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A NOVEL SYSTEM TO STUDY SEED RECALCITRANCE AND DORMANCY - COMPARATIVE PROTEOMICS BETWEEN TWO *SPARTINA* SPECIES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in
The Department of Plant Pathology and Crop Physiology

by
Yi Wang
M.S., Louisiana State University, 2010
B.S., Beijing University of Chemical Technology, 2006
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ABSTRACT

Spartina alterniflora is a dominant salt marsh cordgrass along the U.S. Atlantic and Gulf coasts, and the species is widely used for wetland restoration in Louisiana. *S. alterniflora* seeds are shed dormant from the mother plant. However, long-term preservation of *S. alterniflora* seeds is challenging because the seeds are recalcitrant, losing viability when dried below 45% water content. In this dissertation, I investigated the following three aspects of *S. alterniflora*: recalcitrance, dormancy release by cold stratification and the effect of drying temperature on the critical water content.

Comparative proteomics between *S. alterniflora* and orthodox, desiccation tolerant *Spartina pectinata* seeds identified 83 heat-stable (soluble after 40 min at 95°C) spots that were significantly more abundant in *S. pectinata* or missing in *S. alterniflora*. The association between the presence of those proteins and desiccation tolerance was further confirmed by the evidence that these proteins were still present in the desiccation tolerant, but missing in the desiccation intolerant, *S. pectinata* seedlings. These 83 spots were sequenced and share homologies to known proteins, *e.g.* late embryogenesis abundant proteins (LEAs), molecular chaperones, antioxidants, cystatin, and glyceraldehyde-3-phosphate dehydrogenase. These data suggest that LEA prevention pathways, molecular chaperone rescue pathways, ubiquitin-proteasome and autophagy degradation pathways and ROS-antioxidant systems may account for the desiccation tolerance in *S. pectinata*.

Comparative proteomics was used to identify putative dormancy-breaking associated proteins that exhibit the same protein expression pattern during cold stratification in *S. alterniflora* and *S. pectinata* seeds. *In silico* gel analysis revealed that 9 individual spots were more abundant in dormant, and 7 spots were more abundant in the non-dormant state. These

proteins share homologies with seed storage proteins, glycolytic enzymes, and molecular chaperones. The proteomic results suggest that degradation of storage proteins, increased cellular metabolism, and increased activities of molecular chaperones during cold stratification may be associated with dormancy release in *S. alterniflora* and *S. pectinata*.

Mortality curves (seed viability versus water content) of *Spartina alterniflora* at different drying temperatures (4ºC, 14ºC and 24ºC) suggest that the drying rates are temperature-dependent, but the critical water content is independent of the drying temperature in *S. alterniflora*.

CHAPTER 1
INTRODUCTION

<u>Seed Recalcitrance of *S. alterniflora*</u>

Based on desiccation tolerance, seeds can be categorized as orthodox (desiccation-tolerant) and recalcitrant (desiccation-sensitive) (Roberts, 1973). Orthodox seeds are able to survive extreme water loss (90-95% water removal) (Vertucci and Farrant, 1995). In contrast, recalcitrant seeds die when dried below 40-70% (dry weight basis, dwb) (Berjak and Pammenter, 2008).

Spartina alterniflora is a dominant salt marsh cordgrass along the U.S. Atlantic and Gulf coasts (Valiela *et al.,* 1978). Since it can survive a wide range of salinities, produce a vigorous canopy to reduce wave energy, and provide nursery areas for wildlife, *S. alterniflora* is widely used in wetland restoration (Anderson and Treshow, 1980). Seed-based propagation to restore coastal wetlands needs a large number of seeds; however, long-term storage and transport of *S. alterniflora* seeds is challenging because they exhibit recalcitrance, quickly losing viability when dried below 45% water content (dwb).

Recalcitrant seeds are generally large, non-dormant and cold intolerant (Dickie and Pritchard, 2002), imposing difficulties on seed storage and dictating a small window of time between seed collection and experimentation. In contrast, recalcitrant *S. alterniflora* seeds are shed dormant, are cold insensitive and relatively small (caryopsis, *ca.* 8-10 mm) (Chappell, 2008), and they can be imbibed and stored in water at 2°C for 8-10 months without germinating. The longer time frame of seed storage of *S. alterniflora* greatly benefits experiment planning and implementation. The dormant *S. alterniflora* is not only less metabolically active (which benefits seed storage), but also provides physiological controls of both dormant and non-dormant states. In addition, orthodox *S. pectinata*, which is in the same genus as *S. alterniflora*, can be used as a

closely-related physiological comparator (Baumel *et al.,* 2002) to determine whether the experimental results are a common seed response of the genus to desiccation or due to the recalcitrance.

Three hypotheses have been proposed to explain recalcitrant seed death: physical damage, oxidative stress, and lack of protective molecules (Pammenter and Berjak, 1999). Late embryogenesis abundant proteins (LEAs) are a group of protective proteins associated with desiccation tolerance (Pammenter and Berjak, 1999), and accumulation and degradation of LEAs coincided with acquisition and loss of desiccation tolerance, respectively (Blackman *et al*., 1991; Boudet *et al*., 2006). Up-regulation of LEAs is observed in desiccation tolerant stages of various anhydrobiotes (Rodriguez *et al.,* 2010; Boschetti *et al.,* 2011). Up-regulation of a number of genes/proteins, *e.g.* cystatin, glyceraldehyde-3-phosphate dehydrogenase, superoxide dismutase, is also consistently observed in desiccation tolerant stages of anhydrobiotes (Cornette *et al.,* 2010; Mali *et al.,* 2010). A transcriptomic screen of the anhydrobiotic Antarctic midge revealed that 92 homologs of genes associated with ubiquitin-proteasome, autophagy and programmed cell death were up-regulated during dehydration (Teets *et al.,* 2012).

Seed Dormancy Release by Cold Stratification

Dormancy is defined as the inability of a viable seed embryo to germinate even under favorable germination conditions (Bewley, 1997). Comparative proteomics between dormant and non-dormant *A. thaliana* Cvi (Cape Verde Island) seeds identified a number of proteins that were associated with dormancy release during cold stratification (Arc *et al.,* 2012). A similar proteomic approach was used to examine dormancy breaking associated proteins in tree seeds of beech, Norway maple and sycamore, and proteins involved with energy metabolism, protein degradation and protein synthesis may be associated with dormancy release (Pawlowski, 2010).

Unlike most recalcitrant seed species that are shed non-dormant, *S. alterniflora* seeds are shed dormant from the mother plant. Comparative proteomics was used to identify proteins that are associated with dormancy breaking by cold stratification between dormant and non-dormant *S. pectinata* and *S. alterniflora*. If any differentially expressed proteins are associated with dormancy release, it is expected to see those proteins exhibit the same expression pattern in both *S. pectinata* and *S. alterniflora*.

Effect of Drying Temperature and Drying Rate on the Critical Water Content

If seed recalcitrance is related with biochemical events, which are usually highly temperature dependent, it is expected that the critical water content (a turning point when the recalcitrant seed starts to die during desiccation) for loss of viability of recalcitrant seeds will be affected as drying temperature changes. In order to determine whether the critical water content is temperature dependent, mortality curves of percent viability versus water content were obtained in recalcitrant *Spartina alterniflora* seeds at 4°C, 14°C and 24°C. Based upon the literature, this experiment has not been conducted with any other species possessing dormant, recalcitrant seeds.

In this dissertation, I address the following questions: (1) are there any differences in the two-dimensional proteomic profiles of the heat-stable fraction between *S. pectinata* and *S. alterniflora* seeds? (2) Is there any association between differentially expressed heat-stable proteins and the loss of viability in recalcitrant seeds? (3) Are there any differences in the two-dimensional proteomic profiles of the phosphorylated heat-stable fraction between *S. pectinata* and *S. alterniflora*? (4) Is there any total soluble protein associated with dormancy breaking during cold stratification in *S. alterniflora* and *S. pectinata*? (5) Do the drying temperature and drying rate affect the critical water content of the recalcitrant *S. alterniflora*?

CHAPTER 2
COMPARATIVE PROTEOMICS OF RECALCITRANT SEED DEATH IN *SPARTINA ALTERNIFLORA*

LITERATURE REVIEW

Three hypotheses have been proposed to explain the desiccation intolerance of recalcitrant seeds: physical damage, oxidative stress and lack of protective molecules (Pammenter and Berjak, 1999; Berjak and Pammenter, 2008). Physical damage during drying and subsequent rehydration has been examined in *S. alterniflora*, and no increased leachates, an indicator of physical damage of cell membrane, were observed during the drying of the whole seeds of *S. alterniflora* and *S. pectinata* (Chappell, 2008). For oxidative stress, lipid peroxidation did not increase during desiccation of *S. alterniflora* and *S. pectinata*, and increased protein oxidation during desiccation was not associated with seed death in *S. alterniflora*, but was just a simple response of seeds to the drying (Chappell, 2008; Chappell and Cohn, 2011). These data suggest physical damage and oxidative stress are probably not the cause of recalcitrant *S. alterniflora* seed death during desiccation.

Non-reducing sugars are a family of protective molecular candidates associated with desiccation tolerance (Pammenter and Berjak, 1999). Newly synthesized sucrose and raffinose have been correlated with the acquisition of desiccation tolerance during orthodox seed development (Blackman *et al.,* 1992; Black *et al.,* 1996). However, vitrification of non-reducing sugars occurred at *ca.* 20% water content, which is much lower than the critical water contents of recalcitrant seeds (*ca.* 40-70% water contents) (Williams and Leopold, 1989; Bruni and Leopold, 1992; Leopold *et al.,* 1994; Sun *et al.,* 1994). In addition, no trehalose was identified in desiccation tolerant anhydrobiotic Bdelloid rotifers (Lapinski and Tunnacliffe, 2003; Tunnacliffe *et al.,* 2005). Thus, based on recent reading and materials summarized in earlier reports, non-

reducing sugars alone cannot explain recalcitrance.

Lack of protective proteins may be another possible cause of recalcitrant seed death during desiccation. One candidate is LEA proteins, which have been considered for several decades as a family of protective molecules associated with desiccation tolerance. They are designated as LEAs because their synthesis occurs during the late stage of embryogenesis in seeds (Dure *et al.,* 1981; Galau *et al*., 1986). LEA proteins are not only present in plants, but ubiquitous across kingdoms, *e.g.* microorganisms (Stacy and Aalen, 1998) and invertebrates (Goyal *et al*., 2005b; Hand *et al*., 2011). The concurrence between expression of LEAs and acquisition of desiccation tolerance (Dure *et al.*, 1981; Close *et al*., 1989) implicated conceivable participation of LEAs in desiccation tolerance. Degradation of LEAs coincided with loss of desiccation tolerance in radicles of orthodox *Medicago truncatula* seeds (Boudet *et al*., 2006). Besides LEAs, a number of other proteins have also been reported to be up-regulated under desiccation stress, *e.g.* antioxidants (Schokraie *et al.,* 2010; Wang *et al.,* 2010a), heat-shock proteins (HSPs) (Hayward *et al.,* 2004), cysteine protease inhibitor (Mali *et al.,* 2010), and glyceraldehyde-3-phosphate dehydrogenase (Chen *et al.,* 2009).

To examine whether a lack of protective proteins causes *S. alterniflora* seed death during desiccation, comparative proteomics of heat-stable fractions between the dry seeds of *S. alterniflora* and *S. pectinata* was performed. Several reasons why heat stable fractions were utilized are listed. First, some of previously identified protective proteins, *e.g.* LEAs and superoxide dismutase, have been reported to be heat stable proteins in the literature (Nice *et al.,* 1994). Secondly, heating of the total soluble protein solution followed by centrifugation would denature and precipitate the majority of proteins, serving as a pre-fractionation process to simplify the proteomes before proteins are loaded for 2-D gel separation, because 2-D gel

electrophoresis (2-DE) has limited separation capability (maximally ~3000 proteins on a gel) (Görg *et al.,* 2004). In addition, desiccation is a severe stress, removing the majority of cytoplasmic water and shell water off macromolecules and cell membranes (Hoekstra *et al.,* 2001) and causing cytosolic crowding, which increases the chances of macromolecular interaction, resulting in protein denaturation and aggregation (Ellis and Minton, 2006). Heating is a similar harsh stress as desiccation, which is also able to denature proteins. It is speculated that proteins that are able to survive heating can also resist desiccation stress. Although only heat-stable proteins have been studied so far, I do not exclude the possibility of any non-heat sensitive proteins may be associated with recalcitrance of *S. alterniflora*, and the future work will examine any differentially expressed proteins of total fractions between *S. alterniflora* and *S. pectinata*.

MATERIALS & METHODS

Seed Materials

S. alterniflora seeds were harvested in November 2008, 2009, and 2010 from wild plants in the marshes of Port Fourchon, Louisiana (N 29º08'2.79" W90º14'51.9"). Seeds were collected by hand shattering and immediately sealed in plastic zipper bags. After transport to the laboratory (Louisiana State Univ., Baton Rouge, LA), seeds were scattered on clean germination papers (Anchor Paper) and air dried for 2 weeks (water content, *ca.* 20%, dwb). After drying, seeds were freeze-clamped in liquid N_2, sealed in 50 ml plastic tubes (Corning Co., Lowell, MA) and then stored at -80ºC. Dry *S. pectinata* seeds were purchased from Western Native Seed (Coaldale, CO, USA). After delivery to the laboratory, they were stored dry and in tightly sealed Mason jars at -20ºC until use (Wang, 2011).

Drying Method

The flash dryer consists of a Nalgene jar (Thermo Fisher Scientific, Rochester, USA), a 12 V (0.16 A) computer fan (Radioshack, Fort Worth, USA) connected to 12 V (1000 mA) power adapter (Radio Shack, Fort Worth, USA), $CaSO_4$ desiccant (Drierite, W.A. Hammond Drierite Company, Xenia, USA) and a Ball jar rim (Ball Corporation, Broomfield, USA) lined with mesh as a seed holder. The seeds, placed on the mesh/rim assembly, are rapidly dried by air that is pulled up by the computer fan. Fresh desiccant (*ca.* 30 g) was used for each dry down experiment. If desiccant became moisture saturated (indicated as pink color) during an experiment, it was replaced by fresh Drierite (blue color) (Chappell, 2008; Wang, 2011).

Desiccation Tolerance Test of *S. pectinata* Seedlings

Bottles of *S. pectinata* seeds, which were stored dry at -20ºC, were taken from the freezer and warmed to room temperature (24ºC). Dormancy of *S. pectinata* seeds was released by cutting the seed 1/3 of the way down from the apex. For each experiment, three biological replicates of twenty cut seeds were placed on two pieces of Anchor germination paper in a plastic Petri dish containing 8 ml of distilled water, and a folded Kim-Wipe disposable tissue was placed over the seeds. The Petri dish was placed in a box that was filled with moist paper towels on the bottom. The cut seeds were germinated at 27ºC for 3-5 days. The *S. pectinata* seedlings were divided into three sets based on the shoot lengths ($\leq$2 mm, 2mm-5 mm, $\geq$5 mm). These three sets of seedlings were flash dried in different drying units for 2 days at room temperature, re-imbibed and incubated at 27ºC for 7 days of seedling growth to examine whether the shoots would continue to elongate. If the shoots of *S. pectinata* seedlings continued to elongate, they were considered desiccation tolerant, and intolerant if they failed to elongate.

Protein Sample Preparation

Heat-stable protein extraction in *S. alterniflora* and *S. pectinata* was thoroughly optimized as described in Wang (2011). All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., St. Louis, USA) unless otherwise stated. Fifty seeds of *S. alterniflora* or *S. pectinata* were ground in liquid nitrogen, and ground powders were transferred to a pre-chilled glass homogenizer and then homogenized in 5 ml buffer [50 mM HEPES pH 7.5, protease inhibitor cocktail (P9599) (1% v/v)] at 4°C. The total homogenate was centrifuged at 14,000 g, at 4°C for 20 min, twice. For the heat-stable fraction, the supernatant was heated at 95°C for 40 min, cooled in ice for 30 min, and then centrifuged at 20,000 g at 4°C for 40 min. Heat soluble proteins were precipitated by adding 100% trichloroacetic acid [TCA, (w/v)] to make a final concentration of 10% TCA (w/v), and washed with 10 ml cold acetone (-20°C), three times. The pellet was vacuumed dried and stored at -80°C until used for fractionation by two-dimensional gel electrophoresis.

Electrophoresis

For 2-D electrophoresis, protein pellets (~800 µg) were dissolved in 40 µl lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) (w/v) and 20 mM dithiothreitol (DTT), 2% (w/v) IPG buffer NL 3-10] at room temperature (23°C) overnight (Görg *et al.,* 2004). Protein samples were desalted with the Mini Dialysis Kit (GE Healthcare). Dialysis tubes and caps were thoroughly rinsed with distilled water to make sure that the tube storage solution was removed. After dissolving in lysis buffer, proteins were pipetted into dialysis tubes, and then the dialysis cap, which contained the dialysis membrane (cut-off molecular weight-1 kDa), was positioned on the tube, screwed firmly and inverted to let all protein samples rest on the dialysis membrane. The inverted dialysis tube and

caps were secured on a float and placed on the dialysis solution, which contained the same concentration of lysis buffer that was used to dissolve the protein pellets, with continuous stirring. During dialysis overnight, salts and small molecules (≤ 1 kDa) were exchanged through the membrane, and the contents inside the dialysis tube were mixed by inverting the tube twice. After dialysis, the tube was centrifuged at 500 g for 20 sec to make sure the protein solution was fully recovered at the tube bottom. Finally, the dialyzed protein solution was carefully pipetted and transferred to a clean tube for the subsequent 2-D use (PlusOne Mini Dialysis Kit Manual, GE Healthcare). Five microliters of lysis buffer containing the dialyzed proteins were diluted to 50 microliters (10x) with distilled water for protein concentration determination using the bovine serum albumin as the standard (Bradford, 1976).

A recipe to prepare 1200 ml of 12.5% polyacrylamide gels [150.0 g acrylamide, 4.032 g bis-acrylamide, 1.5 M Tris-HCl pH 8.8, 10% SDS (w/v), 10% ammonium persulfate (APS) (w/v) and 340 µl Tetramethylethylenediamine (TEMED)] (the stirred gel solution was degassed under vacuum for 20 minutes, and APS and TEMED were not added until gels were ready to pour) was used to make 12 gels. A volume (~30 µl) of lysis buffer that contained 800 µg of proteins was added to the rehydration solution [7 M urea, 2 M thiourea, 0.001% (w/v) bromophenol, 2% (w/v) CHAPS, 20 mM DTT and 0.5% (w/v) IPG buffer NL 3-10] to make the final volume of 450 µl. The 450 µl rehydration buffer was applied to 24 cm immobilized pH gradient dry strips pH 3-10 (nonlinear) (GE Healthcare), which were then rehydrated overnight at room temperature. After 12-hour rehydration, pH strips were gently rinsed with distilled water to remove crystallized urea and excess cover fluid. In the IEF dimension, pH strips were placed into the electrophoresis unit (Ettan IPGphor 3, GE Heatlthcare), which was set up at a stable temperature of 20°C. Isoelectric focusing ran at 500 V constant for 1.5 h, 1,000 V constant for 1.5 h, 8,000 V gradient for 3 h,

8,000 V constant for 3 h, 10,000 V constant for 1 h. After the first dimension, pH strips were equilibrated twice. In the first equilibration step, 7 ml of SDS equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol (v/v), 2% SDS (w/v), 0.002% (w/v) bromophenol and 0.5% DTT (w/v)] was pippetted onto each IPG strip in a rehydration tray that was then gently shaken for 40 min at room temperature. In the second equilibration step, iodoacetamide [1.25% (w/v)] was used instead of DTT, and strips were gently shaken for 40 min at room temperature. After equilibration, IPG strips were carefully loaded into corresponding gel cassettes and sealed with agarose solution. The bottom tank of the SDS-PAGE dimension apparatus (Ettan DAL twelve system separation unit, Amersham) was filled with 8 L of 1x SDS running buffer [2.5 mM Tris-base pH 8.3, 19.2 mM glycine and 0.01 % (w/v) SDS], and the top tank was filled with 2 L of 2x SDS running buffer [5 mM Tris-base pH 8, 38.4 mM glycine and 0.02% (w/v) SDS] once the glass plates were in. SDS-PAGE ran at 40 V for 1 hour first, and at 110 V until 2,050 Vhrs (it normally takes overnight) at constant 18ºC.

Gels were carefully loaded onto staining racks of a DodecaTM stainer (Bio-Rad)for subsequent colloidal Coomassie Brilliant Blue G-250 (Sigma-Aldrich, Sigma-Aldrich Company Ltd., St. Louis, USA) staining (analytical and preparative) (Neuhoff *et al.,* 1988). Gel trays were shaken in the staining tank filled with 10 L fixation solution (40% ethanol, 10% acetic acid and 50% distilled water) at 250 rpm overnight. The fixative was removed, and gels were washed with 10 L distilled water, three times. The staining solution (10 L) contained 2% H_3PO_4 (ACS grade, Fisher Scientific, US) (v/v), 10% $(NH_4)_2SO_4$ (Sigma-Aldrich, Sigma-Aldrich Company Ltd., St. Louis, USA) (w/v), 20% methanol (HPLC grade, Fisher Scientific, US) (v/v) and 0.1% Coomassie G-250 (w/v). The preparation of the staining solution (10 L) was by sequential addition as follows: 235 ml of phosphoric acid (85%, w/v) was added to 2 L of distilled water; 1

kg of ammonium sulfate powder was added to 4 L of distilled water. After these were fully dissolved, phosphoric acid and ammonium sulfate solutions were combined and poured into the staining tank together with 1.5 L of distilled water. Ten grams of Coomassie Brilliant Blue G-250 were dissolved in 500 ml distilled water, and then added to staining tank. Two liters of methanol were not added to staining tank until the gel staining began. The staining took place for 2 days, and gels were de-stained with distilled water until the background was clear (8-10 hours in most cases).

For the phosphorylated protein staining with Pro-Q Diamond (Steinberg *et al.,* 2003), each gel was fixed in a 1 L solution that contains 50% (v/v) methanol and 10% (v/v) acetic acid in a Nalgene staining box (Z358304, Sigma-Aldrich), with gentle agitation (250 rpm) overnight (~12 h). After fixation, the gel was washed with 1 L distilled water for 10 minutes, 3 times. The gel was then incubated in 250 ml Pro-Q Diamond phosphoprotein gel stain solution (used as supplied), with gentle agitation (200 rpm) for 90 min. The Nalgene staining box was wrapped with aluminum foil to prevent gel exposure to light. After staining, each gel was destained in a 1 L solution that contains 5% (v/v) 1 M sodium acetate (pH 4.0), 75% (v/v) distilled water and 20% (v/v) acetonitrile for 30 min. The destaining step was repeated 3 times (total staining time = 1.5 h). The staining box was also wrapped with aluminum foil during destaining.

After de-staining, Coomassie stained gels were scanned with Magicscan (UMAX). Gel analysis was performed with Progenesis SampleSpots (Nonlinear Dynamics, Newcastle, UK). Pro-Q Diamond stained gels were scanned with Typhoon Trio scanner (GE Healthcare). Gels were visualized using an excitation wavelength of 532 nm and an emission wavelength of 580 nm. For gel analysis, five biological replicates were included for either *S. pectinata* or *S. alterniflora*.

In-gel Trypsin Digestion

The protocol of in-gel trypsin digestion was described in Shevchenko *et al.* (2007). Everything involved in the digestion experiments was rinsed with 70% ethanol at the beginning. Gels were rinsed in distilled water for 1 hour, and a plastic tray together with the gel was put onto a light box. The desired protein bands or spots were punctured with a 1 ml pipette tip head, and spots were cut into smaller pieces (2 mm x 2 mm) with a clean scalpel. The gel pieces were transferred to a clean 1.5 ml plastic tube, and 100 µl of freshly-made 100 mM ammonium bicarbonate/acetonitrile (Sigma-Aldrich, Sigma-Aldrich Company Ltd., St. Louis, USA) (1/1, v/v) washing solution was added to cover the gel pieces at 4°C for 30 min with occasional gentle vortexing, and the washing step was repeated several times until the dye was completely removed from the gels. Then, the staining solution was carefully pipetted out, and 500 µl of 100% acetonitrile was added to gel pieces. The tubes containing gel pieces were gently shaken at room temperature, and acetonitrile was removed until gel pieces became white and shrunken. The gels were vacuum dried (SpeedVac, SAVANT) at room temperature, and then 30-60 µl of trypsin (Proteomics Grade, Sigma-Aldrich) solution (0.02 µg/µl) was added depending on the gel size and staining intensity. Gels were submerged in trypsin solution for 4 hours on ice first, with subsequent addition of 30 µl of 100 mM ammonium bicarbonate, and incubated in a water bath at 37°C overnight. After overnight digestion, tubes were cooled to room temperature and micro-centrifuged (200 x g) to spin down gel pieces. The trypsin and ammonium bicarbonate solution was carefully pipetted into a clean tube, and 100 µl extraction buffer (1:2, v/v) (5% formic acid/acetonitrile) was added to each tube containing the gel pieces, which was then incubated for 10 minutes at 37°C. The extraction buffer was finally pipetted out and combined with the trypsin

solution. The mixture was vacuum dried with the refrigeration on, and peptide pellets were stored at -80°C for LC-MS/MS sequencing.

LC-MS/MS Based Peptide Sequencing

Liquid chromatography electrospray tandem mass spectrometry (LC MS/MS) was used to analyze the peptide mixture extracted from gel spots (Patterson and Aebersold, 1995). Dry tryptic digests of gel spots were dissolved in 10 µl 0.1% formic acid and then injected manually onto a 0.3 x 1 mm trapping column (PepMap C18, Dionex Corporation, Salt Lake City, UT) using a nano LC system equipped with Switchos and Ultimate 2000 pumps (Dionex Corporation), at a flow rate of 10 µl/min. The Switchos valve was set on loading position prior to sample loading. After sample loading, the trapping column was washed with 0.1 % formic acid at flow rate of 5 µl/min for an additional 5 min and then the valve was switched to the inject position. Peptides were then eluted at 200 nl/min and separated on a 75 µm x 15 cm C18 column (Biobasic, Vydac HPLC Columns, Grace Davison, IL), with a gradient of 5-40% acetonitrile over 60 min, followed by 80% acetonitrile for 5 min. The eluent was directed into a quadrupole time-of-flight mass spectrometer (QStar, Applied Biosystems) and ionized immediately using an electrospray source (Nano spray II, Applied Biosystems) at high voltage of 2.5 kv with nebulizer gas. The mass spectrometer was operated in IDA (information dependent acquisition) mode with the three most intense ions in each survey scan subjected to MS/MS analysis using collision energies ranging from 20 eV to 50 eV.

MS/MS data obtained from Q-Star was processed by Mascot MS/MS ion search (Matrix Science) (Perkins *et al.,* 1999) to identify proteins based on MS/MS data from one or more peptides. The Mascot parameters were set as the defaults, which include: peptide tolerance ±1.2 Da; MS/MS tolerance ±0.6 Da; peptide charge 2+, 3+, 4+; 1 missed cleavage allowed. The

taxonomy was selected as Virdiplantae (green plants). Databases of NCBI, SwissProt and Plant ESTs were all examined for each protein sample. Only a peptide, the score of which was above the significance threshold that Mascot MS/MS gives each search, was counted a 'good' peptide. Proteins that contained at least two identified unique peptides were counted as confident identification. The MS/MS data were also subjected to *de novo* sequencing using two software programs, Mascot Distiller (Matrix Science) (version 2.4.2) and Bio-Analyst 1.1 (Applied Biosystems). To avoid possible errors in *de novo* sequencing, MS/MS results were also validated manually. The peptides obtained from *de novo* sequencing with high ion score were subjected to BLAST search for protein homologies restricted to green plants. The protein homology with a lowest E value (Expect value) was selected. The E value of a significant threshold was set below 0.01 (default value = 10).

Searching for Known Cis-regulatory Element Motifs

Identified *S. pectinata* proteins were BLAST (Basic Local Alignment Search Tool) searched for the orthologous rice genes using tblastn program (Ouyang *et al.,* 2007) (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml). The 1000 base pairs upstream region of the ATG starting site of each targeted gene was used for searching annotated cis-regulatory element motifs. Two databases of plant cis-acting regulatory DNA elements were utilized: PLACE (http://www.dna.affrc.go.jp/PLACE/index.html) (Higo *et al.,* 1999) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Rombauts *et al.,* 1999). Information of putative transcription factors that may bind to those cis-regulatory elements was traced from the literature provided by PLACE and PlantCARE databases. Family classification of transcription factors was based on PlnTFDB (http://plntfdb.bio.uni-

potsdam.de/v3.0/) (Perez-Rodriguez *et al.,* 2010) and AtTFDB (http://arabidopsis.med.ohio-state.edu/AtTFDB/) (Davuluri *et al.,* 2003).

RESULTS

Desiccation Intolerance of *S. alterniflora*

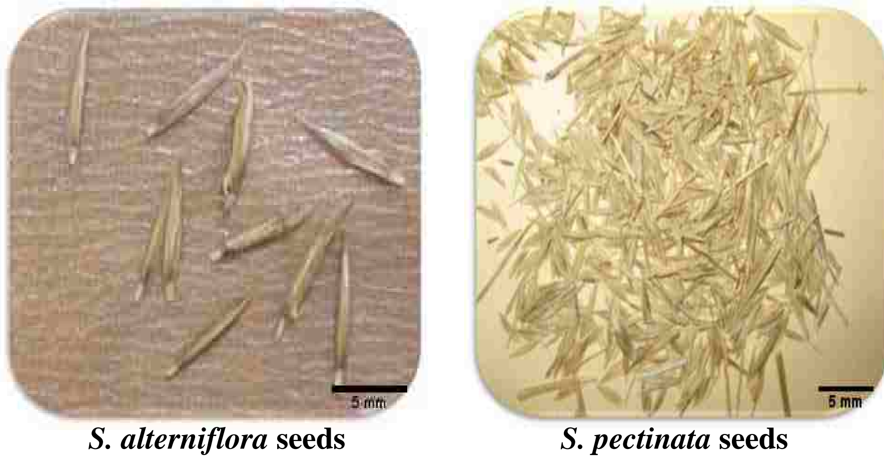
S. alterniflora seeds exhibited recalcitrant behavior (Fig. 2.1b). When *S. alterniflora* seeds were dehydrated below 43 ± 2% (dwb), they began to lose viability rapidly (% viability = 50% at 30% water content). In contrast, orthodox *S. pectinata* seeds were still 100% alive when their water contents dropped to ~10% (dwb).

Two-dimensional Gel Comparison of Heat-stable Fractions

A workflow of comparative proteomics is shown in Figure 2.2. Heat-stable proteins of dry seeds of *S. pectinata* and *S. alterniflora* were resolved on 2-D gels (pH 3-10NL) and stained with Coomassie Blue G-250 (Fig. 2.3). Since dry *S. pectinata* (10%, dwb) seeds were viable and air dried *S. alterniflora* (17%, dwb) seeds were dead, differentially expressed proteins between those two species may contribute to the desiccation tolerance in *S. pectinata*.

For the *S. pectinata* gel, the majority of spots are located in the molecular mass range of 10-30 kDa and pI range of 5-8, while most spots are focused in the range of 20-60 kDa and pI 3-5 for *S. alterniflora* (Fig. 2.3). Based on the software detection (Progenesis SameSpots), the 2-D gels of *S. alterniflora* and *S. pectinata* contain 231 and 297 individual spots of heat-stable proteins, respectively. Comparison of 2-D gels between the two *Spartina* species identified 103 spots with significant changes of protein abundance, among which 83 spots had more abundance in dried *S. pectinata* (fold ≥ 1.6) (Table S-9) and 20 spots more abundant in dried *S. alterniflora* (fold > 2). Two examples of gel software analysis are shown in Figs 2.4 and 2.5.

a



b

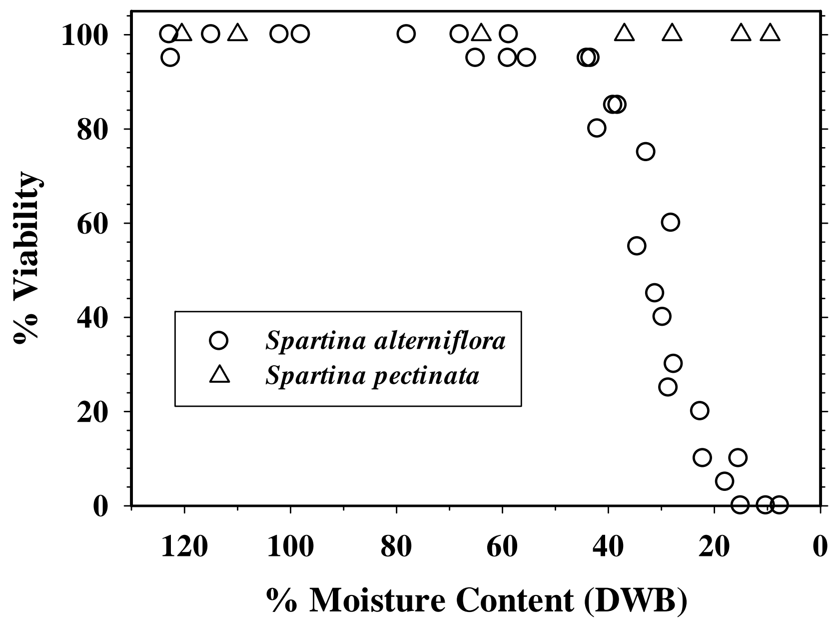


Figure 2.1. a, images of *S. alterniflora* and *S. pectinata* seeds. b, viability of *S. alterniflora* and *S. pectinata* seeds as a function of moisture content during desiccation. *S. alterniflora* seeds were imbibed in water after harvest (2006) and then air dried. *S. pectinata* seeds (2007) were imbibed after dry storage at -20ºC and then air dried at 23ºC.

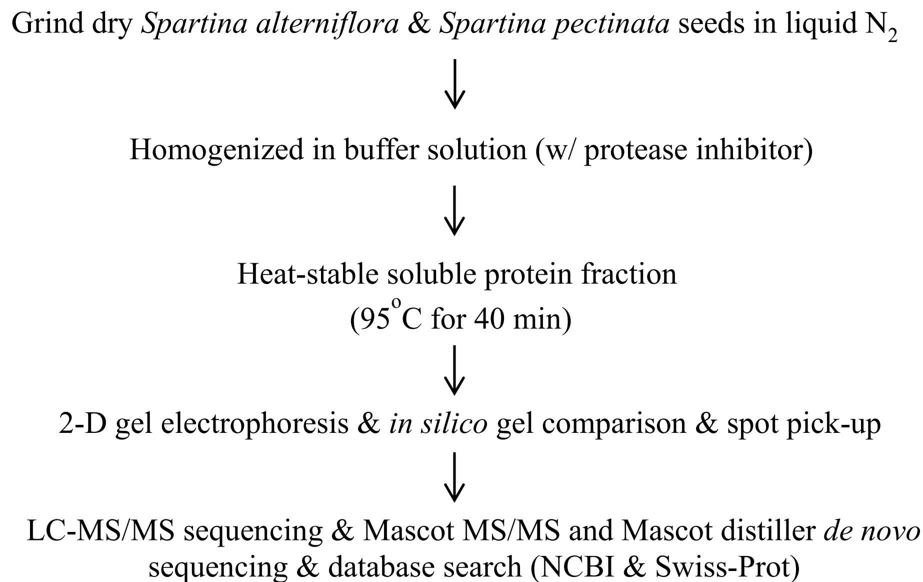


Figure 2.2. A workflow for using comparative proteomics to identify differentially expressed proteins between orthodox *S. pectinata* and recalcitrant *S. alterniflora*.

The 83 spots that were more abundant in dry *S. pectinata* were sequenced with LC-MS/MS. MS/MS data were processed by Mascot MS/MS ion search (Fig. 2.7) for probability-based protein identification, and Mascot Distiller for *de novo* peptide sequencing (Fig. 2.6), which were then subjected to a BLAST search. Proteins identified by Mascot MS/MS ion search and BLAST searches of peptides obtained from *de novo* sequencing are summarized in Table 2.1. The criteria for judging a confident identification by Mascot MS/MS ion search are that at least two unique peptides belong to the same protein, and the score of each match was above the significant Mascot scores threshold. If only one peptide was identified, the peptide was further confirmed with *de novo* sequencing by Mascot Distiller. Of the 83 spots, homologies were identified for 70, while 13 spots did not match any known sequences (Table S-8).

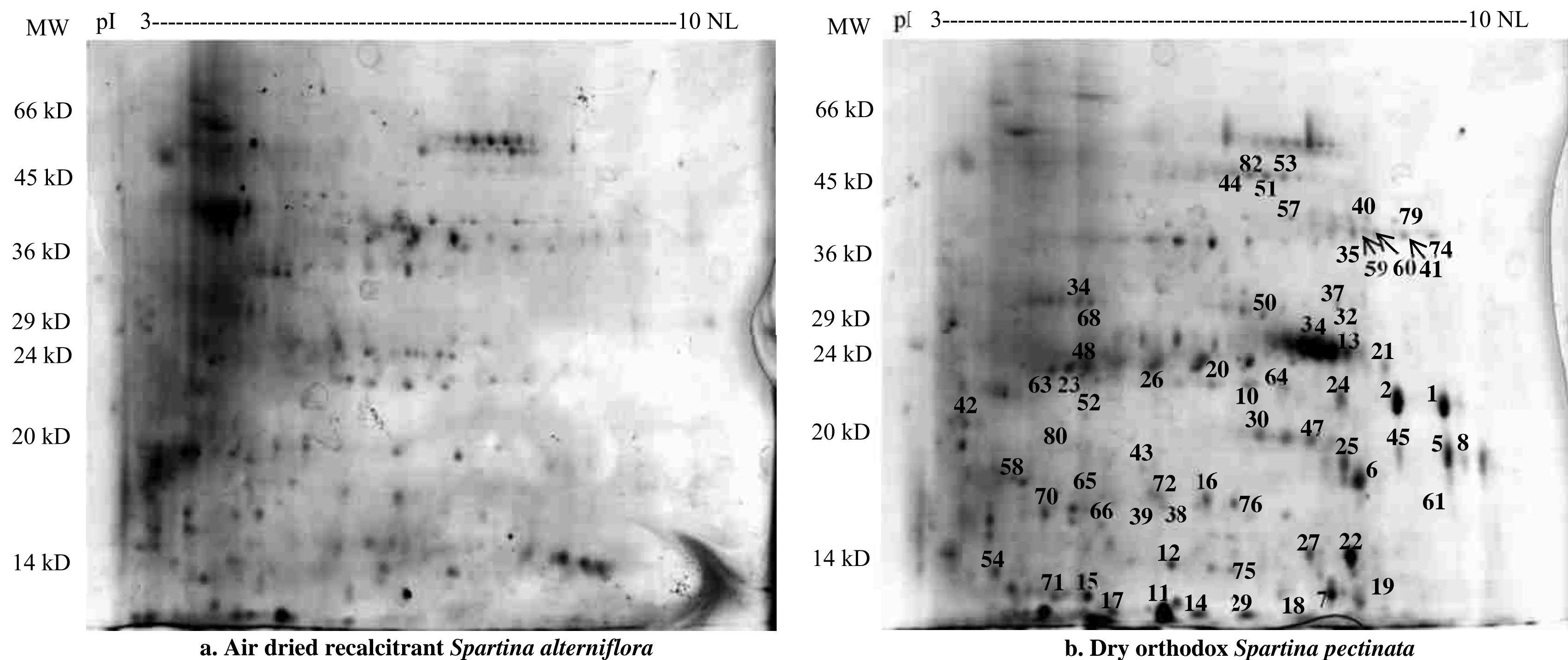


Figure 2.3. Comparison of two-dimensional gels of heat-stable protein fractions between dry recalcitrant (a) *S. alterniflora* and (b) dry orthodox *S. pectinata*. Gels were stained with Colloidal Coomassie Blue G-250. Protein spots, whose abundance was significantly higher in *S. pectinata*, are labeled (b), and were subjected to LC-MS/MS sequencing (Table 2.1). *S. alterniflora* seeds were harvested from Port Fourchon, LA (2009) and air dried at 23ºC for 2 weeks [MC=17% (DWB), G=0%, V=0%]. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), and stored dry at -20ºC for 20 months [MC=10% (DWB), G=0%, V=95%]. MC = moisture content; G = germination percentage; V = viability.

Table 2.1. Heat stable proteins that were more abundant in *S. pectinata* or missing in *S. alterniflora*. Proteins were identified using a Mascot search of MS/MS data or a BLAST search of peptides obtained from *de novo* sequencing by Mascot Distiller from *S. pectinata* peptide digest with NCBI and Swiss-Prot databases.

Spot[a]	Experimental $M_r(x10^3)/pI$[b]	Protein Homology	Accession #	Theoretical $M_r(x10^3)/pI$[c]	Mascot[d]	E value[e]
		Responsive to abiotic stimulus				
		LEA proteins				
1	23/7.9	Group-3 late embryogenesis abundant protein (LEA) (*Sorghum bicolor*)	ACV91271	24/7.9	-	$4e^{-7}$
2	23/6.9	Group-3 LEA (*Ampelocalamus calcareus*)	ADC55280	20/5.9	-	0.001
3	28/6.2	Dehydrin (*Sporobolus stapfianus*)	CAA71750	8/7.1	-	$3e^{-6}$
8	20/8.3	Group-3 LEA (*Zea mays*)	NP_001105298	23/8.6	-	$9e^{-4}$
13	26/6.6	LEA-1 (*Sorghum bicolor*)	XP_002465340	38/6.9	-	$5e^{-10}$
24	23/6.6	Group-3 LEA (*Pogonatherum paniceum*)	ADF36680	15/8.4	-	$3e^{-4}$
32	33/6.6	LEA-1 (*Sorghum bicolor*)	XP_002465340	38/6.9	-	$3e^{-9}$
37	33/6.6	LEA-1 (*Sorghum bicolor*)	XP_002465340	38/6.9	-	$3e^{-9}$
38	17/5.6	LEA-14 (*Zea mays*)	NP_001152646	16/5.7	-	$2e^{-6}$
47	21/6.2	Dehydrin (*Zea mays*)	CAA33364	17/8.0	-	$2e^{-4}$
50	33/6.0	LEA-1 (*Sorghum bicolor*)	XP_002465340	38/6.9	-	$6e^{-9}$
72	17/5.6	LEA-14 (*Zea mays*)	NP_001152646	16/5.7	-	$2e^{-6}$
		Chaperones				
23	25/5.2	Chaperonin (*Zea mays*)	ACF78291	26/8.5	6 (37)	-
39	17/5.5	Type-II heat shock protein (*Brachypodium distachyon*)	XP_003564488	18/6.0	3 (12)	-
42	23/3.8	Putative nascent polypeptide associated complex alpha chain (*Oryza sativa*)	AAT01337	14/4.9	4 (50)	-
43	20/5.6	Small heat shock protein (*Hordeum vulgare*)	BAK00191	18/5.6	-	$8e^{-17}$
48	26/5.3	Chaperonin (*Zea mays*)	ACF78291	26/8.5	4 (15)	-
57	42/6.0	Heat shock protein 60 kDa (*Oryza sativa*)	NP_001054641	59/6.2	4 (11)	-
63	25/5.1	Chaperonin (*Oryza sativa*)	CI007522	21/5.9	1 (9)	-
75,81	13/5.7	Small Type-I heat shock protein (*Oryza sativa*)	ABY52935	18/5.8	1 (9)	-
80	22/5.1	Nascent polypeptide associated complex alpha chain (*Brachypodium distachyon*)	XP_003568578	22/4.2	2 (16)	-

[a] Spot identification number (Fig. 2.3). [b] Obtained manually based on 3-10 NL IPG strip and molecular markers. [c] Obtained from Mascot. [d] Number of identified unique peptides by Mascot MS/MS ion search. The values in parentheses indicate the percentage of sequence coverage of identified peptides. [e] Lowest E-value of BLAST search for *de novo* sequenced peptides. The E value of a significant threshold was set below 0.01 (default value = 10).

Table 2.1. (Continued from the previous page).

Spot[a]	Experimental $M_r(x10^3)/pI^b$	Protein Homology	Accession #	Theoretical $M_r(x10^3)/pI^c$	Mascot[d]	E value[e]
		Responsive to Abiotic Stimulus				
		Metabolism				
5	20/7.9	Fructose-bisphosphate aldolase (*Zea mays*)	NP_001105336	39/7.5	2 (7)	-
25	20/6.6	Glyceraldehyde-3-phosphate dehydrogenase (*Magnolia liliiflora*)	P26518	37/7.1	2 (6)	-
35	37/6.6	Glyceraldehyde-3-phosphate dehydrogenase (*Aeluropus lagopoides*)	AEP31954	36/7.1	8 (37)	-
40	41/6.7	Glyceraldehyde-3-phosphate dehydrogenase (*Antirrhinum majus*)	P25861	37/8.0	2 (9)	-
41	37/7.0	Glyceraldehyde-3-phosphate dehydrogenase (*Aeluropus lagopoides*)	AEP31954	36/7.1	7 (37)	-
59	37/6.7	Glyceraldehyde-3-phosphate dehydrogenase (*Brachypodium distachyon*)	XP_003573318	36/6.6	4 (16)	-
60	37/6.8	Glyceraldehyde-3-phosphate dehydrogenase (*Hordeum vulgare*)	P26517	37/6.6	6 (21)	-
61	17/7.9	Fructose-bisphosphate aldolase (*Zea mays*)	NP_001105336	39/7.5	2 (7)	-
74	37/7.6	Glyceraldehyde-3-phosphate dehydrogenase (*Aeluropus lagopoides*)	AEP31954	36/7.1	6 (29)	-
79	41/7.0	Fructose-bisphosphate aldolase (*Zea mays*)	P08440	38/7.5	4 (20)	-
		Antioxidants				
10	24/5.8	Glutathione transferase (*Spartina alterniflora*)	EH277202	24/6.0	2 (17)	-
10	24/5.8	Mitochondrial Mn superoxide dismutase (*Dactylis glomerata*)	HO174410	21/6.4	2 (12)	-
16	17/5.7	Superoxide dismutase [Cu/Zn] (*Aeluropus lagopoides*)	Q9SQL5	15/5.3	4 (27)	-
17	12/5.4	Glutaredoxin (*Sorghum bicolor*)	XP_002446766	13/6.6	-	$1e^{-10}$
18	12/6.3	Glutaredoxin (*Eragrostis curvula*)	EH188284	22/8.3	1 (8)	
26	26/5.6	Glutathione peroxidase (*Helianthus annuus*)	ABG36000	22/6.7	-	$5e^{-9}$
29	12/5.7	1- Cys Peroxiredoxin (*Triticum aestivum*)	ACE82290	-	-	$5e^{-5}$
36	17/5.2	Putative superoxide dismutase [Cu-Zn] (*Oryza sativa*)	BAD09607	20/5.8	5 (31)	-
58	19/5.0	Peroxiredoxin-2 (*Oryza sativa*)	EAY84834	23/6.2	2 (12)	-
65	17/5.2	Superoxide dismutase [Cu-Zn] (*Oryza sativa*)	BAD09607	21/5.8	3 (20)	-
66	16/5.3	2-Cys Peroxiredoxin (*Oryza sativa*)	NP_001043845	17/5.6	3 (41)	-
68	33/5.3	1-Cys Peroxiredoxin (*Triticum aestivum*)	ACE82290	24/6.4	-	$4e^{-6}$
70	17/5.0	Putative superoxide dismutase [Cu-Zn] (*Oryza sativa*)	BAD09607	20/5.8	5 (31)	-

[a] Spot identification number (Fig. 2.3). [b] Obtained manually based on 3-10 NL IPG strip and molecular markers. [c] Obtained from Mascot. [d] Number of identified unique peptides by Mascot MS/MS ion search. The values in parentheses indicate the percentage of sequence coverage of identified peptides. [e] Lowest E-value of BLAST search of *de novo* sequenced peptides. The E value of a significant threshold was set below 0.01 (default value = 10).

Table 2.1. (Continued from the previous page).

