Scientific RevertAid™ reverse transcriptase (#EP0441) (San Diego, CA, USA) manufacturers.
specifications. For the cDNA reaction mix, Oligo-dT<sub>18</sub> (RT-008) and RNase Inhibitor (C0024) (BioPioneer, San Diego, CA, USA) from a different manufacturer was used.

**Real-Time Quantitative PCR of Rice Cold Experiment**

Genes to be analyzed for Real-Time Quantitative PCR (RT-qPCR) expression were assembled in MicroAmp® Fast Optical 96-well reaction plates (Life Technologies, Carlsbad, CA, USA) or in Avant TempPlate 96-well semi-skirt (USA Scientific, Ocala, FL, USA) reaction plates. The plate design consisted in a standard dilution series with duplicates (1/4, 1/40, 1/400, 1/4000), a template negative control (reaction mix only), and a set of triplicate samples in a 1/40 dilution. The 96-well plates were loaded with 5 µl of each sample dilution, and 20 µl of reaction mix. The SYBR® green fluorescent dye mix (with ROX™ dye) used was composed of 10X qPCR Super Mix (BioPioneer, San Diego, CA, USA), 5.5 µl of ultrapure water, 1 µl forward primer, and 1 µl reverse primer. The real-time PCR instrument used was StepOne™ from BioPioneer (San Diego, CA, USA), and CFX96™ from Bio-Rad (Hercules, CA, USA). The amplification program was set to manufacturers specifications (35 cycles and 25 µl reaction volume). The endogenous control used to normalize gene expression data was 18s rRNA (forward primer, 5’-CGGCTACCACATCCAGGAA-3’; reverse primer, 5’-TGTCACTACCTCCCCGCTGTA-3’). The qPCR primers for LOC_Os07g43670 (forward primer, 5’-CTGTTCCAGGTACCAGTG-3’; reverse primer, 5’-GTGGGCAGCTTGACCTT-3’) and LOC_Os12g43380 (forward primer, 5’-GCTACAACGTCGCCATGA-3’; reverse primer, 5’-CTGCAGGCGTGTGTCTT-3’) were designed according to IDT’s PrimerQuest© software specifications (www.idtdna.com/PrimerQuest). The relative quantification of gene expression analysis was calculated using the comparative C<sub>T</sub> method (ΔΔ C<sub>T</sub> Method) (Schmittgen and Livak, 2008). Primer efficiency was calculated using the arithmetic formula E=(10<sup>-1/slope</sup>-1)x100.

**Rice Tissue Protein Extracts and Quantification**

Approximately 300-500 mg of tissue powder (previously homogenized in liquid nitrogen with mortar and pestle) was used to prepare plant-protein extracts. Samples with plant tissue powder were resuspended in a 1:1 ratio of packed volume to SII buffer (100 mM NaPhosphate, pH 8.0; 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, and 0.1 % Triton X-100).
The tissue powder was resuspended slowly by rotation at 4°C for 10 minutes. Samples were kept on ice, and then two rounds of sonication were conducted at 10% power for 10 seconds (0.5 seconds intervals) using a Fisher Scientific Model 505 Sonic Dismembrator (Hampton, NH, USA) with a 1/8” tapered microtip. Sample extracts were clarified twice by centrifugation at 20,000 x g for 10 minutes at 4°C. Small aliquots were made from the clarified extracts, and the remainder was stored at -80°C to prevent protein degradation.

Protein concentration from the rice tissue extracts was determined with the Bradford protein assay (Bradford, 1976). Protein concentration of rice tissue samples and BSA (Bovine Serum Albumin) standard curve were determined according to Fermentas Bradford reagent (#R1271) manufacturers specifications (Thermo Scientific, San Diego, CA, USA).

**SDS-PAGE AND WESTERN BLOT ANALYSIS**

Protein samples were normalized and prepared for gel electrophoresis. The amount of protein loaded for each sample was normalized and diluted in 1X PBS buffer to a final volume of 15 µl. Protein samples were mixed with 4 µl of 5X SDS-PAGE loading buffer and 1µl of 2 M DTT. Samples were boiled for 5 minutes in a water bath, then loaded into handcast 10 % or 12 % SDS-PAGE gels assembled on SDS-PAGE electrophoresis running cassettes containing 500 ml 1X SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS). The SDS-PAGE gels were run at 94 volts for 2 hours in a Bio-Rad PowerPac™ HC Power Supply (Hercules, CA, USA).

After SDS-PAGE gel electrophoresis, transfer buffer and samples were prepared for western blot transfer according to Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell instruction manual specifications (Hercules, CA, USA). Proteins were transferred to a Thermo Scientific Nitrocellulose Membrane (#88018) (San Diego, CA, USA) at 100 volts for 1:15 hour on ice-cold transfer buffer with constant stirring. After incubation, nitrocellulose membranes were incubated in 1:2000 anti-FLAG® M2 (Sigma-Aldrich, St. Louis, MO, USA) blocking solution (10 ml per membrane). Primary hybridizations were put on a rocker shaker and incubated overnight at 4°C in the dark. The following day, membranes were incubated with 10 mls of fresh 5% blocking solution with 1:2000 Goat anti-mouse IgG (H+L) HRP (Millipore, Temecula, CA, USA) for 1:30 hour on a rocker-shaker incubator at room temperature. After secondary antibody hybridization, nitrocellulose membranes were
washed 3x with 1X TBS + 0.05% Tween 20, and then rinsed 5x with DI water. After washing, membranes were soaked for 1 minute with 10 ml of ECL reagent (100 mM Tris-HCl pH 8.5, 1.25 mM Luminol, and 0.198 mM p-Coumaric acid) with 2 µl of 30% H2O2 for chemiluminescence substrate activation. Membranes were brought to the developer room where they were exposed to X-OMA™8x10 inches autoradiography film (Kodak, Rochester, NY, USA) in the range of 20 seconds to 1 minute depending on the nature of the protein sample and concentration. For some of the nitrocellulose membrane transfers, a Pierce Reversible Protein Stain (Thermo Scientific, San Diego, CA, USA) was conducted according to manufacturers specifications to determine protein transfer efficiency before primary antibody hybridization.

**SDS-PAGE and Silver Staining Analysis**

SDS-PAGE gels of TAP-tagging protein samples were silver stained before sending the samples for mass spectrometry analysis. Protein gels were silver stained according to Sigma-Aldrich silver stain kit for proteins (St. Louis, MO, USA) manufacturer’s specifications.

**Yeast Two-Hybrid of Proteins Identified by TAP-Tagging Coupled with Tandem Mass Spectrometry (LC-MS/MS)**

Proteins identified from Arabidopsis’ TAP-Tagging experiments were subjected to protein-protein interaction analysis using a yeast two-hybrid system. The yeast strain EGY-48 and vectors, pB42AD+Trp, pB42AD-T+Trp, pLexA+His, pLexA-53+His, pLexA-pos+His, pLexA-lam+His, and p80p-LacZ+Ura were kindly provided by Dr. Christopher Glembotski from San Diego State University. The OsSPX1 gene (Os06g40120) was subcloned into the pLexA+His vector; the rest of the genes, ERD2 (At1g56410), BIP2 (At5g42020), Zinc Finger (At5g24870), CORI3 (At4g23600) and phosphatidylinositol 3,4 kinase (At1g64460) were PCR amplified from rice cDNA of cold treated tissue using gene specific primers (Appendix A) and subcloned into the pB42AD+Trp vector.

All yeast transformations into EGY-48 yeast strain were conducted according to Matchmaker™ LexA Two-Hybrid System (#K1609-1, p.38) transformation protocol (Clontech/Takara Bio, Otsu, Japan). The p80p-LacZ+Ura vector was first transformed into
EGY-48, and selected on SD (-Ura) plates (SD= Synthetic dropout media). Following the generation of EGY-48 [p80p-LacZ^+Ura] yeast construct, the vectors pLexA-53^+His, pLexA-pos^+His, pLexA-lam^+His, and pLexA-OsSPX1^+His were individually transformed into EGY-48 [p80p-LacZ^+Ura] and selected on SD (-Ura -His) plates. Yeast transformants were then tested for galactosidase activity as an indicator of LacZ transcription activation. Colonies from EGY-48 [p80p-LacZ^+Ura] + pLexA-OsSPX1^+His and EGY-48 [p80p-LacZ^+Ura] + pLexA-pos^+His were streaked on SD (-Ura –His +1x BU salts +X-Gal 80mg/L) and assessed for formation of blue colonies.

The activation-domain vectors, pB42AD^+Trp (empty), pB42AD+At1g56410^+Trp, pB42AD+At5g42020^+Trp, pB42AD+At5g24870^+Trp, pB42AD+At4g23600^+Trp, pB42AD+At1g64460^+Trp, and pB42AD-T^+Trp were transformed into EGY-48 [p80p-LacZ^+Ura]+pLexA-OsSPX1^+His and selected on SD (-Ura –His -Trp) plates. To test interaction of binding and activation domain (AD), colonies from SD (-Ura –His -Trp) plates were streaked on SD induction plates (Gal/Raf/-His –Leu –Trp –Ura + X-gal 80mg/L +1xBU salts) to test reporter gene activation (LacZ and LEU2) and expression induction of AD fusion proteins. Streaked colonies were grown at 30°C for 24 to 96 hours before assessing for blue-colony formation.

**RICE cDNA LIBRARY SCREENING**

The RNA extraction and mRNA purification was conducted as described below. Rice seedlings used for cDNA library preparation were treated at 4°C for 5 days under regular rice growing conditions (10 day-old at harvesting). A total of 800 mg of rice tissue powder was used to prepare RNA with TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer specifications (Chomczynski, 1993). RNA was quantified with a GeneQuant II™ RNA/DNA (Pharmacia Biotech, Piscataway, NJ, USA) spectrophotometer, and RNA integrity (quantity and quality) was assessed in a formaldehyde-based agarose denaturing gel with ethidium bromide (1.5% agarose in 40 mls of 1X MOPS-EDTA; with addition of 9 ml formaldehyde and 3 µl of ethidium bromide right before gel solidification). Messenger RNA (mRNA) was purified 3X times from 75 µg of total RNA (260/280 ratio=1.8; concentration= 0.721 µg/µl) using Invitrogen Dynabeads®
mRNA purification kit (Life Technologies, Carlsbad, CA, USA) according to manufacturers specifications.

The first strand cDNA synthesis was conducted as described below. The enriched rice mRNA was used to generate first strand cDNA synthesis and amplification by long distance PCR using “Mate & Plate™” library system (Clontech/Takara Bio, Otsu, Japan). According to “Mate & Plate™” manufacturers specifications, a total of 2 µls of poly A+ RNA purified with Dynabeads® mRNA purification kit (Life Technologies, Carlsbad, CA, USA) was used in a 100 µl reaction volume (2 µl first strand cDNA, 70 µl water, 10 µl 10X Advantage 2 PCR buffer, 2 µl 50x dNTP mix, 2 µl 5’-PCR primer, 2 µl 3’-PCR primer, 10 µl 10X melting solution, and 2 µl 50X Advantage 2 polymerase mix). The cDNA was amplified according to the following program: 95°C, 30 sec; 26X (95°C, 10 seconds; 68°C, 6 minutes (+5 sec increments/cycle)); 68°C, 5 minutes; 4°C, hold.

