PURIFICATION AND PARTIAL CHARACTERIZATION OF GENETICALLY ENGINEERED THIOL PROTEASE OVER-EXPRESSED IN *ESCHERICHIA COLI* AND ANALYSIS OF PRIMING EFFECTS ON FREE AMINO ACID ACCUMULATION IN *PINUS TAEDA* SEEDS

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the Degree of
MASTER OF SCIENCE
July, 1997

OKLAHOMA STATE UNIVERSITY

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PREFACE

The overall objective of an ongoing project is to determine the physiological and genetic mechanisms responsible for the invigoration of loblolly pine (*Pinus taeda* L.) seeds subjected to controlled water stress through solid matrix priming (SMP). It is hypothesized that the invigoration is due to a change in the sequence of events in the germinating seed where storage protein degradation and mobilization which normally commence after germination are stimulated to occur before radicle emergence. The mobilization of protein reserves provides: osmotically active substances for osmotic adjustment leading to increased capacity of the embryo to grow and the components for synthesis of new proteins for the growing plant. It is also hypothesized that thiol protease (TP) is a major enzyme involved in the degradation of storage proteins when seeds are primed, as it is known to degrade reserve proteins, to be active during germination and to be up-regulated by water stress.

As one effort toward the overall objective, the first chapter concerns TP antigen production to make anti-TP antibodies so that it becomes possible to further elucidate TP functions at the translation and post-translation levels cellularly and subcellularly. In addition, the over-expressed TP protein provides a way to efficiently purify the enzyme by immunoaffinity chromatography from loblolly pine. The second chapter, without direct relations to Chapter I but as another step toward the overall goal, concerns SMP effects on free amino acid accumulation and the relationships among free amino acid accumulation, TP activity increase and decrease of water potential.

ACKNOWLEDGMENTS

I express my sincere appreciation to my advisors, Dr. Richard Essenberg, Dr. Stephen Hallgren and Dr. Andrew Mort for their intelligent supervision, constructive guidance, encouragement and friendship. My sincere appreciation is extended to Dr. Yinghua Huang, who graciously provided advice anytime and generously extended his laboratory to accommodate my research in the part of gene expression.

I also express my special gratitude to Mr. David M. Ferris and Mr. Zhiquan Zhang for their technical suggestions and assistance in this project.

Moreover, I thank the Department of Biochemistry and Molecular Biology, the Department of Forestry and OCAST for providing the research opportunity and an assistantship to support this work. I am grateful to the Oklahoma Division of Forestry and Weyerhaeuser Company for providing loblolly pine seeds.

Finally, heartfelt thanks go to my understanding wife, Yunrong, daughter, Julie, and son, Fei, for their sacrifice, help, love and support. I also express appreciation to fellow students for beneficial discussions and to all the residents of Stillwater, Oklahoma, who have made this stay a memorable one.

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Chapter I

Purification and partial characterization of genetically engineered thiol protease over-expressed in *Escherichia coli*

Abstract

Thiol protease cDNA was successfully over-expressed in *Escherichia coli* and the gene products were purified and partially characterized. More importantly, anti-thiol protease antibodies were generated by using the purified protein as antigen. A loblolly pine cDNA clone *C4-1* coding for thiol protease was inserted into *pMAL™-c2* expression vector after the *malE* gene, which encodes the maltose binding protein (MBP). Restriction enzyme digestion showed the construct had the correct size of insert and vector. Northern blotting demonstrated that the recombinant plasmid was transformed into *Escherichia coli DH5α* and that the thiol protease mRNA was only expressed after IPTG induction. DNA sequencing indicated no nucleotide errors in the fusion section of recombinant plasmid. Selected colonies containing the hybrid plasmid expressed a 85 kDa fusion protein of MBP-thiol protease inducible by IPTG. Both soluble and insoluble fusion proteins were purified by amylose affinity chromatography and preparative PAGE to homogeneity and used as antigens to make polyclonal antibodies. MBP-specific antibodies, separated from the antibody mixture, were used as a control. The remaining thiol protease-specific antibodies successfully recognized thiol proteases expressed in the *E. coli* and the thiol proteases expressed in the embryo of loblolly pine seeds by Western blotting. The purified fusion protein was cleaved with Factor Xa between the MBP and thiol protease to remove the MBP. The enzyme expressed in *E.*

coli cell, either the purified fusion protein or the purified thiol protease fragment from the fusion protein did not show any thiol protease activity. Possibilities causing lack of activity of the expressed thiol protease are discussed and future studies were proposed.

Introduction

Thiol proteases (EC3.4.22) in protein degradation serve a wide variety of roles during the plant life cycle such as hydrolysis of storage protein for seed germination, reallocation of organic nitrogen and detoxification of abnormal proteins. Differential regulation of protein stability also represents a potential mechanism for modulating gene expression, since cell protein level is determined by rates of both synthesis and degradation.

The thiol proteases from prokaryotes and eukaryotes characterized thus far fall into about 20 evolutionarily related families (Rawlings and Barret 1994); three of these are represented by streptopain from *Streptococcus*, calpain (calcium-dependent) from birds and mammals, and papain from *Carica papaya* (Rawlings and Barret 1994, Barret 1986). The papain superfamily seems to be the predominant family in eukaryotes. The sequences of cDNA and genomic clones have been determined for many members of this superfamily, including cathepsins of animal cells (Tahio et. al. 1983, Chan et. al. 1986, Ishdoh et. al. 1987 1989), thiol proteases expressed in slime molds (Williams et. al. 1985, Pears et. al. 1985, Datta and Firtel 1987), plant cell thiol proteases including papaya papain (Cohen et. al. 1986), barley aleurain EP-A, EP-B, EP-1 (Rogers et. al. 1985, Hong et. al. 1988, Koehler and Ho 1988 1990, Marttila et. al. 1995), rice oryzain α, ß, γ (Watanabe et. al. 1991), mung bean sulfhydrylendopeptidase (Mitsuhashi et. al. 1986, Akasofu et al. 1989), soybean protease K1, K2, G1 (Wilson et. al. 1988,

Papastoitsis and Wilson 1991), *Brassica napus* COT44 (Harada et. al. 1989, Dietrich et. al. 1989), *Arabidopsis thaliana* A1494, RD19, RD21 (Yamaguchi-Shinozaki and Shinozaki 1993) *Vicia sativa* (Nong and Munitz 1994), ice plant (Forsthoefel, et al. 1996) and loblolly pine thiol protease (Neale; Huang, personal communication). Sequence comparisons between the protein and deduced sequences from cDNA clones show considerable similarity in several domains, which indicates this gene family has been highly conserved. The sequence similarity among thiol proteases extends from the 5' to 3' ends of the open reading frames, including not only the mature polypeptide but also the signal sequence and precursor domains (North 1986).

Thiol proteases can be divided into two groups according to function: 1) creation of biologically active molecules, and 2) degradation of storage and biologically active proteins and peptides (see review of Bound and Butler 1987). The first group includes enzymes that process precursors and activate enzymes. For example, thiol proteases are involved in processing a number of protein precursors *in vivo*, both intracellularly, as with proinsulin (Docherty et. al. 1982), and extracellularly, as with proapolipoprotein A-II (Gordon et. al. 1984). During germination, a thiol protease might be responsible for the activation of latent, protein-bound forms of beta-amylase. Protein-bound forms of β-amylase exist in the mature megagametophyte primarily in the highly cross-linked fraction (Roswell and Goad 1962). Extraction of barley grain with papain resulted in the appearance of two β-amylase forms with pI's identical to those of the predominant forms found in germinating barley (LaBerge and Marchylo 1986).

The second group includes lysosomal and ATP-dependent thiol proteases that are involved in degrading or committing active molecules to extensive hydrolysis. These catabolic proteases: 1) degrade defective, 'abnormal' or normal polypeptides; 2) control

the concentration of potent enzymes; and 3) have nutrient functions through the creation of amino acids from proteins.

There is a close correlation between synthesis of thiol proteases and seed germination in many species such as barley (Enari and Mikola 1977), rice (Watanabe et. al. 1991), soybean (Wilson et. al. 1988, Papastoitsis and Wilson 1991), *Brassica napus* (Harada et. al. 1988, Dietrich et. al. 1989), and mung bean (Mitsuhashi et. al. 1986, Akasofu et al. 1989). Thiol protease may play a major role in protein turnover permitting the constant renewal of cellular contents in germinating seeds. Miller and Huffaker (1981) have extensively purified and characterized a thiol protease from primary leaves of barley seedlings. This major vacuolar protease (EP-1) has been shown to hydrolyze both the large and small subunits of ribulose 1,5-bis-phosphate carboxylase into discrete polypeptides (Thomas and Huffaker 1981).

Thiol protease may be responsible for mobilization of seed storage proteins during seed germination and postgermination. A major thiol protease partially purified from wheat seeds germinated for four days is capable of hydrolyzing wheat gluten (aggregates of prolamins and glutelins) into small peptides of a few amino acids (Shutov et. al. 1984). Protease G1 from soybean (which appears by 4 days after imbibition) specifically cleaves the acidic polypeptides of glycinin yielding a product of approximately 1.5 kilodaltons smaller (Wilson et. al. 1988). Soybean thiol protease K1 is active toward α, α' and b subunits of ß-conglycinin (Papastoitsis and Wilson 1991). Kunitz-type and Bowman-Birk-type trypsin inhibitors can be degraded by thiol protease K1 in soybean seed germination (Wilson et. al. 1988, Papastoitsis and Wilson 1991). Shutov et. al. (1984) and Wilson (1986) pointed out that only after this initial cleavage

does the protein become susceptible to further extensive proteolysis by other proteases.

Thiol proteases as hydrolytic enzymes not only appear temporally and spatially to accompany seed germination, but thiol protease genes can also be induced by water stress at other stages of the plant life cycle. Guerrero et. al. (1990) cloned a turgor-responsive *15a* gene in pea shoots that encoded a 363 amino acid protein with high similarity to the thiol protease papain. mRNAs *RD19* and *RD21* from *Arabidopsis thaliana* which are expressed in response to dehydration have high similarity to pea *15a* and tomato thiol protease *C14* (induced by low temperature) and rice oryzain α and β (Koizumi et. al. 1993). Williams et. al. (1994) also isolated a clone *A1494* from wilting *Arabidopsis thaliana* leaves that encodes a protein which has 80% similarity to RD19 and RD21. The putative protein was 86% identical to pea 15a. *A1494* is ABA-responsive, whereas *rd19* and *rd21* are not. Frosthoefel et al. (1996) isolated a cDNA clone *SEP1* that belongs to the thiol protease family from ice plant that is induced under drought and NaCl stress. However, little is known concerning the physiological role of these changes in thiol protease mRNAs (Callis 1995).

Purification of thiol protease has been proven to be inconsistent from species to species. A number of groups have now isolated and purified this enzyme which has been shown to be stable even at elevated temperature (Cohen et al. 1986, Morikawa et al. 1994), but for some species many obstacles hamper the use of conventional techniques to take the large step from partially pure to highly pure enzymes (Buttle 1994). Size exclusion chromatography and ion exchange or affinity chromatography have proven relatively inefficient at separating the enzyme from a background of proteins. Moreover a property of the enzyme in some species such as *Vicia faba* (Yu

and Greenwood 1994) and loblolly pine (unpublished data) is autodigestion. Thus, any purification procedure must be reasonably rapid.