Spot[a]	Experimental $M_r(x10^3)/pI^b$	Protein Homology	Accession #	Theoretical $M_r(x10^3)/pI^c$	Mascot[d]	E value[e]
		Responsive to Abiotic Stimulus				
		DNA or RNA binding protein				
15	13/5.3	Glycine-rich RNA-binding protein (*Spartina alterniflora*)	EH277379	23/8.2	3 (20)	-
52	23/5.3	Transcription factor BTF3 (*Sorghum bicolor*)	XP_002466010	18/5.8	3 (37)	-
		Cellular recycling				
19	13/6.7	Autophagy-related protein-13 (*Arabidopsis thaliana*)	NP_190528	68/8.8	-	0.003
31	21/6.1	Tetra-ubiquitin (*Saccharum* hybrid cultivar H32-8560)	AAC67551	34/6.2	8 (28)	-
45	20/6.9	Ubiquitin (*Solanum lycopersicum*)	CAA51679	60/7.1	5 (10)	-
83	7/6.2	Ubiquitin precursor (*Musa acuminate*)	AAM69293	9/6.5	3 (44)	-
		Protease inhibitor				
3	28/6.2	Putative cystatin (*Zea mays*)	CAG29024	27/6.1	3 (13)	-
4	27/6.3	Putative cystatin (*Zea mays*)	CAG29024	27/6.1	2 (8)	-
9	26/6.5	Putative cystatin (*Zea mays*)	CAG29024	27/6.1	3 (13)	-
20	26/5.7	Cystatin (*Chloris virgate*)	DC997921	21/6.3	2 (18)	-
22	14/6.6	Trypsin inhibitor (*Triticum monococcum*)	ACR83573	16/7.2	-	$8e^{-6}$
		Stress related proteins				
11	12/5.6	Stress-responsive protein (*Oryza sativa*)	NP_001043194	17/8.4	2 (15)	-
14	12/5.6	Stress responsive protein (*Oryza sativa*)	AAP92753	13/5.0	-	$2e^{-5}$
30	21/5.8	Abscisic stress ripening protein 2 (*Oryza sativa*)	ABR25748	21/7.2	2 (22)	-
54	14/4.9	Truncated copper binding protein (CutA) (*Oryza sativa*)	2ZOM_A	19/9.1	2 (15)	-
		Protein Synthesis Inhibitor				
21	25/6.8	Translation-inhibitor protein (*Gentiana triflora*)	BAC66487	20/7.6	2 (13)	-
		Storage Proteins				
44	50/5.7	Vicilin (*Eleusine coracana*)	CX264721	21/5.5	2 (22)	-
51	50/6.0	Globulin-like protein (*Oryza sativa*)	AAM33459	52/6.8	-	$5e^{-6}$
53	50/6.1	Globulin-like protein (*Oryza sativa*)	AAM33459	52/6.8	-	$4e^{-7}$
73	50/6.2	Globulin-like protein (*Oryza sativa*)	AAM33459	52/6.8	-	$4e^{-7}$
82	50/5.8	Globulin-like protein (*Oryza sativa*)	AAM33459	52/6.8	-	$4e^{-7}$

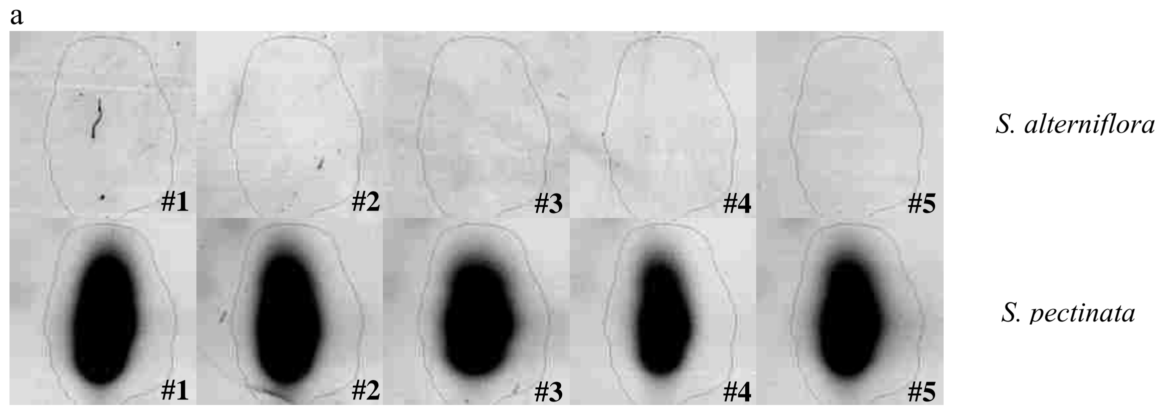
[a] Spot identification number (Fig. 2.3). [b] Obtained manually based on 3-10 NL IPG strip and molecular markers. [c] Obtained from Mascot. [d] Number of identified unique peptides by Mascot MS/MS ion search. The values in parentheses indicate the percentage of sequence coverage of identified peptides. [e] Lowest E-value of BLAST search of *de novo* sequenced peptides. The E value of a significant threshold was set below 0.01 (default value = 10).

Table 2.1. (Continued from the previous page).

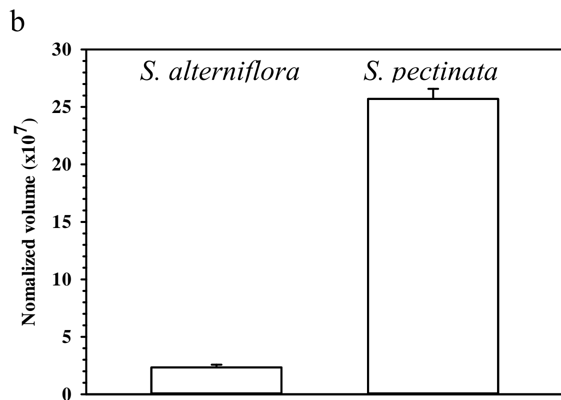
Spot[a]	Experimental $M_r(x10^3)/pI$[b]	Protein Homology	Accession #	Theoretical $M_r(x10^3)/pI$[c]	Mascot[d]	E value[e]
		Other proteins				
6	17/6.6	CBS-domain (Cystathionine Beta Synthase) Arabidopsis protein targeted to mitochondrion protein (*Saccharum officinarum*)	CBB36496	22/8.7	-	0.005
12	14/5.6	Hypothetical protein (*Selaginella moellendorffii*)	XP_002963754	88/6.1	-	$9e^{-5}$
33	33/5.8	SOUL heme-binding domain containing protein (*Triticum aestivum*)	CBH32522	24/6.0	2 (9)	-
34	33/5.2	Oxygen-evolving enhancer protein 1 (*Zea mays*)	ACG31595	34/5.6	2 (8)	-
71	13/5.0	Probable calmodulin, calcium-binding protein (*Zea mays*)	NP_001148586	17/4.8	-	$1e^{-9}$
76	17/5.7	FKBP FK506 binding protein-2 (*Oryza sativa*)	EAZ09641	16/5.4	2 (17)	-

[a] Spot identification number (Fig. 2.3). [b] Obtained manually based on 3-10 NL IPG strip and molecular markers. [c] Obtained from Mascot. [d] Number of identified unique peptides by Mascot MS/MS ion search. The values in parentheses indicate the percentage of sequence coverage of identified peptides. [e] Lowest E-value of BLAST search of *de novo* sequenced peptides. The E value of a significant threshold was set below 0.01 (default value = 10).

a



b



c

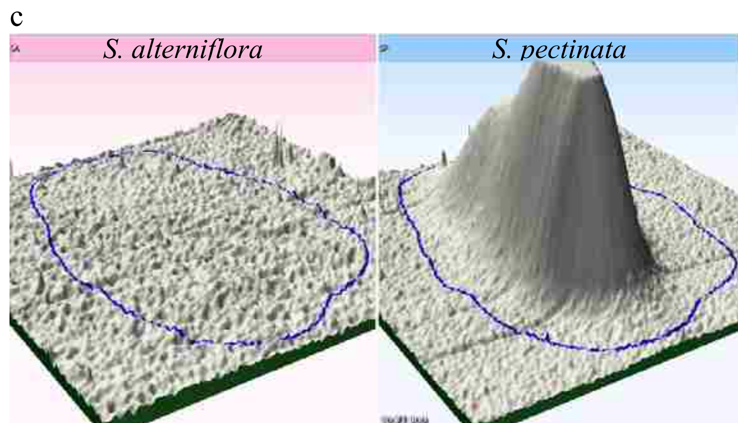
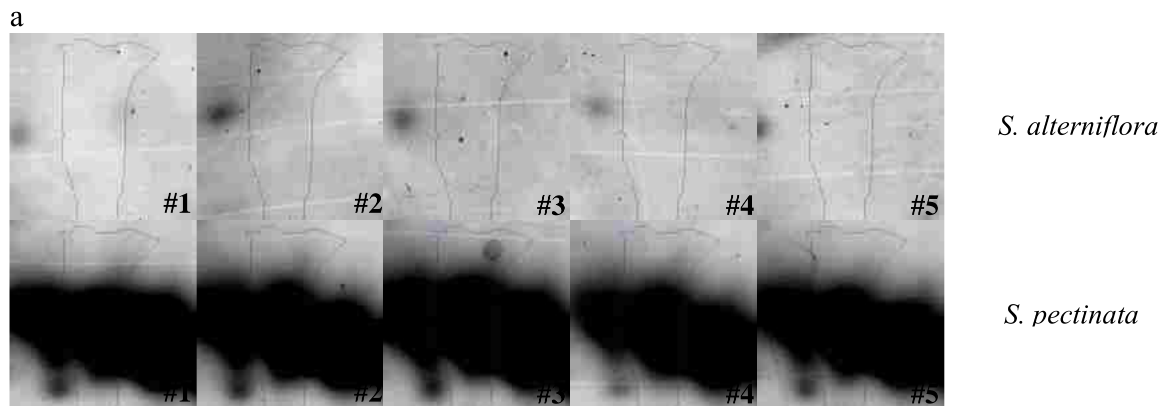
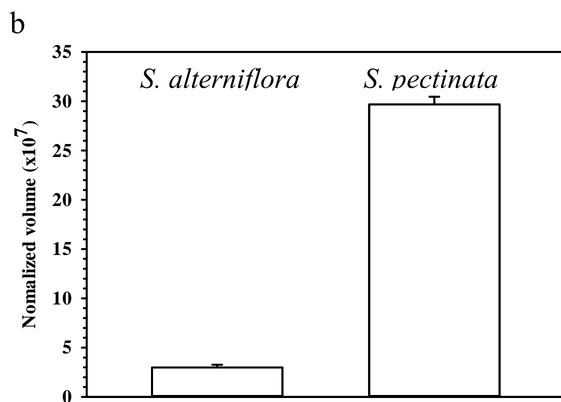


Figure 2.4. *In silico* 2-D gel analysis by Progenesis Samespots. a, group-3 LEA (spot 2, figure 2.3) is present in *S. pectinata* but missing in *S. alterniflora*. Five biological replicates were included for both *S. pectinata* and *S. alterniflora*. b, spot volume comparison of group-3 LEA in Fig. 2.4a (n=5). c, 3D montage comparison of group-3 LEA in Fig. 2.4a.

a



b



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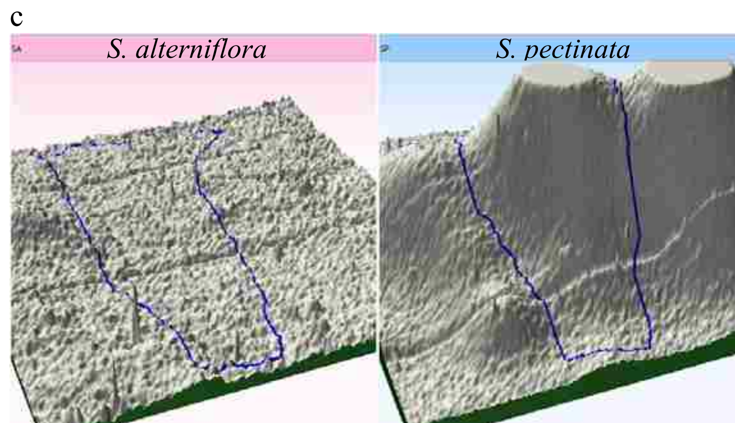


Figure 2.5. *In silico* 2-D gel analysis by Progenesis Samespots. a, cystatin (spot 3, Figure 2.3) is present in *S. pectinata* but missing in *S. alterniflora*. Five biological replicates were included for both *S. pectinata* and *S. alterniflora*. b, spot volume comparison of cystatin in Fig. 2.5a (n=5). c, 3D montage comparison of cystatin in Fig. 2.5a.

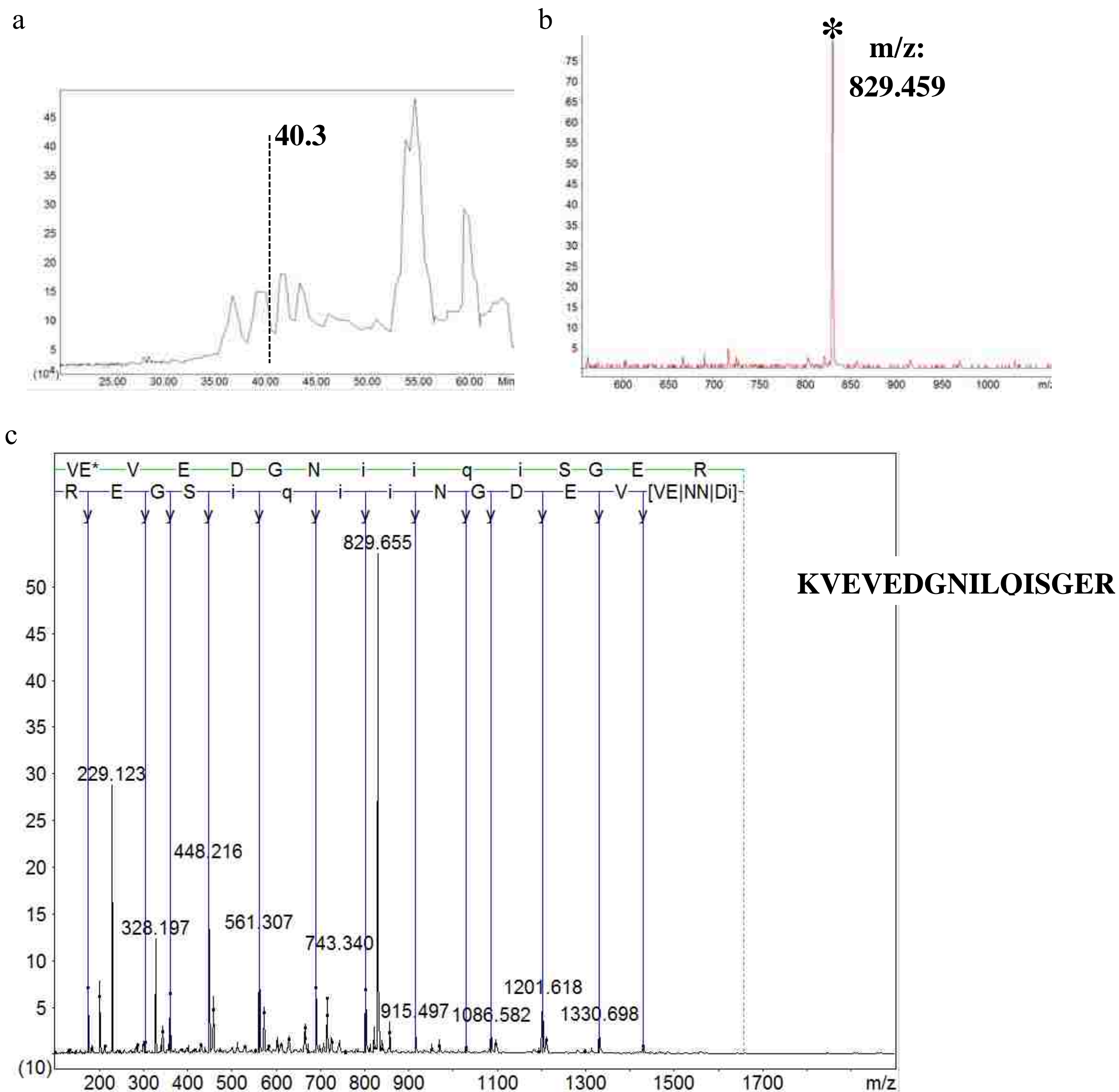


Figure 2.6. Mass spectrometry data for peptide *de novo* sequencing. a, total ion chromatogram of all the mass spectra during a 70 min gradient LC separation. Dash line represents an eluted peptide at 40.30 minute. b, mass spectrum of the eluted peptide at 40.30 min. c, MS/MS spectrum of the peptide eluted at 40.30 minutes. The mass differences between different "y" ions is used to predict the amino acid sequence. Direction from amino side to carboxylic side is from right to left.

A number of proteins shared homologies with LEAs, which include group-3 LEAs (#1, 2, 8, 24, 32, 37, 50), group-2 LEAs (dehydrin) (#3, 47) and LEA-14 (#38, 72). *De novo* identified peptides, DETGNVIqqATEqV and DAVMNTEGMSGDAG, belonged to motif 1 and motif 2 of group-3 LEAs, respectively (#1, 2, 24). Chaperonins (spots #23, 48, 57, 63), small heat shock proteins (#39, 43, 75), group-2 LEAs (dehydrin) (#3, 47), nascent peptide associated complex proteins (#42, 80) and FK506 binding protein (#76), which belong to the group of putative molecular chaperones, were present in dry *S. pectinata*. Since only one peptide was identified for dehydrin (#47), the peptide was further manually confirmed by *de novo* sequencing with Mascot Distiller and Blast search (Table 2.1). Identified proteins include a number of antioxidants, such as superoxide dismutase [Cu-Zn] (#16, 36, 65, 70), mitochondrial manganese superoxide dismutase (#64), peroxiredoxin (#29, 58, 66, 68), glutathione transferase (#10), glutathione peroxidase (#26), and glutaredoxin (#17, 18). Interestingly, spots for glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#25, 35, 40, 41, 59, 60, 74) (Fig. 2.8) and fructose bisphosphate aldolase (#5, 61, 79) were more abundant in *S. pectinata* or missing in *S. alterniflora*. Protease inhibitor, cystatin (a cysteine protease inhibitor, #3, 4, 9 and 20) and trypsin inhibitor (#22) were only present in *S. pectinata*. Ubiquitin (#31, 45 and 83) and autophagy-related protein (#19) were associated with cellular recycling of degraded proteins. Two putative DNA and RNA binding proteins were identified, which are glycine-rich binding protein (#15) and transcription factor BTF3 (#52). A number of spots share homology with known stress related proteins, *e.g.* stress-responsive proteins (#11, 14), abscisic acid stress ripening protein-2 (#30) and copper binding protein (#54). Other identified proteins associated with stress based on the GO terminology include translation-inhibitor protein and globulin-1 proteins (#44, 51, 53, 82). A number of identified proteins were not categorized as the stress

responsive proteins based on the GO terminology, and those proteins include CBS-domain Arabidopsis protein target to mitochondrion protein (#6), hypothetical protein (#12), SOUL heme binding domain containing protein (#33), oxygen evolving enhancer protein (#34), calmodulin/calcium binding protein (#71), and FK506 binding protein (#76).

Proteomic data have been deposited to Tranche (https://proteomecommons.org/dataset.jsp?id=x8O52IsPvhZyRxzhBwU1RkxL%2F2UUKETUzg2%2FB3VKRFfTAFwa23Lj0IVSdf0aYyfW5pqkb9i%2BWWfLoA2u%2BKQTMBAXhfoAAAAAAAcOg%3D%3D) and PeptideAtlas (ftp://PASS00103:LG8339u@ftp.peptideatlas.org/).

Desiccation Tolerance Test of *S. pectinata* Seedlings

The germination speed of cut *S. pectinata* varied. After 3 days of germination, *S. pectinata* seedlings were divided into 3 groups (<2 mm, 2mm-5 mm, ≥5 mm) based on the lengths of shoot emergence. *S. pectinata* seedlings with a shoot length that was shorter than 2 mm were still desiccation tolerant, because the shoots continued to elongate (11/12 in Fig. 2.9) after flash drying and re-imbibition for growth at 27°C (Fig. 2.9). Fig. 2.10 shows majority of *S. pectinata* seedlings of the shoot length that was between 2 mm and 5mm continued to grow (12/20 in Fig. 2.10) after flash drying and re-imbibition. However, when the shoot length of *S. pectinata* seedlings grew longer than 5 mm, they became desiccation intolerant. Seedlings stopped growing, and fungal growth started to be noticed on seedlings (Fig. 2.11).

2-D Gel Comparison among *S. pectinata* Seedlings of Different Developmental Stages

Two-dimensional gels between desiccation tolerant *S. pectinata* seeds and *S. pectinata* seedlings of differential developmental stages were compared. If proteins that were present in *S. pectinata* seeds, but missing in *S. alterniflora* seeds, are associated with desiccation tolerance, it

a

1. gi|348161388 **Mass:** 36472 **Score:** 562 **Matches:** 9(5) **Sequences:** 6(5) **emPAI:** 0.54
glyceraldehyde-3-phosphate dehydrogenase [Aeluropus lagopoides]
☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	50	710.8474	1419.6802	1419.6704	0.0098	0	71	0.00032	1		K.DAPMFVVGVNEDK.Y
☑	52	717.8995	1433.7845	1433.7514	0.0331	0	(31)	3.3	1		R.AASFNIIPSSTGAAK.A
	53	717.9055	1433.7965	1433.7514	0.0451	0	(23)	21	3		R.AASFNIIPSSTGAAK.A
☑	54	718.0000	1433.9854	1433.7514	0.2340	0	44	0.12	1		R.AASFNIIPSSTGAAK.A
☑	56	749.9402	1497.8659	1497.8403	0.0256	0	90	4e-06	1		R.VPTVDVSVVDLTVR.I
☑	57	750.4348	1498.8550	1497.8403	1.0147	0	(20)	36	1		R.VPTVDVSVVDLTVR.I
☑	66	894.9112	1787.8078	1787.7903	0.0175	0	91	2.7e-06	1		K.LVSWYDNEWGYSNR.V
☑	79	1088.9982	2175.9818	2175.9631	0.0188	0	151	1.6e-12	1		K.GIMGYTDEDLVSTDFVGDSR.S
☑	92	871.1341	2610.3804	2610.3476	0.0328	0	119	2.1e-09	1		K.VIHDNFGIVEGLMTTVHSITATQK.T

b

Protein sequence coverage: 29%

Matched peptides shown in ***bold red***.

```
  1 MGKIKIGING FGRIGRLVAR VALQSEDVEL VAVNDPFITT DYMTYMFKYD
 51 TVHGQWKHSD IKVKDAKTLL FGQKPVTVFG IRNPEEIPWA EAGAEYVVES
101 TGVFTDKEKA AAHLKGGAKK VVISAPSKDA PMFVVGVNED KYTSDLNIVS
151 NASCTTNCLA PLAKVIHDNF GIVEGLMTTV HSITATQKTV DGPSAKDWRG
201 GRAASFNIIP SSTGAAKAVG KVLPDLNGKL TGMAFRVPTV DVSVVDLTVR
251 IEKAASYEDI KNAIKAASEG KLKGIMGYTD EDLVSTDFVG DSRSSIFDAK
301 AGIALNDHFV KLVSWYDNEW GYSNRVVDLI RHMAKTQ
```

c

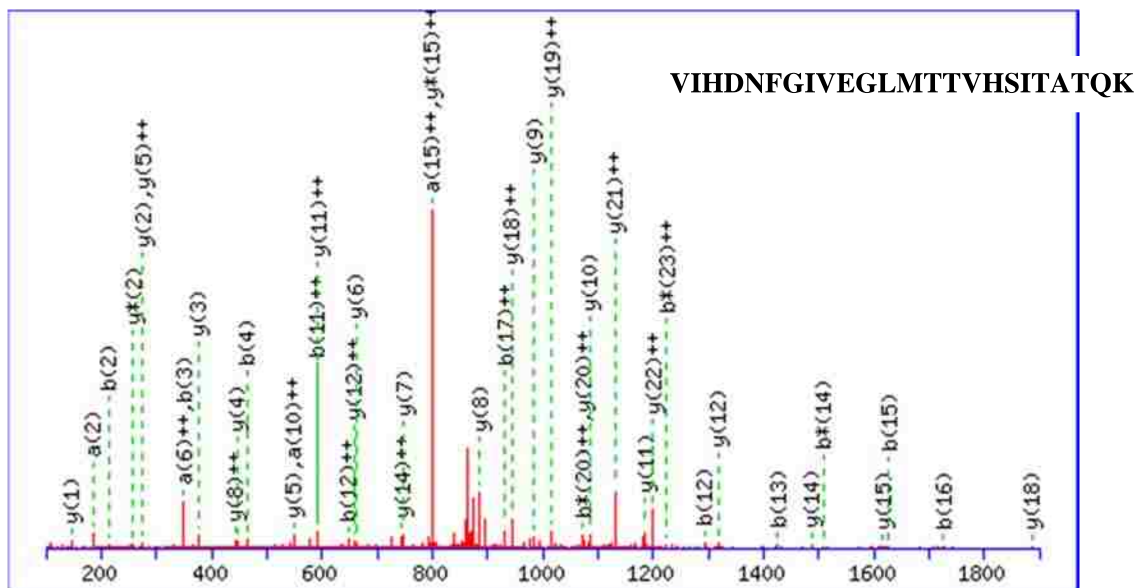


Figure 2.7. Protein identification based on a Mascot MS/MS ion search. a: identified protein homology with highest Mascot protein score. b: sequence coverage of identified peptides amino acid number divided by the total amino acid number. c: MS/MS spectrum of the peptide "VIHDNFGIVEGLMTTVHSITATQK".

was expected that these proteins would continue to be present in desiccation tolerant seedlings, but disappear in desiccation intolerant *S. pectinata* seedlings.

S. pectinata seedlings of shoot length that was *ca.* 2-3 mm were still desiccation tolerant (Figs 2.9 and 2.10), and 2-D gel profile of their heat-stable fraction looked closely similar (Fig. 2.12). Computational comparison of Progenesis SameSpots between desiccation tolerant *S. pectinata* seeds and fresh desiccation tolerant seedlings did not identify any significant expression changes of those spots in Table 2.1 (Fig. 2.12), except spots #34 and #68. Oxygen-evolving enhancer protein (#34), peroxiredoxin (#68) and several other proteins that exhibited similar pI and molecular weight to spots #34 and #68 were more abundant in *S. pectinata* fresh seedlings (b) than seeds (a).

When *S. pectinata* seedlings continued to grow to the point when the shoot length was longer than 5 mm, they made the transition from desiccation tolerant to desiccation intolerant. The majority (270 ± 2 of 289 ± 8 spots; average of three replicates ± represents standard error) of heat-stable proteins disappeared in the 2-D gel profiles of fresh, undried desiccation intolerant *S. pectinata* seedlings (Fig. 2.13a). Heat-stable proteins from flash dried desiccation intolerant *S. pectinata* seedlings almost completely vanished (280 ± 2 of 289 ± 8 spots) (Fig. 2.13b).

Two-dimensional Gel Comparisons of Phosphorylated Heat-stable Proteins

Figure 2.8 shows the 2-D gel comparison of phosphorylated heat-stable proteins between *S. alterniflora* and *S. pectinata*. Gels were stained with PRO-Q Diamond that specifically binds to phosphorylated proteins. Molecular markers that contained known phosphorylated proteins (ovalbumin and β-casein) were used as positive controls, and these two phosphorylated proteins (45 kDa and 24 kDa) in the marker mixture were stained and visualized (Fig. 2.8). A number of phosphorylated proteins were observed in the molecular range of 20-25 kDa and pI range of 5.7-

6.4 in *S. pectinata*, while no phosphoproteins were visualized with PRO-Q Diamond staining in *S. alterniflora*. Those differentially expressed phosphorylated heat-stable proteins in *S. pectinata* (Fig. 2.3b) were sequenced with LC-MS/MS and shared homologies with cystatin, abscisic acid ripening protein, tetra-ubiquitin, and dehydrin (Table 2.2).

Effect of Drying and Rehydration on the Proteomic Profiles of Heat-stable Fractions in *S. alterniflora* and *S. pectinata*

Freshly harvested and air dried *S. alterniflora* were compared to examine the effect of dehydration on the 2-D proteomic profiles of heat-stable fractions, but software did not detect significant changes of proteomic profiles (Fig. 2.14). Computational analysis did not detect significant changes of proteomic profiles of heat-stable fractions between dry and fully hydrated (re-imbibed) dormant *S. pectinata* (Fig. 2.15).

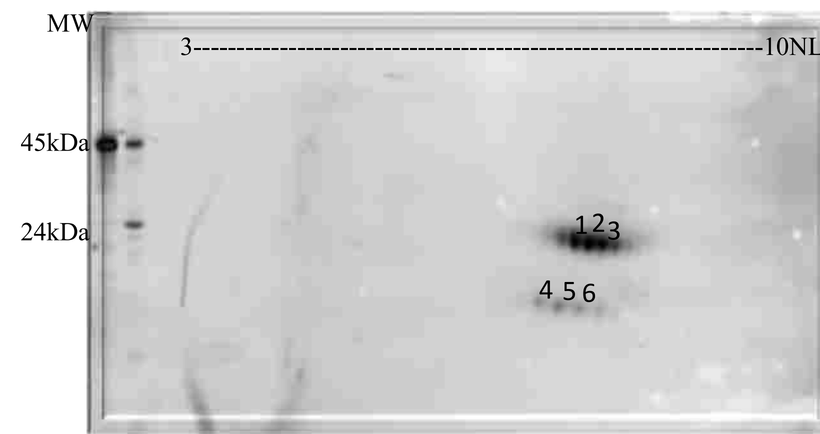
Identification of Enriched Cis-regulatory DNA Elements

The 38 differentially expressed (versus *S. alterniflora*) *S. pectinata* proteins (Table 2.1) were BLAST searched for the orthologous rice genes (38) using the tblastn program in the database of Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). The 1 kb nucleotide sequence of the upstream promoter region of each gene was subjected to the *in silico* identification of *cis*-regulatory element motifs, and 215 and 100 *cis*-element motifs were identified by PLACE and PlantCARE, respectively. Motifs that were present in over 50% of those 38 orthologous rice genes were considered as enriched *cis*-element motifs: 57 by PLACE and 13 by PlaceCARE (Tables 2.6 and 2.7). Transcription factors that may putatively bind to those *cis*-regulatory DNA elements are also listed; categorization of transcription factors was based on PlnTFDB and AtTFDB (Tables 2.6 and 2.7).

DISCUSSION

One of the hypotheses of the recalcitrant seed death during desiccation is the absence of protective proteins (Berjak and Pammenter, 2008). Recent evidence supporting this association came from transcriptomic and proteomic changes observed during the dehydration of desiccation tolerant anhydrobiotes or the development of orthodox seeds (Mali *et al.,* 2010; Rodriguez *et al.,* 2010). Various mRNAs/proteins were up-regulated significantly in desiccation tolerant stages; consequently, those differentially expressed macromolecules were putatively associated with desiccation tolerance. Proteomic comparison between hydrated and desiccated recalcitrant *Camellia sinensis* embryos was performed to identify putatively recalcitrance-associated proteins, *e.g.* catalase, ascorbate peroxidase (Chen *et al.,* 2011). However, the interpretation of results of this one-species experimental system, whose protein expression patterns were compared before and after desiccation in the absence of an orthodox seed comparison, is ambiguous because the observed differential expression pattern during dehydration could be either associated with a lack of desiccation tolerance or just a simple response to the drying. Proteomic analysis of only a recalcitrant-seeded species could not detect proteins

a. Air dried recalcitrant *Spartina alterniflora*



b. Dry orthodox *Spartina pectinata*

Figure 2.8. Comparison of two-dimensional gels of phosphorylated, heat-stable proteins between dry recalcitrant *S. alterniflora* (a) and dry orthodox *S. pectinata* (b). Phosphorylated proteins were stained with PRO-Q Diamond. Differentially expressed spots in *S. pectinata* were labeled and subjected to LC-MS/MS sequencing. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2009) and air dried at 23ºC for 4 weeks [MC=17% (DWB), G=0%, V=0%]. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), and stored dry at -20ºC for 23 months [MC=10% (DWB), G=0%, V=95%]. MC = moisture content; V = viability; G = germination percentage.

Table 2.2. Identification of phosphorylated proteins using Mascot and BLAST searches. Analytical analysis was performed with PRO-Q Diamond stained gels, and differentially expressed phosphorylated proteins labeled in *S. pectinata* (Fig. 2.6) were excised from Colloidal Coomassie Blue stained gels for identification using a Mascot search of MS/MS data with NCBI and SWISSPROT databases, or a BLAST search of peptides obtained from *de novo* sequencing (Mascot Distiller) of MS/MS data with the NCBI database. a: peptide that was identified by Mascot MS/MS with the Mascot score above the significant threshold was reported in the table. b: Protein homology search was accomplished with Mascot MS/MS ion search (version 2.3.x) (http://www.matrixscience.com/). c: Theoretical molecular weight (MW) and isoelectric point (pI) were reported in the Mascot program. d: Experimental molecular weight (MW) and isoelectric point (pI) were calculated manually based on 3-10 NL IPG strip and known molecular markers. e: a Mascot score of the significant threshold was given in the output after each Mascot MS/MS search. Mascot score for identified peptides, which was above the significant threshold of each Mascot search, was reported.

Spot Number	Identified Sequences[a]	Protein Homology[b]	Accession Number	Theo. MW /pI[c]	Expt. MW /pI[d]	Peptide Coverage (%)	Mascot Score[e]
1, 2	KFDILMK GDATAFTNADLGAK SNSLFPYELLEILR	Putative cystatin (*Zea mays*)	CAG29024	25 kDa/6.1	28 kDa/6.2	13	157
3	FDILMK GDATAFTNADLGAK	Putative cystatin (*Zea mays*)	CAG29024	25 kDa/6.1	28 kDa/6.2	8	70
4	HKEHLGEMGALAAGAFALYEK ITEEIAATAAVGAGGYAFHEHHEK	Abscisic stress ripening protein 2 (*Oryza sativa*)	ABR25748	21 kDa/5.8	22 kDa/5.9	22	164
5	ESTIHLVLR TLSDYNIQK IQDKEGIPPDQQR TITLEVESSDTIDNVK TLADYNIQKESTLHLVLR	Tetra-ubiquitin (*Saccharum* hybrid cultivar H32-8560)	AAC67551	34 kDa/6.2	22 kDa/6.2	28	616
6	TGGILHR	Dehydrin (*Zea mays*)	CAA33364	17 kDa/8.0	21 kDa/6.4	4	58

Table 2.3. Summary of presence or up-regulation of glyceraldehyde-3-phosphate dehydrogenase in organisms under different stresses.

Species	Tissues	Differential Expression	Stresses	Citation
Agrostis scabra	Roots	Protein↑	Heat	Xu and Huang, 2008
Agrostis stolonifera	Leaves & roots	Protein↑	Salinity	Xu *et al.,* 2010
Arabidopsis thaliana	Seeds	Protein↑	Desiccation	Gallardo *et al.,* 2001
Arabidopsis thaliana	Seedlings	mRNA↑	Heat, anaerobic	Yang *et al.,* 1993
Artemia franciscana	Cysts	mRNA↑	Desiccation	Chen *et al.,* 2009
Cichorium intybus	Roots	Protein↑	Cold	Degand *et al.,* 2009
Craterostigma plantagineum	Leaves	mRNA↑	Desiccation	Rodriguez *et al.,* 2010
Craterostigma plantagineum	Leaves	mRNA↑	Desiccation, ABA	Velasco *et al.,* 1994
Medicago sativa	Seedlings	mRNA↑	Salinity	Jin et al., 2010
Milnesium tardigradum	Adult	Protein↑	Desiccation	Schokraie *et al.,* 2010
Nicotiana tabacum	Seedlings	mRNA↑	Drought	Ziaf *et al.,* 2011
Oryza sativa	Panicles	Protein↑	Salinity	Dooki *et al.,* 2006
Oryza sativa	Anthers	Protein↑	Drought	Liu and Bennett, 2011
Oryza sativa	Seedlings	mRNA↑	Drought	Minhas and Grover, 1999
Oryza sativa	Seedlings	mRNA↑	Drought, ABA	Pillai *et al.,* 2002
Oryza sativa	Seedlings	mRNA↑	Drought	Reddy *et al.,* 2002
Physcomitrella patens	Plant Tissues	Protein↑	Desiccation	Wang *et al.,* 2009

Table 2.3. Continued from the previous page.

Species	**Tissues**	**Differential Expression**	**Stresses**	**Citation**
Pleurotus sajor-caju	Strains	mRNA↑	Drought, salinity	Jeong *et al.,* 2000
Populus tremula	Shoots	Protein↑	Drought	Pelah *et al.,* 1997
Populus trichocharpa	Plant tissues	mRNA↑	Drought	Plomion *et al.,* 2006
Selaginella lepidophylla	Leaves	mRNA↑	Desiccation	Iturriaga *et al.,* 2006
Selaginella tamariscina	Leaves	Protein↑	Desiccation	Wang *et al.,* 2010a
Solanum luherositm	Seedlings	mRNA↑	Salinity	Jeong *et al.,* 2001
Triticum aestivum	Roots	Protein↑	Salinity	Wang *et al.,* 2008
Triticum durum	Seeds	Protein↑	Heat	Laino *et al.,* 2010
Vitis vinifera	Shoot tips	mRNA↑	Drought, salinity	Cramer *et al.,* 2007

Table 2.4. Summary of presence or up-regulation of fructose bisphosphate aldolase in organisms under different stresses.

Species	Tissues	Differential Expression	Stresses	Citation
Agrostis scabra	Roots	Protein↑	Heat	Xu and Huang, 2008
Agrostis stolonifera	Leaves & roots	Protein↑	Salinity	Xu *et al.,* 2010
Arabidopsis thaliana	Cell culture	Protein↑	Salinity	Ndimba *et al.,* 2005
Avena sativa	Leaves	Protein↑	Heat	Michelis and Gepstein, 2000
Medicago sativa	Seedlings	mRNA↑	Salinity	Jin et al., 2010
Nicotiana paniculata	Roots	mRNA↑	Salinity	Yamada *et al.,* 2000
Oryza sativa	Roots	Protein↑	Salinity	Chitteti and Peng, 2007
Oryza sativa	Seedlings	mRNA↑	Drought	Minhas and Grover, 1999
Oryza sativa	Leaves	Protein↑	Drought	Salekdeh *et al.,* 2002
Plectus murrayi	N/A	mRNA↑	Desiccation	Adhikari *et al.,* 2009
Populus trichocharpa	Plant tissues	mRNA↑	Drought	Plomion *et al.,* 2006
Selaginella lepidophylla	Leaves	mRNA↑	Desiccation	Iturriaga *et al.,* 2006
Selaginella tamariscina	Leaves	Protein↑	Desiccation	Wang *et al.,* 2010a
Sesuvium portulacastrum	Roots	mRNA↑	Salinity	Fan *et al.,* 2009
Triticum aestivum	Roots	Protein↑	Salinity	Wang *et al.,* 2008
Vitis vinifera	Shoot tips	mRNA↑	Drought, salinity	Cramer *et al.,* 2007

Table 2.5. Summary of presence or up-regulation of cystatin (cysteine protease inhibitor) in organisms under different stresses.

Species	Tissues	Differential Expression	Stresses	Citation
Amaranthus hypochondriacus	Seeds & roots	mRNA↑	Desiccation, drought, salinity, cold	Valdés-Rodríguez *et al.,*2007
Arabidopsis thaliana	Siliques	mRNA↑	Developing siliques, oxidative and nitrosative stresses	Belenghi *et al.,* 2003
Arabidopsis thaliana	Seeds	Protein↑	Desiccation	Hwang *et al.,* 2009
Arabidopsis thaliana	Plants	mRNA↑	Drought, cold	Seki *et al.,* 2001
Arabidopsis thaliana	Seedlings	mRNA↑	Drought, salinity, cold	Zhang *et al.,* 2008
Arabidopsis thaliana	Plant	mRNA↑	Heat	Hwang *et al.,* 2010
Artemia franciscana	Cysts	mRNA↑	Desiccation	Chen *et al.,* 2009
Cakile maritime	Leaves	mRNA↑	Drought, salinity	Megdiche *et al.,* 2009
Castanea sativa	Leaves & roots	mRNA↑	Salinity, cold	Pernas *et al.,* 2000
Cichorium intybus	Roots	Protein↑	Cold	Degand *et al.,* 2009
Craterostigma plantagineum	Leaves	mRNA↑	Desiccation	Rodriguez *et al.,* 2010
Hordeum vulgare	Leaves	mRNA↑	Cold, anaerobiosis	Gaddour *et al.,* 2001
Milnesium tardigradum	Adult animals	mRNA↑	Desiccation	Mali *et al.,* 2010
Panax ginseng	Roots	mRNA↑	Salinity	Jung *et al.,* 2010
Polypedilum vanderplanki	Larvae	mRNA↑	Desiccation	Cornette *et al.,* 2010
Triticum aestivum	Shoots & roots	mRNA↑	Drought, salinity, cold, ABA	Christova *et al.,* 2006

Table 2.5. Continued from the previous page.

Species	**Tissues**	**Differential Expression**	**Stresses**	**Citation**
Triticum aestivum	Leaves	mRNA↑	Cold	Houde *et al.,* 2006
Transgenic tobacco	Plants	Protein↑	Cold	Van der Vyver *et al.,* 2003
Vigna unguiculata	Leaves	mRNA↑	Drought	Diop *et al.,* 2004
Vitis vinifera	Shoot tips	mRNA↑	Drought, salinity	Cramer *et al.,* 2007
Zea mays	Roots	mRNA↑	Cold	Massonnaeu *et al.,* 2005

a

< 2 mm shoots, before drying

b

< 2 mm shoots, after drying

Figure 2.9. Desiccation tolerance test of *S. pectinata* seedlings with shoot length shorter than 2mm. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), and stored dry at -20°C until use. a, *S. pectinata* seeds were cut, and incubated for 3 days at 27°C for seed germination. b, fresh *S. pectinata* seedlings (a) were flash dried for 2 days and re-imbibed for germination for 7 days.

a

2-5 mm shoots, before drying

b

2-5 mm shoots, before drying

Figure 2.10. Desiccation tolerance test of *S. pectinata* seedlings with shoot length between 2mm and 5mm. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), and stored dry at -20°C until use. a, *S. pectinata* seeds were cut, and incubated for 3 days at 27°C for seed germination. b, fresh *S. pectinata* seedlings (a) were flash dried for 2 days and re-imbibed for a germination test for 7 days.

a

> 5 mm shoots, before drying

b

> 5 mm shoots, after drying

Figure 2.11. Desiccation tolerance test of *S. pectinata* seedlings with shoot length longer than 5mm. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), and stored dry at -20°C till use. a, *S. pectinata* seeds were cut, and incubated for 3 days at 27°C for seed germination. b, fresh *S. pectinata* seedlings (a) were flash dried for 2 days and re-imbibed for a germination test for 7 days.

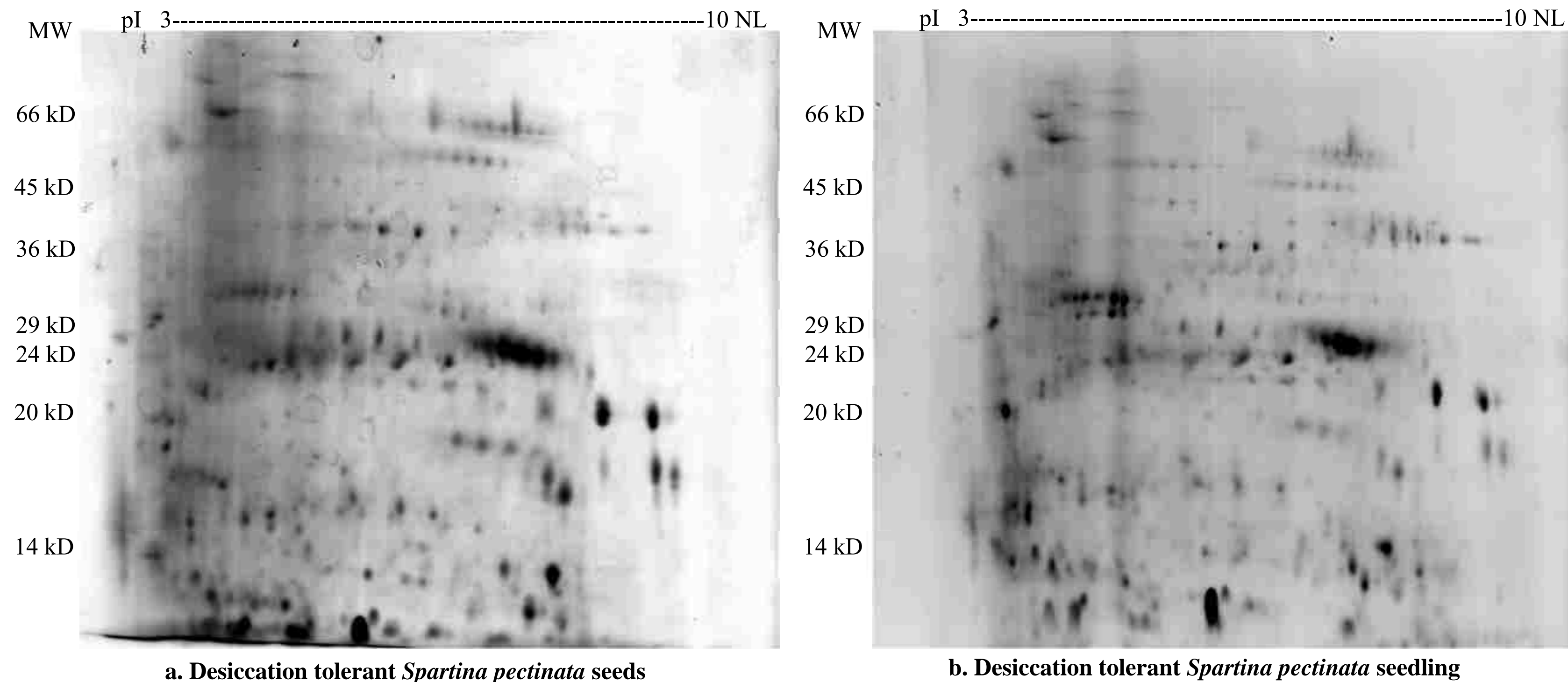
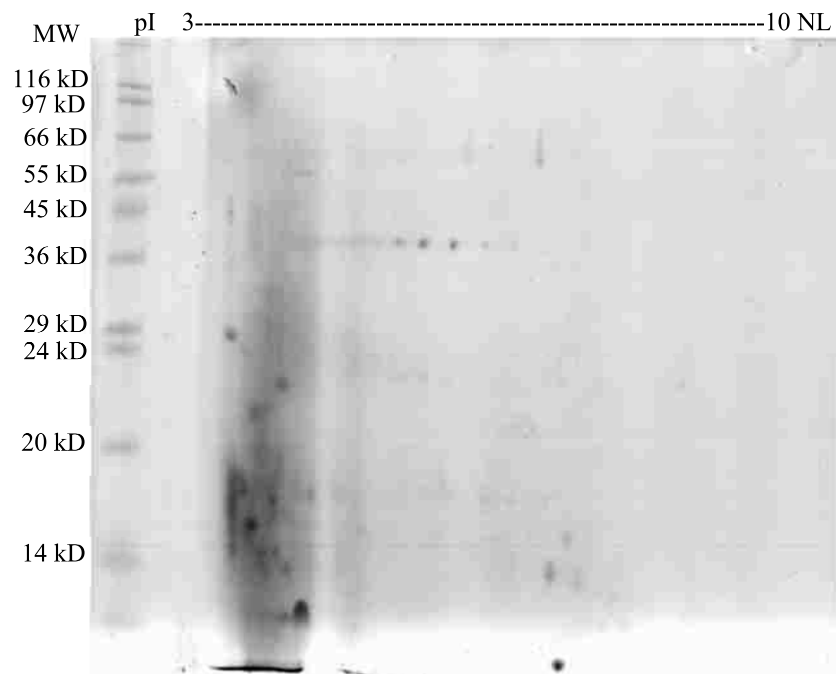
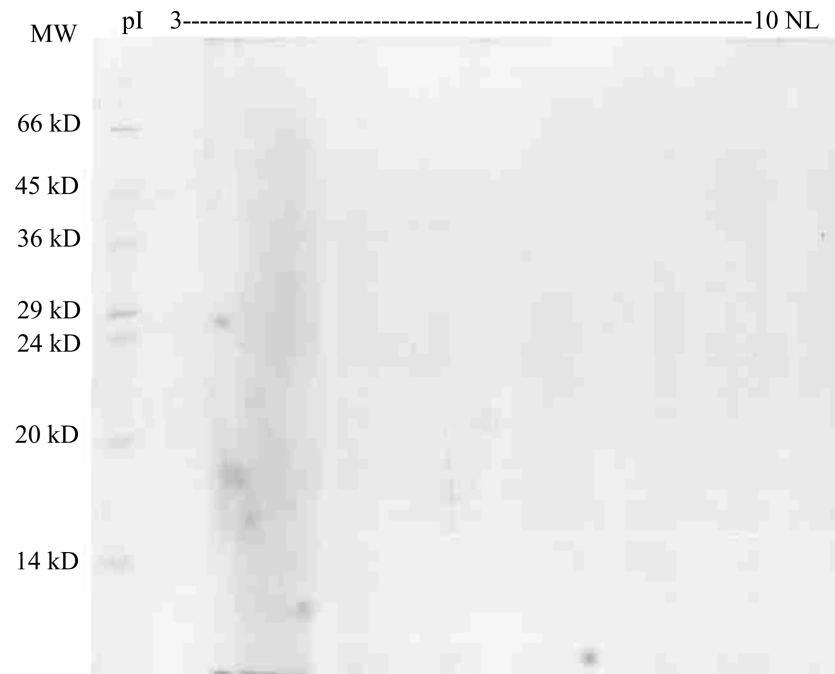


Figure 2.12. Comparison of two-dimensional gels of heat-stable protein fractions between (a) dry desiccation tolerant *S. pectinata* seeds and (b) fresh desiccation tolerant *S. pectinata* seedlings. Gels were stained with Colloidal Coomassie Blue G-250. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), and stored dry at -20ºC. *S. pectinata* seeds were cut and incubated at 27ºC, and seedlings of shoot length that was *ca.* 2-3 mm were selected for protein extraction.



a. Fresh desiccation intolerant *S. pectinata* seedling



b. Flash dried desiccation intolerant *S. pectinata* seedling

Figure 2.13. Two-dimensional gels of heat-stable protein fractions from (a) Fresh desiccation intolerant *S. pectinata* seedlings (shoot > 5mm) (b) flash dried desiccation intolerant *S. pectinata* seedlings. Gels were stained with Colloidal Coomassie Blue G-250. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), and stored dry at -20ºC. *S. pectinata* seeds were cut and incubated at 27ºC, and seedlings of shoot length that was longer than 5 mm were selected for protein extraction.