Yeast constructs pGBKT7, pGADT7, and rice cDNA library yeast transformation were constructed as described below. The cDNA library was transformed into yeast strain Y187^Leu according to Yeastmaker™ Transformation System 2 (Clontech/Takara Bio, Otsu, Japan) manual specifications for library scale transformations. Four days after transformation, colonies were resuspended with sterile glass beads, harvested in 5 ml of freezing medium (YPDA broth + 25 % Glycerol), and stored at -80°C. The number of cells per ml was counted with a hemocytometer and calculated to be 54.1x10^7 cells/ml. The number of independent clones, 1.2 million, was calculated by counting the number of colonies from a 1/100 library dilution-spread on a SD (-Leu) plate times the volume of 0.9 % NaCl resuspension.

Generation of pGBKT7 constructs and Y2H gold/Y187 yeast strains transformation were conducted as described below. The OsSPX1 gene was cloned into the pGBKT7 binding domain vector (Appendix A). The control vectors pGBKT7-53^trp, pGBKT7-lam^trp, and pGBKT7-OsSPX1^trp bait were transformed separately into Y2H^trp gold yeast strain. The activation domain control plasmid pGADT7-T^Leu was transformed into Y187^1Leu yeast strain. Transformants’ colonies were saved in their corresponding selective medium (SD, – Leu or –Trp) with 25% glycerol. Transformations were done with Yeastmaker™ Transformation System 2 (Clontech/Takara Bio, Otsu, Japan) and according to manual specifications for small-scale transformations.
Yeast mating of Y2H gold and Y187 rice cDNA library was conducted as described below. Rice cDNA library experiments were conducted according to Matchmaker™ Gold Yeast Two-Hybrid System user manual specifications (Clontech/Takara Bio, Otsu, Japan). A total of four independent rice cDNA library yeast matings were conducted. The only differences were in the mating incubation time (20 hrs to 30 hrs), shaking speed (20 rpm to 50 rpm), and bait-to-library ratio (Appendix B).

Identification of OsSPX1 putative interacting proteins from Y2G mating was conducted as described below. Blue colonies from the rice cDNA library and bait yeast matings were inoculated overnight at 30°C in 2 ml of SD (-Ade –His –Leu –Trp) liquid broth (QDO broth). The following day, minipreps were conducted according to Zymoprep™ Yeast Plasmid Miniprep (Zymo Research, Irvine, CA, USA) manufacturer’s specifications. The resulting yeast plasmid minipreps were transformed into DH5α competent cells, and spread on LB + Amp [100µg/µl] to select for the pGADT7 recombinant vector carrying a cDNA library insert. Resulting colonies from DH5α transformation were inoculated overnight on LB + Amp [100 µg/µl] liquid broth at 37°C, 275 rpm. Minipreps were conducted on overnight inoculums using Fermentas GeneJet™ Plasmid Miniprep kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturers’ specifications. Plasmid DNA minipreps were subjected to a single restriction enzyme digestion with HindIII. The pGADT7-Rec vector contains two HindIII restriction sites flanking the CDS III terminus and SMART™ III terminus region. Once the pGADT7-Rec vectors were confirmed to contain a cDNA insert, minipreps were sequenced at the SDSU Microchemical Core facility using the forward T7 promoter primer (TAATACGACTCACTATAGGGCGA) and the reverse 3’-AD primer (AGATGGTGCACGATGCACAG). Sequencing data, from all plasmid minipreps, was aligned against NCBI’s *Oryza sativa* (rice) nucleotide BLAST database in order to identify matching rice cDNA transcripts.

Testing of putative interacting proteins with OsSPX1 via mating or co-transformation was conducted as described below. Identified rice cDNA transcripts were PCR amplified from rice enriched mRNA (4°C, 5 days) and subcloned into the pGADT7 empty vector using the restriction sites EcoRI and BamHI for Os03g0681900, EcoRI and XhoI for Os06g0646600, and EcoRI and XhoI for Os09g0270900. The pGADT7 constructs generated and pGBK77- OsSPX1 bait were co-transformed into Y2H gold yeast strain using
Yeastmaker™ Transformation System 2 (Clontech/Takara Bio, Otsu, Japan) and according to manufacturers’ specifications for small-scale transformations. Alternatively, pGADT7 constructs were transformed into Y187 yeast strain and mated with pGBKTY-\textit{OsSPX1} in Y2H gold following Matchmaker™ Gold Yeast Two-Hybrid System user manual specifications (Clontech/Takara Bio, Otsu, Japan). Co-transformations or matings were plated on DDO plates, and re-streaked on QDO plates following blue colony formation to confirm protein interaction.

OsINP1 domain dissection was conducted as described below. A domain dissection study was conducted on Os06g0646600 (OsINP1) and OsSPX1. Felis Wolven generated all \textit{OsSPX1} and OsINP1 domain fragments and constructs (pGBK7 and pGADT7) for domain interaction study. The OsINP1 gene was cloned into three different pGADT7 constructs: Original blue colony, ELK-Homeodomain only, and full length. Similarly, the \textit{OsSPX1} gene was cloned into three different pGBK7 constructs: SPX domain only, C-terminus domain only, and full length. Five co-transformation pairs were generated and transformed into Y2H gold: Pair 1, \textit{OsSPX1} full length + OsINP1 ELK-Homeodomain only; pair 2, \textit{OsSPX1} SPX domain only + OsINP1 full length; pair 3, \textit{OsSPX1} SPX domain only + OsINP1 ELK-Homeodomain only; pair 4, \textit{OsSPX1} C-terminus domain only + OsINP1 ELK-Homeodomain only; and pair 5, \textit{OsSPX1} C-terminus domain only + OsINP1 full length (Appendix A). Yeast co-transformations were conducted with Yeastmaker™ Transformation System 2 (Clontech/Takara Bio, Otsu, Japan) and according to manufacturer’s specifications. Co-transformations were plated on DDO plates, and blue colonies were streaked on QDO plates to confirm protein interaction.

\textbf{ARABIDOPSIS COLD STRESS EXPERIMENT FOR TAP-TAGGING EXPERIMENT}

Approximately, 100 to 200 seeds were sown on Whatman® filter paper (Sigma-Aldrich, St. Louis, MO, USA) in 150mm plant-tissue culture plates (½X Murashige & Skoog (MS) Medium™ (MP Biomedicals, France), 15 g/l agar, pH= 5.7) with or without 30 µg/µl of Kanamycin (Kan^{30}) or 5 % sucrose. Seeds were vernalized in the dark for 3 days at 4°C. Seeds were grown at regular growth chamber conditions specific for Arabidopsis. After two and half weeks of growth, seedlings were cold-treated at 4°C for 24 to 48 hours. Whole plant tissue was harvested and flash frozen in liquid nitrogen at the corresponding time point.
Frozen samples were homogenized in liquid nitrogen for approximately 15 minutes using a pestle and mortar. Samples were saved in -80°C until aliquot preparation.

**ARABIDOPSIS TANDEM AFFINITY PURIFICATION OF 2xSTREP-3xFLAG TAGGED PROTEINS FOR MASS SPECTROMETRY**

This protocol was developed by Dr. Steve Briggs lab (UCSD, La Jolla, USA) in collaboration with Dr. Steve Kay (UCSD, La Jolla, USA). The protocol included in this thesis was optimized with minor modifications for our *in vitro* protein purification experiments.

Aliquots of approximately 2 g to 4 g of homogenized Arabidopsis tissue were transferred to 2 ml eppendorf tubes. The tissue powder was resuspended in a 1:1 packed volume ratio with 1X SII Buffer (100 mM NaPhosphate, pH 8.0; 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, and 0.1% Triton X 100) + 1 mM PMSF (serine protease inhibitor) + 1X complete protease inhibitor tables (Roche, Basel, Switzerland) + 1X Phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) + 50 µM MG132 (Peptides International, Louisville, KY, USA). Samples were quickly vortexed (<5 sec) and incubated at 4°C in a rotating incubator. Fully resuspended tissue samples were sonicated twice at 10% power for 10 seconds (0.5 on/off), on ice, with a Fisher Scientific 505 Sonic Dismembrator using a 1/8” tapered microtip (Waltham, MA, USA). Extracts were clarified three times at 20,000 x g for 10 minutes at 4°C on an Eppendorf 5417K bench top cold centrifuge (Hamburg, Germany). Protein concentration was quantified with Fermentas Bradford reagent and according to manufacturer’s specifications (Thermo Scientific, San Diego, CA, USA).

Extracts were incubated overnight in a rotator incubator at 4°C with 80 µl of cross-linked anti-FLAG® M2 (Sigma-Aldrich, St. Louis, MO, USA) magnetic Invitrogen Dynabeads® (Life Technologies, Carlsbad, CA, USA) per 50 mg of protein extract. The following day, the magnetic beads were captured with a magnetic tube holder, and the flow-through was decanted. The beads were washed 3x with 500 µl 1X SII Buffer with no protease or phosphatase inhibitors, and transferred in the last wash to low retention eppendorf tubes. Two washes of 900 µl of 1X FLAG to Strep buffer (100 mM NaPhosphate, pH 8.0; 150 mM NaCl, 0.05 % TX-100) were quickly done at room temperature to equilibrate the beads for elution. The beads were eluted twice with 200 µl of 1X FLAG to Strep buffer +
500 ng/µl of FLAG® peptide (Sigma-Aldrich, St. Louis, MO, USA). The first elution was done on a rotating incubator at 4°C for 15 minutes and the second elution at 30°C. At the end of each elution, the 200 µl flow-through was combined to a total of volume of 400 µl. The resulting 400 µl flow-through was incubated with rotation at 4°C with 150-200 µl of Strep-Tactin magnetic beads (washed 3x with FLAG to Strep buffer) (Qiagen, Venlo, Netherlands). Beads were quickly washed first with FLAG to Strep buffer, and then twice with 25 mM Ammonium Bicarbonate buffer (prepared fresh). The final wash was removed and beads were flash frozen in liquid nitrogen and stored in -80°C.

Magnetic bead samples were either sent intact in dry ice to mass spec analysis; or denatured in an SDS-PAGE gel and sent to mass spec analysis in the form of three gel pieces corresponding to different molecular weight bands. Peptide sequence data from mass spec analysis was used to identify potential interacting protein candidates based on percent coverage and number of unique identified peptides.
CHAPTER 3

RESULTS

The goal of this project is to understand the molecular mechanisms by which OsSPX1 functions in plant cold signaling pathways, by identifying potential OsSPX1 interacting proteins using different molecular biology and biochemistry techniques. I hypothesized that upon low temperature stress, over-expression of OsSPX1 activates cold responsive signaling pathways through protein-protein interactions.