To obtain highly purified thiol protease, Holwerda et al. (1990) expressed aleurain in *E. coli*. Hara-Nishimura et al. (1993) also expressed a thiol protease from a cDNA clone of *Schistosoma mansoni* in *E. coli* and Götz and Klinkert (1993) expressed the same cDNA clone in insect cells. The cDNA clone for loblolly pine thiol protease (*C4-1*) has recently been identified by Yinghua Huang in the Forestry Genetics Laboratory, Oklahoma State University. The clone shows a high degree of nucleotide sequence similarity to *Arabidopsis RD19*.

Our current project is to determine the physiological and genetic mechanisms responsible for the invigoration of loblolly pine seeds subject to controlled water stress through solid matrix priming. We hypothesize that the invigoration is due to a change in the sequence of events in the germinating seed where storage protein degradation and mobilization which normally commence after germination are stimulated to occur before radical emergence. We hypothesize thiol protease is a major enzyme involved in the degradation of reserve proteins when seeds are primed, as it is known to degrade reserve proteins, to be active during germination and to be up-regulated by water stress.

The objectives of this study were: 1) to overexpress the loblolly pine specific thiol protease in *E. coli* using *pMAL* expression system, 2) to purify the expressed thiol protease, 3) to partially characterize the purified thiol protease and 4) most importantly, to prepare antibodies against the thiol protease of loblolly pine for future studies in thiol protease abundance, localization and post-translational regulation of reserve protein proteolysis during priming and germination.

Materials and Methods

A full length of thiol protease cDNA clone was overexpressed in *E. coli* to produce the precursor of the thiol protease as an antigen to make antibody and to characterize further. In this study the *pMAL™-c2* vector (New England Biolabs, Beverly , MA) was used. The cloned thiol protease gene C4-1 from loblolly pine organgenic tissues was inserted downstream from the *malE* gene of the vector, which encodes maltose binding protein (MBP), resulting in the expression of an MBP fusion protein in *E. coli* (Guan et al. 1987, Maina et al. 1988). The expression system uses the strong "tac" promoter and the *malE* translation initiation to give high-level production of the fusion protein (Amann and Brosius 1985).

Construction of a fusion plasmid

All the DNA manipulations were performed following the standard techniques in Current Protocols in Molecular Biology (Ausubel et al. 1995). To produce a fusion protein in the *pMAL™-c2* vector (6,646 base pairs), the open reading frame of thiol protease was inserted into the *pMAL™-c2* vector so that it is in the same translation reading frame as the vector's *malE* gene. The vector was digested by *Bam* H I (Promega, Madison, WI) and *Sal* I (GibcoBRL, Gaithersburg, MD) at the polylinker site, resulting in a 6,634 base pairs linear fragment with both ends sticky. The insert containing the thiol protease open reading frame was prepared from a pBluescript SK plasmid. The pBluescript SK plasmid was digested by *Bam* H I and *Xho* I (Promega), resulting in a fragment containig the thiol protease insert with exactly complementary overhangs to the sticky ends of the vector. The large linearized fragment of *pMAL™-c2* vector and the 1522 bp cDNA fragment containing of thiol protease open reading frame were purified from 0.8% agarose gels using the Prep-a-Gene system (Bio-Rad,

Hercules CA) and were ligated using T4 DNA ligase (Stratagene, La Jolla, CA) so as to insert the cDNA sequence right after *malE* gene.

Transformation and growth of E. coli DH5α

The constructed fusion plasmid, and *pMAL™-c2* vector without insert as a control, were transformed into competent cells of *E. coli DH5α*. Transformed cells were spread on LB agar plates containing 100 mg/ml ampicillin. After incubation at 37°C overnight, colonies were picked onto master LB amp plate and a plate containing 80 mg/ml Xgal and 0.1 mM IPTG. *Lac* phenotype was determined on the Xgal plate and the 'white' clones were recovered from the corresponding patch on the master plate after 8-16 h incubation at 37°C.

Clone screening

Screening for the presence of insert and for correct reading frame were performed in three steps:

1) DNAs were prepared and digested with restriction nucleases of *Bam* H I and *Hind* III (Promega) and analyzed to determine the presence of the insert.
2) Using selected clones expression of the fusion protein was induced by IPTG and the cell proteins were analyzed by SDS-PAGE.
3) The DNAs were sequenced from positive clones using *malE* Primer and M13 Primer (New England Biolabs) to check both the 5' and 3' ends, respectively.

IPTG induced expression of the MBP-thiol protease fusion protein

The isolated clone containing the thiol protease peptide-coding DNA sequence in the correct reading frame was termed as *pMALEx-C4-1*. In a pilot experiment individual colonies of *pMALEx-C4-1* and also colonies of *E. coli DH5α* transformed with *pMAL™-c2* vector were used to inoculate 2 ml aliquots of LB medium containing 100 μg/ml ampicillin. The LB media were incubated at 37°C for 4 to 5 h with shaking at 225 rpm until the A_{600} of the culture reached 0.3. The cells were pelleted by centrifugation at 1,000 x *g*, 4°C for 1 min, resuspended in fresh media containing the antibiotic ampicillin to inoculate 20 ml aliquots of the same media. The cultures were then shaken at 37°C until the A_{600} reached 0.4, whereupon induction of fusion protein was achieved by the addition of IPTG to a final concentration of 0.3 mM. Incubation of the cultures was then continued for a further 1.5 h at 37°C.

Optimization of expression temperature and cell harvesting time

Immediately prior to and at 30 min (for 37 °C; 60 min for 28 °C; 120 min for 17°C) intervals following induction, the A_{600} values of the culture were measured and 1.5 ml samples of the cells were collected by centrifugation (5,000 x *g*, 22°C, 1 min). The pelleted cells were resuspended in 0.3 ml SDS-PAGE sample buffer and stored at -20°C. The resuspended cells were sonicated 6 times in 15 s pulses and centrifuged at 4°C, 9,000 x *g* for 20 min. The supernatants were designated as crude soluble fraction. The pellets were resuspended in 0.3 ml SDS-PAGE sample buffer and designated as insoluble fraction. Fifty μl soluble (or insoluble) fraction was mixed with 50 μl amylose resin (New England Biolabs) slurry and incubated on ice for 15 min. The mixture was centrifuged for 1 min, supernatant was discarded, and the pellet was washed with 1 ml column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM azide, 10

mM β-mercaptoethanol). The suspension was re-centrifuged for 1 min and the resin then suspended in 50 µl SDS-PAGE sample buffer as "protein bound to amylose". The samples were heated in a boiling water bath for 5 min and centrifuged for 1 min. The supernatants from uninduced cells, cells at different induced time or temperature, and the resin samples were loaded on 10 - 20% SDS-PAGE gels.

Purification of soluble fusion protein and thiol protease protein

The pilot experiment was scaled up from 20 ml to 250 ml culture medium. Cells were grown at 37°C until A_{600} reached 0.4 and continue to grow another 1.5 h after IPTG induction. The cells were harvested by centrifugation (3,000 x *g*, 4°C, 10 min) and the cell pellet was resuspended in 50 ml of 50 mM Tris-HCl pH 8.0, and 2 mM EDTA and recentrifuged. The cell pellet was immediately frozen in liquid nitrogen and stored at -70°C. Cells were extracted for total protein and for shearing DNA by sonication in short pulse of 9 x 15 s in 20 ml column buffer and centrifuged (10,000 x *g*, 4°C, 10 min). The supernatant was filtered through a MILLEX-HA 0.45 mm membrane filter unit (Millpore). The soluble protein extract was diluted 1:3 with column buffer. The pellet with inclusion bodies of fusion protein and cell debris was resuspended in 10 ml detergent buffer (200 mM NaCl, 1% deoxycholic acid (w/v), 1% IGEPAL (Sigma), 1 mM EDTA) and centrifuged (5,000 x *g*, 4°C, 10min). The supernatant was discard and the pellet was resuspended with 10 ml Triton (0.5%)-EDTA (2mM) and centrifuged (5,000 x *g*, 4°C, 10min). This step was repeated twice. The supernatant from the final centrifugation was removed and the pellet resuspended in 5 ml column buffer containing 6 M urea. The protein was incubated on ice for 1 h. Any remaining insoluble material was removed by centrifugation at 39,000 x *g*, 4°C for 20 min. The supernatant

was filtered through a 0.45 mm Millex-HA micron membrane before it was loaded on the column.

The prepared soluble protein extract was applied at 4°C to a 2.5 ml volume column (Novagen, Madison, WI) with amylose resin (New England Biolabs) previously equilibrated with column buffer. After application of the protein extract, the column was washed at 4°C with 12 volumes of column buffer. The bound proteins were eluted from the column with elution buffer (column buffer plus 10 mM maltose) and 0.5 ml fractions were collected. The fractions containing the fusion protein, as determined by standard Bradford's assays and SDS-PAGE, were combined and desalted, concentrated by ultra filtration (Centricon 30, Amicon, Beverly, MA). The desalted proteins were stored at -20°C.

Purification of the denatured fusion protein from inclusion bodies was similar to purification of soluble fusion protein except that 6 M urea was added to the column buffer and elution buffer for column equilibration, washing and elution.

Cleavage of fusion protein with Factor Xa and thiol protease purification

The MBP of the fusion protein was cleaved by the addition of Factor Xa (New England Biolabs) to a final concentration of 1% the amount of the fusion protein. The incubation was conducted at 4°C for 24 h. The cleavage was checked by SDS-PAGE. The thiol protease was then purified away from the cleaved MBP by a second passage over the amylose resin column. The purified thiol protease was eluted in the flow through fraction, was desalted, analyzed as before, assayed for activity and stored at -20°C.

Protein analysis by SDS-PAGE

Total proteins from *E. coli DH5α* transformed with *pMAL-C4-1* or with *pMAL™-c2* vector before and after induction with IPTG and proteins eluted from the amylose resin column before and after Factor Xa cleavage were analyzed by denaturing electrophoresis in 0.1% (w/v) SDS, 10 to 20% (w/v) polyacrylamide gels (Laemmli 1970). Protein samples were heated at 100°C for 5 min in 1x SDS-PAGE sample buffer (25 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.005% (v/v) bromophenol blue), centrifuged at 12,000 x *g* for 1 min and the supernatants were applied to the gels. The molecular weights were determined by the method of Weber et al. (1972). Molecular markers included: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; glutamate dehydrogenase 55.0 kDa; ovalbumin 42.7 kDa; aldolase, 40.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor 21.5 kDa and lysozyme 14.4 kDa (Promega). The gels were run according to Laemmli (1970) on a Bio-Rad Mini-Protean II (Hercules, CA), stained in 0.025 % (w/v) Coomassie Blue R-250, destained in 30% (v/v) methanol, 10% (v/v) acetic acid, dried on a Slab Gel Dryer (Model SGD4050, Savant, Germany) and scanned on HP Deskscan II (Hewlett, Packard).

Northern analysis

Total RNAs were extracted from induced *E. coli* cells that was transformed with the insert thiol protease coding sequence (*pMAL-C4-1*) or without the insert of the thiol protease (*pMAL™-c2* vector). The cultured cells of 50 ml (A_{600} = 0.5)were collected by centrifugation at 10,000 x *g*, 4°C, 10 min and resuspended in 10 ml protoplasting buffer (15 mM Tris-HCl pH8.0, 0.45 M sucrose, 8 mM EDTA). The cell wall was digested by addition of 80 µl of 50 mg/ml lysozyme and incubation on ice for 15 min.

Then the lysate was collected by centrifugation at 6,000 x g, 4°C for 5 min, resuspended in 0.5 ml lysing buffer (30 mM Tris pH 7.4, 0.1 M NaCl, 5 mM EDTA, 1% SDS (w/v), 100 mg/ml protease K (add just before use)) and incubated at 37°C for 5 min. After chilling on ice, 0.25 ml saturated NaCl was added to the suspension. After another 10 min incubation on ice, the suspension was microcentrifuged at high speed, 4°C for 10 min to remove the pellet. Two volumes 100% ethanol was added to the supernatant and precipitated at -20°C overnight. The RNA was pelleted at high speed microcentrifugation for 15 min, rinsed with 75% ethanol, dissolved in DEPC-treated water, and stored at -70°C.