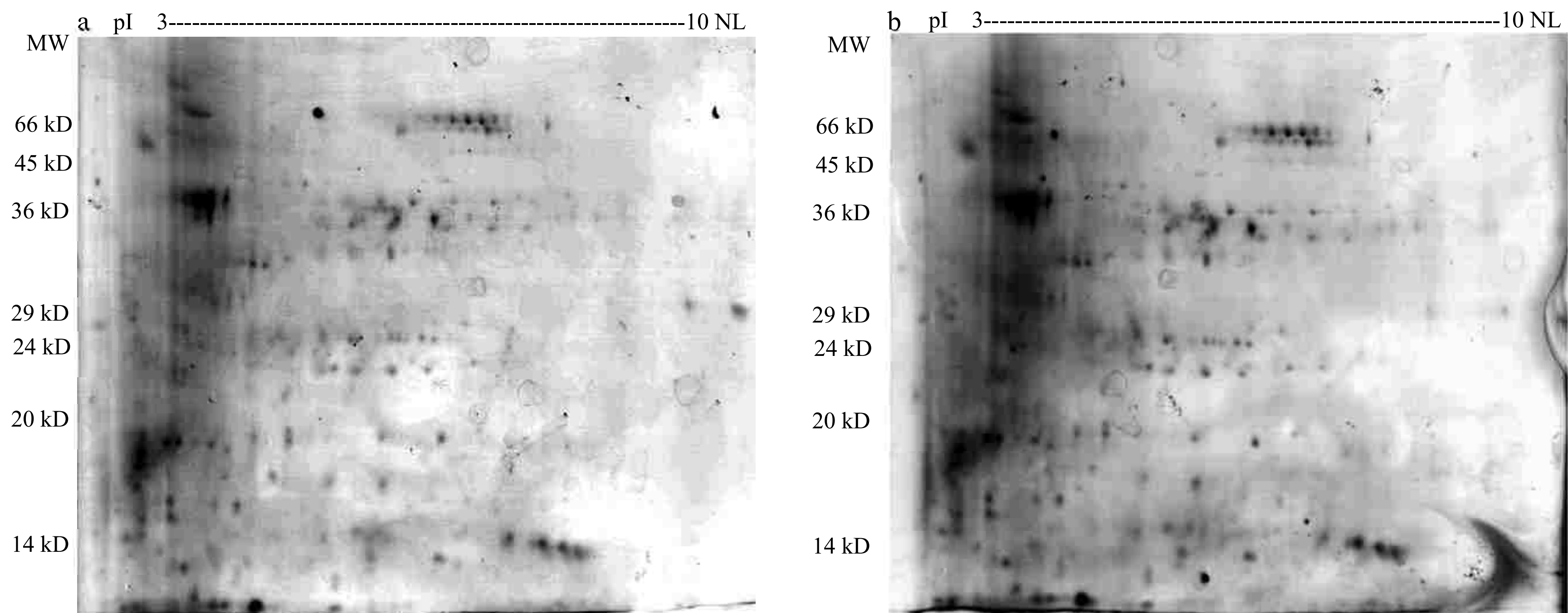


Figure 2.14. Comparison of two-dimensional gels (24 cm nonlinear immobiline pH gradient strip 3-10 NL) of heat-stable proteome fractions between (a) fresh harvested *S. alterniflora* (MC = 45 ± 1%, dwb; G = 0%; V = 100%) and (b) air dried *S. alterniflora*. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2009). Seeds for (b) were air dried at 23ºC for 2 weeks [MC=17% (DWB), G=0%, V=0%]. MC = moisture content; G = germination percentage; V = viability.

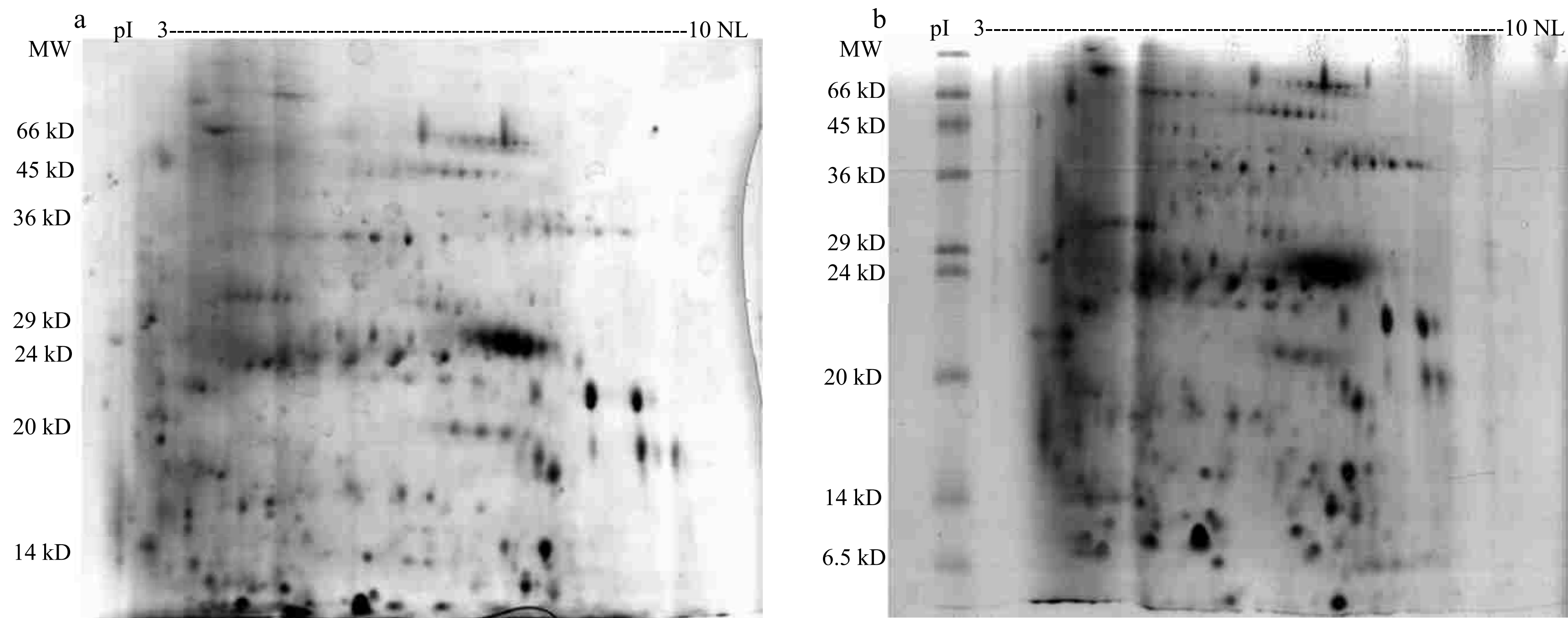


Figure 2.15. Comparison of two-dimensional gels of heat-stable proteome fractions between (a) dry *S. pectinata* (24 cm nonlinear immobiline pH gradient strip 3-10 NL) and (b) rehydrated *S. pectinata* (18 cm nonlinear immobiline pH gradient strip 3-10 NL). *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), and stored dry at -20ºC. Dormant seeds were rehydrated for 7 days at 23°C.

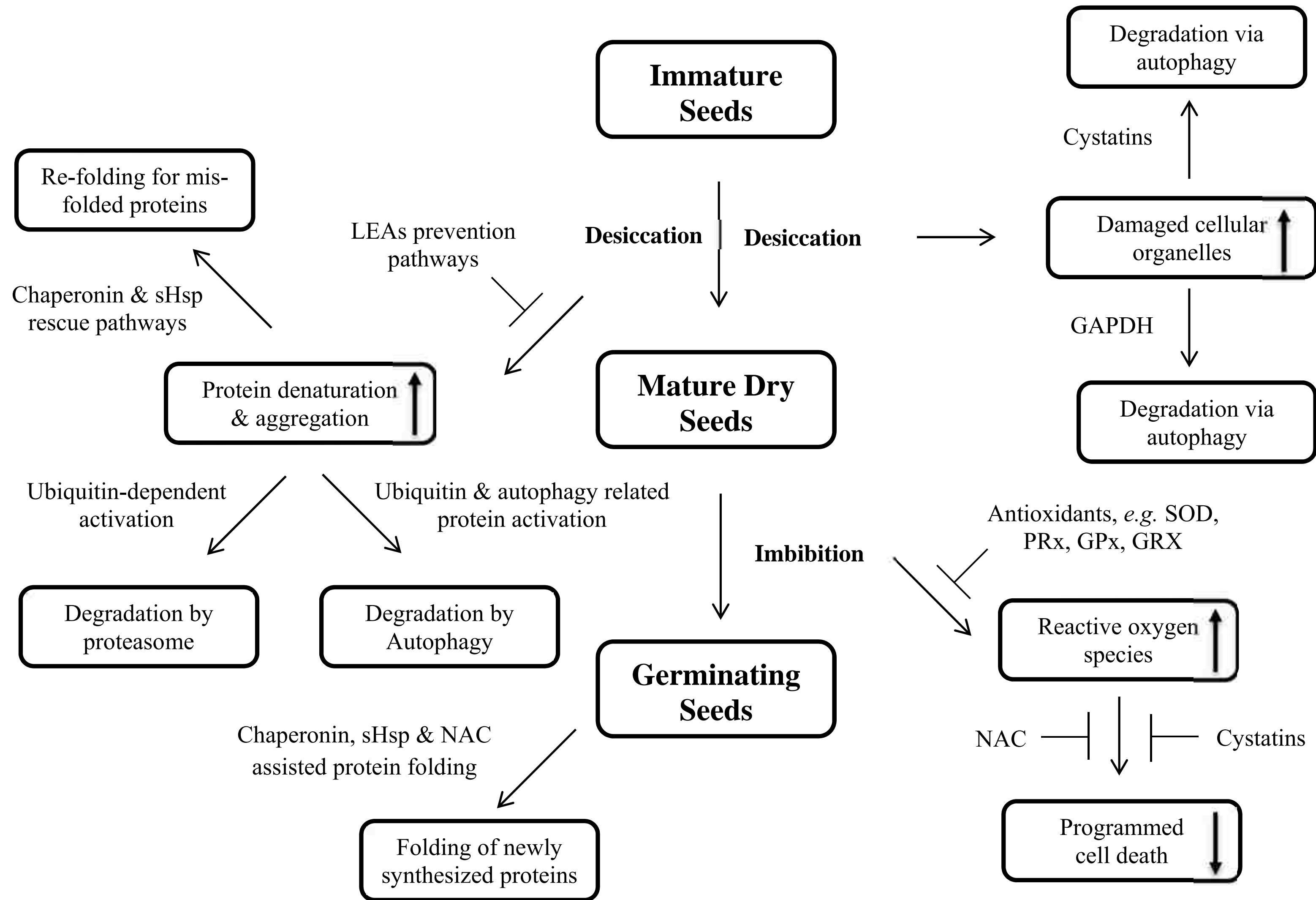


Figure 2.16. A model of desiccation tolerance derived from differentially expressed proteins in desiccation tolerant *Spartina pectinata* seeds that are not present in recalcitrant *Spartina alterniflora* seeds.

Table 2.6. List of enriched *cis*-regulatory element motifs identified by the PLACE database (http://www.dna.affrc.go.jp/PLACE/index.html) (Higo *et al.,* 1999). Proteins listed in Table 2.1 were tblastn-searched for orthologous rice genes in the rice genome, and 1 kb upstream promoter regions of those genes were searched for cis-element motifs. Motifs that were identified in over 50% of these genes were considered as enriched motifs. Motifs that were reported to be associated with dehydration in literature are also listed in the table. B = C, G or T; D = A, G or T; H = A, C, or T; K = G or T; M = A or C; N = A, C, G or T; R = A or G; S = C or G; V = A, C or G; W = A or T; Y = C or T. TF: transcription factor. Classification of putative TF protein was based on PlnTFDB and AtTFDB. n/a: no putative TF or family was found in the literature. Desiccation stress associated *cis*-regulatory motifs are in bold type.

***Cis*-regulatory motifs**	**DNA Sequence**	**Putative TFs**	**Family of Putative TFs**	**Occurrence**
ARR1AT	NGATT	ARR1	ARR-B	38/38
CAATBOX1	CAAT	NF-Y	CCAAT	37/38
WRKY71OS	TGAC	WRKY71	WRKY	37/38
EBOXBNNAPA	CANNTG	E box binding TF	bHLH	36/38
GT1CONSENSUS	GRWAAW	GT-1	Trihelix	36/38
GTGANTG10	GTGA	n/a	n/a	36/38
CACTFTPPCA1	YACT	n/a	n/a	35/38
DOFCOREZM	AAAG	DOF	C2C2-DOF	35/38
GATABOX	GATA	GATA, ASF-2	C2C2-GATA	35/38
MYBCORE	**CNGTTR**	**MYBs**	**MYB**	**33/38**
ACGTATERD1	**ACGT**	**ABREs**	**bZIP**	**32/38**
CCAATBOX1	CCAAT	NF-Y	CCAAT	32/38
MYCOONSENSUSAT	**CANNTG**	**ATMYC2, rd22BP1, ICE1**	**bHLH**	**32/38**
NODCON2GM	CTCTT	n/a	n/a	32/38
OSE2ROOTNODULE	CTCTT	n/a	n/a	32/38
ROOTMOTIFTAPOX1	ATATT	n/a	n/a	32/38
SEF4MOTIFGM7S	RTTTTTR	SEF 4	MADS	32/38
CURECORECR	GTAC	SBP	SBP	31/38
RAV1AAT	CAACA	RAV1	AP2-EREBP	31/38
POLLEN1LELAT52	AGAAA	n/a	n/a	30/37
TAAAGSTKST1	TAAAG	DOF1	C2C2-DOF	29/38
SORLIP1AT	GCCAC	n/a	n/a	29/38
GT1GMSCAM4	GAAAAA	GT-1	Trihelix	29/38
WBOXATNPR1	TTGAC	WRKY18	WRKY	29/38
POLASIG1	AATAAA	n/a	n/a	28/38
BIHD1OS	TGTCA	OsBIHD1	HB	28/38
WBOXNTERF3	TGACY	WRKY1, WRKY2, WRKY4	MYB	27/38
MYB1AT	**WAACCA**	**MYB**	**MYB**	**27/38**
ABRELATERD1	**ACGTG**	**ABREs**	**bZIP**	**27/38**
EECCRCAH1	GANTTNC	LCR1	MYB	27/38
IBOXCORE	GATAA	LeMYBI	MYB	27/38
SORLIP2AT	GGGCC	n/a	n/a	26/38
TATABOX5	TTATTT	TBPs	n/a	26/38
CGCGBOXAT	VCGCGB	AtSR1-6	CAMTA	25/38
INRNTPSADB	YTCANTYY	INR	-	25/38
SURECOREATSULTR11	GAGAC	ARFs	ARF	25/38
ABRERATCAL	**MACGYGB**	**ABREs**	**bZIP**	**24/38**
OSE1ROOTNODULE	AAAGAT	n/a	n/a	23/38

Table 2.6. (Continued from the previous page).

Cis-regulatory motifs	DNA Sequence	Putative TFs	Family of Putative TFs	Occurrence
ASF1MOTIFCAMV	TGACG	ASF, TGA	bZIP	23/38
NODCON1GM	AAAGAT	n/a	n/a	22/38
PRECONSCRHSP70A	SCGAYNRNNNN NNNNNNNNNN NHD	n/a	n/a	22/38
REALPHALGLHCB21	AACCAA	n/a	n/a	22/38
MYB2CONSENSUSAT	**YAACKG**	**MYB2**	**MYB**	**22/38**
MYBPZM	**CCWACC**	**MYB-P, MYB-C1**	**MYB**	**22/38**
WBOXHVISO1	TGACT	SUSIBA2, SURE	WRKY	22/38
RHERPATEXPA7	KCACGW	RHF	n/a	22/38
MYBST1	**GGATA**	**MybSt1**	**MYB**	**21/38**
DPBFCOREDCDC3	**ACACNNG**	**DPBP-1, DPBP-2**	**bZIP**	**21/38**
SITEIIATCYTC	TGGGCY	TCP domain TFs	TCP	21/38
CGACGOSAMY3	CGACG	n/a	n/a	21/38
MYBCOREATCYCB1	**AACGG**	**CYC**	**MYB**	**21/38**
10PEHVPSBD	TATTCT	n/a	n/a	20/38
MYCATRD22	**CACATG**	**MYC2, rd22BP1**	**bHLH**	**19/37**
CBFHV	**RYCGAC**	**CBF1, CBF2, DREs, CRT**	**AP2-EREBP**	**19/38**
MYCATERD1	**CATGTG**	**MYC2, rd22BP1**	**bHLH**	**17/38**
DRECRTCOREAT	**ACCGAC**	**CBF1, DREB1, DREB2**	**AP2-EREBP**	**14/38**
DRE2COREZMRAB17	**ACCGAC**	**CBF1, DREB1, DREB2**	**AP2-EREBP**	**13/38**
ACGTABREMOTIFA2 0SEM	**ACGTGKC**	**ABREs**	**bZIP**	**12/38**
RYREPEATBNNAPA	**CATGCA**	**B3 domain, ABI3**	**ABI3VP1**	**10/38**
MYB2AT	**TAACTG**	**MYB**	**MYB**	**9/38**
ABREOSRAB21	**ACGTSSSC**	**ABREs**	**bZIP**	**7/38**
ABREATCONSENSUS	**YACGTGGC**	**ABREs**	**bZIP**	**5/38**

required for desiccation tolerance if those proteins were not present. The novel benefit of my system is the use of orthodox *S. pectinata* seeds as a physiological 'control' to compare with recalcitrant *S. alterniflora*. Proteomic comparison between air dried *S. alterniflora* and dry *S. pectinata* was performed to identify any differentially expressed heat-stable proteins, and those spots that had significantly more abundance in the orthodox *S. pectinata* were putatively associated with desiccation tolerance.

Table 2.7. List of enriched *cis*-regulatory element motifs identified by the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Rombauts *et al.,* 1999). Proteins listed in Table 2.1 were tblastn-searched for orthologous rice genes in the rice genome, and 1 kb upstream promoter regions of these genes were searched for cis-element motifs. Motifs that were identified in over 50% of the genes were considered as enriched motifs. Motifs that were reported to be associated with dehydration in literature were also listed in table.TF: transcription factor. Classification of putative TF protein was based on PlnTFDB and AtTFDB. n/a: no putative TF or family was found in the literature. Desiccation stress associated *cis*-regulatory motifs are in bold type.

***Cis*-regulatory motifs**	**DNA Sequence**	**Putative TFs**	**Family of Putative TFs**	**Occurrence**
TATA-box	TTTTA, TATAA, TATA, TATAAAT	TBPs	n/a	38/ 38
Unnamed__4	CTCC	n/a	n/a	37/38
CAAT-box	CAAAT, TGCCAAC, CAAT, TGCAAATCT	NF-Y	CCAAT	36/38
G-Box	CACGTT, CACGTA, CACGTG, GACACGTAGT, GCCACGTGGA, TACGTG	GBPs	bZIP	31/38
Sp1	CC(G/A)CCC, GGGCGG	n/a	n/a	30/38
Unnamed__1 Unnamed__2	CGTGG	n/a	n/a	28/38
ARE	**TGGTTT**	**AtMYB2**	**MYB**	**24/38**
circadian	CAANNNNATC	n/a	n/a	24/38
TGACG-motif	TGACG	CREB	bZIP	23/38
CGTCA-motif	CGTCA	CREB	bZIP	22/38
MBS	**TAACTG, CAACTG**	**MYBs**	**MYB**	**19/38**
ABRE	**CGCACGTGTC, TACGTG, GCAACGTGTC, CACGTG, ACGTGGC**	**ABREs**	**bZIP**	**18/38**

The reason dry *S. pectinata* and dry *S. alterniflora* seeds were selected for comparative proteomics is that they have the same value of protein oxidation based on Chappell's (2008) data, which found that protein carbonylation was constant and high in *S. pectinata* regardless desiccation and rehydration. But protein carbonylation increased during the dehydration of *S. alterniflora*, eventually reaching to the same value of dry *S. pectinata* seeds. Because *S.*

alterniflora seeds do not undergo the maturation drying process on the mother plant, the increased protein carbonylation during dehydration of *S. alterniflora* was a response of the seeds to the drying. In order to have an equal physiological comparison, the fresh harvested *S. alterniflora* seeds were air dried to mimic the maturation drying process to let *S. alterniflora* and *S. pectinata* have the same values of protein oxidation.

Chaperones

LEAs

A number of spots were identified as sharing homologies with LEAs (group-2, group-3, and LEA14), which have been proposed to be a family of putative desiccation-tolerance associated proteins for decades (Berjak and Pammenter, 2008). Up-regulated expression of group-3 LEAs was observed in a maturing orthodox seed (Boudet *et al.,* 2006) and desiccation tolerant stages of various anhydrobiotes, *e.g.* nematode (Chakrabortee *et al.,* 2007), bdelloid rotifer (Boschetti *et al.,* 2011) and chironomid (Cornette *et al.,* 2010) species. Disappearance of dehydrin and group-3 LEAs coincided with loss of desiccation tolerance in *Medicago truncatula* radicles, and re-induction of desiccation tolerance recovered the expression of those LEAs (Boudet *et al.,* 2006). The association between the presence of LEAs and desiccation tolerance was also strengthened by transgenic and mutant studies. *Group-3 LEA*-silenced nematode *Caenorhabditis elegans* experienced a higher mortality compared to the wild type during dehydration (Gal *et al.,* 2004). Transgenic rice plants over-expressing group-3 barley LEA cDNA exhibited improved tolerance to drought and salinity stresses (Xu *et al.,* 1996; Rohila *et al.,* 2002); whether or not such plants would survive extreme desiccation, similar to seeds, is an open question. One of the putative functions of LEAs is that they might behave as a chaperone-like molecular shield during dehydration (Tunnacliffe and Wise, 2007). Shrinkage of the

cytoplasmic space increases the incidence of protein aggregation and membrane folding during drying, but the hydrophilic LEAs might interact with the dehydration-sensitive proteins, keeping the shells hydrated and hydrophobic peptides apart to prevent destabilization and denaturation (Hoekstra *et al.,* 2001). *In vitro* and *in vivo* experiments indicated group-3 LEAs and dehydrins were capable of protecting proteins from aggregation induced by desiccation (Chakrabortee *et al.,* 2007; Kovacs *et al.,* 2008). A high ratio (5:1) of LEAs to desiccation-sensitive proteins was required to achieve only partial protection during desiccation (Chakrabortee *et al.,* 2007); however, LEAs only constitute ~7% of total proteins in mature cotton embryos (Roberts *et al.,* 1993). One possibility could be explained by the observations of Goyal *et al.* (2005b; 2005c), who found that Aav-LEA-1, a group-3 LEA from *Aphelenchus avenae*, was sliced to many shorter peptide fragments during dehydration. Those smaller fragments could also behave the same as the intact LEA in anti-aggregation, and the auto-cleavage was not observed in hydrated protein extracts. In addition to full-length LEAs, smaller LEA peptide fragments were also observed in mature maize kernels (Chen *et al.,* 2002). The implication from those studies is auto-cleavage of LEAs was active upon desiccation, providing a maximum number of protective molecules under water stress. Except for the observations of Chen *et al.* (2002), there is no additional evidence in plants for a similar maturation-activated LEA fragmentation process.

Group-3 LEAs and dehydrins are highly hydrophilic (Grand average of hydrophathicity: -1.233 and -1.139), and computational prediction using FoldIndex (Prilusky *et al.,* 2005) indicates that they are highly unfolded and unstructured. Given a certain size of a protein, the property of lacking traditional secondary structure may provide LEAs a large contacting surface for binding to various targets (Tompa, 2002), and *in silico* calculation predicted that a group-3 LEA from *Aphelenchus avenae* was able to bind 20 times more water than the same-sized globular protein

(Goyal *et al.,* 2003). More interesting findings came from observations that LEAs were able to adopt conformational changes from disordered structures in solution to secondary structures under dehydration (Wolkers *et al.,* 2001; Soulages *et al.,* 2002; Goyal *et al.,* 2003), and this occurence has been speculated to be associated with their putative protective functions in anhydrobiosis.

LEA14 was first recognized in water-stressed cotton leaves, and it was classified as an atypical group of LEAs since it exhibited different hydrophobicity characteristics compared to other LEAs (Galau *et al.,* 1993). NMR spectroscopy revealed the three dimensional structure of *Arabidopsis* LEA14 protein, consisting of one α-helix and seven β-strands and sharing closest structural similarity with fibronectin of animal cells (Singh *et al.,* 2005). The homolog of *LEA14*, *pcC27-45*, was highly abundant in desiccated leaves of resurrection plant, *Craterostigma plantagineum* (Piatkowski *et al.,* 1990) and drought-treated soybean leaves (Maitra and Cushman, 1994). mRNA expression was greatly up-regulated in dehydrated roots of sweetpotato, and transgenic calli overexpressing *LEA14* exhibited greater tolerance to drought and salt stresses (Park *et al.,* 2011).

However, LEAs cannot be the solely determinant factor in desiccation tolerance, because LEAs have been also detected in recalcitrant seeds (Finch-Savage *et al.,* 1994; Gee *et al.,* 1994). A complete protection against desiccation-induced protein aggregation requires a fairly high ratio of LEAs to targeted proteins (5:1), and merely a partial protection could be achieved even at a high ratio (Goyal *et al.,* 2005a; Chakabortee *et al.,* 2007). *Arabidopsis thaliana* mutant seeds of *lea14* exhibit reduced longevity, but they are still desiccation tolerant (Hundertmark *et al.,* 2011). A hypothetical combination role of LEAs and sugars in desiccation tolerance has been proposed in the literature: only half of the sugars could be removed by dialysis from a heat-stable

extract containing both LEAs and sugars (Walters *et al.,* 1997). In addition, a mixture of sucrose and dehydrin exhibited stronger hydrogen bond strength and a higher glass transition temperature compared to sucrose alone (Wolkers *et al.,* 2001). The role of protective sugars, especially the raffinose family oligosaccharides (RFO), is still controversial and requires further investigation, especially by a comparative physiology approach.

<u>Chaperonins and small heat-shock proteins</u>

Chaperonins (Cpn) and small heat shock proteins (sHsp) are two sets of molecular chaperones required for the folding of newly synthesized and stress-denatured peptides, assisting peptide assembly and translocating newly synthesized peptides (Ellis, 2006). The chaperonin (Cpn60 & Cpn10) family of proteins is homologous to *Escherichia coli* proteins (GroEL & GroES), and co-chaperonin-10 (GroES) cooperates with Cpn60 (GroEL) to assist the folding and assembly of proteins (Hartl, 1996).

Highly abundant chaperonins were observed in dehydrated anhydrobiotes (Adhikari *et al.,* 2009; Farrant *et al.,* 2009; Gusev *et al.,* 2011), and protein expression was up-regulated by 7.5 fold during desiccation of a resurrection plant *Xerophyta viscosa* (Abdalla *et al.,* 2010). Introduction of a plant *Cpn* cDNA fragment to *E. coli* generated transformants with higher salt-stress tolerance (Yamada *et al.,* 2002). During dehydration of rat liver cells, Cpn60 was observed to quickly partition from the cytosol to mitochondria, and they may assist denatured proteins to acquire native states by providing a shield under crowding conditions of cellular space during dehydration (Itoh *et al.,* 2002). In addition to desiccation, Cpn60 may also have a significant protective role during rehydration, being able to assist the folding and refolding of proteins that are denatured or newly synthesized (Wang *et al.,* 2004).

In addition to mitochondrial chaperonins, Cpn60 was also observed in chloroplasts (Hartl, 1996). Antisense expression of chloroplast Cpn60-β produced transgenic tobacco plants with slow growth and delayed flowering (Zabaleta *et al.,* 2002), and deletion of chloroplast *Cpn60-β* caused cell death in heat stressed *A. thaliana* mutants compared to wild types (Ishikawa *et al.,* 2003). Similarly, mutants of *A. thaliana* chloroplast Cpn-60α exhibited an abnormal and defective embryo development; however, the mutated seeds were still desiccation tolerant, being able to undergo the normal maturation drying process, and the germinated seedlings established a similar phenotype compared to wild type (Apuya *et al.,* 2001), which suggests that chaperonins are not the sole factor determining desiccation tolerance.

Small heat shock proteins can act as molecular chaperones to stabilize non-native state proteins and prevent aggregation of denatured proteins during desiccation stress (Waters *et al.,* 1996). The association between sHsp and desiccation tolerance is not conclusive based on the literature. On the one hand, a type I sHsp was abundantly presently in recalcitrant chestnut seeds (Collada *et al.,* 1997). On the other hand, up-regulation of sHsp has been observed in desiccated orthodox seeds (Wehmeyer *et al.,* 1996), a resurrection plant (Alamillo *et al.,* 1995), nematodes (Adhikari *et al.,* 2009) and a midge (Cornette *et al.,* 2010). Transgenic rice seedlings expressing a small heat shock protein cDNA exhibited enhanced drought stress tolerance (Sato and Yokoya, 2008). Similar to LEAs, accumulation of sHsp occurred during the late seed maturation process (Wehmeyer *et al.,* 1996; Huang *et al.,* 2012). Their expression level increased as seed drying proceeded (Wehmeyer *et al.,* 1996) but decreased once the germination event was observed (Huang *et al.,* 2012). *A. thaliana* seed mutants with Δ*abi3-1* deletion, which were non-dormant and desiccation tolerant, had 1/10 sHsp protein content compared to wild type, but expression level of sHsp protein was undetectable in several mutants with the Δ*abi3-6* deletion, exhibiting

desiccation sensitive and non-dormant phenotypes (Wehmeyer *et al.,* 1996). A similar study found that desiccation sensitive mutants of *abi3-6*, *fus3-3* and *lec1-2* showed little expression of sHsp, while a desiccation tolerant but embryo-defective mutant of *lec2-1* exhibited the same level of sHsp as the wild type (Wehmeyer and Vierling, 2000). A comparative study of sHsp expression between stressed and non-stressed wild type and *abi3-6*, *fus3-3* and *lec1-2* mutants found nearly no accumulation of sHsp occurred in *abi3-6*, *fus3-3* and *lec1-2* mutants, but expression of sHsp could be observed in those mutants under stress (Wehmeyer and Vierling, 2000), which implies that expression of sHsp during seed development and stress is probably regulated by alternative pathways.

Several issues may have been overlooked in the literature. Some types of Hsp, like Hsp-70 and Hsp-90, are constitutively present in non-stressed organisms and considered as housekeeping proteins in the cell, while a sHsp was up-regulated under desiccation stress (Wang *et al.,* 2004). Therefore, several issues need consideration: (1) which groups of Hsps are really associated with desiccation tolerance? (2) Cells may die not only during dehydration but also during rehydration, and few papers have discussed the roles of those stress associated proteins in rehydration.(3) Which groups of Hsps are involved in desiccation and which groups of Hsps participate in rehydration? Various groups of Hsps exhibited different expression patterns during dehydration and rehydration in *Sarcophaga crassipalpis* (Hayward *et al.,* 2004), where Hsp-70 and sHsp were greatly induced in desiccated non-diapausing pupae but were already highly abundant in diapausing pupae. Hsp-90 expression did not change during desiccation but was up-regulated upon rehydration. Comparative studies of protein expression of various Hsps between desiccation tolerant *Sphincterochila zonata* and desiccation sensitive *S. cariosa* found higher Hsp-72, Hsp-74 and Hsp-90 expression in desiccation sensitive *S. cariosa*, while different small

size molecular Hsps (~20 kDa) were more abundantly present in desiccation tolerant *S. zonata* (Mizrahi *et al.,* 2010). The implication from these studies is that different Hsps, although they all act as molecular chaperones, may be involved in various cellular pathways for different physiological events, *e.g.* dehydration and rehydration. Some groups of Hsp may assist the folding and refolding of non-native state proteins, or stabilize and prevent the denatured proteins from aggregation during desiccation. When rehydration proceeds, protein synthesis starts and proteins that are denatured during desiccation need to be refolded or degraded. Different sets of Hsp may stabilize the newly synthesized peptides, assist the folding of denatured proteins and participate in degradation of protein aggregates.

Nascent polypeptide associated complex

Two nascent polypeptide associated complex-alpha chain (NACA) proteins and one basic transcription factor-3 (BTF-3) were present in *S. pectinata*, but missing in recalcitrant *S. alterniflora* seeds. NAC consists of two subunits, βNAC (~20 kDa), which was originally named BTF-3 (Rospert *et al.,* 2002), and αNAC (~30 kDa) (Wang *et al.,* 2010b). αNAC and BTF-3 (βNAC) share low similarity of amino acid sequences but they exhibit similar folding. NAC expression was up-regulated under desiccation (Wang *et al.,* 2010b), drought and salt (Jiang *et al.,* 2007). Both αNAC and BTF-3 are putative molecular chaperones, functionally close to Hsp-70 that binds to and stabilizes newly synthesized peptides on ribosomes, and then the NAC-peptides complex is passed to Cpn-60/Cpn-20 for the final folding, preventing those polypeptides from mis-folding, proteolysis and falsely interacting with other macromolecules (Preissler and Deuerling, 2012). NAC may also participate in preventing and degrading protein aggregation based on the evidence from yeast cells, in which Δ*nac* Δ*ssb* double mutants exhibited higher protein aggregation compared to Δ*ssb* alone (Koplin *et al.,* 2010). These

putative functions of NAC suggest that NAC may be able to prevent aggregation of denatured proteins induced by desiccation, protect those newly synthesized polypeptides and assist the translocation of some of those polypeptides to mitochondria after rehydration.

Glycolytic Enzymes, Cystatin, Ubiquitin and Autophagy-related Protein-13

In my study, two identified proteins were glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose bisphosphate aldolase. GAPDH, a *ca.* 37 kDa protein, catalyzes the reversible reaction between glyceraldehyde-3-phosphate and 1,3-bisphosphate glycerate.

The abundance of mRNA/proteins of GAPDH and aldolase has been observed in desiccation tolerant stages of various anhydrobiotes, *e.g. C. plantagineum* (Velasco et al., 1994; Rodriguez *et al.,* 2010), *Milnesium tardigradum* (Schokraie *et al.,* 2010), and *Artemia franciscana* (Chen *et al.,* 2009). Although an up-regulated expression of GAPDH and aldolase coincided with desiccation and other stresses (Tables 2.3 and 2.4), a possible pivotal role of these enzymes in conferring protection against stress was little investigated based on existing literature. Recent studies of GAPDH in non-plant systems indicated that GAPDH probably behaves not only simply as a 'house-keeping' enzyme in glycolysis, but as a "moonlighting" protein involved in multiple cellular pathways (Tristan *et al.,* 2011). Such pathways include a link of metabolic activities to gene transcription between cytosol and nucleus (Zheng *et al.,* 2003), apoptosis (Tarze *et al.,* 2007), metabolic switching (Ralser *et al.,* 2007), inhibiton of caspase-independent cell death (Rathmell and Kornbluth, 2007), vesicle transport from the ER to the Golgi (Tisdale, 2001), pollen development (Munoz-Bertomeu, 2010) and a target of inhibition by hydrogen peroxide (Hancock *et al.,* 2005).

Fructose bisphosphate aldolase (FBA) catalyzes the reversible breakdown of fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone-3-phosphate. Literature suggests that FBA, like GAPDH, may also act as a moonlighting protein (Lorenzatto *et al.,* 2012). Aldolase was reported to directly interact with three subunits of vacuolar H^+-ATPase, and mutants with aldolase gene deletion could only grow in acid medium buffer (Lu *et al.,* 2001; Lu *et al.,* 2004). Multiple functions of FBA, such as parasite surface localization, adhesion and invasion (Jewett and Sibley, 2003; Pomel *et al.,* 2008; Tunio *et al.,* 2010), were reported in pathology studies. Up-expression of FBA genes and proteins has been observed in desiccation, drought, heat or salt stressed plants (Table 2.4).

Cystatins were present in dry *S. pectinata* and missing in air dried *S. alterniflora*. Cystatins are part of a family of papain-like cysteine protease inhibitors with a conserved Gln-Xaa-Val-Xaa-Gly peptide motif (Xaa: any amino acid) (Benchabane *et al.,* 2010). They exist across various plant families, *e.g.* Arabidopsis (Hwang *et al.,* 2009), maize (Massonneau *et al.,* 2005), and cowpea (Diop *et al.,* 2004). *A. thaliana* and *Oryza sativa* contain 12 and 7 cystatins, respectively (Martinez and Diaz, 2008). Phylogenetic analysis of cystatins in *Arabidopsis*, rice and barley revealed that these cystatin clusters might share a similar origin (Martinez *et al.,* 2005). Cystatins have multiple putative functions in plants, *e.g.* inhibitory effects in seeds, regulation of protein turnover in germinating seeds, and defense responses to biotic stresses (Benchabane *et al.,* 2010). The concurrence between up-regulation of cystatins and various abiotic stresses is summarized in Table 2.5. Up-regulation of cystatins has been observed in desiccation tolerant stages of various anhydrobiotes, *e.g. M. tardigradum* (Mali *et al.,* 2010), *A. franciscana* (Chen *et al.,* 2009), and *C. plantagineum* (Rodriguez *et al.,* 2010). During maize embryo development, cystatin accumulated abundantly during the maturation process but

decreased after germination (Huang *et al.,* 2012). Dehydration was able to induce the expression of cystatin in both desiccation intolerant and desiccation tolerant embryos, but cystatin accumulated considerably more in desiccation tolerant ones (Huang *et al.,* 2012). The *cis*-regulatory element region of two cystatin genes, *AtCYSa* and *AtCYSb*, contained a dehydration-responsive element and an abscisic acid responsive element, which implicates a possible role of the cystatins in water stress (Zhang *et al.,* 2008). Overexpression of the two cystatin genes in yeast and *Arabidopsis* enhanced their resistance to various abiotic stresses, *e.g.* drought, salinity and cold (Zhang *et al.,* 2008).

Genes of calpain, a calcium-dependent cysteine protease, have been identified in plants and may be involved in multiple plant processes (Margis and Margis-Pinheiro, 2003). Necrosis in root tips of maize, a type of programmed cell death stimulated by anoxia stress, coincided with calcium dependent induction of calpain (Subbaiah *et al.,* 2000). The presence of cystatin may be able to inhibit the activities of calpain and induced programmed cell death under stress.

Two ubiquitin and one tetra-ubiquitin proteins were uniquely present in *S. pectinata* (Table 2.1). Ubiquitin is a protein involved in protein cycling, and it directs targeted proteins to the proteasome for degradation (Kimura and Tanaka, 2010). Tetra-ubiquitin mRNAs, similar to *LEAs*, were up-regulated during late embryogenesis stage and most abundantly expressed in dry sunflower seeds (Almoguera *et al.,* 1995). Up-regulation of ubiquitin was seen in a desiccated tardigrade and a cryptobiotic midge (Cornette *et al.,* 2010; Mali *et al.,* 2010).

One common function of GAPDH, cystatin and ubiquitin is that they can putatively participate in autophagy (Solomon *et al.,* 1999; Tristan *et al.,* 2011). Autophagy exists in ordinary cells, and it is able to remove the protein aggregates, maintaining the cellular turnover homeostasis (Kopito, 2000); but enhanced autophagy activities were observed under stress

conditions in mammalian cells and plants (Ratnakumar *et al.,* 2010; Tizon *et al.,* 2010). In desiccation tolerant yeast *Saccharomyces cerevisiae,* autophagy related genes (*ATGs*) were abundantly present in air dried strains (Ratnakumar *et al.,* 2010). Several *ATG* genes were up-regulated during seed maturation and desiccation in *A. thaliana* (Liu and Bassham, 2012).

Cystatin C in human cells was able to induce autophagy under stress, and induced autophagy putatively served as a cellular protection against an unfavorable cellular environment (Tizon *et al.,* 2010). GAPDH could serve as a negative mediator in cell death by targeting the mitochondria, recovering the mitochondrial outer-membrane permeabilization (Tristan *et al.,* 2011). Cystatin has also been proposed to possibly regulate the removal of damaged mitochondria and protect cells from caspase-independent cell death through increased autophagy activities (Colell *et al.,* 2007).

A possible connection between ubiquitin and autophagy is also implicated in the literature. *ATG* genes from yeasts (*e.g. ATG5*, *ATG7*, *ATG10*, *ATG12*, and *ATG16)* putatively participate in ubiquitin conjugation pathways and share homologies with proteins in *A. thaliana* (Liu and Bassham, 2012). Increased chances of macromolecular interaction under water stress cause protein denaturation (Hoekstra *et al.,* 2001), and protein aggregates induced by desiccation were observed *in vitro* (Goyal *et al.,* 2005a). Those induced protein aggregates could not be degraded directly via the ubiquitin directed proteasome pathway; meanwhile they could potentially block the ordinary function of ubiquitin-proteasome cycling (Bence *et al.,* 2001). However, the presence of ubiquitin seemed to act as a signal, triggering the degradation of protein aggregates by activating the directing of the protein aggregates to the lysosome through the autophagy pathway (Kim *et al.,* 2008a; Riley *et al.,* 2010).

Although caspases, a family of cysteine proteases pivotal in apoptosis in animals cells (Kumar, 2007), have not been detected in plants yet, a homolog of caspase, metacaspase, is present in plants (Uren *et al.,* 2000). Unlike caspases, plant metacaspases do not have Asp specificity and cleave targets at Arg and Lys residues (Tsiatsiani *et al.,* 2011). Activation of caspase-like metacaspase was associated with PCD of the nucellus during *Sechium edule* seed development (Lombardi *et al.,* 2007). *A. thaliana* contains six genes of metacaspases, and a cytosolic metacaspase, *AtMCP2d*, played a positive regulating role in the fungal toxin and oxidative stress induced cell death (Watanabe and Lam, 2011). *AtMCP2d* overexpressing transgenic *Arabidopsis* plants exhibited increased cell death induced by fungal toxin and oxidative stress, while *AtCP2d* mutants displayed reduced sensitivity to those cell death inducers (Watanabe and Lam, 2011). Oxidative stress induced cell death coupled with a number of cysteine proteases was also observed in soybean cells, and cystatin was effective in partially inhibiting the PCD (Solomon *et al.,* 1999). Overexpression of a cystatin gene in transgenic *Arabidopsis thaliana* and tobacco partially inhibited avirulent pathogen and oxidative stress induced cell death (Belenghi *et al.,* 2003). Based on this information, cystatin could hypothetically act as a negative mediator in inhibiting cysteine protease activity under oxidative stresses, protecting the cell from cysteine protease triggered death. Other types of protease inhibitors, *e.g.* serine protease inhibitor, have also been observed in water stressed vegetative plants (Wang *et al.,* 2003; Huang et al., 2007; Contour-Ansel *et al.,* 2010), and trypsin inhibitor (#22 in *S. pectinata*) from a heat stable *Adnanthera pavonina* seed extract was able to protect the enzyme activity from desiccation (Lam *et al.,* 1999), implicating a conceivable combination role of diverse types of protease inhibitors in desiccation tolerance.

The association between GAPDH, ubiquitin and desiccation tolerance is also supported from studies of mutants. A GAPDH knockout strain of desiccation tolerant *S. cerevisiae* (*tdh1*Δ) turned the yeast to desiccation intolerant (2% survival), and a ubiquitin mutant yeast had only 49% survival after drying, compared to wild type (Ratnakumar *et al.,* 2010). Interestingly, another GAPDH knock out strain (*tdh3*Δ) exhibited a significantly higher desiccation tolerance (68% survival) compared to the *tdh1*Δ mutant (Ratnakumar *et al.,* 2010), implicating a differential effect of the *GAPDH* gene family in desiccation tolerance.

Antioxidants

Reactive oxygen species (ROS), *e.g.* superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and singlet oxygen ($^1O_2$), originate from electron transport chains; excessive accumulation of ROS, which can irreversibly modify and oxidize lipid, protein and DNA, is harmful to the cell (D'Autréaux and Toledano, 2007; Chappell, 2008). Antioxidants, *e.g.* superoxide dismutase (SOD), peroxiredoxin, glutaredoxin and glutathione peroxidase, are able to scavenge these free radicals and maintain the homeostasis of the cell against oxidative stress.

SOD is a family of enzymes that catalyze conversion of superoxide into oxygen and hydrogen peroxide. Two isozymes of SOD, [Mn] and [Cu-Zn], were present in *S. pectinata* and their cellular location has been found in mitochondria [Mn], cytosol and chloroplast [Cu-Zn] (Alscher *et al.,* 2002). Glutaredoxin, similar to thioredoxin, is a small redox protein, and it contains a reversible dithiol-disulfide active site for electron transfer (Holmgren, 1989), and glutathione and NADPH are cofactors (Holmgren, 1989). In *S. pectinata*, another antioxidant utilizing glutathione is glutathione peroxidase, which catalyzes reduction of hydrogen peroxide

to water, lipid hydroperoxides to alcohol, and oxidation of glutathione to glutathione disulfide (Arthur, 2000). Navrot *et al.* (2006) found that glutathione peroxidases are glutathione independent and do not react with glutathione, suggesting that glutathione peroxidases should be classified as peroxiredoxins. Peroxiredoxins are another group of thiol-based peroxidases, and four subfamilies of peroxiredoxin (including glutathione peroxidase) were defined: 1-Cys, 2-Cys, PRX Q and PRX II (Dietz, 2011).

For a long period of time, a deficient antioxidant system in recalcitrant seeds has been considered as a cause of seed death during desiccation (Berjak and Pammenter, 2008). Evidence supporting that argument included two parts, an unbalanced oxidative status in recalcitrant seeds during desiccation and an accumulation of various antioxidants in desiccation tolerant anhydrobiotes. Increased lipid peroxidation and reduced peroxidase activities coincided with a loss of viability of recalcitrant *Theobroma cacao* axes death during desiccation (Li and Sun, 1999). The death of *Quercus robur* axes during desiccation was associated with a decline of antioxidant concentration and activity (Hendry *et al.,* 1992). Activities of SOD and peroxidases gradually increased during a 7-day dehydration period in desiccation-tolerant resurrection plant *Selaginella tamariscina* (Wang *et al.,* 2010a). Abundant accumulation of SOD mRNAs was observed in desiccated stages of a nematode (Adhikari *et al.,* 2009) and a tardigrade (Mali *et al.,* 2010; Schokraie *et al.,* 2010). *Glx* (glutaredoxin) was one of the three genes up-regulated in the dehydrated anhydrobiotic nematode *Aphelenchus avenae* (Browne *et al.,* 2004). *GPx* (glutathione peroxidase) was up-regulated by ~30 fold in desiccated *A. avenae* compared to control, and *GPx* cross-species silencing via RNAi significantly reduced the viability of *A. avenae* under desiccation (Reardon *et al.,* 2010). Up-regulation of peroxiredoxins was also

associated with desiccation (Iturriaga *et al.,* 2006; Wang *et al.,* 2009) and drought (Seki *et al.,* 2001; Liu and Bennett, 2011) stress.

It is noted that up-regulation of various antioxidants was not only observed in desiccation stress, but also under cold, drought and oxidative stresses (Seki *et al.,* 2001; McKersie *et al.,* 2000; Plomion *et al.,* 2006). Not restricted to various stresses, antioxidants are universally present across both desiccation intolerant and tolerant species. The ubiquitous presence and up-regulation of antioxidants in various stresses raise the question if there is a group of antioxidants specifically associated with desiccation stress. This question was addressed in a study by comparing antioxidant genes that were associated with acquisition of desiccation tolerance during orthodox seed maturation, differentially expressed in desiccation intolerant *A. thaliana* plants responding to water stress, and up-regulated in resurrection plants responding to desiccation (Illing *et al.,* 2005). The results showed a majority (72%) of *A. thaliana* antioxidants were also abundantly present in control plants, and only five antioxidant genes were highly expressed in dry seeds, *i.e.* 1-Cys PRx (At1g48130), [Cu-Zn] SOD (At2g28190) and [Mn] SOD (At3g56350), along with catalase and ferritin (Illing *et al.,* 2005). In order to see if the identified SOD and PRx in *S. pectinata* share close homologies with those seed specific protein in *A. thaliana*, they were 'blasted' against the *A. thaliana* database. [Cu-Zn] SODs (#36 & #70) (BAD09607 & P93407) in *S. pectinata* have closest homology and share 70% and 68% mRNA similarity with seed specific SOD in *A. thaliana* (At2g28190), respectively. [Mn] SOD (#10) (HO174410) shares 86% mRNA similarity with seed specific SOD in *A. thaliana* (At3g56350). 1-Cys peroxiredoxins (#29 & 68) (ACE82290) have the closest homology and 100% mRNA similarity with seed specific 1-Cys PRx in *A. thaliana* (At1g48130).

Since most recalcitrant seed species are typically large, embryonic axes of those seeds had to be excised to obtain an appropriate quantity of samples for drying experiments and expediting drying rate. However, excision of embryonic axes artificially caused a burst of ROS generation, regardless of whether embryonic axes were excised before (Roach *et al.,* 2008) or after (Roach *et al.,* 2010) a drying experiment. Those studies suggest that increased oxidative stress and antioxidant activities in desiccated, excised recalcitrant seed axes were probably artifacts and had nothing to do with recalcitrant seed death. Oxidative stress and antioxidants were not associated with recalcitrant *S. alterniflora* seed death during desiccation compared to *S. pectinata* (Chappell, 2008); and artifacts created by axis excision were first identified. In addition, in a comparison of two desiccation-intolerant *Eragrostis* species and one desiccation-tolerant *E. nindensis* revealed that antioxidants were all active in the hydrated state and exhibited the same response to early dehydration, but antioxidant activities were only maintained in desiccation tolerant *E. nindensis* (Illing *et al.,* 2005). However, it is unknown whether decreased antioxidant activity in desiccation-sensitive species was a result of the death or caused death. The maintenance of antioxidant activities in the dry state may be a consequence of programmed cell protection, preventing denaturation by molecular chaperones during the desiccation period and utilization for the future imbibition and seed germination. Thus, the presence of antioxidants in dry state may not be associated with desiccation tolerance, but rather protection against the stress of subsequent metabolic reactivation during imbibition.

If those antioxidants are not associated with desiccation tolerance, why are they abundantly present in the dry state of desiccation tolerant species and what roles do they participate in? One possibility of recalcitrant seed death is somewhat neglected in the literature: recalcitrant seeds may die during rehydration (Walters *et al.,* 2002). In order to judge whether

seeds are viable or not after dehydration, they must undergo a viability test, either the tetrazolium (TZ) or germination test, in which seed hydration is inevitably required. A number of studies have reported that fused cell membranes in desiccation sensitive species would undergo an irreversible conformational change upon rehydration, leaving 'gaps' on cell membranes and causing cellular solutes to leach out (Hoekstra et al., 2001). However, such leakage was not observed in desiccated *S. alterniflora* whole seeds during rehydration (Chappell, 2008). In orthodox seeds, the level of free radicals increases during seed germination, and hydrogen peroxide concentration also rose sharply (Garnczarska and Wojtyla, 2008). Meanwhile, the enhanced activity of antioxidant SOD has also been observed during the seed germination (Gidrol *et al.,* 1994; Garnczarska and Wojtyla, 2008). Redox activities of peroxide generation and various antioxidants are active at the early stage of germination in soybean axes (Puntarulo *et al.,* 1988) and radish seeds (Schopfer *et al.,* 2001). An immediate burst of extracellular superoxide and peroxide production accompanied seed imbibition in orthodox pea seeds, and superoxide content increased as germination proceeded (Kranner *et al.,* 2010). The presence of antioxidants in the dry orthodox seeds could potentially protect the seeds against increased oxidative stress upon seed rehydration, and the lack of antioxidative proteins in recalcitrant *S. alterniflora* seeds may contribute to their mortality during imbibition.