The specific aims of this study are the following:

1. To characterize the transgenic Arabidopsis plants that over-express the rice OsSPX1 gene.
2. To identify plant proteins that interact with OsSPX1 following low temperature stress treatment. The two approaches we employed are:
   b. In vitro: Tandem Affinity purification (TAP)-tagging, coupled with liquid chromatography and tandem mass spectrometry (LC-MS/MS).

The experimental results from the two specific aims above are listed in this section under the following subheadings: Developmental phenotypes of the transgenic Arabidopsis plants that over-express OsSPX1, freezing and drought stress treatments, inorganic phosphate profile following cold stress treatment, soluble sugars profile following cold stress treatment, rice transcriptome profile following cold stress treatment, in vivo identification of OsSPX1 interacting proteins with a yeast two-hybrid system, and in vitro identification of OsSPX1 interacting proteins using Tandem Affinity Purification (TAP)-tagging coupled with Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS).

DEVELOPMENTAL PHENOTYPES OF THE TRANSGENIC ARABIDOPSIS PLANTS THAT OVER-EXPRESS OsSPX1

Transgenic Arabidopsis plants over-expressing OsSPX1 (OsSPX1-OX) displayed some interesting developmental phenotypes. One of the differences between the wild type Arabidopsis plants (WT) and OsSPX1 over-expression (OsSPX1-OX) transgenic plants is that the OsSPX1-OX transgenic plants produced larger seeds than WT plants. Figure 1 shows
sizes of the seeds from WT and OsSPX1-OX plants. Seeds of OsSPX1-OX plants were approximately 2.5x larger than WT seeds.

In addition, seeds of OsSPX1-OX plants had a darker brown color as opposed to the lighter brown color in WT seeds. After seed germination, leaf morphology was also affected in the OsSPX1-OX plants. Wild type leaves showed the classic long blade-like structures and an overall light-green color. In contrast, OsSPX1-OX lines showed a shorter and serrated-like structure, with an overall darker green color (Figure 2). There was a difference in flowering time between WT and OsSPX1-OX lines. After 3 ½ weeks of growth under long day conditions (22°C, 16/8 photoperiod, light intensity =~100 m²sec⁻¹), transgenic plants showed delay in flowering time and slower elongation of the first node as compared to WT plants (Figure 3). The number of trichomes in the leaves was also significantly affected in the OsSPX1-OX plants, and differences were visible without the need of magnification (Figure 4).
Figure 2. Leaf morphology of OsSPX1 over-expression line. Two pictures were taken of 3½ week old Arabidopsis seedlings with a Canon Powershot A560 camera. The picture on the left panel corresponds to an Arabidopsis WT seedling, and right panel picture corresponds to OsSPX1-Ox. Both pictures were taken at approximately same height (~10 cm), but at different pot positions.

Figure 3. Late flowering. WT (top) and OsSPX1-Ox (bottom) seedling pictures were taken 3½ weeks after germination with a Canon Powershot A560 camera. Both pictures were taken at approximately same distance (~20cm from pot).
FREEZING AND DROUGHT STRESS TREATMENTS

Preliminary results showed that over-expression of OsSXP1 in rice (*Oryza sativa japonica*) resulted in an overall increase in recovery after 5 days of 4°C cold treatment. When grown under 4°C cold condition, the growth of both WT and transgenic rice seedlings over-expressing OsSPX1 were stunted. However, transgenic rice seedlings quickly resume their growth right after being moved back to regular growth condition, and grew taller than WT seedlings (Figure 5).

![Figure 4. Trichome morphology. Leaf from WT plant (panel A) and OsSPX1-Ox (panel B). Both pictures were taken at the San Diego State University Electron Microscope Facility by Felis Wolven.](image)

**Figure 4.** Trichome morphology. Leaf from WT plant (panel A) and OsSPX1-Ox (panel B). Both pictures were taken at the San Diego State University Electron Microscope Facility by Felis Wolven.

![Figure 5. Rice seedling recovery after 5 days of 4°C cold treatment. Measurements were taken at day 4 after cold treatment from the tip of the longest leaf to root-shoot junction (n=3/sample, except #2, n=2).](image)

**Figure 5.** Rice seedling recovery after 5 days of 4°C cold treatment. Measurements were taken at day 4 after cold treatment from the tip of the longest leaf to root-shoot junction (n=3/sample, except #2, n=2).
In order to investigate the role of OsSPX1 during plant freezing response, we compared the transgenic Arabidopsis plants, which either over-express OsSPX1 in wild type Arabidopsis background (OsSPX1-OX), or over-express OsSPX1 in the atspx1 mutant background (OsSPX1-OX/atspx1). AtSPX1 shares ~60% sequence similarity at the amino acid level, thus is considered to be an Arabidopsis homologue of the rice OsSPX1 gene. Figure 6 shows two-week old Arabidopsis thaliana seedlings after freezing treatments. Plants were subjected to 72 hours of -8°C freezing treatment at dark, followed by 4°C thawing at dark, and 48 hours of recovery under regular growth conditions. Results from freezing experiments showed that over-expression of OsSPX1 in both wild type and atspx1 mutant backgrounds enhanced plant’s freezing tolerance when compared to wild type and atspx1 mutant, which failed to recover after freezing stress.

![Figure 6. Freezing experiment of OsSPX1 over-expression in Arabidopsis. The numbers on the top left corner correspond to the following genotypes: #1, WT; #2, atspx1 mutant; #3, AtSPX1/039445C (atspx1 mutant complement); #4, OsSPX1-Ox/atspx1 line 6-3; and #5, OsSPX1-Ox (line 7-4). Two week old seedlings were treated at -8°C for 72 hours. Seedlings were thawed at 4°C overnight, and recovered at regular growth conditions. Plant recovery was assessed after 48 hours.](image)

We also generated transgenic plants that over-expressed the AtSPX1 gene in the atspx1 mutant background (AtSPX1-OX/atspx1). Intriguingly, these transgenic plants were freezing sensitive (Figure 6, picture #3). However, when AtSPX1 was over-expressed in the
WT Arabidopsis plant (AtSPX1-OX), similar level freezing tolerance as seen in the OsSPX1-OX transgenic plants was observed (Figure 7).

![Image of freezing experiment]

**Figure 7.** Freezing experiment of AtSPX1 over-expression in Arabidopsis. The numbers on the top left corner correspond to the following genotypes: #1, WT; #2, AtSPX1-Ox in WT (line 1-2); and #3, AtSPX1-Ox in WT (line 2-5). Two week old seedlings were treated at -8°C for 72 hours. Seedlings were thawed at 4°C overnight, and recovered at regular growth conditions. Plant recovery was assessed after 48 hours.

Due to the overlapping of plant cold stress signaling pathways and drought stress pathways shown by a number of previous studies (Seki et al., 2002), we wanted to test if over-expression of OsSPX1 had an effect on plant drought tolerance. WT and various transgenic plants were deprived of water for two weeks, followed by replenishing each pot with water until saturation. Drought stress tolerance was assessed by observing plant recovery such as turgidity at 6-hour and 24-hour time points. We found that the OsSPX1-OX transgenic plants, not the OsSPX1-OX/atspx1 plants, were able to survive a two-week drought stress, and recovered to full turgidity after addition of water (Figure 8). The WT and the atspx1 mutant plants failed to survive the two-week drought treatment.

**INORGANIC PHOSPHATE PROFILE FOLLOWING COLD STRESS TREATMENT**

To test the correlation between plant’s phosphate levels and low temperature tolerance, we analyzed the total leaf inorganic phosphate levels in various Arabidopsis plants after low temperature stress treatment. Our data showed that the inorganic phosphate level in the OsSPX1-OX transgenic plants was slightly lower when compared to WT and transgenic plants that only contained an empty plant transformation vector, either before (22°C) or after 48 hour cold (4°C) treatment (Figure 9). In contrast, the atspx1 mutant, transgenic plants
Figure 8. Arabidopsis drought stress. Two weeks of dry soil conditions. Two biological replicates (1 and 2) per genotype (A-D). Panel A corresponds to WT, panel B corresponds to OsSPX1-Ox, panel C corresponds to atspx1 mutant, and panel D corresponds to OsSPX1-Ox/atspx1.

Figure 9. Inorganic phosphate profile of Arabidopsis constructs during control and cold stress treatment. This graph represents a physiological analysis of OsSPX1-OX and AtSPX1-OX transgenic constructs in WT or atspx1 background in response to low temperature stress, 4°C treatment for 48hrs. The samples analyzed are: WT (6673), vector WT (T4 vector 6673), OsSPX1-OX (T4 OsSPX1-OX in 6673), AtSPX1-OX/WT (T4 AtSPX1-Ox in 6673), atspx1 (039445C), vector atspx1 (T4 vector 039445C), OsSPX1-OX/atspx1 (T3 OsSPX1-OX in 039445C #12-5), and AtSPX1-OX/atspx1 (T4 AtSPX1 in 039445C #7-6). The malachite green assay was used to measure total inorganic leaf phosphate levels of 2.5 to 3 week-old control and cold samples (n=3 biological replicates per construct, 6-9 seedlings/pot).
either contained an empty vector in the atspxl mutant background, OsSPX1-OX/atspx1, and the AtSPX1-OX/atspx1 transgenic plants showed an overall higher inorganic phosphate levels than that in the WT plants. Interestingly, the OsSPX1-OX transgenic plants and the AtSPX1-OX transgenic plants, although displayed similar tolerance to freezing stress, showed a different inorganic phosphate profile. Inorganic phosphate level in the AtSPX1-OX transgenic plants is very similar to that in the WT plants. In addition, the OsSPX1-OX/atspx1 transgenic plants, which was also freezing tolerant, showed an overall higher level of inorganic phosphate than OsSPX1-OX and AtSPX1-OX plants.

**Soluble Sugars Profile Following Cold Stress Treatment**

To test the correlation between plant’s soluble sugar levels and low temperature tolerance, we measured the total amount of soluble sugars in leaves after low temperature stress. Leaf tissues from three-week old Arabidopsis plants were harvested before and after 48hrs of 4°C cold treatment. We found that the levels soluble sugar in all the plants tested increased after cold treatment (Figure 10). Similar to what was observed for the leaf inorganic phosphate level, different freezing tolerant plants were found to have different soluble sugar profiles. The freezing tolerant OsSPX1-OX transgenic plants (#7-4) showed higher soluble sugar levels after cold treatment than WT, atspxl mutant, and the OsSPX1-OX/atspx1 transgenic plants. The soluble sugar levels of the OsSPX1-OX/atspx1 after cold treatment was more or less similar to that of the WT plants, but higher than that of the atspxl mutant. The atspxl mutant also displayed the lowest basal soluble sugar levels, while the basal soluble sugar levels from the other plants were similar.