Each RNA samples (20 μg) was subjected to denaturing electrophoresis with formaldehyde in (1%) agarose gels (Ausubel et al. 1995) and blotted onto Zeta-Probe membrane (Bio-Rad) according to the manufacture instruction. The bound RNA was hybridized at 42.5°C for 20 h with approximately 200 ng of PCR-[^{32}P]dCTP-labeled (5.4 x 10^{10} dpm mg^{-1}) 3' untranslated region of *C4-1* cDNA insert. After hybridization, the filters were washed successively in 100 ml of 2 x SSC (0.3 M NaCl, 0.03 M Trisodium citrate)/0.1% SDS (w/v) and 0.5 x SSC/0.1% SDS at 42.5°C for 15 min each, 0.1 x SSC/0.1% SDS at 65.0°C for 15 min and then rinsed in another 100 ml 2 x SSC at room temperature. The rinsed filters were subjected to autoradiography with X-OMAT (AR) X-ray film (Kodak).

Enzyme activity of thiol protease analysis

General protease activity was measured following Mitsuhashi et al. (1986) and Yu and Greenwood (1994) with modifications. 0.1 ml of enzyme solution (crude extract, purified fusion proteins before and after Factor Xa cutting) was pre-incubated with 10 μl

L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E64) to a final concentration of 150 μM or with 10 μl water on ice for 30 min. The solution was then mixed with 0.1 ml 4% (w/v) azocasein in McIlvaines buffer (20 mM citric acid, 60 mM NaH_2PO_4, pH 5.6, 0.02% [w/v] NaN_3) and incubated at 37°C for 2 h. The reaction was stopped by adding 0.8 ml 10% (w/v) TCA and vortexing. After standing at room temperature for 20 min, the solution was centrifuged at 16,000 x *g* for 10 min. Soluble peptides in the supernatant were determined by A_{340}. Controls for each sample were similarly treated, but without the 2 h incubation. One unit of enzyme activity is the amount of enzyme required to cause an increase of 1.0 in absorbency across a 1 cm path length, compared to the blank. Thiol protease activity is the difference between the enzyme activity with E64 incubation and that without E64 incubation.

Antibody production and immunoblotting

Denatured antigen of purified fusion protein was used for antibody preparation. The preliminarily purified fusion protein was further cleaned by preparative SDS-PAGE. After electrophoresis, the gel was soaked in 100 ml 0.25 M KCl in ice bath to show protein bands. The band of ~85 kDa was cut out, frozen in liquid nitrogen, and then ground into powder. The protein was eluted from the powder by addition of 1.5 ml PBS and shaking overnight at 4°C . The solution was centrifuged to remove gel residuals and the supernatant was concentrated by ultra filtration (Centricon 30). Polyclonal murine antibodies to the purified fusion protein from the soluble form and insoluble form were produced by induction of ascites fluid in John Sherwood's lab, Plant Pathology, Oklahoma State University, using 50 μg fusion protein dissolved in Freund's incomplete adjuvant over four weekly injections. After screening for antibody production by ELISA,

booster injections were conducted. Anti-fusion protein ascites were collected two weeks after the last injection.

The antibodies are a mixture mainly against both MBP and thiol protease. Polyclonal antibody was purified by first removing MBP-specific antibodies by repeated passage through a MBP-Sepharose column before isolation of thiol protease -specific antibody through a thiol-protease-MBP-Sepharose column according to the method of Holwerda et al. (1990). The affinity supports were made by coupling either 10 mg of crude MBP (purified from *E. coli* cells transformed with *pMAL™-c2* vector after IPTG induction) or 10 mg of SDS-PAGE purified MBP-thiol protease fusion protein to 1 g of cyanogen bromide-activated Sepharose (Pharmacia LKB Biotechnology Inc. Piscataway , NJ) in 0.1 M sodium borate, pH 9.0, containing 0.5 M NaCl, and 0.1% SDS. After extensive washing with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.9, and 150 mM NaCl), the antibodies were removed from their respective affinity columns by elution with 0.2 M glycine, pH 2.0, at room temperature. Eluted fractions were neutralized, followed by concentration and dialysis against TBS containing 0.1% sodium azide. Antibodies eluted from MBP-Sepharose and shown to be specific to MBP protein on immunoblots were used as a control.

An Immun-Lite chemiluminescent Assay kit (Bio-Rad) was used in Western blot analysis. Total proteins or purified proteins were separated by SDS-PAGE described as above. Mini Trans-Blot® system (Bio-Rad) was used to transfer the proteins to Immun-Lite membrane (Bio-Rad). Location of bound antibodies was determined using alkaline phosphate-conjugated secondary antibody Goat Anti-Mouse IgG-AP (Bio-Rad). Visualization of the antibody location was provided by the light signal emitted by a reaction of alkaline phosphorylase with chemiluminescent substrate (Disodium 3-(4-

methoxyspiro[1,2-dioxetane-3,2-(5'-chloro)-tricyclo[3.31.$1^{3,7}$]decan]-4-yl)phenyl phosphate). The blot was then exposed to X-OMAT (AR) X-ray film (Kodak) for 3 min.

Results

0.8% agarose gel electrophoresis of the digests of *pMAL™-c2* vector by *Bam* H I and *Sal* I yielded a 6,634 bp linearized fragment of vector and the digests of *p*BlueScript SK by *Bam* H I and *Xho* I yielded a 1532 bp cDNA fragment containing *C4-1* thiol protease open reading frame. These two fragments were ligated to form a recombinant termed *pMAL-C4-1*. Restriction digestion of the recombinant plasmid *pMAL-C4-1* with *Bam*H I and *Hind* III showed a ~600 bp and a ~900 bp fragments for the insert and a 6.6 kb fragment for the vector. Sequencing this construction from both the 5'end and the 3' end of the insert showed the full coding sequence of the cDNA fragment flanked by the *malE* gene and *lacZa* gene with no errors (data not shown). Transformation of *E. coli DH5α* cells with this *pMAL-C4-1* yielded the clone termed *pMALEx-C4-1* which contained the plasmid construct oriented correctly for expression of the maltose binding-thiol protease fusion protein.

Following induction by IPTG, *E. coli* cells of *pMALEx-C4-1* expressed a ~85 kDa protein on SDS-PAGE (Figure 1). Twenty-seven out of 30 selected clones transformed with the recombinant plasmid showed 85 kDa fusion protein by protein expression on SDS-PAGE. *E. coli DH5α* cells transformed *pMAL™-c2* vector alone expressed a ~51 kDa MBP-β-gal-α fusion protein (Figure 1). Non-induced cells failed to express fusion proteins (Figure1). The total proteins extracted at different times following the induction of the *E. coli DH5α* accumulated gradually during the 3 h tested period (Figure2). After centrifugation, however, protein samples from the supernatant and the pellet on SDS-PAGE showed that most of the fusion protein was in the form of inclusion bodies at 3 h

induction, and the maximum yield of soluble fusion protein appeared at 1.5 h induction (Figure 2).

To increase the yield of soluble fusion protein, cells were induced by IPTG at temperatures of 17°C, 28°C and 37°C for another 6 h, 3h, and 1.5 h, respectively. After induction, the A_{600}s remained the same as before induction. However, expression levels of the fusion protein in either soluble form or the form of inclusion bodies were very low at 17°C and 28°C (Figure 3).

Northern analysis of total RNA extracted from non-transformed *E. coli DH5α* and from *E. coli DH5α* transformed either *pMAL™-c2* vector or *pMAL-C4-1* construct, after induction with IPTG for 1.5 h, demonstrated that the synthesis of the fusion protein correlated with the synthesis of a novel 2.6 kb mRNA. This mRNA appeared only in *E. coli DH5α* transformed with *pMAL-C4-1* and only after IPTG induction (Figure 4).

The fusion protein of interest was mainly in the form of inclusion bodies. After sonication of the cells and centrifugation of the solution, the pellet was washed by detergent buffer and Triton-EDTA solution. The detergent buffer greatly washed out membrane bound proteins and Triton-EDTA also had certain effects to remove background proteins (Figure 5). Purification of the denatured fusion protein from the inclusion bodies or from the soluble form in supernatant by amylose column affinity chromatography yielded a dominant product of 85 kDa polypeptide and a number of minor products with lower molecular weights as determined by SDS-PAGE (Figure 6).

The time course of Factor Xa cleavage of the fusion protein is shown in Figure 7. Digestion was almost completed within 24 h. Cleavage of the MBP sequence with Factor Xa and removal of the MBP sequence and contaminating *E. coli* proteins by a second round of amylose affinity chromatography yield a ~ 42 kDa product.

Western blot analysis showed that the polyclonal antibodies of against the thiol protease protein recognized the fusion protein and the thiol protease purified after cleavage by Factor Xa (Figure 8). The antibodies also recognized three forms of thiol proteases in loblolly pine seeds with molecular weight ~150 kDa, 45 kDa and 31 kDa (Figure 9).

There was no significant difference of thiol protease activity between lysates of non-transformed *E. coli DH5α* cells and lysates of *E. coli* cells transformed with *pMAL-C4-1* when using azocasein as substrate. Neither was there between lysates of the transformed cells without insert and that with *C4-1* insert. (Table 1). Preliminarily purified fusion protein after amylose column and purified thiol protease from second round of amylose column after Factor Xa cut did not have thiol protease activity. This was true for either from soluble source or insoluble source (data not shown). The expressed thiol protease could not digest loblolly pine storage protein either (Figure 10).

Discussion

This study accomplished the expression, identification and characterization of recombinant gene products in *E. coli* system which resulted in several meaningful findings. The experiment showed that choosing proper restriction enzymes to obtain the complementary sticky ends makes gene construction much simpler and reduces error in the procedure of recombination. Using *pMAL* expression system, the tough thiol protease protein was fruitfully expressed in a fusion protein form. The purified product of thiol protease was used as an antigen against which thiol protease antibody was prepared. The antibody successfully recognized thiol protease protein in loblolly

pine, which provides one possible approach to study reserve protein mobilization involved in thiol protease and post-translational modification of thiol protease.

Complementary overhangs digested by different restriction enzyme simplified recombinant procedure and decreased error in construction. There were no available same restriction enzyme pairs to generate complementary overhangs in both pMAL-c2 vector and pBlueScript SK with thiol protease open reading frame insert. In this study, however, we used *Bam*H I and *Sal* I to restrict *pMAL™-c2* vector and *Bam*H I and *Xho* I to digest pBlueScript SK plasmid. The linearized vector and the prepared insert had exactly complementary sticky ends which increased ligation efficiency. Ninety percent of clones transformed with the recombinant plasmid by protein expression on SDS-PAGE showed 85 kDa fusion protein. All three sequenced constructs from selected clones showed no error in nucleotide sequences. However, an adapter ligated onto a created blunt end showed very low efficiency of ligation and transformation. And the selected white clones showed low percentage of expression of the fusion protein (unpublished data). Another strategy we tried was PCR (data not shown) to amplify the insert with the complementary overhangs to linearized vectors, which showed no expression of the fusion protein though they had right insert size observed on 1% agarose gel in five selected clones. Sequencing one of the clones demonstrated one base pair was missing that resulted in reading frame shift.