Other Stress Associated Proteins

Water-stress-responsive protein

Two spots sharing homologies with rice water-stress-responsive proteins (#11: NP_001043194; #14: AAP92753) were highly expressed in *S. pectinata*. Both proteins (NP_001043194 and AAP92753) contain 113 amino acids, and sequence alignment indicates that they share 97% amino acid sequence similarity with the rice protein. Analyzing the amino

acid sequences predicts they should contain a stress responsive A/B barrel domain (pfam07876, ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=203789). Up-regulation of this protein family has been observed in water- and salt-stressed condition, but their function is unknown (Gu *et al.,* 2004). BLAST analysis shows that rice water-stress-responsive protein shares 84% and 92% amino acid similarity with stable protein (SP1) of *Populus tremula* (AJ276517) and Pop3 of *A. thaliana* (At3g17210). SP1 is a highly heat-stable protein up to boiling in *P. tremula*, and SP1 was classified as a novel group of stress-related protein since it did not exhibit any structural similarity with other classic stress associated proteins (Dgany *et al.,* 2004). *sp1* was up-regulated in response to salt, cold and desiccation stresses (Wang *et al.,* 2002). Up-regulation of *sp1* was also observed in drought stressed citrus (Torres *et al.,* 2007).

Abscisic acid stress ripening protein-2 (ASR)

Abscisic acid (ABA) is a pivotal regulator in desiccation tolerance, since increased ABA coincides with a decline of seed water content, acquisition of desiccation tolerance during orthodox seed development, and accumulation of putative protective proteins (Meurs *et al.,* 1992). Some ABA- related mutants, *e.g. abi3* and *abi5*, are desiccation sensitive (Finkelstein, 2010). ASR was first discovered in tomato, and the expression of *asr* genes is ABA-responsive (Lusem *et al.,* 1993). Three genes of *asr* have been identified in tomato, *asr-1*, *asr-2*, *asr-3* (Maskin *et al.,* 2001). Characterization of ASRs showed they are highly hydrophilic and rich in His, Lys, Glu and Ala (Maskin *et al.,* 2001), which may contribute to their heat-stability. An ASR homologue-LLA was associated with desiccation tolerance in *Lilium longiflorum* pollen, since accumulation of the protein was only observed during the late stage of pollen maturation (Wang *et al.,* 1998). mRNA of *asr-1* and *asr-2* was undetectable in hydrated leaves of tomato but up-regulated under water stress (Maskin *et al.,* 2001). Up-regulation of *asr* genes in response to

drought stress was also observed in several other studies (Silhavy *et al.,* 1995; Padmanabhan *et al.,* 1997; Riccardi *et al.,* 1998). Transgenic *A. thaliana* overexpressing *lla* genes exhibited increased drought and salt tolerance (Yang *et al.,* 2005).

ASR1 was shown to be primarily located in the nucleus by subcellular fractionation experiments (Lusem *et al.,* 1993), and it may be a putative DNA binding protein (Padmanabhan *et al.,* 1997). ASR1 does not share any structural similarity with other DNA binding proteins; however, similarly to LEAs, ASR1 is intrinsically unstructured and exhibits conformational changes from unstructured to structured state upon zinc addition and desiccation (Goldgur *et al.,* 2007). The induced structural transition may suggest a possible regulatory role upon desiccation; folded ASR may bind to DNA, initiating signaling pathways to protect cells from desiccation.

Glycine rich RNA binding protein-2

The glycine-rich RNA binding protein (GRPs) family is ubiquitously present in plants, and its members play putative roles in post-transcriptional regulation of gene expression under various abiotic stresses (Kim *et al.,* 2005). There are 27 glycine-rich and small RNA recognition motif (RRM)-containing proteins in *A. thaliana*, and proteins that contain both RRM and high number of glycines were named GPRs (Lorković and Barta, 2002). Each of the eight members of GRPs seems to function differently under various stresses, according to the literature. The *Grp-3* gene was up-regulated during drought stress in *A. thaliana* (Seki *et al.,* 2001), but gene expression of *grp-4* decreased in *A. thaliana* under water stress (Kwak *et al.,* 2005). Transgenic *A. thaliana* plants overexpressing *grp-4* exhibited slow growth under drought and salinity stresses (Kwak *et al.,* 2005).

GRPs have a major impact on plant cold tolerance. Up-regulation of GRP-2, GRP-4 and GRP-7 was observed in cold stressed *A. thaliana*, and transgenic *A. thaliana* plants

overexpressing various GRPs showed increased cold tolerance (Kwak *et al.,* 2005; Kim *et al.,* 2005, 2007, 2008b; Kim and Kang, 2006). Considering the habitat of *S. pectinata*, which is grown in Colorado and would experience cold night weather during October to December, the presence of GRP-2 in *S. pectinata* could be associated with cold-tolerance rather the desiccation tolerance; this issue could be resolved by proteomic analysis of orthodox *S. spartinae* seeds, which grow in close proximity to *S. alterniflora* in Louisiana.

Oxygen evolving enhancer protein-1

Oxygen evolving enhancer (OEE) proteins are a family of thylakoid membrane proteins functionally involved in photosystem II, and they contain 3 subunits: OEE-1 (33 kDa), OEE-2 (23 kDa) and OEE-3 (16 kDa) (Sugihara *et al.,* 2000). OEE-1 extracted from the green alga *Scendesmus obliquus* was heat-stable, and exhibited thioredoxin activity (Heide *et al.,* 2003). Expression of OEEs, along with 1-Cys peroxiredoxin and SOD, was up-regulated in rice (*Oryza sativa* L. cvs. Nipponbare) under drought stress (Ali and Komatsu, 2006), but the higher expression of OEE was unable to rescue the rice plants in drought stress, which could be interpreted that the differential proteomic expression of OEEs is just a normal response of the plant to water stress or the up-regulated amount of OEE was still deficient. In a comparison between drought sensitive Nipponbare and drought tolerant Zhonghua 8 rice varieties, OEE was expressed higher in Zhoughua 8 than Nipponbare (Ali and Komatsu, 2006). Up-regulation of OEE-2 was also observed in drought tolerant leaves of *Elymus elongatum* under severe water stress (Gazanchian *et al.,* 2007). Thus, the presence of OEE may help *S. pectinata* against mild water stress but not severe desiccation. Another putative role of OEEs is their protection against salt stress, and the up-regulation of OEEs was seen in salt-stressed rice (Abbasi and Komatsu, 2004) and mangrove (Sugihara *et al.,* 2000). Since *S. pectinata* is a halophyte (Warren *et al.,*

1985), OEEs could possibly play a role in anti-salt stress in *S. pectinata*. However, *S. alterniflora* is also a halophyte and able to tolerate high salt stress; therefore, the missing OEEs in *S. alterniflora* indicates OEE may play multiple roles in *S. pectinata* in addition to protection against salt stress.

FK506 binding protein-2 (FKBP)

FKBP16-2, a *ca.* 16 kDa protein, belongs to a family of proteins exhibiting prolyl isomerase activity similar to cyclophilin (Siekierka *et al.,* 1989). Prolyl isomerase and cyclophilin are enzymes that catalyze the isomerization between *cis*- and *trans*- isomers of peptide bonds of the amino acid proline, and they may function as molecular chaperones (Ahn *et al.,* 2010).

The presence of FKBPs is conserved in various eukaryotes, and they behave as molecular chaperones in yeast (Kuzuhara and Horikoshi, 2004) and mammals (Ishikawa *et al.,* 2008). In plants, two FK506 (FK506-73 and FK506-77) could bind to Hsp90, suggesting that they are components of Hsp90 complex (Reddy *et al.,* 1998). In rice, 29 putative genes encoding FKBPs have been identified (Gollan and Bhave, 2010), and *FKBP16* was highly expressed in maize seeds (Yu *et al.,* 2012). Various *FKBP* genes are up-regulated in rice seedlings responding to salt and drought stress, but the expression pattern of each *FKBP* gene varied during a 24 h water stress treatment (Ahn *et al.,* 2010). Similar results were observed for maize *FKBP* genes (Yu *et al.,* 2012). *FKBP16* gene expression remained unchanged in response to drought stress, and *FKBP16-2* and *FKBP16-4* were up-regulated under heat stress (Yu *et al.,* 2012). ABA may be involved in regulation of FKBP gene expression, since three abscisic acid responsive elements (ABREs) have been identified in wheat *FKBP* (Kurek *et al.,* 2000).

Storage Proteins

Globulin-1 proteins are seed storage proteins. Two types of seed globulin proteins can be defined, which are 7S vicilin-type globulin-1 and 11S legumin-type globulin-2 (Shewry *et al.,* 1995). Globulin-1 normally consists of three polypeptide subunits, and the molecular weight of each subunit is *ca.* 43 kDa, 47 kDa and 53 kDa (Brown *et al.,* 1980). Storage protein accumulation occurs during embryo maturation, decreases during germination and increases in desiccation tolerant embryos in response to desiccation (Huang *et al.,* 2012). *Arabidopsis* seed development mutants, *fus2* and *abi3*, are desiccation-intolerant and contain greatly reduced amounts of storage proteins compared to the wild type (Finkelstein *et al.,* 2002; Finkelstein, 2010). However, those observations are not sufficient to support the association between storage proteins and desiccation tolerance. The *fus2* and *abi3* mutants are also compromised in another major seed developmental event, seed dormancy. Storage protein deposition, as well as induction of dormancy and desiccation tolerance, is a major feature of the maturation phase of orthodox seed development (Kermode and Finch-Savage, 2002). Therefore, these mutants are prevented from generally proceeding from embryogenesis to seed maturation. Thus, it is inappropriate to conclude that accumulation of storage proteins alone is tightly associated with desiccation tolerance. Although *abi-3* mutants are desiccation intolerant and show reduced storage protein accumulation, the mutant exhibit phenotypes of other severe defects, *e.g.* loss of dormancy, reduced seed size and seed filling, reduced accumulation of chlorophyll and production of green seeds (Finkelstein *et al.,* 2002). Based upon current genetic screens, it is difficult to conclude that the loss of desiccation tolerance in the mutants is a result of reduced accumulation of storage proteins, or reduced expression of storage proteins is caused by lack of dormancy, or synthesis of

some other desiccation-tolerance association proteins, which are regulated by ABI3, is turned off.

The difference in desiccation tolerance between *S. alterniflora* and *S. pectinata* cannot be simply explained by absence of one of these important transcription factors, *e.g. fus, lec* or *abi3*, because seed mutants of those transcription factors are non-dormant (Finkelstein *et al.,* 2008), but both *S. alterniflora* and *S. pectinata* seeds are shed dormant at maturity.

Other Proteins

Other identified proteins include calmodulin, a copper binding protein, translation inhibitor, SOUL-heme binding domain protein, and a CBS-domain protein, as well as several unidentified hypothetical proteins. Calmodulin is a highly conserved protein across the kingdoms of life, which participates in signal transduction by binding to the second messenger Ca^{2+} (Chin and Means, 2000). Calcium-coupled phosphorylation cascades are essential for plants in response to environmental abiotic stresses (Xiong *et al.,* 2002). Dehydrins exhibit calcium-binding dependent phosphorylation (Alsheikh *et al.,* 2003), which is discussed below in the protein phosphorylation section. Gene ontology mapping of copper binding protein, CBS-domain protein, and hypothetical proteins showed they belong to GO:0006950 (response to stress) and GO:0009628 (response to abiotic stimulus). More information concerning the association between these proteins and desiccation stress will be further investigated.

Protein Phosphorylation

Protein phosphorylation is one of the major post-translational modifications in plants, affecting protein stability, function and translocation, and modulating signaling pathways during plant growth, development and responses to abiotic stress. PRO-Q Diamond staining revealed a number of phosphorylated proteins only present in *S. pectinata* but missing in *S. alterniflora*

(Fig. 2.8). LC-MS/MS coupled with Mascot searches identified these proteins to be a dehydrin, ubiquitin, abscisic acid stress ripening protein and cystatin (Table 2.2).

Dehydrin phosphorylation has been well characterized in the literature. ERD14, belonging to a family of acidic dehydrins in *A. thaliana*, could be phosphorylated, and increased ERD14 phosphorylation was observed in cold-treated tissues, suggesting that dehydrin phosphorylation may be a stress-triggered event, regulated via stress-dependent kinases (Alsheikh *et al.,* 2003). In addition, the phosphorylated ERD14 exhibited more Ca^{2+} binding capability than the non-phosphorylated protein (Alsheikh *et al.,* 2003), suggesting the phosphorylation dependent Ca^{2+} binding of dehydrins may be associated with their putatively protective functions in stress, since Ca^{2+} is known as a secondary messenger pivotal in regulating signaling pathways under various abiotic stresses (Xiong *et al.,* 2002). Induced protein phosphorylation was also observed in the resurrection plant *C. plantagineum*, in which a dehydrin and an abscisic inducible protein underwent phosphorylation upon desiccation (Röhrig *et al.,* 2006). The function of phosphorylated dehydrin was assessed by Rahman *et al.* (2011), who reported phosphorylated dehydrins from *Thellungiella salsuginea* (salt cress) were able to polymerize actin filaments and putatively stabilize the cellular cytoskeleton under stress. In a comparison between drought resistant and drought sensitive wheat varieties, phosphorylated dehydrin DHN-5 accumulated more abundantly in resistant than sensitive varieties (Brini *et al.,* 2007).

Cystatins from mammalian chicken are phosphoproteins (Laber *et al.,* 1989). Calpastatin, an inhibitor of calcium-dependent cysteine proteases, is present predominantly as aggregated granules close to cell nucleus; but phosphorylation on serine residues was able to mediate the redistribution of calpastatin to intracellular cytosol (Averna *et al.,* 2001). Phosphorylation and

de-phosphorylation may regulate the interconversion of calpastatin between active and inactive states (Salamino *et al.,* 1997). Little information about phosphorylated plant ubiquitin and ASR proteins are reported in literature. In order to predict computationally whether plant ubiquitin and ASR proteins can be phosphorylated or not, I tested *in silico* prediction using the NetPhos 2.0 server (Blom *et al.,* 1999), and dehydrin, cystatin, ASR and ubiquitin putatively contain 10, 6, 5 and 7 phosphorylation sites, respectively.

Comparison of 2-D Proteomes from *S. pectinata* Seedlings of Differential Desiccation-tolerance Stages

Desiccation tolerance decreases as germination of the orthodox seed proceeds. In order to examine the timing of the loss of desiccation tolerance in *S. pectinata*, fresh germinating *S. pectinata* seedlings of different developmental stages were subjected to 2-day flash drying and re-imbibed at 27°C to test whether the shoot or radicles will continue to elongate. Flash dried seedlings that had ≤2 mm shoots continued to grow under favorable growing condition, and after 3-4 days incubation, radicles started to emerge. No differences, *e.g.* shoot lengths, radicle lengths and fungal growth on seedlings, were observed between unstressed and flash dried *S. pectinata* seedlings after a 14-day growth. All of flash dried *S. pectinata* seedlings with ≥5 mm shoots stopped growing under favorable growing condition, and massive fungal growth, which is not observed in the healthy seedlings, was seen in dead *S. pectinata* seedlings. Seedlings that had >2 mm & <5 mm shoots were also tested for desiccation tolerance, and Fig. 2.10 showed that 11/20 shoots continued to elongate after 7-day incubation under favorable growing condition. Those 9 non-elongated seedlings failed to grow when the incubation extended to 14 days, and noticeable fungal growth on those seedlings was observed. Experimental results of the seedling desiccation tolerance test suggest that *S. pectinata* seedlings start to lose desiccation tolerance when the shoot lengths grow over a point of between 2 mm and 5 mm.

If those *S. pectinata* proteins (Table 2.1) that are missing in *S. alterniflora* are associated with desiccation tolerance, it is expected to see those proteins will be still present in desiccation tolerant *S. pectinata* seedlings but disappear in desiccation intolerant seedlings. *In silico* comparison of 2-D gels revealed very similar proteomic profiles between desiccation tolerant *S. pectinata* seeds and desiccation tolerant *S. pectinata* seedlings, except two spots, oxygen-evolving enhancer protein (#34) and peroxiredoxin-1 (#68), which were significantly up-regulated in seedlings. Oxygen-evolving enhancer protein is functional in photosystem II of chloroplast (Sugihara *et al.,* 2000), and its gene expression dramatically increased as the seed germinates to seedlings based on the RNA-Seq FPKM Expression Values from Rice Genome Annotation Project (Ouyang *et al.,* 2007). The up-regulation of oxygen-evolving enhancer proteins may be due to biogenesis of chloroplasts in growing seedlings. More abundant accumulation of peroxiredoxin-1 in germinating seedlings was a surprise because peroxiredoxin-1 was highly present in dry seeds (Illing *et al.,* 2005). RNA-Seq FPKM Expression Values from Rice Genome Annotation Project shows gene expression of peroxiredoxin is highest in seeds but drops almost to zero in germinated seedlings (Ouyang *et al.,* 2007). One possible explanation is peroxiredoxin-1 proteins that were functional in seed germination were not newly synthesized. Those peroxiredoxin-1 proteins that were present in dry seeds were not degraded during seed germination, and they may function as antioxidants, required for balancing the level of oxidative stress during the seed germination.

S. pectinata seedlings started to lose desiccation tolerance when seedlings grew to a certain shoot length between 2 mm and 5 mm. The seedlings that had ≥5 mm shoots were selected for comparison, because those seedlings were tested to be desiccation intolerant. In addition, starting from seed germination to seedlings growth took only 3 days so that they may

not experience too much seedling development. Two-dimensional gel comparison between desiccation tolerant dry *S. pectinata* seeds, fresh seedlings (≤ 2 mm) and fresh desiccation intolerant *S. pectinata* seedlings (≥5 mm) revealed that the majority of heat-stable proteins disappeared in desiccation intolerant seedlings, except some acidic proteins (Fig. 2.13). Almost all of the heat-stable proteins disappeared in flash dried desiccation intolerant *S. pectinata* seedlings (Fig. 2.13), and the further degradation of those heat-stable proteins in flash dried seedlings may be a consequence of seedling death.

The presence of the 38 unique proteins in desiccation tolerant *S. pectinata* seedlings, which disappeared in desiccation intolerant seedlings, is consistent with a role for these proteins in seed desiccation tolerance, and the lack of the 38 proteins in *S. alterniflora* may be the cause of recalcitrant seed death.

Identification of Putative Cis-regulatory Elements

Several reasons may explain why the 38 *S. pectinata* heat stable proteins are not present in *S. alterniflora*. One possibility is the genes of those proteins are mutated and they are not present in *S. alterniflora*. At this stage, we do not have any molecular evidence to support this hypothesis. Another possibility is these genes are present in *S. alterniflora*, but they are not transcribed; the transcription factors, a group of proteins that bind to specific DNA sequences of *cis*-regulatory motifs and control the transcription of those genes (Latchman, 1997), are missing or nonfunctional. An approach of computational analysis was reported to identify *cis*-regulatory elements of various groups of genes that were associated with different types of ROS (reactive oxygen species) stress (Petrov *et al.,* 2012). A number of databases of *cis*-regulatory elements are available, *e.g.* ATCOECIS (Vandepoele *et al.,* 2009), AtcisDB (Palaniswamy *et al.,* 2006), AthaMap (Steffens *et al.,* 2005), AtProbe (http://exon.cshl.org/cgi-bin/atprobe/atprobe.pl),

DATF (Guo *et al.,* 2005), DoOP (Barta *et al.,* 2005), ppdb (Yamamoto *et al.,* 2007), PLACE and PlantCARE. Unfortunately, most of these search programs are specific for *Arabidopsis thaliana* genes. *Spartina pectinata* from the Chloridoideae and *Oryza sativa* from the Bambusoideae both belong to Poaceae family, and they are more phylogenetic close than *Spartina pectinata* vs. *Arabidopsis thaliana* (Zhang, 2000). Thus, the amino acid sequences of each of the 38 *S. pectinata* proteins were BLAST-searched for the closest rice orthologous genes using tblastn, and those rice orthologous genes were used for *cis*-regulatory element identification. This approach, using orthologous genes from a different species to examine putative enriched *cis*-regulatory elements of *S. pectinata*, can be validated by the study of *in silico* genomic analysis of the non-coding DNA, which revealed that a *cis*-regulatory element was present in promoter regions of different genes across various plant species (Picot *et al.,* 2010). The study indicated that transcription factor binding to specific *ci*s-regulatory elements was evolutionarily conserved.

To examine whether genes of these 38 *S. pectinata* proteins contain common transcription factor binding motifs, 1 kb upstream nucleotide sequences of the promoter region of the rice orthologous genes were subjected to *in silico* identification for known *cis*-regulatory motifs using the search programs PLACE and PlantCARE. Since the two search programs use different algorithms and they have their own limitations (Bailey, 2008), a combinational implemention of two programs should greatly increase the confidence of identification.

Thirty-eight rice orthologous genes were identified, and the promoter regions of those 38 genes were subjected to search programs to identify *cis*-regulatory elements. PLACE and PlantCARE databases identified a total of 215 and 100 known *cis*-regulatory elements, respectively, in the 38 rice genes. To identify motifs that are commonly present in the promoter regions of those genes, *cis*-regulatory elements that were present in over half of genes (≥18/38)

were considered as enriched motifs. In total, 57 (PLACE) and 13 (PlantCARE) enriched cis-element motifs and the putative transcription factors that bind to those motifs were identified (Tables 2.6 and 2.7). Three major groups of cis-regulatory elements were found to be associated with water stress based on the literature and are discussed below.

Abscisic acid (ABA) is a plant hormone that plays a pivotal role in acquisition of seed desiccation tolerance and plant response to environmental abiotic stresses (Campalans *et al.*, 1999). Increased ABA content coincides with acquisition of desiccation tolerance and an accumulation of desiccation stress-associated mRNA and proteins in orthodox seeds (Johnson-Flanagan *et al*., 1992; Still *et al*., 1994). ABA induces the expression of water stress-associated genes in vegetative tissues (Moons *et al*., 1995; Bartels, 2005). Up-regulation of these ABA-responsive stress genes suggested the presence of common cis-regulatory elements in the promoter regions of those genes. One example of an ABA-associated cis-regulatory element is the abscisic acid responsive element (ABRE). An 8-base pair sequence (CACGTGGC) of the ABRE was identified as the specific recognition site for a wheat DNA binding transcription factor (Guiltinan *et al.,* 1990). A similar ABRE motif (RTACGTGGR, R = A or G) was identified in the promoter region of ABA-responsive *rab16* gene (Mundy *et al.,* 1990). A PlantCARE screening revealed that ABRE motifs were commonly present in the promoter regions of 18 of the 38 *S. pectinata* gene homologues. A PLACE screening also identified ABRE-like motifs to be enriched in the promoter regions, ABRELATERD1 (ACGTG) (26/38), ABRERATCAL (MACGYGB, M: A or C; Y: C or T; B: C, G or T) (23/38) and ACGTATERD1 (ACGT) (31/38).

In terms of cellular compartmentation, putative nucleus genes (GO: 0005634) of group-3 LEA, GAPDH and calmodulin-Ca^{2+} binding protein all contained ABRE motifs, except that

calmodulin was not identified in PlantCARE. Enriched ABRE motifs present in nucleus genes suggests they may be involved in ABA-dependent functions of protection and transcriptional activation during desiccation. The enriched presence of ABRE motifs was not seen in putative cytosol (GO: 0005829), plasma membrane (GO: 0005886), mitochondria (GO: 0005739) and plastid (GO: 0009536) genes from the group of 38 genes evaluated.

In the dicot *Arabidopsis,* ABRE binding factor (ABF) and ABA-responsive element binding protein (AREB) are the transcription factors that are able to directly interact with ABREs (Jakoby *et al.,* 2002). They both belong to one of the ten groups of basic region/leucine zipper motif (bZIP) transcription factors (Jakoby *et al.,* 2002). AREB proteins screened by the yeast one-hydrid system were annonated as AREB-1, AREB-2 and AREB-3 (Uno *et al.,* 2000), and ABF proteins were designated as ABF-1, ABF-2 (AREB-1), ABF-3 and ABF-4 (AREB-2) (Choi *et al.,* 2000). In the monocot wheat, the DNA binding protein (EmBP-1) exhibited specific ABRE-binding activity (Guiltinan *et al.,* 1990). ABI5 (ABA insensitive) and AREBs/ABFs from *A. thaliana* (Lopez-Molina and Chua, 2000) and TRAB from rice (Hobo *et al.,* 1999) were also identifed. Four *lea* genes of *A. thaliana*, *AtEm1*, *AtEm6*, *Rab18* and *Cor47*, contained ABRE motifs, and were significantly down-regulated in the *abi5* mutant (Lopez-Molina and Chua, 2000). A yeast two-hybrid assay showed that ABI5 was able to directly interact with ABI3, which is an embryo-specific transcription factor mediating ABA signaling pathways (Nakamura *et al.,* 2001). Mutant screening identified ABI3 and ABI5 from dicots and their homologs VP1 (vivipary) and TRAB1 from monocots, which are essential transcription factors involved in ABA signaling pathways (McCarty *et al.,* 1991; Giraudat *et al.,* 1992; Hobo *et al.,* 1999). TRAB1 is localized in the cell nucleus, and the binding of TRAB1 to ABRE is ABA-induced phosphorylation dependent (Kagaya *et al.,* 2002). Since the seed mutants of *vp1* and *abi3* were

desiccation intolerant (McCarty *et al.,* 1991; Parcy *et al.,* 1994), the enriched ABRE motif present in the promoter regions indicates the putative role of those genes in desiccation tolerance.

It was reported that *ABI5* could rescue ABA-insensitivity of *abi3-1* mutant, but *ABI3* failed to rescue the ABA-insensitivity of *abi5-4* mutant (Lopez-Molina *et al.,* 2002), which implicated that ABI3 is the upstream transcription factor of ABI5. Ectopic expression of *ABI3* in transgenic *A. thaliana* plants induced a number of seed specific mRNAs (Parcy *et al.,* 1994). But the expression of seed specific genes could be due to an interaction between ABI3 and the downstream transcription factor ABI5 that then induced gene expression. Thus, it would be interesting to see if ectopic expression of ABI3 is still able to induce those seed specific mRNAs in *abi5* mutants. Yeast two-hybrid screening identified a rice transcription factor TRAB1 from rice that directly bound to an ABRE element via the interaction with VP1 (Hobo *et al.,* 1999), which is a transcription factor of ABA-insensitivity loci in monocots orthologous with ABI3 (McCarty *et al.,* 1991).

Seed mutants of *abi3* and *abi5* exhibit phenotypes of desiccation intolerance and non-dormancy (Kucera *et al.,* 2005). However, *S. alterniflora* seeds are desiccation sensitive but dormant, which implicates possible separate signaling pathways of those transcription factors in dormancy and desiccation tolerance in the monocot *S. alterniflora*. Such a model is suggested by mutant phenotypes of the monocot homolog genes, VP1 and TRAB1. In the monocot maize, mutants of *vp1*, the monocot homolog of *abi3*, exhibited a phenotype with neither dormancy nor desiccation tolerance; however, transgenic lines overexpressing *trab1* and mutants lacking *trab1*, which is the monocot homolog of *abi5*, failed to exhibit significant changes of stress tolerance (Xiang *et al.,* 2008). The result implicates that multiple basic leucine zip transcription factors may exist and act as downstream transcription factors of VP1, and those transcription factors

have separated roles in determining desiccation tolerance and dormancy in monocots. Several other bZIP transcription factors of ABI5 and TRAB1 homologs have also been reported, *e.g.* ABL (ABI5-like) (Yang *et al.,* 2011) and OsbZIP23 (Xiang *et al.,* 2008) in rice.

The second group of highly enriched cis-regulatory motifs that are associated with water stress includes MYB (myeloblastosis) and MYC (myelocytomatosis) -related motifs, *e.g.* MYBCORE (33/38), MYCOONSENSUSAT (32/38), MYB1AT (27/38), MYCATRD22 (19/37). The association between MYB and MYC-related motifs in plants and water stress was observed from the drought responsive gene *rd22*, which could be induced by ABA but does not contain the ABRE motif (Yamaguchi-Shinozaki and Shinozaki, 1993). The MYB and MYC motifs in the promoter region of *rd22* could be recognized by transcription factors of rd22BP1 (MYC) and ATMYB2 (MYB), and transgenic lines overexpressing MYC and MYB transcription factors exhibited ABA hypersensitivity compared to wild types (Abe *et al.,* 1997; Abe *et al.,* 2003). *ATMYBR1* transcripts, a *MYB* gene from *A. thaliana*, accumulated during the late stage of seed embryogenesis and were abundant in the dry seed of wild type *A. thaliana* (Kirik *et al.,* 1998). But, the *ATMYBR1* gene expression disappeared in the *fus3*, *lec1* and *abi3* mutants (Kirik *et al.,* 1998), implicating a possible role of the *MYB* gene in desiccation tolerance or seed maturation. NAC (different from nascent peptide-associated complex) transcription factor was reported to be able to recognize the MYC specific motif of the promoter region of *ERD1* gene (early response to dehydration stress), and transgenic plants with NAC trans factor overexpression exhibited increased drought tolerance (Tran *et al.,* 2004).

DRE (dehydration responsive element) motifs, *e.g.* DRECRTCOREAT (13/38) and CBFHV (19/38), which contain a 9 bp nucleotide sequence TACCGACAT, were another group of *cis*-regulatory motifs associated with water stress. Different from ABRE and MYB/MYC

motifs, DRE was classified as the ABA-independent cis-regulatory motif (Lata and Prasad, 2011). The DRE motif was identified in the promoter regions of Arabidopsis *rd29* (Yamaguchi-Shinozaki and Shinozaki, 1994), *kin1* and *cor6.6* (Wang *et al.,* 1995) and *BN115* from *Brassica napus* (Jiang *et al.,* 1996), which were associated with water and cold stress. The activity of a reporter gene that was attached to the promoter region of *kin1* and *cor6.6* increased under water stress (Wang *et al.,* 1995), suggesting some transcription factors might bind to the DRE motifs and mediate the gene transcription in response to dehydration. A DRE binding transcription factor, CBF1 (C-repeat/DRE binding factor 1), which was identified in *A. thaliana* and contained an AP2 domain, was able to activate the expression of the marker gene with the DRE recognition site in the promoter region, but could not activate the reporter gene expression that was without DRE motif (Stockinger *et al.,* 1997). Transcripts of DRE binding protein in maize, DBF1, accumulate during maize embryogenesis, and the gene expression was up-regulated in drought stressed seedlings (Kizis and Pages, 2002). Transactivation experiments in *Arabidopsis* leaf protoplasts showed that DRE binding proteins (DREB1A and DREB2A) were able to activate the reporter gene expression that contained the DRE recognition motif in the promoter region (Liu *et al.,* 1998). But, they exhibited differential gene expression in response to abiotic stresses, *DREB2A* being induced by dehydration, while *DREB1A* being induced under cold stress (Liu *et al.,* 1998). Similar results were observed in rice *DREB* homologs, where *OsDREB2A* expression was dehydration inducible, but *DREB1A* and *DREB1B* were induced by cold (Dubouzet *et al.,* 2003). Transgenic *A. thaliana* plants overexpressing *DREB1A* surprisingly not only exhibited better cold tolerance, but also increased tolerance to drought stress and exhibited severe retarded growth (Kasuga *et al.,* 1999), which implicates a pleiotropic effect of DREB1A or possible cross-talk between DREB1A and DREB1B.

These results suggested that multiple transcription factors may be involved and function together in regulating dehydration stress associated gene expression. Future work will be to screen for those stress associated transcription factors that would be present in orthodox *S. pectinata* but missing in recalcitrant *S. alterniflora*.

SUMMARY

Comparative proteomics between the recalcitrant *S. alterniflora* and orthodox *S. pectinata* identified 38 heat-stable proteins putatively associated with desiccation tolerance, and those proteins may participate in various ways to cope with desiccation. In contrast to previous proposals in the literature, the proteomics data obtained in this study suggest that desiccation tolerance may be the result of the action of a suite of proteins with various functions, rather than a single class of desiccation protective proteins. First, LEAs can protect desiccation sensitive proteins from denaturation, prevent the denatured proteins from precipitation and assist re-folding of denatured proteins. Secondly, the presence of Cpn and sHsp contributes to the rescue pathways, and Cpn, sHsp and NAC are able to assist the folding and refolding of denatured proteins induced by desiccation and newly synthesized peptides during desiccation and rehydration. Thirdly, when the prevention and rescue pathways may be unable to confer sufficient protection to specific proteins under severe desiccation stress, those denatured proteins can be removed via degradation pathways of the ubiquitin-proteasome and autophagy. Ubiquitin, cystatin, GAPDH and ATG may be involved in the autophagy in *S. pectinata*. Fourthly, a burst of oxidative stress occurs once dry seeds are rehydrated, and ROS increase as seed germination proceeds. The presence of antioxidants, *e.g.* SOD, Prx, GPx and GRx, would be able to scavenge

those ROS and maintain cell redox homeostasis. A model for roles of identified proteins in seed desiccation tolerance in *S. pectinata* is presented in Fig. 2.16.

It is noted that GAPDH (Table 2.3), aldolase (Table 2.4) and cystatin (Table 2.5) are also up-regulated under drought stress in vegetative tissues such as leaves. If such expressed proteins were able to confer sufficient protection against desiccation, one would expect that vegetative tissues should survive severe desiccation stress (~10%, dwb); however, leaves die at much higher water contents. How can the two sets of different outcomes be resolved? One possible explanation is that those identified proteins in *S. pectinata* are just a small group of protective proteins, considering only heat-stable proteins have been studied so far. The 2-D gel electrophoresis also has restricted resolving capability so that non-abundant proteins, *e.g.* transcription factors, might not be seen on gels. Missing information may account for the difference between desiccation tolerance and drought tolerance. Secondly, although factors determining the differences between desiccation- and drought- tolerance are unknown, those proteins identified in *S. pectinata* may ubiquitously participate in protective pathways under various stresses, such as desiccation, drought, salinity, and cold. *In silico* analysis of the cis-regulatory elements of those genes from *A. thaliana* and *Zea mays* revealed that majority of genes share common cis-regulatory elements, *e.g.* abscisic acid responsive elements (ABRE) (32/38) and dehydration responsive elements (21/38) (Cuming *et al.,* 2007). Transcription factors, which interact with those cis-regulatory elements, can be triggered under various stress conditions; thus, up-regulation of those genes and proteins would be observed universally under different types of stress. Thirdly, since proteins have multiple isoforms, some of those proteins may be specifically expressed in dry seeds. Only 5 antioxidant genes were highly expressed in dry seeds by comparative studies (Illing *et al.,* 2005). It is very possible that only those seed

specific proteins are the key for desiccation tolerance. Fourthly, water contents gradually decrease after reserve deposition during orthodox seed development, but recalcitrant seeds are shed off mother plants at high water contents. Orthodox seeds have to experience drought stress first and then desiccation stress when water content drops to very low levels. Rationally, if seeds are not able to survive drought stress, they are already dead before they become desiccation tolerant. The presence of those protective proteins is likely to help *S. pectinata* seeds survive the drought stress during seed development. For recalcitrant seeds, they might not survive the initial drought stress because of the absence of those proteins.

Further work is needed to provide additional evidence to support the association between these expressed proteins and desiccation tolerance. *S. spartinae*, which is also orthodox but naturally grown in close spatial proximity to *S. alterniflora*, can be utilized as another control. Unfortunately, *S. spartinae* seeds that either I collected from marshes of Port Fourchon or purchased commercially germinated poorly, exhibiting very low seed set (i.e. many 'empty' seeds).

Based on the preliminary data, three majors groups of water stress-associated cis-regulatory elements, ABREs (32/38), DREs (21/38) and MYCs/ MYBs (33/38, 32/38), were enriched in the promoter regions of rice homologous genes of unique *S. pectinata* proteins. The highly enriched presence of these stress associated cis-regulatory elements implicated that some transcription factors may be present in desiccation tolerant *S. pectinata* but missing in recalcitrant *S. alterniflora*, and an absence of only one or two transcription factors might account for the missing proteins in *S. alterniflora*. In order to confirm this hypothesis, future work should include comparative transcriptomics or Northern blotting to screen for differential expression of stress-association transcription factors between *S. pectinata* and *S. alterniflora*.

CHAPTER 3
COMPARATIVE PROTEOMICS TO EXAMINE THE SEED DORMANCY IN *SPARTINA ALTERNIFLORA* & *SPARTINA PECTINATA*

LITERATURE REVIEW

Dormancy is defined as the inability of the seed embryo to germinate under favorable conditions (Bewley, 1997). A dynamic balance of two important plant hormones, abscisic acid (ABA) and gibberellin (GA), controls the seed dormancy and germination (Kucera *et al.,* 2005). By screening the mutants of *Arabidopsis thaliana* that exhibited a non-dormant phenotype, a number of genes have been identified to be associated with induction of dormancy during seed maturation, *e.g. ABA-insensitive 3* (*ABI3*), *Fusca3* (*FUS3*) and *Leafy Cotyledons* (*LEC1* and *LEC2*) (Kucera *et al.,* 2005), and *Delay of Germination 1* (*DOG1*) (Bentsink *et al.,* 2006). Release of seed dormancy can be achieved by one or several factors, *e.g.* after-ripening, chilling, light and temperature (Bewley and Black, 1994). However, the mechanism of the dormancy release by moist chilling is still unknown. Gibberellin is essential for the seed germination, and *GA Arabidopsis* mutants exhibited defective seed germination (Koornneef and van der Veen, 1980). It was proposed that dormancy release by cold stratification may be due to an increased GA biosynthesis and sensitivity upon chilling (Derkx and Karssen, 1993). A slight increase of endogenous GA content, which coincided with dormancy release, was observed in cold stratified hazel seeds (Ross and Bradbeer, 1971). But, Arabidopsis GA biosynthesis deficient mutants still required chilling for dormancy release (Debeaujon and Koornneef, 2000). Endogenous GA contents did not increase during cold stratification in hazel seeds, but the dormancy state was released, which suggests that the sensitivity of the seeds to GA changed upon chilling (Williams and Bradbeer, 1974). The molecular mechanism of the GA signaling pathways upon chilling was

examined in *A. thaliana*, and a cold inducible *GA oxidase* gene, *AtGA3ox1*, may play an essential role in seed imbibition upon chilling (Yamauchi *et al.,* 2004).

Comparative proteomics between dormant and non-dormant *A. thaliana* Cvi seeds identified a number of proteins that were associated with dormancy release during cold stratification, *e.g.* storage proteins (cruciferin), embryonic cell protein and LEAs were down regulated, while some metabolism and stress related proteins, *e.g.* heat shock protein-60, heat shock protein-90, alanine aminotransferase and enolase, accumulated during cold stratification (Arc *et al.,* 2012). Changes of proteome profiles during dormancy release by cold stratification were studied in tree seeds of beech, Norway maple and sycamore, and proteins involved in energy metabolism, protein degradation and protein synthesis may be associated with dormancy breaking (Pawlowski, 2010). *S. pectinata* and *S. alterniflora* seeds are a good system to study seed dormancy. First, *S. pectinata* and *S. alterniflora* caryopses (attached with lemma and palea) are relatively small and easy to manipulate. Second, both *S. pectinata* and *S. alterniflora* mature seeds exhibit deep dormancy when they shed off the mother plants, and their dormancy state can be released by moisture chilling for 1-2 months. Third, *S. pectinata* seeds can be dried to ca. 10% (DWB) and stored at -20ºC for several years without losing dormancy. *S. alterniflora* seeds cannot be stored dry because of the recalcitrance, but they were shed dormant off the mother plant, and seeds could be harvested from wild Louisiana marshes every November. Comparative proteomics was used to identify proteins that are associated with dormancy breaking by cold stratification between dormant and non-dormant *S. pectinata* and *S. alterniflora*. If any differentially expressed proteins are associated with dormancy release any proteins, it is expected to see those proteins exhibit the same expression pattern in both *S. pectinata* and *S. alterniflora*.

MATERIALS & METHODS

Seed Materials

S. alterniflora seeds were harvested in November 2009 from wild plants in marshes of Port Fourchon, Louisiana. Seeds were collected by hand shattering and immediately sealed in plastic zipper bags. After transport to the laboratory (Louisiana State Univ., Baton Rouge, LA), 10 gram lots of *S. alterniflora* seeds were placed in 250 ml of distilled water in plastic GA-7 culture containers (Magenta vessels) (Sigma Aldrich, St. Louis, USA), and stored at 2°C. Dry *S. pectinata* seeds were purchased from Western Native Seed (Coaldale, CO, USA). After delivery to the laboratory, they were stored dry and in tightly sealed Mason jars at -20°C until use. Ten grams of *S. pectinata* seeds were imbibed with 250 ml of distilled water in the Magenta vessel, and placed at 2°C. The initial point of seed germination tests was 1 week after imbibition, when the seeds are fully hydrated. The germination and viability tests were performed every week after seed imbibition until the seeds become totally non-dormant.

Germination and Viability Tests

Vessels of *S. alterniflora* and *S. pectinata* were taken from 2°C and placed in an ice bath to minimize the warming. Filled seeds of *S. alterniflora* and *S. pectinata* were selected on a light table for experiments. Three replicates, each containing 20 seeds on two pieces of germination paper (Anchor Paper Co., St. Paul, Minnesota, USA) secured by placing a Kim-Wipe™ disposable tissue over them, and 8 ml of distilled water, were used for each of the germination test. The plastic Petri dishes (9 x 9 cm) were incubated at 27°C for 14 days, and emergence of the shoots and radicles was recorded at 7 and 14 days. The seeds that failed to germinate after 14 days were forwarded to the viability test. In the viability test, the upper third of coleoptile of the

seed was cut off with a razor blade, a procedure that breaks dormancy, if present (Cohn and Gatz, 2002; Chappell, 2008).

Total Soluble Protein Preparation

Dormant *S. alterniflora* seeds (2009) were imbibed in dH_2O at 2°C for 1 week immediately after harvest. Non-dormant *S. alterniflora* seeds (2009) were obtained after incubation in dH_2O at 2°C for 12 weeks immediately after harvest. Dormant *S. pectinata* seeds were harvested in 2007 (lot #10950, purchased from Western Native Seeds in 2007), stored dry at -20°C and imbibed in dH_2O for 1 week. Non-dormant *S. pectinata* seeds (lot #10950, purchased from Western Native Seeds in 2007) were obtained after incubation in dH_2O for 12 weeks. Fifty *S. alterniflora* or *S. pectinata* seeds of three independent biological replicates were ground in liquid nitrogen. Ground powders were transferred to a pre-chilled glass homogenizer and homogenized in 5 ml buffer [50 mM HEPES pH 7.5, protease inhibitor cocktail (1% v/v)] at 4°C. The total homogenate was centrifuged at 14,000 g at 4°C for 20 min, twice. Total proteins were precipitated by adding 100% TCA (w/v) to make a final concentration of 10% TCA (w/v), and washed with 10 ml cold acetone (-20°C), three times. The pellet was vacuumed dried and stored at -80°C until used for fractionation by two-dimensional gel electrophoresis.

Electrophoresis, In-gel Trypsin Digestion, LC-MS/MS Based Peptide Sequencing

Protein pellets (~ 800 μg) were dissolved in 40 μl lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS (w/v) and 20 mM dithiothreitol (DTT), 2% (w/v) IPG buffer NL 3-10] at room temperature (23°C) overnight. Protein concentration was determined according to the Bradford method using bovine serum albumin as the standard (Bradford, 1976). Protocols of protein separation by two-dimensional gel electrophoresis, gel staining, *in silico* gel analysis and LC-MS/MS peptide sequencing are the same as described in the part of MATERIALS & METHODS

of Chapter 2. The spot of fold change value (≥1) and p value (≤0.05) were reported. For the silver stained gels, 120 µg of total protein were applied for each replicate of *S. alterniflora* and *S. pectinata*. Gel trays were shaken in the staining tank (Dodeca^{TM} stainer, Bio-Rad) filled with fixation solution (40% ethanol, 10% acetic acid and 50% distilled water) at a speed of 6 for 2 hours in the fixation step. Next, gels were shaken in the sensitizing solution (0.5 M potassium acetate, 0.2% sodium thiosulphate, 30% ethanol) at speed of 6 for 30 minutes. The gels were washed three times with distilled water for 10 minutes each time after the sensitizing step. Then, gels were stained (0.2M silver nitrate) at 400 RPM. After a 30-minute staining, gels were washed once with distilled water for 1 minute. In the developing step, gels were shaken in a solution containing 3% potassium carbonate, 0.0001% sodium thiosulphate (w/v) and 0.008% formaldehyde (v/v) at speed of 6 for 5 minutes. In the stopping step, gels were incubated in 5% acetic acid (v/v) and shaken at 300 rpm for 10 minutes. Finally, gels were rinsed with distilled water for 10 minutes.

RESULTS

S. alterniflora and *S. pectinata* produce dormant seeds, but the dormant state can be alleviated by cold stratification at 2ºC. Every week during cold stratification, three replicates of twenty seeds were tested for germination at 27ºC. Under optimal conditions, both *S. pectinata* and *S. alterniflora* seeds began to germinate after 4 weeks of cold stratification, and they were both 100% germinable after 12 weeks of imbibition at 2ºC (Fig. 3.1) .

To study the effect of cold stratification on the alleviation of seed dormancy, comparative proteomics was used to identify differentially expressed proteins between dormant (cold stratification for 1 week) and non-dormant (cold stratification for 12 weeks) *S. pectinata* and

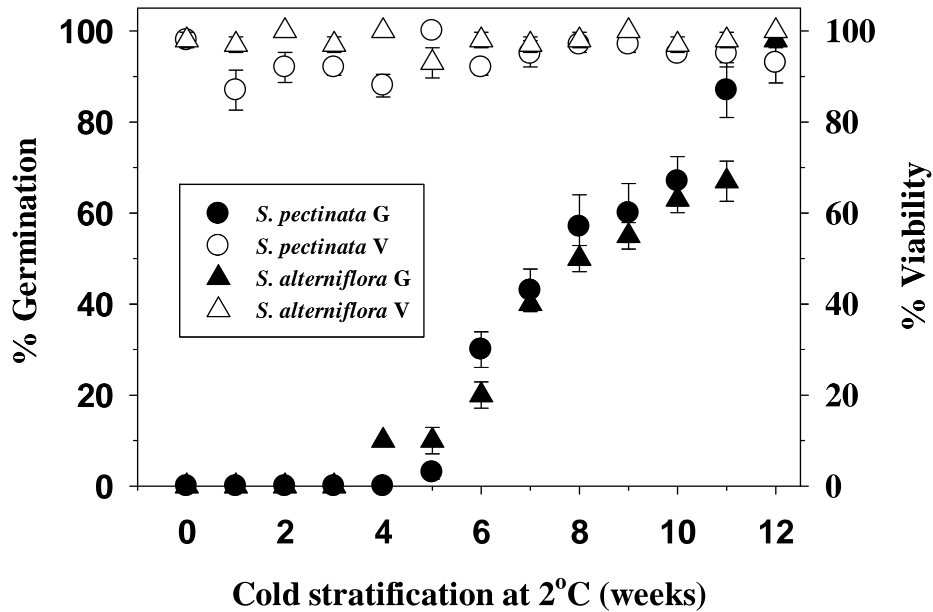


Figure 3.1. Relationship between time of cold-stratification and germination and viability for *S. alterniflora* and *S. pectinata*. *S. alterniflora* seeds were submerged in dH_2O at 2°C without drying after harvest at Port Fourchon, LA (2009). *S. pectinata* seeds were harvested in 2007 (lot #10950, purchased from Western Native Seed, Coaldale, USA), stored dry at -20°C, and then submerged in dH_2O at 2°C. Every week, three replicates were tested for germination at 27°C for 14 days. Emergence of shoots was the indicator for germination. G = germination; V = viability. Error bars: standard error.

S. alterniflora seeds. At the beginning, proteins extracted from dormant and non-dormant *S. alterniflora* were separated on 2-D gels with pI 3-10 (non-linear), and majority of protein spots were resolved in the pI range from 4 to 8 (Fig. 3.2). For a better gel separation, proteomes from *S. alterniflora* and *S. pectinata* were separated on a narrower pH DryStrip 4-7 (Figs 3.3 and 3.4). Based on software analysis (Progenesis SameSpots) and manual verification, a total of 671 and 624 spots were detected on 2-D gels in dormant *S. alterniflora* and *S. pectinata*, respectively. In non-dormant seeds, 658 spots were detected in *S. alterniflora* and 610 spots in *S. pectinata*. In *S. alterniflora*, 92 spots were more abundant in dormant seeds and 87 spots were more abundant in non-dormant seeds (Table 3.1). Thirty seven spots were down regulated, and 36 spots were up-regulated in non-dormant *S. pectinata* seed during cold stratification (Table 3.1). If any differentially expressed proteins were associated with dormancy release, it is speculated that those proteins would exhibit the same changing pattern in both *S. alterniflora* and *S. pectinata*. Nine spots that were down-regulated and seven spots that were up-regulated during cold stratification had the same molecular mass and pI on gels of both *S. alterniflora* and *S. pectinata* (Table 3.1, Figs 3.5 and 3.6). In both *Spartina* species, statistical analysis of the difference in spot abundance between dormant and non-dormant gels showed no 'dormant' spots completely disappeared after cold stratification, and no new spots appeared after dormancy was broken. Several spots have relative low fold change (D/ND or ND/D) of 1.2-1.4 (*e.g.* SA30, SP19 and SA50), but the CV (coefficient of variation) value, which shows the variation of spot volume of each 3 biological replicates, is relative low (3%-14%) (Tables S-3 and S-4).

