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Characterization and Expression of the Douglas-fir Luminal Binding Protein

by

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B.Sc. Hon., University of New Brunswick, 1993

A Dissertation Submitted in Partial Fulfillment for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

We accept this dissertation as conforming to the required standard

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ABSTRACT

The endoplasmic reticulum (ER) molecular chaperone, BiP, plays a role in the translocation and subsequent folding and assembly of newly synthesized proteins targeted to the ER and secretory pathway. The sequence encoding a Douglas-fir (*Pseudotsuga menziesii* [Mirb] Franco) BiP homologue (PmBiP) was identified by differential screening of a seedling cDNA library. Southern blotting indicated that PmBiP is most likely present as a single copy although other BiP alleles likely exist within a given seedlot. The deduced amino acid sequence of PmBiP contains a HEEL tetrapeptide sequence which functions to retain PmBiP in the ER and is different from HDEL commonly found in angiosperm plant BiPs. Amino acid sequence alignment and phylogenetic analysis show that PmBiP is highly similar to other plant BiPs yet forms a distinct phylogenetic subgroup separate from angiosperm BiPs. Northern and western blotting revealed that PmBiP is subject to developmental regulation during seed development, germination, and early seedling growth and is seasonally regulated in needles of young seedlings. The expression of PmBiP is developmentally regulated during seed development with higher amounts present in seeds prior to embryo development and the deposition of storage proteins. Increased PmBiP expression correlates with seedling growth and development and the mobilization of seed storage proteins. Increased synthesis during germination is likely due to increased synthesis of cell wall proteins and other secretory traffic. This idea is supported by immunolocalization of PmBiP in root tip cells showing staining around the new cell wall in telophase cells and at the periphery of cells in the elongation zone.

PmBiP may also play a role in mediating homotypic ER and nuclear envelope membrane fusion during mitosis in actively dividing tissues. PmBiP is seasonally regulated in the needles of young seedlings and increased expression was observed in tissues treated with low temperature suggesting that PmBiP plays an important role in the adaptation of seedlings to low temperatures. This is most likely accomplished through the maintenance of secretory traffic through the ER necessary for the synthesis of proteins with a more direct role in cold acclimation. Proteins were associated with PmBiP in an ATP dependent manner in mature seeds and 2-day-old seedlings but were only detectable in minute amounts. ATP associated proteins were more readily detectable in embryonal suspensor mass (ESM) cultures but only in small amounts unsuitable for N-terminal sequencing and identification.

The Douglas-fir BiP promoter (PmBiPPro1) contains a variety of cis-acting regulatory elements commonly found in the promoters of storage protein genes, light regulated genes, and phenlypropanoid and cell wall protein genes. The presence of different cis-element groups suggests the transcriptional regulation of PmBiP is controlled by a variety of signal transduction pathways depending upon the developmental and/or physiological state of a given tissue.

Transient expression analysis showed that PmBiPPro1 is functional in germinating Douglas-fir embryos. The expression of PmBiPPro1 in transgenic *Arabidopsis* is associated with actively dividing and secretory tissues. Deletion analysis showed that minimal promoter elements lie within a 263 bp region directly upstream of the PmBiP cDNA sequence although upstream flanking sequences are necessary for higher level expression. G-box motifs residing

within the 263 bp fragment together with a quantitative activator region (QAR) and a negative regulatory region (NRR) present in upstream areas are likely involved in transcriptional control in young seedlings. PmBiPPro1 was also wound inducible in transgenic *Arabidopsis* cotyledons that correlated with similar experiments conducted in Douglas-fir seedlings. Elements involved in conferring wound inducibility are located in PmBiPPro1-5 but upstream elements are necessary for higher level expression. G-box motifs may also play a role in the wound inducibility of the Douglas-fir BiP promoter.

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AFP, antifreeze protein

Amino acids,

A : alanine	M : methionine
C : cysteine	N : asparagine
D : aspartic acid	P : proline
E : glutamic acid	Q : glutamine
F : phenylalanine	R : arginine
G : glycine	S : serine
H : histidine	T : threonine
I : isoleucine	V : valine
K : lysine	W : tryptophan
L : leucine	Y : tyrosine

ATP, adenosine triphosphate

BiP, luminal binding protein

bp, base pair(s)

CaM, Calmodulin

CBF, C-repeat-binding factor

cDNA, complementary DNA

CoIp, co-immunoprecipitation

CHS, chalcone synthase

CRT, C repeat

DRE, dehydration responsive element

DREB, DRE-binding protein

ER, endoplasmic reticulum

ERSE, ER stress element

ESM, embryonal suspensor mass cells

GA_3, gibberellic acid

GUS, ß-glucuronidase

gDNA, genomic DNA

h, hours

HSP, heat shock protein

IOD, integrated optical density

JA, jasmonic acid

kb, kilobase(s) or 1000 bp

LEA, late embryogenesis abundant
LMW, low molecular weight
M, molar
MDH, malate dehydrogenase
MM, millimolar
MG, megagametophyte
min, minutes
µl, microlitres
mRNA, messenger RNA
NNPP, neural network promoter prediction
NRR, negative regulatory region
PAGE, polyacrylamide gel electrophoresis
PAL, phenylalanine ammonia lyase
Pipes, 1,4- piperazinediethanesulfonic acid
PmBiP, Douglas-fir BiP
PmBiPPro1, Douglas-fir BiP promoter
PR, pathogenesis related
PVP, polyvinylpyrrolidone
QAR, quantitative activator region
rmsd, relative mean square deviation
rRNA, ribosomal RNA
rbcS, ribulose-1,5-bisphosphate carboxylase
RT, room temperature
SDS, sodium dodecyl sulfate
TSS, transcriptional start site
UPRE, unfolded protein response element
UTR, untranslated region(s)

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DEDICATION

I would like to dedicate this thesis to my mother, Brenda Lynn Walsh, whose lack of formal education was more than compensated for by her practical life experience. I thank her for her constant encouragement and love.

CHAPTER 1: LITERATURE REVIEW

Introduction

Seed development and germination represent important stages in the life-history of plants as the genetic, biochemical, and molecular composition of a seed will ultimately determine a seedlings ability to become established, thrive, and reproduce in its environment. Once a seedling becomes established in its environment, it must often overcome many stress challenges to survive and reproduce. Since plants are non-motile, they cannot evade stress simply by moving, hence, plants have developed a variety of stress responses that allow them to survive. A major focus of research in the past decade has focused on understanding the molecular events underlying seed development, germination, post germination, and the stress responses of plants. The following research represents an examination of the expression of the Douglas-fir luminal binding protein (BiP) (Tranbarger and Misra, 1995) during these processes. This research is significant in that it not only elucidates molecular events of conifer seed development, germination, and stress response but it also highlights the similarity and differences which exist at a molecular level between the gymnosperms and angiosperms, two evolutionarily distinct plant groups with obvious differences in their life histories.

Douglas-fir

Pseudotsuga menziesii [Mirb.] Franco, more commonly known as Douglas-fir, is a diploid (2n=26) and monoecious member of the *Pinaceae* family. The reproductive life cycle of Douglas-fir extends over a period of 17 months (Allen

and Owens, 1972). Axillary buds appear in April and differentiate into vegetative, pollen, or seed-cone buds within 10 weeks. Development of these tissues proceeds until late fall when buds become dormant until spring. Bud burst, revealing male and female cones, occurs in late March to early April and is followed by pollination. The pollen is engulfed within the micropyle where it germinates and fertilizes the ovule after about 9 weeks. Seed development proceeds over the summer with seed shed occurring in late summer/early fall.

The natural range of Douglas-fir extends from British Columbia south to Mexico and east to the Rocky Mountains. Favorable growth characteristics and superior wood quality make it one of the most economically important conifer species in the Pacific Northwest. Consequently, reforestation initiatives have created a significant demand for high quality seed and superior performing genotypes. Conventional breeding and selection practices are routinely used to maintain and enhance seed stocks and produce genetically superior Douglas-fir trees (Silen, 1978). Progress is slow due to the long reproductive cycle and unpredictable seed crops resulting from failed reproductive processes that can include a lack of cone bud development, low pollination success, premature embryo abortion and seed immaturity (Owens et al., 1991).

The germination capacity of Douglas-fir and other conifer tree seeds is under strong maternal control, is genotype specific, and has been observed to decrease with time in stored seed stocks (Chaisurisri et al., 1993; El-Kassaby et al., 1992). This decline in seed viability is associated with deterioration of cell membranes, decreased respiration and a reduced rate of protein synthesis (Delouche and Baskin, 1973). Genotypic loss of seed viability can compromise the genetic diversity of stored seed stocks. Ex-situ gene conservation through

seed storage in crop plants relies on frequent rejuvenation of stored seeds. Ex-situ conservation in forestry, especially with conifers, cannot accommodate this rejuvenation component because of the long time required to reach sexual maturity and the difficulty in controlling the dynamic genetic structure of conifers as a result of high genetic heterozygosity and individual heterogeneity resulting from an open-pollinated mating system. To understand better the genetic control of germination and dormancy among various genotypes it is necessary to elucidate the molecular events that occur during seed storage, stratification and germination. Such knowledge will aid in the development of more effective gene conservation practices and help maintain the genetic diversity of stored seed stocks.

Seed Development and Germination

The development of a seed follows successful pollination and fertilization. The origin and development of angiosperm seed tissues is different from that of the gymnosperms (Bewley and Black, 1985). In angiosperms, one of two male nuclei released from the pollen tube fuses with the egg nucleus to form a diploid zygote. The triploid endosperm seed storage tissue arises from the fusion of a second pollen tube nucleus with two polar nuclei from the embryo sac. In nonendospermic dicot angiosperms, the endosperm is consumed by the developing embryo and storage reserves are reorganized into the cotyledons. The developing embryo undergoes a series of morphological and cellular changes (embryogenesis) that ultimately result in the formation of a dormant mature embryo consisting of an embryonic axis with cotyledons and both root and shoot apices. Embryogenesis in angiosperms occurs in three roughly

discernable phases. During early embryogenesis, cell division and pattern formation occur to produce an embryo with specified root and shoot apices, cotyledons, and provascular tissue (West and Harada, 1993). The maturation phase includes cell expansion accompanied by the accumulation of storage reserves such lipids, carbohydrates, and storage proteins (Higgins, 1984). Storage reserves deposited within seed tissues serve as an energy and nitrogen source for postgerminative seedling growth (Bewley and Black, 1985). During late embryogenesis, seed tissues become dehydrated and the embryo becomes metabolically and developmentally inactive (Kermode, 1990). This state of dormancy is necessary to inhibit precocious germination and allow the seed to germinate only when favorable conditions are encountered.

Gymnosperm seeds differ both anatomically and genetically from angiosperm seeds. In contrast to angiosperm storage tissues that originate and develop after fertilization, the main storage tissue in conifer seeds is the haploid, maternally derived megagametophyte (MG) that is formed prior to fertilization (Misra, 1994). Fusion of a single male gamete released from the pollen tube with the female egg cell forms a diploid zygote and initiates embryo development. Embryo development in conifers can be divided into two stages that consist of a short proembryo phase followed by a longer embryo phase that occurs after the proembryo elongates into the surrounding MG (Singh, 1978). Development proceeds through the embryo phase in three morphologically distinct stages that include: early-, mid- (maturation), and late-embryogenesis stages. Unlike angiosperms, accumulation of storage reserves in the MG occurs shortly after fertilization and in the embryo during mid-embryogenesis (Misra, 1994). As with angiosperms, the last stages of

development involve water loss and the transformation of the embryo into an inactive dormant state.

The composition and deposition of seed protein reserves has received much attention due to its contribution to seedling establishment during postgermination and its importance to both human and animal nutrition (Bewley and Black, 1985). Based on their solubility, seed storage proteins can be grouped into albumins (soluble in water and dilute buffer at neutral pH), globulins (insoluble in water but soluble in salt solutions), glutelins (soluble in dilute alkali or acid solution), and prolamins (soluble in 70-90% aqueous alcohol). Albumins and globulins are the major storage types in many monocot and dicot seeds while prolamins are mostly found in cereal grains (Galili et al., 1998). Storage proteins are synthesized on the RER and translocated to the lumen where they can undergo postranslational modification by glycosylation, proteolytic processing, oligomeric association, and inter/intra-molecular disulfide bond formation. Following modification, storage proteins can be transferred to the Golgi apparatus and on to vacuoles or they can be retained within the ER. Globulins are stored in vacuoles whereas the prolamins can be stored in vacuoles or ER delimited protein bodies (Galili et al., 1998). The study of seed proteins in conifers have revealed that the crystalloids are the major type of storage protein (Misra, 1994). The Crystalloids are a detergent soluble subgroup of the 11S globulins and are localized to 0.5-5 μm protein bodies found in both the MG and embryonic axis of mature Douglas-fir seeds (Green et al., 1991). Immunolocalization of a 42 kDa globulin storage protein from Norway spruce showed that transport to storage vacuoles was mediated by the

Golgi (Hackman et al., 1990). This is in agreement with the transport pathway of angiosperm globulin storage proteins.

Germination begins with elongation of the embryonic axis following water uptake by the seed (imbibition) and is complete once the radicle has emerged (Bewley, 1997). Imbibition stimulates the resumption of metabolic activities and initiates the repair of damaged membranes, DNA, and mitochondria and elicits the synthesis of proteins from stored and newly synthesized mRNA. The emergence of the radicle through surrounding seed tissues is primarily a turgor driven event that results from expansion of cells that lie between the root cap and base of the radicle. Some seeds require a cool moist treatment (stratification) prior to exposure to germination conditions to overcome dormancy. This is especially true for Douglas-fir and other conifers (Edwards, 1986). Dormancy, roughly defined as the failure to complete germination under favorable conditions, can result from coat imposed dormancy in which the embryo is constrained by surrounding seed structures or the embryos themselves may be dormant (Bewley, 1997). Following emergence of the radicle, postgerminative growth begins. The mobilization of storage reserves is a critical event which permits the seedling to become an established and photoautotrophic organism (Bewley, 1997; Misra, 1994). Storage reserves are mobilized from the endosperm or cotyledons in angiosperms or from the MG of conifers and are transported to the embryo where they are used for growth. In angiosperms, cysteine proteinases are thought to catalyze the initial stages of storage protein mobilization during seed germination (Shutov and Vaintraub, 1987). The degradation of storage protein proceeds via several steps involving the action of multiple proteinases that participate in a specific order during

germination (Mitsuhashi and Oaks, 1994; Segundo et al., 1990; Shutov and Vaintraub, 1987; Yamauchi et al., 1996). Very little is known about the proteinases responsible for the hydrolysis of storage proteins during conifer germination. Cysteine proteinase and pepsin-like acid proteinase activity is associated with protein mobilization during germination in Scots Pine (Salmia 1981a, Salmia 1981b) and aminopeptidases are proposed to be involved in mobilization of storage reserves of Lodgepole pine (Gifford et al. 1988). Stratification elicits the synthesis or activation of metalloproteinase activity in Douglas-fir seeds (Forward et al., 2000).

Developmentally Regulated Gene Expression during Seed Development and Germination

A variety of prevalent mRNA sets are expressed during seed development through post-germination (Goldberg et al., 1989). The expression of specific gene sets is coincident with changes in development and thought to respond to particular regulatory signals. The constitutively expressed mRNAs are common among all stages and include house keeping or structural genes like actin and tubulin. Embryo specific mRNAs are expressed throughout embryogenesis but others such as storage protein mRNAs are confined to the mid maturation phase. The late embryogenesis abundant (LEA) mRNAs are expressed during the late stage of embryogenesis and may play an important role in stabilizing proteins and membranes against dehydration (Bewley and Marcus, 1990). Other mRNAs are expressed during late embryogenesis to germination and are stored in the seed. Genes involved in the mobilization of

storage reserves are generally confined to the germination-postgermination phase of development.

Gene expression during seed development and germination in angiosperms has been studied extensively and is beyond the scope of this review. Hence, developmental gene expression in conifers is emphasized.

Developmental gene expression in conifers

In recent years, significant progress has been made in identifying and characterizing genes that are expressed during seed development and germination in conifers (Chatthai and Misra, 2000; Dong and Dunstan, 2000; Misra, 1994). Many of the genes identified to date have been isolated through screening of cDNA libraries prepared from particular developmental stages. Recently, differential display has been used to identify genes expressed during embryogenesis in pine (Cairney et al., 2000). Much information regarding genes expressed during embryogenesis has come from work using somatic embryos (Dong and Dunstan, 2000). Somatic embryos develop through morphological phases similar to that of zygotic embryos and provide a readily available source of experimental material. Work in this area has also been accelerated by the need for molecular markers to gauge and improve the quality of somatic embryos for use in reforestation initiatives.

Conifer seed storage protein cDNAs representing the albumins, vicilins, and legumins have been isolated and characterized in the past decade from both zygotic and somatic embryos (Dong and Dunstan, 2000). Expression of many of the seed storage protein genes occurs shortly after fertilization in the MG and occurs in both zygotic and somatic embryos during mid-maturation

(Chatthai and Misra, 1998; Dong and Dunstan, 1999; Flinn et al., 1993; Leal and Misra, 1993b). ABA and osmoticum appear to be important regulators of storage protein gene expression in somatic embryos and suggests these factors are important for regulating storage protein gene expression in developing seeds. Multiple LEA genes have been characterized in conifers and are expressed during the later stages of embryo development (Close et al., 1993; Dong and Dunstan, 1996a; Dong and Dunstan, 1997b; Dong and Dunstan, 1999; Leal and Misra, 1993a). Upon germination of spruce somatic embryos, LEA mRNAs rapidly disappear (Dong and Dunstan, 1996a). As in angiosperms, conifer LEA genes are believed to protect cells from dehydration stress experienced during seed desiccation.

A novel cDNA encoding a metallothionein protein was isolated from a seed development cDNA library and its expression was similar to that of the Douglas-fir 2S albumin seed storage protein genes (Chatthai et al., 1997; Chatthai and Misra, 1998). Expression occurred in the MG shortly after fertilization and was first detected in precotyledonary somatic and zygotic embryos. Transcripts decreased during the late stages of seed development and were not detected in mature seeds (Chatthai et al., 1997). Transcript amounts were very low in germinating and young seedlings. The modulation of its expression by metal ions in stratified Douglas-fir seeds suggested a role in regulating microelement availability during seed development and seedling growth. Metallothionein cDNAs have also been isolated from somatic embryos of white spruce and show similar developmental expression patterns (Dong and Dunstan, 1996a).

A number of heat shock proteins have been isolated from white spruce somatic embryos that belong to the HSP70 and low molecular weight (LMW) HSP families (Dong and Dunstan, 1996a; Dong and Dunstan, 1996a). Two of the LMW HSPs are most likely localized to the cytoplasm while another was predicted to be localized to the mitochondria. The expression of the LMW HSPs was inducible by heat treatment and were temporally regulated in spruce somatic embryos (Dong and Dunstan, 1996b). However, the HSP70 homologue was expressed in all stages of developing somatic embryos (Dong and Dunstan, 1996b).

Two cDNAs encoding pathogenesis related (PR) proteins were identified through differential screening of a spruce somatic embryo cDNA library (Dong and Dunstan, 1997a). The cDNAs were similar to other secreted plant chitinases and ß-1,3-glucanases that can hydrolyze the cell wall components of many fungi. Expression of both genes was high at the beginning of maturation but then declined only to increase again during late maturation stages. It was suggested that the expression of these proteins in somatic embryos may be part of a developmentally programmed defense mechanism or the result of stress imposed by tissue culture conditions.

Several other cDNAs isolated from a white spruce somatic embryo cDNA library encode for proteins with unclear functions during embryogenesis as they are not homologous to any known proteins (Dong and Dunstan, 1996a). Hence, future study is needed to identify the functions of these proteins during embryo development.

Germination in conifers occurs as a result of the synergistic activities of proteins encoded by three distinct genomes: the diploid embryo, the haploid

maternally derived MG and maternal seed coat (El-Kassaby et al., 1992; Misra, 1994). Many molecular and biochemical processes occur during imbibition and stratification. Major changes in mRNA populations have been observed in loblolly pine during stratification, germination, and post-germinative growth (Mullen et al., 1996). The mRNA, protein, and enzymatic activity of two isocitrate lyase genes, involved in lipid metabolism, increased following imbibition and reached maximal levels prior to MG senescence (Mullen and Gifford, 1997). In Douglas-fir, stratification elicits the synthesis of new mRNA and protein (Taylor and Davies, 1995; Taylor et al., 1993). Two of these genes were identified as encoding homologues of histone H1 and the ß-subunit of the 20S proteasome. Further screening of a stratification cDNA library identified three Douglas-fir LEA genes that showed marked increases in transcript amounts during stratification and it was suggested that they play a role in the chilling-induced breakage of dormancy (Jarvis et al., 1996). Expression was observed in both the MG and embryo but transcripts accumulated first in the MG (Jarvis et al., 1997). While treatment with ABA inhibited germination of nondormant seeds, expression of LEA genes was unaffected. Differential screening of a Douglas-fir seedling cDNA library identified several genes with identities and expression patterns that suggested important roles in germination and post-germinative growth (Tranbarger and Misra, 1995). Transcripts of a cysteine proteinase were present in the MG of mature and stratified seeds and increased significantly by 4 days after exposure of stratified seeds to germination conditions (Tranbarger and Misra, 1996). The temporal and tissue specific pattern of CysP transcript accumulation suggested a role in storage protein mobilization. Molecular chaperones from the HSP70, HSP60,

and low molecular weight HSP families showed increased expression following stratification and germination and were suggested to be involved in protein biogenesis (Kaukinen et al., 1996; Tranbarger and Misra, 1995).

Stress responses

Once established in their environment, plants can face a variety of stresses which they must endure to thrive and reproduce. Such stresses can include: heat stress, cold stress, desiccation, insect and pathogen attack, wounding, and UV stress. Since cold stress and wounding have been investigated in this dissertation, an overview of these two stress responses is presented.

Wound response

Wounding is a continual threat to plants in the environment and can result from wind, rain, hail, sand, and herbivores. Plants respond by expressing many defense genes in the area of the wound site as a deterrent to herbivores or barrier to potential invading microorganisms. Plants can also induce wound response genes systemically to protect other plant parts from potential damage or infection.

Using a cDNA microarray technique, Reymond et al. (2000) examined the expression of 150 different genes in response to mechanical wounding in *Arabidopsis*. Genes showing increased expression included several PR proteins such as ß-1,3-glucanase which attack fungal cell walls, and phenylpropanoid biosynthesis enzymes such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS). Evidence is now accumulating that cell wall proteins also play an important role in plant defense. The cell wall protein extensin has

been shown to increase in response to wounding in several species and is believed to help reinforce the cell walls near wound sites (Elliott and Shirsat, 1998; Merkouropoulos et al., 1999; Wycoff et al., 1995). A maize proline-rich protein involved in secondary cell wall formation is also induced in response to wounding (Vignols et al., 1999).

Wounding of tomato plants elicits the systemic synthesis of over 20 defense related proteins (Ryan, 2000). Among these, a broad spectrum of proteinase inhibitors is expressed which interfere with herbivore digestion. Signaling pathway genes such as prosystemin, lipoxygenase, calmodulin, and systemin receptor are also induced and have been suggested to amplify the defense response to provide maximal protection (Ryan, 2000). A variety of proteinases are induced by wounding but a role in defense response remains to be established.

The expression of many wound response genes can be induced by jasmonic acid (JA) or its precursor oxophytodienoic acid (Reymond and Farmer, 1998). Other signaling molecules such as oligosaccharides, ABA and ethylene are also believed to play a role in signaling (Birkenmeier and Ryan, 1998; Doares et al., 1995; O'Donnell et al., 1996; Rojo et al., 1999). The systemic induction of wound responsive genes in tomato leaves is mediated by systemin (Ryan, 2000). Systemin is an 18 amino acid peptide produced by proteolytic processing of a 200 amino acid precursor protein, prosystemin. Prosystemin is present at low levels in unwounded plants but increases in response to wounding (McGurl et al., 1992). Wounding activates processing and systemin is released from damaged plant cells where it travels via the phloem to other areas of the plant. Interaction of systemin with its receptor activates the synthesis of wound

response genes via a JA signaling pathway (Bergey et al., 1996; Meindl et al., 1998; Ryan and Pearce, 1998; Scheer and Ryan, 1999). Systemin homologues have also been identified in other members of the Solanaceae family (Constabel et al., 1998). Wounding also stimulates gene expression through a JA independent pathway in *Arabidopsis* (Rojo et al., 1998; Titarenko et al., 1997). Oligosaccarides from damaged tissues induce a specific set of wound response genes while locally repressing JA induced genes that are activated in systemic tissues (Rojo et al., 1999). The local repression of the JA signaling pathway appears to be mediate by ethylene in the damaged tissue.

Similar wound response genes are expressed in tree species upon wounding or insect attack. In poplar, genes encoding endochitinases, protease inhibitors and vegetative storage proteins show increased expression in response to leaf wounding both locally and remotely (Clarke et al., 1994; Davis et al., 1993; Hollick and Gordon, 1993). Lignin biosynthesis enzymes are also wound inducible in poplar and likely play a role in strengthening cell walls to prevent pathogen infiltration (Chen et al., 2000). In response to wounding, many conifer species synthesize terpenoid resins that contain compounds to inhibit microbial growth and insect feeding (Bohlmann and Croteau, 1999). These resins also perform the additional function of sealing the wound site through evaporative hardening. Characterization of this response in grand fir has revealed that several terpenoid synthases show increased expression in response to wounding (Bohlmann et al., 1997; Gijzen et al., 1992; Gijzen et al., 1991; Steele et al., 1998a; Steele et al., 1998b). In Norway spruce, the activities of CHS and stilbene synthase increase in response to wounding that lead to the synthesis of tannins and insoluble polymers (Brignolas et al., 1995). In white

spruce somatic embryo derived plantlets, PR proteins such as endochitinase and ß-1,3-glucanase genes were expressed 1 hour after wounding (Dong and Dunstan, 1997a). The recent isolation of a wide variety of genes from grand fir should facilitate the elucidation of signal transduction pathways leading to the expression of wound response genes in conifers (Bohlmann and Croteau, 1999).

Cold response

When plants of a freezing tolerant genetic disposition are exposed to a period of low, non freezing temperature, biochemical changes occur which allow the plant to survive a subsequent exposure to freezing temperatures. This period of acclimation is essential for the development of freezing tolerance as unacclimated plants are readily killed by exposure to freezing temperatures. Freezing temperatures are a dominant factor limiting the geographical location of important crop species and periodically account for major losses in plant productivity.

The stress imposed by freezing is similar to that imposed by drought in that freezing of extracellular water causes a net flux of water out of the cell across the plasma membrane (Steponkus, 1991; Steponkus, 1984). For example, freezing of winter cereal seedlings over a range of -2 to -20 °C imposes water potentials of -2.4 to -24 MPa and causes losses of up to 90% of the osmotically active water from cells. The primary source of injury during the course of such osmotic excursions is manifested in the plasma membrane (Steponkus, 1984). Several forms of membrane damage result from freeze-induced dehydration and include expansion-induced-lysis, lamellar-to-hexagonal-II phase transitions, and fracture jump lesions (Steponkus et al., 1993). Recent evidence

has suggested that protein denaturation is another significant form of cellular injury that can occur in plants at low temperature (Guy et al., 1998; Guy et al., 1994). The conformational stability of most globular proteins is low with maximal stability occurring at a specific temperature (Pace, 1990). Deviations above or below this temperature can result in protein instability and denaturation. Cold denaturation results in the inactivation of many enzymes through subunit dissociation (Privalov, 1990).

Although a variety of biochemical changes have been observed during cold acclimation, the biochemical mechanisms of cold acclimation are still poorly understood (Thomashow, 1990; Thomashow, 1998). The cold tolerant phenotype is conferred by multiple genes and involves the modification of existing cellular components and the synthesis of specific factors that function to modify the cell and escape freezing damage. Changes in lipid composition, increased levels of organic acids, the appearance of new isozymes, and increases in soluble sugar and protein have been observed in plants during cold acclimation (Thomashow, 1990). Within the last decade many studies have discovered that a variety of genes are expressed during cold acclimation. Early evidence that changes in gene expression were necessary for cold acclimation came from studies which showed that plants treated with the protein synthesis inhibitor cyclohexamide were unable to become frost tolerant (Thomashow, 1990).

A large number of genes have now been isolated that show increased expression within hours of exposure to low, non-freezing temperatures (Gilmour et al., 1992; Gilmour and Thomashow, 1991; Lin and Thomashow, 1992; Thomashow, 1999; Thomashow, 1998). The role of many cold acclimation

genes is unclear, but for some, function can be inferred from sequence identity to proteins of known function. The variety heat shock protein (HSP) family members up-regulated in response to cold treatment likely play an important role in preventing freeze-induced denaturation and aggregation of cellular proteins (Guy et al., 1998; Krishna et al., 1995; Pareek et al., 1995; Ukaji et al., 1999). Other genes such as the *Arabidopsis* chloroplast cold regulated desaturase, fad8, may function to alter the lipid composition during cold acclimation. Expression of several signal transduction proteins such as MAP kinases and calmodulin-related proteins may be important in controlling the expression of cold acclimation genes or regulating the activity of proteins involved in freezing tolerance (Mizoguchi et al., 1993; Polisensky and Braam, 1996). However, for the majority of cold regulated proteins, function cannot be inferred from amino acid sequence similarity. One such group are proteins with homology to the LEA proteins expressed during the late stages of embryogenesis in developing seeds (Thomashow, 1999). These proteins are very hydrophilic and remain soluble upon boiling in dilute buffer. They possess a simple amino acid composition and contain a number of repeat sequences predicted to form amphipathic α-helicies (Thomashow, 1998). Due to their similarity to the LEA genes it has been suggested that these proteins function to protect against the effects of freeze-induced dehydration. This appears true for the *Arabidopsis* chloroplast COR15a gene as constitutive expression increases the stability of the plasma membrane during freezing (Artus et al., 1996). Subsequent experiments indicated that COR15am decreased the incidence of freeze-induced lamellar-to-hexagonal II phase transitions that occurred in locations where the plasma membrane came into

close apposition with the chloroplast envelope as a result of freeze-induced dehydration (Steponkus et al., 1998). Moreover, the COR15am polypeptide appeared to accomplish this by altering the intrinsic curvature of the chloroplast envelope inner membrane. Whether other cold acclimation proteins of this type perform similar functions in other cellular locations is unknown. Plant antifreeze proteins (AFPs) are another group of proteins for which activity has been demonstrated. AFPs possess thermal hysteresis properties in which they lower the freezing point of water without affecting its melting temperature (Griffith and Antikainen, 1996). Plant AFPs also alter the growth and morphology of ice crystals by binding to the surface of forming ice crystals to inhibit their growth and ability to fuse with other ice crystals. A number of AFPs have been isolated from the extracellular space (apoplast) of winter rye and a variety of other monocot and dicot plant species (Antikainen and Griffith, 1997; Griffith et al., 1992; Griffith et al., 1997). These proteins have been suggested to function in modifying ice crystal formation in the apoplast to prevent rupture of the plasma membrane during freezing stress. They may also perform other functions as they are similar to some PR proteins. Future work is required to elucidate the function of many other cold regulated genes in the acclimation of plants to low temperature.