**Rice Transcriptome Profile Following Cold Stress Treatment**

To identify genes that are potentially regulated by OsSPX1, we analyzed rice GeneChip® Microarray data generated by Dr. Zhen Su’s laboratory in China (State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China). The microarray data were obtained from wild type rice (Oryza sativa jap.) and two independent OsSPX1-antisense rice lines treated with 4°C for 24 hours. The microarray
Figure 10. Arabidopsis soluble sugars profile before and after cold treatment. Leaf tissue from three week-old seedlings were harvested before (22°C) and after 48 hours of 4°C cold treatment. Samples analyzed: WT (6673), *OsSPXI*-OX (T3 *OsSPXI*-OX in 6673 #7-4), *atspx1* (039445C), and *OsSPXI*-OX/*atspx1* (T3 OsSPXI-Ox in 039445C #12-5) Whole tissue extracts were made from homogenized tissue in liquid nitrogen. Extracts were sugar-hydrolyzed, and digested in a sulfuric acid-anthrone reagent mix. Samples were measured at O.D. 620 nm and soluble sugar concentration measured thereafter.

Signal intensities were normalized and probe sets with signal intensities lower than 50 were filtered out for further analysis.

Then a 2-fold cut-off was applied to make the following comparisons: WT cold vs. control; *OsSPXI*-antisense line 1, cold vs. control; *OsSPXI*-antisense line 2, cold vs. control; These 3 comparison tables were combined to generate an expression table of 13,260 genes, used for hierarchical clustering analysis.

Figure 11 shows the expression patterns of genes being up- and down-regulated following cold stress treatment. After 24 hours of 4°C treatment, 8,464 genes from WT rice showed differential expression patterns. Of these genes, 3,246 were up-regulation by 2-fold, while 5,218 genes were down-regulation by 2-fold. The two independent *OsSPXI*-antisense lines, A1 and A2 respectively, contained similar numbers of genes with differential expression patterns. The A1 line contained 3,799 genes that were up-regulated and 4,174 that were down-regulated following cold treatment. Similarly, 3,802 genes were up-regulated and 4,182 genes were down-regulated in the A2 line.

The rice microarray data revealed interesting gene expression patterns. Particularly, I was interested in *OsSPXI* (LOC_Os06g40120) and other two genes with distinct expression patterns in response to cold stress (Figure 11). The first gene, which encodes a ribonuclease
Figure 11. Heat map of WT and *OsSPX1*-antisense rice during cold stress. Hierarchical clustering analysis by TreeView of 13,260 genes from 24 hours 4°C cold treatment microarray data. Each cluster represents the Log2 transformation of a particular probeset. “Green” color represents down-regulation (<2) and “Red” color presents up-regulation (>2). Both values and color relationship represent the Log2 ratio between Cold/Control (Ck) treatments. “Ni” corresponds to WT, “A1” to antisense line-1, and “A2” to antisense line-2. Dendrogram branch length corresponds to relationship between each data set (short= closer relationship, long= distant relationship).
T2 domain containing protein (LOC_Os07g43670), displayed a relatively high expression in the WT background and no significant change under both control and cold condition. The basal expression of this gene in both of the OsSPX1-antisense lines was also similar to that in the WT background. However, the expression was down-regulated after cold treatment (Figure 12). The second gene, which encodes a thaumatin-like protein (LOC_Os12g43380), had a relatively low basal expression in the WT, and its expression was further down-regulated by cold stress. On the other hand, the expression of this gene was constitutively higher in both of the antisense lines than that in the WT, and was not affected by cold stress treatment (Figure 12). Interestingly, expression of OsSPX1 in WT and both antisense-OsSPX1 lines was up-regulated (Figure 12), possibly due to incomplete inhibition of OsSPX1 transcription from antisense RNA. Non-specific hybridization of OsSPX1 probe sets with other rice SPX-domain containing genes could be another possible reason for such induced expression.

![Figure 12](image)

**Figure 12. Microarray gene expression after 24 hour of cold treatment.** Gene expression Log2 ratio (Cold/Control) of probesets corresponding to the genes Os07g43670, Os12g43380, and Os06g40120. Intensity ratio corresponds to microarray data signal ratio of control over cold.

In order to validate the microarray data, we designed primers and employed quantitative polymerase chain reaction (qPCR) to test the mRNA expression of the LOC_Os07g43670, LOC_Os12g43380, and LOC_Os06g40120 genes in the WT and rice transgenic plants which over-express the OsSPX1 gene (rice OsSPX1-OX). Ideally, RNA samples from the two antisense lines should be used for this validation experiment. However, due to the availability of the seeds from the two antisense line, instead, we used the rice
OsSPX1-OX lines to track the gene expression changes of the above genes in a time-course experiment.

Seeds of the WT and rice OsSPX1-OX (T3 line 10.2-1 #2) plants were germinated under the 12:12 photoperiod (28°C Day, 25°C night). Five-day old seedlings were cold treated at 4°C and harvested at 0-hour, 6-hour, 24-hour, and 5-day time points. Plant tissue samples were flash-frozen in liquid nitrogen and homogenized using a mortar and pestle. Total RNA was extracted using the standard protocol as suggested by manufacturer, and treated with RNase-free DNaseI to remove genomic DNA contamination. Reverse transcription was conducted with 1 microgram of total RNA, and the resulting cDNA was used for qPCR analysis (refer to Experimental Methods section for complete details).

Real-time qPCR analysis on the LOC_Os12g43380 and LOC_Os07g43670 genes across all the time points is shown in Figure 13. The relative expression of LOC_Os12g43380 in the WT background follows a similar trend to what was observed in the microarray data, where its expression was down-regulated after 24 hours of cold treatment (Figure 12). Interestingly, in rice OsSPX1-OX, the basal expression of LOC_Os12g43380 appears to be much lower than that in the WT plants, consistent with our expectation since its basal expression was much higher in the two antisense plants, suggesting that the expression of this gene is potentially regulated by OsSPX1. Cold treatment resulted in a significant down-regulation of the LOC_Os12g43380 expression at multiple time points in this experiment.

![Figure 13. Rice RT-qPCR expression during cold stress. Relative expression levels of Os07g43670 (ribonuclease T2 domain containing protein) and Os12g43380 (thraumatin-like gene). Expression levels were normalized to 18s rRNA. The results are the mean values ± re the mean valuentd biological replicates.](image-url)
The expression of the LOC_Os07g43670 gene in the WT background shows a different pattern from the qPCR experiment when compared to that from the microarray data, where no significant difference in expression was observed (Figure 12). In contrast, our qPCR data indicated that the expression of this gene is down-regulated following the 24-hour cold treatment (Figure 13). Furthermore, the gene expression patterns of the LOC_Os07g43670 gene were very similar between WT and the rice OsSPX1-OX transgenic plants (Figure 13), suggesting that the expression of this gene is not regulated by OsSPX1.

The qPCR result of the OsSPX1 (LOC_Os06g40120) gene validates the microarray data (Figure 14), where a significantly higher expression was obtained at 0-hr time point, and the expression was slightly higher at 6-hour and 24-hour time points. Although we expected that the expression of the OsSPX1 gene remains constant in the transgenic plants, since the expression is controlled by the Cauliflower Mosaic Virus (a double 35S) promoter, the increased expression can be possibly explained by the contribution from the endogenous OsSPX1 expression, which was picked up by the same qPCR primers (Figure 14).

![Figure 14. Rice OsSPX1 RT-PCR expression during cold stress. The relative expression levels of LOC_Os06g40120 treated at 4°C and collected at 0-hour, 6-hour, 24-hour, and 5-day time points. Expression levels were normalized to 18s rRNA. The results are the mean values ± SD of two independent biological replicates.](image-url)
We also tested the OsSPX1 protein expression levels in the one of the rice OsSPX1-OX lines (T3 10.2-1 #2), to test if the protein level correlates with the mRNA level. Protein extracts of WT and the T3 10.2-1 #2 plants were subjected to SDS-PAGE electrophoresis and Western blot analysis, with the anti-FLAG® M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) to detect FLAG epitope-tagged OsSPX1 in the transgenic plants (T3 10.2-1 #2).

It appears that the OsSPX1 protein expression did not correlate with the mRNA expression. The OsSPX1 protein level remains fairly constant after 0-hour, 6-hour, and 24-hour cold treatment (Figure 15, panels A and B, lanes #5-7) However, OsSPX1 protein level increased to a much higher degree after 5 days of cold treatment, as seen from the two independent biological replicates (Figure 15, panels A and B, lane #8).

Figure 15. Exogenous Nter-TAP-Tag-OsSPX1 protein levels in rice during cold stress. Sample order is the following for A and B immunoblots (top) and nitrocellulose membrane Pierce stain (C): Lanes 1-4 = WT (0-hour, 6-hour, 24-hour, 5-days); Lanes 5-8= 10.2 (0-hour, 6-hour, 24-hour, 5-days). Immunoblot A corresponds to biological replicate #1, and immunoblot B corresponds to biological replicate #2. Pierce stain (C) is a nitrocellulose membrane stain before primary antibody (anti-FLAG® M2) hybridization. OsSPX1 MW= 59.42kDa. Red band= ~70KdA, Blue band= ~60kDa. Film exposure: 45 seconds.
**IN VIVO IDENTIFICATION OF OSSPX1 INTERACTING PROTEINS WITH A YEAST TWO-HYBRID SYSTEM**

A rice cDNA library was constructed from tissues collected from 10-day old rice seedlings treated with 4°C cold for 5 days. By using the Clontech™ Mate & Plate® system, we constructed a cDNA library with about 1.2 million independent clones that contained pGADT7 recombinant vectors with different rice genes in the Y187 yeast strain (Leu⁻ autotrophic marker). The bait OsSPX1 gene was subcloned into the pGBKKT7 vector and transformed into the Y2H gold yeast strain (Trp⁻ autotrophic marker). Four independent mating experiments were conducted between Y187, which contained the cDNA library, and Y2H gold, which contained the pGBKKT7-OsSPX1 fusion gene (Appendix B). Potential positive colonies, showing blue on double dropout plates (DDO: SD – Leu – Trp + Aureobasidin A [AbA] + X-α-Gal) were re-screened on the more stringent quadruple dropout plates (QDO: SD – Ade – His – Leu – Trp + AbA + X-α-Gal). Yeast minipreps were conducted with positive colonies obtained from QDO plates, followed by DNA sequencing analysis to identify the cDNA inserts in the recombinant pGADT7 vectors.

We identified 8 protein candidates that potentially interact with OsSPX1 (Table 1). Most of the identified proteins were annotated to be involved in various metabolism processes, except for one (Os06g0646600) that belongs to the family of Knox homeodomain transcription factors. Sequencing of the cDNA inserts revealed that of the 8 genes identified, most only contained part of the predicted cDNA towards the 3’-end, and a poly-A tail following the 3’-UTR of the gene in the pGADT7 recombinant vector. We suspected that this was probably due to the optimization during our cDNA library construction.

One of the positive clones identified from the cDNA library screening encodes a potential Knox family transcription factor (Os06g0646600). This clone contained partial sequence of the Os06g0646600 gene, and the corresponding amino acid sequence encompasses the potential ELK and Homeodomains (Figure 16). We named this clone OsINP1, which stands for OsSPX1-interacting protein 1. Knowing that the clone encodes a potential transcription factor, we selected this gene for further analysis.