LC-MS/MS peptide sequencing coupled with database search was used to identify these 16 common differentially expressed spots in the two species (Tables 3.2, 3.3 and 3.4). LC-MS/MS sequencing of the nine down regulated spots (more abundant in dormant seeds) during

cold stratification showed that most of them were the same proteins (Tables 3.2 and 3.3). Five globulin proteins, which had very similar molecular mass of 64-65 kDa and pI 6.8-6.9, were more abundant in dormant than non-dormant *S. alterniflora* and *S. pectinata* (Table 3.2). *De novo* sequencing of spots, SA#5 and SP#5, identified three peptides NPESiVSSFSK, VqGEGVVATiENGER, APAGAVTYFANTDGR, which were BLAST searched and shared homologies with globulin-1 (Fig. 3.7). Cysteine synthase (SA#30/SP#7) was also down regulated during cold stratification. Other down regulated proteins included elongation factor Tu, phosphoglycerate kinase, tubulin, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and serpin. A number of spots had multiple identifications (indicating more than one protein was localized to these spots) based on database searching, *e.g. de novo* sequencing of spot SA#5 identified one peptide, which was BLAST searched to share homology with alcohol dehydrogenase, as well as other peptides homologous to globulin-1 in the same spot. Two peptides (SP#2) were identified as peroxidase, and SA#10 contained one peptide that shared homology with dihydrolipoamide dehydrogenase. SA#21 and SP#19 were identified to share homologies with proteins of both elongation factor Tu and phosphoglycerate kinase. SA#34 and SP#4 shared homologies with proteins of tubulin and Rubisco. SA#29 and SP#8 were identified to be different proteins, SA#29 contained two proteins, tubulin and Rubisco and SP#8 was identified as serpin.

Up-regulated proteins during cold stratification (more abundant after dormancy-breaking) were identified as enolase, ketol-acid reductoisomerase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, heat shock protein-70 kDa, V-type proton ATPase, luminal binding protein 2, ricin type lectin-domain protein, ATP synthase, chaperonin 60 kDa, mannosidase, and protein disulfide-isomerase (Table 3.4). Mascot MS/MS ion search of LC-MS/MS data of spot

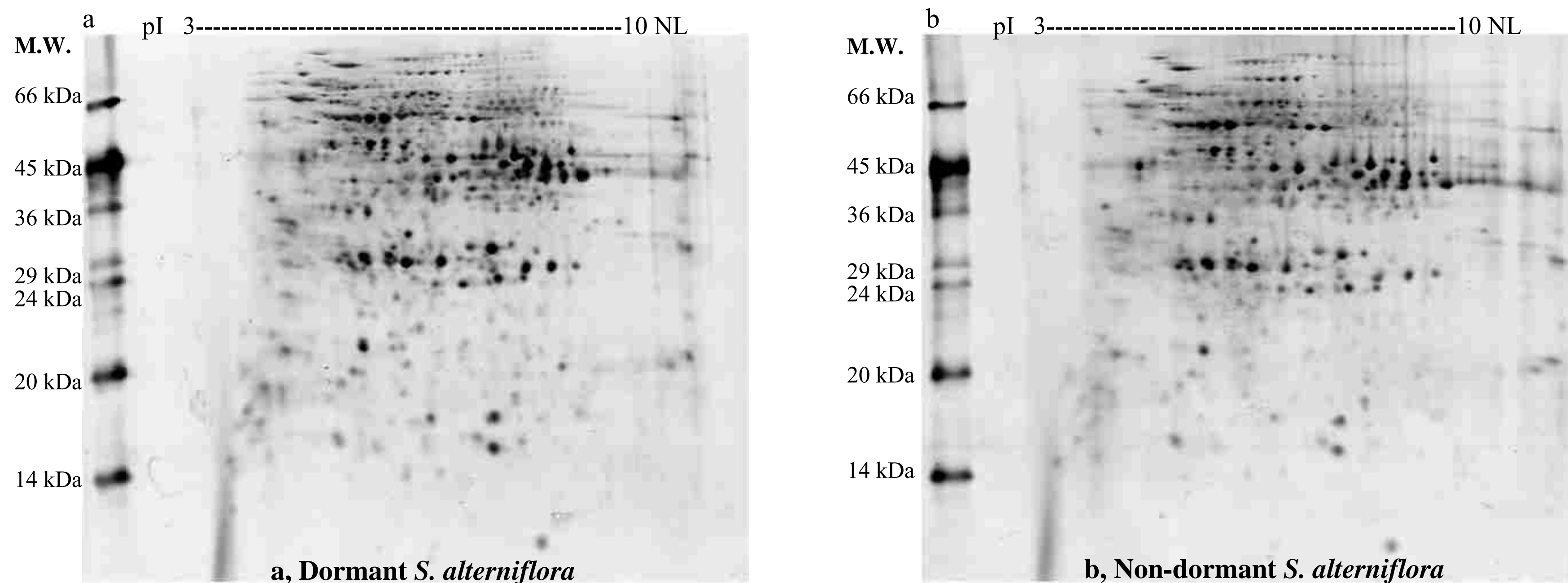


Figure 3.2. Comparison of silver stained 2-D (pI 3-10 NL) profiles between seeds of dormant (a) and non-dormant (b) *S. alterniflora*. Dormant *S. alterniflora* seeds (2009) were incubated in dH_2O at 2°C for 1 week after harvest (% germination = 0%; % viability = 100%). Non-dormant *S. alterniflora* (2009) seeds were obtained after incubated in dH_2O at 2°C for 12 weeks immediately after harvest ((% germination = 100%).

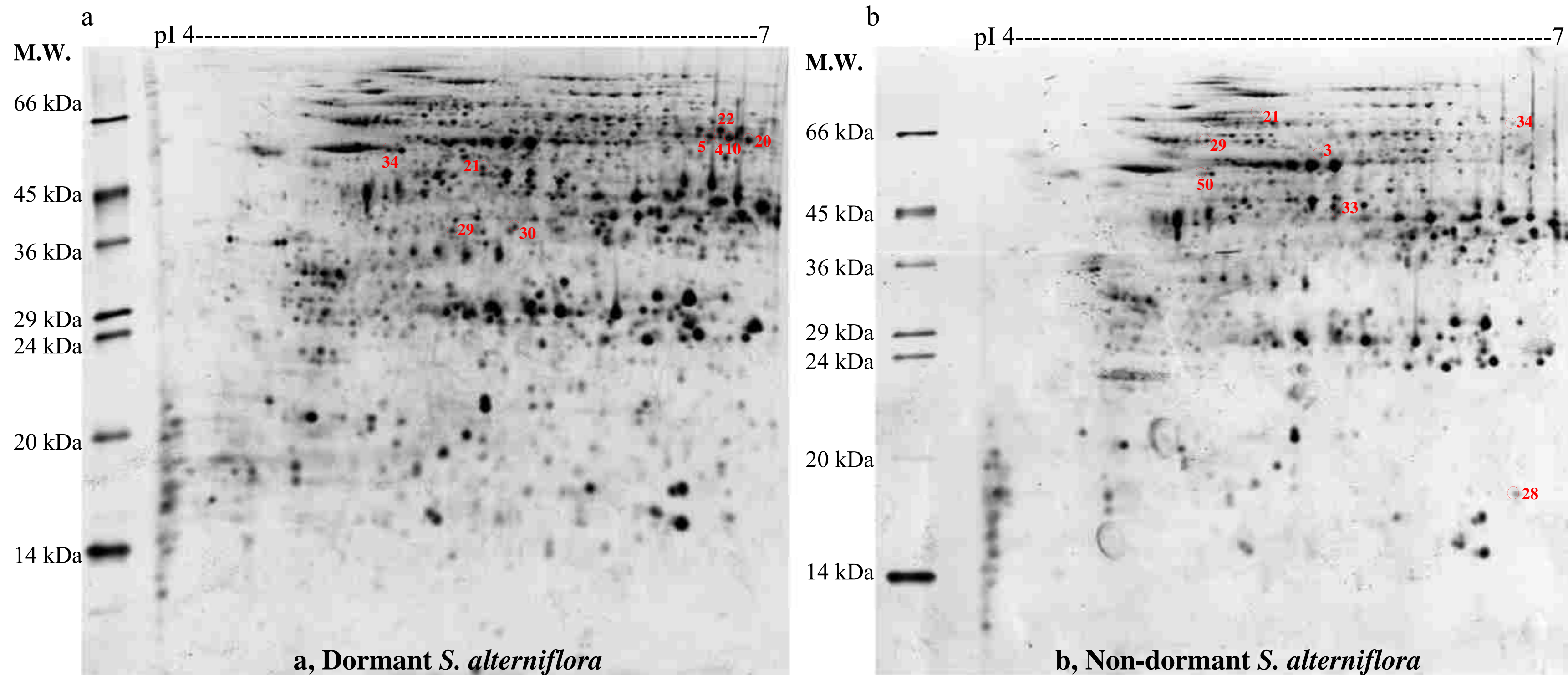


Figure 3.3. Comparison of Colloidal Coomassie Blue G-250 stained 2-D (pI 4-7) profiles between seeds of dormant (a) and non-dormant (b) *S. alterniflora*. Dormant *S. alterniflora* seeds (2009) were imbibed in dH_2O at 2ºC for 1 week immediately after harvest (% germination = 0%; % viability = 100%). Non-dormant *S. alterniflora* (2009) seeds were obtained after imbibed in dH_2O at 2ºC for 12 weeks immediately after harvest (G%=100%). Numbers on the gels were the spots selected for peptide sequencing.

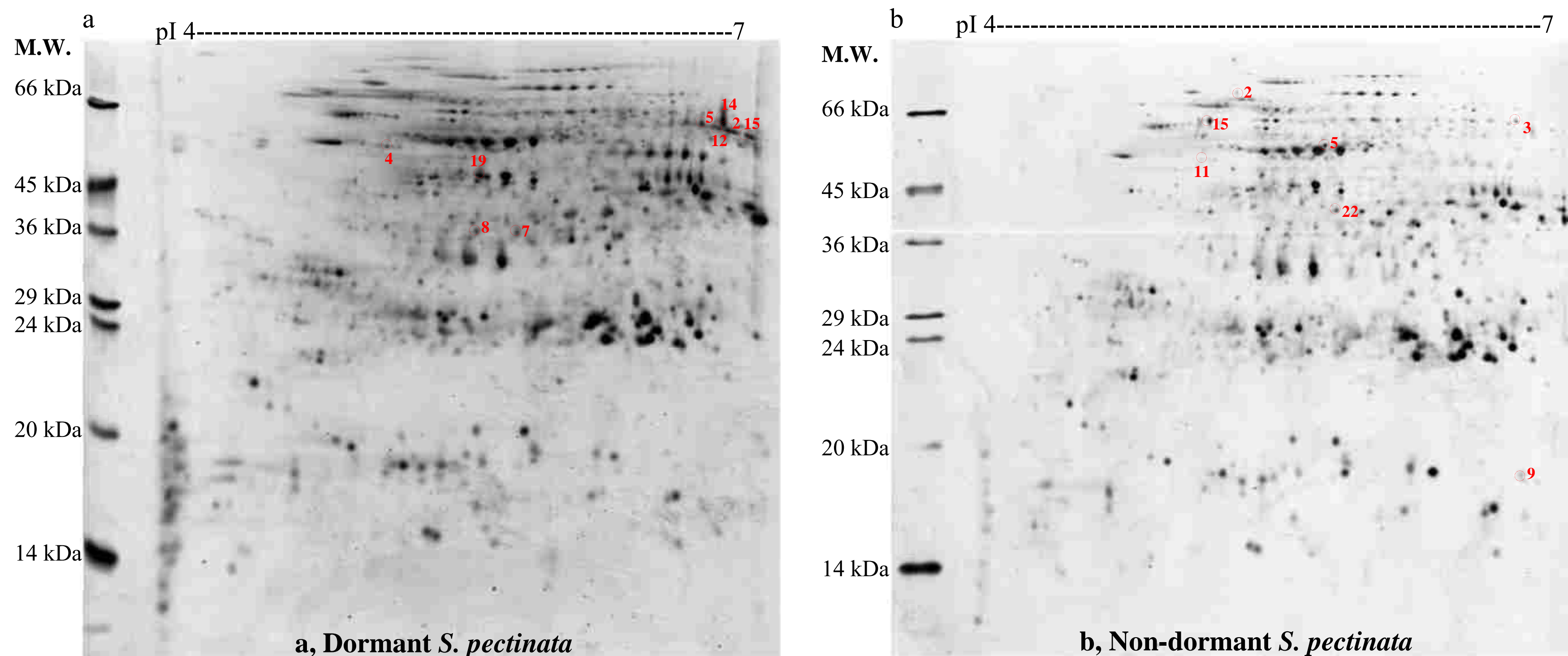


Figure 3.4. Comparison of Colloidal Coomassie Blue G-250 stained 2-D (pI 4-7) profiles between seeds of dormant (a) and non-dormant (b) *S. pectinata*. Dormant *S. pectinata* seeds were harvested in 2007 (lot #10950, purchased from Western Native Seeds in 2007), stored dry at -20°C and imbibed in dH_2O for 1 week (% germination = 0%; % viability = 100%). Non-dormant *S. pectinata* seeds (plot #10950, purchased from Western Native Seeds in 2007, stored dry at -20°C) were obtained after imbibed in dH_2O at 2°C for 12 weeks (% germination = 100%). Numbers on the gels were the spots selected for peptide sequencing.

Table 3.1. A summary of *in silico* analysis of 2-D gels of dormant and non-dormant seeds of *S. alterniflora* and *S. pectinata* during cold-stratification. The number of total spots on 2-D gels (pI 4-7) were detected by Progenesis SameSpots software with manual verification. a, spots that were more abundant in dormant than non-dormant seeds of *S. alterniflora* and *S. pectinata* had the same molecular mass and pI on 2-D gels. b, spots that were more abundant in non-dormant than dormant seeds of *S. alterniflora* and *S. pectinata* had the same molecular mass and pI on 2-D gels.

Spot Number	*S. alterniflora*	*S. pectinata*
Total spots (dormant)	671	624
Total spots (Non-dormant)	658	610
Spots more abundant in dormant seeds	92	37
Shared protein spots [a]	9	
Spots more abundant in non-dormant seeds	87	36
Shared protein spots [b]	7	

Table 3.2. Proteins, which were more abundant in dormant than non-dormant seeds of both *S. alterniflora* and *S. pectinata*, were identified by *de novo* sequencing and BLAST searched. a, spot number of *S. alterniflora* and *S. pectinata* which had the same molecular weight and pI values. b, identified peptides by *de novo* sequencing with Mascot Distiller (Matrix Science, 2.4.2). c, peptides were searched for closest homologies (lowest E value) against NCBI and SwissProt databases. The E value of a significant threshold was set below 0.01 (default value = 10). d, the ratio of average normalized spot number of dormant to non-dormant seeds. The spot of fold change value (≥1) and p value (≤0.05) was reported. i: I or L; q: Q or K.

Spots # [a]	*De novo* Peptides [b]	Homology [c]	Accession # [c]	Theo. MW/pI	Expt. MW/pI	E-Value [c]	Fold Change[d]
SA #22	VVqGEGVVATGADGER VTYFANTDGR NiqiVGCFEiqAE	Globulin-1 (*Sorghum bicolor*)	XP_002464118	69 kDa /7.3	65 kDa /6.7	3E-05	2.4
SP #14	NPESiVSSFSK YVVqGEGVVATiENGER QiVGCFEVqAER	Globulin-1 (*Sorghum bicolor*)	XP_002464118	69 kDa /7.3	65 kDa /6.7	2E-10	1.7
SA #4	GVVATNENGER GAVTYFANTDGR NPESiVSSYSK	Globulin-1 (*Zea mays*)	ABS89082	28 kDa /7.9	64 kDa /6.7	4E-09	4.4
SP #12	VqGEGVVATiEDGER AVTYFANTDGR qiVGCFEVqAER	Globulin-1 (*Sorghum bicolor*)	XP_002464118	69 kDa /7.3	64 kDa /6.7	6E-10	1.9
SA #5	QqEGVVATiEDGER APAGAVTYFANTDGR NPESiVSSFSK	Globulin-1 (*Sorghum bicolor*)	XP_002464118	69 kDa /7.3	64 kDa /6.6	3E-11	4.1
SA #5	iiGVDiNSPR	Alcohol dehydrogenase (*Malus domestica*)	AEL75218	41 kDa /6.9	64 kDa /6.6	6E-04	4.1
SP #5	VqGEGVVATiENGER NPESiVSSFSK	Globulin-1 (*Sorghum bicolor*)	XP_002464118	69 kDa /7.3	64 kDa /6.6	2E-08	2.2

Table 3.2. Continued from previous page.

Spots # [a]	*De Novo* Peptides b	Homology [c]	Accession # [c]	Theo. MW/pI	Expt. MW/pI	E-Value [c]	Fold Change[d]
SA #10	qGEGVVATiENGER NPESiVSSFSK EEGqGYETVR	Globulin-1 (*Sorghum bicolor*)	XP_002464118	69 kDa /7.3	64 kDa /6.8	2E-04	3.5
SA #10[e]	KFVSPSEVSVDiiDGGSTTVKG	Dihydrolipoamide dehydrogenase (*Oryza sativa*)	EEC78570	53 kDa /7.6	64 kDa /6.8	9% /120 [e]	3.5
SP #2	VqGEGVVATiENGER NPESiVSSFSK	Globulin-1 (*Sorghum bicolor*)	XP_002464118	69 kDa /7.3	64 kDa /6.8	2E-08	2.6
SP #2	QViDAAK NiqViTGTqSViR	Peroxidase (*Zea mays*)	NP_001141196	38 kDa /5.2	64 kDa /6.8	9E-05	2.6
SA #20	qGEGVVATiEDGER NPESiVSSYSK	Globulin-1 (*Sorghum bicolor*)	XP_002464118	69 kDa /7.3	65 kDa /6.9	1E-06	2.4
SP #15	VqGEGVVATiEDGER NPESiVSSFSK	Globulin-1 (*Zea mays*)	XP_002464118	69 kDa /7.3	65 kDa /6.9	2E-04	2.0

Table 3.3. Proteins, which were more abundant in dormant than non-dormant seeds of both *S. alterniflora* and *S. pectinata*, were identified by MASCOT MS/MS ion search.

Spots # [a]	Homology [b]	Accession # [b]	Theo. MW/pI	Expt. MW/pI	Peptide # [c]	Sequence Coverage [d]	Mascot Score [c]	Fold Change [e]
SA #21	Elongation factor Tu (*Oryza sativa*)	EEC73516	55 kDa/5.7	50 kDa/5.5	11	26	797	1.4
SA #21	Phosphoglycerate kinase (*Zea mays*)	NP_001142404	42 kDa/5.7	50 kDa/5.5	9	30	653	1.4
SP #19	Phosphoglycerate kinase (*Zea mays*)	NP_001142404	42 kDa/5.7	50 kDa/5.5	17	50	1196	1.2
SP #19	Elongation factor Tu (*Zea mays*)	ACN28515	51 kDa/6.2	50 kDa/5.5	6	22	585	1.2
SA #34	Tubulin (*Vitis vinifera*)	XM_002263786	50 kDa/4.9	58 kDa/5.0	10	31	674	2.2
SA #34	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit RUBISCO (*Crypsis schoenoides*)	ABP01431	52 kDa/6.3	58 kDa/5.0	7	17	370	2.2
SP #4	Tubulin (*Zea mays*)	P14640	50 kDa/4.9	58 kDa/5.0	9	29	700	1.6
SP #4	RUBISCO (*Cynodon transvaalensis*)	ABP01425	52 kDa/6.1	58 kDa/5.0	8	20	426	1.6
SA #30	Cysteine synthase (*Oryza sativa*)	AAD23907	34 kDa/5.4	38 kDa/5.6	3	14	291	1.3
SA #30	Legumin (*Hordeum vulgare*)	BAJ94492	38 kDa/5.7	38 kDa/5.6	4	16	273	1.3
SP #7	Cysteine synthase (*Oryza sativa*)	AAD23907	34 kDa/5.4	38 kDa/5.6	5	20	389	2.1
SA #29	Tubulin (*Zea mays*)	P14640	50 kDa/4.9	41 kDa/5.5	7	30	750	2.2
SA #29	RUBISCO (*Peridictyon sanctum*)	CAA90006	52 kDa/6.0	41 kDa/5.5	4	18	404	2.2
SP #8	Serpin (*Sorghum bicolor*)	XP_002466824	50 kDa/5.7	41 kDa/5.5	6	17	521	1.5

a, spot number of *S. alterniflora* and *S. pectinata* which had the same molecular weight and pI values. b, identified protein homologies by MASCOT MS/MS ion search (Matrix Science). c, the number of identified peptides and probability based scoring. a Mascot score of the significant threshold was given in the output after each Mascot MS/MS search. Mascot score from identified peptides, which was above the significant threshold of each Mascot search, was reported. d, the ratio of identified peptide sequences to the whole sequence of proteins. e, the ratio of average normalized spot number of dormant to non-dormant seeds. The spot of fold change value (≥1) and p value (≤0.05) was reported.

Table 3.4. Proteins, which were more abundant in non-dormant than dormant seeds of both *S. alterniflora* and *S. pectinata*, were identified by MASCOT MS/MS ion search.

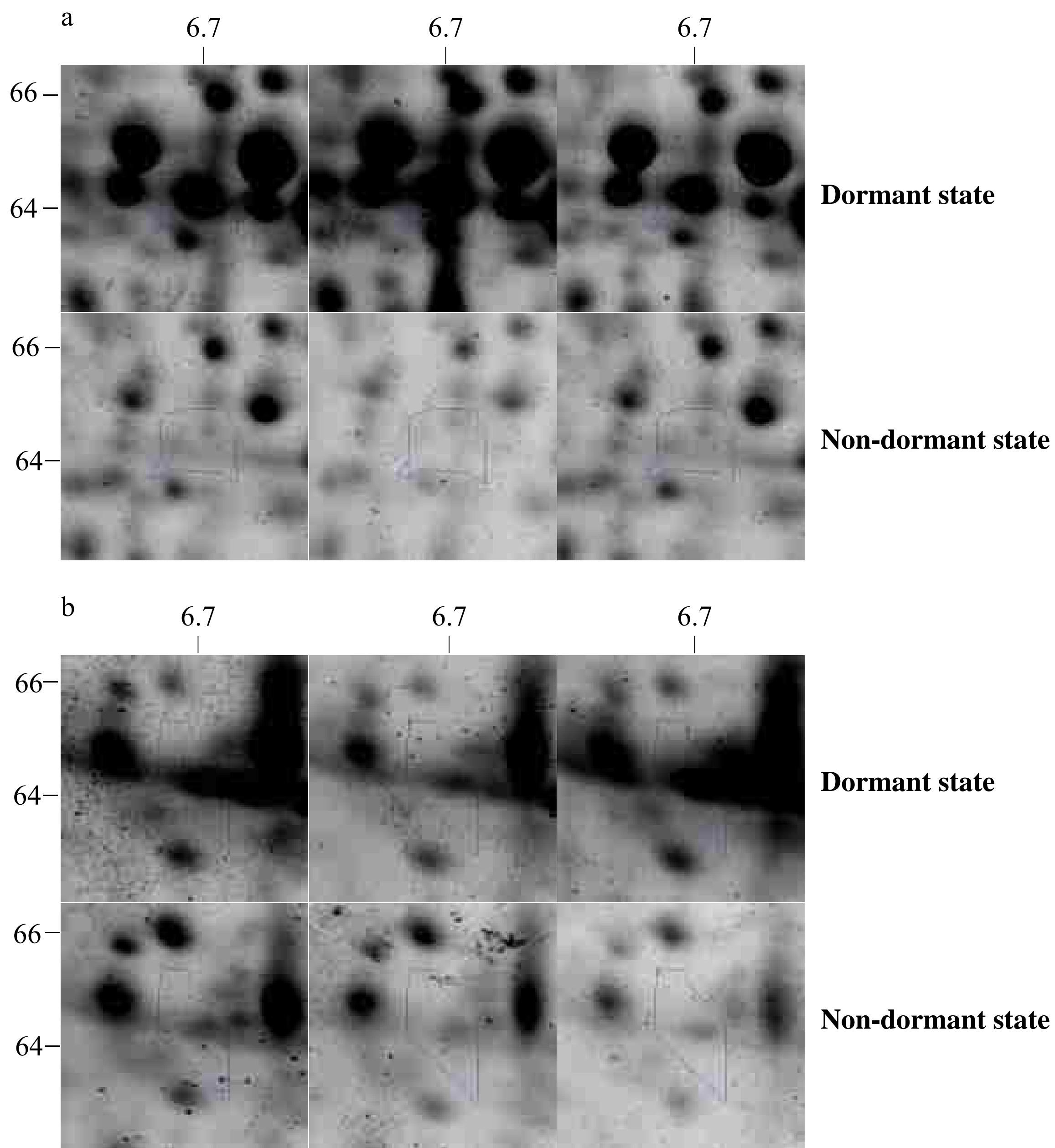
Spots # [a]	Homology [b]	Accession # [b]	Theo. MW/pI	Expt. MW/pI	Peptide # [c]	Sequence Coverage [d]	Mascot Score [c]	Fold Change [e]
SA #3	Enolase (*Brachypodium distachyon*)	XP_003573806	48 kDa/5.6	64 kDa/5.7	6	20	567	1.7
SA #3	Ketol-acid reductoisomerase (*Oryza sativa*)	NP_001056384	62 kDa/6.0	64 kDa/5.7	4	12	362	1.7
SP #5	Enolase (*Brachypodium distachyon*)	XP_003573806	48 kDa/5.6	64 kDa/5.7	8	27	718	3.4
SP #5	Ketol-acid reductoisomerase (*Oryza sativa*)	NP_001043738	60 kDa/5.7	64 kDa/5.7	3	5	219	3.4
SA #29	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (*Zea mays*)	NP_001167944	60 kDa/5.5	66 kDa/5.4	6	28	510	1.8
SP #15	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (*Zea mays*)	NP_001167944	60 kDa/5.5	66 kDa/5.4	6	21	281	2.1
SA #21	Heat shock protein 70 kDa (*Zea mays*)	NP_001130314	73 kDa/5.5	68 kDa/5.5	8	19	838	2.0
SA #21	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (*Brachypodium distachyon*)	XP_003564482	60 kDa/5.5	68 kDa/5.5	6	20	578	2.0
SA #21	V-type proton ATPase catalytic subunit A (*Brachypodium distachyon*)	XP_003570412	69 kDa/5.1	68 kDa/5.5	4	10	372	2.0
SP #2	Luminal-binding protein 2 precursor (*Zea mays*)	NP_001105893	73 kDa/5.1	68 kDa/5.5	13	27	1173	4.2
SP #2	Heat shock protein 70 kDa (*Sorghum bicolor*)	XP_002465468	71 kDa/5.2	68 kDa/5.5	11	22	872	4.2

a, spot number of *S. alterniflora* and *S. pectinata* which had the same molecular weight and pI values. b, identified protein homologies by MASCOT MS/MS ion search (Matrix Science). c, the number of identified peptides and probability based scoring. a Mascot score of the significant threshold was given in the output after each Mascot MS/MS search. Mascot score from identified peptides, which was above the significant threshold of each Mascot search, was reported. d, the ratio of identified peptide sequences to the whole sequence of proteins. e, the ratio of average normalized spot number of dormant to non-dormant seeds. The spot of fold change value (≥1) and p value (≤0.05) was reported.

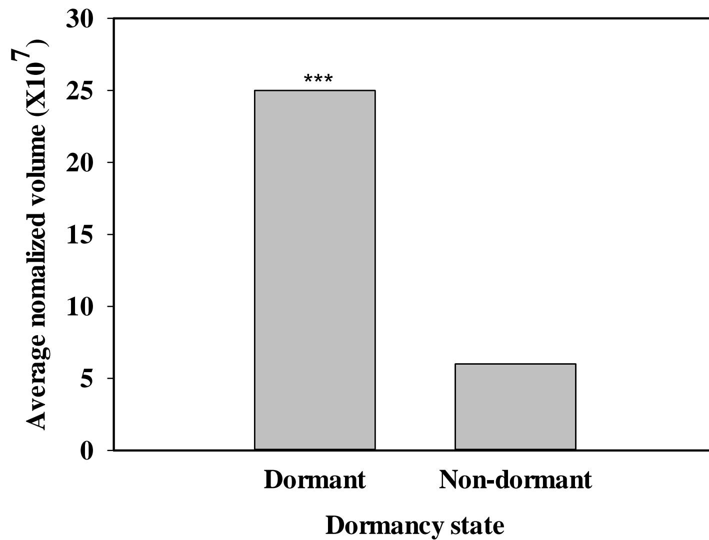
Table 3.4. Continued from previous page.

Spots # [a]	Homology [b]	Accession # [b]	Theo. MW/pI	Expt. MW/pI	Peptide # [c]	Sequence Coverage [d]	Mascot Score [c]	Fold Change [e]
SA #28	Ricin type lectin-domain protein (*Sorghum bicolor*)	XP_002465329	39 kDa/6.3	17 kDa/6.7	3	11	194	1.8
SP #9	Protein disulfide-isomerase (*Zea mays*)	P52588	57 kDa/5.2	17 kDa/6.7	3	7	163	1.5
SA #34	Lysosomal alpha-mannosidase (*Hordeum vulgare*)	BAJ95309	113 kDa/5.9	66 kDa/6.6	5	6	450	1.5
SP #3	Chaperonin 60 kDa (*Oryza sativa*)	NP_001054641	59 kDa/6.2	66 kDa/6.6	4	11	340	1.7
SA #50	F1-ATP synthase, beta unit (*Sorghum bicolor*)	CAA75477	49 kDa/5.3	53 kDa/5.3	14	49	1242	1.2
SA #50	Enolase (*Oryza sative*)	NP_001056727	48 kDa/5.4	53 kDa/5.3	7	34	837	1.2
SP #11	Enolase (*Brachypodium distachyon*)	XP_003558351	48 kDa/5.5	53 kDa/5.3	12	58	1300	1.5
SA #33	Phosphoglycerate kinase (*Zea mays*)	NP_001142404	42 kDa/5.7	42 kDa/5.7	4	20	371	1.4
SP #22	Hexokinase (*Oryza sativa*)	AAZ93624	50 kDa/5.2	42 kDa/5.7	2	4	94	1.7

a, spot number of *S. alterniflora* and *S. pectinata* which had the same molecular weight and pI values. b, identified protein homologies by MASCOT MS/MS ion search (Matrix Science). c, the number of identified peptides and probability based scoring. a Mascot score of the significant threshold was given in the output after each Mascot MS/MS search. Mascot score from identified peptides, which was above the significant threshold of each Mascot search, was reported. d, the ratio of identified peptide sequences to the whole sequence of proteins. e, the ratio of average normalized spot number of dormant to non-dormant seeds. The spot of fold change value (≥1) and p value (≤0.05) was reported.


a
6.7
6.7
6.7
66
64
Dormant state
66
64
Non-dormant state
b
6.7
6.7
6.7
66
64
Dormant state
66
64
Non-dormant state

c



d

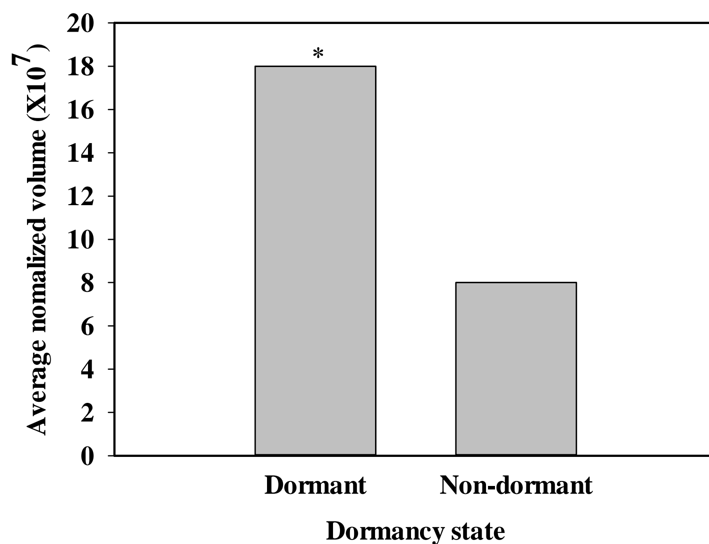
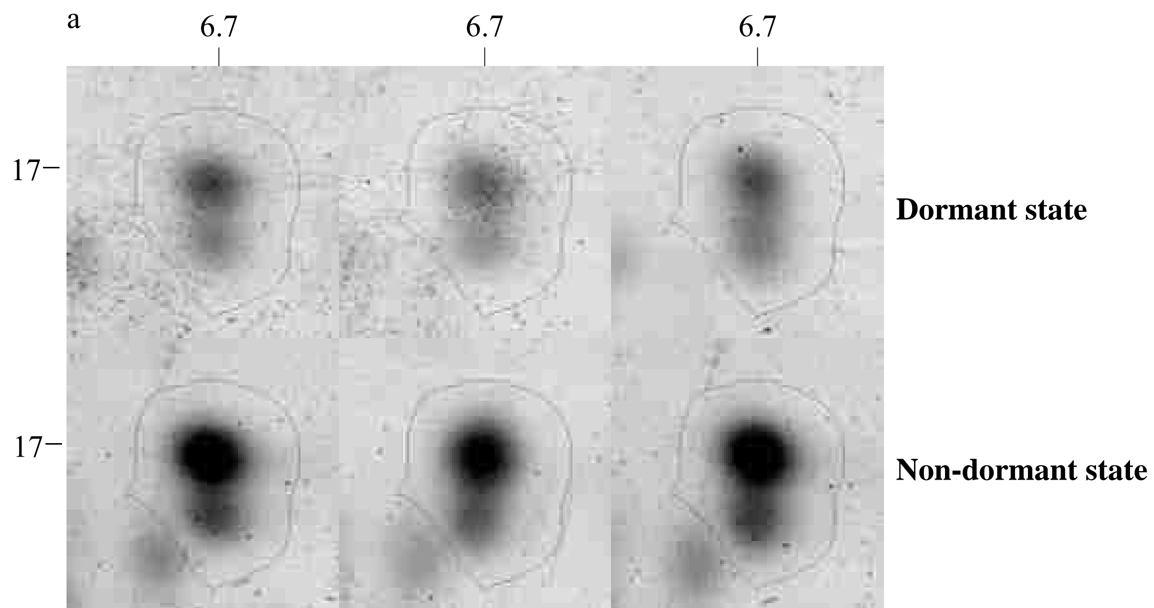
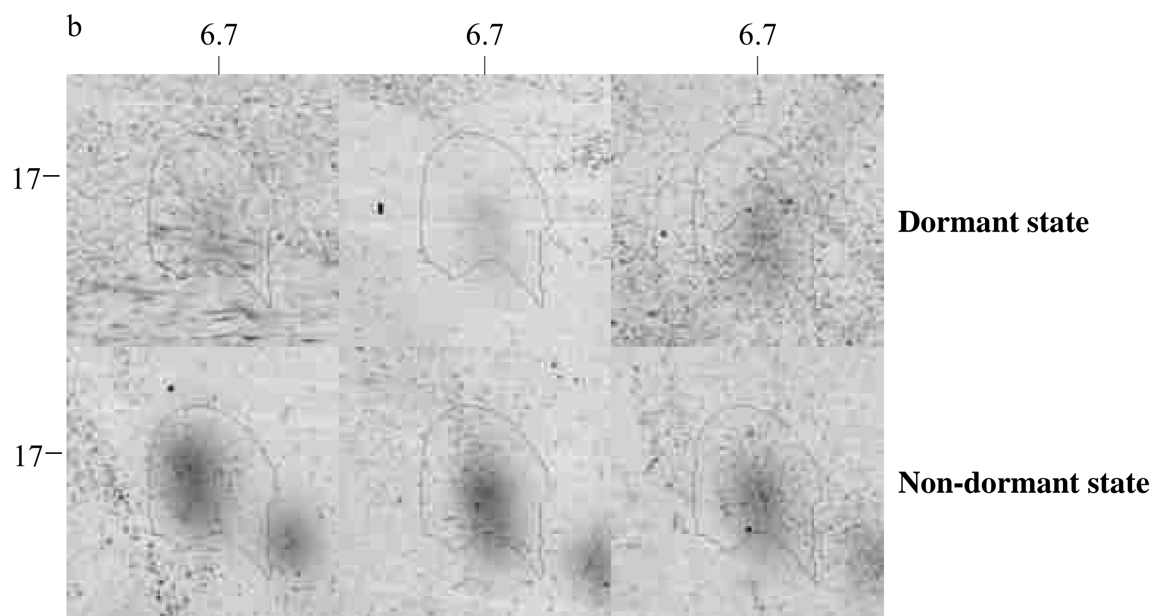
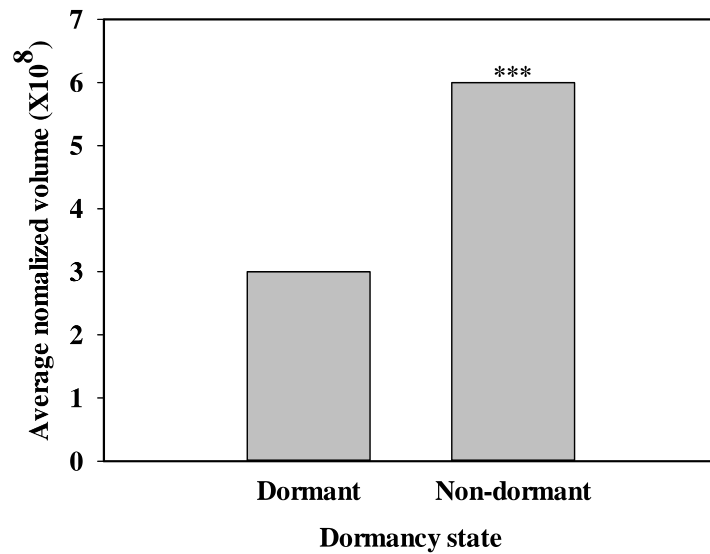


Figure 3.5. Changes in proteins identified as globulin-1 which were more abundant in dormant than non-dormant seeds of *S. alterniflora* (SA #5) (a, c) and *S. pectinata* (SP #5) (b, d) during cold stratification. (a, c), 2-D gels of three biological samples. Molecular mass (kDa) and pI are shown in the images. (b, d), comparison of average normalized volume of spots between dormant and non-dormant seeds. ***, significant ($p<0.001$). *, significant ($p<0.05$).


a
6.7
6.7
6.7
17
17
Dormant state
Non-dormant state


b
6.7
6.7
6.7
17
17
Dormant state
Non-dormant state

c



d

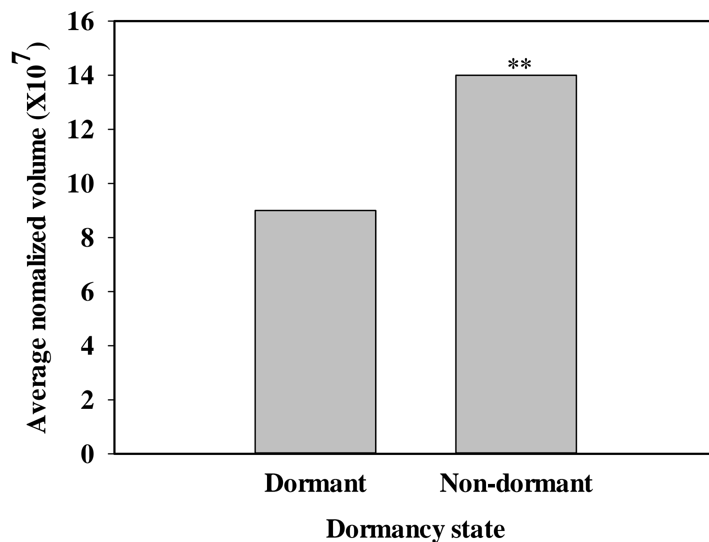


Figure 3.6. Changes in proteins identified as ricin type domain protein (a, c) and disulfide-isomerase and heat shock protein-70 (b, d) which were more abundant in non-dormant than dormant seeds of *S. alterniflora* (SA #28) (a, c) and *S. pectinata* (SP #9) (b, d) during cold stratification. (a, c), 2-D gels of three biological samples. Molecular mass (kDa) and pI are shown in the images. (b, d), comparison of average normalized volume of spots between dormant and non-dormant seeds. ***, significant ($p<0.001$). **, significant ($p<0.01$).

SA#21 identified eight peptides belonging to heat shock protein-70 kDa (Fig. 3.8). SA#33 and SP#22 were identified to be different proteins, phosphoglycerate kinase and hexokinase, respectively. SA#34 and SP#3 were mannosidase and chaperonin 60 kDa, and SA#28 and SP#9 were also different proteins: ricin type lectin-domain protein, and protein disulfide-isomerase and heat shock protein-70 kDa.

DISCUSSION

A Suite of Proteins Exhibiting Same Expression Pattern in Both *S. alterniflora* and *S. pectinata* During Cold Stratification

The dormancy state of *S. alterniflora* and *S. pectinata* seeds can be alleviated by cold stratification. Comparative proteomics was used to attempt to identify the differentially expressed proteins that may be associated with the dormancy release by cold stratification. Ninety-two and 37 spots were down regulated, and 87 and 36 spots accumulated during the moisture chilling in *S. alterniflora* and *S. pectinata*, respectively. Those proteins, which exhibited the same expression pattern during the moisture chilling in both *S. alterniflora* and *S. pectinata*, were selected and sequenced. Nine down-regulated and seven up-regulated spots during cold stratification fell into this category.

Degradation of Storage Proteins in Non-dormant Seeds

A number of *ca.* 64 kDa globulin-1 proteins were sharply down-regulated during dormancy release in both *S. alterniflora* and *S. pectinata*. Two types of globulin proteins can be defined based on sedimentation coefficients, which included 7S vicilin-type globulin-1 and 11S legumin-type globulin-2 (Shewry *et al.,* 1995). Globulin-1 normally consists of three polypeptide subunits, and the molecular weight of each subunit is *ca.* 43 kDa, 47 kDa and 53 kDa (Brown *et al.,* 1980). Down-regulation of storage proteins was also reported in cold stratified *A. thaliana*

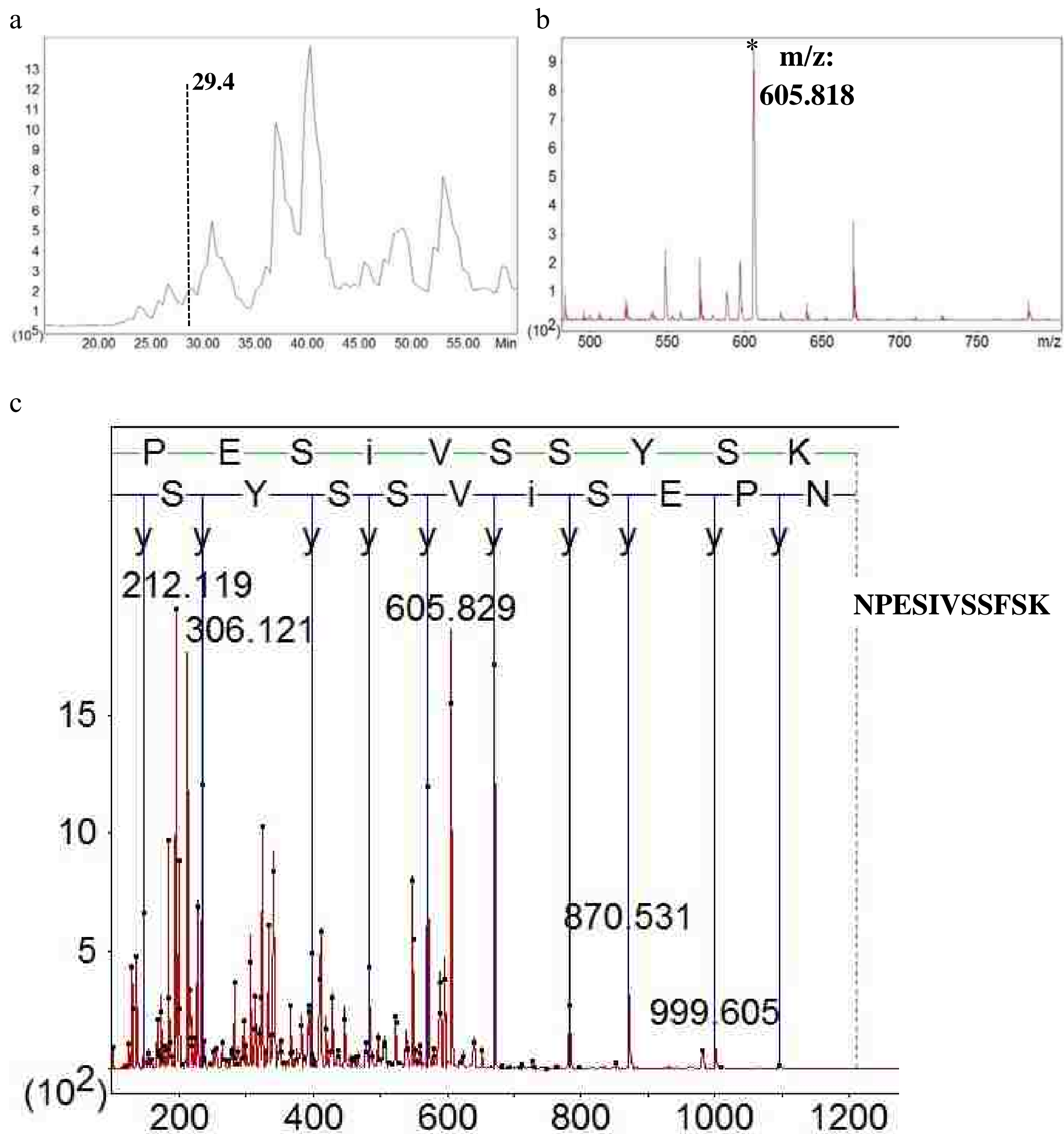


Figure 3.7. Mass spectrometry data of protein identified as globulin-1 by *de novo* sequencing. a, total ion chromatogram of all the mass spectra during a gradient 70 min gradient LC separation. Dash line represents an eluted peptide at 29.4 minute. b, mass spectrum of the eluted peptide (m/z: 605.818, 2+) at 29.4 min. c, MS/MS spectrum of the peptide eluted at 29.4 minutes. The mass differences between different "y" ions is used to predict the amino acid sequence. Direction from amino side to carboxylic side is from right to left.

a

```
1.    gi|212275400    Mass: 72618    Score: 838    Matches: 10(8)   Sequences: 10(8)   emPAI: 0.42
      uncharacterized protein LOC100191408 [Zea mays]
      Check to include this hit in error tolerant search
```

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	49	484.2995	966.5843	966.5611	0.0233	0	32	2.8	1		K.HLNITLTR.S
	75	617.3601	1232.7057	1233.6605	-0.9548	0	20	39	2		K.VQEVVSEIFGK.S
☑	93	678.3647	1354.7148	1354.6616	0.0532	0	75	0.00013	1		R.NTADTTIYSIEK.S
☑	105	731.3507	1460.6868	1459.6579	1.0289	0	56	0.0094	1		K.APNGDAWVETTDGK.Q
☑	115	776.9116	1551.8087	1551.7682	0.0405	0	67	0.00066	1		R.QAVTNPQNTFFGTK.R
☑	130	590.0024	1766.9853	1766.9414	0.0439	0	53	0.013	1		K.IPAEVASEIEAAIADLR.Q
☑	132	903.9875	1805.9604	1805.8981	0.0623	0	145	1e-11	1		R.IINEPTAAALSYGMNNK.E
☑	133	911.4918	1820.9691	1820.9017	0.0674	0	146	7.4e-12	1		K.SQVFSTAADNQTQVGIR.V
☑	137	970.0359	1938.0572	1937.9993	0.0579	0	161	2.2e-13	1		K.GVNPDEAVAMGAAIQGGILR.G
☑	140	671.0526	2010.1361	2010.0633	0.0728	1	90	2.6e-06	1		R.DKIPAEVASEIEAAIADLR.Q

b

Protein sequence coverage: 19%

Matched peptides shown in ***bold red***.

```
  1 MAASLLLRAV RRRELASPLG SLGANLQSTC AANICSKWGN FARPFSAKAA
 51 GNEVIGIDLG TTNSCVAVME GKNPKVIENA EGARTTPSVV AFTQKGERLV
101 GTPAKRQAVT NPQNTFFGTK RMIGRRFDDP QTQKEMKMVP YKIVKAPNGD
151 AWVETTDGKQ YSPSQVGAFV LTKMKETAES YLGKSVSKAV ITVPAYFNDA
201 QRQATKDAGR IAGLDVERII NEPTAAALSY GMNNKEGLIA VFDLGGGTFD
251 ISILEISNGV FEVKATNGDT FLGGEDFDNT LLEFLVSDFK KTEGIDLSKD
301 RLALQRLREA AEKAKVELSS TSQTEINLPF ITADASGAKH LNITLTRSKF
351 ESLVHNLIER TRDPCKNCLK DAGISTKEVD EVLLVGGMTR VPKVQEVVSE
401 IFGKSPSKGV NPDEAVAMGA AIQGGILRGD VKELLLLDVT PLSLGIETLG
451 GIFTRLINRN TTIPTKKSQV FSTAADNQTQ VGIRVLQGER EMAADNKLLG
501 EFDLVGIPPA PRGLPQIEVA FDIDANGIVT VAAKDKATGK EQNITIRSSG
551 GLSEADIQKM VQEAELHAQK DQERKALIDI RNTADTTIYS IEKSLGEYRD
601 KIPAEVASEI EAAIADLRQE MASDDIEKIK AKLEAANKAV SKIGQHMSGG
651 GSGDSQSGSG PQGGGDQAPE AEYEEVKK
```

c

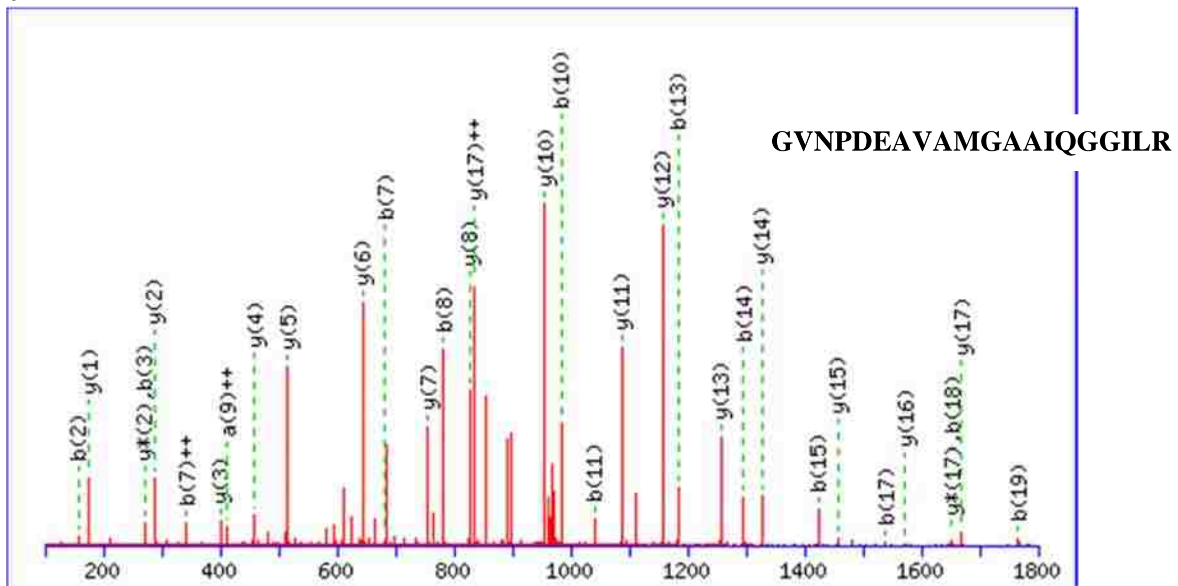


Figure 3.8. Protein identified as heat shock protein-70 based on Mascot MS/MS ion search. a: identified protein homology with highest Mascot protein score. b: sequence coverage of identified peptides amino acid number divided by the total amino acid number. c: MS/MS spectrum of the peptide “GVNPDEAVAMGAAIQGGILR”.