Temperature decrease during induction with IPTG did not increase the soluble proportion of the fusion protein. The expression system adopted in this study produced a large portion of insoluble inclusion bodies of the fusion protein when induction was conducted at 37°C. Lauritzen et al. (1991) and Takaki et al. (1988) increased soluble portion by lowering induction temperature. In contrast, this study showed lower temperature (17°C and 28°C) inhibited expression of the fusion protein

(Figure 3). TB1 *E. coli* host cells provided by the expression system did not work (data not shown).

Affinity chromatography in the purification of the fusion protein was highly efficient. The MBP-thiol protease fusion protein expressed in *E. coli DH5α* was purified nearly 90% from the other *E. coli* proteins in a crude cell lysate with a single passage over the amylose column followed by elution with maltose. Some smaller proteins from the *E. coli* crude cell lysate were found to be co-purified with the 85 kDa MBP-thiol protease protein after passage through the amylose column (Figure 7). It is speculated that some may have bound to the fusion protein through protein/protein interaction. That a second round over the amylose column yielded purer fusion protein supports this speculation. Some might be products of protein degradation during purification. A fact that protein of 28 ~ 29 kDa appeared before Factor Xa digestion and disappeared after the digestion (Figure 10) indicates this small protein might be the middle fragment of the fusion polypeptide containing a Factor Xa recognizing site. Indirect evidence to support the protein degradation is that only one band appeared on the Northern blot, which suggests the smaller proteins may not be from the partial transcription of the recombinant DNA. After Factor Xa digestion, the thiol protease protein was separated from the MBP by passing the mixture over amylose column again.

Antibodies against the MBP-thiol protease protein expressed in *E. coli DH5α* transformed with *pMAL-C4-1* construct were successfully used to analyze thiol protease protein levels in loblolly pine seed tissues. Antibodies against the denatured fusion proteins from both soluble source and insoluble source had high titers to the thiol protease expressed in transformed *E. coli* shown by ELISA analysis. However, the specific antibody titer to the thiol protease was lower from the insoluble antigen than

that from the soluble antigen. This indicates that the improper folding of the fusion protein during induction still has effects on the later proper refolding after denaturation. And this misfolding further influenced the production of antibodies that were against thiol protease protein conformation. Removal of anti-MBP and other supposed antibodies from the ascites fluid did not increase antibody specificity to thiol protease in loblolly pine tissues (Figure 10), demonstrating that the fusion protein was relatively pure.

Neither the crude extract from the transformed *E. coli* cells, the purified fusion protein, nor the purified thiol protease after Factor Xa digestion had thiol protease activity, even though the fusion proteins were from the soluble source (Table 1). There are several possibilities for the fusion protein being non-functional. First, misfolding might be responsible for non-functionality (Lauritzen et al. 1991, Takaki et al. 1988). We tried denaturation and then carefully refolding of the fusion protein but we failed to obtain a functional protein. Second, the MBP might interfere with the thiol protease function in the fusion protein. The fact that purified thiol protease after Factor Xa cutting could not digest protein suggests that the problem is within the thiol protease itself. The third possibility is that the expressed thiol protease may be a non-functional precursor of the mature enzyme. Direct and indirect evidences from various studies support the last possibility.

Most proteases are synthesized as high molecular-mass precursors which are post-translationally processed to the mature forms (Mach et al. 1994). The prosequences function in covering the active site (James and Sielecki 1986), intracellular transport (Chrispeels and Raikhel 1992) and correct folding of proteases (Vernet et al. 1991). Thiol proteases are also synthesized as precursors (Okamoto and Minamikawa 1995) which have N-terminal sequences (Mitsuhashi and Minamikawa 1989, Holwerda et al.

1990, Koehler and Ho 1990) and C-terminal sequence (Hara-Nishimura et al. 1993). Götz and Klinkert (1993) reported that, in insect cells expressing the complete cDNA sequence of *Schistosoma mansoni,* the human parasite, the putative thiol protease showed no proteolytic activity on hemoglobin and was not further cleaved to produce an active form. Hara-Nishimura et al. (1993) did not obtain the active thiol protease either with the full length cDNA expressed in *E. coli.* However, an active form of 36 kDa was observed in an *E. coli* expression system when the N-terminal signal sequence was deleted before transformation. In the current study, three forms of thiol proteases from loblolly pine seed tissues were shown on Western blot: ~150 kDa, 45 kDa and 31 kDa. It is speculated that the 31 kDa might be the active form, the 45 kDa might be the precursors and the 150 kDa might be multimers of the thiol protease.

Conclusions

The thiol protease of loblolly pine was expressed in *E. coli* in the form of a fusion protein with MBP. MBP-thiol protease fusion protein was purified to homogeneity on an amylose affinity column. After cleaving the MBP leader sequence, the fusion protein yielded a recombinant enzyme containing 15 extra amino acids (ISEFGSPGLQEFGTR) before the start codon and 386 amino acids (42.169 kDa) derived from the open reading frame. Neither the fusion protein nor the thiol protease after cleavage showed thiol protease activity using azocasein or storage protein of loblolly pine seeds as substrates. Antibodies against the expressed fusion protein were made and they recognized three thiol protease members from loblolly pine seeds.

Future studies

In conclusion, we summarize briefly some areas in which there is the most potential for future progress. First, regarding thiol protease, it is synthesized as a precursor as reported in numerous studies and the antibodies made against the expressed recombinant protein have led to the ability to approach post-translational processing to a mature enzyme in loblolly pine. Whether the proform of the thiol protease is processed autocatalytically or requires proteolytic activity from another protease or needs glycosylation could be elucidated.

Second, although the expressed thiol protease is inactive, the antibodies to it provide a way to efficiently purify the enzyme by immunoaffinity chromatography from loblolly pine tissues. Large amount purified enzyme would make it possible to crystallize the protein to analyze the three dimensional structure and catalytic properties.

Third, *E. coli* expressing the recombinant with deleted leading sequence might be an alternative to obtain large amount active thiol protease.

Finally, we hypothesize that solid matrix priming (SMP) treatment (a kind of water stress) of loblolly pine seeds involves the degradation of storage protein before germination and thiol protease is involved in the process. Since numerous articles report thiol protease activity in seed germination and induction by water stress. The antibodies in this study are beginning to bear fruit in studies of thiol protease protein changes during SMP and subsequent germination by Western blotting. The antibodies could also be applied to immunolocalization of the thiol protease to clarify its function, and be helpful to dissect the post-translation process at sub-cellular levels.

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Table 1. Azocaseinase activities of *E. coli* cell lysates and purified MBP-thiol protease fusion protein. One unit of enzyme activity is the amount of enzyme required to cause an increase of 1.0 in absorbency across a 1 cm path length, compared to the blank. The values in the table represent for means and standard deviation of three replicates of each treatment.

Treatments	Azocaseinase activity (units)	
	+E64	-E64
Non-transformed cells	1.05±0.12	1.09±0.08
Transformed with PMAL-c2 (-IPTG)	0.96±0.07	0.99±0.13
Transformed with PMAL-c2 (+IPTG)	1.18±0.19	1.03±0.14
Transformed with PMAL-C4-1 (-IPTG)	1.08±0.24	1.15±0.16
Transformed with PMAL-C4-1 (+IPTG)	1.23±0.16	1.02±0.08
MBP-thiol protease fusion protein (-Factor Xa)	0.07±0.06	0.10±0.12
MBP-thiol protease fusion protein (+Factor Xa)	0.02±0.01	0.03±0.12

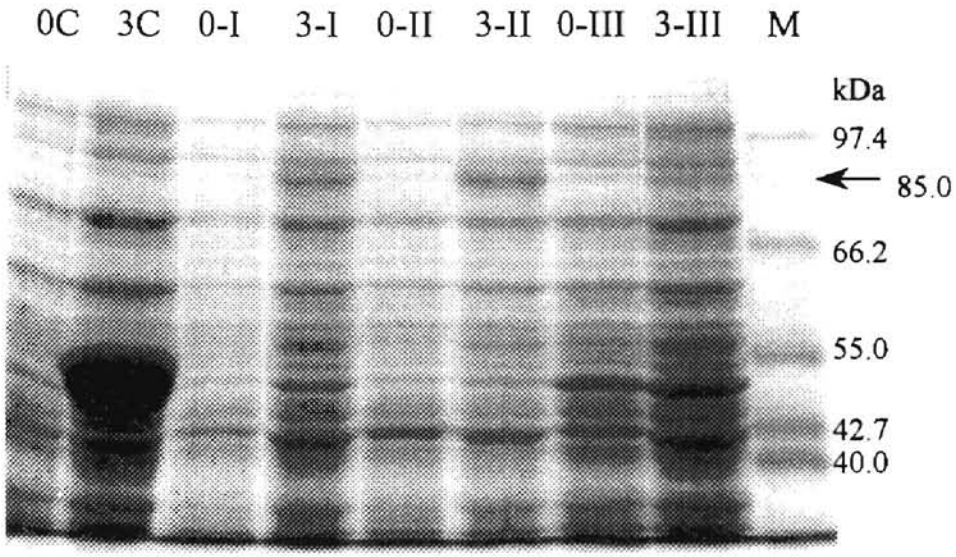

Figure 1. Clone screening by fusion protein expression . Value of label 0 and 3 represents before IPTG induction and 3 hours after IPTG induction, respectively. Letter C, *E. coli* cells transformed with *pMAL-c2* vector. I, II, III, different clones transformed with *pMAL-C4-1* construct. M, protein standard. Total protein from 50 μl cultures was loaded onto 10 % SDS-PAGE each lane and followed by Coomassie Blue staining.

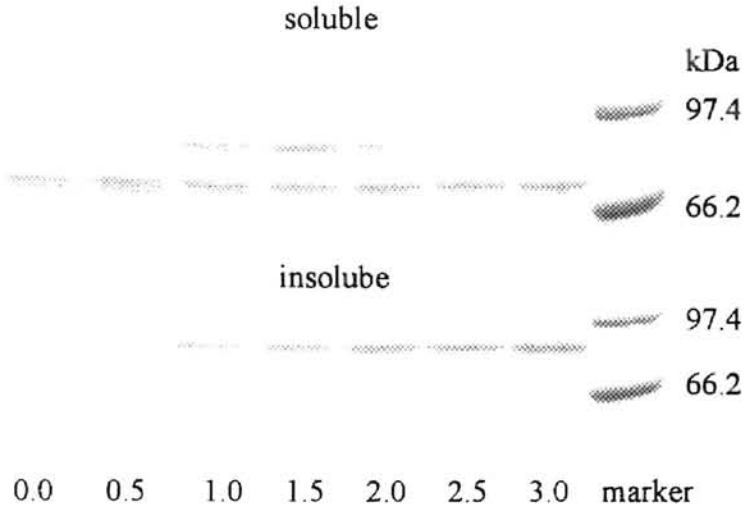

Figure 2. Time courses of soluble and insoluble MBP-thiol protease fusion protein induction by IPTG in *E. coli DH5α* transformed with recombinant plasmid of *pMAL-C4-1*. Total protein from 50 μl cultures was loaded onto 10 % SDS-PAGE and followed by Coomassie Blue staining.