We conducted a domain-dissection experiment to test if there is also an interaction between OsSPX1 and the full length OsINP1 protein, and to map the interaction domains on
Table 1. Y2H Coupled with a Rice cDNA Library Screening. Identified Genes from 4 Independent cDNA Library Matings, pGBKT7-OsSPX1 [Y2H] + pGADT7-cDNA library [Y187]. All Positive Blue Screening Colonies Were Screened on DDO (SD –Leu –Trp +AbA + X-a-Gal) and QDO (SD –Ade –His – Leu –Trp +AbA + X-a-Gal) Plates to Increase Stringency

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<th>Gene</th>
<th>Description</th>
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<td>Blue (4) Y4 mini-#1</td>
<td>Os09g0270900</td>
<td>FeS Suf E</td>
</tr>
<tr>
<td>4</td>
<td>Blue (2) Y7 mini-#1</td>
<td>Os05g0562200</td>
<td>Drought induced</td>
</tr>
<tr>
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<td>Blue (2) Y7 mini-#2</td>
<td>Os09g0270900</td>
<td>FeS Suf E</td>
</tr>
<tr>
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<td>Os12g0589100</td>
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<td>3</td>
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<td>1</td>
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<td>Os06g0646600</td>
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**HOMEO DOMAIN** (237-298)
**ELK** (214-235)
**KNOX2** (112-158)
**KNOX1** (48-81)

Blue1-1 new (206-298) **short poly A sequence**
OsSINP1 ELK/Homeodomain (204-310)

Figure 16. Os06g0646600 amino acid sequence and domains. The full-length Os06g0646600 amino acid sequence (317aa, 34.6kDa) with KNOX1 domain highlighted in grey, KNOX2 domain in turquoise blue, ELK domain in green, and Homeodomain in yellow. The first highlighted dark-yellow sequence (top to bottom, forward) and second (bottom to top, reverse) corresponds to Blue1-1 screening positive clone. Amino acid sequences in red (top to bottom, forward; and bottom to top, reverse) correspond to OsSINP1 ELK/Homeodomain generated by Felis Wolven.
both proteins. Figure 18 shows the result of this experiment. Three recombinant pGBK7 vectors were constructed; each contained either the full-length OsSPX1 (OsSPX1 Full), or the N-terminal SPX-domain of the OsSPX1 (OsSPX1 SPX domain only), or the C-terminus of the OsSPX1 (OsSPX1 Cter only) (Figure 17). Meanwhile, two recombinant pGADT7 vectors were constructed too, which either contained the full length OsINP1 (OsINP1 Full), or just the ELK/Homeodomain of the OsINP1 (OsINP1 ELK/Homeodomain only). The original miniprep from the cDNA screening was also included as a positive control. Co-transformation with different combination of recombinant pGBK7 and pGADT7 constructs into Y2H gold yeast strain was performed and the transformants were first screened on DDO (SD –Leu –Trp) plates and then on QDO/X/A (SD –Ade –His –Leu –Trp +AbA + X-α-Gal) plates. Protein interaction was confirmed when blue colonies appeared on the QDO plates. This experiment yielded important information about the interaction between OsSPX1 and OsINP1 (Figure 18). The full-length OsSPX1 protein interacts with all three versions of the OsINP1 protein, including the Blue1 Y2H screening, the OsINP1 ELK/Homeodomain only, and the full-length OsINP1 protein (Figure 18), validating the results from our yeast two-hybrid screening. In addition, it appears that of the C-terminal domain of the OsSPX1 is sufficient for the interaction since it could also interact with all 3 versions of the OsINP1 proteins (Figure 18). However, the SPX domain of the OsSPX1 itself failed to interact with any of the OsINP1 proteins.

![OsSPX1 amino acid sequence and domains. The full length OsSPX1 amino acid sequence (295aa, 33.1kDa) with SPX domain highlighted in yellow, and C-ter domain in green are shown. SPX-only and C-ter only constructs in the pGBK7 vector were generated by Felis Wolven.](image-url)

Yellow = SPX domain only

Green = Cter only
Figure 18. Domain-dissection of OsSPX1 and OsINP1 constructs. Yeast colony resuspension drop of OsINP1 interaction with OsSPX1. Full length OsINP1, ELK/Homeodomain only, and original Y2H blue (+) colony were co-transformed into the pGBK7-OsSPX1, SPX domain only, and C-terminus only of OsSPX1 in Y2H gold yeast strain. Blue colonies correspond to (+) interaction between bait protein linked to DNA binding domain (pGBK7-BD) and prey protein linked to Activation Domain (pGADT7-AD). Constructs were grown on QDO (SD–Ade–His–Leu–Trp +AbA + X-a-Gal) plates at 30°C.

In Vitro Identification of the OsSPX1 Interacting Proteins Using Tandem Affinity Purification (TAP)-Tagging Coupled with Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

Construct used in the TAP-tagging system is shown in Figure 19. Three repeats of Flag tags and two repeats of Strep were translationally fused to the N-terminus of the OsSPX1 protein (N-ter-tag-OsSPX1). The N-ter-tag-OsSPX1 fusion gene was then subcloned into the plant binary vector pCHF3 that contains a constitutive CaMV 35s promoter to drive the expression of the fusion gene. The rationale of using the epitope-tagged OsSPX1 protein was to utilize anti-epitope tag antibodies to capture in vitro protein-protein interactions from the Arabidopsis plant extracts. The purpose of using both Flag and Strep epitope tags was to minimize non-specific interaction among different proteins through sequential purification steps using anti-flag and Strep-Tactin.

The WT Arabidopsis plants, and the two freezing tolerant transgenic Arabidopsis plants, OsSPX1-OX and OsSPX1-OX/atspx1 were treated with 4°C cold for 24 hours.
Tissues from whole seedlings, including leaves and roots, were harvested and frozen in liquid nitrogen, and then ground to fine powder. Protein extracts were prepared by re-suspending tissue powder in SII buffer containing protease and phosphatase inhibitors, followed by two rounds of sonication to further lyse the plant cells. The suspension was cleared by high-speed centrifugation (refer to “Experimental Methods” for details). Clear supernatants, which contained protein extracts, were subjected to sequential purification steps as shown in Figure 20. The first step involved the overnight incubation of protein extracts with magnetic Dynabeads® (Life Technology) cross-linked with the anti-FLAG® M2 antibody (SigmaAldrich, St. Louis, MO, USA). The second step was to elute bound proteins from magnetic beads using the competing FLAG® peptide (Sigma-Aldrich, St. Louis, MO, USA), followed by the incubation of the eluted proteins with Strep-Tactin magnetic beads (Qiagen, Venlo, Netherlands). The presence of the bait, which is the epitope-tagged OsSPX1 protein after each purification step was confirmed by a Western blot analysis using the anti-FLAG® M2 antibody against the N-ter-tag-OsSPX1 protein (Figure 21, panel A). Loading with an equal amount of protein in each lane of the SDS-PAGE gel (8%) helped us determine if bait protein was well captured and concentrated at each step, and to compare protein expression in the two transgenic lines.

Silver staining was also used to visualize the captured proteins along with the bait (Figure 21; panel B). After confirming the presence of N-ter-tag-OsSPX1 from either the Western blot analysis, or the silver staining, all the samples were sent for LC-MS/MS (from final purification step) either in the forms of Strep-Tactin beads, or in denatured SDS-PAGE gel pieces. We have conducted 4 independent TAP-tagging experiments with Arabidopsis seedlings. The mass spectrometry analyses were conducted at UCSD Biomolecular/Proteomics Mass Spectrometry Facility (Experiment 2), Dr. Steve Briggs lab at UCSD (Experiment 1), and UCSF Mass Spectrometry Facility (Experiment 3 and 4).
Our first TAP-tagging experiment identified peptides which belong to 26 Arabidopsis proteins (Table 2; top). Our bait N-ter-tag-OsSPX1 contained the highest amount of peptides identified from this experiment, confirming the success of the TAP-tagging experiment. Besides the bait protein, majority of the proteins identified were heat shock (HS) proteins, proteins that are closely-related HS proteins, and a few uncharacterized proteins. However, the peptide coverage (percent of the full length protein that is covered by the identified peptides) of all the proteins was really low. This led us to conduct two more TAP-tagging experiments, which identified additional proteins with slightly higher peptide coverage. Table 2 (bottom) shows a list of proteins identified from the second TAP-tagging experiment. Similar to the first experiment, majority of proteins identified were related to HS proteins. One of them, which is closely related to HSP70 protein, was the early response to dehydration 2 (ERD2) protein that had been shown previously to be stress responsive (Taji et al., 1999).

We decided to validate the interaction between ERD2 and OsSPX1 in a different yeast-two hybrid system. Two other proteins were selected based on the percentage peptide coverage and their biological importance for the validation (Table 2, bottom list): one protein...
Figure 21. Visualization of bait and captured proteins after TAP-tagging. Panel A corresponds to Western blot against Nter-Tag flag epitope with FLAG® M2 antibody. Lanes 1–7 are Flag beads and 8–14 Strep beads (1, 8= vector 6673; 2, 9= OsSPX1-Ox 6673 (7-4); 3, 10= OsSPX1-Ox 6673 (5-5); 4, 11=vector 039445c; 5, 12= OsSPX1-Ox 039445C (12-5); 6, 13= OsSPX1-Ox 039445C (13-2); 7, 14= 6673. Panel B corresponds to Silver stain gel. Lanes 1–6 are Flag beads and 10–15 Strep beads (1, 10= vector 6673; 2, 11= OsSPX1-Ox 6673 (7-4); 3, 12= OsSPX1-Ox 6673 (5-5); 4, 13=vector 039445c; 5, 14= OsSPX1-Ox 039445C (12-5); 6, 15= OsSPX1-Ox 039445C (13-2); 8= MW marker.
Table 2. LC-MS/MS Protein Identification Results from TAP-Tagging experiments 1 and 2. Proteins Identified from Two Independent TAP-Tagging Experiments Are Show in Both Tables. Percent Coverage is the Number of Peptides Identified by LC-MS/MS that Correspond to a Specific Protein. Highlighted Protein Names (Yellow) Correspond to Proteins Selected for a Yeast Two-Hybrid System-Based Protein Interaction Study