Components involved in the signal transduction cascade have been identified in recent years. The initial events in the signal transduction of cold acclimation appear to be mediated through calcium influx across the plasma membrane in alfalfa, *Arabidopsis* and tobacco (Knight et al., 1996; Knight et al., 1992; Monroy and Dhindsa, 1995; Tahtiharju et al., 1997). In *Arabidopsis* calcium release from the vacuole may also play an important signaling role. Stretch calcium

channels characterized from onion epidermis are modulated by cold and represent possible candidates for the plasma membrane calcium channels (Ding and Pickard, 1993a; Ding and Pickard, 1993b). It has been suggested that structures which transmit force to the channel gating mechanism may become stiffer at low temperatures and induce channel opening. The increase in cytosolic calcium functions to activate calmodulin (CaM) and calcium dependent kinases which could phosphorylate transcription factors and activate gene transcription (Monroy and Dhindsa, 1995; Poovaiah and Reddy, 1993; Roberts and Harmon, 1992; Tahtiharju et al., 1997). Low temperature, calcium dependent repression of protein phosphatase 2A activity is another mechanism proposed to lead to the phosphorylation of proteins necessary for induction of cold regulated genes (Monroy et al., 1998). The activation of an alfalfa MAP kinase within 10 minutes of cold treatment and increased transcript accumulation within 20 minutes suggests a role for this protein in early signaling events (Jonak et al., 1996). The transcriptional activation of several cold responsive genes in *Arabidopsis* is mediated by the dehydration response element binding protein/C-repeat-binding factor (DREB1/CBF1) though interaction with the dehydration response element/C repeat (DRE/CRT) promoter element (Baker et al., 1994; Liu et al., 1998; Stockinger et al., 1997; Yamaguchi-Shinozaki and Shinozaki, 1994). Over-expression of the CBF1 transcription factor in transgenic *Arabidopsis* plants causes a significant increase in freezing tolerance of non-acclimated plants through the activation of several cold response genes (Jaglo-Ottosen et al., 1998). The accumulation of CBF1 transcripts in response to cold treatment is proposed to be a secondary early response in a two step pathway (Gilmour et al., 1998). An unknown

upstream activator designated ICE (inducer of CBF expression) is proposed to be present in non-acclimated cells and following cold treatment becomes activated to simulate CBF1 transcription though an ICE BOX promoter element. Despite the fact that conifers retain their foliage during winter, little is known about the genes expressed during cold acclimation. In plants that retain their foliage, decreased temperatures can cause an energy imbalance between absorbed incident light energy and that required for photosynthesis (Huner et al., 1998). Such conditions can lead to the formation of damaging reactive oxygen species. During cold acclimation in Scots pine, elevated levels of reactive oxygen scavenging enzymes such as ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, and dehydroascorbate reductase are produced and correlate with freezing tolerance (Tao et al., 1998). A specific glutathione reductase isozyme was synthesized in the needles of cold hardened red spruce needles (Hausladen and Alscher, 1994b). The independence of the isozyme Km to temperature was proposed to provide necessary enzyme activity during cold temperatures (Hausladen and Alscher, 1994a). Two-dimensional SDS-PAGE of western white pine foliage proteins showed that a number of proteins increased to high levels during the winter months (Ekramoddoullah and Taylor, 1996). N-terminal sequencing identified one of these proteins as a small subunit of ribulose biphosphate carboxylase. A 19 kDa protein designated Pin M III was also identified and its abundance correlated with frost hardiness in needles (Ekramoddoullah et al., 1995). The sugar pine homologue Pin l I was also found in increasing amounts in needles during the fall. The function of white and sugar pine Pin proteins is unknown although Pin m III shows some sequence similarity to PR proteins (Yu et al.,

2000). The protein profiles of interior spruce needles, roots and buds and Douglas-fir buds show increases in a 30 kDa protein during the winter months (Roberts et al., 1991). In white spruce, a dehydrin and glycine-rich RNA binding protein gene are expressed upon exposure of plants to cold (Richard et al., 1999; Richard et al., 2000). Although the function of the RNA binding protein is unclear, the dehydrin may help protect against stress imposed by freeze-induced dehydration. A 70 kDa secreted white spruce protein gene of unknown function, AF70, is expressed in seedlings when exposed to cold acclimation conditions (Sabala et al., 1997). The expression of several genes, including three LEA protein genes, is enhanced by cold treatment during stratification in Douglas-fir seeds (Jarvis et al., 1997; Jarvis et al., 1996; Taylor et al., 1993).

Heat Shock Proteins

HSPs play an important and essential role in the development, physiology and stress response of plants and are located in virtually every plant organelle (Boston et al., 1996; Vierling, 1991). HSPs are heat inducible and are part of a larger multi-gene superfamily containing members that are not regulated by heat. These members are highly homologous to heat inducible members and are often referred to as heat shock cognate (HSC) proteins. HSPs are also considered molecular chaperones as they can assist in the folding of newly synthesized proteins, prevent the aggregation of misfolded proteins during stress, translocate proteins across membranes, target proteins for degradation, and regulate the activity of receptors and transcription factors (Becker and Craig, 1994; Hendrick and Hartl, 1993). Homologues of plant molecular

chaperones are well studied in mammals and bacteria. Several classes of HSPs have been described and are grouped according to their apparent molecular mass in kDa and include HSP100, HSP90, HSP70, HSP60, and the low molecular weight (LMW) HSPs (15-30 kDa) (Lindquist and Craig, 1988; Miernyk, 1999; Vierling, 1991). The isolation of several HSPs that are differentially expressed during germination in Douglas-fir emphasizes the importance of these proteins in the development and physiology of Douglas-fir (Tranbarger and Misra, 1995). Douglas-fir homologues of the HSP70, HSP60, and LMW HSPs showed increased expression during germination and early seedling development (Kaukinen et al., 1996; Tranbarger and Misra, 1995).

Eukaryotic members of the HSP70 family are located in the cytosol, nucleus, mitochondrial matrix, chloroplasts, and ER lumen and are homologous to the *E. coli* DnaK protein (Bardwell and Craig, 1984; Boston et al., 1996; Hendrick and Hartl, 1993). Some family members are expressed constitutively while others are expressed only under stress conditions. HSP70 proteins bind to misfolded or denatured proteins caused by heat stress to prevent aggregation and promote ATP dependent refolding. Cytosolic HSP70 participates in folding by shielding the hydrophobic regions of nascent and incompletely folded polypeptide chains (Agashe and Hartl, 2000). Organelle HSP70 homologues are also involved in the translocation and folding of newly synthesized polypeptides across mitochondrial, chloroplast, and ER membranes (Gray and Row, 1995; Haas, 1994; Martinus et al., 1995; Stuart et al., 1994). HSP70 proteins are composed of two major domains, the N-terminal ATPase domain with a molecular mass of ~45 kDa and the C-terminal peptide binding domain of ~25 kDa. The 3D structure of bovine HSP70 ATP binding

domain has been solved and shows that it is highly similar to that of hexokinase and actin (Bork et al., 1992; Flaherty et al., 1990; Flaherty et al., 1991). The 3D crystal structure of *E. coli* DnaK peptide binding domain has been solved and it was suggested that the eukaryotic HSP70 proteins share the same structure (Zhu et al., 1996). This structure turned out to be significantly different from the MHC I peptide binding cleft that was originally predicted (Flajnik et al., 1991; Rippmann et al., 1991). An NMR solution structure of a mammalian HSC70 peptide binding domain lacking the C-terminal 10 kDa variable region is practically identical to the DnaK structure in the ß-sandwich peptide binding pocket (Morshauser et al., 1999). The ATPase activity of HPS70s is stimulated by peptides (Flynn et al., 1989) and the peptide binding specificity of different HSP70 family members share general characteristics but differences have been reported (Blond-Elguindi et al., 1993a; Fourie et al., 1994; Gragerov and Gottesman, 1994). HSP70 family members often function in concert with other molecular chaperones and/or accessory proteins. Such is the case for the *E. coli* DnaK protein which requires the chaperone activating protein DnaJ and the nucleotide exchange factor GrpE (Miernyk, 1997). Several DnaJ homologues have been identified in mammals but the existence and requirement for GrpE homologues remains controversial (Miernyk, 1999). HSP70 has also been implicated in the uncoating of clathrin coated vesicles in mammals, yeast, and plants (Chappell et al., 1986; Gao et al., 1991), a reaction that also requires the DnaJ homologue auxilin (Jiang et al., 1997; Ungewickell et al., 1997; Ungewickell et al., 1995). HSP70s may also be involved in cell to cell transport in plants as a viral specific HSP70 homologue was necessary for the cell to cell translocation of the beet yellows closterovirus (Peremyslov et al.,

1999). Cytosolic HSC70s are expressed constitutively in a variety of plant species although additional cytosolic HSP70 is produced upon heat stress (Vierling, 1991). Plant cytosolic HSP70 forms part of protein import complex which facilitates the import of nuclear encoded proteins into mitochondria and chloroplasts by maintaining them in a translocation competent conformation (May and Soll, 2000). The characterization and expression of HSP70 genes has been studied extensively in spinach which contains up to 12 different family members localized to various cellular compartments (Guy and Li, 1998). Expression patterns of individual family members can vary depending on tissue type, temperature stress, wounding, and development. The expression pattern of many HSP70 members suggests they play an important role in the normal cellular biogenesis of proteins. This is supported by the finding that expression of chloroplast and cytosolic HSP70 showed diurnal regulation in spinach leaves that paralleled the diurnal cycle of total cell protein synthesis (Li et al., 2000). The ER HSP70 family member, BiP, has been extensively studied in animals and yeast. A significant body of research also exists in plants. The Douglas-fir BiP homologue was isolated through differential screening of a Douglas-fir seedling cDNA library (Tranbarger and Misra, 1995).

BiP

The ER is one of the largest cellular organelles and performs functions vital for cell viability including lipid and sterol synthesis. The ER is also the gateway to the secretory pathway and is responsible for the synthesis, assembly, and glycosylation of proteins destined for secretion, cell wall, plasma membrane, vacuole, Golgi, and protein storage vacuoles. The ER retention of BiP and other

ER resident proteins is mediated by the presence of a carboxy-terminal tetrapeptide signal sequence. The identity of this signal sequence is commonly H/KDEL. However, other variations of this signal sequence have been identified: SDEL in *Plasmodium falciparum* (Kappes et al., 1993), MDDL in *Trypanosoma brucei* (Bangs et al., 1993), *and* KEEL in *Echinococcus multilocularis* (GENBANK: M63604). An ER retention receptor has been identified in mammals (Lewis and Pelham, 1990; Lewis and Pelham, 1992; Lewis et al., 1990), yeast (Semenza et al., 1990), *Plasmodium falciparum* (Elmendorf and Haldar, 1993) and *Arabidopsis thaliana* (Lee et al., 1993) that is localized in a post ER, pre/cis-golgi compartment and is thought to retrieve escaping BiP and other ER resident proteins and return them to the ER.

BiP assists in the cotranslational and posttranslational translocation of newly synthesized polypeptides across the ER membrane (Brodsky et al., 1995; Panzner et al., 1995). During posttranslocational translocation, BiP acts as a Brownian molecular ratchet by binding nonspecifically to translocating polypeptides and minimizing passive backward diffusion that results in a net forward translocation (Matlack et al., 1999). BiP also maintains the permeability of the ER by covering both nontranslocating and active translocon pores (Hamman et al., 1998). The luminal DnaJ domain of Sec63p mediates recruitment of BiP to the translocon in yeast and stimulates its ATPase activity (Corsi and Schekman, 1997). BiPs interaction with the DnaJ domain is only transient but sufficient to stimulate ATP hydrolysis and activate BiP for peptide binding (Misselwitz et al., 1999; Misselwitz et al., 1998). Activation by the DnaJ domain is suggested to allow BiP to interact with peptides it would not normally bind and that the DnaJ domain is the primary determinant of

substrate specificity. This is despite the preferred peptide binding specificity of BiP as determined by affinity panning of a phage display library which showed a preference for a seven residue sequence Hy(W/X)HyXHyXHy, where Hy is a large hydrophobic amino acid, W is tryptophan, and X is any amino acid (Blond-Elguindi et al., 1993a).

It has been suggested that BiP exists in two different pools, one inactive oligomeric phosphorylated, or ADP ribosylated pool and another unmodified monomeric active pool able to bind unfolded or unassembled proteins (Freiden et al., 1992). More recent evidence showed that oligomeric forms can also bind peptides and stimulate their ATPase activity which induced their conversion to active monomers (Blond-Elguindi et al., 1993b). BiP binds to a variety of unfolded nascent polypeptides and participates in their folding and maturation (Hendershot et al., 1996; Simons et al., 1995). For polypeptides that are unable to attain their mature conformation, due to misfolding (Schmitz et al., 1995) or lack of a subunit component (Knittler et al., 1995), BiP remains associated with the polypeptide until it is transported via a retrograde transport system to the proteasome for degradation (Sommer and Wolf, 1997). Despite the presence of BiP binding sequences (Blond-Elguindi et al., 1993a) in most proteins, it appears that some secretory proteins do not normally bind to BiP (Graham et al., 1990; Hurtley et al., 1989; Morris et al., 1997). This may be explained by the observation that although immunoglobulin light chains contain multiple potential BiP binding sites (Knarr et al., 1995), BiP only binds to sites in domains that fold slowly (Hellman et al., 1999). Thus, the association of BiP with newly synthesized proteins during folding is not solely dependent on the presence of potential BiP binding sequences but is determined by the rate and

stability of folding. BiP has been shown to participate in protein folding in concert with other ER molecular chaperones such as calnexin, protein disulfide isomerase, and glucose regulated protein 94 (Kim and Arvan, 1995; Melnick et al., 1994; Puig and Gilbert, 1994).

The signal transduction pathways controlling BiP expression in normal unstressed cells has not been characterized but may be mediated through a growth factor mediated pathway (Brewer et al., 1997). Far more progress has been made characterizing signaling pathways controlling BiP expression in ER stressed cells, particularly in yeast and mammals. Increased expression of BiP and other ER chaperones in response to misfolded protein accumulation in the ER is mediated by the unfolded protein response (UPR) (Mori, 2000). The UPR is an intracellular signaling pathway leading from the ER to nucleus mediated through an ER transmembrane serine/threonine kinase, Ern1p/Ire1p (Cox et al., 1993; Mori et al., 1993) and a basic-leucine zipper transcription factor, Ern4p/Hac1p, in yeast (Cox and Walter, 1996; Mori et al., 1996). Ern1p/Ire1p N-terminal domain is located in the ER lumen while the C-terminal kinase/endonuclease domain faces the cytosol. Hac1p is produced in UPR activated cells and its levels are controlled by regulated splicing of its mRNA. Splicing replaces its C-terminal tail with a different peptide that renders it more resistant to rapid ubiquitin-dependent degradation (Cox and Walter, 1996). Splicing of HAC1 mRNA is mediated by the Ern4p/Ire1p C-terminal site-specific endoribonuclease that cleaves HAC1 mRNA (Sidrauski and Walter, 1997). Following translocation to the nucleus, binding of the Ern4p/Hac1p transcription factor to the 22 bp unfolded protein response element (UPRE) stimulates transcription (Mori et al., 1992). A transcriptional coactivator

complex composed of Gcn5p, Ada2p, Ada3p, and Ada5p that interacts with Ire1p and Hac1p is essential for UPR activation (Welihinda et al., 1997; Welihinda et al., 1999). Transcriptional activation is facilitated by targeting the histone acetylase, Gcn5p to promote histone acetylation of ER chaperone genes. In mammalian cells ER stress causes the reversible dissociation of BiP from the luminal domain of mammalian IRE1 resulting in the oligomerization, in *trans* autophosphorylation, and activation of IRE1 (Bertolotti et al., 2000). Despite the conservation of IRE1 between animals and yeast, subsequent downstream signaling events may be different. Mammalian IRE1 is capable of cleaving yeast HAC1 mRNA in the same sites as yeast Ire1p, however, no mammalian HAC1 homologue has yet been identified (Niwa et al., 1999). Further, following ER stress, IRE1 undergoes proteolytic cleavage that releases the C-terminal kinase/nuclease domains to accumulate in the nucleus where they are postulated to regulate splicing events leading to transcriptional enhancement of ER chaperones. In other observations, ATF6, a 90 kDa protein localized to the ER membrane undergoes proteolytic cleavage following ER stress that liberates an N-terminal 50 kDa fragment which localizes to the nucleus (Haze et al., 1999). The ATF6 50 kDa fragment is a bZIP transcription factor which stimulates transcription through a tripartite ER stress response element (ERSE), $CCAATN_9CCACG$ (Yoshida et al., 1998). Several other transcription factors also interact with the ERSE under ER stress conditions and are deemed necessary for transcriptional activation. NF-Y/CFB is a bZIP protein which recognizes the distal CCAAT motif in the human BiP promoter (Roy and Lee, 1995; Roy et al., 1996) and the p70CORE/YY1 recognizes the proximal CCACG

motif (Li et al., 1997; Li et al., 1994). The central N_9 bears a GGC trinucleotide important for interaction with the ER stress factor (ERSF) (Roy and Lee, 1999).

In contrast to other eukaryotes, BiP is encoded by multigene families in many plant species. In Tobacco BiP is encoded by a multigene family comprising approximately six or more members (Denecke et al., 1991) whereas in *Arabidopsis* BiP is encoded by only two genes (Koizumi, 1996). At least two BiP genes are present in maize and Southern blot analysis of maize genomic DNA indicated the family may contain as many as 8 members (Wrobel et al., 1997). Soybean BiP is encoded by a multigene family of at least 4 members (Figueiredo et al., 1997; Kalinski et al., 1995) but spinach BiP is encoded by only a single gene (Anderson et al., 1994a). The significance of BiP multigene families in plants is unknown.

In angiosperms, the expression of BiP is subject to developmental, hormonal, stress-induced, and diurnal regulation (Anderson et al., 1994a; Anderson et al., 1994b; Denecke et al., 1995; Denecke et al., 1991; Figueiredo et al., 1997; Jones and Bush, 1991; Kalinski et al., 1995). The expression pattern of BiP in plants is suggestive of a role in protein translocation and folding. For example, tobacco BiP transcripts are more abundant in tissues with high rates of cell division and in secretory tissues. Treatment of plants with tunicamycin, which inhibits N-linked glycosylation and proper protein folding, also results in increased levels of BiP expression (D'Amico et al., 1992; Denecke et al., 1995; Denecke et al., 1991; Koizumi, 1996). High levels of expression are associated with the accumulation of protein intermediates that are unable to attain their proper folded conformation due to mutations. The maize mutants *floury*-2, *Defective endosperm*-B30, and *Mucronase* are characterized by having reduced zein storage

protein accumulation in the protein bodies of maize endosperm. Increased BiP amounts in the *floury*-2 mutant are coincident with the appearance of a zein protein with a mutant signal peptide cleavage site which causes it to be aberrantly anchored in the ER membrane (Gillikin et al., 1997). BiP protein amounts are elevated in barley aleurone layers treated with GA_3 relative to those treated with ABA (Jones and Bush, 1991). The increased secretion of α-amylase following GA_3 treatment suggests a role for BiP in its folding. BiP has been implicated in the folding and assembly of several plant storage proteins. BiP levels are enhanced during seed storage protein deposition in soybean, rice, and pumpkin cotyledons (Hatano et al., 1997; Kalinski et al., 1995; Muench et al., 1997). BiP has been shown to associate with nascent prolamines during protein body formation in rice and is thought to retain them in the ER for assembly into protein bodies (Li et al., 1993; Muench et al., 1997). BiP has also been shown to associate with phaseolin in bean cotyledons treated with tunicamycin and with mutant phaseolin monomers that were unable to assemble into trimers (D'Amico et al., 1992; Pedrazzini et al., 1994). BiP also associates with normal phaseolin monomers but cannot be detected in association with fully assembled trimers (Vitale et al., 1996). Assembly of monomers into trimers abolished BiP binding presumably through masking of BiP binding sites during assembly. BiP may also play a role in the mobilization of storage proteins as increased amounts of BiP were observed in pumpkin microsomes during the period of storage protein mobilization (Hatano et al., 1997). It was suggested that BiP participates in the folding and secretion of hydrolytic enzymes responsible for storage protein mobilization.

Little is known about the signal transduction pathways regulating BiP expression in plants. It can be inferred that plants also possess a UPR due to the increased transcript amounts observed in tissue treated with tunicamycin. Using tobacco plants overexpressing BiP, it has been shown that BiP transcription is regulated by a feedback mechanism that monitors BiP protein levels (Leborgne-Castel et al., 1999). These findings are consistent with the existence of a plant UPR pathway. Recent evidence has shown that increased BiP expression in response to fungal elicitors in leaves is independent of the UPR (Jelitto-Van Dooren et al., 1999). Further, the elicitor response of BiP was shown to occur systemically in advance and independent of PR gene expression. Thus, it has been suggested that a novel transduction pathway exists which can anticipate increased ER protein traffic in response to pathogen attack (Jelitto-Van Dooren et al., 1999). That BiP expression is high in actively dividing tissues, secretory tissues, seed storage tissues during reserve accumulation and mobilization, and shows regulation by a variety of stimuli suggest that multiple regulatory pathways exist.

Objectives

The objectives of this thesis were:

(A) To isolate and characterize a cDNA containing a full-length open reading frame and to determine if a BiP gene family exists in Douglas-fir.

(B) To develop antiserum to the deduced amino acid sequence and examine expression of PmBiP during seed development, germination and early seedling development using northern and western blot analysis. To examine seasonal variations in the needles of one-year-old seedlings using

western blot analysis. To examined changes in PmBiP mRNA and protein amounts in young seedlings subjected to various treatments to determine possible stimuli regulating the expression of PmBiP.

(C) To confirm the subcellular localization of PmBiP predicted from the deduced amino acid sequence by subcellular fractionation and immunolocalization.

(D) To isolate substrate proteins of PmBiP during different phases of seedling development or during responses to stress via co-immunoprecipitation and to determine their identity through N-terminal amino acid sequencing.

(E) To isolate and characterize the PmBiP promoter region and to examine its expression in Douglas-fir and transgenic *Arabidopsis* when fused to the ß–glucuronidase (GUS) reporter gene.

CHAPTER 2: MATERIAL AND METHODS

Plant Material

Coastal Douglas-fir (*Pseudotsuga menziesii* [Mirb] Franco) seeds (seed-lot #952) were grown as previously described (Tranbarger and Misra, 1995). Germinating and young seedlings were collected at midday at the times indicated, frozen in liquid nitrogen, and stored at -80°C until further use. Growth of young Douglas-fir seedlings (high elevation seed-lot #6485) used for seasonal expression analysis was as described in Ekramoddoullah et al. (1995). One needle from each of 112 trees was collected and pooled on the morning of the dates indicated, frozen in liquid nitrogen, freeze dried, ground to a powder and stored at -20°C until further use. Developing seeds were collected from an open-pollinated seed orchard during midday on the dates indicated at Pacific Forest Products Ltd., Saanichton, B.C., Canada. Developing seeds were promptly dissected from cones, frozen on dry ice, and stored at -80°C until further use.

Douglas-fir embryonal suspension mass (ESM) cultures used in this work were provided by Dr. Pramod K. Gupta, Weyerhaeuser Company, Tacoma, WA, USA. ESM cultures were grown in the dark at RT in 250 ml screw-cap flasks containing a proprietary liquid media and subcultured every 10 days. The protocol for maintaining ESM cultures and generating somatic embryos is described in detail by Gupta and Pullman (1991).

Growth of *Arabidopsis* plants used for transformation was as follows. Approximately 10-20 *Arabidopsis thaliana* (L.) Heynh. seeds of ecotype

Columbia were placed on nylon screen covering moistened Sunshine mix #3 soil (Sun Gro Horticulture, Bellevue, Washington, USA) on 10 cm diameter pots and then covered with saran wrap secured with an elastic band. Pots were placed at 4°C for 2 days to promote uniform germination then transferred to a growth chamber with an 18 h 24°C day/6 h 22°C night cycle with 150 $\mu Em^{-2}s^{-1}$ of light. Saran wrap was removed when plants began to push against its surface (approximately 2 days).

Growth of Douglas-fir embryos used for transient expression was as follows. Interior Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) seeds (seedlot 8912) were imbibed for 2 days at 4°C then surface sterilized in 50% industrial bleach (6% hypochlorite) for 20 min at room temperature followed by 3 rinses in sterile dH_2O. Embryos were aseptically dissected from seeds and placed on woody plant medium (WPM; Lloyd and McCown (1980)) at 22°C in dark for 16 h before particle bombardment.

Tobacco (Xanthi) and potato (Desiree) plants for use in transformation were grown in Majenta jars on hormone free MS medium (Murashige and Skoog, 1962) under 16 h light/8h dark photoperiod at a constant temperature of 23°C.

Isolation of Full Length BiP cDNA's

A partial length BiP cDNA clone from a Douglas-fir cDNA library prepared from poly A+ RNA isolated from 4 & 6 day old seedlings was used as a probe (Tranbarger and Misra, 1995). The cDNA was ^{32}P-labelled with a random primers DNA labeling kit (GIBCO BRL, Burlington, Ontario, Canada) and used to rescreen the cDNA library, as per the manufacturers instructions (Stratagene, La Jolla, California, USA), to obtain a full length cDNA. *In vivo* excision of

recombinants showing a positive signal into the pBluescript plasmid contained within XL1-Blue *E. coli* was done as per the manufacturer instructions (Stratagene). Plasmid DNA from each positive clone was digested with EcoR I and electrophoresed on a 1% agarose gel and transferred to Zeta Probe membrane (BioRad, Mississauga, Ontario, Canada) for Southern blotting. Clones containing a BiP cDNA insert of an appropriate size were selected for DNA sequencing.

Inverse PCR and Cloning

Inverse PCR was conducted based on the method described in Ochman et al. (1990) as follows. Douglas-fir genomic DNA was extracted from spring-flush needles by a modification of the CTAB method (De Verno et al., 1989). Approximately 18 µg of DNA was digested with Xba I or Sac I overnight at 37°C. Each reaction was heat inactivated at 65°C for 20 min then suspended in 10 ml of ligation buffer (50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP) with 0.02 Weiss units/µl T4 DNA ligase for 16 h at 15°C. Circularized DNA was precipitated by the addition of 1/10 volume of 2.5 M ammonium acetate followed by 2 volumes of -20°C 100% ethanol on ice then centrifuged 25 000 x g for 10 min at 4°C. Precipitated DNA was suspended in 60 µl of sterile dH_2O of which 5 µl was subjected to PCR using Taq PCR MasterMix (QIAGEN, Mississauga, Ontario, Canada) and 250 pmol of the following primers in a 100 µl reaction. Xba I digest used primer combinations p5-3z8 (5'-AAT GAA AGC GAA GTG ACA CC-3') and p14-5a4 (5'-CAG AAC CAT TAA CAA GAG CAA GAT 3') or p14-5z1.1 (5'-AAC CAG CAG TGA TAA ACG CC-3') and p14-5a4. Sac I digest used primer

combinations p5-3z8 and p14-5a3 (5'-TAT GGT TTG GAT AAA AAG GGA G-3') or p14 5z1.1 and 14-5a3. Conditions for PCR consisted of 1 cycle of denaturing at 95°C for 5 min and 1 min at 75°C; 30 cycles of denaturing at 94°C for 1 min, primer annealing at 56°C for 1 min and an extension of 72°C for 2 min followed by a final elongation step of 72°C for 5 min. Aliquots of each reaction (20 µl) were separated on an agarose gel and subjected to Southern blotting to identify potential promoter fragments. PCR reactions containing positive fragments were cloned into the pCR®2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California, U.S.A.) according to the manufacturer instructions. Colonies containing an appropriately sized insert were screened using PCR with the appropriate primers followed by Southern blotting. Plasmid DNA for colony screening using PCR was obtained by suspending colonies in 200 µl of dH_2O followed by incubation at 85°C for 5 min. Samples were centrifuged at 16,000 x g for 5 min and 36 µl was removed and used as template for PCR.

DNA Sequencing

Both strands of all cDNA clones were manually sequenced using Sequenase according to the manufacturer instructions (United States Biochemical, Cleveland, Ohio, USA) and oligo primers (Appendix I) synthesized on a PCR MATE 391 DNA synthesizer (Applied Biosystems, Mississauga, Ontario, Canada). PmBiP promoter and expression constructs were sequenced in both directions using the Big Dye™ Terminator Cycle sequencing Ready Reaction (Perkin Elmer) and oligo primers (Appendix II) with the ABI Prism automated 377 DNA Sequencer (Perkin Elmer). Plasmid DNA for sequencing was isolated

using the Wizard™ 373 DNA Purification System (Promega, Madison, Wisconsin, U.S.A.). DNA sequence trace files were assembled using the DNASTAR program SeqMan (DNASTAR Inc, Madison, Wisconsin, U.S.A.).