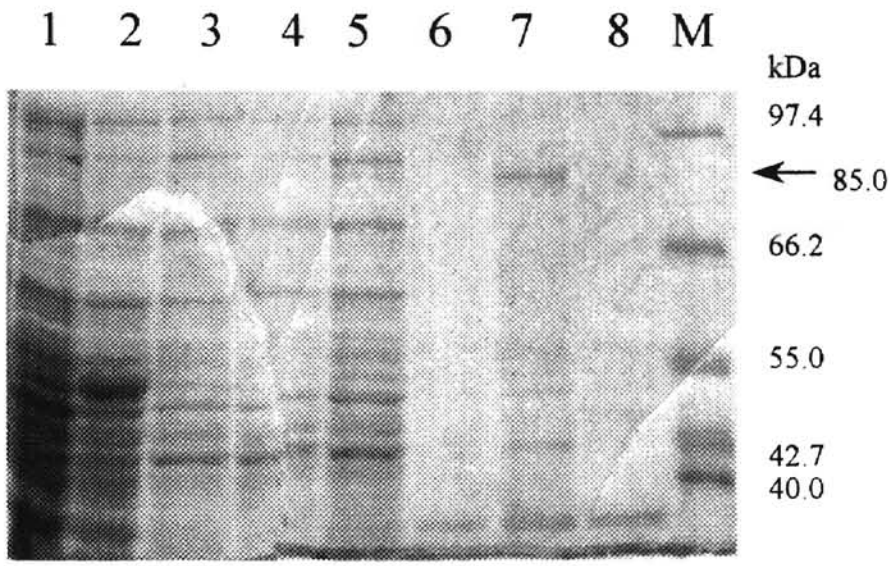

Figure 3. Soluble and insoluble fusion protein induction by IPTG at different temperatures. Lane 1, lysate of *E. coli* transformed with *pMAL-c2* vector before IPTG induction. Lane 2, lysate of *E. coli* transformed with *pMAL-c2* vector after 1.5 h IPTG induction. Lane 3, 4, 5, soluble protein extracted from *E. coli* transformed with *pMAL-C4-1*. Lane 6, 7, 8, insuluble protein extracted from E. coli transformed with *pMAL-C4-1*. Lane3, 6, cell cultured at 17°C for 6 h. Lane 4, 8, cell cultured at 28°C for 3 h . Lane 5, 7, cell cultured at 37°C for 1.5 h.Total protein from 50 μl cultures was loaded onto 10 % SDS-PAGE and followed by Coomassie Blue staining.

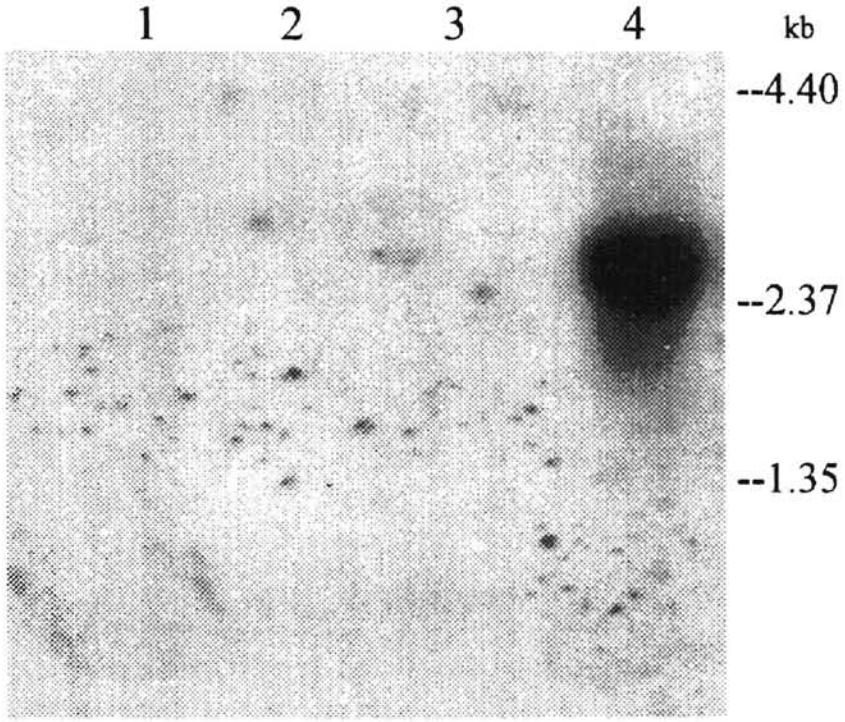

Figure 4. Northern analysis of the RNA extracted from (1) Nontransformed *E.coli DH5α* cells, (2) transformed with *pMAL-c2* vector, (3) transformed with *pMAL-C4-1* construct beforeIPTG induction, (4) transformed with *pMAL-C4-1* construct after 1.5 hours of IPTG induction. 20 μg of total RNA was loaded onto each lane. The *Zeta-Probe membrane* was hybridized to PCR radiolabeled 3' untranslated region of loblolly pine *C4-1* thiol protease cDNA insert.

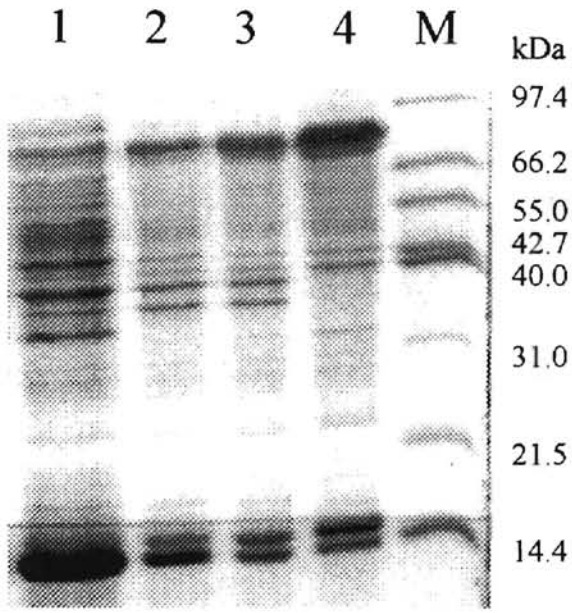

Figure 5. Pre-column purifications of fusion inclusion bodies by washing steps. 1. suspension of pellet from cell lysate, 2. after washing by detergent buffer, 3. after washing by Triton-EDTA solution, 4. supernatant of pellet suspension in 1% SDS.

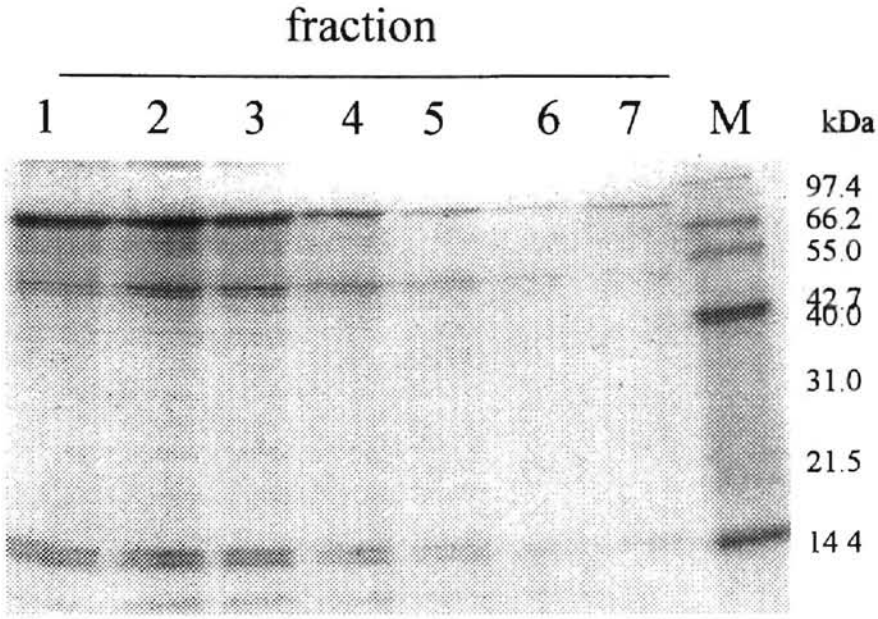

Figure 6. SDS-PAGE analysis of fusion protein fractions eluted from amylose column with column buffer with 10 mM maltose. M, Protein standard.

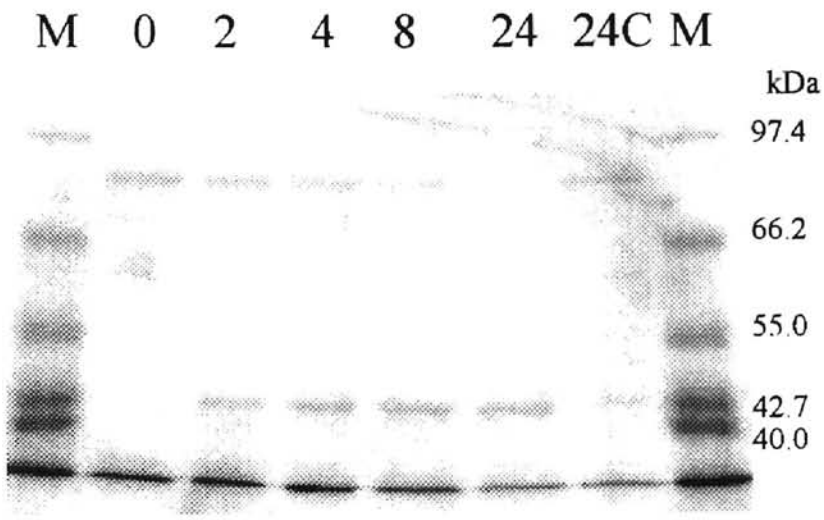

Figure 7. Time course of Factor Xa digestion of MBP-thiol protease fusion protein. The values are the hours of digestion at 4°C . M, Protein standards. 24C, 24 hours digestion without Factor Xa.

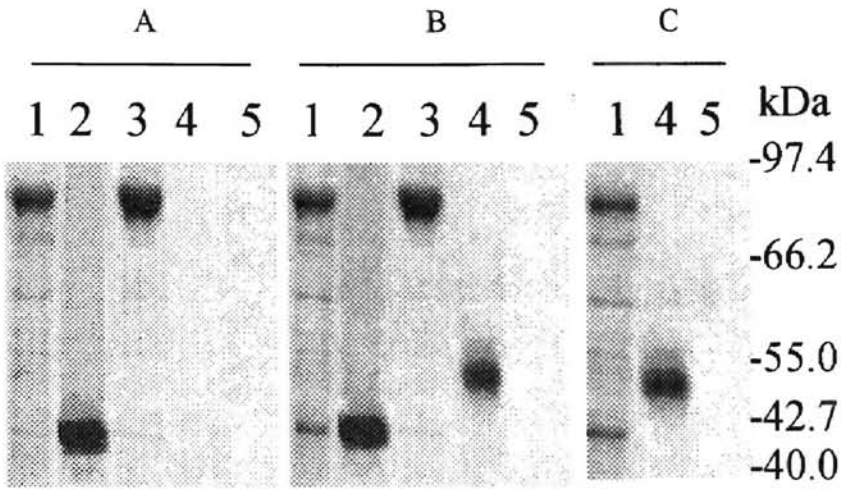

Figure 8. Western blot analysis of maltose binding fusion protein and Factor Xa cleaved thiol protease protein. A: antibodies against protease; B: antibodies against fusion protein; C: antibodies against MBP. A and B were transblotted one after another from the same gel. Lane 1: 100μg of total protein from induced *E. coli* cells transformed with *pMAL-C4-1*; lane 2: 2 μg of thiol protease protein purified from second round amylose column after digestion by Factor Xa; lane 3: 2 μg of fusion protein before Factor Xa digestion, lane 4: 25 μg of total protein from induced *E. coli* cells transformed with *pMAL-c2* vector, lane 5; 100 μg of total protein from induced E. coli cells without transformation.