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>% Coverage</th>
</tr>
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<tbody>
<tr>
<td><strong>TAP-Tagging Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>Heat shock cognate 70 kDa protein 3</td>
<td>2.5</td>
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<tr>
<td>Heat shock protein 101</td>
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</tr>
<tr>
<td>Heat shock cognate 70 kDa protein 3</td>
<td>2.5</td>
</tr>
<tr>
<td>Heat shock protein 101</td>
<td>2.5</td>
</tr>
<tr>
<td>Heat shock cognate 70 kDa protein 3</td>
<td>2.5</td>
</tr>
<tr>
<td>Putative uncharacterized protein</td>
<td>8</td>
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<tr>
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<td>3.5</td>
</tr>
<tr>
<td>GAPCP-2</td>
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<tr>
<td>Putative uncharacterized protein</td>
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</tr>
<tr>
<td>Cysteine proteinase RD21a</td>
<td>6.3</td>
</tr>
<tr>
<td>Heat shock protein 101</td>
<td>1.1</td>
</tr>
<tr>
<td>Histone H4</td>
<td>9.7</td>
</tr>
<tr>
<td>E3 ubiquitin-protein ligase ATL4</td>
<td>5.1</td>
</tr>
<tr>
<td>Putative uncharacterized protein</td>
<td>2</td>
</tr>
<tr>
<td>Ribulose bisphosphate carboxylase large chain</td>
<td>6.3</td>
</tr>
<tr>
<td>Heat shock cognate 70 kDa protein 3</td>
<td>2.5</td>
</tr>
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<tr>
<td>Heat shock cognate 70 kDa protein 3</td>
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<td>GAPCP-2</td>
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<tr>
<td>Putative uncharacterized protein</td>
<td>4.5</td>
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<tr>
<td>Cysteine proteinase RD21a</td>
<td>4.3</td>
</tr>
<tr>
<td><strong>TAP-Tagging Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>BIP (LUMINAL BINDING PROTEIN); ATP binding</td>
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</tr>
<tr>
<td>Luminal binding protein 1 (BiP-1) (BP1)</td>
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<tr>
<td>BIP (LUMINAL BINDING PROTEIN); ATP binding</td>
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</tr>
<tr>
<td>HSP70 (heat shock protein 70); ATP binding</td>
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<td>heat shock cognate 70 kDa protein 2 (HSC70-2) (HSP70-2)</td>
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<td>heat shock cognate 70 kDa protein 3 (HSC70-3) (HSP70-3)</td>
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<td>ERD2/HSP70T-1 (EARLY-RESPONSIVE TO DEHYDRATION 2); ATP binding</td>
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<tr>
<td>HSC70-1 (heat shock cognate 70 kDa protein 1); ATP binding</td>
<td>18.59</td>
</tr>
<tr>
<td>HSP70B (heat shock protein 70B); ATP binding</td>
<td>19.5</td>
</tr>
<tr>
<td>Zinc finger (C3HC4-type RING finger) family protein</td>
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belongs to the Zinc Finger family and the other is the luminal binding protein 2 (BIP2-ATP) binding protein.

Interestingly, the same set of HS proteins, as well as the ERD2 and the BIP2-ATP were also identified from the third TAP-tagging experiment (Table 3) In addition, two new proteins were identified from experiment #3: a jasmonic acid responsive-related protein, CORI3, and a Phosphatidylinositol 3- and 4-kinase family protein (PPI kinase). Both of these proteins were included for the validation.

**Table 3. LC-MS/MS Protein Identification Results from TAP-Tagging Experiment 3. Proteins Identified from a Third TAP-Tagging Experiment. The Mascot Score Represents the Summed Score of the Individual Peptides Identified. Matching Peptides Were Shared Among BIP, HSPs and ERD2 Proteins. Highlighted Protein Names in Yellow Correspond to Proteins Identified on TAP-Tagging Experiment #2. Light-Green Highlighted Proteins Correspond to New Proteins Identified in TAP-Tagging Experiment #3. These Proteins Were Selected for a Yeast Two-Hybrid System-Based Protein Interaction Study**

<table>
<thead>
<tr>
<th>Mascot Score</th>
<th>Protein Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>BIP (LUMINAL BINDING PROTEIN); ATP binding</td>
</tr>
<tr>
<td>64</td>
<td>luminal binding protein 1 (BiP-1) (BP1)</td>
</tr>
<tr>
<td>64</td>
<td>BIP (LUMINAL BINDING PROTEIN); ATP binding</td>
</tr>
<tr>
<td>64, 80, 90</td>
<td>HSP70 (heat shock protein 70); ATP binding</td>
</tr>
<tr>
<td>64, 80</td>
<td>heat shock cognate 70 kDa protein 2 (HSC70-2) (HSP70-2)</td>
</tr>
<tr>
<td>64</td>
<td>heat shock cognate 70 kDa protein 3 (HSC70-3) (HSP70-3)</td>
</tr>
<tr>
<td>64, 80</td>
<td>ERD2/HSP70T-1 (EARLY-RESPONSIVE TO DEHYDRATION 2); ATP binding</td>
</tr>
<tr>
<td>64, 80</td>
<td>HSC70-1 (heat shock cognate 70 kDa protein 1); ATP binding</td>
</tr>
<tr>
<td>64</td>
<td>HSP70B (heat shock protein 70B); ATP binding</td>
</tr>
<tr>
<td>81, 108</td>
<td>Bait (OsSPX1)</td>
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<tr>
<td>33</td>
<td>Phosphatidylinositol 3- and 4-kinase family protein</td>
</tr>
<tr>
<td>30</td>
<td>Single strand-binding family protein</td>
</tr>
<tr>
<td>28</td>
<td>Kinesin motor protein-related</td>
</tr>
<tr>
<td>31</td>
<td>CORI3 (Coronatine Induced 1, Jasmonic acid responsive 2); transaminase</td>
</tr>
<tr>
<td>30</td>
<td>Unknown protein</td>
</tr>
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</table>

We conducted a different yeast two-hybrid study with the proteins identified from the TAP-tagging system. We cloned OsSPX1 into the pLexA^{(4His)} vector, which contains the LexA binding domain, and the candidate genes from the TAP-tagging system into the pB42AD^{(+Trp)} vector, which contains the LexA activation domain. The 5 candidate genes were: ERD2 (At1g56410), BIP2-ATP (At5g42020), Zinc Finger (At5g24870), CORI3
(At4g23600) and phosphatidylinositol 3,4 kinase (At1g64460). The recombinant pB42AD construct, which contained one of the candidate genes, was co-transformed with pLexA-OsSPX1 into EGY-48 [p80p-LacZ^+Ura] yeast strain (His, and Trp autotrophic marker), and the transformants were selected on SD (-Ura -His -Trp) plates. Colonies able to grow on SD (-Ura -His -Trp) plates were then streaked on the SD-Gal/Raf/-His –Leu –Trp –Ura + X-gal\(^{80}\text{mg/L}\) +1xBU salts plates to induced the expression of the LexA-BD-OsSPX1 and the LexA-AD-candidate genes (refer to “Experimental Methods” section for more details). Blue colonies indicate the interaction between OsSPX1 and the candidate proteins.

Results from the above yeast two-hybrid study indicate that there was no detectable protein-protein interaction between OsSPX1 and any of the candidate proteins identified from the TAP-tagging system. Co-transformation of OsSPX1 and the individual candidate proteins, ERD2, Zinc Finger, BIP2-ATP, CORI3, and PPI kinase, did not result in any blue colonies when grown on SD +GAL +RAF –His –Leu –Trp + 1X BU salts + X-gal (Figure 22, Figure 23), as compared to positive control, pB42AD-T + pLexA-p53. Results from the positive control and negative control, pLexA-Lam + prey, were used to ensure that the yeast two-hybrid system was working properly. For example, some colonies obtained from the co-transformation of pLexA-OsSPX1 and pB42AD-ZincFinger, or pLexA-OsSPX1 and pB42AD-CORI3, or pLexA-OsSPX1 and pB42AD-PPI kinase did become blue (Figure 23). However, the appearance of those blue colonies was quite similar to that from our negative control, and the amount of blue colonies was very limited, suggesting that the weak interaction detected from this experiment is probably an artifact.
Figure 22. Yeast two-hybrid protein interaction assay of ERD2, Zinc Finger, and BIP2. Induction plate interaction of pB42AD (activation domain) and pLexA (binding domain) in SD BASE (GAL+RAF) + CSM (-Ura-His-Leu-Trp) +1X BU salts + X-gal [80mg/L]. Left top corner corresponds to positive control interaction, pB42AD-T + pLexA-p53. Right top corner corresponds to false positive control with pLexA-Lam vector. Bottom plates correspond to OsSPX1 interaction test with ERD2, BIP2 and Zinc Finger (plate not shown). Blue colonies correspond to positive interaction between bait and prey.
Figure 23. Yeast two-hybrid protein interaction assay of CORI3, Phosphatidylinositol 3- and 4- kinase, and Zinc Finger. Induction plate interaction of pB42AD (activation domain) and pLexA (binding domain) in SD BASE (GAL+RAF) + CSM (-Ura-His-Leu-Trp) +1X BU salts + X-gal [80mg/L]. Right middle plate corresponds to positive control interaction, pB42AD-T + pLexA-p53. Bottom set of plates corresponds to co-transformation with false positive control pLexA-Lam vector. Top and middle plates correspond to two independent OsSPX1 co-transformations with Zinc Finger, CORI3 and Phosp3,4 kinase. Blue colonies correspond to positive interaction between bait and prey.
CHAPTER 4

DISCUSSION

OVER-EXPRESSION OF OsSPX1 IN Arabidopsis thaliana RESULTS IN ABERRANT MORPHOLOGICAL AND DEVELOPMENTAL PHENOTYPES

Studies have suggested that larger seed size is associated with greater tolerance to seedling establishment, as opposed to smaller seeds, thus increasing plant fitness during growth (Westoby et al., 2002; Moles et al., 2005). One of the developmental phenotypes resulting from the over-expression of OsSPX1 and AtSPX1-OX in Arabidopsis thaliana was the production of larger seeds by these transgenic plants (Figure 1). Genetic analysis has identified multiple genes be associated with seed size. For instance, the Arabidopsis EOD3 gene, which encodes the P450/CYP78A6 protein, when over-expressed in the wild-type Arabidopsis thaliana, increased seed size. In contrast, the loss-of-function mutant, eod3, produced smaller seeds (Fang et al., 2012). Another study conducted with Oryza sativa (rice) found that over-expression of the growth-promoting TIFY11b gene also resulted in the production of larger seeds, longer leaves with higher sucrose and starch levels than that from the wild-type rice plants (Hakata et al., 2012).

Over-expression of OsSPX1 also affected leaf morphology. Leaves of OsSPX1-OX transgenic plants are shorter and serrated (Figure 2). Similar serrated-leaf phenotypes have been observed in other Arabidopsis mutants. For instance, the KNAT1 homeobox gene, when over-expressed (Lincoln et al., 1994), caused an extremely serrated phenotype. A similar phenotype was also reported in the gain- or loss-of-function mutants in which the CUU2 and CUC3 genes, involved in the regulation of leaf serration were mutated (Hasson et al., 2011). Leaf morphogenesis in Arabidopsis is complex and has been studied extensively, and multiple leaf-shape phenotypes resulting from gain- or loss-of-function mutations of a number of important genes have been documented (Tsukaya, 2005).

Total number of trichomes per leaf was also affected in the OsSPX1-OX transgenic Arabidopsis. Trichome is a single, large epidermal cell with 3 to 4 symmetrical branches in Arabidopsis (Huelskamp et al., 1994). The trichome morphology in the OsSPX1-OX plants
resembles that of the Arabidopsis *itb* (*Irregular Trichome Branch*) mutant, more specifically the *itb4-1*, of which branch length was asymmetrical, and 2 to 5 branches per trichome is common (Zhang et al., 2005). The spatial distribution of trichomes in the *OsSPX1*-OX plants was found to be lower than wild-type plants, as well as the number of trichomes per leaf (Figure 4). The number of trichomes distributed along the plant leaf is controlled by complex regulatory mechanisms. A clear example of trichome spatial distribution can be seen in the *Arabidopsis thaliana* ecotype Landsberg *erecta*, which has a higher spacing and lower number of trichomes per leaf than the Columbia ecotype (Larkin et al., 1996).