Cvi seeds, where a dozen of cruciferins, which are a major group of storage proteins in Brassica seeds, decreased during cold stratification (Arc *et al.,* 2012). Down-regulation of gliadin, a class of storage protein in wheat, was associated with dormancy release by cold stratification in beech seed (Pawlowski, 2010). Major hydrolysis of storage protein typically coincides with seed germination; proteomic studies showed a decreased protein expression of globulins during germination in seeds of rice (Yang *et al.,* 2007) and Arabidopsis (Gallardo *et al.,* 2001). Products from degradation of globulin-1 proteins in *S. alterniflora* and *S. pectinata* during cold stratification may be used for other events associated with dormancy breaking, *e.g. de novo* protein synthesis. Serpin, which is a class of proteins that is able to inhibit serine protease activities (Whisstock and Bottomley, 2006), is only more abundant in dormant *S. pectinata* seeds. The presence of serpin may be important in keeping storage proteins from degradation in dormant seeds. However, if this is true, it is surprising that it was not detected in *S. alterniflora* seeds.

Proteins Involved in Energy Metabolism Accumulated in Non-dormant Seeds

Up-regulation of a number of metabolism-related proteins coincided with dormancy breaking in seeds of *S. alterniflor*a and *S. pectinata* during cold stratification. Proteins associated with glycolytic pathways, *e.g.* enolase, phosphoglycerate mutase and phosphoglycerate kinase, were more abundant in non-dormant *S. alterniflora* and *S. pectinata* seeds. Similar accumulation of glycolytic enzymes during cold stratification was also observed in *A. thaliana* Cvi (Arc *et al.,* 2012), beech, Norway maple and sycamore seeds (Pawlowski 2010). Hexokinase, enolase, phosphoglycerate mutase and phosphoglycerate kinase are four glycolytic enzymes, which catalyze the 1st step of glycolysis and the chemical reactions from phosphoenolpyruvate (PEP) to 1,3-bisphosphoglycerate. PEP, 2-phosphoglycerate and 3-phosphoglycerate could be transported

in and out between the cytosol and chloroplast, and they can be converted and enter the amino acid and protein biosynthesis pathways in chloroplasts. Ketol-acid reductoisomerase, which catalyzes the chemical reaction from 2,3-dihydroxy-3-methylbutanoate to 2-hydroxy-2-methyl-3-oxobutanoate and produces one molecule of NAPDH, is essential for the valine, leucine and isoleucine biosynthesis (Satyanarayana and Radhakrishnan, 1965). However, cysteine synthase, which is a transferase catalyzing cysteine biosynthesis, was more abundant in dormant than non-dormant *S. alterniflora* and *S. pectinata* seeds. These *de novo* biosynthesized amino acids and proteins are probably used for dormancy release events or seed germination. Mannosidase is able to hydrolyze mannose, which can be a sugar in glycoproteins, and alpha-mannosidase is mandatory for glycan processing, as reported in *A. thaliana* (Liebminger *et al.,* 2009). Accumulation of alpha-mannosidase in non-dormant seeds implies that polysaccharides and glycoproteins start to be broken down during cold stratification and used for events of dormancy-breaking.

V-type (vacuolar) proton ATPase and ATP synthase were up-regulated in non-dormant *S. alterniflora* and *S. pectinata* seeds. Accumulation of ATP synthase was also observed in non-dormant beech, Norway maple, and sycamore seeds during cold stratification (Pawlowski, 2010). ATP is one of the major energy sources for cell metabolism, biosynthesis and active transport of cellular substrates (Nelson and Cox, 2004). V-type proton ATPase, whose role is different from F-ATPase that function in ATP synthesis, is able to acidify cellular organelles by using the energy of ATP hydrolysis to pump protons across the plasma membranes (Nelson *et al.,* 2000). V-ATPase is associated with seed germination, in which mRNA expression of *LVA-P1*, a *vacuolar ATPase*, was greatly up-regulated in the micropylar region prior to radicle emergence in response to GA addition in *gib-1* seeds (Cooley *et al.,* 1999), and increased ATPase activity was observed

in germinating pumpkin seeds (Maeshima *et al.,* 1994). In a comparison of Norway maple seeds stratified at 3ºC to break dormancy versus incubation at 15ºC (dormancy maintained), all types of ATPase were more active in seeds that were stratified at 3ºC (Krawizarz and Szczotka, 2000). Up-regulation of glycolytic enzymes, ATP synthase and ATPase indicate that energy metabolism and acidification and softening of seed tissues may be associated with the dormancy breaking during cold stratification.

Molecular Chaperones May Assist the Folding of Newly Synthesized Proteins

Molecular chaperones, *e.g.* heat shock protein-70 and chaperonin, which are essential for the protein folding (Ellis, 2006), accumulated in non-dormant seeds during cold stratification. Highly abundant glycolytic enzymes in non-dormant seeds produces glycolytic substrates, which may be transported from cytosol to chloroplast for amino acid and peptide biosynthesis, and the presence of heat shock protein-70 and chaperonin may assist the folding of those newly synthesized peptides. However, up-regulation of heat shock protein-70 and chaperonins were also associated with cold stress (Wang *et al.,* 2004), and both *S. alterniflora* and *S. pectinata* were cold stratified at 2ºC for 3 months. Therefore, accumulation of those molecular chaperones could be either associated with the dormancy release or a result of cold stress or both. Without a physiological control provided by a *Spartina* species, which produces dormant seeds that could not be released after 3 months of cold stratification, it may not be possible to differentiate between the effects of cold *per se* versus dormancy-breaking.

Other Identified Proteins

Accumulation of lectin-domain protein and disulfide isomerase was found in non-dormant seeds. Lectins are a group of proteins that exhibit specific binding for sugar moieties (Komath *et al.,* 2006). It may be involved in some processes of dormancy breaking events

associated with carbohydrate binding. Disulfide isomerase is pivotal in assisting protein folding by catalyzing the formation and breakage of disulfide bonds of two cysteines (Wilkinson and Gilbert, 2004). More abundance of disulfide isomerase in non-dormant seeds indicates that *de novo* protein synthesis and protein folding are taking place during cold stratification, and those newly synthesized proteins may be used for the dormancy breaking event.

SUMMARY

Comparative proteomics was used to identify total soluble proteins that may be associated with dormancy release during cold stratification in *S. alterniflora* and *S. pectinata* seeds. Both mature *S. alterniflora* and *S. pectinata* seed are dormant, and the dormancy state could be alleviated by cold stratification. If any proteins are associated with dormancy breaking, it is expected to see those proteins exhibit the same expression pattern in both *S. alterniflora* and *S. pectinata* during 3 months cold stratification because such moist chilling breaks dormancy of both species. There are 9 spots that were more abundant in gels of dormant seeds, and 7 spots that were more abundant in gels of non-dormant *S. alterniflora* and *S. pectinata* seeds. Nevertheless, some of the spots contained peptides that were homologous to a protein in only one *Spartina* species. LC-MS/MS sequencing did identify many proteins that share homologies with storage proteins, phosphoglycerate mutase, enolase, heat shock protein-70, chaperonins, *etc*. The proteomic results suggest that degradation of storage proteins, increased cellular metabolism, and increased activities of molecular chaperones during cold stratification may be associated with dormancy release in *S. alterniflora* and *S. pectinata* seeds.

However, there are also some limitations of this comparative proteomics approach. Proteomics has limited separation and resolution capability; only *ca.* 3000 proteins can be

visualized on a 2-D gel (Görg *et al.,* 2004), but total proteins are known to be at least one order of magnitude greater. In this case, the majority of proteins are neglected. This may explain why non-abundant proteins, *e.g.* transcription factors or metabolic enzymes associated with ABA and GA synthesis and catabolism, were not identified in this study. In addition, the abundance of proteins was compared between dormant and non-dormant seeds. But, it is also necessary to examine the enzymatic activity of those identified proteins. Therefore, the future work would include: (1) sub-fractionation of cellular proteins, *e.g.* a heat stable fraction, mitochondrial fraction or nucleus fraction, to simplify the proteome profiles; (2) use more sensitive molecular techniques, *e.g.* comparative transcriptomics, to investigate if any transcription factors are associated with dormancy breaking during cold stratification.

CHAPTER 4
CRITICAL WATER CONTENT FOR LOSS OF VIABILITY IS INDEPENDENT OF DRYING TEMPERATURE AND RATE FOR RECALCITRANT *SPARTINA ALTERNIFLORA* SEEDS

LITERATURE REVIEW

Spartina alterniflora seeds lose viability when dried below 43 ± 2% (DWB). If loss of viability is caused by biochemical events, it is expected that the critical water content (CWC) for loss of viability will be affected by a change of drying temperature, because biochemical reactions are usually temperature dependent - the rate of biochemical reaction halves for every 10°C temperature decrease for biological system (Laidler, 1997).

The CWCs were found to be independent of drying temperature observed in cocoa embryonic axes (Liang and Sun, 2002) and whole seeds of *Araucaria hunsteinii* (Tompsett, 1982). Since temperature is able to significantly affect drying rate, the influence of temperature on CWC can also be considered as the influence of drying rate. Although the effects of drying rate on the CWC of recalcitrant seeds have been vigorously studied at single temperatures, there is incongruity of both experimental designs and results (reviewed by Berjak and Pammenter, 2008; Pammenter and Berjak, 1999). The definition of slow and rapid drying used in many studies varied greatly. It took days to 'rapidly' dry seeds below CWCs (Farrant *et al.,* 1989; Tompsett 1982), while only hours were required with flash drying method (Ajayi *et al.,* 2006; Wesley-Smith *et al.,* 2001). The disparity of drying rates used in literature makes it difficult to make comparisons among studies, and the prolonged dehydration that needed days or weeks to bring the seeds down below CWCs also complicated the data interpretation because seed aging and deterioration should be taken into consideration in highly metabolic-active non-dormant recalcitrant seeds. Long duration dehydration can allow seeds to initiate the germination process, which made seeds become more desiccation sensitive (Farrant *et al.*, 1985); therefore, the death

of the slowly dried seeds possibly happened at a higher water content because the seeds were more desiccation-sensitive as germination proceeded.

Inconsistent results can also be found in literature. Most studies reported that flash drying was able to retain the viability of embryonic axes/whole seeds of recalcitrant species to a lower CWC compared to slow drying (Berjak *et al.,* 1990; Pammenter *et al.,* 1991; Pritchard, 1991; Finch-Savage, 1992; Berjak *et al.,* 1993; Farrant *et al.,* 1993; Walters *et al.,* 2001; Wesley-Smith *et al.,* 2001; Makeen *et al.,* 2005; Kioko *et al.,* 2006). However, slow and rapid drying was reported to have no significant effects on the embryonic axes of *Castanea sativa* Mill. in terms of loss of viability, cytoplasmic viscosity and membrane permeability (Leprince *et al.,* 1999). The CWCs of the embryos of recalcitrant *Inga vera* subsp. *Affinis* remained the same (105%, dwb) under slow, intermediate and rapid drying (Faria *et al.,* 2004); however, people would argue that the different drying rates were achieved by changing the relative humidity of the atmosphere using different saturated salts, which is still relatively slow compared to the flash drying method. A similar case was found in the whole seeds of *Aquilaria agallocha* Roxb (the authors concluded there was a significant difference, but it was not supported by their Fig. 2 data) (Kundu and Kachari, 2000), which indicated that the CWCs of the whole seeds were independent of the drying rates. Surprisingly, a relationship between CWC and drying rate was shown in cocoa embryonic axes, and rapid drying, however, killed seeds at higher water contents (Liang and Sun, 2002).

In order to determine the relationship between drying temperature/rate and CWC, *S. alterniflora* seeds were dried at 4°C, 14°C, and 24°C in the flash drying unit, and seed moisture contents and percent viability at various dehydration point were determined.

MATERIALS AND METHODS

Seed Materials

S. alterniflora seeds were harvested in November 2008 from marshes of Port Fourchon, Louisiana. Seeds were collected by hand shattering. Seeds were immediately sealed in plastic zipper bags when harvested, put in a cool place after harvest and transported to lab. Ten gram aliquots of seeds were put in Magenta vessels (Sigma Aldrich, St. Louis, USA) with 250 ml of distilled water. They were placed at 2ºC and stored for 1-2 months. Before each experiment, filled seeds were selected on the backlight board and gently patted with Kim-Wipe disposable tissues to remove excess free water (Chappell, 2008).

Seed Germination and Viability Tests

Three replicates were done for each germination test, in which twenty filled seeds were chosen and put on two pieces of germination paper in a plastic Petri dish containing 8 ml of distilled water. The seeds were secured by placing a Kim-Wipe™ disposable tissue over them. The plastic Petri dishes were incubated at 27ºC for 14 days for the germination test and another 14 days for a viability test. Emergence of the shoots and radicles was recorded at 7 and 14 days. The seeds that failed to germinate after 14 days were forwarded to the viability test. In the viability test, one-third part of the seed was cut off by a razor blade, a procedure that breaks dormancy (Chappell, 2008).

Drying Methods

The flash dryer consists of a Nalgene jar (Thermo Fisher Scientific, Rochester, USA), a 12 V (0.16 A) computer fan (Radioshack, Fort Worth, USA) connected to 12 V (1000 mA) power adapter (Radioshack, Fort Worth, USA), $CaSO_4$ desiccant (W.A. Hammond Drierite Company, Xenia, USA) and a ball jar rim (Muncie, USA) lined with mesh as a seed holder. The seeds,

placed on the computer fan, are rapidly dried by air that is pulled up by the computer fan. Four flash drying units were evenly placed in two incubators whose temperatures were set at 4ºC and 14ºC. Another two flash drying units were placed on bench at room temperature (24ºC). Fresh desiccant (*ca.* 30 g) was used for each dry down experiment (Dried recycled desiccant cannot be used). If the desiccant became moisture saturated (indicated as pink color) during each experiment, it was replaced by fresh, undried material (blue color). After rapid drying, the seeds were immediately forwarded to the germination and moisture content determination tests. The moisture content calculation was based on dry weight basis. Seed dry weight was obtained by placing seeds at 105ºC for 7 days (Chappell, 2008).

RESULTS

A change of temperature (4ºC, 14ºC, 24ºC) significantly influenced the drying rate of *S. alterniflora* seeds (Fig. 4.1). Dehydration took place fastest at 24ºC and slowest at 4ºC. Dehydrated *S. alterniflora* seeds needed 15, 20, and 50 days to reach the equilibrium moisture content at 24ºC, 14ºC, and 4ºC, respectively (Fig. 4.1). A relationship between percent viability and drying duration is presented in Figure 4.2, which shows that the rate of viability loss is faster at 24ºC than 14ºC and 4ºC. It took 2 d, 5 d and 20 d to achieve 50% viability loss at 24ºC, 14ºC, and 4ºC, respectively (Fig. 4.2). For almost complete loss of viability (<10%), 15 d, 30 d, and 60 d at 24ºC, 14ºC, and 4ºC were required. Fig. 4.3 shows the mortality curves of percent viability versus water content at 4ºC, 14ºC, and 24ºC. *S. alterniflora* began to rapidly lose their viability when dehydrated below 43 ± 2% (dwb), which indicates that CWC is independent of temperature and drying rate in *S. alterniflora* seeds.

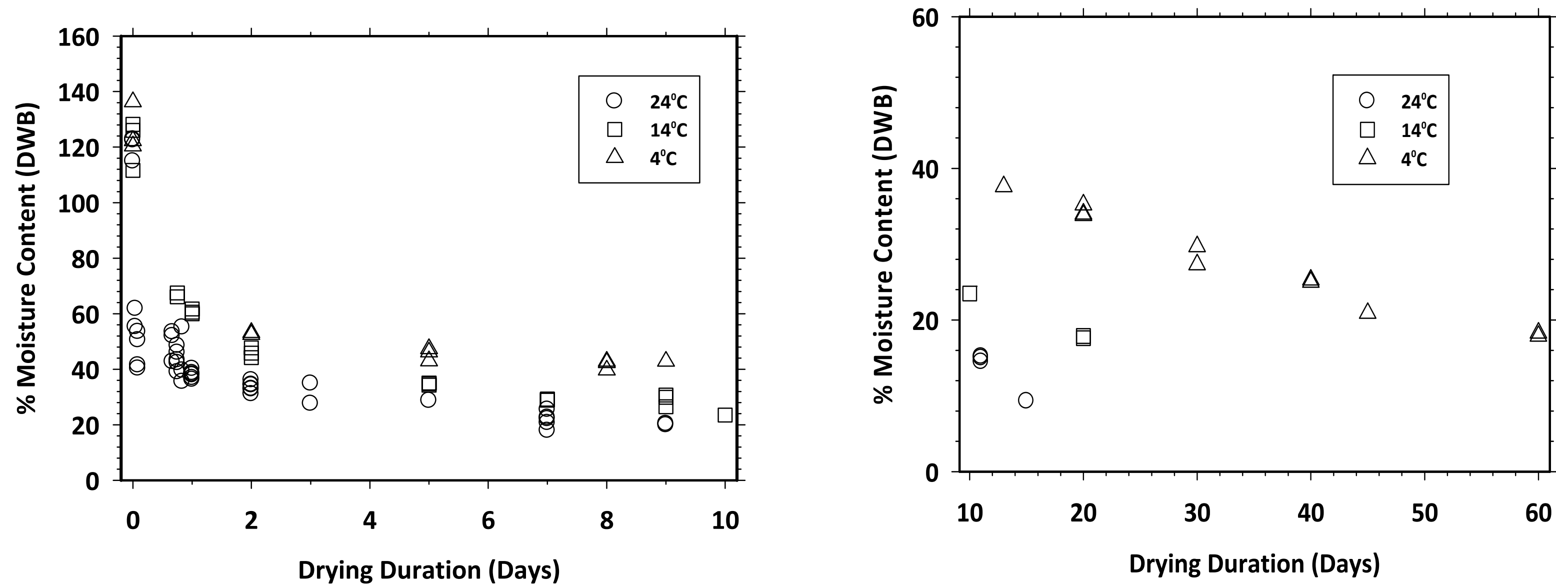


Figure 4.1. Relationship between moisture content of flash dried *S. alterniflora* seeds and drying duration at 4°C, 14°C and 24°C. *S. alterniflora* seeds were harvested from Port Fourchon, Louisiana in 2008 and cold stratified for 1-2 months at 2°C before use.

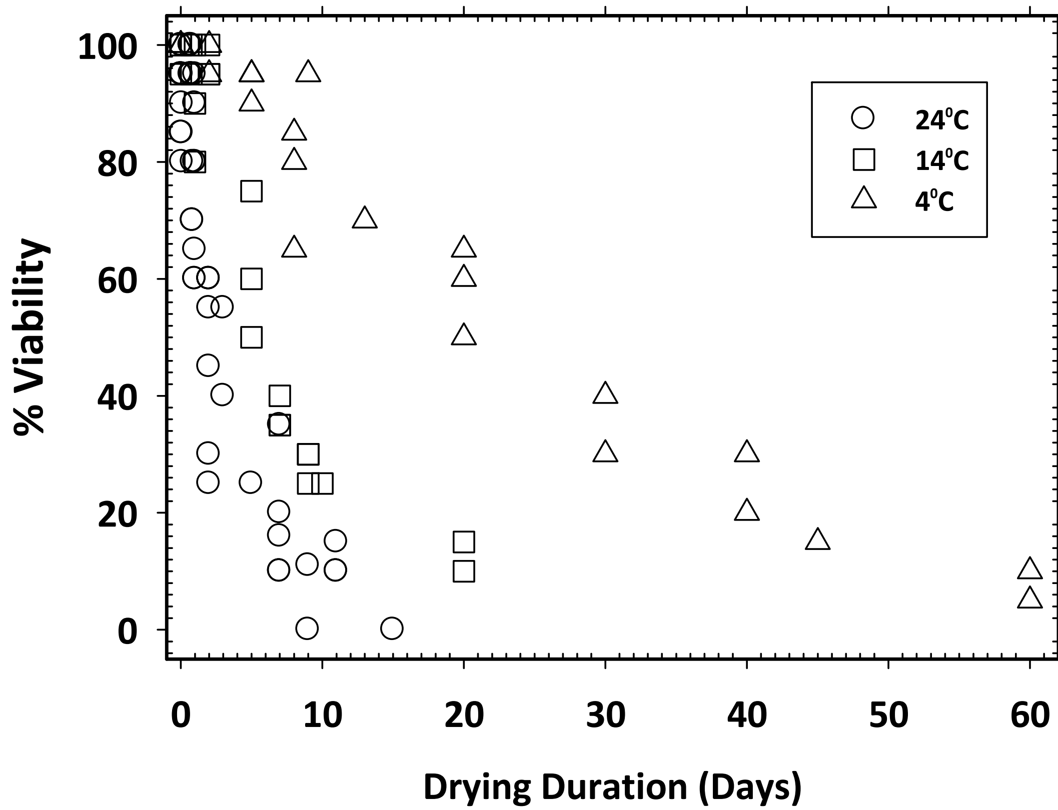


Figure 4.2. Relationship between viability of flash dried *S. alterniflora* seeds and drying duration at 4°C, 14°C, or 24°C. Seeds were considered viable if shoot emergence was observed. *S. alterniflora* seeds were harvested from Port Fourchon, Louisiana in 2008 and cold stratified for 1-2 months at 2°C before use.

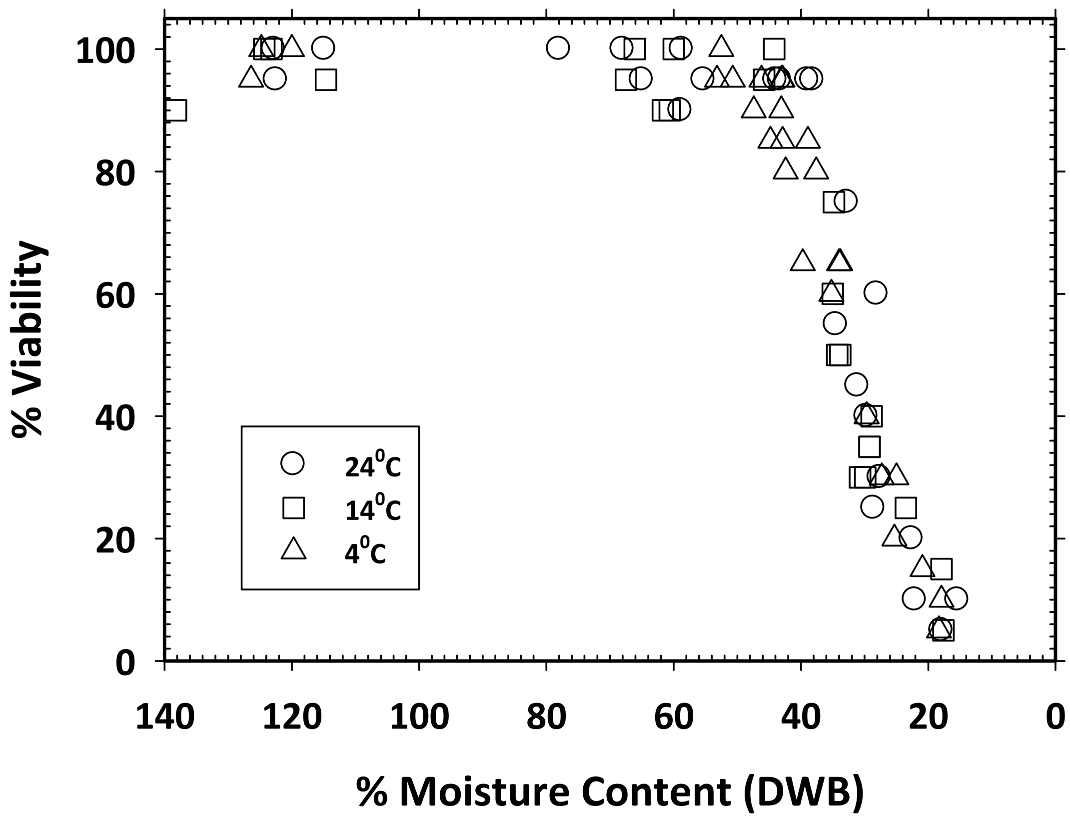


Figure 4.3. Viability of flash dried *S. alterniflora* seeds as a function of moisture content after flash drying at 4ºC, 14ºC and 24ºC. Seeds were considered viable if shoot emergence was observed. *S. alterniflora* seeds were harvested from Port Fourchon, Louisiana in 2008 and cold stratified for 1-2 months at 2ºC before use.

DISCUSSION

One factor that determines the rate of water loss is the gradient in water potential between seed tissue and the vapor pressure of external air, which depends on temperature (Ntuli and Pammenter, 2009). A significant difference of drying rate was observed as drying temperature changed in *S. alterniflora* seeds (Fig. 4.1) - drying took place fastest at 24ºC and slowest at 4ºC. Since temperature significantly affects the drying rates, the effects of temperature on CWC can also be viewed as the effects of drying rates on CWCs.

Results of the effect of drying rate on the CWC of recalcitrant seeds are incongruous. Many studies have reported that flash drying is able to retain the viability of recalcitrant seeds to a lower CWC, and slow drying kills the seeds at higher water contents (reviewed by Berjak and Pammenter, 2008; Pammenter and Berjak, 1999). However, the dependence of drying rate on CWC was not observed in *S. alterniflora* seeds, and they all began to lose their viability when dehydrated below 43 ± 2% (dwb) at 4ºC, 14ºC and 24ºC. This result is consistent with previous work by Chappell (2008), in which CWCs remained the same in both dormant and non-dormant *S. alterniflora* seeds that were dried at different rates at constant temperature. Similar results have also been reported in seeds of *Inga vera* subsp. *Affinis* (Faria et al., 2004), seeds of *A. agallocha* (Kundu and Kachari, 2000) and embryonic axes of *C. sativa* Mill. (Leprince *et al.,* 1999).

Several reasons may explain the inconsistent drying rate results reported in the literature. Some studies conducted the comparison between the slowly dehydrated whole seed and rapidly dehydrated excised embryonic axes before or after drying (Berjak *et al.,* 1993; Kioko *et al.,* 2006; Makeen *et al.,* 2005), which is, however, an improper physiological comparison. The drying rate is greatly affected by the seed coverings, size and component structure (Berjak and

Pammenter, 2006), all of which are able to make the drying rate of whole seeds and embryonic axes greatly different. The water contents of the component structures excised from the whole seed varied after dehydration (Pritchard, 1991; Probert and Longley, 1989) and component structures also had different drying rates during drying (Pritchard and Prendergast, 1986). Most observations of a notable effect of flash drying on survival to the lower critical water content were found in excised embryonic axes (Ajayi *et al.,* 2006; Wesley-Smith *et al.,* 2001) but not whole seeds. A burst of reactive oxygen species was observed during excision of embryonic axes of recalcitrant sweet chestnut seeds (Roach *et al.,* 2008); such oxidative stress may contribute additionally to loss of viability at slower drying rates. Recalcitrant seeds are generally large, which makes them difficult to dehydrate in flash drying units in light of the sample size and even drying across the tissue. Uneven drying of the whole seed and seed mass variation have been found to produce a range of CWCs and potentially mask a true CWC (Daws *et al.,* 2004). Compared to the whole seed, the embryonic axes are small in size and mass, and easier to handle within flash drying units. However, the mass and size of *S. alterniflora* seeds are not an issue, because they are much smaller compared to most other recalcitrant seeds. It takes 24 hours to dehydrate fully hydrated seeds of *S. alterniflora* to around their CWC. Furthermore, the water content of cotyledons instead of the water content of embryonic axes has been suggested to be the determining factor of recalcitrant *Quercus robur* seed death (Finch-Savage, 1992), and cotyledons seemed to be more desiccation sensitive than embryonic axes (Finch-Savage, 1992; Leprince *et al.,* 1999). Finally, *S. alterniflora* seeds are shed dormant and are less metabolically active compared to other recalcitrant species, which are shed non-dormant. The non-dormant status and active metabolism of recalcitrant seeds might cause potential problems because seed aging and deterioration during drying may change seed desiccation sensitivity, thus affecting the

CWC. *S. alterniflora* seeds used in this study were submerged and stratified in H_2O for 1-2 months, during which seeds were in a transition from dormant to non-dormant stage. Previous experiments showed that the CWCs remained the same in both dormant and non-dormant *S. alterniflora* seeds that were dried at different rates at constant temperature (Chappell, 2008).

Only limited studies have reported the effects of drying temperature on the CWC, and CWCs remained unchanged under different drying temperatures (Liang and Sun, 2002; Tompsett, 1982). In our system, *S. alterniflora* seeds were dehydrated in the flash drying units, which consisted of the fan of the same electric power and the same amount of fresh desiccant; thus temperature was the controlling variable of drying rate. In addition, studies of the effects of drying temperature on recalcitrant seed viability cannot be performed at chilling temperature for most other species because loss of viability and ultrastructural damage have been found in chilling stored recalcitrant seeds (Ajayi *et al.,* 2006; Kioko *et al.,* 2006). *S. alterniflora* seeds are chilling tolerant (stored at 2^oC for almost a year without germinating in water and no loss of viability), which makes the experimental design feasible at 4^oC.

The independence of the CWC on drying temperature seems to be inconsistent with a general previous assumption that temperature is able to affect the CWC if recalcitrant seed death is related with biochemical events, which are temperature dependent. In biological systems, the rate of biochemical reaction halves for every 10^oC temperature decrease, but the unchanged CWC at three different drying temperatures suggests that seed death is probably not caused by biochemical events in *S. alterniflora*. Another possible explanation is that the rates of biochemical events of both damage and attempted repair, *e.g.* ROS and antioxidant, changes simultaneously as a function of drying temperature. The synchronous change of the rates lets either damage or repair outweigh the other, and keeps the CWC remain unchanged.

SUMMARY

The effect of drying temperature and drying rate on the critical water content of *S. alterniflora* seeds was examined. Temperature is able to significantly affect the drying rate in *S. alterniflora*, but an effect of temperature and drying rate on the CWC was not observed in *S. alterniflora*.

CHAPTER 5
CONCLUSIONS AND PROSPECTS FOR FUTURE WORK

Recalcitrance of *S. alterniflora*

Comparative proteomics between the recalcitrant *S. alterniflora* and orthodox *S. pectinata* seeds identified a number of proteins that were putatively associated with desiccation tolerance. More than 30 proteins that were detected in *S. pectinata* were missing or much less abundant in *S. alterniflora*. The association between these proteins and desiccation tolerance was further confirmed by the disappearance of these proteins when *S. pectinata* seeds germinated and the seedlings lost desiccation tolerance. Based on the literature, these proteins may be involved in multiple cellular pathways to confer protection against desiccation. First, LEAs can protect desiccation sensitive proteins from denaturation, prevent the denatured proteins from precipitation and assist re-folding of denatured proteins. Secondly, the presence of Cpn and sHsp contributes to the rescue pathways, and Cpn, sHsp and NAC could assist the folding and refolding of denatured proteins induced by desiccation and newly synthesized peptides during desiccation and rehydration. Thirdly, when the prevention and rescue pathways may be unable to confer sufficient protection to specific proteins under severe desiccation stress, those denatured proteins can be removed via degradation pathways of the ubiquitin-proteasome and autophagy. Ubiquitin, cystatin, GAPDH and ATG may be involved in the autophagy in *S. pectinata*. Fourthly, a burst of oxidative stress occurs once dry seeds are rehydrated, and ROS increase as seed germination proceeds. The presence of antioxidants, *e.g.* SOD, Prx, GPx and GRx, would scavenge those ROS and maintain redox homeostasis of the seed tissues.

The promoter regions of those 38 genes were subjected to searching programs of *cis*-regulatory elements by using the PLACE and PlantCARE databases. Screening for the cis-regulatory elements with high occurrence (≥18/38), 57 and 13 enriched cis-element motifs were

identified for each of the databases. A number of those enriched cis-regulatory motifs were associated with water stress, based on the literature, *e.g.* ABRE, ABRELATERD1, MYCATRD22, MYCATERD1. Those cis-regulatory motifs, such as ABREs, MYB and MYC, could bind to transcription factors to regulate the gene transcription under water stress. The future work would use comparative transcriptomics to examine if any desiccation stress-associated transcription factors are differentially expressed between *S. pectinata* and *S. alterniflora*.

Seed Dormancy Release by Cold Stratification

Comparative proteomics between dormant and non-dormant seeds was used to identify any dormancy-breaking associated proteins, which exhibit the same expression pattern in both *S. alterniflora* and *S. pectinata* during 3 months cold stratification. There are 9 spots that were more abundant in gels of dormant seeds, and 7 spots that were more abundant in gels of non-dormant *S. alterniflora* and *S. pectinata* seeds. LC-MS/MS sequencing identified proteins share homologies with storage proteins, serpin, phosphoglyerate mutase, enolase, heat shock protein-70, chaperonins, *etc*. The proteomic results suggest that degradation of storage proteins, increased cellular metabolism, and increased activities of molecular chaperones during cold stratification may be associated with dormancy release in *S. alterniflora* and *S. pectinata* seeds. Moist chilling may change the seed sensitivity in response to plant hormones (Schmitz *et al.,* 2002), thus inducing the expression of genes that are responsible for dormancy breaking. Unfortunately, no ABA- or GA- associated proteins were identified in my study. The reason may be due to the limited separation and resolution capabilities of 2-D electrophoresis. The future work should include use of more sensitive molecular techniques, *e.g.* microarray or

transcriptomics, to screen for transcription factors or genes of non-abundant proteins that are associated with dormancy breaking.

Effect of Drying Temperatures and Drying Rates

Temperature altered the drying rates of *S. alterniflora* seeds. Dehydrated *S. alterniflora* seeds needed 15, 20, and 50 days to reach the equilibrium moisture content at 24°C, 14°C, and 4°C, respectively. However, the drying temperature did not alter the critical water content at which *S. alterniflora* lost viability. Since enzymatic activity is usually temperature dependent, the experimental results imply that the recalcitrant seed death is not due to metabolic processes. However, the proteomic data in Chapter 2 suggest that recalcitrance of *S. alterniflora* is due to a lack of protective proteins, degradation of denatured proteins and antioxidant enzymes. The simplest model for this apparent conflict is that recalcitrant seed death occurs both during drying and subsequent rehydration. Seed desiccation tolerance may require physical protection during drying, which could be temperature-independent, while metabolic protection would be needed during rehydration. The resolution of this important issue will be a challenge for future research.

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APPENDIX A
SUPPORTING FIGURES AND TABLES

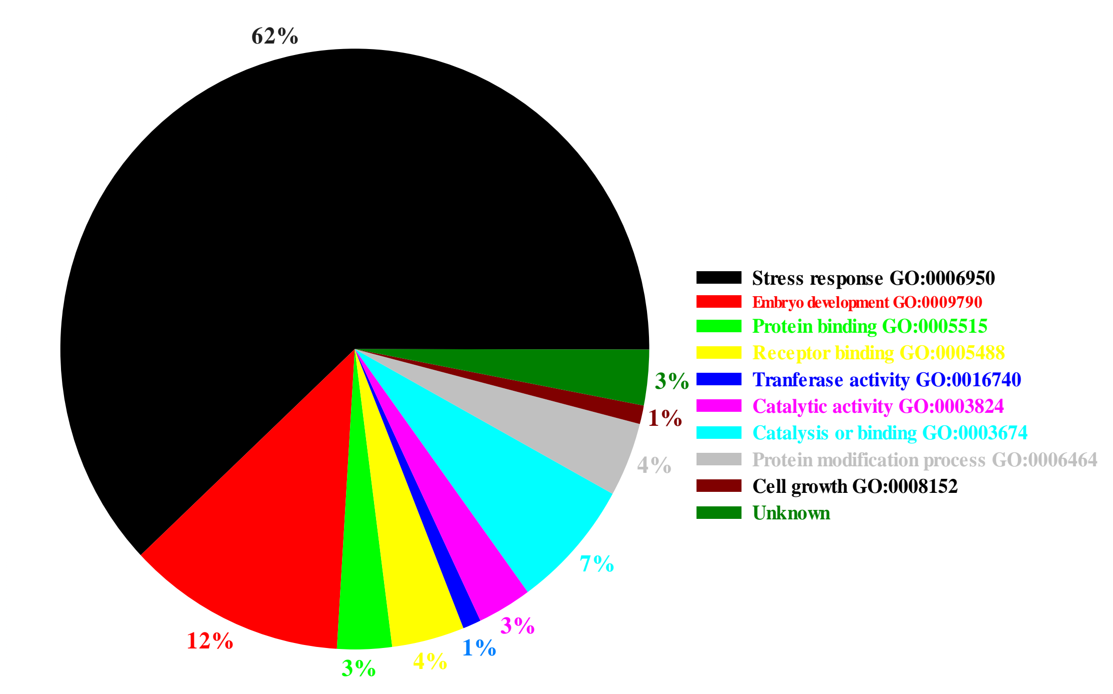


Figure S-1. Representation of Gene Ontology (GO) mapping of identified *Spartina pectinata* heat stable proteins shown in Table 2.1. GO term annotation tool is on Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/).

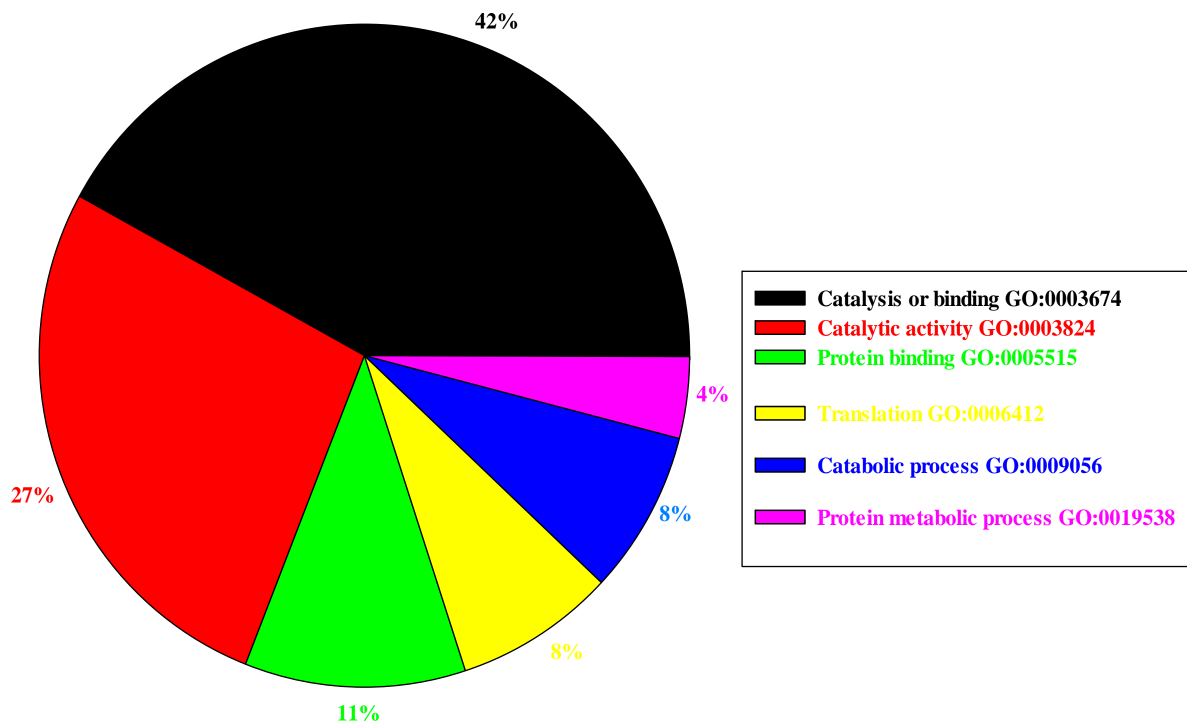


Figure S-2. Representation of Gene Ontology (GO) mapping of identified *Spartina pectinata* and *S. alterniflora* proteins shown in Tables 3.2 and 3.3. GO term annotation tool is on Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/).

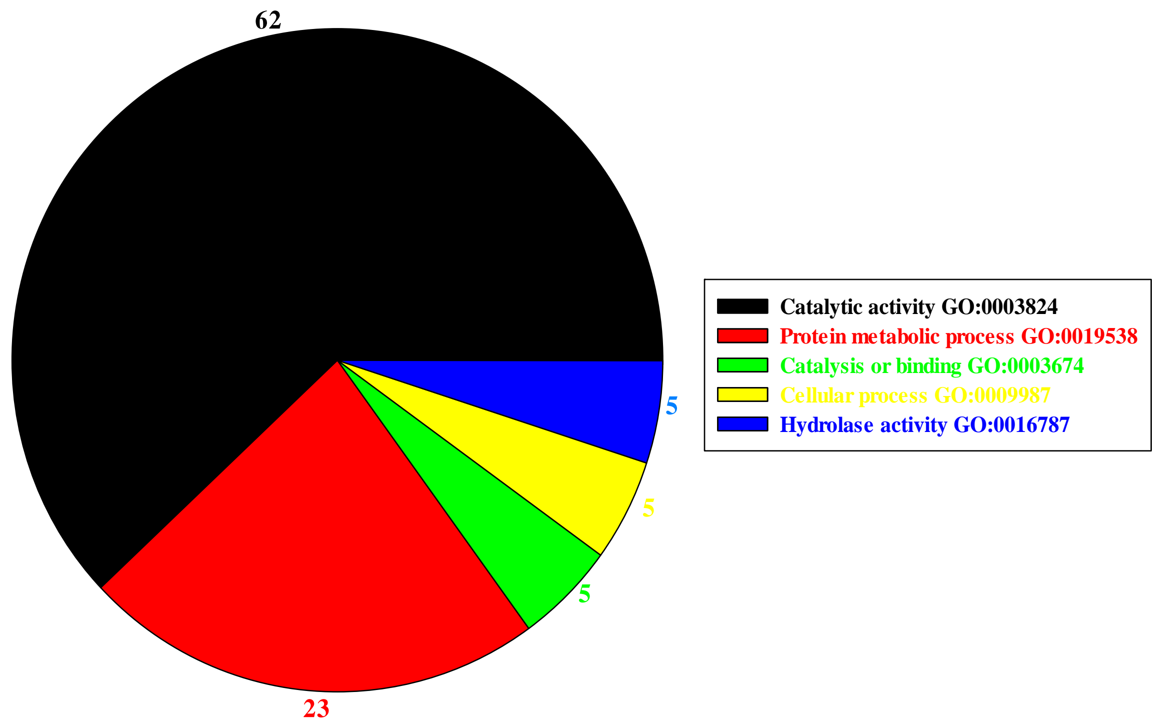
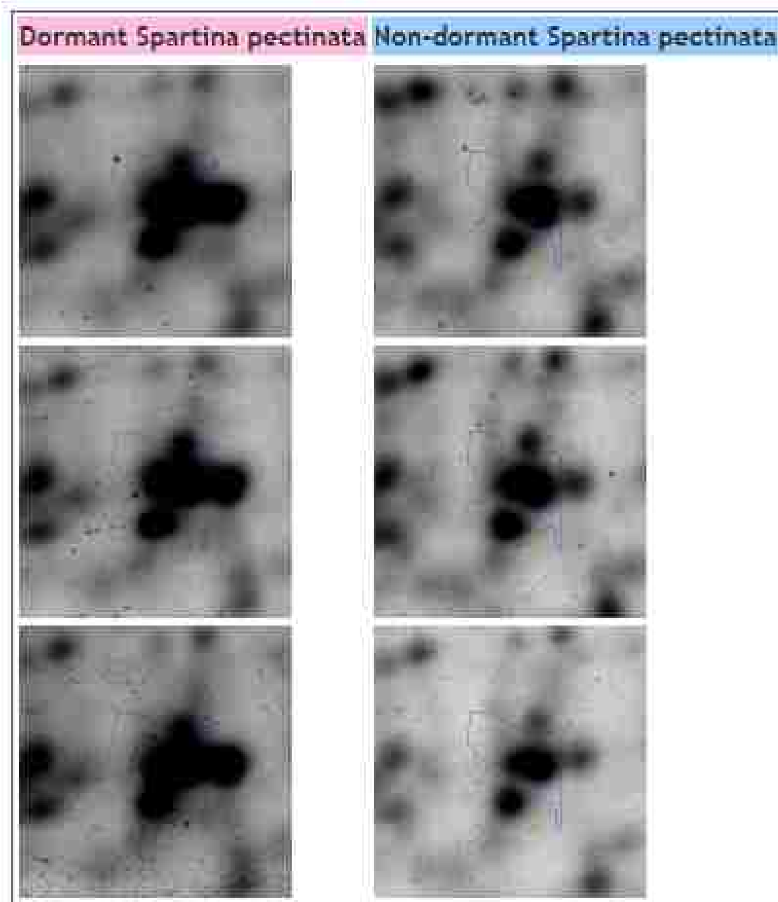


Figure S-3. Representation of Gene Ontology (GO) mapping of identified *Spartina pectinata* and *S. alterniflora* proteins shown in Table 3.4. GO term annotation tool is on Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/).

a



b

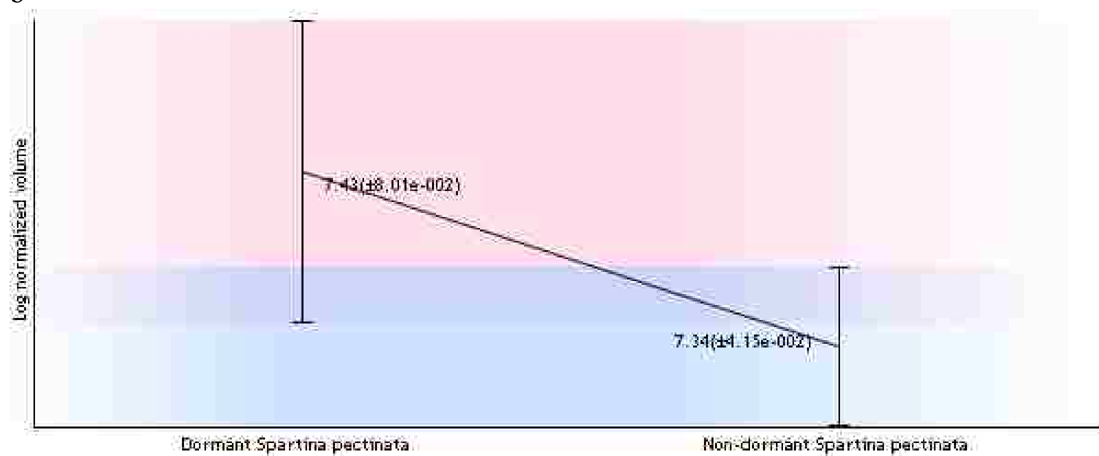
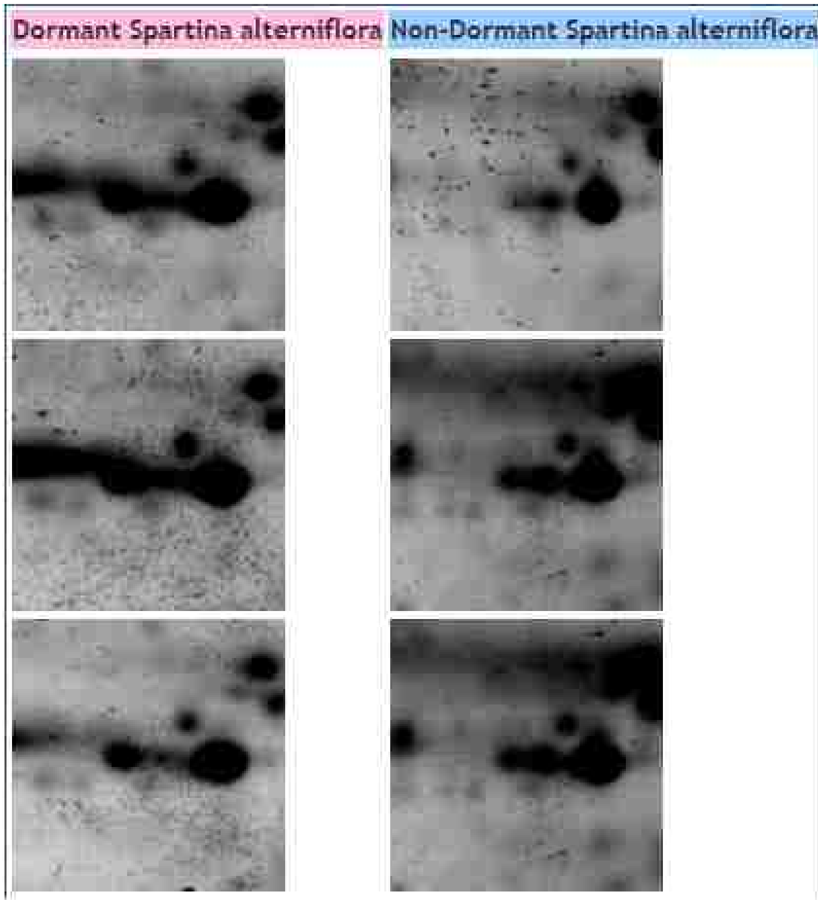


Figure S-4. Sub-section of protein gels showing the SP#19 from dormant and non-dormant *S. pectinata*. a, comparison of spots between dormant and non-dormant states from 3 independent biological replicates; b, statistical analysis of the logarithm values of spot average normalized volume. SP19: phosphoglycerate kinase and elongation factor Tu, fold=1.2.

a



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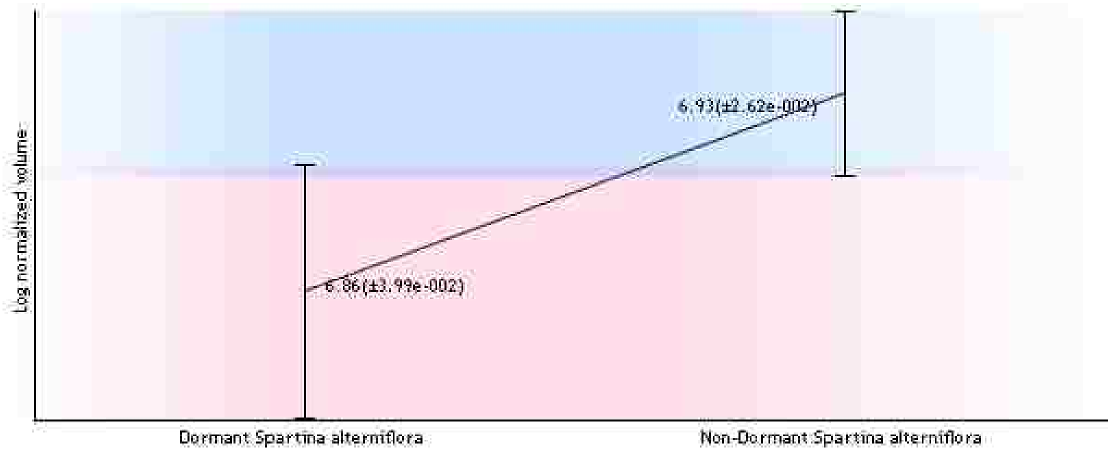


Figure S-5. Sub-section of protein gels showing the SA#50 from dormant and non-dormant *S. alterniflora*. a, comparison of spots between dormant and non-dormant states from 3 independent biological replicates; b, statistical analysis of the logarithm values of spot average normalized volume. SA50: enolase, fold=1.2.