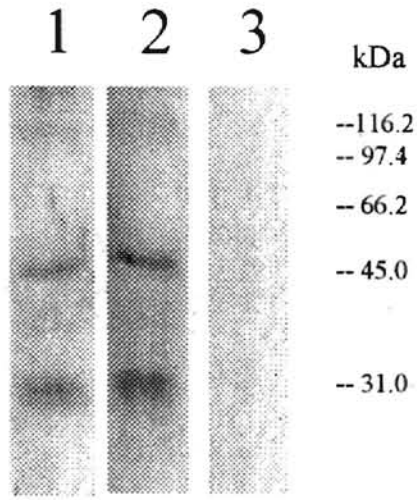

Figure 9. Western analysis of thiol protease protein in seed embryos of loblolly pine. All three lanes were identically loaded 100 mg total protein extracted from loblolly pine embryos and were fractioned by 20% SDS-PAGE. Each lane was cut from the transblotted membrane and was treated with MBP-thiol protease fusion antibody (lane 1), thiol protease antibody (lane 2) or MBP antibody (lane 3).

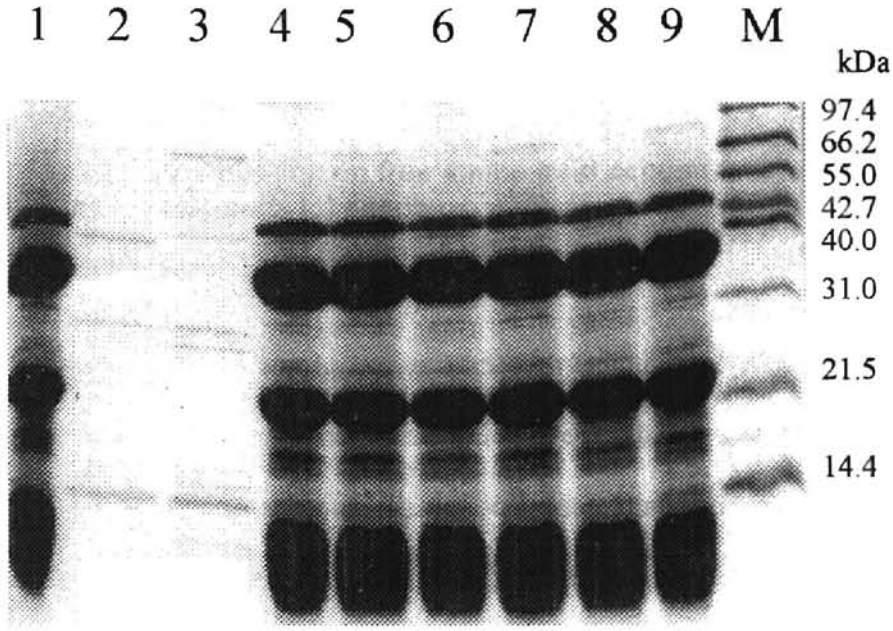

Figure 10. Digestion effects of purified MBP-thiol protease protein by Factor Xa on crystalloid protein of loblolly pine seed embryos. Lane 1: control, no fusion protein was added; lane 1, 4, 5, 6, 7, 8, 9 were loaded with 25 μg crystalloid protein of loblolly pine; lane 2, 4, 6, 8:fusion protein with Factor Xa incubation; lane 3, 5, 7, 9: fusion protein without Factor Xa incubation; lane 6, 7: E64 added to a final concentration of 50 mM before incubation.

Chapter II

Effects of solid matrix priming on free amino acid accumulation and water status in loblolly pine (*Pinus taeda* L.) seeds

Abstract

Several experiments were conducted to approach solid matrix priming (SMP) effects on free amino acid accumulation and water status in seed tissues of loblolly pine (*Pinus taeda* L.). Concentrations of most free amino acids increased after two d SMP and remained high until the late stage of SMP in the embryo. Proline, abundant in non-primed seed tissues, increased 2.5-fold at four d SMP, and contributed more than 50% of the osmotic adjustment generated by total amino acids. Water potential and osmotic potential consistently decreased in embryo during SMP. In addition, the content of all free amino acids increased after four d germination in both embryo and megagametophytes. Prepriming treatment of seeds with thiol protease inhibitors (E64, $CuCl_2$, $HgCl_2$) diminished the SMP effects on free amino acid accumulation, osmotic adjustment, and enhancement of thiol protease activity. The results suggest that thiol protease may be induced by SMP and involved in protein degradation that generates free amino acids for osmotic adjustment and seed invigoration.

Introduction

Improvement of seed germination by solid matrix priming (SMP), a controlled water stress, has been reported in many species (see reviews by Khan 1992, and Pill 1995). SMP delays water absorption of seeds that prepares the seed population towards germination more completely and synchronously (Khan et al. 1980/1981). Numerous

articles have reported that plant cells adapt to water stress by osmotic adjustment (Morgan 1984, Rhodes et al. 1986, Voetberg and Sharp 1991, Good and Zaplachinski 1994). Amino acid accumulation, one of the best characterized osmogulatory responses, has been shown to occur in a variety of monocot and dicot tissues (Good and Zaplachinski 1994). Transcripts of thiol protease (one of the four major proteases in organisms) can be induced by water stress (Guerrero et al. 1990, Koizumi et al. 1993, Williams et al. 1994). Thiol protease is known to initiate the hydrolysis of storage proteins during germination (Samac and Storey 1981, Wilson et al. 1988, Koehler and Ho 1990a, 1990b, Watanabe et al. 1991). Protein rich seeds are characteristic of conifers (Salmia 1981, Groom et al. 1991). However, studies of water stress (SMP) effects on amino acid accumulation, osmotic adjustment and protease activity to enhance seed germination have not been reported.

We hypothesize that thiol protease is a major enzyme involved in the degradation of reserve proteins when seeds are primed, as it is known to degrade storage proteins, to be active during germination and to be up-regulated by water stress. The objectives of this study, as one effort to test our hypothesis, were to determine 1) whether seed tissues accumulate free amino acids during SMP, 2) if so, how much is the amino acid contribution to the water potential, 3) whether osmotic adjustment occurs in embryos during SMP, 4) whether thiol protease activity increases during SMP and 5) whether prepriming treatments of thiol protease inhibitors counteract the effects of SMP on free amino acid accumulation, enzyme activity and embryo water status.

Materials and methods

Seeds. A seed lot produced in a seed orchard from an open-pollinated loblolly pine family (Oklahoma source), designed as OK100, was provided by the Oklahoma Division

of Forestry. The seeds were kept at 8% moisture content in air-tight containers at -20°C until use.

SMP. Before SMP, seeds were imbibed in aerated distilled water at 22°C for 24 h and stratified at 4°C in sealed plastic bags for 60 d in the dark. Carri-All matrix (American Colloid Co., Arlington Heights, IL, USA), was used with 30% moisture content (dry weight base, corresponding water potential was -1.5 MPa). The ratio of seed to matrix was 1:3 (dry weight basis). Seeds were primed for 6 d in darkness at 22°C. Each of SMP experiments had three replicates with 50 seeds per replicate.

Inhibitor treatments. All the inhibitor treatments were conducted before SMP except as specified. A series of inhibitor concentrations for $CuCl_2$, $HgCl_2$ and E64 were tested to see their effects on seed germination. Each treatment had three replicates with 50 seeds per replicate. E64 was first dissolved in 1:1 ethanol to water and the final ethanol concentration was 0.89%. Therefore, all other inhibitors were dissolved in 0.89% ethanol and 0.89% ethanol was used as control. Based on preliminary study of germination performance the optimal concentration of each inhibitor to use for other index measurements was chosen as 5 mM $CuCl_2$, 2 mM $HgCl_2$, 0.05 mM E64. In one treatment, 20 mM cysteine was added to the 2 mM $HgCl_2$ treatment to see reversible effects. Stratified loblolly pine seeds were vacuum infiltrated with each inhibitor for 12 h at room temperature in darkness. After vacuum infiltration, seeds were washed three times in sterile water and blotted dry for SMP, for germination or for protein extraction.

Germination. Seeds were placed in a germination cabinet after stratification with and without SMP according to the experimental designs. The cabinet had a 16 h photoperiod; dark and light temperatures were 20°C and 30°C, respectively. Seeds

were germinated on 7.0 cm diameter Whatman #3 filter paper with 2.0 ml sterile water in a 7.5 cm diameter sterile petri dish.

Free amino acid extraction. Free amino extraction followed the procedures of Rhodes et al. (1986) and Voetberg and Sharp (1991) with modifications. Each treatment had three replicates with 50 seeds per replicate. The embryos and megagametophytes were dissected from the seeds harvested at different times of SMP or subsequent germination and immediately placed separately into liquid nitrogen. After weighing, the dissected embryos or megagametophytes were directly extracted in 5 ml methanol in a scintillation vial for 36 h at 22°C with gentle shaking. The vials stood at 4°C for 12 h, the methanol extract was collected. The same procedure to extract remaining amino acids was repeated with another 5 ml methanol. The two extractions were combined and stored at -20°C prior to amino acid analysis.

Chloroform and water were then added to the aliquots of methanol extracts to give a ratio of 2:2:1 methonal:chloroform:water. The phase was separated by centrifugation at 10,000 ×g at 4°C for 10 min. The aqueous phases were transferred to a new tube and same volume of chloroform was added to the new tube with the same ratio. The above step was repeated.

Free amino acid analysis. L-onrithine, as an internal standard, 0.625 nmols was dissolved in 10 mM HCl and added to 60 µl of the aqueous extracts and processed for amino acid analysis. Prior to derivatization, the sample in a 1.5 ml microcentrifuge tube was dried at 40°C with a SVC-100H Speedvac Concentrator (Savant Instruments Inc., Farmingdale, NY, USA). The dry sample was dissolved in 5 µl 10 mM HCl. The volume was brought up to 30 µl with 20 µl 0.2 M borate buffer, pH 8.8 and 5 µl 10 mM 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in acetonitrile (Cohen and

Michaud 1993). Standard amino acids with known concentration were derivatized in the same way as samples.

The free amino acids were separated by capillary electrophoresis (CE) following the procedure of Zhang et al. (unpublished data). A custom-built instrument for CE included a Spellman (Plainview, NY, USA) model CZE 1000R high voltage power supply with positive and negative polarity and a model FL-750BX fluorescence detector (McPherson Instruments, Acton, MA, USA). The detector was equipped with a cell for on-column capillary detection and a 200 W Xenon-Mercury lamp with the exitation wavelength set to 245 nm; a cut-off filter permitted detection of emission beyond 380 nm. Chromatographic data were collected using custom-built data logger (Merz and Mort 1992) and downloaded into a Power Machintosh 8100/80 computer. Chromatography data were viewed and quantitated by using the program Analog Connection Chrom (Strewberry Tree Computer, Inc., Sunnyvale, CA, USA) and Kaleida-Graph, developed by Abelbeck, software (distributed by Synergy Software, Reading, PA, USA). A 800 mm fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm ID (360 μm OD) was used as the separation column. The length was 500 mm to the detection window. Each new capillary was activated by flushing with 50 μl of 0.1 M NaOH, rinsing with D-H_2O and then running buffer (6% SDS in 30 mM borate buffer, pH 9.0). The running buffer was degased for 10 min before use. Each sample was loaded by gravity-driven flow for 10 s. All separations were conducted at 18 kV (40 μA) at 22°C. The detector conditions were set as: Gain, 5.98; Range sensitivity, 0.03; Time constant, 0.5. Between runs, the column was rinsed with nano-pure water, methanol, 0.1 M NaOH, nano-pure water and running buffer. The buffers in the reservoirs were renewed at an interval of five runs to maintain reproducible separation.