Over-expression of both *OsSPX1* and *AtSPX1* in the wild-type Arabidopsis background affected flowering time. We found that the *OsSPX1* OX lines flowered approximately a week later than WT plants (Figure 3). Similar late flowering phenotypes have been reported from various Arabidopsis mutants. For instance, the over-expression of the *AtCSP2* gene, which encodes a negative regulator with a cold shock domain, results in a decreased freezing tolerance after cold acclimation, shorter siliques, and late flowering (K. Sasaki et al., 2013). One of the central clock components of the Arabidopsis circadian system, the Circadian Clock Associated 1 (*CCA1*) gene, when over-expressed, resulted in disrupted circadian function, long hypocotyls, and late flowering (Lu et al., 2012). It is known that the mechanisms controlling plant flowering are complex. Change in normal flowering time as a result of altered gene expression, such as in the case of over-expression of *OsSPX1*, suggests that the appropriate regulation of gene expression is extremely important.

**OVER-EXPRESSION OF *OsSPX1* IN ARABIDOPSIS ENHANCES TOLERANCE TO FREEZING AND DROUGHT STRESS**

Over-expression of *OsSPX1* in Arabidopsis resulted in an increased survival after freezing stress. The *OsSPX1*-OX and the *OsSPX1*-OX/atspx1 transgenic plants displayed enhanced freezing tolerance after two-day -8°C treatment (Figure 6). Similar results were shown from a previous study with the *OsSPX1* gene in tobacco and Arabidopsis (Zhao et al., 2009). Studies from other gain-of-function experiments have identified novel genes related to the cold signaling pathway. Over-expression of *DREB1/CBF*-type transcription factor genes resulted in the identification of multiple novel transcripts such as the COld-Regulated gene
of which expression had been found to be up-regulated in the CBF1 and CBF3 over-expressing Arabidopsis plants (Kasuga et al., 1999; Gilmour et al., 2000). Despite the fact that the OsSPX1 signaling pathway is not well understood, it is clear that OsSPX1 is either directly or indirectly involved in the plant responses to cold stress.

The Arabidopsis AtSPX1 loss-of-function mutant, the atspx1 mutant displayed enhanced sensitivity to freezing stress. Over-expression of OsSPX1 in the atspx1 mutant background resulted in freezing tolerance (Figure 7). We decided to further investigate the role of AtSPX1. AtSPX1 shares about 60% amino acid similarity with OsSPX1. Thus, I was interested to investigate if, like OsSPX1, over-expression of AtSPX1 had any effects in low temperature stress. Interestingly, we found that the over-expression of AtSPX1 in atspx1 mutant, rescued atspx1 mutant phenotype of being freezing sensitive. Not surprisingly, over-expression of AtSPX1 in the Arabidopsis wild-type background also resulted in the increased freezing tolerance, similar to what was observed in the OsSPX1-OX transgenic plants. Unlike OsSPX1-OX plants, AtSPX1-OX plants showed a number of interesting developmental phenotypes such as abnormal flowers which resembled those from the apetala-1 mutant (Irish and Sussex, 1990), small siliques, and increased rates in embryo abortion. These data suggest the importance of both genes in the cold signaling pathway. Since enhanced freezing tolerance was only observed in OsSPX1-OX/atspx1 (Figure 6, panel 4), not in AtSPX1-OX/atspx1 plants (Figure 6, panel 3), this suggests that there is a fundamental difference in the underling molecular mechanisms by which OsSPX1 and AtSPX1 function during plant cold stress response.

We decided to test for other abiotic stresses in addition to low temperature stress. We subjected our OsSPX1-OX lines to high salt, heat and drought stresses, but only observed consistent results from our drought stress experiments. Over-expression of OsSPX1 and AtSPX1 in the Arabidopsis wild-type background resulted in the increased resistance to drought stress under a two-week dry soil conditions (Figure 8). After the drought treatment, the OsSPX1-OX and AtSPX1-OX transgenic plants were able to regain full turgidity approximately 6 hours after water application. After 24 hours, OsSPX1-OX and AtSPX1-OX plants were completely recovered, and continued to grow towards full maturity. Over-expression of one single gene that results in the tolerance to more than one abiotic stresses has been reported previously. For instance, over-expression of the Spinacia oleracea betaine
aldehyde dehydrogenase, \textit{SoBADH}, gene in sweet potato results in the tolerance to low temperature, salt, and oxidative stress (Fan et al., 2012); over-expression of the wheat transcription factor \textit{TaNAC2} in Arabidopsis results in increased freezing, drought, and salt tolerance (Mao et al., 2012).

\textbf{ARABIDOPSIS PLANTS OVER-EXPRESSIONING \textit{OsSPX1} DISPLAYED A LOWER INORGANIC PHOSPHATE PROFILE THAN WILD-TYPE PLANTS FOLLOWING COLD STRESS}

Over-expression of \textit{OsSPX1} in Arabidopsis resulted in an overall lower level of inorganic phosphate than that in wild-type plants (Figure 9). It was previously reported that the over-expression of \textit{OsSPX1} in Arabidopsis resulted in a dramatic decrease in total leaf inorganic phosphate level after cold treatment in comparison to the phosphate level in wild-type plants (Zhao et al., 2009). Our experiments showed that the overall inorganic phosphate levels in the \textit{OsSPX1}-OX plants are lower than those in wild-type plants, before and after cold stress treatment, suggesting that \textit{OsSPX1} might function as a negative regulator in phosphate homeostasis. However, our results indicated that there was an increase in leaf inorganic phosphate level after cold treatment. The inorganic phosphate data we obtained from the \textit{atspx1} mutant further suggest that \textit{OsSPX1} might act as a negative regulator during phosphate homeostasis. The overall inorganic phosphate level in the \textit{atspx1} mutant was higher than that in the wild-type plants, as well as in the \textit{OsSPX1}-OX plants. Loss of function of the \textit{AtSPX1} gene possibly affected the inorganic phosphate profile, further confirming the involvement of the SPX domain in the regulation of inorganic phosphate (Rouached et al., 2010). Interestingly, the freezing tolerant line, \textit{OsSPX1}-OX/\textit{atspx1} transgenic plants (line #12-5) displayed a different inorganic phosphate profile than that from the \textit{OsSPX1}-OX plants (line #7-4). This suggests that, the rice \textit{OsSPX1} gene may be divergent from the Arabidopsis \textit{AtSPX1} gene regarding their functions in phosphate homeostasis. Interestingly, over-expression of \textit{AtSPX1} in wild-type Arabidopsis resulted in a similar inorganic phosphate profile to that from the wild-type plants, but different than that from the \textit{OsSPX1}-OX plants, suggesting that \textit{AtSPX1} might have different effect on the cellular phosphate homeostasis compared to \textit{OsSPX1}, although these two genes shared about 60\% amino acid similarity. For instance, two orthologous genes from \textit{E. coli} and \textit{S. oneidensis}, both of which encode transcription factors, were found to regulate the same set of
genes, but each orthologous transcription factor had a different influence on transcript regulation (Price et al., 2007). Our data suggests that OsSPX1 might be negatively regulating the cellular inorganic phosphate levels, while the role of AtSPX1 is not clear from our experimental approach, although previous data from other laboratories suggested that AtSPX1 also play a negative role during phosphate starvation. Although a possible correlation between the level of inorganic phosphate and cold stress tolerance has been suggested in a study of the Arabidopsis pho1-2 mutant which contained low inorganic phosphate in leaves, and was more tolerant to low temperature (Hurry et al., 2000), the underlining regulatory mechanisms are still missing.

SOLUBLE SUGARS PROFILE BEFORE AND AFTER COLD STRESS

It has been hypothesized that cold increases soluble sugar synthesis, and the newly synthesized sugar can help lower freezing point and protect the plasma membrane from damage through cold induced crystallization (Koster and Lynch, 1992). We measured the total amount of soluble sugars present in the plant cell after low temperature stress as one of the indication of cold acclimation (H. Sasaki et al., 1996; Theocharis et al., 2012). We found that after 48 hours of 4°C cold treatment, soluble sugar levels of all tested Arabidopsis plants were higher than those before treatment (Figure 10). The OsSPX1-OX plants (line 7-4) showed slightly higher levels than the rest of the plants. A number of studies have shown that soluble sugar levels increase after cold temperature (Guy et al., 1992; Wanner and Juntila, 1999). Interestingly, before cold treatment, the soluble sugar level of atsxp1 mutant was lower than that in the WT, OsSPX1-OX and OsSPX1-OX/atsxp1 transgenic plants. In our freezing experiments, the atsxp1 mutant exhibited higher freezing sensitivity. Low soluble sugar levels in atsxp1 might be responsible for such sensitivities. Low soluble sugar levels have been reported in other freezing-sensitive plants, such as in the Arabidopsis “sensitive to freezing” sfr4 mutant (Uemura et al., 2003), and the Arabidopsis gigantean gi-3 mutant (Cao et al., 2007), where sucrose levels were found to be lower under basal conditions. Our data suggested that plants with higher soluble sugar levels, such as OsSPX1-OX might be advantageous in resisting low temperature stress. However, a higher soluble sugar level might not be the only requirement. The OsSPX1-OX/atsxp1 transgenic plants, which was found to have a similar sugar level compared to that in WT, but lower than the OsSPX1-OX
plants after cold treatment, are still more freezing tolerant. Other mechanisms involving the regulation of osmolyte production and balance might also play a role during cold stress.

**Expression Profile of the Rice OsSPX1-OX Transgenic Plants in Response to Cold Stress**

The analysis of the transcriptome profile of the wild-type rice and the *OsSPX1*-antisense rice plants revealed interesting patterns of gene expression. The hierarchical clustering analysis of the 13,260 rice genes obtained from the comparison between cold and control of the WT and the *OsSPX1*-antisense plants demonstrated a global transcriptome change following cold treatment in these plants (Figure 11). The hierarchical clustering analysis generated a few unique clusters in which genes had different expression patterns in WT compared to those in the *OsSPX1*-antisense plants.