Sequence Analysis

Prediction of signal sequence and signal peptide cleavage site from deduced amino acid sequences was done using SignalP V1.1 World Wide Web Server (Nielsen et al., 1997). Amino acid sequence alignment was constructed using CLUSTAL W v1.7 (Thompson et al., 1994). The phylogenetic tree was constructed using the PHYLIP package (Felsenstein, 1989). The amino acid sequences (and database accession numbers), used for this analysis were: *Aspergillus awamorii* (EMBL: Y12504), *Aplysia californica* (PIR: S24782), *Arabidopsis thaliana 1* (DDBJ: D89341), *Arabidopsis thaliana 2* (DDBJ: D89342), *Caenorhabditis elegans* (GENBANK: U56965), *Drosophila melanogaster* (PIR: JN0666), *Echinococcus granulosus* (GENBANK: M63605), *Echinococcus multilocularis* (GENBANK: M63604), *Eimeria tenella* (EMBL: Z66492), *Gallus gallus* (PIR: I50242), *Glycine max*A (GENBANK: U08384), *Glycine max*B (GENBANK: U08383), *Homo sapiens* (SWISS-PROT: P11021), *Lycopersicon esculentum* (SWISSPROT: P49118), *Mesocricetus auratus* (SWISS-PROT: P07823), *Mus musculus* (SWISS-PROT: P20029), *Neurospora crassa* (EMBL: Y09011), *Nicotiana tabacum*4 (SWISS-PROT: Q03684), *Nicotiana tabacum*5 (PIR: JQ1361), *Oryza sativa* (GENBANK: AF006825), *Phytophthora cinnamomi* (PIR: S38890), *Plasmodium falciparum* (EMBL: X69121), *Phaeodactylum tricornutum* (GENBANK: U29675), *Pseudotsuga menziesii* (GENBANK: Z49764) *Rattus norvegicus* (SWISS-PROT: P06761), *Saccharomyces cerevisiae* (SWISS-PROT: P16474), *Spinacia oleracea*

(GENBANK: L23551), *Trypanosoma brucei* (GENBANK: L14477), *Xenopus laevis* (GENBANK:U62807), *Zea mays*E2 (GENBANK: U58208), and *Zea mays*E3 (GENBANK: U58209).

Analysis of PmBiPPro1 DNA sequence and identification of putative regulatory elements was done by searching the plant cis-acting DNA regulatory database (PLACE) (Higo et al., 1999).

Three-Dimensional Modeling

The three dimensional models of the ATP and peptide binding domains were constructed using the Swiss-PdbViewer and the knowledge based comparative modeling approach as implemented in the SWISS-MODEL server (Guex and Peitsch, 1997; Peitsch, 1996). The following PDB coordinate files (and % identity) were used to construct the ATP binding domain model: 1NGJ.pdb (70.8%), 1ATR.pdb (70.63%), 1NGF.pdb (70.63%), 1ATS.pdb (70.63%), and 1HPM.pdb (70.83%). The following PDB coordinate files (and % identity) were used to construct the peptide binding domain model: 1DKZ.pdb chain A (40.6%), 1DKY.pdb chain A (40.6%), 1DKY.pdb chain B (45.43%), and 1DKX.pdb chain A (40.33%).

Genomic DNA extraction and Restriction Analysis

Douglas-fir genomic DNA was extracted from 40 g spring-flush needles by a modification of the CTAB method (De Verno et al., 1989). Aliquots of 10 µg of DNA were digested for 26 h with restriction enzymes then separated on a 0.7% agarose gel. Hybridization methods were based on those described in Lueders and Fewell (1994) as follows. The gel was incubated at room temperature (RT)

with shaking in denaturing solution (0.5 N NaOH, 150 mM NaCl) for 30 min, rinsed in distilled water, and incubated in neutralizing solution (500 mM Tris-HCl pH 8, 150 mM NaCl) for 30 min. The gel was dried on a vacuum gel drier for 30 min with vacuum only followed by 1 h at 60°C. The dried gel (unblot) was probed with ^{32}P-labelled, random primed, PmBiP cDNA in hybridization solution (0.5 M Na_2HPO_4 pH 7.2, 7% SDS, 100 µg/ml denatured salmon sperm DNA) at 65°C overnight then washed at low stringency twice in hybridization solution for 45 min each at 65°C. The unblot was exposed for 7 days under a phosphorimaging screen and developed using the STORM 820 Phosphorimager (Molecular Dynamics, Sunnyvale, California, USA). Following development, the unblot was washed at high stringency twice in wash buffer (20 mM Na_2HPO_4 pH 7.2, 1% SDS) for 45 min each at 65°C and exposed for 8 days and developed as above. Quantitation was performed using the Image Quant NT software (Molecular Dynamics). Calculation of gene copy number was as described in Pasternak (1993) using a Douglas-fir genome size of 25 pg per haploid nucleus (Ingle et al., 1975).

PCR amplification of BiP from Douglas fir genomic DNA.

Douglas-fir genomic DNA was isolated as described above. PCR amplifications were carried out using *Taq* polymerase according to the directions of Perkin-Elmer Cetus together with synthetic primers, used in DNA sequencing, directed towards the coding region from position 276 to 3' and from position 2251 to 5' (see fig 2). The following amplification strategy was employed: 95°C for 5 min, 75°C for 1 min to add *Taq*. This was followed by 3 cycles of 97°C for 1 min, 58°C for 1 min, and 72°C for 2 min; 30 cycles of 94°C

for 1 min, 61°C for 1 min, and 72°C for 2 min. The reaction was completed by a final extension at 72°C for 5 min. The reaction products were then separated on a 0.7% agarose gel and transferred to Zeta probe membrane (BioRad) for Southern blotting.

Northern Blotting

Total RNA was isolated as described in Kaukinen et al. (1996), separated on a 1% agarose/formaldehyde gel and transferred to Zeta-Probe GT membrane (BioRad). Blots were then probed with ^{32}P-labelled, random primed, PmBiP cDNA following the basic hybridization conditions described in the Zeta-Probe manual. Blots were stripped and reprobed with a PCR amplified genomic fragment representing the Douglas-fir 18S rRNA gene to account for differences in the amount of RNA loaded per lane. Densitometry and adjustment for differences in the amount of RNA loaded per lane (calculation of integrated optical density) was performed as described in Tranbarger and Misra (1996) except for Figure 21E, F, and G. Densitometry for these figures was performed using the ChemiImager™ 4000 system (Alpha Innotech Corporation, San Leandro, California, U.S.A.). Adjustment for differences in the amount of RNA loaded per lane (calculation of integrated optical density) was done by dividing the PmBiP signal density by the fluorescence value of the total ethidium bromide staining in each lane. The result of this calculation was then multiplied by 100 and represented graphically (Figure 21G). Hybridization signals shown in Figure 21A and Figure 23A are from samples blotted on the same membrane and exposed under film overnight. Samples from Figure 21E were exposed under film for 5 days.

Southern Blotting

DNA was electrophoresed on a 1% agarose gel and transferred to Zeta Probe membrane (BioRad) according to the manufacturers instructions. The PmBiP3 cDNA was ^{32}P-labelled with a random primers DNA labeling kit (GIBCO) and hybridization and washing conditions were performed as described under the standard protocol in the Zeta Probe manufacturers instructions. Blots were exposed onto Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, New York, U.S.A.) overnight at -80°C.

Isolation and partial purification of BiP protein

Isolation of BiP was based on the method of Nandan et al. (1994) as follows. Six-day-old Douglas-fir seedlings were ground in liquid nitrogen with a mortar and pestle. The resulting powder was suspended in extraction buffer (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 1mM phenylmethylsulfonyl fluoride (PMSF), 10 µM leupeptin) at a ratio of 1:10 (w:v). The slurry was vortexed vigorously for 2 minutes and centrifuged at 16 000 x g for 25 min. The resulting supernatant was collected with care not to remove fatty layer floating on top of the supernatant. The supernatant was incubated with a 50% solution of gelatin agarose beads (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) in sterile phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) at a ratio of 1:10 (v:v) for 2 hours at 4°C with shaking. The beads were collected and washed 3 times in 4 bed volumes of extraction buffer adjusted to 0.5 M NaCl. The beads were washed once more with 5 mM HEPES pH 7.5. The beads were then incubated twice in 5 bed volumes of release solution (5 mM HEPES, pH 7.5, 3 mM ATP, 1 mM

$MgCl_2$) for 10 min at RT with shaking. The resulting supernatant from the two incubations was pooled and dialyzed overnight at 4°C against distilled water and then lyophilized.

Antibody Production

A synthetic peptide corresponding to the C-terminal 13 amino acids of the PmBiP deduced amino acid sequence was synthesized at the University of Victoria Protein Micro-Chemistry Centre using a Model 430A Applied Biosystems peptide synthesizer (Foster City, California, USA) with the FastMoc chemistry software. The peptide, with an additional cysteine residue added to the N-terminal end, was then conjugated to KLH carrier protein using the Imject kit according to the manufacturer instructions (Pierce, Rockford, Illinois, USA). The conjugated peptide was mixed with Freunds complete adjuvant and injected into New Zealand white rabbits. Subsequent booster injections were given at 2-week intervals using the conjugated peptide prepared in Freunds incomplete adjuvant.

ELISA

ELISA titration of PmBiP antiserum was conducted in Polystyrene microtitre plates coated with 2.5 µg per well of the PmBiP C-terminal peptide prepared in distilled water. After drying overnight at 37°C wells were blocked for 2 h at RT with a solution of 3% powdered milk prepared in PBS then washed 3 times with PBS-Tween (PBS supplemented with 0.05% Tween-20). Antiserum was diluted in PBS-Tween + 1% BSA and added to wells to incubate for 2 h at RT then washed 3 times with PBS-Tween quickly followed by 3 washes for 5 min

each. Wells were incubated with a 1:2000 dilution of goat anti rabbit IgG-Alkaline Phosphatase 2° antibody in PBS-Tween for 2 h at RT followed by 3 quick washes with PBS-Tween and three 5 min washes. Substrate, p-nitrophenyl phosphate (1mg/ml in diethanolamine), was added to each well and allowed to incubate for 10 min at RT in the dark. Absorbance was then measured at 405 nm.

Immunofluorescence Localization

Root tips of 5 day old Douglas-fir seedlings were fixed for 1-2 h at RT with slow agitation in 1% formaldehyde (Polysciences 16% stock, Warrington, Pennsylvania, USA), 0.1% glutaraldehyde (Polysciences 50% stock) in 20 mM Pipes (1,4- piperazinediethanesulfonic acid), then washed 3 times over 1 h in 50 mM Pipes containing 5 mM EGTA and 2 mM $MgSO_4$. Samples were dehydrated to 100% ethanol stepwise at 20 min intervals, 10, 25, and 50% at 0°C and 75, 95, 100 (3 changes) at -20°C. Samples were embedded according to previously published methods as follows (Baskins et al., 1992). Samples were infiltrated in methyl/butyl methacrylate (methyl methacrylate, 1 part; butyl methacrylate 4 parts; benzoin ethyl ether, 0.5%; DTT 10 mM. Gaseous nitrogen was bubbled through the resin mixture for 1 h immediately before use to displace dissolved oxygen). Infiltration was carried out according to the following schedule: 1:2, ethanol:resin, overnight; 1:1, 4 h; 2:1 overnight; pure resin, four changes over 48 h. Samples were then embedded in double ended beam capsules, left a further 24 h and finally exposed to a 365 nm UV lamp as the temperature was gradually raised from -20°C to +20°C over about 8 h to polymerize the resin. Sections of 1-3 μm were cut on dry glass knives and

allowed to dry from a drop of water onto poly-l-lysine coated teflon well slides (Polysciences). To illustrate quality of preservation, some sections were stained with toluidine blue (1% w/v in 1% borax). Sections attached to slides were immersed in acetone for 15 min to remove resin and then rehydrated in decreasing ratios of ethanol: 0.85% NaCl (100%, 80%, 50%, 30%, 1 min each), transferred to PBS (20 min), PBS plus 1% BSA (10 min), and then into primary antibody diluted 1:3000 with PBS or preimmune serum overnight at 4°C followed by 1 h at 37°C. Sections were washed 3 times with PBS (20 min each) and then treated with secondary antibody, anti-rat IgM Cy3-conjugate (Jackson, West Grove, Pennsylvania, USA) diluted 1:400 for 1 h at 37°C. Sections were rinsed thoroughly with PBS, treated with DAPI (1 μg/ml) to stain nuclei and chromosomes and mounted in FluorSave (Calbiochem, La Jolla, California, USA).

Protein Extraction and Western Blotting

Protein extraction from whole developing seeds, mature seeds, germinating seeds, and young seedlings was done by grinding approximately 100 mg of tissue in liquid nitrogen with a mortar and pestle. The powder was suspended in extraction buffer containing 65 mM Tris-HCl, pH 6.8, 1% SDS, 5% glycerol, and 2.5% 2-mercaptoethanol and boiled for 5 min, frozen at -80°C for 1 h, boiled 5 min then centrifuged at 16 000 x g for 25 min. The supernatant was collected and saved for further analysis. For subcellular fractionation (Figure 14), approximately 5 g of tissue was frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The powder was suspended and vortexed in buffer A (100 mM Tris-HCl pH 7.5, 250 mM sucrose, 2 mM $MgCl_2$,

10 mM KCl, 1 mM phenylmethylsulfonylflouride (PMSF), 2.8 mM 2-mercaptoethanol) then filtered through 2 layers of Miracloth (Calbiochem). The filtrate was centrifuged at 25 000 x g for 30 min. The supernatant was collected and centrifuged at 140 000 x g for 1 h. The supernatant (soluble fraction) was saved and the pellet (microsomal fraction) was suspended in buffer B (50 mM phosphate buffer pH 7.5, 20% glycerol and 10 mM 2-mercaptoethanol). Microsomes were separated into soluble and membrane fractions according to Fujiki et al. (1982). To purify nuclei (nuclear fraction), the pellet from the 25 000 x g centrifugation was resuspended in buffer A, layered on a 25%/75% Percoll (Sigma-Aldrich) step gradient and centrifuged at 1000 x g for 20 min. Nuclei were collected from the 25%/75% interface, washed 2X in buffer A, and suspended in buffer B. Sucrose gradient fractionation of microsomes was based on the method described in Coughlan et al. (1996). Microsomes were layered on a 12 ml sucrose step gradient consisting of a 2 ml 20% (w:v) layered on top of 10 ml of a 30-60% (w:v) linear sucrose gradient. Samples were centrifuged in a swinging bucket rotor for 2 h at 112 700 x g at 4°C. One-ml fractions were collected from the bottom of each tube and analyzed by western blotting. Sucrose concentrations were determined using a refractometer. Protein concentrations were determined by the BioRad Reagent protein assay (BioRad). Extraction and quantitation of needle proteins from seasonal samples and densitometry of western blot was as described in Ekramoddoullah et al. (1995). Protein samples were suspended in protein sample buffer (12.5 mM Tris-HCl pH 6.8, 2 % SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue), boiled for 3 min and separated by SDS-PAGE using the Mini-PROTEAN II gel electrophoresis system (BioRad) with a 4% (w/v)

acrylamide stacking gel (80 volts; constant voltage) and an 11% (w/v) acrylamide separating gel (200 volts; constant voltage). The proteins were stained with Coomassie Brilliant Blue R250 or transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, New Hampshire, USA) using a Mini-Trans-Blot cell (BioRad) at 100 volts for 1 h in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol and 0.1% SDS). The membrane was blocked overnight at 4°C in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl; pH 7.5) containing 0.05% Tween-20 (TTBS), incubated with primary antibody (diluted 1:3000 in TTBS) for 90 min at RT then washed two times with TTBS (5 min each). The membrane was then incubated with an alkaline phosphatase conjugated goat anti-rabbit antibody (1:3000 dilution in TTBS) (Cedar Lane Laboratories Ltd., Hornby, Ontario, Canada) for 45 min at RT followed by washing in TTBS (5 min) and TBS (5 min). Immunoreactive bands were visualized by incubating the membrane with 5-bromo-4-chloro-3-indolyl-phosphate (0.165 mg/ml) and nitroblue tetrazolium (0.33 mg/ml) as substrate in buffer containing 100 mM $NaHCO_3$ pH 9.8 and 1 mM $MgCl_2$. Two-dimensional SDS-PAGE was done as described elsewhere (O'Farrell and O'Farrell, 1977).

Immunoprecipitation

PmBiP IgG was purified from whole serum using one-ml Protein A Sepharose 4 fast flow beads (Pharmacia, Peapack, New Jersey, USA) according to the manufacturer's instructions. Immunoprecipitation was done essentially as described in Sambrook et al. (1989). Protein samples were suspended in 70 µl of 1% NP-40 and left on ice for 10 min. Samples were brought up to a final

volume of 500 µl with modified Net-gel buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin) and an appropriate amount of purified PmBiP IgG. Approximately 0.13 µg purified IgG was used per µg of microsomal protein. Samples were incubated for 2 h on a rotating shaker at 4°C. An appropriate amount of a 50% solution of Protein A Sepharose beads (1 µl per µg IgG equilibrated in modified Net-Gel buffer) was added and incubated on a rotating shaker for 90 min at 4°C. Samples were centrifuged briefly to pellet Protein A Sepharose beads. Pellets were washed two times in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and once in buffer containing 10 mM HEPES pH 7.5, 0.1% NP-40 for 20 min each at 4°C in a rotating shaker. Pellets were then suspended and boiled in sample buffer in preparation for SDS-PAGE and silver staining or were washed in release buffer (10 mM HEPES pH 7.5, 3 mM ATP, 4 mM $MgCl_2$) for 15 min at RT with gentle shaking. The supernatant from the release buffer wash was collected and subjected to SDS-PAGE as described above and silver staining as described elsewhere (Sambrook et al., 1989).

Construction of vector pSBIP 3 containing PmBiP coding sequence

Expression vector pSBiP3 (for *Agrobacterium* Ti-mediated plant transformation) was constructed by first removing *gusA* gene from pBISN1 (obtained from S. B. Gelvin, Purdue University) by digesting with Sal I and Not I and inserting the PmBiP3 coding sequence (Figure 1). The PmBiP sequence was PCR amplified from plasmid containing PmBiP cDNA using the following primers: 5'-primer: 5'-GGT TTT GGA TCT AGA **GTC GAC** ATG GGA CGG AAG -3' and 3'-

primer: 5'-CAA CAG AAA CCG CGG CCG CTT AGA GTT CTT C-3' containing Sal I and Not I sites (bold) respectively. In vector pSBiP3, PmBiP is expressed from "super promoter" containing *mas* (mannopine synthase) promoter/activator region preceded by trimer of the *ocs* (octopine synthase) upstream activating sequence.

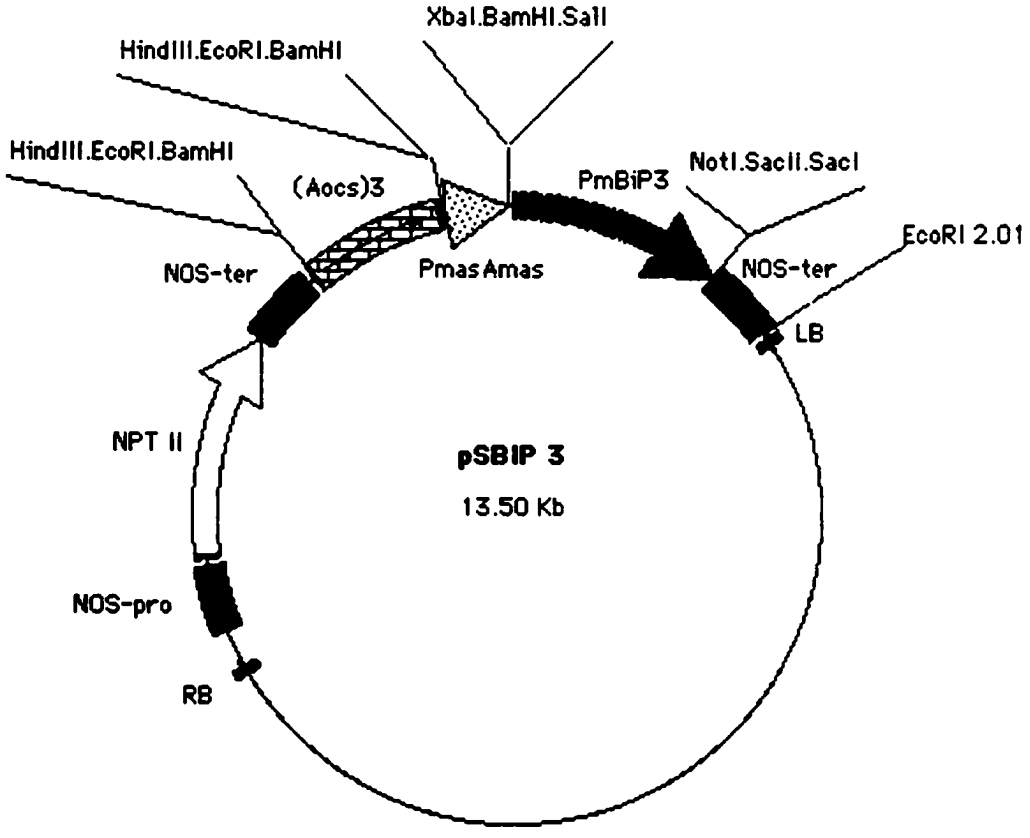

Figure 1. Expression vector construct for PmBiP cDNA

Vector pSBiP for the *Agrobacterium* mediated transformation and stable expression of PmBiP in *Arabidopsis* was constructed as described in Materials and Methods. Various restriction endonuclease sites are indicated. RB = right border, LB =left border NOS-pro = nopaline synthase promoter, NOS-ter = nopaline synthase terminator, NPT II = kanamycin resistance gene neomycin phosphotransferase, $(Aocs)_3$ = trimer of octopine synthase upstream activating sequence, PmasAmas = mannopine synthase promoter/activator region, PmBiP 3= PmBiP cDNA.

Construction of vectors containing PmBiP promoter sequences

The following promoter-gene fusions were constructed for expression in the cytosol. Plasmids were constructed from parent plasmids pBI121 and pBI221 for stable (Figure 2) and transient expression, respectively. Vectors for transient expression are basically as shown in Figure 2 but do not contain the right and left borders and the kanamycin resistance cassette. PmBiP promoter constructs were generated using PCR with either Taq polymerase (PmBiPpro1-1; Qiagen, Mississauga, Ontario, Canada) or DeepVent polymerase (PmBippro1-3 and PmBiPpro1 5; NEB, Beverly, Massachusetts, USA) and PmBiPPro1 clone as template.

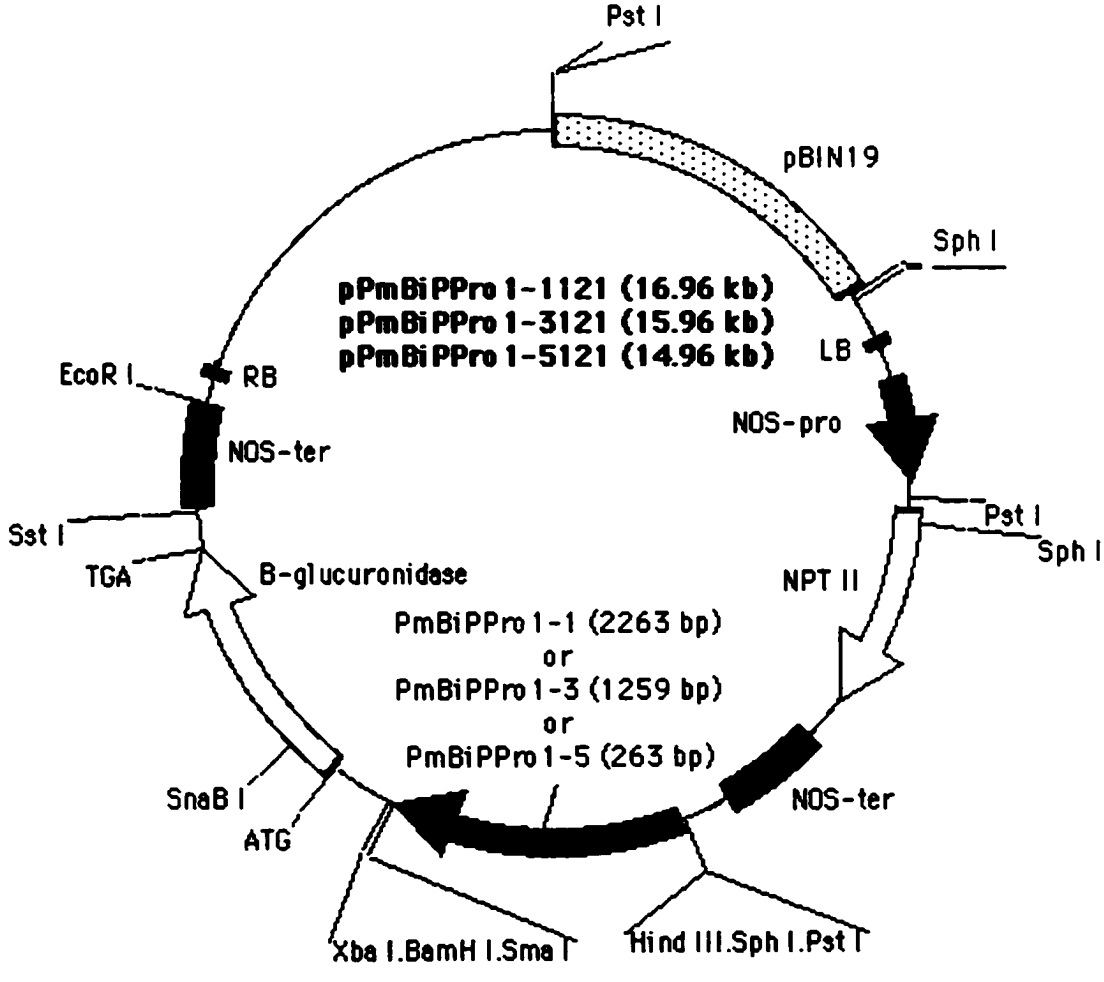

Figure 2. PmBiPPro1 reporter constructs

PmBiPPro1 reporter construct plasmids pPmBiPPro1-1121 (16.96 kb), pPmBiPPro1-3121 (15.96 kb), and pPmBiPPro1-5121 (14.96 kb) containing PmBiPPro1-1 (2263 bp), PmBiPPro1-3 (1259 bp), and PmBiPPro1-5 (263 bp) respectively for *Agrobacterium* mediated transformation were created by successive deletions of the 5' end of the PmBiPPro1 nucleotide sequence and fused to the GUS (β–glucuronidase) reporter gene as described in Materials and Methods. Various restriction endonuclease sites are indicated. RB = right border, LB =left border NOS-pro = nopaline synthase promoter, NOS-ter = nopaline synthase terminator, NPT II = kanamycin resistance gene neomycin phosphotransferase.

The primers, containing Hind III and Xba I sites (bold), used for amplification of the various promoter constructs employed the same 3' primer (5'-TCG AAG CGC AAA **TCT AGA** GTT TAA ACT TCC-3') and the following 5' primers: PmBiPPro1-1 (5'-AAG AAG GC**A AGC TTT** CAA CTA A-3'), PmBiPPro1-3 (5'-GCA TAA GAA **AGC TTC** TAC CCT G-3'), and PmBiPPro1-5 (5'-GCA CTA GG**A AGC TTG** GGA ACT C-3'). Following restriction digestion, the resulting products (PmBiPpro1-1, 2263 bp; PmBiPpro1-3, 1259 bp; PmBiPpro1-5, 263 bp) were cloned into Hind III and Xba I sites of pBI221 replacing the CaMV 35S promoter (~0.8 kb). The resulting plasmids, containing PmBiP promoter sequences were labeled pPmBiPPro1-1221, pPmBiPPro1-3221, and pPmBiPPro1-5221. Replacing the Hind III - Xba I fragment in pBI121 (containing CaMV 35S promoter) by Hind III - Xba I fragments from pPmBiPPro1-1221, pPmBiPPro1-3221, and pPmBiPPro1-5221 respectively, created plasmids pPmBiPPro1-1121, pPmBiPPro1-3121, and pPmBiPPro1-5121.

Transient Expression

Germinated Douglas-fir zygotic embryos were bombarded using the model PDS-1000/He Biolistic® Particle Delivery System (BioRad). DNA (pBI221 plasmid derivatives) was coated onto gold particles (1-3 µm diameter; Sigma-Aldrich) as described by Jefferson (1987) as follows. A gold suspension (60 mg/ml) was prepared in 50% glycerol of which 15 µl was placed in 1.5 ml microfuge tubes with an appropriate amount of either a CaMV 35S::GUS plasmid (pBI221, 5700 bp; Clontech Laboratories Inc, Palo Alto, California, U.S.A.), pPmBiPPro1-1221 (7188 bp), pPmBiPPro1-3221 (6184 bp), or pPmBiPPro1-5221 (5189 bp), 15µl of 2.5 M $CaCl_2$, and 6 µl of 0.1M spermidine with continuous vortexing. The particles were allowed to settle on ice then pelleted by a brief centrifugation. The supernatant was discarded and 70 µl cold 70% ethanol was added without disturbing the pellet. The 70% ethanol was then removed and 70 µl cold 100% ethanol added without disturbing the pellet. This too was removed and the particles suspended in 30 µl cold 100% ethanol with slow vortexing. Aliquots of 10 µl were placed on a macrocarrier disk and allowed to dry in the presence of silica gel desiccant. Each bombardment delivered 1.4×10^{11} constructs and was conducted using the following parameters. The gap distance between the rupture disk and macrocarrier was 0.6 cm, the macrocarrier travel distance was 0.6 cm, the target tissue distance was 8 cm from the microcarrier launch assembly platform, the sample chamber vacuum was 25 inches of mercury, and rupture pressure was 1550 psi. Tissue was then incubated on WPM at 22°C under dark for 48 h prior

to histochemical GUS staining. Following GUS staining (see below) the number of blue spots were counted using a stereo dissecting microscope.

Arabidopsis Transformation

Arabidopsis thaliana plants were transformed according to the method of Clough and Bent (1998). *Agrobacterium tumefaciens* strain MP90 carrying the plasmid CaMV35S::GUS, PmBiPPro1-1::GUS, PmBiPPro1-3::GUS, or PmBiPPro1-5::GUS, was grown to stationary phase in liquid culture at 28°C, 250 rpm, in sterile LB broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.0) containing 50 µg/ml kanamycin and 10 µg/ml gentamycin. Cells were harvested by centrifugation for 20 min at room temperature at 5500 x g then suspended in infiltration medium (5.0% sucrose and 0.05% Silwet L-77 (Lehle Seeds, Round Rock, Texas, U.S.A.)) to a final OD_{600} of approximately 0.8 prior to use. The above ground portions of plants were dipped in infiltration medium containing *Agrobacterium* for 10 seconds with gentle swirling 2 days after removal of the primary bolt. One subsequent dip was made 7 days later. Following each dip, the plants were covered with a plastic bag for 24 h to retain moisture. Plants were grown normally and fed with HI•SOL 18-24-12 soluble plant food (1g/l; Green Valley Fertilizer, Abbotsford, B.C, Canada) once a week via sub-irrigation. Plants were no longer watered once seed-pods began turning brown. When plants were fully dried they were placed in a brown paper bag for 1 week prior to collecting seeds. Seeds were collected by rubbing plants and pods with hands and filtering through a 0.707 mm mesh sieve (W.S. Tyler Company of Canada Ltd., St. Catherines, Ont., Canada) several times until seeds were reasonably free of debris. Seeds were sterilized using vapor phase

sterilization as follows (Clough and Bent, 1998). Collected seeds were placed in 15 ml conical tubes (2-3 ml seeds per tube) with lids attached loosely. Tubes were placed in a rack inside a plastic vacuum dessicator (Bel-art #42025, 240 mm internal diameter) containing a 250 ml glass beaker with 150 ml bleach (5.25% hypochlorite). Five ml of concentrated HCL was placed in a 10 ml glass beaker and floated on top of the bleach solution. The lid was placed on the desiccator and a slight vacuum applied. The desiccator was shaken slightly to spill the concentrated HCL into the bleach and liberate chlorine gas for overnight sterilization. Sterile seeds were sprinkled on 150 x 15 mm^2 selection plates (1/2 MS media, 0.8% agar, 1% sucrose, 50 µg/ml kanamycin, 100 µg/ml ampicillin) and placed in dark at 4°C for 2 days. Plates were removed and placed in a growth chamber with 16h light /8h dark at 22°C for 2 weeks. Healthy green transformants were selected and placed in moist soil in a growth chamber with an 18 h 24°C day/ 6 h 22°C night cycle with 150 $\mu Em^{-2}s^{-1}$ of light covered with saran wrap for the first 2 days of growth.