Contributions of different amino acids and total amino acids to the accumulation of osmotic potential were estimated using measured water content, and differences of amino acid content between primed and non-primed seeds with the conventional formula:

$$\psi_\pi = - nRT / V . \qquad (1)$$

where R is the gas constant (0.0083125 $kJ \cdot K^{-1}mol^{-1}$), T the absolute temperature (295 K), V the volume of water (L), n the number of moles of amino acids, and the unit of ψ_π is MPa. The contributions of different amino acids were calculated on the basis of total water content.

Water relations measurements. Treatments were applied to samples of 30 seeds which were divided into two groups of 15 seeds each. All measurements were conducted on three replicates. Seeds were quickly excised into embryos and megagametophytes to measure moisture content and potential. The small volume embryos were placed in 0.6 ml microcentrifuge tubes and the large volume megagametophytes were placed in 1.5 ml microcentrifuge tubes. Fresh weight (fw) and dry weight (dw, after 72 hours, 70°C) were measured to calculate moisture content (MC = (fw - dw)*100/dw). Embryo water potential was measured with leaf cutter psychrometers and megagametophyte water potential with chambered in situ psychrometers (Merrill Specialty Equipment, Logan, UT) and were read using a Wescor HP-115 water potential data system (Wescor, Logan, UT). All psychrometers were individually calibrated against KCl standards. Measurements were made in a constant temperature water bath. After total water potential (ψ_w) was measured, the chamber was frozen in liquid nitrogen, warmed with hot water and measured for

osmotic potential (ψ_π). Turgor potential was calculated according to the following equation:

$$\psi_w = \psi_\pi + \psi_p \quad (2)$$

Crude enzyme extraction and enzyme activity analysis. Crude endopeptidase extraction and enzyme activity analysis were conducted according to Yu and Greenwood (1994) with modifications. All manipulations were performed at 4°C except where specified. Fifty embryos or megagametophytes at each time point of treatment that were dissected and then kept in liquid nitrogen were ground with liquid nitrogen. The powder of each treatment was homogenized in 5 ml of McIlvaine's buffer (20 mM citric acid, 60 mM NaH_2PO_4, pH 5.6, 0.02% [w/v] NaN_3) containing 1 mM dithiothreitol (DTT). The homogenates were centrifuged at 33,000 ×*g* for 30 min. The supernatant was filtered through Whatman No. 1 filter paper and protein content was immediately measured for enzyme analysis (Bradford 1976).

Endopeptidase activity was measured following Mitsuhashi et al. (1989) and Yu and Greenwood (1994) with modifications. Enzyme solution, 0.3 ml, was mixed with 0.3 ml of 4% azocasein in McIlvaine's buffer and incubated at 37°C for 2 h. The reaction was terminated by addition of 2.4 ml of 10% [w/v] TCA and vortexing. After standing at 22°C for 20 min, the solution was centrifuged at 16,000 ×*g* for 10 min. Soluble peptides in the supernatant were determined by A_{340}. Controls for each sample were similarly treated, but omitting the incubation step. One unit of enzyme activity equals the amount of enzyme required to cause an increase of 1.0 in absorbency across a 1 cm path length, compared to blank. Thiol protease activity was estimated as E64-sensitive endopeptidase activity by pre-incubation with 50.0 mM E64 at 4°C for 30 min.

Results

Solid matrix priming (SMP)

Total free amino acids. Loblolly pine seeds were sampled at different times of SMP or subsequent germination to characterize the free amino acid pattern in embryos or in megagametophytes. The content of total free amino acids in embryo of 4 d primed seeds increased 2.66 fold compared to the embryos from non-primed seeds. There was a transient decrease from 0 to 1 d SMP (Figure 1). After 4 d SMP, content of total free amino acids in embryos decreased and then increased once the seeds were placed under germination conditions; the sharpest increase occurred after 4 d germination (Figure 1) when the radical started to emerge.

The general tendency of free amino acid concentration in megagametophytes was to decline except for a few short increases during SMP and subsequent germination (Figure 1). The first peak appeared at two d priming, the second peak at first d of germination and the last increases occurred after two d of germination. For example, total free amino acid concentration at day two SMP increase 20% compared with that at day one SMP.

Total free amino acid content per embryo almost equaled the amount per megagametophyte (Figure 1). However, amount of free amino acids in megagametophytes per unit weight was far lower than that in embryo, since the weight ratio of embryo to megagametophyte was 1:6.65.

Individual free amino acids. According to the contribution to the decrease of osmotic potential during SMP, the tested free amino acids were artificially divided into two groups: free amino acids in the first group gave more than 4% of total contribution of the osmotic accumulation and free amino acids in the second group contributed less

than 4%. Gly and Gln could not be separated so the two amino acids were put together. The first group included Ala, Asp, Cys, Glu, (Gly+Gln), Pro, Thr, Val, and the second group included Arg, Hyp, Ile, Leu, Lys, Met, Phe, Ser, and Tyr. Regardless of tissue and amount, every individual free amino acid showed increasing tendency in concentration during late stages of germination.

As happened with the total free amino acid content, the amount of each amino acid per embryo was raised by SMP, decreased during late stages of SMP and then increased during germination (Figure 2). Proline was a representative in the first group. While the amount of most free amino acids per megagametophyte showed a transient increase from the first day to the second day of SMP (Figure 3), some amino acids such as Thr, Ile and Phe did not show this increase at this time (Figure 3).

Embryo free amino acids in the second group showed a little increase during SMP except hydroxyproline which sharply increased similar to proline (Figure 2c, d). In the second group only lysine in megagametophyte showed a transient increase at early stages of SMP, while arginine and and serine appeared to increase their concentrations during late stages of SMP (Figure 2d).

Contribution of free amino acids to embryo osmotic potential during SMP. The net increase of total free amino acid content from 0 to 4 d SMP contributed about 0.315 MPa to the decrease of osmotic potential in the embryo (Table 1). This accounted for more than half of the total change of osmotic potential during SMP. Among the free amino acids, proline contributed the most (25%) (Table 1). The next was asparate (14%). The contribution order could be arranged as follows: Pro>Asp>Glu>Ala>Cys>Gly+Gln>His>Val>Thr>Tyr>Phe>Arg>Lys>Ile>Leu.

Water relations. In 4 d SMP, water potential and osmotic potential in embryo decreased 0.59 MPa and 0.65 MPa, respectively. However, water content did not decrease but slightly increased in this period. Turgor potential showed no significant difference between embryos from non-primed and 4 d primed seeds (Table 2).

Thiol protease activity. Thiol protease activity of the embryo extracts from 4 d primed seeds increased 39 fold compared to non-primed seeds (Table 2).

Thiol protease inhibitors

In order to determine the role played by thiol protease in free amino acid increase, thiol protease inhibitors such as E64 and $CuCl_2$ were used to treat the seeds prior to priming. Free amino acids were analyzed both in embryos and megagametophytes.

Free amino acids in embryos. Total free amino acid amounts in embryos from $CuCl_2$ treated seeds was 31% of the control; while the amount in embryo from E64 treated seeds was 40% of the control (Table 3). Table 3 lists inhibited percentages of individual and total amino acids by thiol protease inhibitors. Accumulations of Ala, Asp, Glu, Gly+Gln, His, Lys, Met and Pro were inhibited by either $CuCl_2$ or E64. Accumulation of arginine was the least inhibited by $CuCl_2$ and E64. Increases of Ile, Ser, Thr and Val were inhibited more by $CuCl_2$ than by E64. In contrast, accumulation of phenylalanine was inhibited more by E64 than that by $CuCl_2$ (Table 3). Cysteine could reverse the inhibition in most amino acids; Figure 4 shows asparate as an example.

Free amino acids in megagametophytes. In contrast to the amount in embryos, the accumulation of free amino acid in megagametophytes was relatively less inhibited. $CuCl_2$ inhibited 32% of the total accumulation and E64 inhibited 49% of the total

accumulation (Table 3). Accumulation of arginine in megagametophyte could be stimulated by either $CuCl_2$ or E64. Another thiol protease inhibitor $HgCl_2$ treatment prior to SMP also increased the amount of arginine (data not shown). Different inhibition patterns were observed between embryos and megagametophytes for the individual amino acids (Table 3).

Embryo potentials and water contents. Thiol protease inhibitor E64 negated the effects of SMP on the decreases of water potential and osmotic potential (Table 3). E64 had no influence on the embryo water content and turgor potential (Table 3).

Thiol protease activity. If the seeds were treated with thiol protease inhibitor E64 prior to priming, the activity of thiol protease from embryo extract was decreased 8-fold (Table 2).

Discussion

The main contribution of the research reported here was to show that amount of free amino acids in embryos increased during the SMP process and that they accounted for more than half of the total accumulation of osmotic potential induced by SMP. In addition, the increase of the free amino acids in both embryo and megagametophyte during SMP was inhibited by thiol protease inhibitors. This was the first study to approach the relationships between controlled water stress, thiol protease and free amino acids prior to and during seed germination in tree species.

Most of the amino acids measured demonstrated a characteristic accumulation in embryos of loblolly pine during two to four d of SMP, followed by a transient decline, and then the content of free amino acids increased again during subsequent germination (Figure 1 to 3). The increase in free amino acid concentration in embryo

during SMP was not from simply concentrating the solutes, since embryo moisture content did slightly increase rather than decreasing during SMP. Proline was an actively accumulated amino acid in terms of the total amount, which accounted for 25% at 4 d SMP and for 42% at 6 d SMP out of all the amino acids accumulated. The accumulated proline contributed 25% of the 0.315 MPa change in osmotic potential. The contribution to the decrease of osmotic potential was lower than that that has been found in maize root tips where proline itself accounted for a 0.29 MPa drop in osmotic potential under water stress (Voetberg and Sharp 1991). This suggests that the way proline accumulated in loblolly pine embryo during SMP may differ from maize root tips which was involved an active biosynthesis of proline under water stress (Voetberg and Sharp 1991). It is postulated that the proline accumulated in embryo of loblolly pine might, at least at early stages of SMP, be from digestion of storage proteins. That almost all individual amino acids had the same increasing tendency supports this postulation. Alternatively, we can not rule out the possibility that the accumulation of free amino acids might be transported from the surrounding tissue of megagametophyte. The opposite tendencies of free amino acid increase in embryo and of decline in megagametophyte during 2 to 4 d SMP provides indirect evidence for the second possibility (Figure 1).

Free amino acid analysis using thiol protease inhibitors did not support the second scenario. The inhibition of free amino acid accumulation in embryo during SMP by prepriming treatment with thiol protease inhibitors indicated that digestion of proteins occurred in this period (Table 1). A direct evidence was that E64 negated the SMP effects on enhancement of thiol protease activity. Of the four major classes of proteases described in plant cells, thiol proteases from seed and vegetative tissues have been the most widely investigated. Thiol protease gene expression is not only

developmentally regulated, such as in germinating seeds, but thiol protease mRNAs also accumulate in stressed plants, consistent with a role in proteolytic degradation of polypeptides that have been denatured by exposured to environmental stress. Low or high temperature stress (Schaffer and Fisher 1988, 1990), salinity and drought stress (Guerrero et al. 1990, Koizumi et al. 1993) and exposure to ABA (Williams et al. 1994) are among the factors that induce transcript accumulation. Consistent with these experiments, related studies in our laboratory at levels of transcription, translation and potential substrate and products of thiol protease support SMP induced thiol protease that in turn digested proteins (data not shown). A 1.5 kb thiol protease mRNA accumulation in embryo of loblolly pine peaked at two d SMP. A 31 kDa anti-thiol-protease reactive polypeptide increased its level after 4 d SMP shown on Western blotting. Moreover, buffer soluble protein increase after 2 d SMP and buffer insoluble storage protein decrease during SMP were closely correlated to the accumulation of free amino acids and the increase of thiol protease activity in embryos. In addition, thiol protease inhibitor E64 negated SMP effects on seed germination improvement. With the results of free amino acid analysis, all the above support the overall hypothesis that the invigoration of loblolly pine seeds subjected to controlled drying through SMP may be due to a change in the sequence of events in the germinating seed where storage protein degradation and mobilization which normally commence after germination are stimulated to occur before radical emergence. The accumulation of amino acids from mobilization of reserve proteins provides 1) osmotically active substances for osmotic adjustment leading to increased capacity of the embryo to grow and 2) the components for synthesis of new proteins for the growing plant. It is necessary to determine whether other proteases are involved in the degradation of storage proteins; the close correlations between germination improvement, transcripts, translational products,

enzyme activity and free amino acid levels during SMP suggest thiol protease is a major enzyme involved in the degradation of reserve proteins when seeds are primed.