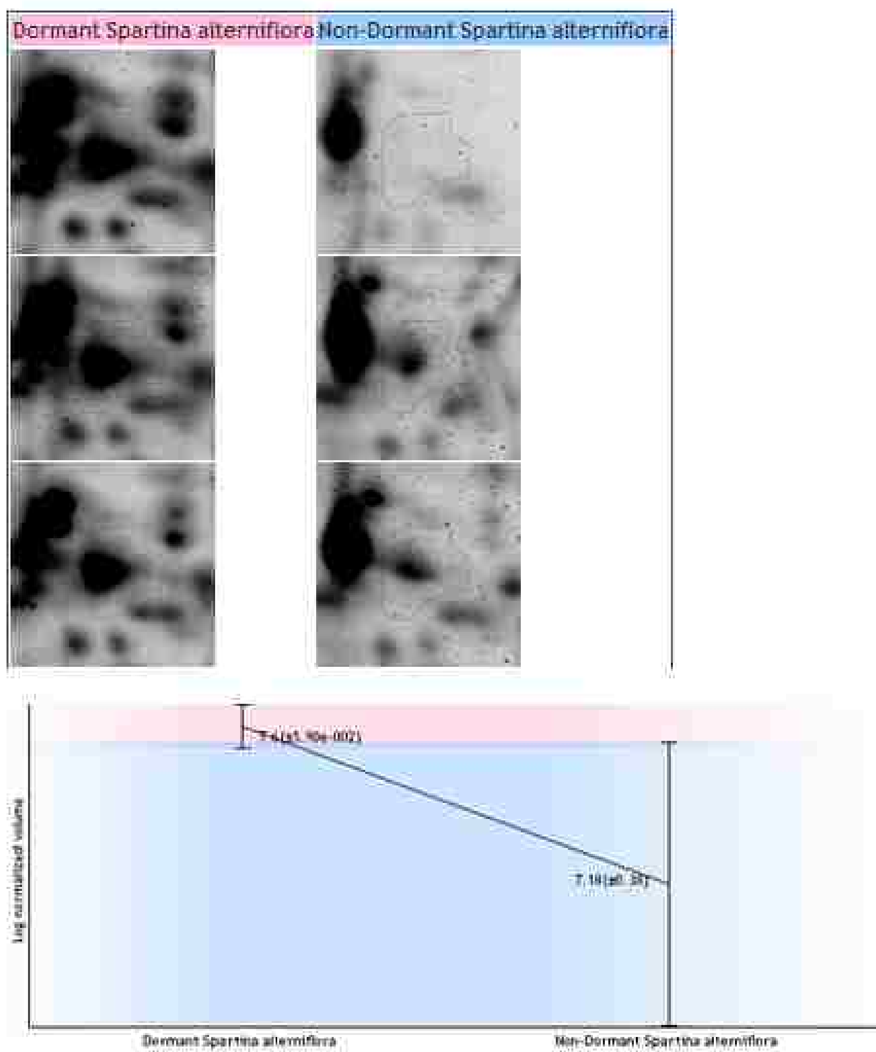


Figure S-6. Sub-section of protein gels showing the SA#20 from dormant and non-dormant *S. pectinata.* Large CV variation of the dormant *S. alterniflora* SA#20 caused by the difference of spot expression in biological replicates (Table S-4). SA#20: globulin-1.

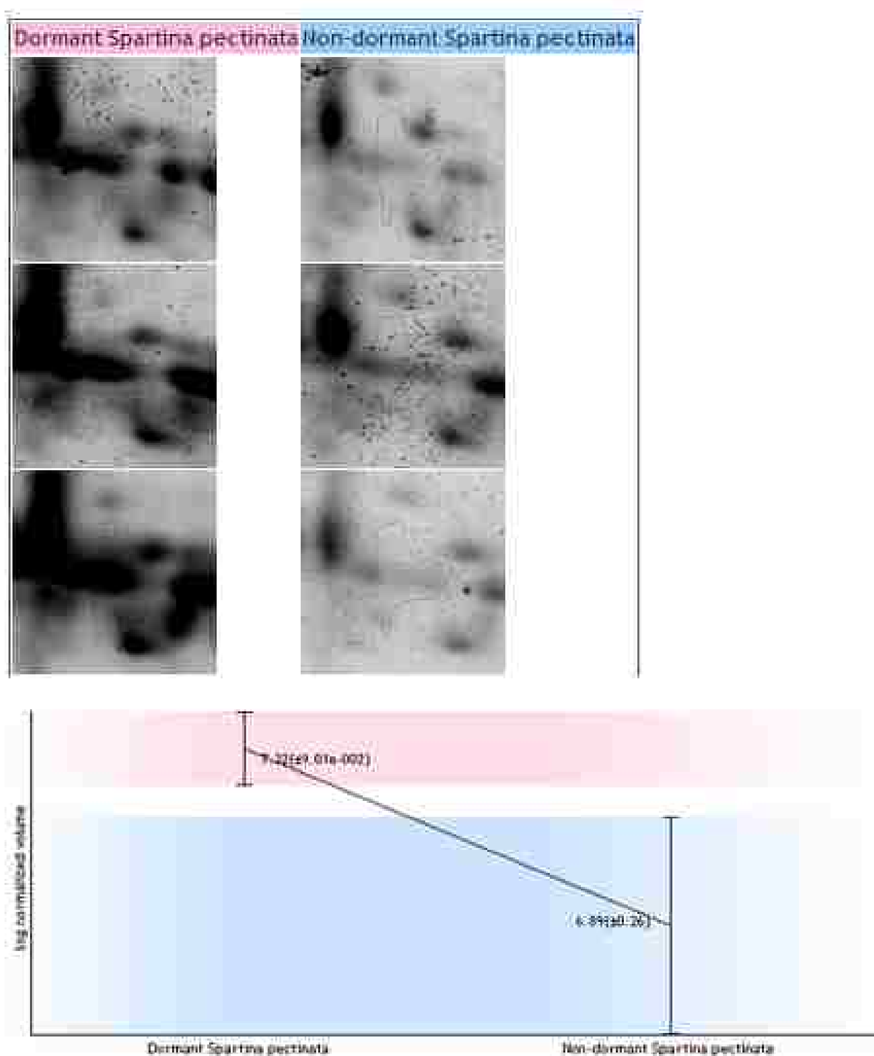
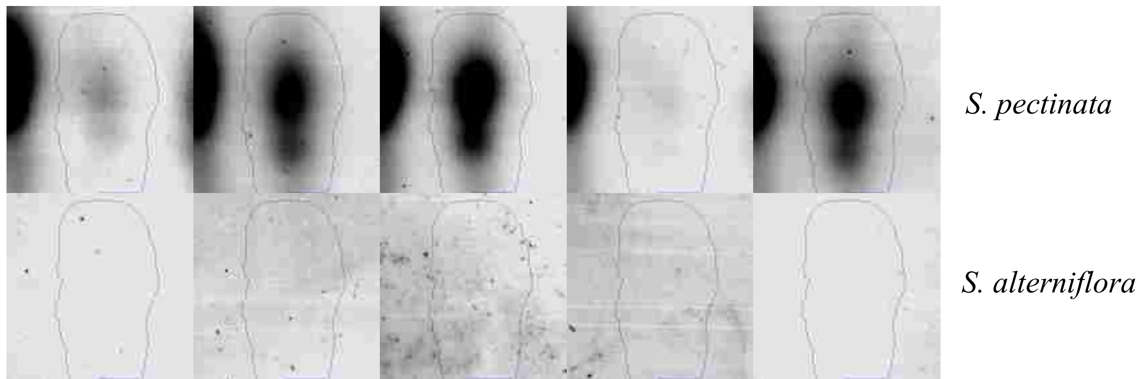


Figure S-7. Sub-section of protein gels showing the SP#2 from dormant and non-dormant *S. pectinata*. Large CV variation of the non-dormant *S. pectinata* SP#2 caused by background noise (Table S-4). SP#2: luminal-binding protein 2 & heat shock protein 70 kDa.



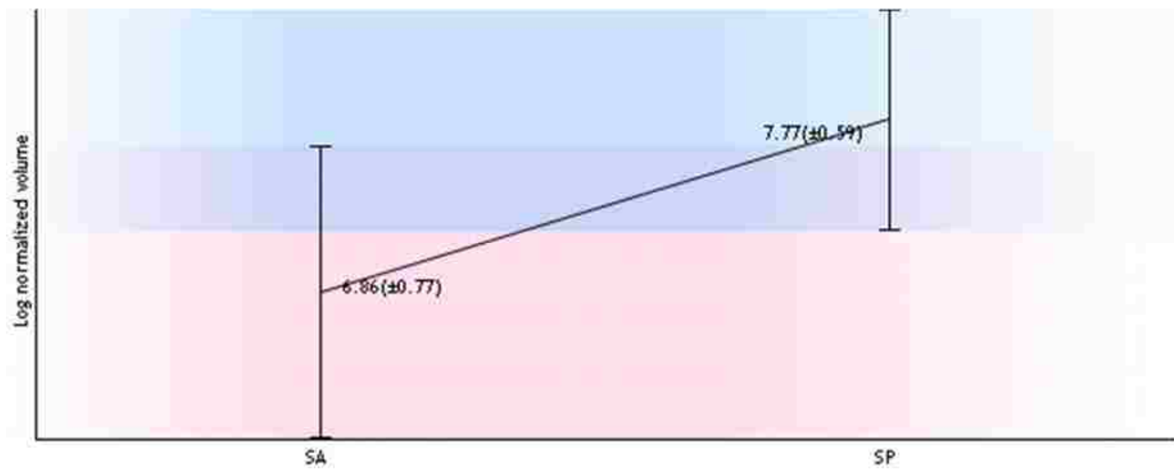
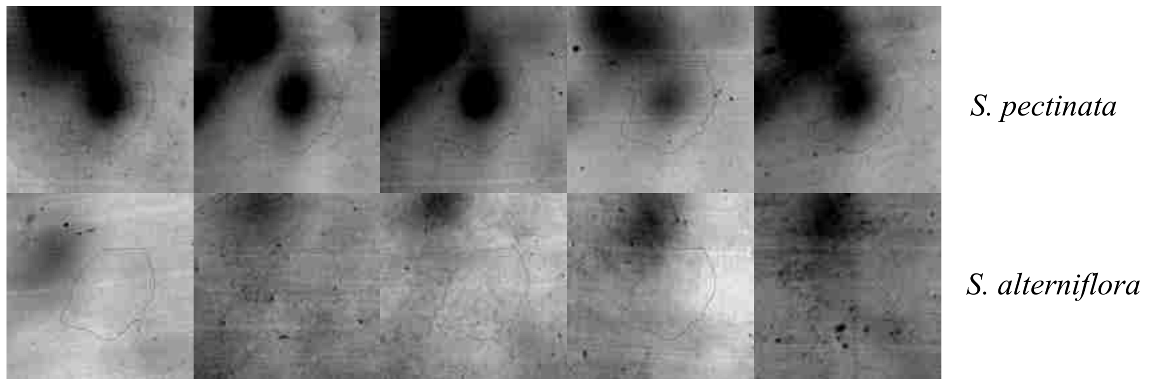


Figure S-8. Sub-section of protein gels showing the SP#8 from *S. pectinata.* Large CV variation of SP#8 caused by the difference of spot expression in biological replicates and SA#8 caused by background noise (Table S-9). SP#8: group-3 LEA.



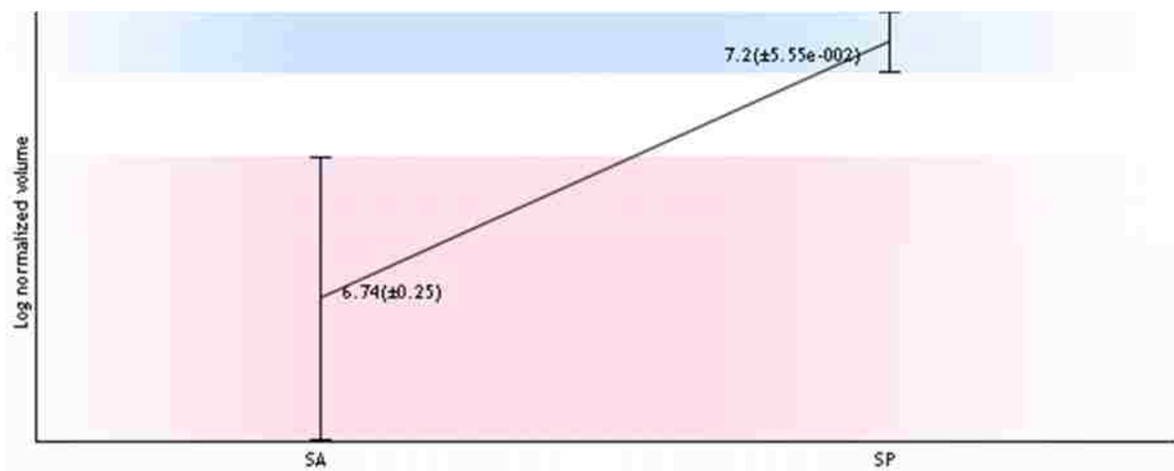
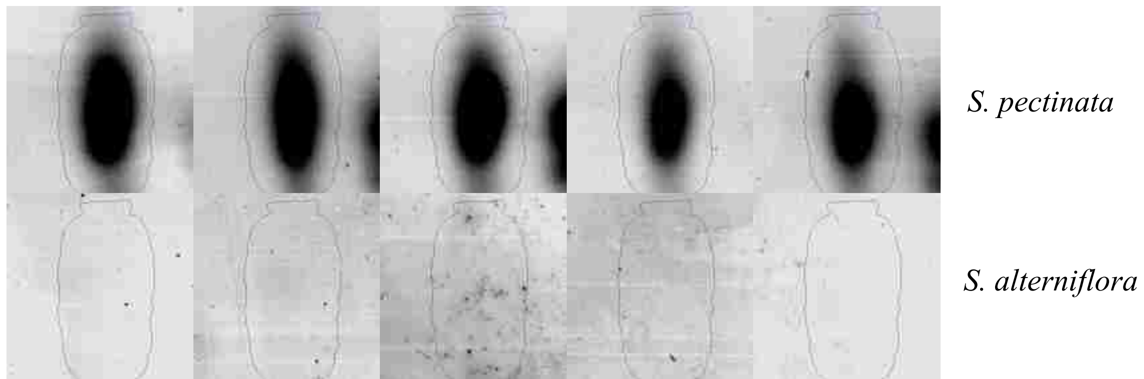


Figure S-9. Sub-section of protein gels showing the SP#38 from *S. pectinata.* Large CV variation of SA#38 caused by background noise (Table S-9). SP#38: LEA-14.



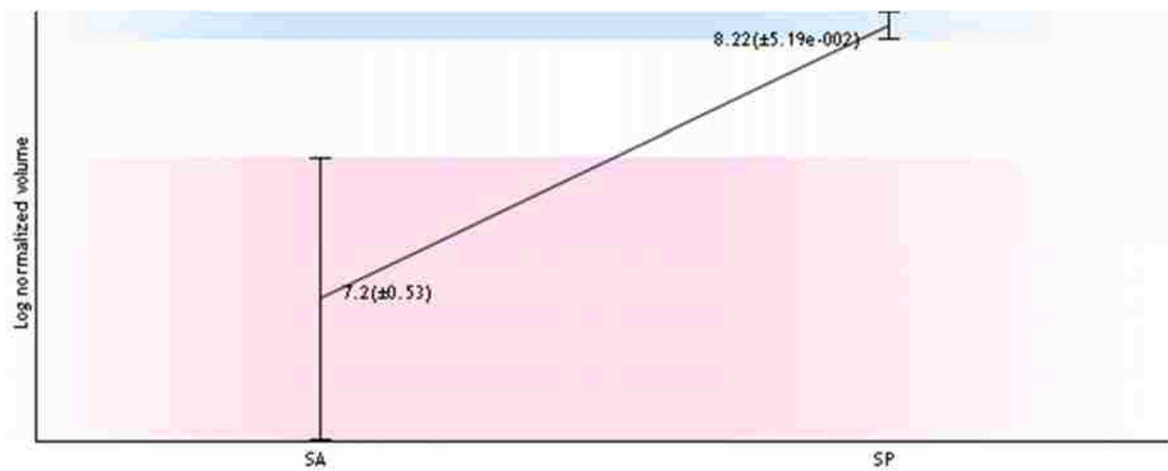
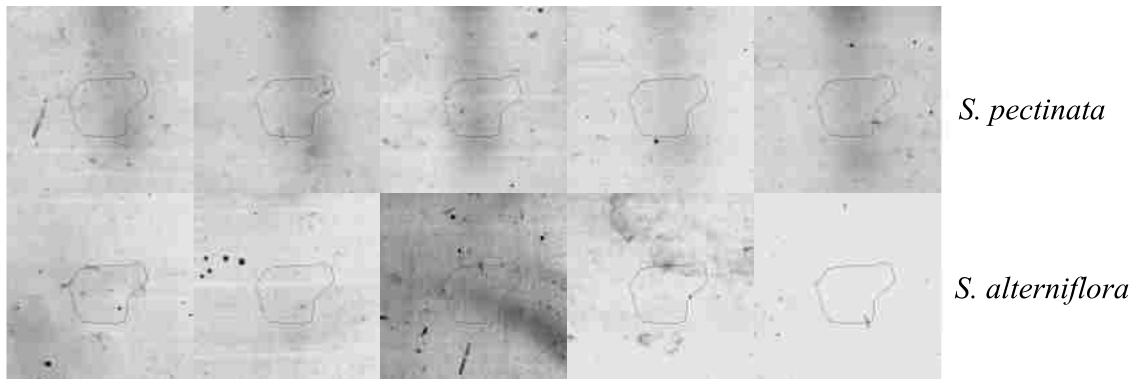


Figure S-10. Sub-section of protein gels showing the SP#5 from *S. pectinata.* Large CV variation of SA#5 caused by background noise (Table S-9). SP#5: fructose-bisphosphate aldolase.



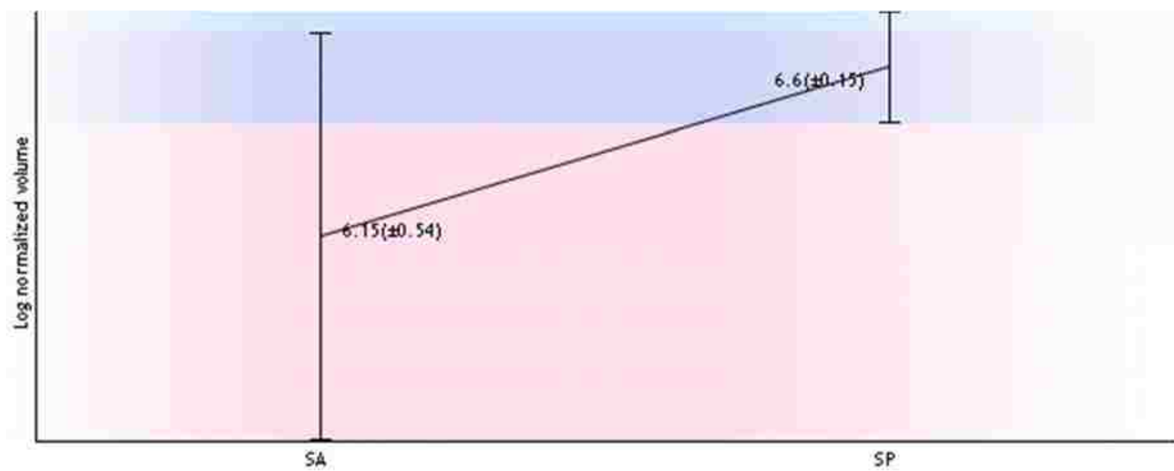
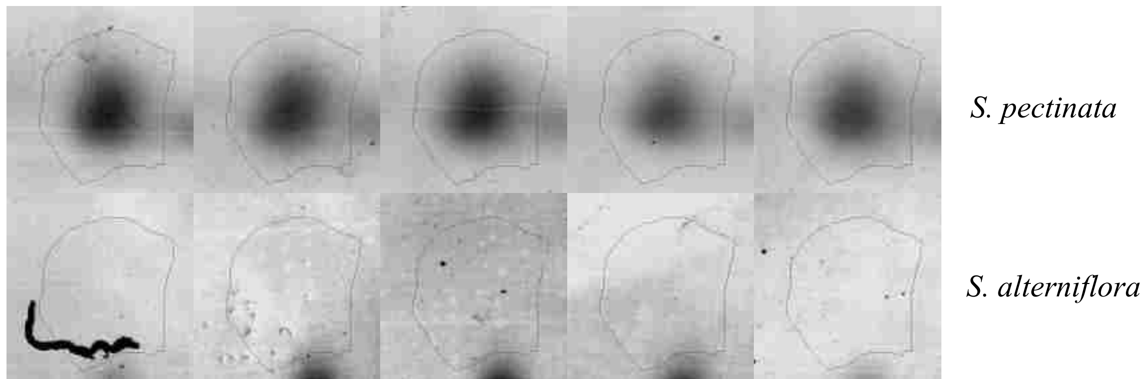


Figure S-11. Sub-section of protein gels showing the SP#61 from *S. pectinata.* Large CV variation of SA#61 caused by background noise (Table S-9). SP#61: fructose-bisphosphate aldolase.



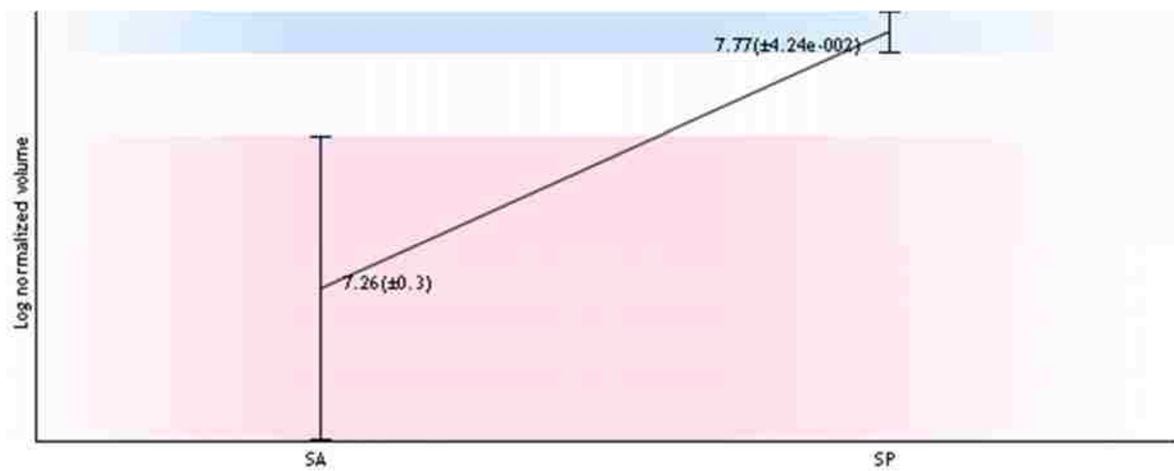
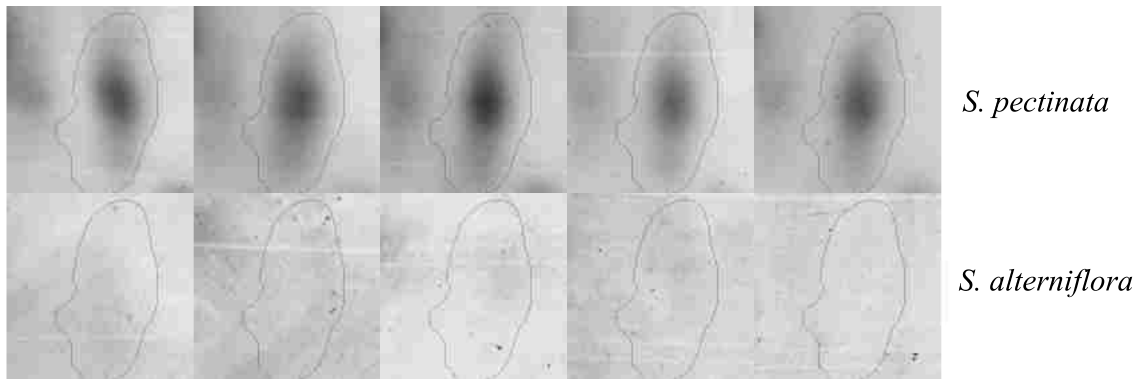


Figure S-12. Sub-section of protein gels showing the SP#30 from *S. pectinata.* Large CV variation of SA#30 caused by background noise (Table S-9). SP#30: abscisic stress ripening protein 2.



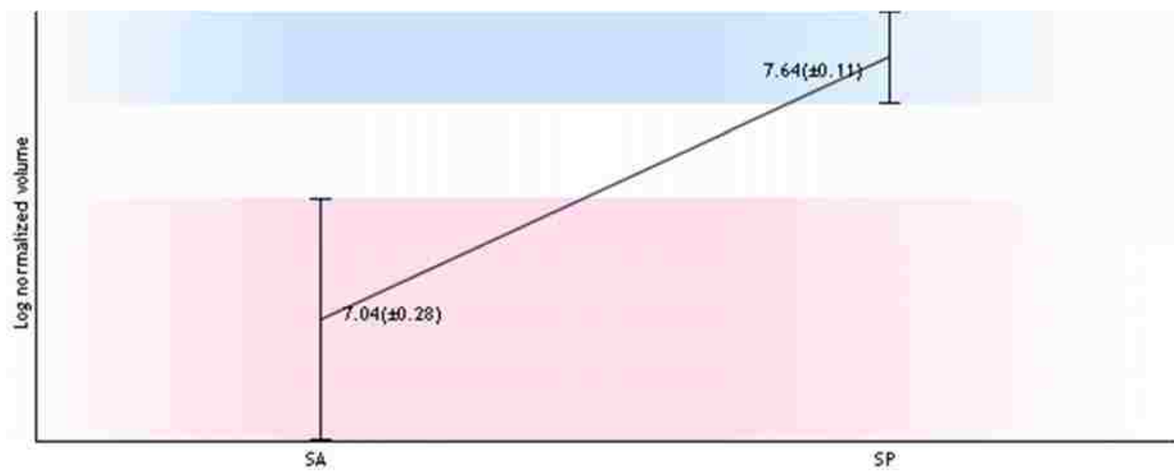
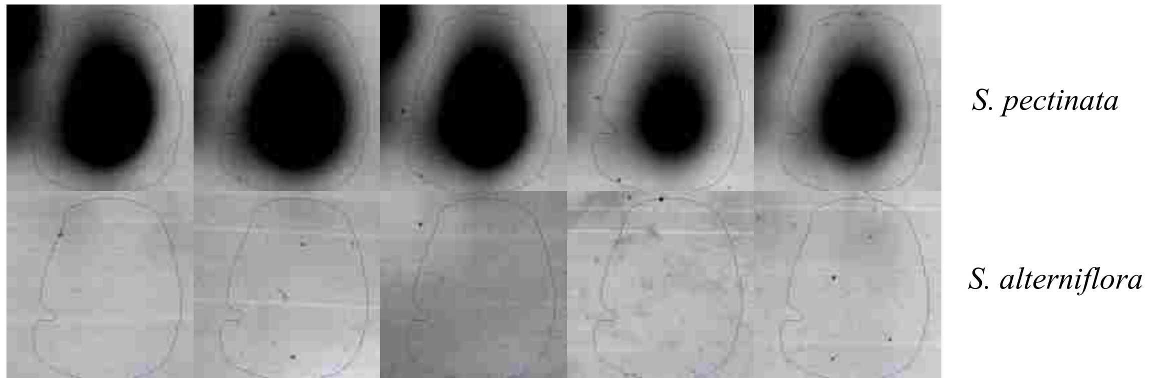


Figure S-13. Sub-section of protein gels showing the SP#21 from *S. pectinata.* Large CV variation of SA#21 caused by background noise (Table S-9). SP#21: translation inhibitor protein.



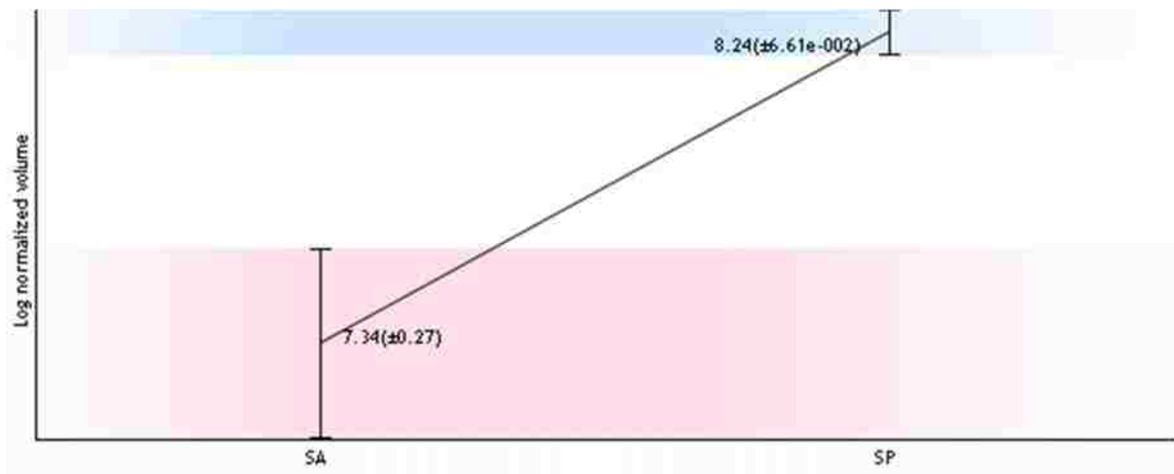


Figure S-14. Sub-section of protein gels showing the SP#6 from *S. pectinata.* Large CV variation of SA#6 caused by background noise (Table S-9). SP#6: CBS-domain Arabidopsis protein targeted to mitochondrion protein.

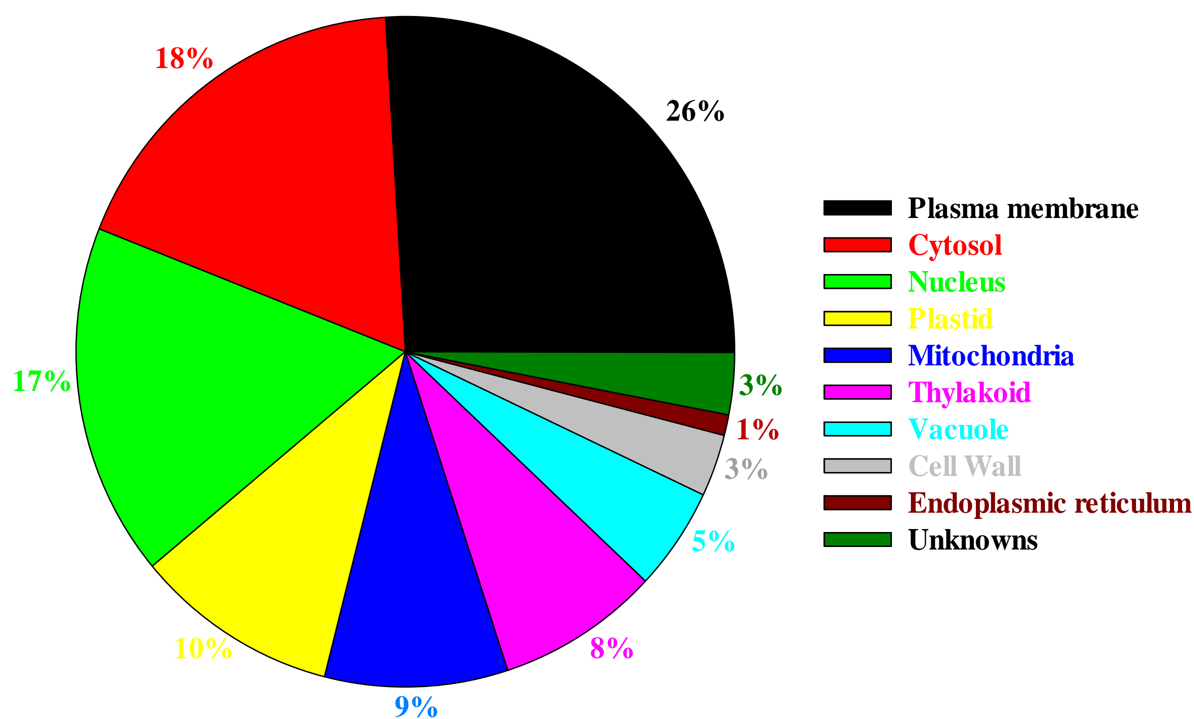


Figure S-15. Putative cellular localization of identified *S. pectinata* proteins of Table 2.1. Putative protein cellular localization was obtained from http://rice.plantbiology.msu.edu/. One protein may be categorized in several cellular locations.

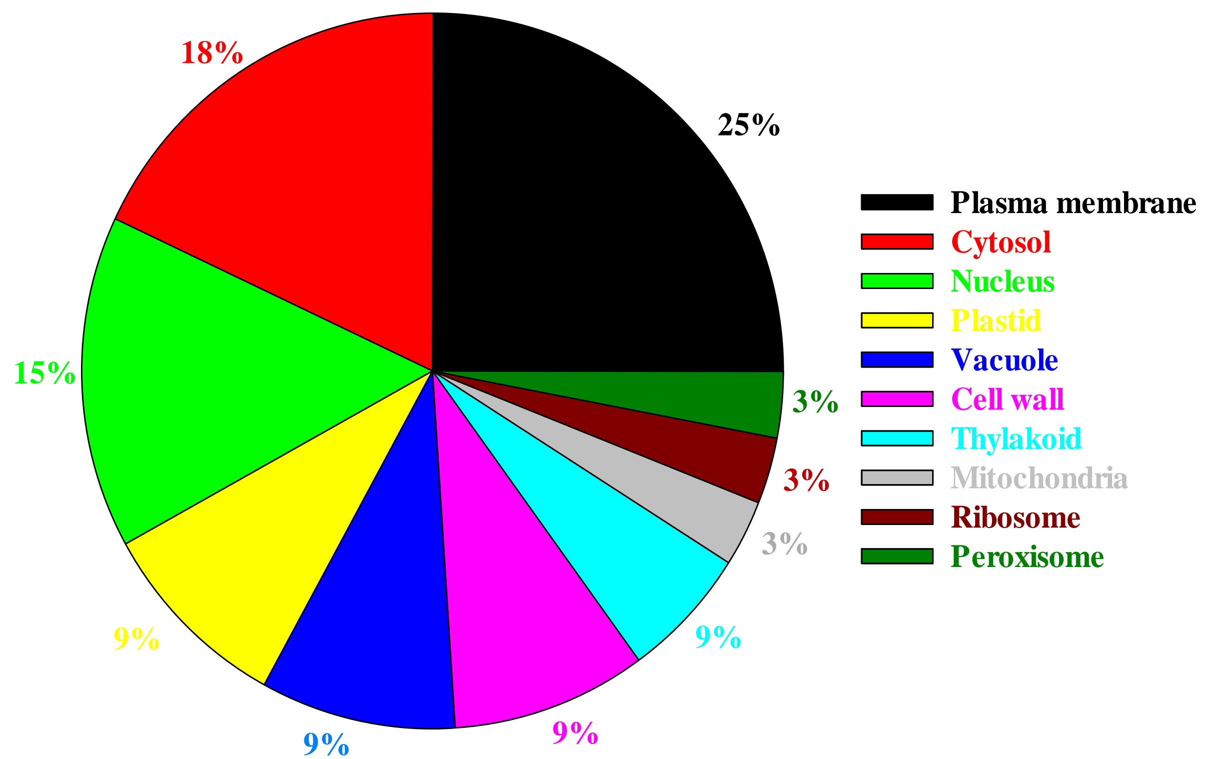


Figure S-16. Putative cellular localization of identified *S. pectinata* and *S. alterniflora* proteins of Tables 3.2 and 3.3. Putative protein cellular localization was obtained from http://rice.plantbiology.msu.edu/. One protein may be categorized in several cellular locations.

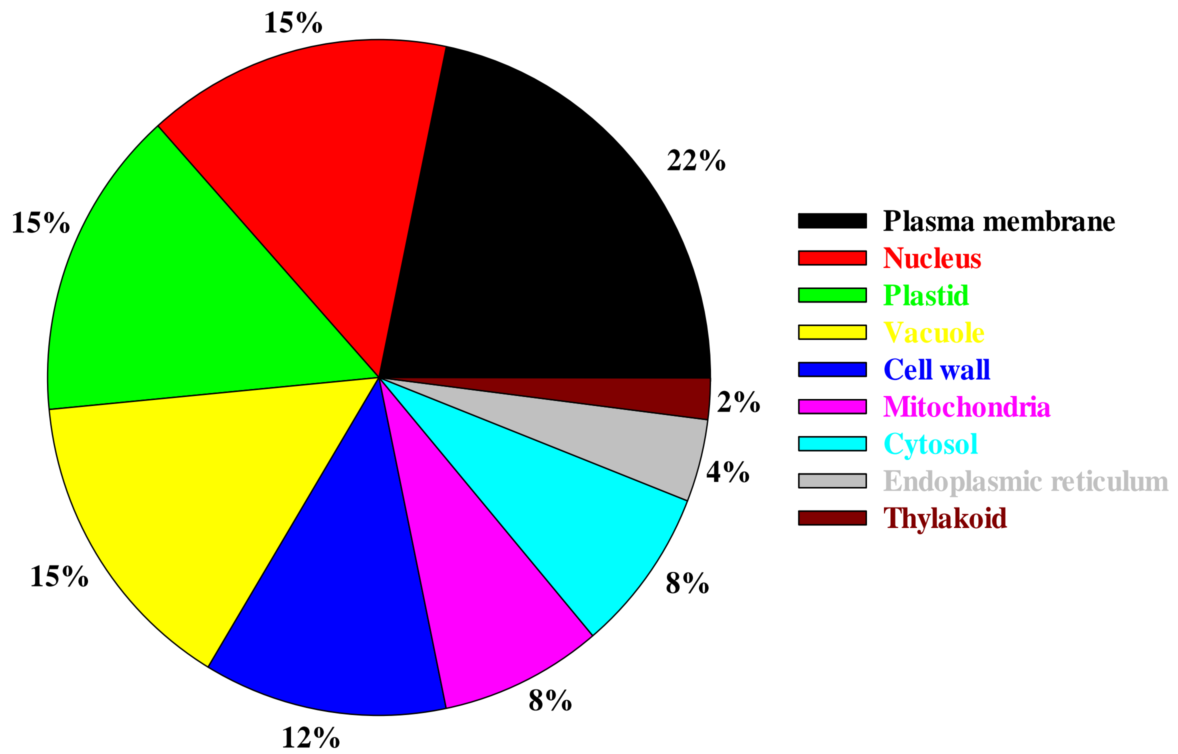


Figure S-17. Putative cellular localization of identified *S. pectinata* and *S. alterniflora* proteins of Table 3.4. Putative protein cellular localization was obtained from http://rice.plantbiology.msu.edu/. One protein may be categorized in several cellular locations.

Table S-1. List of identified *Spartina pectinata* protein spots (Fig. 2.1) belonging to different GO terms in Figure S-1.

GO Term	List of Protein Spots (Table 2.1)
Response to stress GO:0006950	3 (3), 4, 5, 6, 9, 10, 11, 14, 15, 16, 20, 23, 25, 26, 29, 33, 34, 35, 36, 38, 39, 40, 41, 42, 43, 47, 48, 52, 58, 59, 60, 61, 63, 65, 66, 68, 70, 72, 74, 75, 79, 80
Embryo development GO:0009790	1, 2, 8, 13, 24, 32, 37, 50
Protein binding GO:0005515	12, 57
Receptor binding GO:0005488	54, 71, 76
Catalytic activity GO:0003824	17, 18
Catalysis or binding GO:0003674	19, 44, 51, 53, 82
Protein modification process GO:0006464	31, 45, 83
Cell growth GO:0008152	21
Transferase activity GO:0016740	10
Unidentified	22, 30

Table S-2. List of identified *Spartina pectinata* and *Spartina alterniflora* protein spots (Tables 3.2 and 3.3) belonging to different GO terms in Figure S-2.

GO Term	List of Proteins (Table 2.1)
Catalysis or binding GO:0003674	SA#4 (globulin), SA#5 (globulin), SA#10 (globulin), SA#20 (globulin), SA#22 (globulin), SA#30 (legumin), SP#2 (globulin), SP#5 (globulin), SP#12 (globulin), SP#14 (globulin), SP#15 (globulin)
Catalytic activity GO:0003824	SA#5 (alcohol dehydrogenase), SA#29 (RUBISCO), SA#30 (cysteine synthase), SA#34 (RUBISCO), SP#2 (peroxidase), SP#4 (RUBISCO), SP#7 (cysteine synthase)
Protein binding GO:0005515	SA#29 (tubulin), SA#34 (tubulin), SP#4 (tubulin)
Translation GO:0006412	SA#21 (Elongation factor Tu), SP#19 (Elongation factor Tu)
Catabolic process GO:0009056	SA#21 (Phosphoglycerate kinase), SP#19 (Phosphoglycerate kinase)
Protein metabolic process GO:0019538	SP#8 (Serpin)

Table S-3. List of identified *Spartina pectinata* and *Spartina alterniflora* protein spots (Fig. 3.4) belonging to different GO terms in Figure S-3.

GO Term	**List of Proteins (Table 2.1)**
Catalysis or binding GO:0003674	SA#28 (lectin-domain protein)
Catalytic activity GO:0003824	SA#3 (enolase), SA#3 (ketol-acid reductoisomerase), SA#21 (phosphoglycerate mutase), SA#29 (phosphoglycerate mutase), SA#33 (phosphoglycerate mutase), SA#34 (mannosidase), SA#50 (enolase), SP#5 (enolase), SP#5 (ketol-acid reductoisomerase), SP#9 (disulfide-isomerase), SP#11 (enolase), SP#15 (phosphoglycerate mutase), SP#22 (Hexokinase)
Hydrolase activity GO:0016787	SP#21 (V-type ATPase)
Cellular process GO:0009987	SA#50 (F1-ATP synthase)
Protein metabolic process GO:0019538	SA#21 (heat shock protein), SP#2 (luminal-binding protein 2 precursor), SP#2 (heat shock protein), SP#3 (chaperonin), SP#9 (heat shock protein)

Table S-4. Logarithm value of average normalized volume, fold change and ANOVA p value of each spots shown in Tables 3.2 and 3.3. a: Logarithm value of normalized volume from three independent biological replicate gels. Error bar represents standard deviation. CV: coefficient of variance value of each 3 biological replicates. CV values are for the normalized spot volume values and not for the logarithm of these values. D: dormant; ND: Non-dormant. b: spot mis-alignment. c: large variation of spot expression.

Spot #	Dormant Seed Log[volume] [a]	Non-dormant Seed Log[volume] [a]	Fold D/ND	ANOVA P value	CV Value D	CV Value ND
SA22	7.39 ± 0.04	7.01 ± 0.2	2.4	0.005	7%	24%
SA4	6.95 ± 0.07	6.29 ± 0.24	4.4	0.001	9%	29%
SA5	7.17 ± 0.47	6.6 ± 0.16	3.9	0.001	14%	24%
SA10	6.7 ± 0.16	6.15 ± 0.24	3.5	0.005	21%	30%
SA20	7.6 ± 0.06	7.18 ± 0.38	2.4	0.03	8%	42% [c]
SA21	7.13 ± 0.06	6.97 ± 0.12	1.4	0.024	9%	15%
SA34	7.36	7.02 ± 0.03	2.2	$8.3e^{-6}$	0%	5%
SA30	7.14 ± 0.04	7.02 ± 0.1	1.3	0.023	5%	14%
SA29	6.97 ± 0.15	6.63 ± 0.06	2.2	0.003	20%	8%
SP14	7.55 ± 0.07	7.31 ± 0.11	1.7	0.005	9%	14%
SP12	7.02 ± 0.09	6.75 ± 0.16	1.9	0.011	12%	22%
SP5	7.25 ± 0.13	6.91 ± 0.18	2.2	0.001	16%	25%
SP2	7.32 ± 0.09	6.89 ± 0.26	2.6	0.01	12%	36%
SP15	6.77 ± 0.09	6.33 ± 0.61	2.0	0.016	13%	13%
SP19	7.43 ± 0.08	7.34 ± 0.04	1.2	0.038	11%	5%
SP4	6.77 ± 0.03	6.56 ± 0.15	1.6	0.014	5%	19%
SP7	7.51 ± 0.01	7.19 ± 0.08	2.1	$3.1e^{-4}$	2%	10%
SP8	7.17 ± 0.03	6.99 ± 0.02	1.5	$1.0e^{-4}$	3%	3%

Table S-5. Logarithm value of average normalized volume, fold change and ANOVA p value of each spots shown in Table 3.4. a: Logarithm value of normalized volume from three independent biological replicate gels. Error bar represents standard deviation. CV: coefficient of variance value of each 3 biological replicates. CV values are for the normalized spot volume values and not for the logarithm of these values. D: dormant; ND: Non-dormant. b: background noise.

Spot #	Dormant Seed Log[volume] [a]	Non-dormant Seed Log[volume] [a]	Fold ND/D	ANOVA P value	CV Value D	CV Value ND
SA3	7.02 ± 0.04	7.25 ± 0.1	1.7	0.003	5%	14%
SA29	6.78 ± 0.05	7.04	1.8	$8.3e^{-5}$	6%	1%
SA21	6.45	6.74 ± 0.18	2.0	0.009	1%	23%
SA28	7.52 ± 0.06	7.77 ± 0.04	1.8	$5.1e^{-4}$	9%	5%
SA34	6.54 ± 0.05	6.71 ± 0.02	1.5	$7.9e^{-4}$	12%	0%
SA50	6.86 ± 0.04	6.93 ± 0.03	1.2	0.017	5%	3%
SA33	7.33 ± 0.07	7.47	1.4	0.003	9%	0%
SP5	6.07 ± 0.18	6.6 ± 0.16	3.4	0.003	22%	22%
SP15	6.97 ± 0.06	7.28 ± 0.07	2.1	$5.3e^{-4}$	8%	10%
SP2	6.03 ± 0.45	6.69 ± 0.21	4.2	0.016	56% [b]	27%
SP9	6.96 ± 0.08	7.15 ± 0.07	1.5	0.007	11%	10%
SP3	6.83 ± 0.07	7.05 ± 0.21	1.7	0.044	9%	27%
SP11	6.65 ± 0.05	6.84 ± 0.12	1.5	0.014	7%	16%
SP22	6.99 ± 0.04	7.22 ± 0.05	1.7	$2.9e^{-4}$	5%	6%

Table S-6. Identified peptides from Mascot MS/MS or *de novo* sequencing corresponding to Table 2.1. i: I or L. q: Q or K. m: Mascot MS/MS. d: *de novo* sequencing.