Transcriptome profiling is a great tool to measure global gene expression changes under certain conditions (Tarca et al., 2006). The transcriptome data from this analysis comprised of two time points, 0-hour and 24-hour after cold treatment. I was interested to test, during a time course, the expression patterns of the genes that were found to be differentially expressed from the microarray analysis following cold stress treatment. Two genes were selected based on the reasons discussed below, LOC_Os07g43670 and LOC_Os12g43380, and their expression in the time course experiment was analyzed using real time quantitative PCR (RT-qPCR), with WT and the *OsSPX1*-OX rice plants. The LOC_Os07g43670 gene, which encodes a ribonuclease T2 family domain containing protein, shares a 74% sequence similarity at the amino acid level to the Arabidopsis At2g02990 (*AtRNS1*) gene. The transcript of this gene has been reported to be up-regulated in response to the increase of the *AtSPX1* expression (Duan et al., 2008). Interestingly, *AtRNS1* has also been reported to be functional during inorganic phosphate starvation (Miura et al., 2005). However, our data suggested that the expression of LOC_Os07g43670 might not be regulated by *OsSPX1* (Figure 13), since the expression patterns of this gene were very similar between WT and the rice *OsSPX1*-OX transgenic plants. In contrast, the expression of the LOC_Os12g43380 gene was found to possibly be regulated by *OsSPX1*. A tBLASTx search revealed that the LOC_Os12g43380, a thaumatin-like gene, was 56% identical to Arabidopsis At4g11650 (*Osmotin 34*), a gene involved in the responses to several biotic and abiotic stresses (Anzlovar and Dermastia, 2003). Interestingly, our qPCR data showed that
the expression of Os12g43380 increased in the OsSPX1-OX transgenic rice plants, and decreased in the OsSPX1-antisense plants revealed by microarray data suggesting that OsSPX1 regulates the expression of the LOC_Os12g43380 transcript (Figure 13).

A previous study showed that the expression of OsSPX1 was up-regulated by cold treatment, reaching a maximal value at the 24-hour and 48 hour time points (Zhao et al., 2009). Results from our qPCR analysis showed that the expression of OsSPX1 in the wild-type background gradually increased from the 0-hour to the 24-hour time point, and decreased at day-5 time point. Not surprisingly, the OsSPX1 expression in the OsSPX1-OX rice plants was significantly higher than that in WT (Figure 14). The variation in expression in the OsSPX1-OX rice plants is possibly due to the presence of the endogenous OsSPX1 transcripts, which were also picked up by the OsSPX1 primers during qPCR analysis. We also examined the OsSPX1 protein expression to test if the protein expression is consistent with the mRNA expression. We found the OsSPX1 protein level to be relatively constant from time point 0-hour to 24-hour, then accumulated to a higher level at day 5 (Figure 15). Protein accumulation is common during abiotic stress responses. For instance, the increased accumulation of the dehydrin-like proteins under cold and heat stress in Brassica oleracea var. botrytis, Arabidopsis thaliana, and Lupinus luteus has been reported (Rurek, 2010). The accumulation of the OsSPX1 protein at day 5 suggests that certain amount of time is required to synthesize the OsSPX1 protein, although there is already enough amount of OsSPX1 transcripts available. Other mechanisms such as protein turnover, and protein-protein interaction might also be involved, resulting in the accumulation of OsSPX1 at day 5.

**Screening of the Yeast Two-Hybrid Library Resulted in the Identification of OsINP1 from Rice, an OsSPX1 Interacting Protein**

We constructed a yeast two-hybrid library with rice tissues that contained about 1.2 million independent clones to screen for putative OsSPX1 interacting proteins. From a total of four independent cDNA library screenings, we identified a total of 8 potential candidates, mostly involved in various metabolic processes, and one putative transcription factor (Table 1). Sequencing of the 8 cDNA clones revealed that all the transcripts were incomplete and contained a short poly-A tail. Interestingly, cDNA libraries with short transcripts are not uncommon, and preparation of long or full-length transcripts requires optimal amplification
methods (Piao et al., 2001). We optimized our cDNA population to include longer size transcripts; however, we failed to identify any protein candidates. Another possibility is that the SPX domain of OsSPX1 may have affected the yeast mating efficiency. It is known that the yeast SYG1 protein functions by interacting with the G-protein B subunit through the N-terminus, which contains the SPX domain. Such interaction resulted in the inhibition of the yeast pheromone signal required for mating (Spain et al., 1995), and it was shown that the N-terminal SPX domain was required for the inhibition. Regardless of the possible problems, we identified a clone with a partial cDNA sequence corresponding to the rice gene Os06g0646600, which we named OsINP1. The full length OsINP1 protein contains 4 domains: KNOX1, KNOX2, ELK, and Homeodomain (Figure 16). Previous domain dissection analysis of the rice OsH15 protein which also contained KNOX1 and KNOX2 domains revealed that KNOX1 was involved in gene suppression, while KNOX2 was involved in homodimerization (Nagasaki et al., 2001). The ELK domain was hypothesized to serve as a nuclear localization signal (Meisel and Lam, 1996) or in protein-protein interactions (Vollbrecht et al., 1991). The homeodomain has been widely studied; it forms a helix-turn-helix motif, and proteins containing this domain are involved in DNA-binding (Banerjee-Basu and Baxevanis, 2001).

We also conducted a domain dissection experiment to map the protein interaction between OsSPX1 and OsINP1. Our results show that the full length OsSPX1 protein can interact with all three versions of the OsINP1 protein, including the clone that originally identified from the yeast two-hybrid screening, the OsIPN1 that only contains the ELK/Homeodomain, and the full-length OsINP1 protein (Figure 18). These results assured us that the interaction between OsSPX1 and ELK/homeodomain from the yeast two-hybrid screening were real. More interestingly, we found that it was the C-terminus domain of OsSPX1, not the N-terminal SPX domain, which was necessary for the interaction.

The true biological relevance of OsINP1 remains to be elucidated. The yeast two-hybrid system is a great tool for studying protein-protein interaction, but might not be truly representative of the in vivo conditions inside the plant. False-positives are common due to different physiological conditions (Serebriiskii et al., 2000) in yeast compared to those in plant cells. Regardless, the question still remains if the OsINP1 gene has a positive or negative effect on plant responses to abiotic stress. Transgenic plants which either over-
express or with reduced level of OsINP1 would be useful to test the biological relevance of the interaction between OsSPX1 and OsINP1.

**Tandem Affinity Purification (TAP) Coupled with Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Resulted in the Identification of Putative OsSPX1 Interacting Proteins**

The use of epitope tagged bait proteins to identify potential interacting proteins or protein complexes under their natural conditions has certain advantages in protein discovery. We applied a modified TAP-tagging method for working with Arabidopsis tissue, which was developed by Dr. Steve Briggs and Dr. Steve Kay’s laboratories at UCSD.

By using two epitope tags, the Flag and the Strep tags that are translationally fused to the N-terminus of the OsSPX1 protein (Figure 19), we rationalized that a sequential purification of plant extracts from tissues of the OsSPX1-OX transgenic plants could capture potential OsSPX1 interacting proteins with less non-specific protein contamination (Figure 20). We performed a total of 4 TAP-tagging experiments, from which we have identified a number of unique OsSPX1 interacting proteins (Table 2; Table 3). Of the proteins identified by mass spectrometry analysis, the majority were heat shock related proteins. Heat shock proteins are mostly molecular chaperones involved in the processes such as protein folding, stabilization, as well as in the translocation of proteins among others (X. Wang et al., 2004). Heat shock proteins are not only exclusive to heat stress, but are present during other abiotic stressors such as cold, salt, and osmotic stress as well (Swindell et al., 2007). Despite the possibility that heat shock proteins might indeed interact with OsSPX1, we decided to look at other protein candidates in case the presence of heat shock proteins was a result of non-specific interaction due to protein unfolding (Gingras et al., 2007).

We conducted a different yeast two-hybrid experiment to test the *in vivo* interaction between OsSPX1 and proteins identified from the TAP-tagging experiment, including ERD2 (At1g56410), BIP2 (At5g42020), Zinc Finger (At5g24870), CORI3 (At4g23600), and PPI 3,4 kinase (At1g64460). A locus search with the TAIR database (www.arabidopsis.org) revealed that the *ERD2* gene was induced by heat and dehydration; *BIP2* was related to
endosperm development; Zinc Finger was involved in nucleic acid binding; COR13 in amino acid metabolism; and PPI 3,4 kinase in phosphotransferase activity. However, co-transformation of OsSPX1 with each of the above individual protein did not reveal any detectable interactions (Figure 22; Figure 23). We have successfully detected the interaction between the proteins from our positive control, thus excluding the possibility that the problem was in our yeast two-hybrid system.

There is a possibility that the conditions inside the yeast cells do not reflect the optimal physiological conditions that allow the interactions among plant proteins to occur (Szilagyi et al., 2005). It is also possible that the interaction between OsSPX1 and Arabidopsis proteins might be transient and weak since OsSPX1 was originally identified from rice. It would be ideal to perform the TAP-tagging experiment with the rice OsSPX1-OX transgenic plants. More OsSPX1 interacting proteins could be potentially identified. Such experiment would also validate the TAP-tagging experiment in Arabidopsis.

**SUMMARY**

The data generated by this study demonstrated that over-expression of the rice OsSPX1 gene in Arabidopsis enhanced the plant’s ability to survive freezing stress. We also found that over-expression of OsSPX1 in Arabidopsis increased survivability during and after drought stress treatment. Over-expression of OsSPX1 led to a number of noticeable developmental phenotypes such as: larger seeds, short serrated-leaves, abnormal trichome morphology, and late flowering.

We were able to identify a few of potential OsSPX1 interacting proteins by TAP-tagging and yeast two-hybrid approaches. One protein candidate, the OsINP1 protein, was found to interact with OsSPX1, and it was determined that the C-terminus of OsSPX1 was necessary for the interaction. However, the biologically relevance of the interaction between OsINP1 and OsSPX1 needs to be further evaluated for their involvement during low temperature stress responses.
ACKNOWLEDGEMENTS

I would like to thank my thesis chair, Elizabeth Waters, for her guidance and helpful discussions; and to my thesis committee member, Joan Chen, for her unconditional support, patience, expert mentoring, and friendship; and Joseph M. Mahaffy for his advice and encouragement. I also thank Dmitri A. Nusinow (UCSD Kay lab) for training and troubleshooting guidance with the TAP-tagging experiments; thanks to Felis Wolven for her laboratory training and help with scientific writing. Special thanks to all former Chen Lab students that helped with this project: Melisa Cruz, Cristina Alcaraz, Ernestine Miranda, Ming Liu, Aaron Cruz, Chuck Sanchez, and Josh Mock.
REFERENCES


APPENDIX A

PRIMER SEQUENCES
### Appendix A. Primer sequences

This list includes primer sequences used to conduct my experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Primer Sequence</th>
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<td>At1g56410</td>
<td>ERD2/HSP70T-1-5' EcoRI</td>
<td>5'-CCCCGAAATCATGGTCTGTAAGGGAGAAGGT-3'</td>
<td>Y2H: pB42AD vector cloning</td>
</tr>
<tr>
<td>At1g56410</td>
<td>ERD2/HSP70T-1-3' XhoI</td>
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APPENDIX B

MATING EFFICIENCY CALCULATIONS
**Appendix B. Mating efficiency calculations.** Calculations of all yeast mating experiments. A good mating efficiency has a range of 2-5% according to Matchmaker™ Gold Yeast Two-Hybrid System User Manual (Clontech/Takara Bio, Mountain View, CA, USA). Refer to manual for calculations.

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