Tobacco and Potato Transformation

Leaf strips from tobacco and stem segments (5-10 mm pieces) and leaves (cut at the base) from potato were pre-cultured upside down for 3-5 days on MS 104 medium (MS medium supplemented with 1 µg/ml BAP, 0.1 µg/ml NAA, pH 5.7). Explants were incubated in S2 medium (MS medium without agar but supplemented with 0.5 g/l MES and 20 g/l mannitol) inoculated with a 1:200 (v:v) dilution of an overnight culture of *Agrobacterium tumefaciens* strain MP90 for 2-3 days under low light intensity. *Agrobacterium tumefaciens* overnight culture was grown at 28°C in LB media supplemented with 50 µg/ml

kanamycin and 10 µg/ml gentamycin. Explants were incubated at low light intensity on Stage I medium (MS medium supplemented with 6 g/l agarose (instead of 8 g/l agar), 200 mg/l glutamine, 600 mg/l MES, 500 mg/l polyvinylpyrrolidone (PVP), 20 g/l mannitol, 20 g/l glucose, 40 mg/l adenine-SO_4, 2.5 mg/l zeatine-riboside, 0.1 mg/l NAA, and 0.02 mg/l GA_3) for 3-5 days followed by transfer to Stage II medium (Stage I medium supplemented with 100 µg/ml kanamycin and 500 µg/ml cefotaxime) for 7-12 days to initiate callus. To initiate shoots, explants containing callus were transferred to Stage III medium (Stage II medium containing no NAA) for 5-7 weeks. Following shoot formation, plantlets were transferred to rooting medium (MS medium supplemented with 50 µg/ml kanamycin and 250 µg/ml cefotaxim) and grown as described for parent plants.

Histochemical GUS Staining

GUS staining was based on the method described by Jefferson (1987). Tissue from particle bombardment or transgenic plants was immersed in solution containing 1 mM X-Gluc, 100 mM sodium phosphate buffer pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% triton X-100, and incubated overnight at 37°C.

In vitro GUS assay

Fresh plant tissue was placed in a 1.5 ml eppendorf tube containing ice cold lysis buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 10 mM 2-mercaptoethanol, and 0.02 g/ml insoluble PVP) and homogenized using a glass pestle connected to a Barnant series 10 mixer

(Barnant Company, Barrington, Illinois, USA). Homogenates were centrifuged at 16 000 x g for 15 min at 4°C. Supernatants were collected and assayed for protein using the method of Bradford (1976). GUS activity was measured in 100 µl extraction buffer (without PVP) containing 6 µg of total protein and 1mM p-nitrophenyl ß-D-glucuronide as substrate at 37°C using a Thermomax microplate reader and Softmax Pro v3.1 software (Molecular Dynamics Corporation). Absorbance was measured at 405 nm every 5 min or after 18 h.

CHAPTER 3: RESULTS

Isolation of full-length cDNAs and sequence analysis

Characterization of BiP genes from angiosperms has shown that they are highly conserved and can be encoded by multigene families (see above). Nothing is known about the genetic structure of BiP genes in gymnosperms. To determine the primary structure of BiP in Douglas-fir and to further understand its role in development, full-length BiP cDNAs were isolated and characterized.

Rescreening Douglas-fir seedling cDNA library

To isolate full-length clones a partial PmBiP cDNA (Tranbarger and Misra, 1995) was used as a probe to rescreen a cDNA library prepared from poly A^+ mRNA from 4-6-day-old seedlings. Screening of approximately 3.6 x 10^5 phage clones yielded 23 positive signals. Secondary screening of 14 clones yielded 12 positives that were *in vivo* excised to form the pBluescript plasmid in XL1- Blue *E. coli* cells. Full-length cDNA clones were chosen based on a BiP transcript sized of approximately 2.5 kb (Tranbarger and Misra, 1995). Restriction digestion of plasmid DNA from 12 potential clones yielded cDNA inserts of varying sizes (Figure 3).

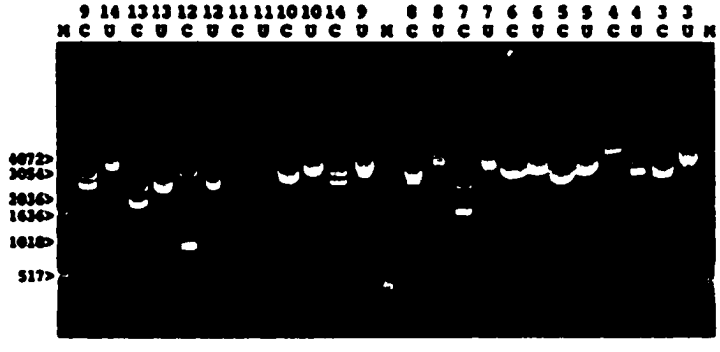

Figure 3. Isolation of full-length PmBiP cDNA clones

Ethidium bromide staining of restriction digests of 12 positive cDNA clones isolated from rescreening of the Douglas-fir cDNA library (Tranbarger and Misra, 1995). Markers (M) are indicated on the left (in bp) and clones are listed along the top. u, uncut; c, cut.

Four cDNA clones, PmBiP3, PmBiP5, PmBiP10, and PmBiP14 contained inserts of an appropriate size and were subjected to Southern blot analysis to further confirm the identity of the isolated inserts (Figure 4). All 4 cDNAs hybridized to the partial length BiP cDNA probe and were selected for DNA sequencing.

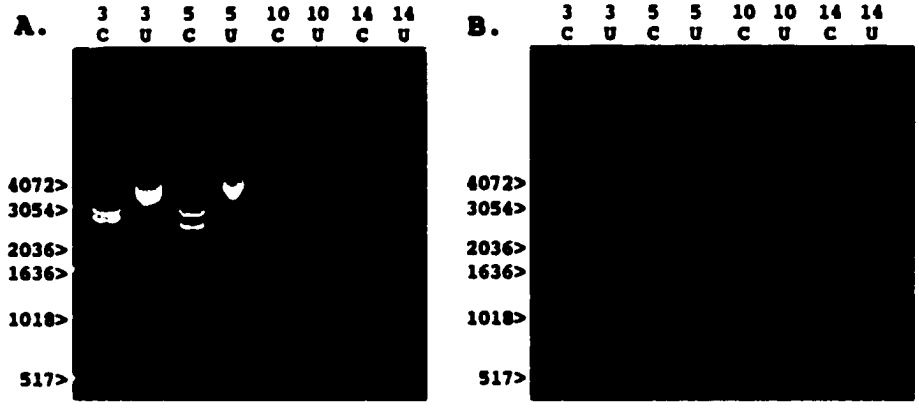

Figure 4. Identification of full length BiP cDNA clones

Plasmid DNA prepared from positive clones obtained through re-screening a Douglas-fir 4 and 6 day germination cDNA library was digested with EcoR1 and electrophoresed on a 1% agarose gel then stained with ethidium bromide (A). Identities of the cDNA inserts were confirmed by Southern blotting (B). Samples include BiP clone 3, 5, 10, and 14, cut (c) or uncut (u), as well as a 1 kb ladder (M). Size markers (given in base pairs) are indicated.

DNA sequencing and analysis

DNA sequencing of the four clones PmBiP3, PmBiP5, PmBiP10, and PmBiP14 indicated they were 2626 bp, 2495 bp, 2552 bp, and 2423 bp in size, respectively. Nucleotide sequence alignment yielded few yet distinct differences between the

4 cDNAs (Table 1). The highest proportion of the sequence differences were in the 5' and 3' untranslated regions (UTR; 7 total) with clones 3 and 10 also containing a deletion in the 3' UTR at position 2429/2430. The differences existing within the coding region (4 total) were, in every case, in the third base pair of the codon and did not result in any differences in the amino acid sequence.

bp position	PmBiP 3	PmBiP 5	PmBiP 10	PmBiP 14	Consensus
176	A	T	T	*	T
185	A	A	G	*	A
258	T	G	T	*	T
331	A	G	A	A	A
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2388	C	T	C	C	C
2429,2430	-	G	-	G	-,G
2453	G	G	G	A	G
2454	A	A	T	A	A

Table 1. Summary of the differences in the nucleotide sequence of four PmBiP cDNAs

Numbering of bp position is relative to that given for the PmBiP3 cDNA sequence. Shaded area indicates differences within the coding region. * indicates no base for that position due to shorter cDNA length.

The nucleotide and deduced amino acid sequence of the largest cDNA clone (2626 bp) is shown in Figure 5 (Genebank accession number Z49764). All future reference to PmBiP will refer to the PmBiP3 cDNA nucleotide and deduced amino acid sequence as it is the largest clone and does not vary significantly with the other cDNAs. The origin of the other cDNA sequences will be discussed below.

```
   1 gaagctgccgtggtcgcagataatgcaattgcaatgctgaggtttctctgagaggatcgatagtcgggac
  71 gattttctctgtttcgatacatatccttttcgcttttcaacgatatcgcttcgttttcagccatttaattc
 141 gcatacgtgaacgaagatcggccgcagtgaaggttatcttgtcgatttcgctgttgtgagctttttgcac
 211 tgcgataacacaccaataggtgtcacttcgctttcattcacgaggtattgaggttgcttctgcttaaaat
 281 ttgatgcgcgagggttttggaaaggcgccagccatgggacggaagcagaaatgcgctgggttcaacaacg
 351 ctggaaaagatttcaacggctttATGTTCCTTGCGGCGTTTATCACTGCTGGTTTTCTTTTCAGCTCTGT
                          M  F  L  A  A  F  I  T  A  G  F  L  F  S  S  V  16
 421 TATTGCTGCAGAAGAAGCAGCAAAGTTAGGAACAGTAATTGGTATAGATCTCGGAACCACGTATTCTTGT
       I  A  *A  E  E  A  A  K  L  G  T  V  I  G  I  D  L  G  T  T  Y  S  C  39
 491 GTTGGTGTTTACAAAAATGGTCATGTTGAAATCATAGCAAATGACCAAGGAAATAGGATTACACCTTCTT
       V  G  V  Y  K  N  G  H  V  E  I  I  A  N  D  Q  G  N  R  I  T  P  S  62
 561 GGGTTGCCTTCACTGATACCGAAAGACTCATCGGAGAGGCTGCCAAAAACCAGGCGGCAATGAATCCTGA
       W  V  A  F  T  D  T  E  R  L  I  G  E  A  A  K  N  Q  A  A  M  N  P  E  86
 631 AAGGACCGTTTTTGATGTGAAACGGTTGATTGGAAGAAAGTATGAGGACAAGGAGGTGCAAAAAGACATC
       R  T  V  F  D  V  K  R  L  I  G  R  K  Y  E  D  K  E  V  Q  K  D  I  109
 701 AAACTTTTGCCCTACAAAATTGTAAACAAAGATGGGAAGCCTTACATTCAGGTGAAGATCAGGGATGGTG
       K  L  L  P  Y  K  I  V  N  K  D  G  K  P  Y  I  Q  V  K  I  R  D  G  132
 771 AAATCAAAGTTTTTAGTCCCGAGGAAATTAGTGCAATGATTTTGTTGAAAATGAAGGAAACAGCTGAGTC
       E  I  K  V  F  S  P  E  E  I  S  A  M  I  L  L  K  M  K  E  T  A  E  S  156
 841 CTACCTTGGAAGGAAAATCAAGGATGCAGTTGTTACAGTTCCAGCATATTTCAATGATGCACAAAGACAG
       Y  L  G  R  K  I  K  D  A  V  V  T  V  P  A  Y  F  N  D  A  Q  R  Q  179
 911 GCCACCAAGGATGCTGGTGTAATTGCTGGGTTAAATGTTGCTCGTATAATAAATGAGCCAACTGCTGCAG
       A  T  K  D  A  G  V  I  A  G  L  N  V  A  R  I  I  N  E  P  T  A  A  202
 981 CAATTGCATATGGTTTGGATAAAAAGGGAGGAGAAAAGAACATTCTTGTTTATGACCTTGGAGGTGGAAC
       A  I  A  Y  G  L  D  K  K  G  G  E  K  N  I  L  V  Y  D  L  G  G  G  T  226
1051 TTTTGATGTCAGTATTCTCACCATTGATAATGGTGTTTTTGAAGTGTTGTCAACCAGCGGGGATACTCAT
       F  D  V  S  I  L  T  I  D  N  G  V  F  E  V  L  S  T  S  G  D  T  H  249
1121 TTAGGAGGAGAGGACTTCGATCAACGAGTTATGGATTACTTCATTAAATTGGTCAAGAAAAAACACAACA
       L  G  G  E  D  F  D  Q  R  V  M  D  Y  F  I  K  L  V  K  K  K  H  N  272
1191 AAGATATTAGCAAGGATAACAGAGCTCTTGGCAAACTTAGGAGGGAGTGTGAGAGGGCCAAAAGAGCTCT
       K  D  I  S  K  D  N  R  A  L  G  K  L  R  R  E  C  E  R  A  K  R  A  L  296
1261 GAGCAGCCAGCATCAAGTTCGTGTTGAAATTGAATCACTTTTTGATGGTGTTGATTTTTCAGAACCATTA
       S  S  Q  H  Q  V  R  V  E  I  E  S  L  F  D  G  V  D  F  S  E  P  L  319
1331 ACAAGAGCAAGATTCGAGGAACTCAATATGGACCTCTTCAAGAAAACTCTTGGGCCAGTAAAGAAGGCTC
       T  R  A  R  F  E  E  L  N  M  D  L  F  K  K  T  L  G  P  V  K  K  A  342
1401 TAGATGATGCTAACTTGCAGAAGACTGAAATTAATGAACTTGTGCTTGTTGGAGGAAGTACTCGCATACC
       L  D  D  A  N  L  Q  K  T  E  I  N  E  L  V  L  V  G  G  S  T  R  I  P  366
1471 AAAGGTTCAGCAATTATTGAAGGACTTATTTGATGGCAAGGAGCCTAACAAAGGTGTTAATCCAGATGAA
       K  V  Q  Q  L  L  K  D  L  F  D  G  K  E  P  N  K  G  V  N  P  D  E  389
1541 GCTGTGGCTTATGGGGCTGCTGTTCAGGGTGGTATTCTGAGTGGTGAGGGAGGTGACGAAACAAAAGATA
       A  V  A  Y  G  A  A  V  Q  G  G  I  L  S  G  E  G  G  D  E  T  K  D  412
1611 TTCTTCTATTGGATGTTGCTCCCCTCAGCCTAGGTATAGAAACTGTTGGTGGAGTAATGACCAAACTTAT
       I  L  L  L  D  V  A  P  L  S  L  G  I  E  T  V  G  G  V  M  T  K  L  I  436
1681 TCCGAGGAACACTGTCATTCCAACAAAGAAGTCACAAGTGTTCACAACTTATCAAGATCAGCAAACCACT
       P  R  N  T  V  I  P  T  K  K  S  Q  V  F  T  T  Y  Q  D  Q  Q  T  T  459
1751 GTTTCAATCAAGGTTTATGAAGGAGAGCGGAGTCTTACAAAGGATTGCCGAGAATTAGGCAAATTTGATC
       V  S  I  K  V  Y  E  G  E  R  S  L  T  K  D  C  R  E  L  G  K  F  D  482
1821 TGTCTGGAATCCCTCCAGCTCCTCGTGGTGTGCCACAGATTGAGGTCACCTTTGAGGTTGATGCCAACGG
       L  S  G  I  P  P  A  P  R  G  V  P  Q  I  E  V  T  F  E  V  D  A  N  G  506
1891 TATCCTCAATGTAAGAGCAGAGGACAAGGGCACCAAGAAAACCGAAAAGATTACCATCACAAATGACAAA
       I  L  N  V  R  A  E  D  K  G  T  K  K  T  E  K  I  T  I  T  N  D  K  529
1961 GGTAGATTGAGCCAGGAAGAAATAGAAAGAATGGTCAAGGAGGCAGAGGAGTTTGCAGAGGAGGATAAGA
       G  R  L  S  Q  E  E  I  E  R  M  V  K  E  A  E  E  F  A  E  E  D  K  552
2031 AAGTGAAGGACAAAATTGATGCGAGGAACAATCTTGAAACATATGTCTACAACATGAAAAGCACCATTAA
       K  V  K  D  K  I  D  A  R  N  N  L  E  T  Y  V  Y  N  M  K  S  T  I  N  576
2101 TGAGAAGGATAAATTGGCAGATAAAATTGATTCCGAAGACAAGGAGAAGATCGAAACTGCTATCAAAGAA
       E  K  D  K  L  A  D  K  I  D  S  E  D  K  E  K  I  E  T  A  I  K  E  599
2171 GCATTGGAATGGCTTGATGACAACCAGTCGGCTGAGAAGGAGGACTTCGAGGAGAAGTTGAAAGAGGTGG
       A  L  E  W  L  D  D  N  Q  S  A  E  K  E  D  F  E  E  K  L  K  E  V  622
2241 AAGCTGTATGCAGTCCCATCATCAAGCAAGTATATGAGAAAACTGGAGGAGGATCTTCTGGAGGCGATGA
       E  A  V  C  S  P  I  I  K  Q  V  Y  E  K  T  G  G  G  S  S  G  G  D  D  646
2311 TGAAGACGAGGACTCGCATGAAGAACTCtaagccatttcagtttctgttgaattttagttgtacaaatca
       E  D  E  D  S  E  E  E  L                                              655
2381 cgatgaactaattctacagaagagatctctgagcataatagggtttatgaggatgattggcaacgaacaa
2451 gagattcaactgatgaaagtcaaatgactgtttgttttttctatcaatcagaatgttattttcacagatt
2521 gaaattggcaacgaacaagagattcaactgatgaaagtcaaatgactatttgtttgttttttctatcaat
2591 cagaatgttattttcacagatttttcaatctgtagt
```

Figure 5. Nucleotide and deduced amino acid sequence of PmBiP3 cDNA

Nucleotide sequence is numbered on the left and amino acid sequence is numbered on the right. Untranslated regions are in lower case letters and the open reading frame is capitalized. The three potential start codons are underlined with the amino acid sequence beginning at the 3rd codon. The predicted signal peptide cleavage site and beginning of the mature PmBiP amino acid sequence is indicated by an asterisk (Nielsen et al., 1997). The ER retention signal sequence is boxed. The 13 carboxy-terminal amino acids used to generate the peptide antiserum are indicated in bold italics.

Three potential start codons were present at positions 284, 314, and 374 in the PmBiP cDNA sequence. Translation at positions 284 and 314 resulted in N-terminal amino acid sequences with characteristics which do not conform to typical signal sequences whereas translation at position 374 resulted in a predicted N-terminal signal sequence of 18 amino acids (Nielsen et al., 1997; von Heijne, 1985). Hence, the PmBiP cDNA contains 5' and 3' untranslated regions of 373 bp and 288 bp respectively and an open reading frame of 1965 bp encoding a protein of 655 amino acids. Cleavage of the signal peptide would result in a predicted mature protein of 637 amino acids with a molecular mass of 70.6 kDa and pI of 5.0. A novel C-terminal tetrapeptide HEEL, which may serve as a retention signal for soluble ER proteins in Douglas-fir, was identified. HEEL was also present in the C-terminal deduced amino acid sequences of the other 3 PmBiP cDNAs.

Restriction analysis and PCR amplification of genomic DNA

To get a better understanding of the organization of PmBiP in the Douglas-fir genome, genomic DNA (gDNA) was digested with *Bam*H I, *Eco*R I, *Kpn* I and analyzed by Southern hybridization (Figure 6).

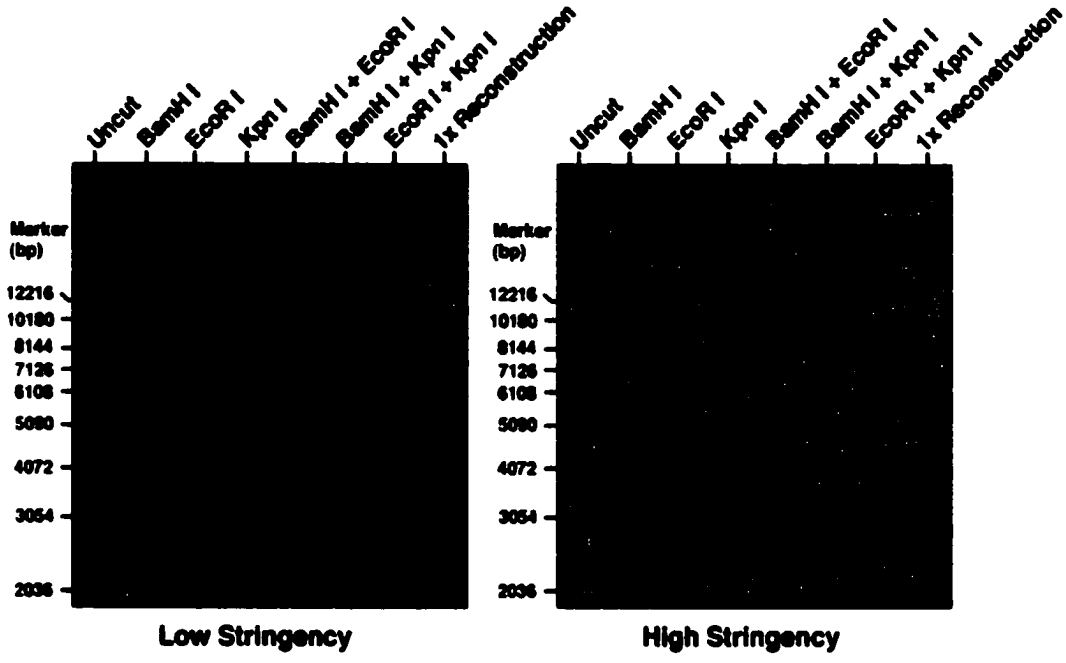

Figure 6. Genomic Southern analysis of PmBiP

Douglas-fir genomic DNA (10 µg/lane) was digested with restriction enzymes as indicated and separated on an agarose gel. The resulting unblot was hybridized to random primed, ^{32}P-labeled, full length PmBiP cDNA and exposed onto film following both low and high stringency wash. A 1x reconstruction lane was also included to estimate the copy number. Calculations for the 1x reconstruction were based on a Douglas-fir genome size of 25 pg per haploid nucleus (Ingle et al., 1975).

Exposure of the unblot (see Materials and Methods) following low stringency wash showed the presence a single strongly reactive band as well as up to 2-3 other weakly reactive bands per lane. Following high stringency washing, weakly reactive bands were no longer detectable and only the strongly reactive band was observed. A 1x reconstruction lane, consisting of the full-length PmBiP cDNA, was also run in order to estimate its copy number in the Douglas-fir genome. Comparison of the signal intensity from the

reconstruction lane to that of the fragment generated by restriction digestion indicated that PmBiP is present as a single copy.

To determine whether the PmBiP gene contained introns, PCR amplification of gDNA was conducted. PCR amplification using primers p5-3z1 and p10-5a1 (see primer map, Appendix C) followed by Southern blotting of the resulting products revealed a band of approximately 5 kb in size (Figure 7). Given that the predicted product from amplification of the cDNA using the same primers is 2021 bp, it is concluded that PmBiP contains introns.

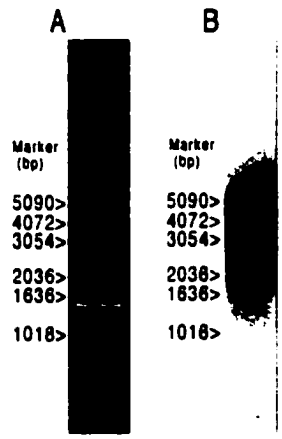

Figure 7. PCR amplification of BiP from Douglas fir genomic DNA

PCR reaction products were separated on a 0.7% agarose gel and stained with ethidium bromide (A) then analyzed by Southern blotting (B). Size markers (in base pairs) are indicated on the left.

Amino acid alignment and phylogenic analysis

PmBiP shared from 60% to 86% amino acid sequence identity with BiPs from animals, plants, protists, and fungi, but shared the highest identity and formed a distinct phylogenetic subgroup with the angiosperm BiPs (Figure 8). The monocot and dicot BiP sequences also formed their own subgroups. Amino

acid sequence alignment showed that PmBiP shared several highly conserved motifs with other plant BiPs (Figure 9).