Spot	Protein Homology	Peptides	Mascot Score
		LEA proteins	
1 [d]	Group-3 late embryogenesis abundant protein (*Sorghum bicolor*)	DETGNViqqATEqV DAVMNTEGMSGDAG	-
2 [d]	Group-3 LEA (*Ampelocalamus calcareus*)	ASETGSYiANK DAVMNTiGMSGD	-
3 [d]	Dehydrin (*Sporobolus stapfianus*)	qGqYGHGTTG TGGiLHR	-
8 [d]	Group-3 LEA (*Zea mays*)	iqqATEqVR	-
13 [d]	LEA-1 (*Sorghum bicolor*)	GqPGiiGAiGNVTGAiK	-
24 [d]	Group-3 LEA (*Pogonatherum paniceum*)	DGVNiqqATEqV MNTiGNCMNTiGMSGD	-
32 [d]	LEA-1 (*Sorghum bicolor*)	GPGiiGAiGNVTGAiK	-
37 [d]	LEA-1 (*Sorghum bicolor*)	GPGiiGAiGNVTGAiK	-
38 [d]	LEA-14 (*Zea mays*)	VPYDFiVSiAK	-
47 [d]	Dehydrin (*Zea mays*)	TGGiiHR	-
50 [d]	LEA-1 (*Sorghum bicolor*)	PGiiGAiGNVTGAiK	-
72 [d]	LEA-14 (*Zea mays*)	VPYDFiVSiAK	-
		Chaperones	
23 [m]	Chaperonin (*Zea mays*)	DGTAYIV DDIIGILE TPGGLILTET VVAVGPGPLDEEGKR PSTAQTKPQGGEVVAVGAG	358
39 [m]	Type-II heat shock protein (*Brachypodium distachyon*)	KFVLPENADMDK FVLPENADMDK VLVISGERR	208
42 [m]	Putative nascent polypeptide associated complex alpha chain (*Oryza sativa*)	LFVISKPDVFK LFVISKPDVFK LSSQLQTQAAEQFK	360
43 [d]	Small heat shock protein (*Hordeum vulgare*)	KVEVEDGNiiqiSGER AAMEDGViTVTVPK	-
48 [m]	Chaperonin (*Zea mays*)	GGLILTETTK IIGILE TPGGLILTETTK DIIGILETDDVK	299
63 [m]	Chaperonin (*Oryza sativa*)	TPGGLLLTETT	88
75 [m]	Type-I heat shock protein (*Oryza sativa*)	VEDGNILQISGER	100
80 [m]	Nascent polypeptide associated complex alpha chain (*Brachypodium distachyon*)	ASDTYVIFGEA LFVISKPDVFK	176
		Metabolism	
5 [m]	Fructose-bisphosphate aldolase (*Zea mays*)	LAADESTGTIG	96
25 [m]	Glyceraldehyde-3-phosphate dehydrogenase (*Magnolia liliiflora*)	IGINGFGR PVTVFG	71

Table S-6. Continued from the previous page.

Spot	Protein Homology	Peptides	Mascot Score
		Metabolism	
35 [m]	Glyceraldehyde-3-phosphate dehydrogenase (*Aeluropus lagopoides*)	TGMSFR VPTVDVSVVDLTVR FGEKPVTVFGVR VSWYDNEWGYSNR	246
40 [m]	Glyceraldehyde-3-phosphate dehydrogenase (*Antirrhinum majus*)	IALNDNFVK SFNIIPSSTGAAK	97
41 [m]	Glyceraldehyde-3-phosphate dehydrogenase (*Aeluropus lagopoides*)	DAPMFVVGVNEDK AASFNIIPSSTGAAK VPTVDVSVVDLTVR LVSWYDNEWGYSNR GIMGYTDEDLVSTDFVGDSR VIHDNFGIVEGLMTTVHSITATQK	624
59 [m]	Glyceraldehyde-3-phosphate dehydrogenase (*Brachypodium distachyo*)	KVVISAPSK AGIALNDNFVK VPTVDVSVVDLTVR LVSWYDNEWGYSNR	364
60 [m]	Glyceraldehyde-3-phosphate dehydrogenase (*Hordeum vulgare*)	PMFVVGVNEDK VPTVDVSVVDLTVR SWYDNEWGYSNR GYTDEDLVSTDFVGDSR EGLMTTVHSITATQK VESTGVFTDKEK	524
61 [m]	Fructose-bisphosphate aldolase (*Zea mays*)	ILAADESTGTIG SSINVENVEE	132
74 [m]	Glyceraldehyde-3-phosphate dehydrogenase (*Aeluropus lagopoides*)	PMFVVGVNEDK SFNIIPSSTGAAK VPTVDVSVVDLTVR LVSWYDNEWGYSNR YTDEDLVSTDFVGDSR EGLMTTVHSITATQK	562
79 [m]	Fructose-bisphosphate aldolase (*Zea mays*)	ILAADESTGTIG LSSINVENVEE LAIDLNAQGLAR	266
		Antioxidants	
10 [m]	Glutathione transferase (*Spartina alterniflora*)	VLDVYEAHLAR IPALQDGDDVLYESR	168
10 [m]	Mitochondrial Mn superoxide dismutase (*Dactylis glomerata*)	EQLDAAVSK ASAVVQLQGAIK	167
16 [m]	Superoxide dismutase [Cu/Zn] (*Aeluropus lagopoides*)	AVLGSNEG GAPEDEIR VHADPDDLGK	180
17 [d]	Glutaredoxin (*Sorghum bicolor*)	PiiTEAGAiAGATSK AiEiDVENDSGDiqDAiK	-
18 [m]	Glutaredoxin (*Eragrostis curvula*)	PLLTEAGAIAGATSK	124

Table S-6. Continued from the previous page.

Spot	Protein Homology	Peptides	Mascot Score
Antioxidants			
26 [d]	Glutathione peroxidase (*Helianthus annuus*)	MFPKGFqTTDiPSNK KiSiqYPASTGR	-
29 [d]	1- Cys Peroxiredoxin (*Triticum aestivum*)	TYPiiADPDR	-
36 [m]	Superoxide dismutase [Cu-Zn] (*Oryza sativa*)	QIPLSGPNSVVGR ELSLSTGNAGGR VVHELEDDLGK VANAEGVAEATIVDK GGHELSLSTGNAGGR	406
58 [m]	Peroxiredoxin-2 (*Oryza sativa*)	YALLADDGVVK LPDATLSYFDPADGELK	155
65 [m]	Superoxide dismutase [Cu-Zn] (*Oryza sativa*)	QIPLSGPNSVVGR VVHELEDDLGK ELSLSTGNAGGR	204
66 [m]	2-Cys Peroxiredoxin (*Oryza sativa*)	FALLADNL EGGQFTISGAEEILK	125
68 [d]	1-Cys Peroxiredoxin (*Triticum aestivum*)	VPNiEiDSTHGR	-
70 [m]	Superoxide dismutase [Cu-Zn] (*Oryza sativa*)	QIPLSGPNSVVGR VVHELEDDLGK VANAEGVAEATIVDK GGHELSLSTGNAGGR	298
DNA or RNA binding protein			
15 [m]	Glycine-rich RNA-binding protein (*Spartina alterniflora*)	FSGFGEVTEARV GFVSFTNSEDAK VGGLAWATDDQSLK	284
52 [m]	Transcription factor BTF3 (*Sorghum bicolor*)	VVIQFQNP GVNTIPGIEEVNIFK ASIPANTWVVSGVPQTK	269
Cellular recycling			
19 [d]	Autophagy-related protein-13 (*Arabidopsis thaliana*)	EESMiiNiPPiSN	-
31 [m]	Tetra-ubiquitin (*Saccharum* hybrid cultivar H32-8560)	LSDYNIQK DKEGIPPDQQR TLEVESSDTIDNVK NIQKESTLHLVLR	321
45 [m]	Ubiquitin (*Solanum lycopersicum*)	TLADYNIQK ESTLHLVLR TLEVESSDTIDNVK	275
83 [m]	Ubiquitin precursor (*Musa acuminate*)	TLADYNIQK ESTLHLVLR TITLEVESSDTIDNVK	211
Protease inhibitor			
3 [m]	Putative cystatin (*Zea mays*)	FDILMK ATAFTNADLG SLFPYELLEILR	157

Table S-6. Continued from the previous page.

Spot	Protein Homology	Peptides	Mascot Score
Protease inhibitor			
4 [m]	Putative cystatin (*Zea mays*)	FDILMK FTNADLGAK	70
9 [m]	Putative cystatin (*Zea mays*)	FDILMK ATAFTNADLGAK NSLFPYELLEILR	151
20 [m]	Cystatin (*Chloris virgate*)	NSLFPYELLEIIR AGTLHHLTLEAIEAGTKK	191
22 [d]	Trypsin inhibitor (*Triticum monococcum*)	AATiVAPAEGCNiqTi	-
Stress related proteins			
11 [m]	Stress-responsive protein (*Oryza sativa*)	FANEFLPALEK IIDYKPTAVN	123
14 [d]	Stress responsive protein (*Oryza sativa*)	FANEFiPAiEK	-
30 [m]	Abscisic stress ripening protein 2 (*Oryza sativa*)	GALAAGAFALYEK GAGGYAFHEHHEK	164
54 [m]	Truncated copper binding protein (CutA) (*Oryza sativa*)	SLLDALTEHVK PEVIALPIK	133
Protein Synthesis Inhibitor			
21 [m]	Translation-inhibitor protein (*Gentiana triflora*)	PAALGPYSQAIK YQVAALPLNA	155
Storage Proteins			
44 [d]	Vicilin (*Eleusine coracana*)	VVMiiSPVSTPGR VAViEAAPR	-
51 [d]	Globulin-like protein (*Oryza sativa*)	VVMiiSPVSTPGR VAViEAAPR	-
53 [d]	Globulin-like protein (*Oryza sativa*)	VVMiiSPVSTPGR TGEEWEqVFENqR	-
73 [d]	Globulin-like protein (*Oryza sativa*)	VAViEAAPR	-
82 [d]	Globulin-like protein (*Oryza sativa*)	VAViEAAPR	-
Other proteins			
6 [d]	CBS-domain (Cystathionine Beta Synthase) Arabidopsis protein targeted to mitochondrion protein (*Saccharum officinarum*)	GFETATVSDViK	-
12 [d]	Hypothetical protein (*Selaginella moellendorffii*)	VLEiiEEDDNGVR	-
33 [m]	SOUL heme-binding domain containing protein (*Triticum aestivum*)	VTMQFLLP RYNPPWTLPPLR	124
34 [m]	Oxygen-evolving enhancer protein 1 (*Zea mays*)	VPFLFTVK PESFGGPFLVPSYR	115
71 [d]	Probable calmodulin, calcium-binding protein (*Zea mays*)	KiTAPFDFPR	-
76 [m]	FKBP FK506 binding protein-2 (*Oryza sativa*)	DVTELQIGVKH EFELGTGQVIKG	171

Table S-7. Identified peptides from Mascot MS/MS corresponding to Tables 3.3 and 3.4.

Spot	Protein Homology	Peptides
Spots more abundant in dormant seeds		
SA #21 SP #19	Elongation factor Tu (*Oryza sativa*)	VGDTVDIVGIR KYDEIDAAPEER TINTATVEYETETR TAALTMVLASVGGSAPK LPFLLAVEDVFSITGR LLQLVELEVR
SA #21 SP #19	Phosphoglycerate kinase (*Zea mays*)	IVVFLN GDTVDIVGIR YDEIDAAPEER TINTATVEYETETR TAALTMVLASVGGSAPK LPFLLAVEDVFSITGR
SA #34 SP #4	Tubulin (*Vitis vinifera*)	IDHKFDLMYAK LVSQVISSLTASLR FVDLEPTVIDEVR SSYAPVISAEK GGGDDAFNTFFSETGAGK GMEEGEFSEAR VDVTEFQTNLVPYPR
SA #34 SP #4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*Crypsis schoenoides*)	AGVGFQAGVK DTDILAAFR EMTLGFVDLLR TFDFKPVDTI ENVNSQPFMR
SA #30 SP #7	Cysteine synthase (*Oryza sativa*)	YLSSVLFQSI IGYSMITDAEE IEPTSGNTGIGLAFMAAAK
SA #30	Legumin (*Hordeum vulgare*)	EVGLGADLVR IFFAPN LQGKGTCGIVLPEATK SPEVLEASFNTTPEMEK
SA #29	Tubulin (*Zea mays*)	HKFDLMYAK LVSQVISSLTASLR VFVDLEPTVIDE LSSYAPVISAEK GGGDDAFNTFFSETGAGK PEQLISGKEDAANNFAR TNSAFEPSSMMAK
SA #29	Ribulose-1,5-bisphosphate carboxylase/oxygenase (*Peridictyon sanctum*)	DILAAFR EMTLGFVDLLR LTYYTPEYETK GPPHGIQVER WSPELAAKCEVWK DDENVNSQPFMR
SP #8	Serpin (*Sorghum bicolor*)	LVLGNALYFK LKLPYQQG LPPGSVDH VAFADGVFVDASLK EAAGKVNSWVEK LPPGSVDHTTR

Table S-7. Continued from the previous page.

Spot	Protein Homology	Peptides
Spots more abundant in non-dormant seeds		
SA #3 SP #5	Enolase (*Brachypodium distachyon*)	GVEVYHNLK HAGWGVMTSHR IEEELGAAAVYAGAK QIGSVTESIEAVK NNVNSIIAPALIGK PSGASTGVYEALELR NFKEENNDGSQK
SA #3 SP #5	Ketol-acid reductoisomerase (*Oryza sativa*)	LAGHDEYIVR SLAGHDEYIVR PAFPMGNIDQTR LEVYNSLTEEGKK GVLGWGSQGPAQAQNLR
SA #29 SP #15	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (*Zea mays*)	DAQIAS YLVSPPEIDR DAQVLGEAPYK GNSEVGHNALGAGR
SA #21 SP #2	Heat shock protein 70 kDa (*Zea mays*)	NTADTTIYSI GDAWVETTDG QAVTNPQNTFFGTK EAAIADLR NEPTAAALSYGMNNK VFSTAADNQTQVGIR PDEAVAMGAAIQGGILR ASEIEAAIADLR
SA #21	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (*Brachypodium distachyon*)	LDQVQLLLK YENDWGVVK DAQVLGEAPYK GSSVGFVETIENDLSQLR PSDDDMGNSEVGHNALGAGR
SA #21	V-type proton ATPase catalytic subunit A (*Brachypodium distachyon*)	LAADTPLLTGQR EDDLNEIVQLVGK FEDPAEGEDVLVAK TTLVANTSNMPVAAR
SP #2	Luminal-binding protein 2 precursor (*Zea mays*)	GVIAGLNVAR FDGKEPNK KDTAEAYLGK EIVLVGGSTR TPSWVAFTDSER VFSPEEISAMILGK FEELNNDLFR VEIIANDQGNR IINEPTAAAIAYGLDK DAVVTVPAYFNDAQR ESLFDGTDFSEPLTR LDVAPLTLGIETVGGVMTK

Table S-7. Continued from the previous page.

Spot	Protein Homology	Peptides
Spots more abundant in non-dormant seeds		
SA #28	Ricin type lectin-domain protein (*Sorghum bicolor*)	NPNYQDESVLWTESR LNFDAFHGDKDHGGVR
SP #9	Protein disulfide-isomerase (*Zea mays*)	TADDIVDFIK KPFDELVVDS TLHANHLP
SA #34	Lysosomal alpha-mannosidase (*Hordeum vulgare*)	ANEPWPLK FIYVEQAFFQR TFGSSADIFAGIFPK QAFFQR VNALYSTPSIYTDAK
SP #3	Chaperonin 60 kDa (*Oryza sativa*)	FLVDGFDVAK VLNPNAEVLNK NSGPIIASQLLLVDEVIR
SA #50 SP #11	F1-ATP synthase, beta unit (*Sorghum bicolor*)	IGLFGGAGVG VVDLLAPYQR AMDGTEGLVR VGLTGLTVAEHFR VLNTGSPITVPVGR LIMELINNVAK TNHFLPIHR LEVAQHLGENMVR HVLGEDHYNTAR GQDVLLFIDNIFR SELGIYPAVDPLDSTSR FVEQATEQQILVTGIK SAVGYQPTLATDLGGLQER PPILTALEVLDNNIR
SA #50 SP #11	Enolase (*Brachypodium distachyon*)	IEEELGAAAVYAGAK QIGSVTESIEAVK NNVNSIIAPALIGK YQHIANLAGNK PSGASTGVYEALELR FKEENNDGSQK SGETEDTFIADLAVGLSTGQIK DATNVGDEGGFAPNIQENK SIEDPFDQDDWVHYAK
SA #33	Phosphoglycerate kinase (*Zea mays*)	IGVIESLLAK YSLKPLVPR LNVPLDDAQK TTIIGGGDSVAAVEK AALPEGGVLLLENVR SLELLEGK
SP #22	Hexokinase (*Oryza sativa*)	LAAAGIVGILR GFTFSFP

Table S-8. Lists of de novo sequenced peptides of spots (present in *S. pectinata* but missing in *S. alterniflora*) that were without a significant homology matching with known proteins from NCBI green plant database (taxonomy: 33090). Identified peptides of contaminants (fibrinogen and keratin) are listed in the table. The E value of a significant threshold was set below 0.01. q: Q or K; i: I or L. N/A: no good quality peptide identified. -: spot was not excised from the gel.

<table>
<tr><th>Spot</th><th>De novo Sequenced Peptides</th></tr>
<tr><td>7</td><td>[ME|Fi] E q q [Nq|Ei] V i q T K</td></tr>
<tr><td>27</td><td>W V N D N E E G F F S A R (fibrinogen)
q S A E N E F V T i K
Y E E i q i T A G R (keratin)</td></tr>
<tr><td>28</td><td>N/A</td></tr>
<tr><td>46</td><td>-</td></tr>
<tr><td>49</td><td>-</td></tr>
<tr><td>55</td><td>-</td></tr>
<tr><td>56</td><td>Y E E i q i T A G R (keratin)
q S A E N E F V T i K</td></tr>
<tr><td>62</td><td>-</td></tr>
<tr><td>64</td><td>W V M N T E [TS|MG] S G D q K</td></tr>
<tr><td>67</td><td>[SS|CA] [VE|PM|NN|Di] N V i D D P T E q V R
[WW] A E [RN] [ND|Tq] V W q q A T E G A V R</td></tr>
<tr><td>69</td><td>E P [TH] M G C F [NG] T F D N D i M i i K
W V N D N E E G F F S A R (fibrinogen)</td></tr>
<tr><td>77</td><td>-</td></tr>
<tr><td>78</td><td>[Ai] E E S N Y E i E [KG] (keratin)
q E Y E q i i A K (keratin)
[ME|Fi] E q q N q V i D N K (keratin)
Y E E i q i T A G R (keratin)
q S A E N E F V T i K (keratin)</td></tr>
</table>

Table S-9. Logarithm value of average normalized volume, fold change and ANOVA p value of each spots shown in Table 2.1. a: Logarithm value of normalized volume from five independent biological replicate gels. Error bar represents standard deviation. CV: coefficient of variance value of each 5 biological replicates. CV values are for the normalized spot volume values and not for the logarithm of these values. b: variation of protein expression; c: background noise; d: gel mis-alignment.

Spot #	SA Log[volume] [a]	SP Log[volume] [a]	Fold SP/SA	ANOVA P value	CV Value SA	CV Value SP
			LEA Proteins			
1	7.18 ± 0.46	8.31 ± 0.04	11.2	8.3e-5	47%	7%
2	7.36 ± 0.14	8.41 ± 0.05	11	2.7e-8	24%	8%
3	7.47 ± 0.12	8.47 ± 0.03	9.9	1.0e-8	21%	6%
8	6.87 ± 0.77	7.77 ± 0.59	6.7	0.022	80% [c]	67% [b]
13	7.41 ± 0.2	8.18 ± 0.06	5.7	3.5e-6	27%	10%
24	7.5 ± 0.22	8.06 ± 0.05	3.5	7.8e-5	34%	8%
32	6.23 ± 0.21	6.7 ± 0.10	2.8	2.8e-4	32%	17%
37	6.5 ± 0.04	6.92 ± 0.08	2.7	4.3e-7	7%	13%
38	6.74 ± 0.25	7.2 ± 0.06	2.7	6.8e-4	52% [c]	10%
47	7.34 ± 0.19	7.75 ± 0.06	2.5	2.4e-4	31%	11%
50	7.24 ± 0.12	7.63 ± 0.09	2.4	6.9e-5	21%	15%
72	7.49 ± 0.11	7.78 ± 0.12	1.9	9.2e-4	19%	22%
			Chaperones			
23	7.52 ± 0.07	8.09 ± 0.05	3.7	4.3e-8	12%	8%
39	6.74 ± 0.25	7.2 ± 0.06	2.7	5.3e-5	14%	22%
42	7.04 ± 0.07	7.45 ± 0.08	2.6	2.5e-6	12%	13%
43	6.89 ± 0.17	7.31 ± 0.15	2.6	6.4e-4	30%	26%
48	8.09 ± 0.11	8.48 ± 0.05	2.4	9.8e-4	19%	8%
57	6.5 ± 0.08	6.86 ± 0.1	2.3	3.5e-5	14%	16%
63	7.77 ± 0.07	8.12 ± 0.05	2.2	1.4e-6	11%	9%
75	6.93 ± 0.06	7.2 ± 0.07	1.7	6.7e-4	9%	8%
80	6.74 ± 0.11	6.94 ± 0.05	1.6	9.0e-4	17%	9%
			Metabolsim			
5	7.2 ± 0.53	8.22 ± 0.05	8.1	4.1e-4	64% [c]	8%
25	7.64 ± 0.17	8.18 ± 0.05	3.4	1.7e-5	27%	9%
35	7.09 ± 0.17	7.53 ± 0.09	2.7	1.2e-4	24%	15%
40	6.6 ± 0.08	7.02 ± 0.08	2.7	4.2e-6	13%	16%
41	6.79 ± 0.07	7.21 ± 0.04	2.6	4.8e-7	13%	7%
59	6.82 ± 0.08	7.17 ± 0.07	2.3	8.4e-6	15%	12%
60	6.85 ± 0.12	7.21 ± 0.05	2.2	3.0e-5	20%	10%
61	6.15 ± 0.54	6.6 ± 0.15	2.2	0.042	61% [c]	24%
74	6.93 ± 0.06	7.2 ± 0.07	1.9	2.6e-5	10%	13%
79	6.71 ± 0.14	6.92 ± 0.06	1.6	0.004	22%	10%
			Antioxidants			
10	7.5 ± 0.12	8.28 ± 0.04	6.0	5.2e-8	21%	8%
16	6.88 ± 0.18	7.52 ± 0.07	4.2	1.0e-5	34%	12%
17	7.46 ± 0.13	8.06 ± 0.13	3.9	1.2e-5	34%	9%
18	7.32 ± 0.12	7.98 ± 0.04	4.5	3.8e-7	24%	8%
26	7.37 ± 0.15	7.89 ± 0.06	3.2	1.1e-5	28%	22%
29	7.39 ± 0.13	7.84 ± 0.29	3.0	0.003	21%	39%
36	7.19 ± 0.2	7.63 ± 0.05	2.7	2.0e-4	34%	8%
58	6.92 ± 0.16	7.29 ± 0.08	2.3	2.4e-4	25%	13%
65	6.9 ± 0.14	7.25 ± 0.03	2.2	9.9e-5	23%	6%
66	7.11 ± 0.12	7.45 ± 0.1	2.1	2.4e-4	20%	16%
68	7.1 ± 0.25	7.46 ± 0.04	2.1	0.003	44%	6%
70	7.49 ± 0.11	7.8 ± 0.03	2.0	4.2e-5	20%	6%

Table S-9. Continued from the previous page.

Spot #	SA Log[volume] [a]	SP Log[volume] [a]	Fold SP/SA	ANOVA P value	CV Value SA	CV Value SP
			DNA or RNA binding protein			
15	7.31 ± 0.16	7.97 ± 0.07	4.5	3.3e-6	29%	11%
52	6.81 ± 0.17	7.2 ± 0.05	2.4	1.6e-4	27%	9%
			Cellular recycling			
19	7.38 ± 0.13	7.97 ± 0.08	3.8	2.9e-6	22%	13%
31	7.36 ± 0.15	7.83 ± 0.04	2.9	0.001	25%	6%
45	7.23 ± 0.4	7.71 ± 0.7	2.6	0.008	55%	12%
83	7.06 ± 0.03	7.52 ± 0.08	2.9	3.2e-6	10%	17%
			Protease inhibitor			
3	7.47 ± 0.12	8.47 ± 0.03	9.9	1.0e-8	21%	6%
4	7.45 ± 0.12	8.41 ± 0.03	8.9	1.2e-8	22%	2%
9	7.66 ± 0.09	8.44 ± 0.05	6.1	1.0e-8	15%	8%
20	7.57 ± 0.10	8.15 ± 0.04	3.8	1.9e-7	17%	6%
22	7.75 ± 0.19	8.34 ± 0.05	3.7	1.7e-5	33%	9%
			Stress related proteins			
11	6.97 ± 0.22	7.77 ± 0.07	6.0	6.2e-6	34%	12%
14	7.28 ± 0.15	7.96 ± 0.05	4.7	9.9e-7	23%	8%
30	7.26 ± 0.3	7.77 ± 0.04	2.9	0.001	54% [c]	7%
54	7.46 ± 0.2	7.85 ± 0.07	2.3	5.9e-4	32%	11%
			Protein Synthesis Inhibitor			
21	7.04 ± 0.28	7.64 ± 0.11	3.7	2.8e-4	44% [c]	17%
			Storage Proteins			
44	6.92 ± 0.14	7.33 ± 0.12	2.6	1.3e-4	24%	18%
51	7.05 ± 0.12	7.42 ± 0.11	2.4	1.1e-4	20%	17%
53	6.91 ± 0.16	7.29 ± 0.09	2.4	2.4e-4	30%	14%
73	6.66 ± 0.25	6.98 ± 0.06	1.9	0.006	39%	11%
82	6.92 ± 0.04	7.44 ± 0.09	3.3	2.6e-7	7%	15%
			Other proteins			
6	7.34 ± 0.27	8.24 ± 0.07	7.3	1.1e-5	46% [c]	11%
12	6.88 ± 0.16	7.66 ± 0.05	5.8	7.7e-7	25%	9%
33	7.09 ± 0.13	7.55 ± 0.07	2.8	1.7e-5	21%	12%
34	6.97 ± 0.12	7.42 ± 0.09	2.8	2.0e-5	22%	15%
71	7.78 ± 0.14	8.09 ± 0.03	2.0	2.1e-4	23%	5%
76	7.03 ± 0.08	7.25 ± 0.1	1.6	0.001	15%	15%

Table S-10. Listed genes for protein spots (Table 2.1) that contain desiccation stress associated *cis*-regulatory motifs listed in Tables 2.6 and 2.7. a: the number corresponds to the spot number listed in the Table 2.1. Some numbers point to the same protein.

***Cis*-regulatory motifs**	**Genes of *S. pectinata* proteins [a]**
MYBCORE	1, 2, 3 (2), 4, 5, 6, 8, 9, 10 (2), 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 26, 29, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 47, 48, 50, 51, 52, 53, 54, 58, 59, 60, 61, 63, 64, 65, 66, 68, 70, 71, 72, 75, 80, 82, 83, 84
ACGTATERD1	1, 2, 3, 4, 5, 8, 9, 10 (2), 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 26, 29, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 47, 48, 50, 51, 53, 58, 59, 60, 61, 63, 65, 66, 68, 70, 71, 72, 75, 80, 82, 83, 84
MYCOONSENSUSAT	1, 2, 3, 4, 5, 6, 8, 9, 10 (2), 11, 13, 14, 16, 17, 18, 19, 20, 21, 22, 25, 26, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 47, 50, 51, 52, 53, 59, 60, 61, 63, 65, 68, 70, 71, 72, 75, 76, 80, 82, 83, 84
MYB1AT	3 (dehydrin), 5, 10 (2), 11, 13, 14, 16, 19, 21, 22, 23, 25, 26, 29, 30, 31, 32, 35, 36, 37, 39, 40, 41, 42, 45, 47, 48, 50, 52, 57, 58, 59, 60, 61, 63, 64, 65, 66, 68, 70, 71, 76, 80, 83
ABRELATERD1	1, 2, 3 (cystatin), 4, 5, 6, 8, 9, 10 (2), 13, 16, 17, 18, 19, 20, 21, 22, 23, 25, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 44, 45, 48, 50, 51, 52, 53, 58, 59, 60, 61, 63, 64, 65, 66, 70, 71, 72, 80, 82, 84
ABRERATCAL	1, 2, 3 (dehydrin), 6, 8, 10 (2), 13, 15, 17, 18, 19, 21, 22, 25, 30, 31, 32, 33, 34, 35, 37, 38, 40, 41, 44, 45, 47, 50, 51, 52, 53, 57, 59, 60, 63, 64, 70, 71, 72, 82, 84
MYB2CONSENSUSAT	1, 2, 3 (dehydrin), 5, 6, 10 (2), 11, 13, 14, 19, 21, 22, 30, 31, 32, 33, 34, 37, 38, 43, 45, 47, 50, 52, 54, 58, 61, 63, 64, 66, 71, 72, 75
MYBPZM	1, 2, 8, 3 (cystatin), 4, 5, 6, 9, 10 (GST), 11, 14, 15, 19, 21, 22, 20, 26, 30, 31, 42, 43, 44, 45, 51, 53, 57, 58, 61, 63, 66, 70, 71, 75, 80, 82
MYBST1	3 (dehydrin), 10 (2), 11, 13, 14, 16, 17, 18, 19, 21, 26, 32, 33, 34, 35, 36, 37, 39, 41, 43, 50, 47, 54, 57, 58, 59, 60, 63, 64, 65, 66, 71, 75, 83, 84
DPBFCOREDCDC3	3 (dehydrin), 5, 6, 10 (2), 13, 16, 19, 22, 25, 30, 32, 33, 34, 35, 36, 37, 38, 40, 41, 42, 43, 44, 47, 50, 51, 52, 53, 59, 60, 61, 64, 65, 70, 72, 75, 80, 82, 83
MYBCOREATCYCB1	6, 10 (2), 11, 13, 14, 16, 17, 18, 19, 21, 22, 25, 31, 32, 33, 36, 37, 38, 39, 40, 42, 43, 44, 45, 50, 51, 53, 59, 63, 64, 65, 66, 72, 75, 76, 80, 82, 84
MYCATRD22	1, 2, 3 (cystatin), 4, 8, 9, 10 (2), 11, 14, 17, 18, 20, 22, 25, 30, 33, 38, 40, 42, 44, 51, 52, 53, 58, 63, 64, 66, 70, 72, 76, 80, 82, 83, 84
CBFHV	1, 2, 3 (dehydrin), 5, 6, 8, 10 (GST), 19, 23, 31, 33, 34, 38, 42, 43, 44, 45, 47, 48, 51, 52, 53, 57, 58, 61, 66, 72, 75, 76, 80, 82, 83
MYCATERD1	1, 2, 3 (cystatin), 4, 8, 9, 10 (GST), 11, 14, 17, 18, 20, 22, 25, 30, 33, 38, 40, 44, 42, 51, 52, 53, 63, 70, 72, 76, 80, 82, 83, 84
DRECRTCOREAT	3 (dehydrin), 6, 10 (GST), 19, 23, 31, 34, 38, 42, 43, 45, 47, 48, 52, 57, 58, 66, 72, 75, 80, 83
ACGTABREMOTIFA20SEM	1, 2, 8, 13, 23, 26, 29, 32, 34, 35, 37, 38, 41, 43, 48, 50, 52, 57, 58, 59, 60, 66, 68, 72, 75, 76
RYREPEATBNNAPA	1, 2, 5, 8, 11, 13, 14, 19, 32, 37, 50, 52, 57, 58, 61, 66, 70, 83
MYB2AT	3 (dehydrin), 10 (SOD), 30, 34, 35, 41, 43, 47, 52, 54, 59, 60, 64, 71, 75
ABREOSRAB21	1, 2, 8, 13, 17, 18, 31, 32, 33, 35, 37, 41, 45, 50, 59, 60, 70, 84
ABREATCONSENSUS	1, 2, 3 (dehydrin), 8, 35, 41, 44, 47, 51, 53, 59, 60, 70, 82
ARE	1, 2, 3 (dehydrin), 5, 8, 10 (GST), 11, 13, 14, 15, 16, 21, 23, 25, 26, 29, 30, 32, 35, 36, 37, 39, 40, 41, 42, 47, 48, 50,52, 57, 58, 59, 60, 61, 63, 65, 66, 68, 70, 71, 80, 83
MBS	3 (dehydrin), 10 (2), 19, 22, 23, 25, 30, 31, 34, 35, 38, 39, 40, 41, 43, 45, 47, 48, 52, 54, 57, 58, 59, 60, 64, 66, 71, 72, 75
ABRE	1, 2, 3 (dehydrin), 5, 8, 10 (GST), 15, 17, 18, 22, 23, 25, 31, 38, 39, 40, 41, 42, 44, 47, 48, 51, 53, 58, 59, 60, 61, 66, 70, 72, 80, 82, 84

Table S-11. An examples of a sample calculation of standard deviation and coefficient of variation of the spot volume of identified proteins corresponding to Tables 2.1 and S-9.

Tables 2.1 and S-9. Spot #61; CV_{SA} = 61%, 5 biological replicates
Spots normalized volume: #1, 1225511.61572769; #2, 2857799.69797463; #3, 1826509.24297377; #4, 2902562.50487096; #5, 309625.326833417
Mean μ = (#1+#2+#3+#4+#5)/5= 1824401.67768
Standard deviation = Square root $[(\#_n - \mu)^2 / n]$, n=1, 2, 3, 4, 5 Standard deviation = 1104939.29458
Coefficient of variation = standard deviation/mean=0.6056 $\approx$ 61%

Table S-12. List of proteins of each cellular component of Figure S-15.

Cellular Components	Proteins
Nucleus	Group-3 LEA (1, 2, 8, 24), dehydrin (4, 47), LEA-1 (13, 32, 37, 50), glycine-rich protein (15), autophagy related protein (19), GAPDH (25, 35, 40, 41, 59, 60, 74), glutathione peroxidase (26), 1-Cys (29, 68), type-II HSP (39), NAC (42, 80), BTF3 (52), Calmodulin (71), hypothetical protein (12)
Mitochondria	FBA (5, 61, 79), CBS-domain protein (6), SOD [Mn] (10, 64), translation inhibitor (21), chaperone (23, 48), GAPDH (25, 35, 40, 41, 59, 60, 74), chaperonin (63),
Cytosol	Cystatin (3, 4, 9, 20), FBA (5, 61, 79), glutathione transferase (10), stress-responsive protein (11, 14), SOD [Cu-Zn] (16, 36, 65), Glutaredoxin (17, 18, 84), GAPDH (25, 35, 40, 41, 59, 60, 74), ubiquitin (31, 45), LEA-14 (38, 72), type-I HSP (43, 75), HSP-60 (57), Calmodulin (71), hypothetical protein (12), ubiquitin precursor (83)
Plasma membrane	Dehydrin (4, 47), FBA (5, 61), stress-responsive protein (11, 14), Glutaredoxin (17, 18, 84), autophagy related protein (19), chaperone (23, 48), GAPDH (25, 35, 40, 41, 59, 60, 74), glutathione peroxidase (26), 1-Cys (29, 68), ubiquitin (31, 45), SOUL heme-binding protein (33), oxygen evolving protein (34), type-II HSP (39), NAC (42, 80), HSP-60 (57), chaperonin (63), Calmodulin (71), FK506 (76), hypothetical protein (12), ubiquitin precursor (83)
Plastid	Translation inhibitor (21), chaperone (23, 48), GAPDH (25, 35, 40, 41, 59, 60, 74), oxygen evolving protein (34), copper binding protein (54), peroxiredoxin-2 (58, 66), chaperonin (63), SOD [Cu-Zn] (70)
Vacuole	Glutaredoxin (17, 18, 84), translation inhibitor (21), GAPDH (25, 35, 40, 41, 59, 60, 74), SOUL heme-binding protein (33),
Thylakoid	Translation inhibitor (21), chaperone (23, 48), oxygen evolving protein (34), peroxiredoxin-2 (58, 66), chaperonin (63), SOD [Cu-Zn] (70)
Endoplasmic reticulum	Type-I HSP (43, 75)
Cell wall	Vicilin (44, 51, 53, 73, 82), peroxiredoxin-2 (58, 66),
Unknown	Trypsin (22), ASR (30)

Table S-13. List of proteins of each cellular component of Figure S-16.

Cellular Components	**Proteins**
Nucleus	Elongation factor Tu (SA21, SP19), phosphoglycerate kinase (SA21, SP19), tubulin (SA34, SA29, SP4), cysteine synthase (SA30, SP7), legumin (SA30)
Mitochondria	Dihydrolipoamide dehydrogenase (SA10)
Cytosol	Alcohol dehydrogenase (SA5), phosphoglycerate kinase (SA21, SP19), tubulin (SA34, SP4), RUBISCO (SA34, SA29, SP4), cysteine synthase (SA30, SP7), serpin (SP8)
Plasma membrane	Alcohol dehydrogenase (SA5), dihydrolipoamide dehydrogenase (SA10), elongation factor Tu (SA21, SP19), phosphoglycerate kinase (SA21, SP19), tubulin (SA34, SA29, SP4), RUBISCO (SA34, SA29, SP4), cysteine synthase (SA30, SP7), legumin (SA30)
Plastid	Elongation factor Tu (SA21, SP19), RUBISCO (SA34, SA29, SP4), cysteine synthase (SA30, SP7)
Vacuole	Phosphoglycerate kinase (SA21, SP19), tubulin (SA34, SA29, SP4), cysteine synthase (SA30, SP7)
Thylakoid	Elongation factor Tu (SA21, SP19), RUBISCO (SA34, SA29, SP4)
Ribosome	RUBISCO (SA34, SA29, SP4)
Cell wall	Globulin-1 (SA22, SA4, SA5, SA10, SA20, SP14, SP12, SP5, SP2, SP15), tubulin (SA34, SA29, SP4), RUBISCO (SA34, SA29, SP4)
Peroxisome	Cysteine synthase (SA30, SP7)

Table S-14. List of proteins of each cellular component of Figure S-17.

Cellular Components	Proteins
Nucleus	Enolase (SA3, SA50, SP5, SP11), phosphoglycerate mutase (SA29, SA21, SP15), ATPase (SA21), luminal-binding protein (SP2), lectin-domain protein (SA28), phosphoglycerate kinase (SA33), hexokinase (SP22)
Mitochondria	Reductoisomerase (SA3, SP5), HSP-70 (SA3, SP2), ATP synthase (SA50), hexokinase (SP22)
Cytosol	Enolase (SA3, SA50, SP5, SP11), phosphoglycerate mutase (SA29, SA21, SP15), chaperonin (SP3), phosphoglycerate kinase (SA33)
Plasma membrane	Enolase (SA3, SA50, SP5, SP11), phosphoglycerate mutase (SA29, SA21, SP15), HSP-70 (SA3, SP2), ATPase (SA21), luminal-binding protein (SP2), lectin-domain protein (SA28), disulfide-isomerase (SP9), mannosidase (SA34), chaperonin (SP3), ATP synthase (SA50), phosphoglycerate kinase (SA33), hexokinase (SP22)
Plastid	Enolase (SA3, SA50, SP5, SP11), reductoisomerase (SA3, SP5), HSP-70 (SA3, SP2), ATPase (SA21), disulfide-isomerase (SP9), ATP synthase (SA50), hexokinase (SP22)
Vacuole	HSP-70 (SA3, SP2), ATPase (SA21), luminal-binding protein (SP2), mannosidase (SA34), ATP synthase (SA50), phosphoglycerate kinase (SA33), hexokinase (SP22)
Thylakoid	Disulfide-isomerase (SP9)
Cell wall	Reductoisomerase (SA3, SP5), HSP-70 (SA3, SP2), ATPase (SA21), luminal-binding protein (SP2), disulfide-isomerase (SP9), mannosidase (SA34)
Endoplasmic reticulum	Luminal-binding protein (SP2), disulfide-isomerase (SP9)

APPENDIX B
LABORATORY NOTEBOOK LOCATIONS FOR DATA IN FIGURES AND TABLES IN THE BODY OF THE DISSERTATION

1. Data corresponding to figure 2.1. Notebook 2: 71-100.
2. Data corresponding to figure 2.2. Notebook 8: 3-6; notebook 7: 91, 99.
3. Data corresponding to figure 2.3. Notebook 8: 7-10; 40-41.
4. Data corresponding to figure 2.4. Notebook 8: 42.
5. Data corresponding to figure 2.5. Notebook 8: 42.
6. Data corresponding to figure 2.6. Notebook 8: 42.
7. Data corresponding to figure 2.7. Notebook 8: 42.
8. Data corresponding to figure 2.8. Notebook 7: 33, 40; notebook: 47-48.
9. Data corresponding to figure 2.9. Notebook 8: 88.
10. Data corresponding to figure 2.10. Notebook 8: 89.
11. Data corresponding to figure 2.11. Notebook 8: 90.
12. Data corresponding to figure 2.12. Notebook 8: 76-79; 81-84; 91-92.
13. Data corresponding to figure 2.13. Notebook 8: 76-79; 81-84; 91-92.
14. Data corresponding to figure 2.14. Notebook 8: 81-83.
15. Data corresponding to figure 2.15. Notebook 8: 81-82, 84.
16. Data corresponding to figure 2.16. not in data books
17. Data corresponding to figure 3.1. Notebook 7: 51-56; Notebook 3: 76, 95-100.
18. Data corresponding to figure 3.2. Notebooks 6: 48-54; 62-63; Notebook 8: 32.
19. Data corresponding to figure 3.3. Notebook 8: 59-66.
20. Data corresponding to figure 3.4. Notebook 8: 59-66.
21. Data corresponding to figure 3.5. Notebook 8: 67.

22. Data corresponding to figure 3.6. Notebook 8: 67.

23. Data corresponding to figure 3.7. Notebook 8: 73.

24. Data corresponding to figure 3.8. Notebook 8: 73.

25. Data corresponding to figure 4.1. Notebook 5: 78-100; notebook 6: 76-80; 84-88.

26. Data corresponding to figure 4.2. Notebook 6: 89.

27. Data corresponding to figure 4.3. Notebook 6: 89.

28. Data corresponding to table 2.1. Notebook 8: 50.

29. Data corresponding to table 2.2. Notebook 8: 43-50.

30. Data corresponding to table 2.3. not in databooks

31. Data corresponding to table 2.4. not in databooks

32. Data corresponding to table 2.5. not in databooks

33. Data corresponding to table 2.6. Notebook 8: 94.

34. Data corresponding to table 2.7. Notebook 8: 94.

35. Data corresponding to table 3.1. Notebook 8: 67.

36. Data corresponding to table 3.2. Notebook 8: 70-73.

37. Data corresponding to table 3.3. Notebook 8: 70-73.

38. Data corresponding to table 3.4. Notebook 8: 70-73.

APPENDIX C
CORRESPONDING TABLES FOR FIGURES IN THE BODY OF THE DISSERATION

Table C-1. Data corresponding to Figure 2.1 (b). Viability of *S. alterniflora* and *S. pectinata* seeds as a function of moisture content during desiccation. Notebook 2: 71-100.

Moisture Content (DWB)	*S. alterniflora* % Seed Viability	*S. pectinata* % Seed Viability
120.5		100
114.9	100	
122.5	95	
122.8	100	
110.2		100
63.8		100
58.9	100	
58.7	100	
55.3	95	
44	95	
43.3	95	
39	85	
38.2	85	
37		100
34.5	55	
32.8	75	
31.1	45	
29.7	40	
28.6	25	
28.1	60	
28.0		100
27.6	30	
22.6	20	
22.1	10	
17.9	5	
15.4	10	
15.2		100
10.2	0	
9.5		100
7.6	0	

Table C-2. Data corresponding to Figure 3.1. Relationship between time of cold-stratification and germination and viability for *S. alterniflora* and *S. pectinata*. Notebook 7: 51-56; Notebook 3: 76, 95-100. Error bar represents standard error.

Weeks	**SP %G**	**SP %V**	**SA %G**	**SP %V**
1	0	98 ± 2	0	98 ± 2
2	0	87 ± 4	0	97 ± 2
3	0	92 ± 3	0	100
4	0	92 ± 2	0	97 ± 2
5	0	88 ± 3	10	100
6	3 ± 2	100	10 ± 3	93 ± 3
7	30 ± 4	92 ± 2	20 ± 3	98 ± 2
8	43 ± 5	95 ± 3	40	97 ± 2
9	57 ± 7	97 ± 2	50 ± 3	98 ± 2
10	60 ± 7	97 ± 2	55 ± 3	100
11	67 ± 5	95	63 ± 3	97 ± 2
12	87 ± 6	95 ± 3	67 ± 4	98 ± 2
1	93 ± 5	93 ± 5	98 ± 2	100

Table C-3. Data corresponding to Figure 4.1. Relationship between moisture content of flash dried *S. alterniflora* seeds and drying duration at 4°C, 14°C and 24°C. Notebook 5: 78-100; notebook 6: 76-80; 84-88.

Days	**%MC (24°C)**	**Days**	**%MC (14°C)**	**Days**	**%MC (4°C)**
0.0833	114.9	0	128.2	0	122.3
0.6667	122.5	0	111.6	0	136.3
0.6667	122.8	0	126	0	120.4
0.6667	55.3	0.75	67.5	2	53.2
0.75	61.8	0.75	66.1	2	52.5
0.75	53.5	1	61.7	5	43
0.75	50.5	1	60	5	46.2
0.75	52	1	60.6	5	47.4
0.75	42.7	2	45.8	8	39.7
0.8333	53.4	2	44.2	8	42.9
0.8333	39.	2	47.7	8	42.4
0.8333	43.3	5	34.8	9	42.9
0.0833	42.3	5	35	13	37.6
0.0833	48.5	5	34.3	20	35.2
1	46	7	29.2	20	34
1	55.1	7	28.9	20	33.8
1	39.6	7	29.2	30	29.7
1	35.5	9	30.7	30	27.3
1	40.3	9	29.9	40	25.3
1	41.5	9	26.5	40	25
1	38.1	10	23.5	45	20.9
2	40.2	20	17.9	60	17.9
2	38.7	20	17.6	60	18.3
2	38.2				
2	36.3				
2	37.9				
2	36.9				
2	33				
3	32.8				
3	36.1				
5	34.3				
7	34.5				
7	31.1				
7	34.30				
7	27.6				
7	34.9				
9	28.7				
9	22.6				
11	22.1				
11	17.9				
15	25.5				

Table C-4. Data corresponding to Figure 4.2. Relationship between viability of flash dried *S. alterniflora* seeds and drying duration at 4ºC, 14ºC, or 24ºC. Notebook 5: 78-100; notebook 6: 76-80; 84-88.

Days	%V (24ºC)	Days	%V (14ºC)	Days	%V (4ºC)
0	100	0	100	0	100
0	95	0	100	0	100
0	100	0	95	0	100
0.0417	95	0.75	95	2	95
0.0417	85	0.75	100	2	100
0.0833	85	1	80	5	95
0.0833	95	1	100	5	95
0.0833	80	1	90	5	90
0.0833	90	2	95	8	65
0.6667	95	2	100	8	85
0.6667	100	2	95	8	80
0.6667	100	5	75	9	95
0.75	95	5	60	13	70
0.75	95	5	50	20	60
0.75	100	7	35	20	65
0.75	95	7	40	20	50
0.75	95	7	35	30	40
0.8333	80	9	30	30	30
0.8333	70	9	30	40	20
0.8333	70	9	25	40	30
1	65	10	25	45	15
1	95	20	15	60	10
1	90	20	10	60	5
1	80				
1	80				
1	60				
1	60				
2	30				
2	25				
2	60				
2	60				
2	55				
2	45				
2	60				
3	40				
3	55				
5	25				
7	20				
7	10				
7	10				
7	35				

Table C-5. Data corresponding to Figure 4.3. Viability of flash dried *S. alterniflora* seeds as a function of moisture content after flash dried at 4ºC, 14ºC and 24ºC. Notebook 5: 78-100; notebook 6: 76-80; 84-88.

%MC	%V (24ºC)	%MC	%V (14ºC)	MC	%V (4ºC)
114.9	100	128.2	100	122.3	100
122.5	95	111.6	100	136.3	100
122.8	100	126	95	120.4	100
55.3	95	67.5	95	53.2	95
61.8	85	66.1	100	52.5	100
53.5	85	61.7	80	43	95
50.5	95	60	100	46.2	95
52	80	60.6	90	47.4	90
42.7	90	45.8	95	39.7	65
53.4	95	44.2	100	42.9	85
39.	100	47.7	95	42.4	80
43.3	100	34.8	75	42.9	95
42.3	95	35	60	37.6	70
48.5	95	34.3	50	35.2	60
46	100	29.2	35	34	65
55.1	95	28.9	40	33.8	50
39.6	95	29.2	35	29.7	40
35.5	80	30.7	30	27.3	30
40.3	70	29.9	30	25.3	20
41.5	70	26.5	25	25	30
38.1	65	23.5	25	20.9	15
40.2	95	17.9	15	17.9	10
38.7	90	17.6	10	18.3	5
38.2	80				
36.3	80				
37.9	60				
36.9	60				
33	30				
32.8	25				
36.1	60				
34.3	60				
34.5	55				
31.1	45				
34.30	60				
27.6	40				
34.9	55				
28.7	25				
22.6	20				
22.1	10				
17.9	10				
25.5	35				

VITA

Yi Wang was born in Pingxiang, a city of Southeast China. He went to Beijing University of Chemical Technology as an undergraduate, and obtained a B.S. degree in summer, 2006. In the same year, he was admitted as a graduate student in Department of Plant Pathology and Crop Physiology at Louisiana State University. He received his M.S. degree in crop physiology in 2010.