Conclusions

The results of the research reported here lead to the following conclusions:

1. Major free amino acid concentration in embryos of loblolly pine increased after 2 d SMP. There was a transient increase of major free amino acids in megagametophytes of loblolly pine in the period of 1 to 2 d SMP.
2. All tested amino acids showed increasing tendencies in both embryos and megagametophytes after 4 d germination.
3. Proline accounted for main part of the increase of detected free amino acids during SMP. The next most important were Asp, Glu and Ala.
4. Water potential and osmotic potential in embryo decreased during SMP, while embryo water content slightly increased.
5. Prepriming treatment of thiol protease inhibitors negated the SMP effects on free amino acid accumulation, water potential and osmotic potential generation and thiol protease activity.

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Table 1. Contributions of amino acids to the accumulation of osmotic potential (ψ_π) and increase of amino acids from zero d to four d solid matrix priming.

Amino acid	Contribution to ψ_π (MPa)	Contribution to ψ_π (%)	Amino acid increase (fold)
Hyp	--	--	--
Ser	--	--	--
Thr	0.0143	4.55	5.61
Pro	0.0789	25.06	2.52
Gly+Gln	0.0162	5.13	3.72
Ala	0.0294	9.34	5.42
His	0.0153	4.87	3.54
Cys	0.0230	7.31	2.60
Glu	0.0297	9.43	1.57
Asp	0.0439	13.94	4.10
Tyr	0.0118	2.23	2.11
Val	0.0149	4.75	159.70
Met	--	--	--
Ile	0.0020	0.64	1.88
Leu	-0.0001	-0.03	0.99
Phe	0.0049	1.58	4.35
Asn	0.0120	3.65	2.16
Lys	0.0002	0.08	1.05
Arg	0.0037	1.18	1.86
Total	0.31	100.00	2.66

Table 2. Effects of Solid matrix priming (SMP) and pre-SMP treatment of loblolly pine seeds with thiol protease inhibitor E64 on embryo water status and thiol protease (TP) enzyme activities.

Index	Non-SMP	SMP	Pre-SMP with E64
Water content (%)	59.60 ± 1.53	62.21 ± 1 13	61.92 ± 1.24
Water potential (MPa)	-1.40 ± 0.09	-1.99 ± 0.07	-1.67 ± 0.05
Osmotic potential (MPa)	-1.47 ± 0.05	-2.12 ± 0.05	-1.75 ± 0.07
Turgor potential (MPa)	0.07 ± 0.06	0.13 ± 0.01	0.08 ± 0.06
TP activity (unit)	0.03 ± 0.06	1.19 ± 0.01	0.08 ± 0.06

Table 3. Inhibition of thiol protease inhibitors on major free amino acid accumulation in embryos and megagametophytes by prepriming treatment of loblolly pine seeds.

Amino acid	Inhibition by $CuCl_2$ (in embryo, %)	Inhibition by E64 (in embryo, %)	Inhibition by $CuCl_2$ (in megagametophyte, %)	Inhibition by E64 (in megagametophyte,,%)
Hyp	–	--	37	66
Ser	55	39	69	51
Thr	67	37	14	46
Pro	77	73	51	64
Gly+Gln	69	51	-38	23
Ala	76	73	61	52
His	84	91	73	48
Cys	74	42	24	72
Glu	69	62	13	39
Asp	54	54	33	53
Tyr	43	29	49	63
Val	58	31	64	66
Met	98	69	-4	85
Ile	88	34	33	52
Leu	20	25	13	8
Phe	14	41	38	79
Asn	78	64	57	82
Lys	62	55	1	-2
Arg	25	7	-482	-541
Total	69	61	32	49

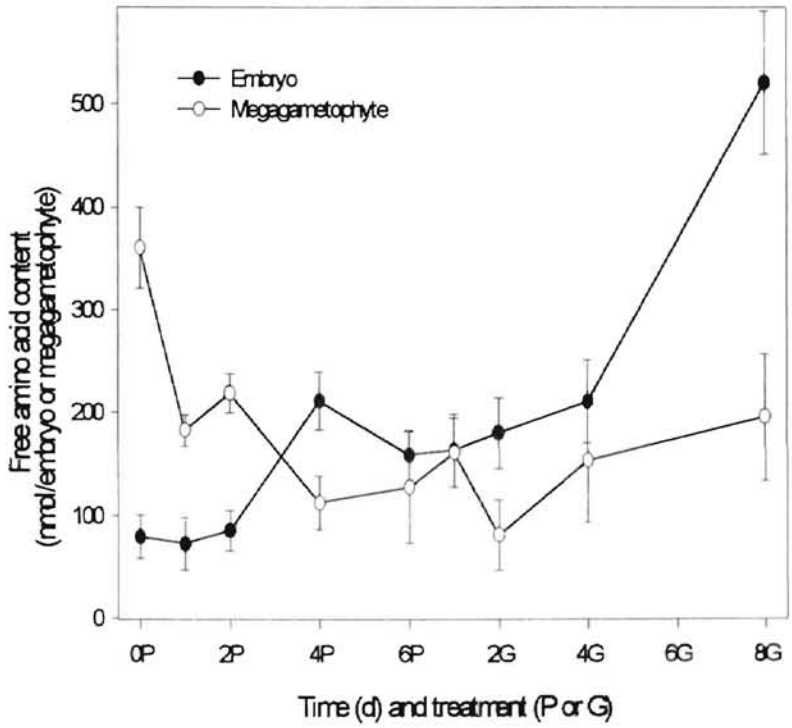

Figure 1. Changes of total free amino acid content in embryo and megagametophyte of loblolly pine during solid matrix priming (P) and subsequent germination (G).

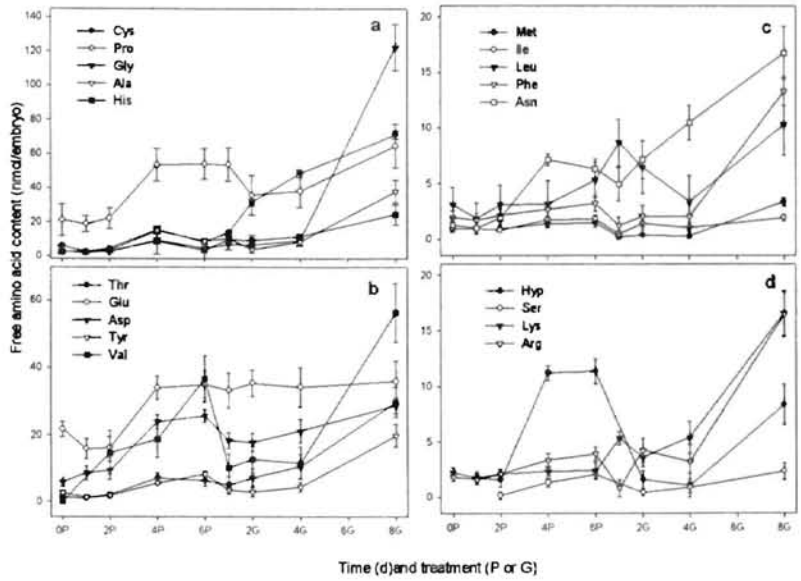

Figure 2. Changes of major free amino acid contents in embryo of loblolly pine during solid matrix priming (P) and subsequent germination (G).

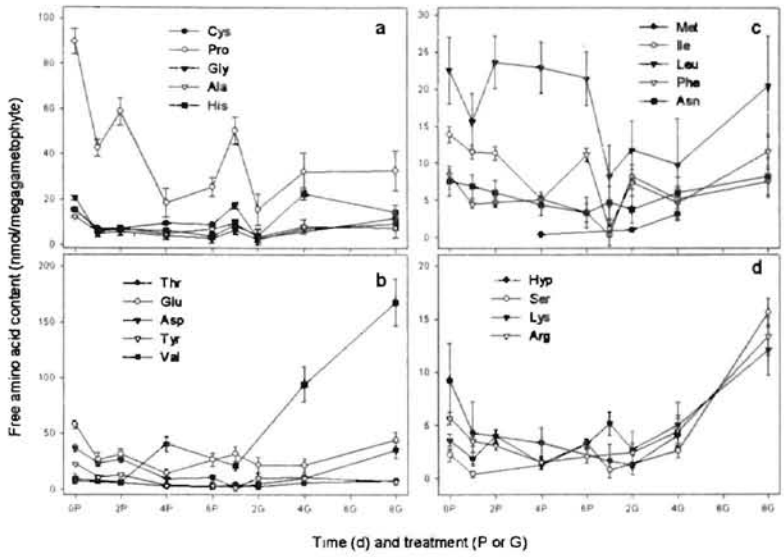

Figure 3. Changes of major free amino acid contents in megagametophyte of loblolly pine during solid matrix priming (P) and subsequent germination (G).

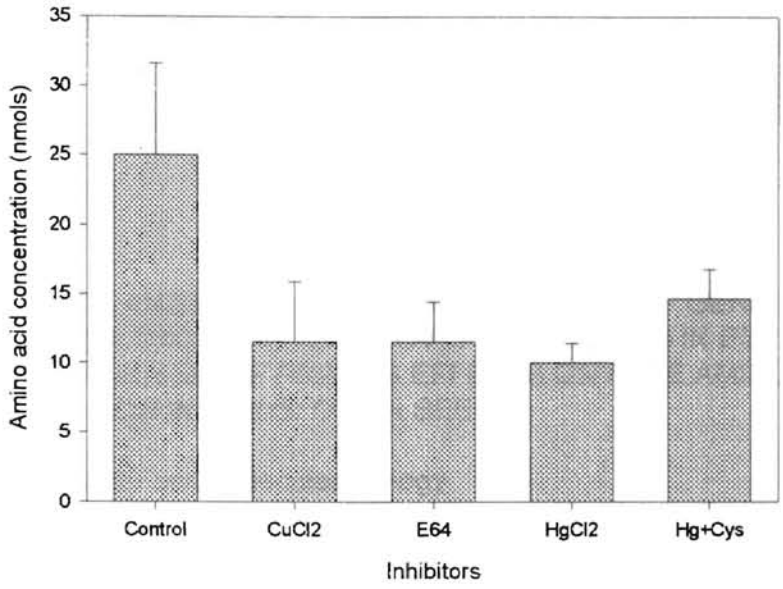

Figure 4. Effects of different thiol protease inhibitors on asparate accumulation in embryos induced by solid matrix priming.

VITA

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Master of Science

Thesis: PURIFICATION AND PARTIAL CHARACTERIZATION OF GENETICALLY ENGINEERED THIOL PROTEASE OVER-EXPRESSED IN *ESCHERICHIA COLI* AND ANALYSIS OF PRIMING EFFECTS ON FREE AMINO ACID ACCUMULATION IN *PINUS TAEDA* SEEDS

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