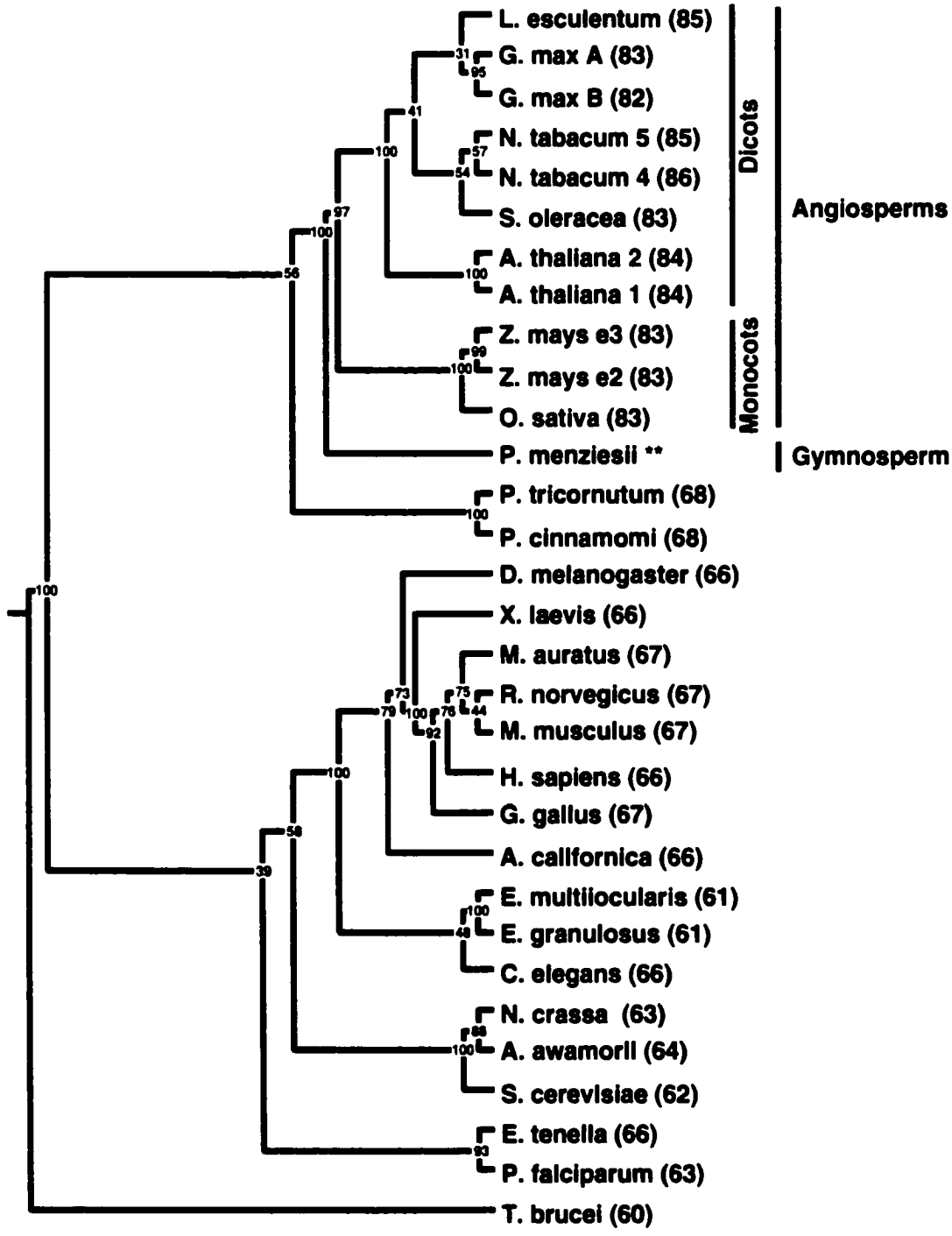

Figure 8. Putative phylogenetic tree of selected BiP amino acid sequences

A phylogenetic tree of a representative sampling of BiP amino acid sequences displayed as a phenogram with midpoint branching. Numbers at branch points are bootstrap values for 100 replicates and numbers in parentheses indicate the percent identity to the Douglas-fir BiP. Amino acid sequences and accession numbers are as described in Materials and Methods.

```
                                                  ↓                  β
P.m.    -----------MFLAAFITAG--FLFSSVIAAEEAAKLGTVIGIDLGTTYSCVGVYKNGH 47
N.t.4   MAGGAWNRRTSLIVFGIVLFG--CLFAFSIATEEATKLGTVIGIDLGTTYSCVGVYKNGH 58
S.o.    -MAVAWKSRASSIAFGIVLLG--SLFAFVSAKDEAPKLGTVIGIDLGTTYSCVGVYKDGK 57
A.t.1   -MARSFGAN-STVVLAIIFFG--CLFALSSAIEEATKLGSVIGIDLGTTYSCVGVYKNGH 56
G.m.A   -MACSFSRGSSCFPLAIIVSLGCLFRDFSIAKEEATKLGTVIGIDLGTTYSCVGVYKNGH 59
Z.m.E3  ---MDRVRG-SAFLLGVLLAG--SLFAFSVAKEETKKLGTVIGIDLGTTYSCVGVYKNGH 54
O.s.    ---MDRVRG-SAFLLGVLLAG--SLFAFSVAKEETKKLGTVIGIDLGTTYSCVGVYKNGH 54
                     .  ..:      :     * :*: ***:******************:*:

P.m.    VEIIANDQGNRITPSWVAFTDTERLIGEAAKNQAAMNPERTVFDVKRLIGRKYEDKEVQK 107
N.t.4   VEIIANDQGNRITPSWVAFTDGERLIGEAAKNLAAVNPERTVFDVKRLIGRKFDDKEVQR 118
S.o.    VEIIANDQGNRITPSWVAFTNDERLIGEAAKNQAAANPERTIFDVKRLIGRKFEDKEVQK 117
A.t.1   VEIIANDQGNRITPSWVGFTDSERLIGEAAKNQAAVNPERTVFDVKRLIGRKFEDKEVQK 116
G.m.A   VEIIANDQGNRITPSWS-FTDSERLIGEAAKNLAAVNPERVIFDVKRLIGRKFEDKEVQR 118
Z.m.E3  VEIIANDQGNRITPSWVAFTDSERLIGEAAKNQAAVNPERTIFDVKRLIGRKFQDKEVQR 114
O.s.    VEIIANDQGNRITPSWVAFTDSERLIGEAAKNQAAVNPERTIFDVKRDIGRKFEEKEVQR 114
        ****************  **: ********** ** ****.:***** ****:::****:

P.m.    DIKLLPYKIVNKDGKPYIQVKIRDGEIKVFSPEEISAMILLKMKETAESYLGRKIKDAVV 167
N.t.4   DMKLVPYKIVNKDGKPYIQVKIKDGETKIFSPEEISAMILTKMKETAEAYLGKKIKDAVV 178
S.o.    DMKLVPYKIVNRDGKPYIQVKVQEGETKVFSPEEISAMILTKMKETAETFLGKKIKDAVV 177
A.t.1   DRKLVPYQIVNKDGKPYIQVKIKDGETKVFSPEEISAMILTKMKETAEAYLGKKIKDAVV 176
G.m.A   DMKLVPYKIVNKDGKPYIQEKIKDGETKVFSPEEISAMILTKMKETAEAFLGKKINDAV- 177
Z.m.E3  DMKLVPYKIINKDGKPYIQVKIKDGENKVFSPEEISAMILGKMKDTAEAYLGKKINDAVV 174
O.s.    DMKLVPYKIVNKIGKPYIQVKIKDGENKVFSPEEVSAMILGKMKETAEAYLGKKINDAVV 174
        * **:**:*:*: ****** *:::** *:*****:***** ***:***::**:**:***
                                                                    γ
P.m.    TVPAYFNDAQRQATKDAGVIAGLNVARIINEPTAAAIAYGLDKKGGEKNILVYDLGGGTF 227
N.t.4   TVPAYFNDAQRQATKDAGVIAGLNVARIINEPTAAAIAYGLDKKGGEKNILVFDLGGGTF 238
S.o.    TVPAYFNDAQRQATKDAGVIAGLNVARIINEPTAAAIAYGLDKRGGEKNILVFDLGGGTF 237
A.t.1   TVPAYFNDAQRQATKDAGVIAGLNVARIINEPTAAAIANVLDKKGGEKNILVFDLGGGTF 236
G.m.A   ---AYFNDAQRQATKDAGVIAGLNVARIINEPTAAAIAYGLDKKGGEKNILVFDLGGGTF 234
Z.m.E3  TVPAYFNDAQRQATKDAGVIAGLNVARIINEPTAAAIAYGLDKKGGEKNILVFDLGGGTF 234
O.s.    TVPAYFNDAQRQATKDAGVIAGLNVARIINEPTAAAIAYGLDKKGGEKNILVFDLGGGTF 234
           ************************************  ***:********:*******

P.m.    DVSILTIDNGVFEVLSTSGDTHLGGEDFDQRVMDYFIKLVKKKHNKDISKDNRALGKLRR 287
N.t.4   DVSILTIDNGVFEVLSTNGDTHLGGEDFDQRIMEYFIKLIKKKHGKDISKDNRALGKLRR 298
S.o.    DVSVLTIDNGVFEVLATNGDTHLGGEDFDQRLMEYFIKLIKKKHTKDISKDNRALGKLRR 297
A.t.1   DVSVLTIDNGVFEVLSTNGDTHLGGEDFDHRVMEYFIKLIKKKHQKDISKDNKALGKLRR 296
G.m.A   DVSILTIDNGVFEVLATNGDTHLGGEDFDQRIMEYFIKLINKKHKKDISKDSRALSKLRR 294
Z.m.E3  DVSILTIDNGVFEVLATNGDTHLGGEDFDQRIMEYFIKLIKKKYSKDISKDNRALGKLRR 294
O.s.    DVSILTIDNGVFEVLATNGDTHLGGEDFDQRIMEYFIKLIKKKYSKDISKDNRALGKLRR 294
        ***:************:*.***********:*:*:*****::**: ******.:**.****
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P.m.    ECERAKRALSSQHQVRVEIESLFDGVDFSEPLTRARFEELNMDLFKKTLGPVKKALDDAN 347
N.t.4   EAERAKRALSSQHQVRVEIESLFDGVDFSEPLTRARFEELNNDLFRKTMGPVKKAMDDAG 358
S.o.    ECERAKRALSSQHQVRVEIESLFDGVDFSEPLTRARFEELNNDLFRKTMGPVKKAMDDAG 357
A.t.1   ECERAKRALSSQHQVRVEIESLFDGVDFSEPLTRARFEELNNDLFRKTMGPVKKAMDDAG 356
G.m.A   EAERAKRALSSQHQVRVEIESLFDGVDFSEPLTRARFEELNNDLFRKTMGPVKKAMEDAG 354
Z.m.E3  EAERAKRALSNQHQVRVEIESLFDGTDFSEPLTRARFEELNNDLFRKTMGPVKKAMEDAG 354
O.s.    EAERAKRALSNQHQVRVEIESLFDGTDFSEPLTRARFEELNNDLFRKTMGPVKKAMDDAG 354
        *.********.***************.*************** ***:**:******::**.

P.m.    LQKTEINELVLVGGSTRIPKVQQLLKDLFDGKEPNKGVNPDEAVAYGAAVQGGILSGEGG 407
N.t.4   LEKTQIDEIVLVGGSTRIPKVQQLLKDYFDGKEPNKGVNPDEAVAYGAAVQGGILSGEGG 418
S.o.    LEKNQIDEIVLVGGSTRIPKVQQLLKEFFNGKEPSKGVNPDEAVAFGAAVQGSILSGEGG 417
A.t.1   LQKSQIDEIVLVGGSTRIPKVQQLLKDFFEGKEPNKGVNPDEAVAYGAAVQGGILSGEGG 416
G.m.A   LQKNQIDEIVLVGGSTRIPKVQQLLKDYFDGKEPNKGVNADEAVAYGAAVQGSILSGEGG 414
Z.m.E3  LEKSQIHEIVLVGGSTRIPKVQQLLKDYFNGKEPNKGVNPDEAVAFGAAVQGSILSGEGG 414
O.s.    LEKSQIHEIVLVGGSTRIPKVQQLLRDYFEGKEPNKGVNPDEAVAYGAAVQGSILSGEGG 414
        *:*.:*.*:*****************:: *:*****.****.*****:******.*******

P.m.    DETKDILLLDVAPLSLGIETVGGVMTKLIPRNTVIPTKKSQVFTTYQDQQTTVSIKVYEG 467
N.t.4   DETKDILLLDVAPLTLGIETVGGVMTKLIPRNTVIPTKKSQVFTTYQDQQTTVTIQVFEG 478
S.o.    EETKEILLLDVAPLTLGIETVGGVMTKLIPRNTVIPTKKSQVFTTYQDQQTTVTIQVFEG 477
A.t.1   DETKDILLLDVAPLTLGIETVGGVMTKLIPRNTVIPTKKSQVFTTYQDQQTTVSIQVFEG 476
G.m.A   EETKDILLLDVAALTLGIETVGGVMTKLIPRNTVIPTKKSQVFTTYQDQQSTVSIQVFEG 474
Z.m.E3  DETKDILLLDVAPLTLGIETVGGVMTKLIPRNTVIPTKKSQVFTTYQDQQTTVSIQVFEG 474
O.s.    DETKDILLLDVAPLTLGIETVGGVMTKLIPRNTVIPTKKSQVFTTYQDQQTTVSIQVFEG 474
        :***:*******.*:*****************************************:**:*:*:**

P.m.    ERSLTKDCRELGKFDLSGIPPAPRGVPQIEVTFEVDANGILNVRAEDKGTKKTEKITITN 527
N.t.4   ERSLTKDCRLLGKFDLTGIAPAPRGTPQIEVTFEVDANGILNVKAEDKASGKSEKITITN 538
S.o.    ERSLTKDCRLLGKFDLTGIAPAPRGTPQIEVTFEVDANGILNVKAEDKASGKSEKITITN 537
A.t.1   ERSLTKDCRLLGKFDLNGIPPAPRGTPQIEVTFEVDANGILNVKAEDKASGKSEKITITN 536
G.m.A   ERSLTKDCRLLGKFELSGIPPAPRGTPQIEVTFEVDANGILNVKAEDKGTGKSEKITITN 534
Z.m.E3  ERSMTKDCRLLGKFDLNGIPSAPRGTPQIEVTFEVDANGILNVKAEDKGTGKSEKITITN 534
O.s.    ERSMTKDCRLLGKFDLSGIPAAPRGTPQIEVTFEVDANGILNVKAEDKGTGKSEKITITN 534
        ***:***** ****:*.**..****.*****************:****.: *:*******

P.m.    DKGRLSQEEIERMVKEAEEFAEEDKKVKDKIDARNNLETYVYNMKSTINEKDKLADKIDS 587
N.t.4   DKGRLSQEEIERMVKEAEEFAEEDKKVKERIDARNSLETYVYNMRNQINDKDKLADKLES 598
S.o.    DKGRLSQEEIERMVREAEEFAEEDKKVKEKIDARNSLETYIYNMKNQISDADKLADKLES 597
A.t.1   EKGRLSQEEIDRMVKEAEEFAEEDKKVKEKIDARNALETYVYNMKNQVNDKDKLADKLEG 596
G.m.A   EKGRLSQEEIERMVREAEEFAEEDKKVKERIDARNSLETYVYNMKNQVSDKDKLADKLES 594
Z.m.E3  EKGRLSQEEIDRMVREAEEFAEEDKKVKERIDARNQLETYVYNMKNTVGDKDKLADKLEA 594
O.s.    EKGRLSQEEIDRMVREAEEFAEEDKKVKERIDARNQLETYVYNMKNTVGDKDKLADKLES 594
        :**********:***:**************::***** ****:***:. :.: ******::.

P.m.    EDKEKIETAIKEALEWLDDNQSAEKEDFEEKLKEVEAVCSPIIKQVYEKTG---GGSSG- 643
N.t.4   DEKEKIETATKEALEWLDDNQSAEKEDYEEKLKEVEAVCNPIITAVYQKSGGAPGGESG- 657
S.o.    DEKEKIEGAVKEALEWLDDNQSAEKEDYDEKLKEVEAVCNPIITAVYQRSG-GPSGESG- 655
A.t.1   DEKEKIEAATKEALEWLDENQNSEKEEYDEKLKEVEAVCNPIITAVYQRSGGAPGGAGGE 656
G.m.A   DEKEKVETAVKEALEWLDDNQSVEKR-YEEKLKEVEAVCNPIISAVYQRSGGAPGGGAS- 652
Z.m.E3  EEKEKVEEALKEALEWLDDNQSAEKEDYEEKLKEVEAVCNPIVSAVYQRSGGAPGGDAD- 653
O.s.    EEKEKVEEALKEALEWLDENQTAEKEEYEEKLKEVEAVCNPIISAVYQRTGGAPGGRRR- 653
        ::***:* * ********:**. **. ::**********.**:. **:::*   .*
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P.m.    GDDEDEDS-HDEL 655
N.t.4   AS-EDDD--HDEL 667
S.o.    ADSEDSEEGHDEL 668
A.t.1   SSTEEEDESHDEL 669
G.m.A   GEDDDEDS-HDEL 664
Z.m.E3  GGVDDD---HDEL 663
O.s.    GRLDDE---HDEL 663
          .  ::.   *:**
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Figure 9. An alignment of selected BiP amino acid sequences

Included are the deduced amino acid sequence of: PmBiP (P.m.), tobacco BiP isoform BLP4 (N.t.4), Spinach BiP (S.o.), *Arabidopsis* BiP isoform 1 (A.t.1), soybean BiP isoform A (G.m.A), Maize BiP isoform E3 (Z.m.E3), and rice BiP (O.s.). (*) indicates an identical amino acid. (:) indicates a highly conserved amino acid change. (.) indicates a moderately conserved amino acid change. The β and γ phosphate binding sites are indicated in bold italics as well as the adenosine binding site (Â). The first residue of the mature amino acid sequence for all sequences is aligned and indicated by an arrow. A putative calmodulin binding site is overlined with identical residues to a calmodulin binding consensus sequence indicated in bold italics. (H) amino acids forming the 5-residue substrate core in *E. coli* DnaK involved in making hydrogen bonds to the peptide substrate backbone. The αB domain of *E. coli* DnaK is overlined with arrows.

The β, γand adenosine binding sites are located in the N-terminal ATP binding domain of PmBiP (residues 1-406) and are responsible for binding ATP and modulating the peptide binding activity of the C-terminal peptide binding domain (Bork et al., 1992; Flaherty et al., 1991). A putative calmodulin binding site was also located in the N-terminal ATP binding domain (Stevenson and Calderwood, 1990).

Several regions within the C-terminal peptide-binding domain of PmBiP (residues 413-629) were highly conserved with angiosperm BiPs. Based on sequence alignments of PmBiP C-terminal peptide-binding domain with *E. coli* DnaK (data not shown), much of the sequence forming the β-sandwich peptide-binding pocket (residues 417-525) is highly conserved (Figure 9; Zhu et al.,

1996). In particular, amino acids that form the 5-residue substrate core and make hydrogen bond contacts to peptide substrate backbones are conserved. Residues which align with the αB domain of the DnaK peptide-binding domain (residues 548-578), a structure proposed to act as a latch to prevent release of peptide substrates from the peptide binding pocket, are also conserved among plant BiPs. The N-terminal signal sequences from the plant BiPs were highly variable and represented the region of greatest sequence divergence. PmBiP contained the shortest predicted signal sequence with 18 amino acids. The monocot sequences each had a predicted signal sequence of 25 amino acids whereas the dicots *Arabidopsis*, spinach, tobacco and soybean sequences had signal sequences of 27, 28, 29,and 30 amino acids respectively. The ER retention tetrapeptide HEEL was different from the HDEL sequence present in angiosperm BiPs and KDEL found in mammalian BiPs.

3D modeling of N and C terminus

Currently no known tertiary structure for BiP exists to allow the visualization of the spatial relationships of conserved sequences within the overall structure. Such information is helpful to validate proposed function with spatial orientation and to identify the relative positions of highly conserved sites identified through amino acid sequence alignment. To visualize the relative positions of highly conserved residues, three dimensional models of the N-terminal ATP binding domain and C-terminal peptide binding domain were constructed using the knowledge based comparative modeling approach (Guex and Peitsch, 1997; Peitsch, 1996).

Figure 10. 3D models of PmBiP ATP and peptide binding domains

(A) Ribbon diagram of the ATP binding domain model composed of amino acids L25-G406. The β and γ phosphate binding sites are indicated in red and green respectively and the adenosine binding site is indicated in yellow. The putative calmodulin binding site is indicated in blue.

(B) Ribbon diagram of the peptide-binding domain composed of amino acids I413-I629. Secondary structures are indicated red for α-helices and yellow for β-sheets and asterisks indicate innermost loops that form the peptide-binding pocket.
(C) Enlarged top view of peptide binding pocket. Amino acids predicted to make hydrogen bonds to peptide substrates, determined from amino acid sequence alignment with *E. coli* DnaK substrate binding domain, are shown in red. Arrow indicates the binding plane of conformationally extended peptide substrates.

The ATP binding domain (Figure 10A) is based on the three-dimensional structure of the bovine heat shock cognate 70, 44 kDa ATP binding domain fragment (Flaherty et al., 1990; Flaherty et al., 1994). The conserved β and γ phosphate and adenosine binding sites are clustered in a central pocket. The putative CaM binding site is exposed on the surface of one side of the bi-lobed ATP binding domain in a position that could be accessible to CaM or other EF-hand protein. The interaction of this site with an EF-hand protein would be expected to affect the ATPase activity as residues E290, K293, and S297 are predicted to interact directly with the adenosine moiety of ATP.

The structure of the peptide-binding domain is based on the tertiary structure of the peptide-binding domain of *E. coli* DnaK (Figure 10B); (Zhu et al., 1996)). Peptides are proposed to bind in an extended conformation between the two innermost loops as in DnaK. From amino acid alignment with DnaK it was possible to identify conserved residues in PmBiP which form hydrogen bond contacts with peptide substrates in *E. coli* DnaK (Figure 10C). Residues M384 and A409 form a hydrophobic arch over the peptide-binding pocket in DnaK and are conserved in V428 and Y453 in the PmBiP structure. Residues Q457, S461, and T451 line the sides of the binding pocket. All residues, excluding S461, are also predicted to make van der Waals contacts and are highly

conserved across all plant BiPs (see Figure 9). Residues making only van der Waals contacts are not shown but are also highly conserved. A second putative adenosine binding site, ETVGG (amino acids 426-430), identified by Anderson et al. (1994a) resides within a loop region whose residues are predicted contact peptide substrates (Figure 10C). It is unclear what possible role the interaction of these residues with the adenosine moiety could play and how it might affect peptide-binding activity.

PmBiP binds to denatured gelatin

Amino acids sequence alignments clearly show a high degree of identity to other BiP proteins and support the identity of PmBiP as a Douglas-fir BiP homologue and member of the HSP70 family of Proteins (Haas, 1994). An inherent property of such proteins is the ability to bind and release denatured proteins in an ATP dependent manner. To determine if PmBiP can bind and release denatured proteins in an ATP dependent manner, total protein from 6 day-old Douglas-fir seedlings was passed over a denatured gelatin-agarose column. Using an antibody against a tomato BiP homologue, the binding and release of PmBiP was followed. Results showed that PmBiP bound denatured gelatin and was released in an ATP dependent fashion (Figure 11). However, the affinity of PmBiP for denatured gelatin, under the experimental conditions, appeared low as a significant proportion of PmBiP remained in the flow-through fraction. It is also possible that a significant proportion of PmBiP was in a conformation unable to bind denatured gelatin.

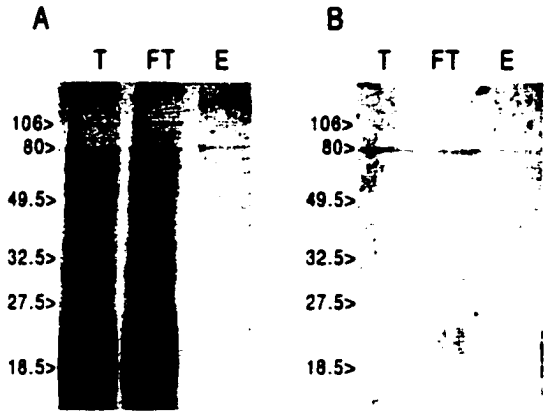


Figure 11. Binding of PmBiP to denatured gelatin

Total protein extract (T) from 6-day-old Douglas-fir seedlings was applied to denatured gelatin-agarose beads. Following washing (FT), bound proteins were eluted (E) with ATP and analyzed using SDS-PAGE and western blotting. Western blotting was done as described in Material and Methods except that an anti-tomato BiP antibody was used (1:1000 dilution; kindly provided by Dr. Maarten Chrispeels).

Antibody development and use in PmBiP analysis

To follow changes in the abundance of PmBiP protein during development and to examine the association of PmBiP with other proteins through immunoprecipitation, it was necessary to develop antiserum to the PmBiP protein.

A number of approaches were followed to obtain PmBiP antiserum. The first approach involved isolating PmBiP protein from tissue extracts using gelatin-agarose chromatography (see above). However, the quantity of protein obtained failed to generate an immune response. The second approach involved the creation of a PmBiP protein fusion with the maltose binding protein for over-expression in *E. coli*. Repeated attempts to obtain recombinant *E. coli* cells containing fusion's targeted to the cytoplasm or periplasm were not

successful. It was concluded that such fusions may have been toxic to *E. coli* cells. The third approach was to construct a synthetic peptide from a region of the PmBiP deduced amino acid sequence and to conjugate it to the highly antigenic KLH protein (Nivison and Hanson, 1987). The conjugated form would serve as antigen to generate an immune response in rabbits. To choose a suitable peptide, the entire PmBiP amino acid sequence was subjected to hydrophilicy, flexibility, and accessibility plots. Of the several possible candidates, the C-terminal 13 amino acids were chosen. The choice of this region was because it contained a different ER retention sequence (see above) and it was hoped that the antiserum generated would allow specific detection of PmBiP when expressed in a heterologous plant.

Evaluation of PmBiP antiserum

To determine if rabbits raised an immune response against the synthetic peptide, an ELISA was conducted using various dilutions of rabbit serum. Results showed a significant response against the peptide and an appropriate dilution factor for use of serum in western blotting applications was determined to be from 1:2000 to 1:4000 (Figure 12).

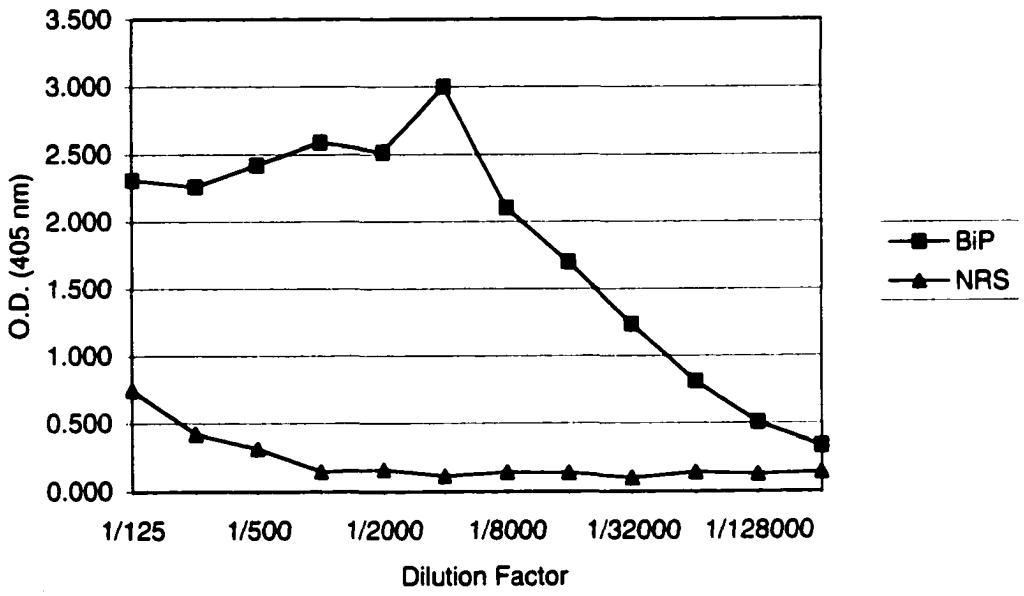


Figure 12. Titration of PmBiP peptide antiserum

ELISA using rabbit antiserum raised against a synthetic peptide derived from the C-terminal 13 amino acids of the deduced PmBiP amino acid sequence. Squares represent reactivity against the PmBiP peptide antiserum (BiP) and triangles represent reactivity against the non-reactive preimmune serum (NRS).

The PmBiP peptide antibody does not recognize other common ER proteins

Examination of PmBiP protein amounts in developing Douglas-fir tissues using western blotting occasionally revealed the presence of lower molecular mass bands. Because the antiserum was generated from a region involved in retaining proteins to the ER, it was possible that the antiserum was detecting other common ER proteins. However, inclusion of several different protease inhibitors within the extraction buffer (buffer A) reduced the appearance of these lower bands. Further, western blotting of protein extracts from tissue which had been freeze dried (immediately frozen in liquid nitrogen then

lyophilized) failed to detect these lower bands (for examples see Figure 17 and Figure 26). Hence, it is most likely that lower molecular mass bands appearing on western blots represent degradation products. To further exclude the possibility that the lower bands represent other ER proteins, N-terminal sequencing was conducted on bands excised from PVDF membranes following SDS-PAGE separation of proteins. Unfortunately, no sequence was obtained due to blockage of the N-terminals. Hence, heterologous antibodies to other ER proteins were obtained for western blotting. The heterologous antibodies anti-Calnexin, anti-Calreticulin, anti-Protein Disulfide Isomerase, anti-HSP47, and anti Heme-Oxygenase-1, obtained from StressGen Biotechnologies (Victoria, B.C., Canada), detected proteins of molecular masses which differed from the lower molecular mass bands detected with the PmBiP antiserum indicating that the PmBiP antiserum does not detect other common ER proteins (Figure 13).

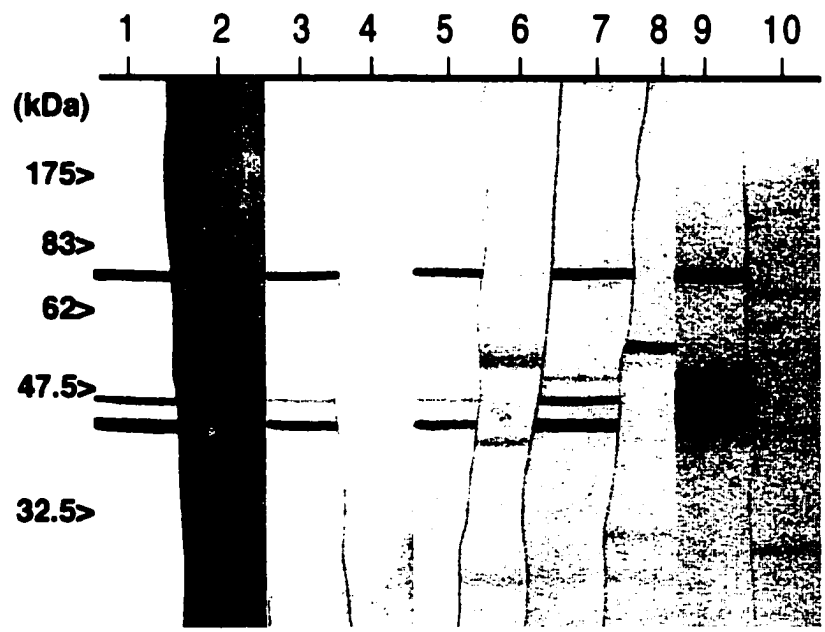


Figure 13. Detection of ER proteins in Douglas-fir using heterologous antibodies

Microsmal proteins from mature Douglas-fir seeds were separated on a preparative SDS-PAGE gel and transferred to a nitrocellulose membrane. Western blotting was conducted as described in Material and Methods on strips excised from the membrane using the 1° antiserum for 3 h against PmBiP (lanes 1, 3, 5, 7, 9; 1:3000 dilution), protein disulfide isomerase (lane 2; 1:1000 dilution), Calreticulin (lane 4; 1:6000 dilution), HSP47 (lane 6; 1µg/ml), Calnexin (lane 8; 1:1000 dilution), and Heme-Oxygenase-1 (lane 10; 1;500 dilution).

Subcellular localization of PmBiP

Subcellular fractionation and western blot analysis using the PmBiP antiserum detected a protein with a molecular mass similar to the predicted molecular mass of mature PmBiP and revealed it to be highly enriched in the microsomal fraction compared to the soluble fraction (Figure 14). Within the microsomal fraction, PmBiP was enriched in the soluble fraction indicating localization within the lumen. PmBiP was also detected in a nuclear fraction.

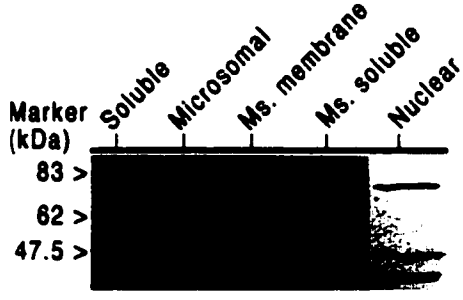


Figure 14. Subcellular localization of PmBiP

Subcellular fractions were prepared as described in Materials and Methods and include soluble (140,000g supernatant), microsomal (140,000g pellet), microsomal membrane (Ms. membrane), microsomal soluble (Ms. soluble; soluble content of microsomes), and nuclear (from different immunoblot). Fifteen micrograms of each fraction was then subjected to western blot analysis.

To determine if PmBiP is localized to the ER of microsomal fractions, microsomes were fractionated using sucrose density centrifugation. Using this

technique, the ER of castorbean endosperm fractionates between 20% and 30% sucrose (Coughlan et al., 1996). In Douglas-fir, the cytochrome P450 reductase and ER marker enzyme activity for NADPH-cytochrome C also localized to the 20% to 30% sucrose fraction (Tranbarger, 1998). Fractionation SDS-PAGE of stratified seed microsomal extracts showed two major diffuse fractions of protein (Figure 15A). The majority of protein was observed in the 10-30% sucrose fractions while a smaller amount was present in the 41% sucrose fraction. Western blotting of the various fractions showed enrichment of PmBiP in the 20-30% sucrose fractions indicating localization to the ER (Figure 15B). PmBiP was also detected in the 34% and 41% sucrose fractions and may be the result of incomplete sedimentation or fraction cross contamination.

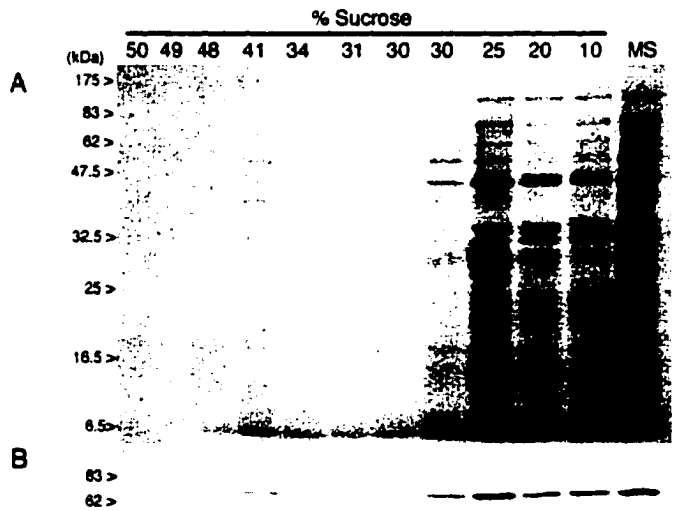


Figure 15. Sucrose gradient fractionation of stratified seed microsomal fraction

Collected fractions were analyzed by SDS-PAGE and staining with Coomassie blue (A) or transferred to nitrocellulose for western blotting with the PmBiP antiserum (B). Eighteen µl of each fraction was loaded per lane. Percent sucrose per fraction is indicated along the top, a crude

microsomal fraction is included as control (MS). Molecular mass markers are indicated on the left.

2D SDS-PAGE analysis of germinating seed tissues

In several angiosperm plant species, BiP is known to be encoded by a multigene family whose members differ in the amino acid sequence (Denecke et al., 1991; Figueiredo et al., 1997; Kalinski et al., 1995; Koizumi and Sano, 1997; Wrobel et al., 1997). To corroborate the results of restriction analysis of gDNA which indicated a single PmBiP gene in Douglas-fir (see above), 2D SDS-PAGE on protein extracts obtained from seeds and seedling tissue derived from an open pollinated seedlot was performed (Figure 16).

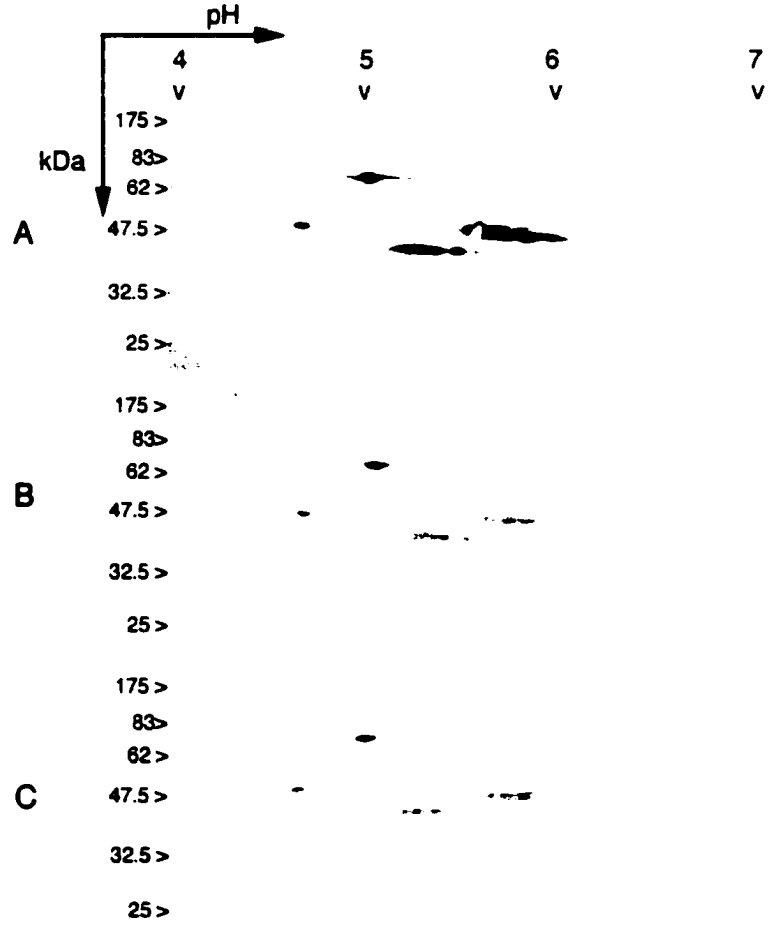


Figure 16. 2D SDS-PAGE analysis of PmBiP in Douglas-fir seeds and seedlings

Protein extract from mature seeds (A), stratified seeds (B) and 2 day old seedlings (C) were separated using 2D SDS-PAGE (30 µg microsomal protein/sample) and subjected to western blot analysis using the PmBiP peptide antiserum. Molecular mass standards are indicated on the left and pH is indicated along the top.

Results indicated that a single protein species was detected in microsomal protein extracts from mature seeds, stratified seeds, and 2 day-old seedlings migrating near the predicted pI and molecular mass. This result indicated and supports restriction analysis data that PmBiP is encoded by a single gene in Douglas-fir. Further, the results show that PmBiP is not present in different post-translationally modified forms and that the amino acid sequence of PmBiP is highly conserved within a given seedlot.

PmBiP protein abundance in tissues of 14-day-old seedlings

To determine the relative abundance of PmBiP in different tissues of 14-day old seedlings, protein was extracted from the freeze-dried roots, shoots, cotyledons, and MG and subjected to western blotting. Results indicated that PmBiP was most abundant in the shoot, with the roots and cotyledons each containing similar amounts (Figure 17). The MG contained very little PmBiP relative to other tissues. It was also noted that the protein extracts from the lyophilized tissues showed no degradation products.

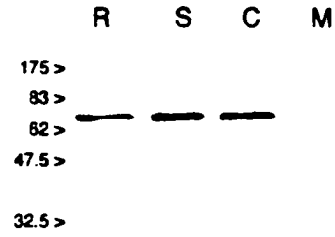


Figure 17. Abundance of PmBiP in tissues of 14-day-old Douglas-fir seedlings
Total protein was extracted from the roots (R), shoots (S), cotyledons (C), and megagametophyte (M) and subjected to SDS-PAGE and western blotting using the PmBiP peptide antiserum (15 µg per lane). Molecular mass markers are indicated on the left.

Species cross-reactivity

To determine if the PmBiP antiserum was capable of detecting BiP in other species, SDS-PAGE and western blotting was conducted on protein extracts collected from a variety of angiosperms and gymnosperms. Results showed that the antiserum does detect proteins with a molecular mass expected of BiP homologues in other plant species (Figure 18).

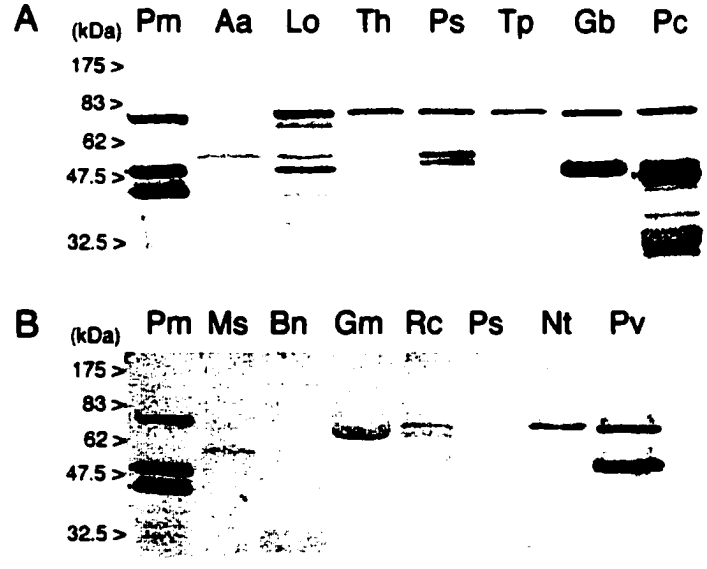


Figure 18. Species cross-reactivity of PmBiP peptide antiserum

Western blotting of protein extracts from a variety of plant species. Extracts from Gymnosperms (A) include *Pseudotsuga menziesii* (Pm; Douglas-fir), *Abies amabilis* (Aa; Amabalis fir), *Larix occidentalis* (Lo; Western larch), *Tsuga heterophylla* (Th; Western hemlock), *Picea sitchensis* (Ps; Sitka spruce), *Thuja plicata* (Tp; Western Red cedar), *Ginkgo biloba* (Gb), *Pinus contorta* (Pc; Logepole pine). Extracts from Angiosperms (B) include *Medicago sativa* (Ms; alfalfa), *Brassica napus* (Bn; oil seed rape), *Glycine max* (Gm; soybean), *Ricinus communis* (Rc; castor bean), *Pisum sativum* (Ps; pea), *Nicotiana tabacum* (Nt; tobacco), *Phaseolus vulgaris* (Pv; bean). Fifteen µg of protein was loaded per lane.

The PmBiP antiserum was more reactive against the gymnosperm BiPs as indicated by both the number of species exhibiting cross reactivity and by the intensity of the reaction. Protein samples from all conifers except Amabalis fir showed reactivity to the PmBiP antiserum. In angiosperms, alfalfa, oil seed rape, and pea, showed no reactivity to the PmBiP antiserum in the molecular mass range expected for BiP. However, it can not be excluded that the failure to detect BiP in some plant species may be due to complete degradation of BiP in those samples as the appearance of lower molecular mass bands on western blots indicates degradation of protein samples has occurred due to storage at -20°C for several years.

Immunofluorescence localization of PmBiP in root tip cells

To confirm results of subcellular fractionation and western blot analysis, immunolocalization of PmBiP in root tip cells of Douglas-fir seedlings was conducted. Sections of methacrylate-embedded plant material provide an ideal material for *in situ* localization of antigens using immunofluorescence. A Douglas-fir root tip section stained with toluidine blue illustrates good preservation of cells at different stages of the cell cycle (Figure 19A). Immuno-

gold labeling of tissue sections was also attempted but was unsuccessful likely due to epitope denaturation during tissue preparation (Larry Fowke, personal communication).

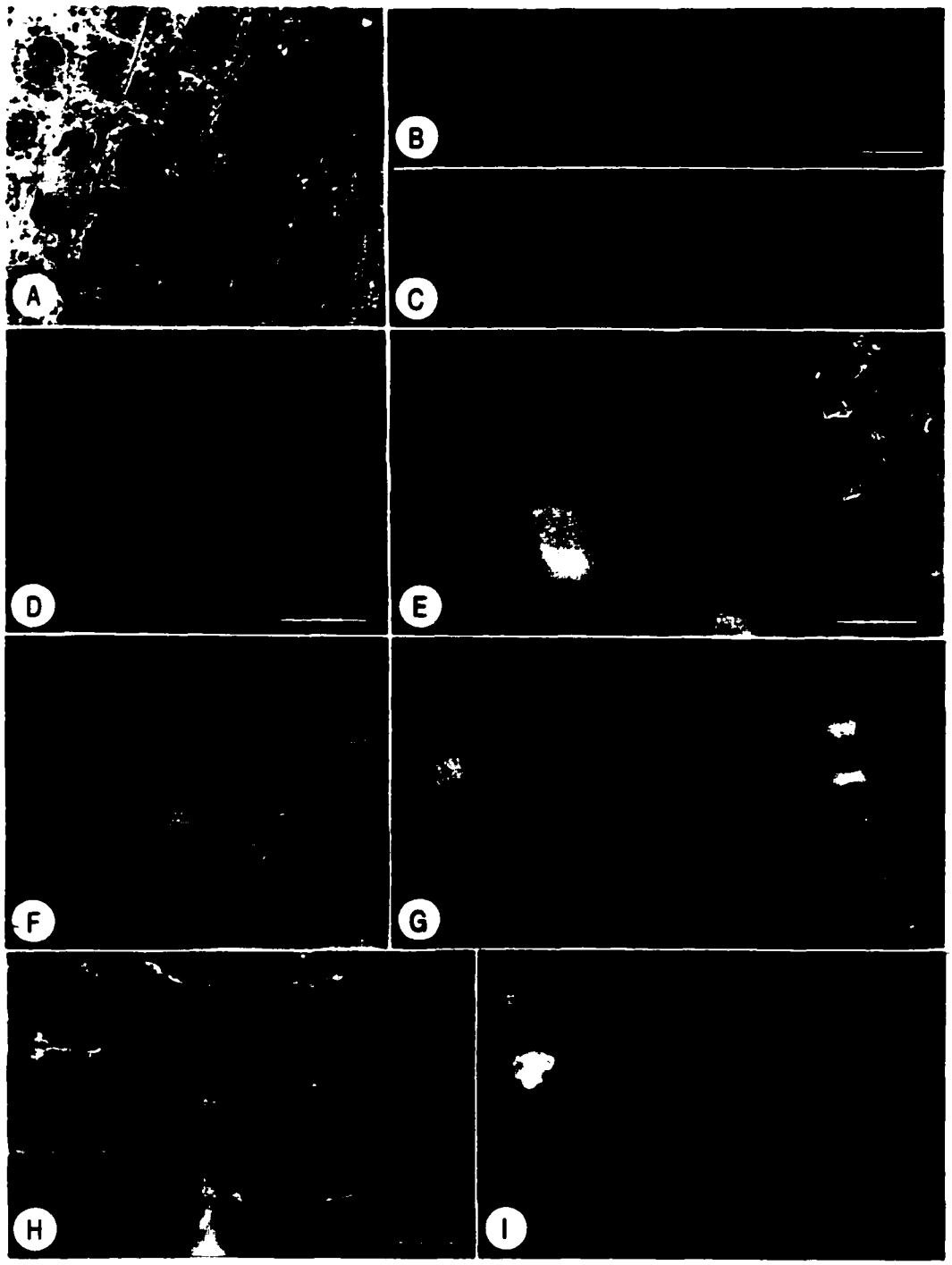


Figure 19. Immunolocalization of PmBiP in Douglas-fir root tip cells

(A) Methacrylate section of Douglas-fir root tip stained with toluidine blue to illustrate quality of cell preservation. Note the cells at late anaphase (LA) and Telophase (T). Bar = 20 μm.

(B) Section of Douglas-fir root tip treated with pre-immune serum as a control. No staining is evident. Bar = 20 μm.

(C) Same section as in (B) showing nuclei and chromosomes stained with DAPI.

(D) Douglas -fir root tip cells stained with PmBiP antiserum. Staining is evident near the cell surface, at the nuclear envelope and on membranous profiles in the cytoplasm. At late prophase (LP) and prometaphase (PM) the nuclear envelope and other membranes penetrating the nucleoplasm are strongly stained (arrowheads). EP = early prophase. Bar = 20 μm.

(E) Same section as in (D) showing nuclei and condensing chromosomes stained with DAPI.

(F) Douglas-fir root tip cells stained with PmBiP antiserum. At early anaphase (EA) stained membranes are present at the pole region (single arrowhead), enclosing the spindle region and penetrating among the chromosomes. By late anaphase (LA) strongly stained membranes are observed at the poles (single arrowhead) and by telophase (T) the developing cell plate and nuclear envelopes are stained. Membranous profiles in the cytoplasm are also stained (double arrowhead). Bar = 20 μm.

(G) Same section as in (F) showing nuclei and chromosomes stained with DAPI.

(H) Vacuolated Douglas-fir root cells stained with PmBiP antiserum. The nuclear envelope and numerous membrane profiles (arrow heads) in the cytoplasm are stained. Bar = 10 μm.

(I) Same section as in (H) showing nuclei stained with DAPI.

Similar sections treated with pre-immune serum did not stain (Figure 19B). In meristem sections stained with PmBiP antiserum, membranes appeared to be labeled at all stages of the cell cycle (Figure 19D, E, and H). At interphase and early prophase, labeling was common near the cell surface, at the nuclear envelope and on short linear profiles, presumably endoplasmic reticulum within the cytoplasm (Figure 19D). By late prophase/prometaphase, the nuclear envelope and membranous elements extending into the nucleus were heavily stained. At metaphase and during early anaphase, membranous elements at the spindle poles and among the chromosomes were stained (Figure 19E). By late anaphase, labeled membranes had accumulated at the

polar regions and at telophase the reforming nuclear envelope and cell plate stained strongly. In the zone of cell elongation adjacent to the meristem, elongating cells with large vacuoles and thin peripheral layers of cytoplasm also exhibited staining with the PmBiP antiserum (Figure 19H). The stain appeared to be restricted to membranous elements in the thin peripheral layer of cytoplasm and the nuclear envelope. Similar results as described above were obtained in pine root tip cells (Dr. Larry Fowke, personal communication).

PmBiP expression

To understand better the developmental regulation of PmBiP, PmBiP mRNA and protein levels during seed and seedling development were examined using northern and western blotting. Western blotting was also used to determine if seasonal variations exist in PmBiP protein levels in the needles of one-year-old seedlings.

PmBiP is developmentally regulated during seed development

Significant changes in the protein profiles of developing seeds are observed following fertilization and the onset of embryo development (Figure 20). The most significant change in protein profile is the appearance of the 45-47 kDa and 30-35 kDa storage proteins (Green et al., 1991). Both before and soon after fertilization, prior to the synthesis of storage proteins (May 31 and June 19, respectively), the amount of PmBiP mRNA observed in developing seeds was approximately 50 to 100 fold higher than that observed during embryogenesis (July 12 - August 15; Figure 21A). During embryogenesis, northern blotting of dissected material showed similar amounts of PmBiP mRNA in both the MG

and developing embryo (Figure 21E and G). Western blot analysis of seeds collected at various stages of seed development showed that the amount of PmBiP protein was also high before and soon after fertilization and decreased thereafter (Figure 21C).

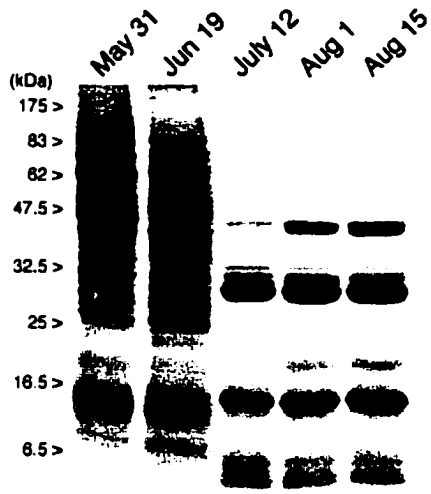


Figure 20. Protein profile of developing Douglas-fir seeds

Total protein was isolated from developing Douglas-fir seeds at the indicated time points. Fifteen micrograms of protein were loaded per lane and stained with Coomassie Blue. Molecular mass markers are indicated on the left. For morphological descriptions of developmental time points see Figure 21.

PmBiP expression during germination and early seedling development

Douglas-fir seeds germinate between 2 to 4 days after exposure of stratified seeds to germination conditions. Following germination, protein profiles changed as the major 45-47 kDa and 30-35 kDa storage proteins were mobilized and seedling growth commenced (Figure 22). Complete mobilization of storage proteins occurred between 6 and 8 days after exposure of stratified

seeds to germination conditions. Between 8 to 14 days, the expended MG was shed as the cotyledons expanded.

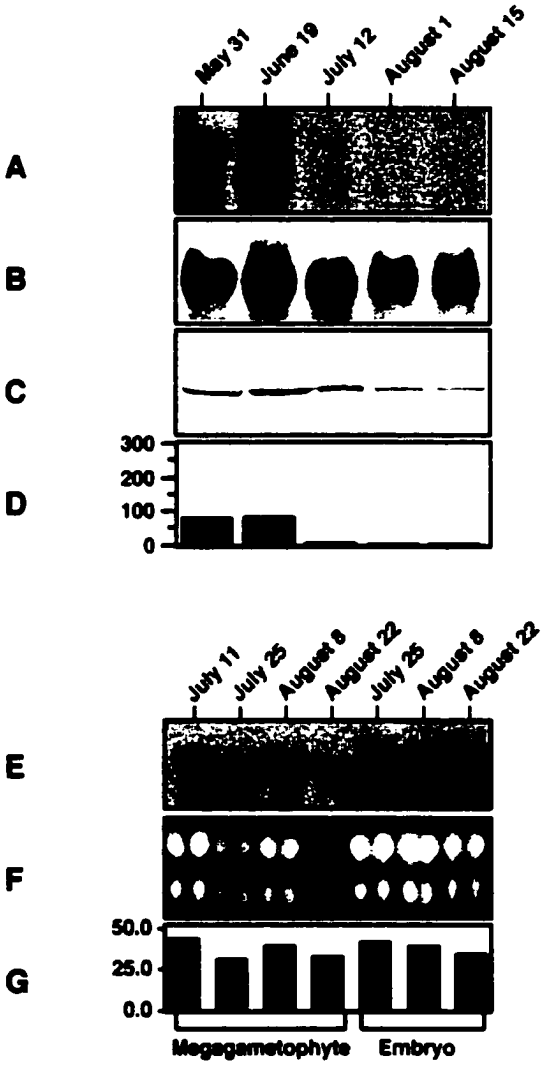


Figure 21. Expression of PmBiP during seed development

Total RNA was isolated from whole developing seeds collected at the indicated time points and subjected to northern blot analysis (20 µg per lane) using the PmBiP cDNA as probe (A). Time points indicated correspond to the following developmental stages based on embryo morphological characteristics established by (Allen and Owens, 1972): May 31 – pre-fertilization, June 19 – proembryo, July 12 – early to mid cotyledonary embryo, August 1 – mid to late embryo, August 15 – late to mature embryo. The same membrane was stripped and reprobed with a genomic DNA probe encoding the Douglas-fir 18S rRNA subunit to account for differences in the

amount of RNA loaded per lane (B). Total protein was isolated from developing seeds and subjected to western blot analysis (15 µg per lane) (C). Differences in the amount of PmBiP transcript represented graphically following adjustment for differences in the amount of RNA loaded per lane (given in units of integrated optical density; D). Total RNA was isolated from the megagametophyte and embryo and subjected to northern blot analysis (20 µg per lane) using the PmBiP cDNA as probe (E). Time points indicated correspond to the following embryonal developmental stages: July 11 – early to mid cotyledonary embryo, July 25 – mid cotyledonary embryo, August 8 – late cotyledonary embryo, August 22 – mature embryo. Ethidium bromide staining of total RNA showing 28S and 18S ribosomal RNA (F) was examined to adjust for differences in the amount of RNA loaded per lane. Differences in PmBiP mRNA represented graphically following adjustment in the amount of RNA loaded per lane (given in units of integrated optical density; G).

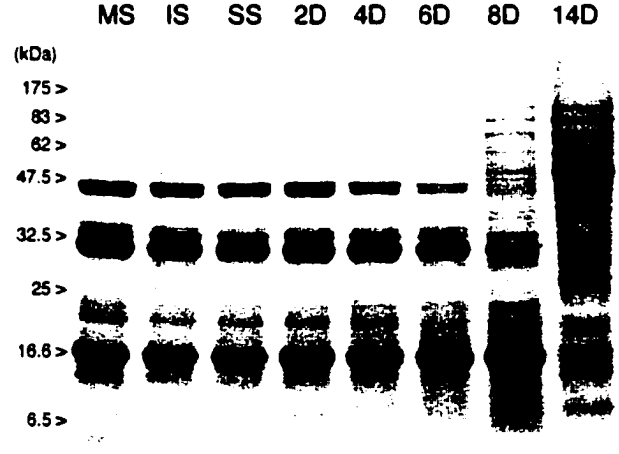


Figure 22. Protein profile of Douglas-fir seeds during germination and early seedling growth.

Total protein was extracted from mature Douglas-fir seeds (MS), imbibed seeds (IS), stratified seeds (SS), and young seedlings at the indicated time points (2D-14D). Fifteen micrograms of total protein was loaded per lane and stained with Coomassie Blue. Molecular mass markers are indicated on the left.

Changes in PmBiP expression occur concurrently with these development periods. Following imbibition and stratification PmBiP mRNA increased

slightly (Figure 23A and C). Upon exposure of the stratified seeds to germination conditions, the amount of PmBiP mRNA increased to levels greater than that observed during the early stages of seed development (Figure 23A and Figure 21D vs. Figure 23C).

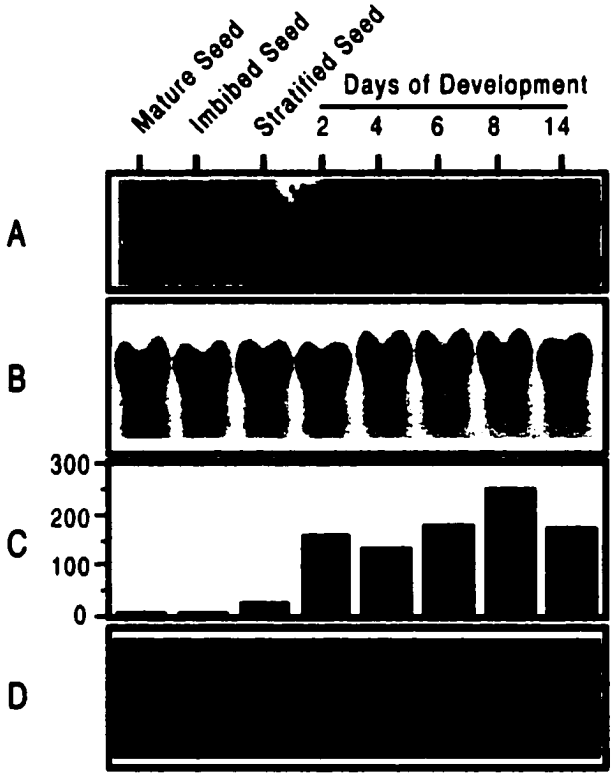


Figure 23. Expression of PmBiP during germination and early seedling development

Total RNA was isolated from tissue collected at the indicated time points and subjected to northern blot analysis (20 µg per lane) using the PmBiP cDNA as probe (A). The same membrane was stripped and reprobed with a genomic DNA probe encoding the Douglas-fir 18S rRNA subunit to account for differences in the amount of RNA loaded per lane (B). Differences in the amount of PmBiP transcript represented graphically following adjustment for differences in the amount of RNA loaded per lane (given in units of integrated optical density; C). Total protein was isolated from the same tissues and subjected to western blot analysis (15 µg per lane) (D).

Levels increased 150 to 200 folds over those observed in mature or imbibed seeds after only a 2-day exposure to germination conditions. This large increase correlated with the onset of visible germination, when the radicle emerged from the seed. PmBiP mRNA amounts were highest after 8 days, approximately 250 fold higher than observed in mature seeds. The amount of PmBiP protein did not show an increase until 8 days after exposure of the stratified seeds to germination conditions with the highest amounts appearing after 14 days (Figure 23D). To determine if PmBiP expression correlated with size or age of developing seedlings, expression was examined in 4-day-old and 14-day-old seedlings of different sizes. Examination of total protein profiles showed a correlation between germination and seedling growth and the mobilization of the major storage proteins (Figure 24). Decreased amounts of the 45-47 kDa storage protein are observed in seeds exposed to germination conditions for 4 days and have germinated compared to those that have not germinated. Further, in seedlings exposed to germination conditions for 14 days, there was a corresponding decrease in the amounts of the 45-47 kDa and 30-35 kDa storage proteins with increased seedling development. Concurrent with seedling growth and development and the mobilization of storage proteins, PmBiP mRNA and protein amounts showed significant changes. Upon stratification of mature seeds, a significant increase in PmBiP transcript amounts was observed (Figure 25A and C). After exposure of stratified seeds to germination conditions for 4 days, the amount of PmBiP transcript did not increase in ungerminated seeds. In visibly germinated seeds (seed coat cracked open and radicle visible), there was a slight increase in PmBiP transcript amounts.

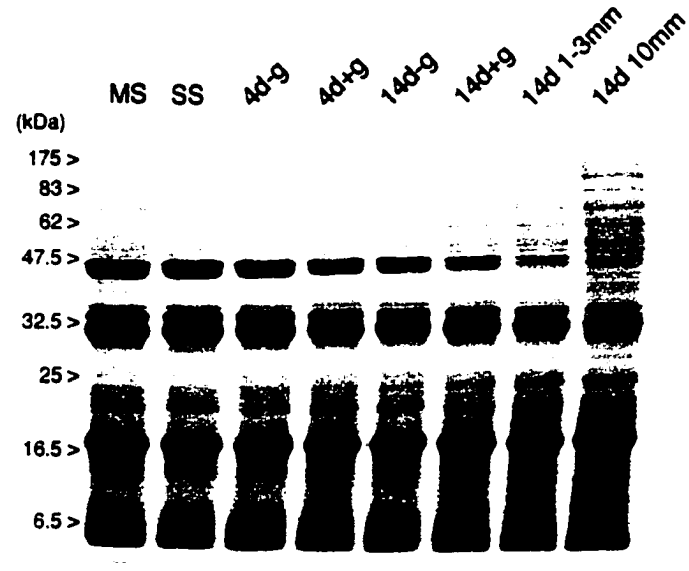


Figure 24. Protein profiles from seedlings at different stages of development

Total protein was isolated from mature seeds (MS), stratified seeds (SS), stratified seeds exposed to germination conditions for 4 days and are ungerminated (4d-g), or germinated (4d+g), stratified seeds exposed to germination conditions for 14 days and ungerminated (14d-g), germinated (14d+g), seedlings with radicle length of 1-3 mm (14d 1-3mm), and radicle length of 10 mm (14d 10mm) and stained with Coomassie Blue. Molecular mass markers are indicated on the left.

In stratified seeds exposed to germination conditions for 14 days but showing no signs of germination, PmBiP transcript amounts were only slightly higher than those observed in mature seeds. Transcript amounts were higher in seeds that had just completed visible germination. Seeds with increased radicle growth did show increased amounts of PmBiP transcript as observed when seeds with 1-3 mm radicles were compared to those with 10 mm radicles. PmBiP protein amounts were similar in mature, stratified seeds as well as in stratified seeds exposed to germination conditions for 4 days irrespective of their germination status (Figure 25). In stratified seeds exposed to germination

conditions for 14 days, increasing amounts of PmBiP protein were observed as seedling development (radicle elongation) proceeded.

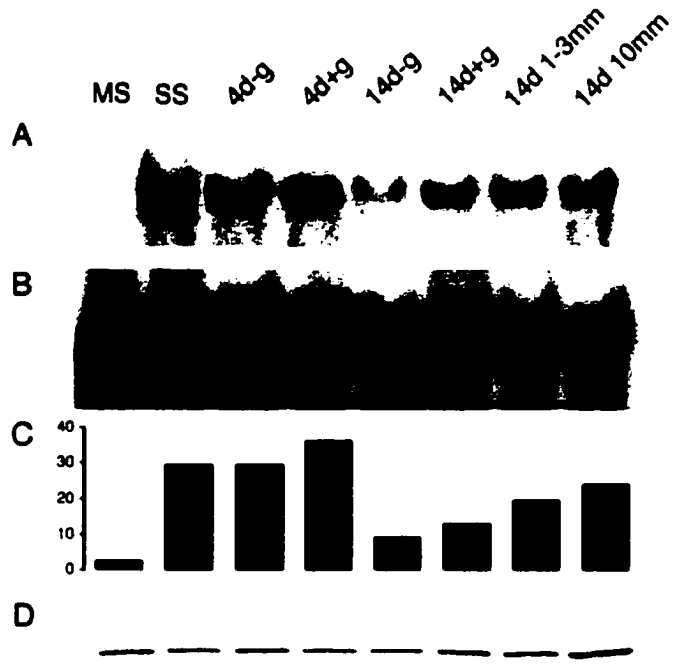


Figure 25. Expression of PmBiP during seedling development in seedlings of different sizes

Total RNA was isolated from tissue of mature seeds (MS), stratified seeds (SS), stratified seeds exposed to germination conditions for 4 days and are ungerminated (4d-g), or germinated (4d+g), stratified seeds exposed to germination conditions for 14 days and are ungerminated (14d-g), germinated (14d+g), seedlings with radicle length of 1-3 mm (14d 1-3mm), and radicle length of 10 mm (14d 10mm) and subjected to northern blot analysis (20 µg per lane) using the PmBiP cDNA as probe (A). The same membrane was stripped and reprobed with a DNA fragment representing the Douglas-fir 18S rRNA subunit to account for differences in the amount of RNA loaded per lane (B). Differences in the amount of PmBiP transcript represented graphically following adjustment for differences in the amount of RNA loaded per lane (given in units of integrated optical density) (C). Total protein was isolated from the same tissues and subjected to western blot analysis (15 µg per lane) (D).

PmBiP protein is seasonally regulated in needles

In northern temperate climates, conifers are unique among many plants in that they retain their foliage during the winter months. As a result, the tissues are exposed to freezing stress and risk damage to proteins, membranes, and photosynthetic apparatus due to photoinhibition and therefore must adapt accordingly (Huner et al., 1998). BiP has been implicated in playing a role in the adaptation of plants to cold by ensuring that secretory traffic is not disrupted by misfolding of proteins caused by low temperatures (Anderson et al., 1994a; Guy et al., 1998). To determine if a correlation exists between low temperature and the expression of PmBiP, the abundance of PmBiP protein was followed over a one-year period in needles collected from 1-year-old Douglas-fir seedlings kept under natural day length and temperature in an outdoor shelter house. PmBiP protein levels showed seasonal variation with the highest amounts occurring in needles collected from seedlings during the winter when the monthly average temperature was below 10 °C (Figure 26).

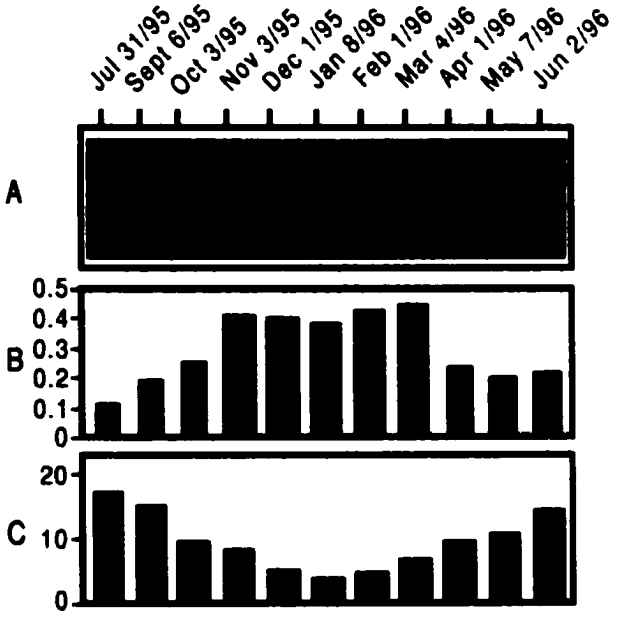


Figure 26. Seasonal variation of PmBiP protein in needles of 1-year-old seedlings

Total protein was isolated from the needles of one year old Douglas-fir seedlings at the indicated times and subjected to western blot analysis (15 µg per lane) (A). Following blot development, immunoreactive bands were quantitated using scanning densitometry and displayed graphically (given in units of arbitrary density; B) to allow direct comparison with the monthly average temperature (given in °C) at each time point (C).

PmBiP expression is affected by various treatments in young seedlings

To gain insight into the regulation of PmBiP with respect to the signals that may control expression, young seedlings were exposed to a variety of conditions and examined for changes in PmBiP mRNA and protein. Results indicated that after 48 hours, NaCl and ABA treatment decreased the amount of PmBiP mRNA compared to the water treated control (Figure 27A and C). Desiccation, however, greatly decreased the amount of PmBiP mRNA. Although less RNA was loaded for the wounding sample, IOD analysis indicated that this treatment increased the level PmBiP mRNA. Heat shock did not appear to have a large effect on the amount of PmBiP mRNA. Cold treatment, however, resulted in an increase in PmBiP mRNA. The amount of PmBiP protein appeared to decrease in response to NaCl and GA_3 (Figure 27D). The lack of detectable protein in wounded and desiccated seedlings may have resulted from sample degradation. No apparent change in PmBiP protein was observed in response to 48 h ABA treatment. A slight increase in PmBiP protein was observed in seedlings exposed to heat shock and cold treatment.

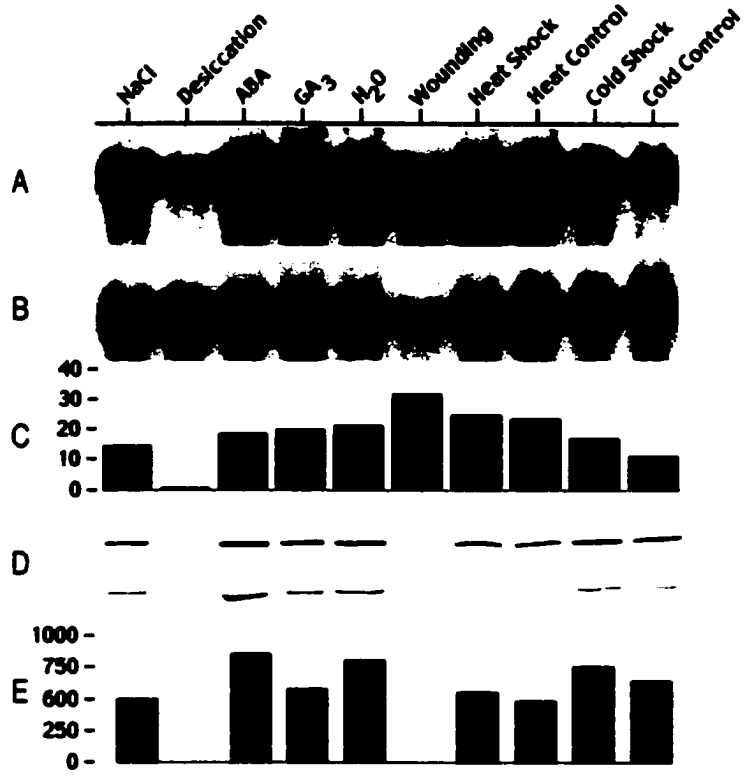


Figure 27. Expression of PmBiP in response to various treatments

14-day-old seedlings were subjected to 250 mM sodium chloride (NaCl), desiccation, 100 µM abscisic acid (ABA), 100 µM gibberellic acid (GA_3), water (control), and wounding (by pinching with forceps) for 48 hours. 14-day-old seedlings were also subjected to heat and cold treatment as follows. Heat shocked seedlings were placed in the dark at 40° C for 3 h while control seedlings were kept in the dark at 24° C for 3 h. Cold treated seedlings were placed in the dark at 4° C for 16 h while control seedlings were placed in the dark at 24° C for 16 h. Total RNA was isolated from the above treated seedlings as well as stratified seeds then subjected to Northern blot analysis (20 µg per lane) using PmBiP cDNA as a probe (A). The same membrane was reprobed with a genomic DNA probe encoding the Douglas-fir 18S rRNA subunit to discern differences in the amount of RNA loaded per lane (B). The integrated optical density (IOD) was then calculated to account for variations in the observed levels of PmBiP mRNA resulting from differences in the amount of RNA loaded per lane (C). Total protein was isolated from the same tissues and subjected to western blot analysis (15 µg per lane) (D). The western blot in C was scanned and density values are represented graphically in units of arbitrary density.

Examination of NaCl and ABA treatment on PmBiP transcript amounts over a 96 h period indicated that dehydration stress imposed by NaCl caused a reduction in PmBiP transcript levels within 24 h and was capable of maintaining reduced amounts after 96 h (Figure 28). The effect of ABA was distinctly different as it caused a significant increase in PmBiP amounts after 24 h but caused a reduction after 48 and 96 h.

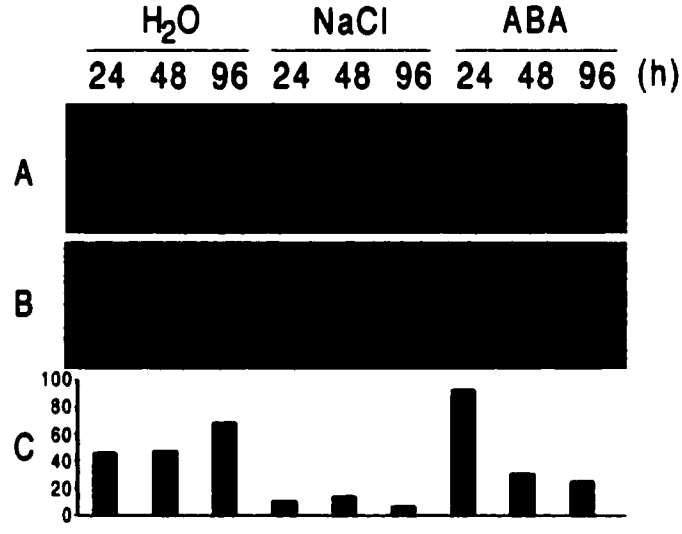


Figure 28. Effect of NaCl and ABA treatment on PmBiP mRNA amounts in 14-day-old seedlings

Seedlings were treated with either 250 mM NaCl or 100 µM ABA for the times indicated. Control seedlings were incubated with water. RNA isolated from each treatment was subjected to northern blot analysis (20 µg per lane) using the PmBiP cDNA as probe (A). Blots were stripped and reprobed with a DNA fragment representing the Douglas-fir 18S rRNA to account for differences in the amount of RNA loaded per lane (B). PmBiP mRNA amounts were then represented graphically as IOD following adjustments for differences in the amount of RNA loaded per lane (C).

The effect of ABA decreasing PmBiP amounts after 48 h is consistent with that observed in Figure 27 although the effect is more pronounced.

Analysis of PmBiP association with substrate proteins

During germination and in response to various treatments, increases in PmBiP expression were observed. Increased expression is believed to support an increase in secretory protein traffic through the ER (Denecke et al., 1991) and BiP is known to associate with proteins targeted to the secretory pathway in plants (Li et al., 1998; Li et al., 1993; Pedrazzini et al., 1994). To support this idea and identify secretory proteins involved in the development and cold response of Douglas-fir, PmBiP was isolated by co-immunoprecipitation (CoIp) using the PmBiP antiserum and treated with ATP to release associated proteins. To ensure the purified PmBiP antiserum immunoprecipitated PmBiP, immunoprecipitation (Ip) was conducted on microsomal proteins isolated from mature seeds. Coomassie blue staining of both the immunoprecipitate pellet and supernatant showed the presence of a protein band with a similar molecular mass predicted for PmBiP present in the pellet and not in the supernatant (Figure 29A). Western blotting of both fractions confirmed the identity of the precipitated protein as PmBiP (Figure 29B). The large protein band migrating around 47.5 kDa (Figure 29A) represents the heavy chain IgG and was detected by the secondary antibody on western blots (Figure 29B). Both PmBiP and IgG were not detected in the supernatant by western blotting indicating that the conditions used for Ip successfully removed all PmBiP from protein extracts. This was confirmed in all subsequent experiments.

Figure 29. Immunoprecipitation of PmBiP from mature seed microsomal protein fractions

Immunoprecipitation of PmBiP from mature seed microsomes was as described in Materials and Methods. Both the supernatant (S) and pellet (P) were subjected to SDS-PAGE and staining with Coomassie blue (A) or transferred to nitrocellulose for western blotting using the PmBiP peptide antiserum (B). Pellet fractions represent 50% of the proteins precipitated from 200 µg of mature seed microsomal protein. Molecular mass markers are indicated on the left.

Association of PmBiP with proteins during germination

To examine the association of PmBiP with proteins during germination, microsomal extracts were prepared from seedlings at various stages of development and subject to CoIp. Pellets from each developmental stage were washed with buffer containing ATP or with buffer without ATP as a control. Results showed that very few high molecular mass proteins were associated with PmBiP in mature seeds and 2 day-old seedling microsomes (Figure 30). However, the amounts were very small and could only be detected by silver staining. Proteins detected in the 14D -ATP sample are likely the result of incomplete washing of the immunoprecipitate. A low abundance and number of ATP dependent associated proteins detected may be due to the complex

tissue structure of the conifer seedlings and dilution of proteins from specific tissues when whole seedlings were used to obtain microsomal fractions.

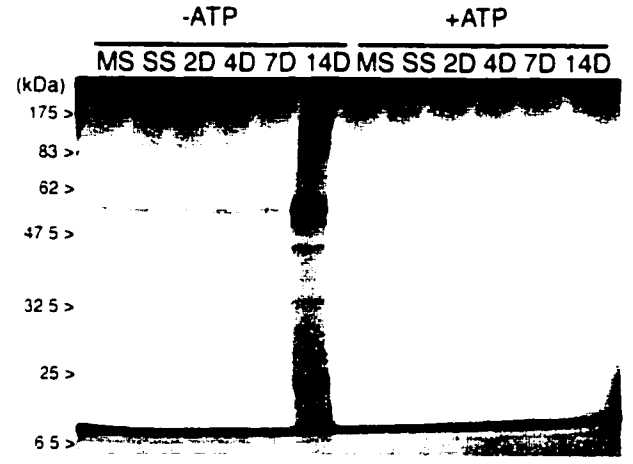


Figure 30. ATP dependent association of PmBiP with proteins during seedling development

PmBiP was immunoprecipitated from approximately 200 µg of microsomal protein from mature seeds (MS), stratified seeds (SS), 2-day old seedlings (2D), 4-day old seedlings (4D), 7-day old seedlings (7D), and 14-day old seedlings (14D). Immunoprecipitated pellets were incubated with ATP (+ATP) or without ATP (-ATP) and the supernatants collected and subjected to SDS-PAGE and silver staining. Bracket indicates ATP dependent associated proteins. Molecular mass markers are indicated on the left.

To try to increase the abundance of PmBiP-substrate protein complexes, seedlings were treated with tunicamycin. Fourteen-day-old Seedlings were treated with tunicamycin for 30 min to 24 h and examined for PmBiP protein. Results showed no difference in the abundance of PmBiP protein between treated seedlings and controls (Figure 31). These results suggested that the seedlings may not have taken up tunicamycin or were resistant to the effects of the applied concentration. Hence, co-immunoprecipitation was not conducted.

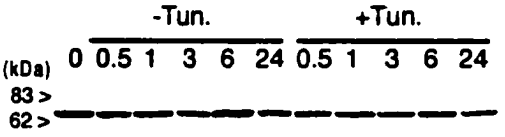


Figure 31. Expression of PmBiP in response to tunicamycin treatment in Douglas-fir seedlings

Western blotting of total protein isolated from 14-day-old seedlings treated with 20 µg/ml tunicamycin for varying periods of time (indicated in hours) using the PmBiP antiserum. Fifteen µg of protein was loaded per lane. Molecular mass markers are indicated on the left.

To avoid the problems associated with tissue complexity of whole seedlings and the possible exclusion of tunicamycin, embryonal suspensor mass (ESM) culture was used in further experiments. ESM cultures are derived from an early stage of Douglas-fir embryo development and represent a source of relatively homogeneous culture that can be readily propagated in liquid media (Durzan and Gupta, 1987).

To determine if PmBiP in ESM cultures associated with proteins in an ATP dependent manner, CoIp was performed on ESM microsomal proteins. Results indicated that PmBiP was associated with approximately 3-5 proteins of different apparent molecular mass (Figure 32).

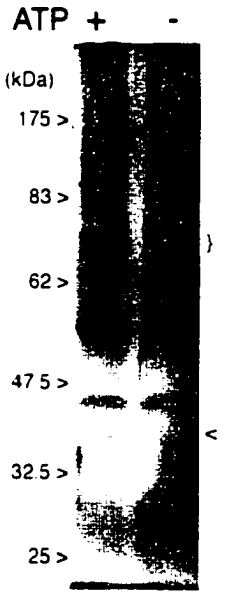


Figure 32. ATP dependent association of PmBiP with proteins in microsomal extracts of ESM culture

Immunoprecipitation was conducted on approximately 400 µg of ESM microsomal protein using purified PmBiP antiserum. Immunoprecipitates were incubated with ATP (+ATP) or without ATP (-ATP) and the supernatants collected and subjected to SDS-PAGE and silver staining. Bracket indicates ATP dependent associated proteins. Molecular mass markers are indicated on the left.

The abundance of such proteins, however, was low and detectable only by silver staining. Hence it was of interest to determine if the abundance of PmBiP/protein complexes could be increased by treatment of cells with tunicamycin and if the association profile could be changed by treatment of ESM cultures with cold. Northern and western blotting was conducted to determine if ESM cultures were responsive to cold and tunicamycin treatment. Results indicated that tunicamycin caused an increase in PmBiP transcript amounts following a 3 and 6 hour treatment (Figure 33A and C).

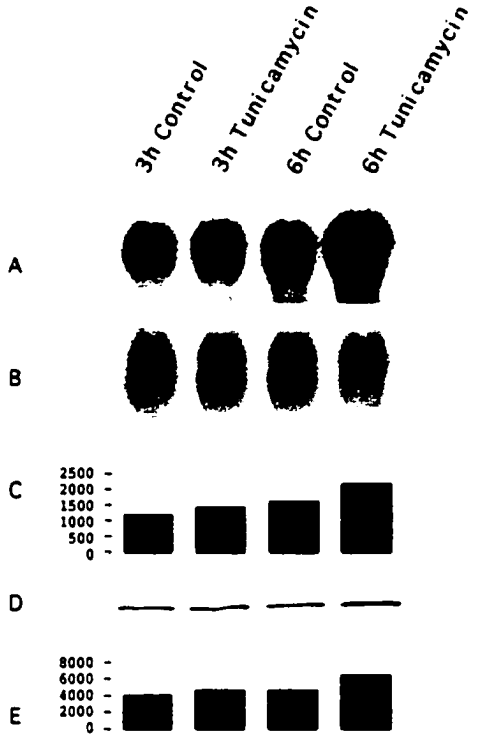


Figure 33. Expression of PmBiP in ESM culture treated with tunicamycin

Total RNA was isolated from ESM culture treated with 20 µM tunicamycin for 3 and 6 h and subjected to northern blot analysis (20 µg per lane) using the PmBiP cDNA as probe (A). The same blot was stripped and reprobed with a DNA fragment representing the Douglas-fir 18S rRNA subunit to account for differences in the amount of RNA loaded per lane (B). PmBiP mRNA amounts represented graphically as IOD following adjustments for differences in the amount of RNA loaded per lane (given in units of integrated optical density) (C). Total protein was isolated from the same tissues and subjected to western blot analysis (15 µg per lane) (D). Following blot development, immunoreactive bands were quantitated using scanning densitometry and displayed graphically (given in units of arbitrary density) (E).

Increases in PmBiP protein amounts were also observed following treatment with tunicamycin, with a larger increase observed in culture treated for 6 h (Figure 33D and E). Cold treatment resulted in only a marginal increase in PmBiP mRNA and protein (Figure 34).

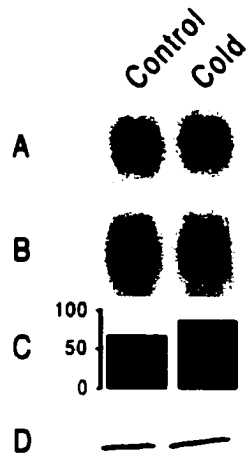


Figure 34. Expression of PmBiP in ESM culture treated with cold

Total RNA was isolated from ESM culture cold treated for 24 h at 4°C and subjected to northern blot analysis (20 µg per lane) using the PmBiP cDNA as probe (A). The same blot was stripped and reprobed with a DNA fragment representing the Douglas-fir 18S rRNA subunit to account for differences in the amount of RNA loaded per lane (B). PmBiP mRNA amounts were represented graphically as IOD following adjustments for differences in the amount of RNA loaded per lane (given in units of integrated optical density) (C). Total protein was isolated from the same tissues and subjected to western blot analysis (15 µg per lane) (D).

Due to the effect of both treatments on the expression of PmBiP CoIp was conducted on protein fractions isolated from treated tissue. Results indicated that treatment of ESM culture with tunicamycin or cold had no significant effect on the ATP dependent association of PmBiP with other proteins detectable by silver staining (Figure 35A and B). It was noted that CoIp of total soluble protein extracts showed an additional band that was not present in PmBiP complexes from microsomal fractions (Figure 35B). The significance of this is unknown.

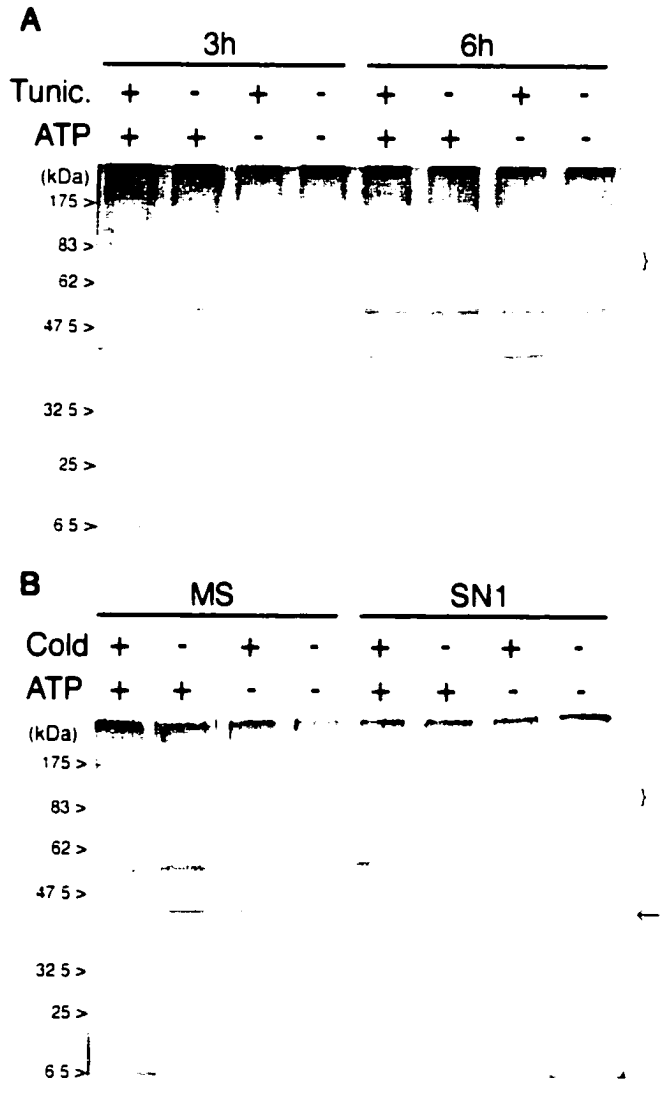


Figure 35. Co-Immunoprecipitation analysis in treated ESM culture

ATP dependent association of proteins with PmBiP in ESM culture treated with tunicamycin (A) or cold (B). Immunoprecipitation using purified PmBiP antiserum was conducted on approximately 400 µg of ESM microsomal protein isolated from control (-) and cultures treated with 20 µM tunicamycin for 3 and 6 h (+) or from control (-) and culture treated with cold (+) for 24h at 4°C. Immunoprecipitated pellets were incubated with ATP (+) or without ATP (-) and the supernatants collected and subjected to SDS-PAGE and silver staining. Molecular mass markers are indicated on the left and brackets indicate PmBiP associated proteins. Arrow indicates proteins associated with PmBiP in SN1 protein fractions but not in microsomal fractions.

Isolation and characterization of PmBiP promoter

It has been shown that the abundance of PmBiP mRNA is developmentally regulated and influenced by a variety of treatments. To begin elucidation of signaling pathways controlling the transcriptional activation of PmBiP, attempts were made to isolate its promoter sequence. Screening of 1.25×10^6 genomic clones from a Douglas-fir genomic library using PmBiP3 cDNA as probe failed to detect PmBiP gDNA sequence. This was most likely due to the fact the PmBiP is present as a single copy in the genome of Douglas-fir (Figure 6). Hence, inverse PCR (iPCR) (Ochman et al., 1990) was employed to directly amplify DNA sequence 5' to the PmBiP coding sequence from Douglas-fir gDNA.

Douglas-fir gDNA was digested with *Sac* I or *Xba* I and circularized to form templates for iPCR. DNA was digested with two different restriction enzymes to increase the probability of producing templates containing a significant portion of the 5' upstream sequence. PCR was conducted using multiple primer sets directed outward from the coding region to increase the probability of amplifying the target sequence. The resultant PCR products were separated on an agarose gel and subjected to Southern blot analysis using the PmBiP cDNA as probe. Results indicated that iPCR with template prepared from Xba I digested gDNA and primer p14-3z1.1 and p14-5a4 generated a fragment of approximately 3 kb in size (Figure 36A). Because the amplified fragment was present in relatively low abundance, the entire iPCR reaction was used for cloning to avoid potential losses due to purification. The resultant colonies

were screened for the ~3 kb fragment using PCR. Initial screening identified three possible clones which were confirmed by Southern blotting (Figure 36B).

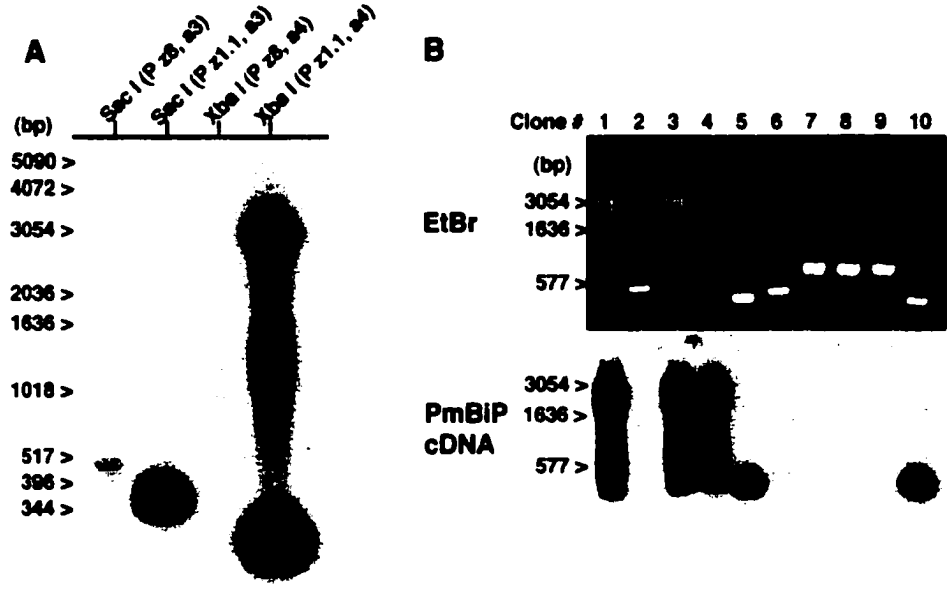


Figure 36. Isolation of PmBiP promoter using iPCR

Douglas-fir genomic DNA was digested with the enzymes indicated, circularized, and subjected to PCR using primers flanking the 5' end of the cDNA, directed upstream, and 3' end of the cDNA, located 5' to the restriction site, directed downstream. Reaction products were then subjected to Southern blot analysis to identify positive target sequences (A). PCR reaction products from Xba I (P z1.1, a4) were cloned then screened using PCR and Southern blotting to identify clones with an appropriately sized insert (B).

DNA sequencing and analysis

The iPCR product was 2760 bp in size and contained 2277 bp of sequence immediately upstream of the PmBiP3 cDNA sequence (Figure 37). The 2277 bp sequence is hereby designated PmBiPPro1. The cloned iPCR product also contained the expected 80 bp from the 3' end and 403 bp from the 5' end of the

PmBiP cDNA sequence as well as the *Xba* I restriction site used to digest Douglas-fir gDNA.

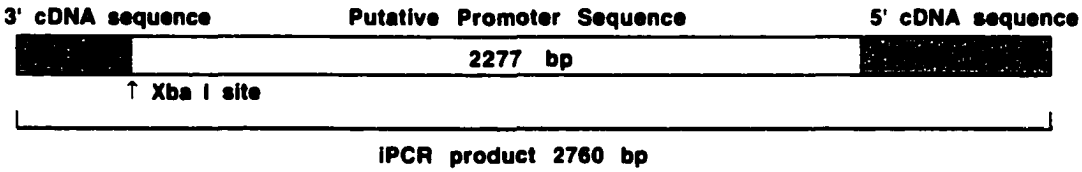
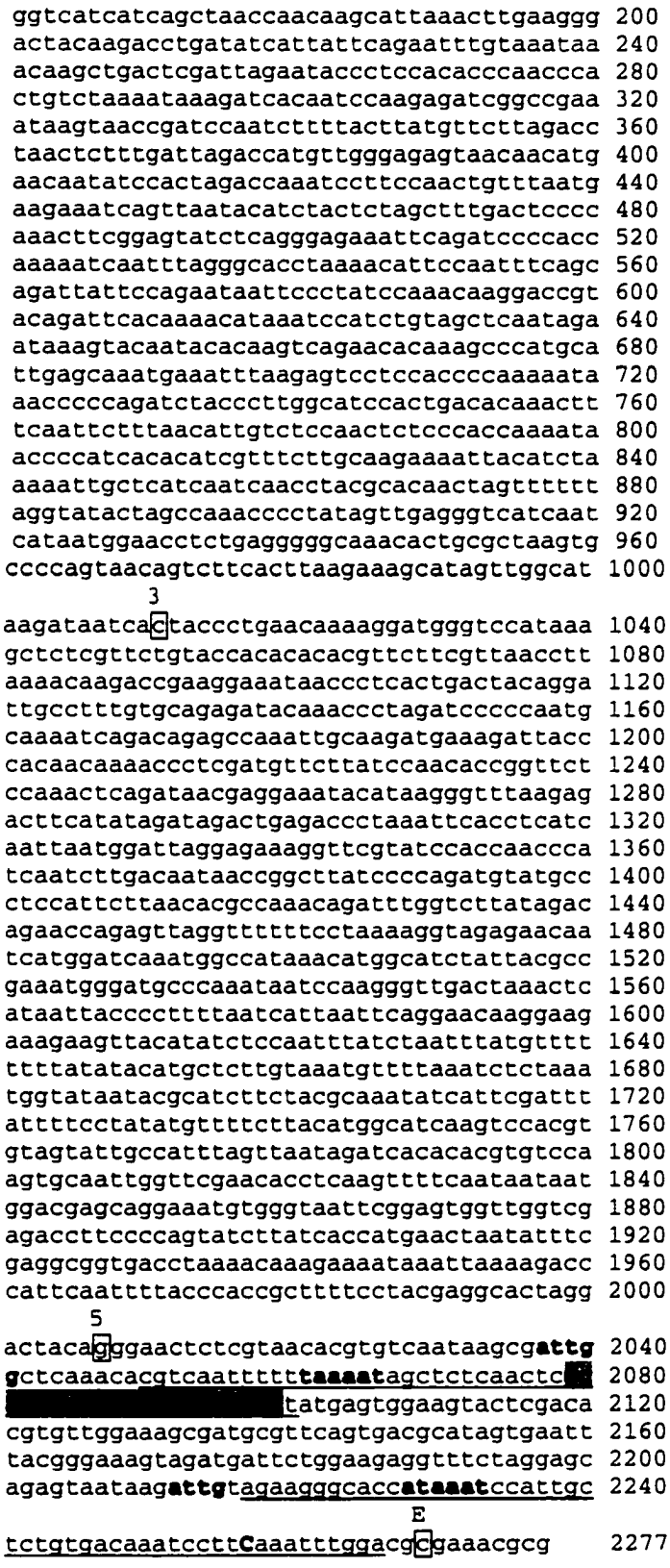


Figure 37. Structural characteristics of PmBiP iPCR product

DNA sequence analysis of clone 1 indicated that the product of iPCR was 2760 bp in size and contained 2277 bp of DNA sequence immediately upstream of the 5' end of the PmBiP cDNA sequence.

Primer extension failed to determine the transcriptional start site (TSS). Hence, the numbering of PmBiPPro1 DNA sequence is not relative to the TSS (Figure 38). Using the Neural Network Promoter Prediction program (NNPP), two potential TSS were identified (Reese and Eeckman, 1995; Reese et al., 1996). The NNPP program examines DNA sequence for potential TATA-box and initiator regions in the DNA sequences of eukaryotic and prokaryotic DNA. Examination of the 5' TSS revealed a possible overlapping UPRE-like sequence that contained a high degree of identity with the yeast UPRE (Figure 38 and Figure 39) (Mori et al., 1998). Despite the high degree of identity over this region, the partial palindromic half sites were not completely conserved. An element similar to the mammalian ERSE (Roy and Lee, 1999; Yoshida et al., 1998) was not identified in PmBiPPro1.

```
                1
tctagatcaac[t]aagtccttaatgtcccgagctttttct 40
tcaccgaccaagatataaacacatcttttaaactcttaag 80
gttcacaacaaactgagatgcaggagatacctctgaactg 120
gcatcataaactttccaggatgcttttaataatgctacca 160
```


ggtcatcatcagctaaccaacaagcattaaacttgaaggg 200
actacaagacctgatatcattattcagaatttgtaaataa 240
acaagctgactcgattagaataccctccacacccaaccca 280
ctgtctaaaataaagatcacaatccaagagatcggccgaa 320
ataagtaaccgatccaatcttttacttatgttcttagacc 360
taactctttgattagaccatgttgggagagtaacaacatg 400
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aagaaatcagttaatacatctactctagctttgactcccc 480
aaacttcggagtatctcagggagaaattcagatccccacc 520
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aacccccagatctacccttggcatccactgacacaaactt 760
tcaattctttaacattgtctccaactctcccaccaaaata 800
accccatcacacatcgtttcttgcaagaaaattacatcta 840
aaaattgctcatcaatcaacctacgcacaactagtttttt 880
aggtatactagccaaacccctatagttgagggtcatcaat 920
cataatggaacctctgaggggcaaacactgcgctaagtg 960
ccccagtaacagtcttcacttaagaaagcatagttggcat 1000
3
aagataatcacctaccctgaacaaaaggatgggtccataaa 1040
gctctcgttctgtaccacacacacgttcttcgttaacctt 1080
aaaacaagaccgaaggaaataaccctcactgactacagga 1120
ttgcctttgtgcagagatacaaaccctagatcccccaatg 1160
caaaatcagacagagccaaattgcaagatgaaagattacc 1200
cacaacaaaaccctcgatgttcttatccaacaccggttct 1240
ccaaactcagataacgaggaaatacataagggtttaagag 1280
acttcatatagatagactgagaccctaaattcacctcatc 1320
aattaatggattaggagaaaggttcgtatccaccaaccca 1360
tcaatcttgacaataaccggcttatccccagatgtatgcc 1400
ctccattcttaacacgccaaacagatttggtcttatagac 1440
agaaccagagttaggtttttcctaaaaggtagagaacaa 1480
tcatggatcaaatggccataaacatggcatctattacgcc 1520
gaaatgggatgcccaaataatccaagggttgactaaactc 1560
ataattacccctttaatcattaattcaggaacaaggaag 1600
aaagaagttacatatctccaatttatctaatttatgtttt 1640
ttttatatacatgctcttgtaaatgttttaaatctctaaa 1680
tggtataatacgcatcttctacgcaaatatcattcgattt 1720
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gtagtattgccatttagttaatagatcacacacgtgtcca 1800
agtgcaattggttcgaacacctcaagttttcaataataat 1840
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agaccttccccagtatcttatcaccatgaactaatatttc 1920
gaggcggtgacctaaaacaaagaaaataaattaaaagacc 1960
cattcaattttacccaccgcttttcctacgaggcactagg 2000
5
actacaggaactctcgtaacacgtgtcaataagcgattg 2040
gctcaaacacgtcaatttttttaaaatagctctcaactc 2080
tatgagtggaagtactcgaca 2120
cgtgttggaaagcgatgcgttcagtgacgcatagtgaatt 2160
tacgggaaagtagatgattctggaagaggtttctaggagc 2200
agagtaataagattgtagaagggcaccataaatccattgc 2240
E
tctgtgacaaatccttCaaatttggacgcgaaacgcg 2277

Figure 38. Nucleotide sequence of PmBiP promoter

PmBiPPro1 consists of 2277 bp upstream of the PmBiP3 cDNA sequence and is numbered from 5' to 3'. Numbering is not relative to the transcriptional start site as the exact position has not been experimentally determined. Predicted promoter regions (-40 to +10) using the Neural Network Promoter Prediction program (Reese and Eeckman, 1995; Reese et al., 1996) are underlined with the putative transcriptional start site indicated in bold capital. A putative UPRE is shaded (Mori et al., 1998). Possible TATA-boxes identified using the Hamming-clustering method are indicated in bold (Milanesi et al., 1996). The nearest upstream CAAT boxes to each TATA-box are also indicated in bold. The first residue of PmBiPPro1-1 (1), PmBiPPro1-3 (3), and PmBiPPro1-5 (5) promoter reporter construct is boxed as is the 3' end of each construct (E).

```
Yeast UPRE          GGAACTGGACAGCGTGTCGAAA
PmBiPPro1 UPRE      CGAACGGG-TAACGTGGCGAAA
                     **** **  * **** *****
```

Figure 39. Alignment of the Yeast UPRE and PmBiPPro1 putative UPRE.

Critical residues for Yeast UPRE function are boxed or underlined. Boxed residues represent partial palindromic half sites separated by a single nucleotide spacer. Asterisks indicate identical residues.

Analysis of the PmBiP promoter for plant cis-acting regulatory elements

To determine if the PmBiPPro1 DNA sequence contains any known plant cis-acting regulatory elements, the nucleotide sequence was examined using the Plant Cis Acting Regulatory Database (PLACE) (Higo et al., 1999; Prestridge, 1991). This analysis revealed a variety of different cis-acting regulatory elements (Table 2). It is noteworthy that many of the cis-elements control the expression of similar types of genes and can be grouped according to the type of gene they are found in. Group A elements are the most abundant and found in the promoter regions of plant storage protein genes or are somehow associated with the regulation of genes during seed development in

angiosperms (e.g. 2SSEEDPROTBANAP, (Stalberg et al., 1996) or RYREPEATLEGUMIN, (Fujiwara and Beachy, 1994)). Such elements can confer embryo or endosperm specific expression (e.g. CANBNNAPA, (Ellerstrom et al., 1996)). Group B are the second most common type of cis-element and are found in the promoters of light regulated genes such as rubisco (e.g. GT1CONSENSUS, (Terzaghi and Cashmore, 1995; Villain et al., 1996) or IBOX, (Donald and Cashmore, 1990; Giuliano et al., 1988)). Group C elements are commonly found in the promoters of genes involved in cell wall synthesis or pathogen defense such as the phenylpropanoid and flavonoid biosynthesis genes CHS and PAL or cell wall protein extensin (e.g. MYBPLANT, (Sablowski et al., 1994) and MYBPZM, (Grotewold et al., 1994) or QARBNEXTA (Elliott and Shirsat, 1998)). Other cis-elements of note are those found in the promoters of cold responsive genes (e.g LTRECOREATCOR15, (Baker et al., 1994; White et al., 1994) or LTRE1HVBLT49 (Dunn et al., 1998), those responding to GA (PYRIMIDINEBOXHV, (Cercos et al., 1999)), auxin (ARFAT, (Ulmasov et al., 1999a; Ulmasov et al., 1999b), and a quantitative enhancer element (QELEMENTZMZM13, (Hamilton et al., 1998). Two putative matrix attachment sites are present in PmBiPPro1 and may be involved in the attachment and organization of the PmBiP gene locus to the nuclear matrix (Gasser et al., 1989). A total of 11 identical pollen specific elements were identified in PmBiPPro1. However the motif identified (AGAAA) represents only one half of a bipartite motif necessary to confer minimal pollen specific expression during mid-late pollen development (Bate and Twell, 1998). PLACE search was unable to identify the other regulatory sequence of this bipartite structure (TCCACCATA) which is located 7 bp downstream from AGAAA.

Examination of the downstream nucleotides from each AGAAA sequence identified only one region containing the nucleotides TCCACCAAC spaced 8 bp from the AGAAA at position 1336. The high degree of identity suggests that both sequences may function together to confer pollen specific expression. Interestingly the second half of this bipartite motif also contains the consensus-binding site for plant Myb transcriptional activators MACCWAMC (Table 2) (Sablowski et al., 1994).

Site Name	Loc.(Str.)	Sequence	Description
[redacted]			Element is present in the promoter of the barley B-hordein gene and the alpha-gliadin, gamma-gliadin, and low molecular weight glutenin genes of wheat (Thomas and Flavell, 1990).
2SSEEDPROTBANAP	2044	(+) CAAACAC	Conserved in many storage-protein gene promoters and may be important for high activity of the napA promoter (Stalberg et al., 1996).
[redacted]			ARF (auxin response factor) binding site found in the promoters of primary/early auxin response genes of *Arabidopsis thaliana*. Many different ARFs are known and can act as activators and repressors (Ulmasov et al., 1999a; Ulmasov et al., 1999b).
ASF1MOTIFCAMV	2145	(+) TGACG	TGACG motifs are found in many promoters and are involved in transcriptional activation of several genes by auxin and/or salicylic acid (Lam et al., 1989; Terzaghi and Cashmore, 1995).
ASF1MOTIFCAMV	2050	(-) TGACG	
[redacted]			Light-induced transcriptional repression of the pea asparagine synthase (AS1) gene (Ngai et al., 1997).
			Box II found in the tobacco plastid atpB gene promoter and is conserved in several NCII (nonconsensus type II) promoters of plastid genes (Kapoor and Sugiura, 1999).

BOXIIPCCHS	2089	(+)	ACGTGGC	Core of "Box II/G box" found in the parsley CHS genes and is essential for light regulation (Terzaghi and Cashmore, 1995).
CACGTGMOTIF	2021	(+)	CACGTG	Represents the core of "G-box" (Foster et al., 1994) and is found in the promoter region of light-responsive genes such as rbcS and CHS. It is also commonly found in other unrelated genes (Williams et al., 1992).
CACGTGMOTIF	2119	(+)	CACGTG	
CACGTGMOTIF	2021	(-)	CACGTG	
CACGTGMOTIF	2119	(-)	CACGTG	
CANBNNAPA	2044	(+)	CNAACAC	Comprises the core of the"(CA)n element" in storage protein genes in *Brasica napus*. Confers embryo and endosperm-specific transcription of napin storage protein gene (napA). Can possibly function in activation and repression to confer seed specificity (Ellerstrom et al., 1996).
CANBNNAPA	2122	(-)	CNAACAC	
				Referred to as the "cereal glutenin box" and is found in the pea legumin gene (legA). The sequence is homologous to the cereal glutenin gene control element ("-300 element" see above) (Shirsat et al., 1989).
				Core site is required for binding of Dof DNA binding proteins in maize. Surrounding nucleotides can influence binding. Dof proteins are so far found only in plants and contain a single zinc finger. Four Dof proteins, Dof1, Dof2, Dof3 and PBF, have been isolated from maize and PBF is an endosperm specific Dof protein that binds to the prolamin box (Yanagisawa and Schmidt, 1999).

DOFCOREZM	2129 (+) AAAG	
DOFCOREZM	2167 (+) AAAG	
DPBFCOREDCDC3	2020 (+) ACACNNG	Consensus binding core sequence for a novel class of bZIP transcription factors, DPBF-1 and 2 (Dc3 promoter-binding factor-1 and 2). Elements are found in the carrot Dc3 gene (a LEA gene) which is expressed in an embryo-specific and ABA inducible manner (Kim et al., 1997).
DPBFCOREDCDC3	2118 (+) ACACNNG	
DPBFCOREDCDC3	2021 (-) ACACNNG	
DPBFCOREDCDC3	2119 (-) ACACNNG	
		Comprises the E-box of storage-protein gene napA of *Brassica napus* (Stalberg et al., 1996).

[redacted]	
EBOXBNNAPA 2021 (+) CANNTG	
EBOXBNNAPA 2119 (+) CANNTG	
[redacted]	
EBOXBNNAPA 2021 (-) CANNTG	
EBOXBNNAPA 2119 (-) CANNTG	
[redacted]	GATA motif found in the Cauliflower Mosaic Virus 35S promoter and binds the transcription factor ASF-2 (Gilmartin et al., 1990; Lam and Chua, 1989)

		Consensus GT-1 transcription factor binding site found in many light-regulated genes (Terzaghi and Cashmore, 1995; Villain et al., 1996).
		Critical element for binding of GT-1 transcription factor to box II of rbcS gene (Green et al., 1988; Terzaghi and Cashmore, 1995; Villain et al., 1996).
HEXMOTIFTAH3H4	2049 (+) ACGTCA	Element found in the promoter of wheat histone genes H3 and H4 and binds the HBP-1A and HBP-1B transcription factors (Mikami et al., 1989; Mikami et al., 1987).
		Conserved motif located in the promoter region of light-regulated genes such as tomato and *Arabidopsis* rbcS (Donald and Cashmore, 1990; Giuliano et al., 1988).

[redacted]		Conserved sequence located in the promoter region of light-regulated genes of both monocots and dicots (Terzaghi and Cashmore, 1995). Also see IBOX above.
		A low-temperature-responsive element located in the barley blt4.9 (a non-specific lipid transfer protein) gene promoter (Dunn et al., 1998).
		Core of the low temperature responsive element (LTRE) of cor15a gene from *Arabidopsis* and involved in the cold induction of the BN115 gene of *Brassica napus* (Baker et al., 1994; White et al., 1994).
		Involved in the attachment of genes to the nuclear matrix (Gasser et al., 1989).
MNF1ZMPPC1	2219 (-) GTGCCCTT	Recognition site of the MNF1 DNA binding protein is located in the maize phosphoenolpyruvate carboxylase (Ppc1) gene promoter and is involved in light induction (Morishima, 1998).
[redacted]		Binding site for Myb26 at the c-Myb and P-box-like binding sites located in the promoter regions of several phenylpropanoid biosynthetic genes. This site is identical to P-box in maize, and to Myb305 binding site in snapdragon (Uimari and Strommer, 1997).
		The binding site for ATMYB2, an *Arabidopsis* MYB homolog involved in regulation of genes that are responsive to water stress (Urao et al., 1993).
		Binding site for MYB (ATMYB2) in dehydration-responsive gene rd22 of *Arabidopsis* and is involved in induction by ABA (Abe et al., 1997; Busk and Pages, 1998).

	Binding site for at least two plant MYB proteins, ATMYB1 and ATMYB2, from *Arabidopsis* (See Above). Also involved in the regulation of flavonoid biosynthesis in petunia by another MYB protein (MYB.Ph3) (Solano et al., 1995; Urao et al., 1993).
	Consensus plant MYB binding site related to box P in promoters of phenylpropanoid biosynthetic genes such as PAL and CS (Sablowski et al., 1994).
	Core of consensus maize P myb homolog binding site. Maize P gene specifies red pigmentation of the kernel pericarp, cob, and other floral organs by activating a subset of phenylpropanoid biosynthetic genes (Grotewold et al., 1994).
	Core motif of a potato MYB homolog, MybSt1, binding site involved in transcriptional activation (Baranowskij et al., 1994).
	A negative regulatory region in the promoter region of the *Brassica napus* extensin gene, extA. Removal of this element led to expression in all tissues within the stem internode, petiole and root (Elliott and Shirsat, 1998).
	NtBBF1 (Tobacco Dof protein homologue) binding site in the *Agrobacterium rhizogenes* rolB gene and is required for tissue-specific expression and auxin induction (Baumann et al., 1999).
	One of two co-dependent regulatory elements responsible for pollen specific activation of the lat52 gene (a cysteine-rich protein) in tomato. Another element, POLLEN2LELAT52 (TCCACCATA) is required for pollen specific expression (Bate and Twell, 1998).

POLLEN1LELAT52 2190 (-) AGAAA	
	The Pyrimidine box found in the barley cysteine proteinase gene, EPB-1, promoter and is required for GA induction (Cercos et al., 1999).
	Quantitative activator region (QAR) in the promoter of the *Brassica napus* extensin gene, extA (Elliott and Shirsat, 1998).
	A quantitative-element in the pollen-specific maize ZM13 gene promoter involved in expression enhancing activity (Hamilton et al., 1998).
	Ribulose-1,5-bisphosphate carboxylase (rbcS) general promoter consensus sequence (Donald and Cashmore, 1990; Manzara and Gruissem, 1988).
ROOTMOTIFTAPOX1 2098 (-) ATATT	Motif found in the promoters of rolD and the root-specific wheat peroxidase (POX1) (Elmayan and Tepfer, 1995).
	"RY repeat" required for the seed specific expression of the *Brasica napus* napA gene. This element is part of an RY/G box complex containing two RY repeats and a G-box) (Ezcurra et al., 1999).
	Found in the promoter region of the soybean glycinin gene (Gy2) (Lelievre et al., 1992).
	Another "RY repeat" found in seed-storage protein genes in legumes such as soybean (Fujiwara and Beachy, 1994).

	"S1F box" conserved in the spinach RPS1 and RPL21 genes that encode the plastid ribosomal proteins S1 and L21, respectively. Possibly a negative regulator decreasing the promoter activity of both genes (Lagrange et al., 1993; Zhou et al., 1992).
	Motif found in the promoter region of beta-conglycinin (7S globulin) gene of soybean (Allen et al., 1989; Lessard et al., 1991). Element binds SEF3 (soybean embryo factor 3).
	Soybean consensus sequence found in the promoter region of beta-conglycinin gene (Allen et al., 1989; Lessard et al., 1991). Element binds SEF4 (soybean embryo factor 4).1
	This motif is found in the rice alpha-amylase gene (RAmy3D) promoter. This motif and a G motif (TACGTA) are responsible for sugar repression (Toyofuku et al., 1998).

Table 2. Putative cis-acting elements located in PmBiPPro1

Putative cis-acting elements were identified using the PLACE (Higo et al., 1999; Prestridge, 1991). Cis-elements are grouped according to type. Elements deleted from PmBiPPro1-1 to form PmBiPPro1-3 are darkly shaded. Elements deleted from PmBiPPro1-3 to form PmBiPPro1-5 are lightly shaded. Elements remaining in PmBiPPro1-5 construct are not shaded. Y=T/C, R=A/G, W=A/T, M=A/C, N=any base.

Transient expression of PmBiP promoter::GUS fusions in Douglas-fir

To determine if PmBiPPro1 can function as a promoter, reporter gene constructs were made by fusing the full-length PmBiPPro1 (PmBiPPro1-1, 2263 bp) sequence to the GUS reporter gene. Two PmBiPPro1 1000 bp deletion GUS fusions were also constructed (PmBiPPro1-3, 1259 bp and PmBiPPro1-5, 263 bp) to identify a minimal promoter region. Douglas-fir germinating embryos were

chosen as the target tissue for transient expression as the amount of PmBiP mRNA rapidly increased upon germination in whole seeds (Figure 23). Hence, germinating embryos were a likely source of trans-acting factors necessary for the transcriptional activation of the putative PmBiP promoter. The results showed that all three constructs had promoter activity and that the average number of expression foci per embryo was approximately the same (Figure 40).

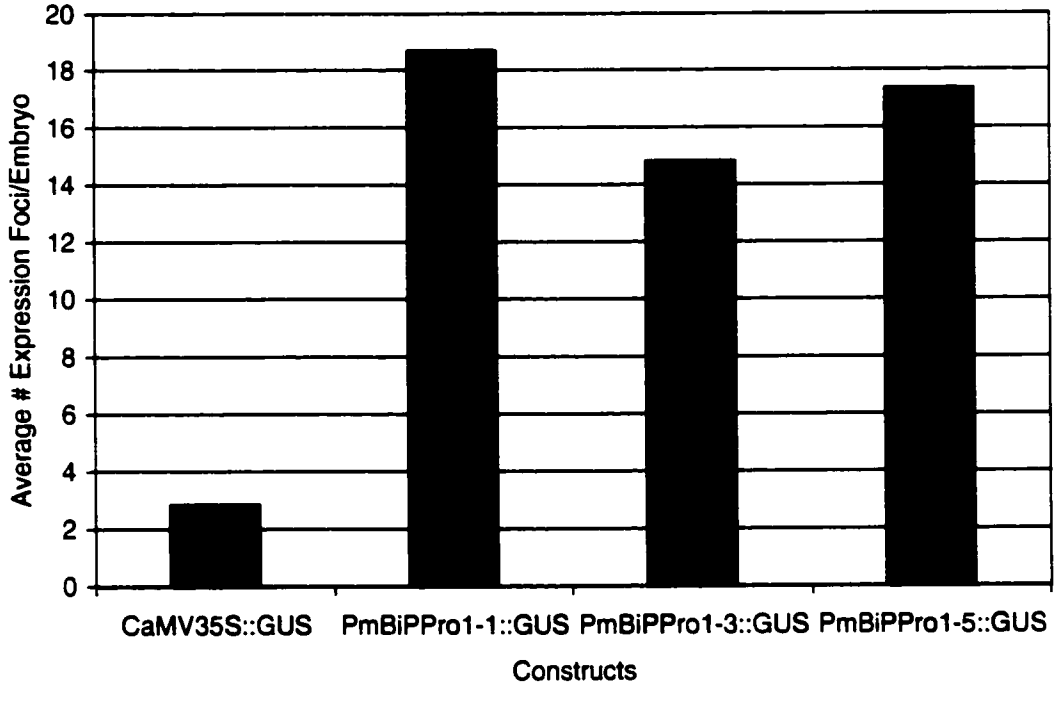

Figure 40. Transient Expression analysis of PmBiPPro1 constructs

Transient expression was conducted as described in Materials and Methods. Ten embryos were bombarded per trial and results are reported as the average number of expression foci per embryo over two trials.

All three PmBiPPro1 constructs showed a greater number of expression foci when compared to the CaMV35S::GUS promoter construct. PmBiPPro1-1 appeared to be the most active construct as most expression foci were darker and relatively larger in diameter than expression foci created by either of the other two constructs. PmBiPPro1-5 produced the smallest and most faint expression foci with PmBiPPro1-3 showing an intermediate expression foci size.

Stable expression of Douglas-fir PmBiP promoter::GUS fusions in *Arabidopsis*

To examine the expression of the PmBiP promoter, *Arabidopsis thaliana* plants were transformed using the floral dip method (Clough and Bent, 1998). *Arabidopsis* was chosen for transformation due to the simplicity and speed of the transformation protocol as well as the fact that *Arabidopsis* is one of the most studied plants. Detailed knowledge exists regarding developmental morphology and molecular responses to stresses such as wounding and cold acclimation (Taiz and Zeiger, 1998; Thomashow, 1998).

Expression of PmBiPPro1 in Arabidopsis seedlings

Following transformation and harvesting of T1 seeds, transgenic seedlings were selected on tissue culture media containing kanamycin. Transgenic seedlings were readily distinguishable following 10-14 days of growth. The transformation efficiency for this protocol was approximately 0.2-0.8%. *In vitro* GUS assays revealed that all PmBiPPro1 constructs were functional in *Arabidopsis* and that plants containing PmBiPPro1-3 constructs had slightly

higher levels of GUS activity than PmBiPPro1-1 but PmBiPPro1-5 seedlings contained significantly lower GUS activity (Figure 41).

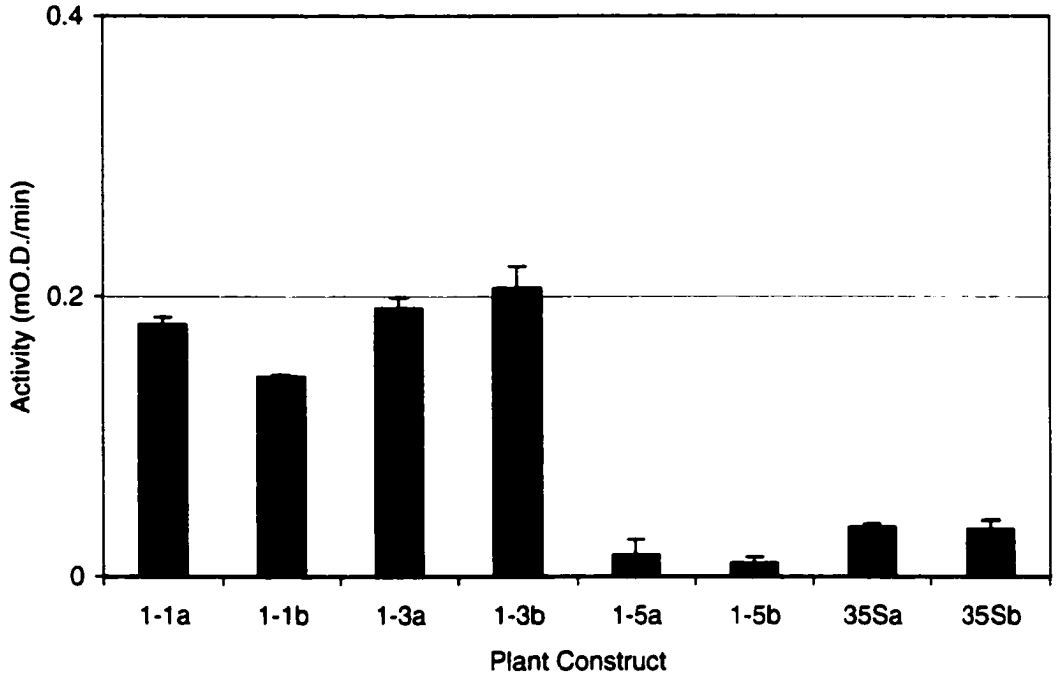

Figure 41. *In vitro* GUS activity in 19-day-old transgenic *Arabidopsis* plants containing various PmBiPPro1 constructs

GUS activity was measured in 6 µg of total protein extracted from whole seedlings as described in Materials and Methods. Two transformants were examined for each construct. Results shown represent the average and standard deviation of three replicates for each plant extract.

Histochemical localization of GUS expression in 2-week-old PmBiPPro1 transgenic *Arabidopsis* seedlings showed strong staining associated with actively dividing/expanding tissues such as the primary root elongation zone (Figure 42G), expanding leaves and leaf primordia (Figure 42A-C). Expression was also associated with the hydathode, a secretory tissue that exudes xylem sap in response to positive root pressure generated in well-watered plants

grown in high humidity (Figure 42J-L). Expression in actively dividing/expanding and secretory tissues was consistently observed in transgenic plants containing each PmBiPPro1 construct. Occasional staining was also observed in the guard cells of the stomata (Figure 42D) or in the core of the transition zone (Figure 42F) for each PmBiPPro1 construct.

Figure 42. Histochemical localization of PmBiPPro1 construct expression in *Arabidopsis* seedlings.

Histochemical staining of 14-day-old seedlings was performed as described in Materials and Methods. Samples include PmBiPPro1-1 (A, D, G, J), PmBiPPro1-3 (B, E, H, K), and PmBiPPro1-5

(C, F, I, L). Expression was examined in shoot apical region (A, B, C), hypocotyl (D, E, F), and roots (G, H, I) and cotyledons (J, K, L).

Deletion analysis of PmBiPPro1 revealed noticeable changes in expression patterns. The effect of the first deletion (PmBiPPro1-3 plants) caused increased expression in all parts of the plant and extended expression to vascular tissue, lateral roots, and root tip (Figure 42B, E, H, K). Further deletion (PmBiPPro1-5 plants) reduced expression in hypocotyl and cotyledons to levels lower than observed in PmBiPPro1-1 plants (Figure 42C, F, I, L) and abolished expression in lateral roots and vascular tissue. PmBiPPro1-5 plants also displayed spurious staining midway between the root collar and root tip (Figure 42I). A short localized area of staining near the vascular tissue was observed in the hypocotyl of a PmBiPPro1-1 plant (Figure 42D). Closer examination revealed this to be a wound site caused by accidental pinching with forceps during transfer of the seedling to fresh media.

Expression in response to wounding

Increased expression of BiP in response to wounding and fungal elicitors has been shown in tobacco and soybean (Jelitto-Van Dooren et al., 1999; Kalinski et al., 1995) and in Douglas-fir (Figure 27). Also, accidental wounding of a PmBiPPro1-1 transgenic *Arabidopsis* plant hypocotyl with forceps suggested that the PmBiP promoter is wound inducible. Hence, it was of interest to determine if the PmBiPPro1 constructs showed wound inducibility in cotyledons of transgenic *Arabidopsis* plants. One cotyledon of 18-day-old transgenic *Arabidopsis* plants was pinched with forceps while the other served as control. Plants were stained histochemically 18 h after wounding and

examined under a stereo dissecting microscope. Results showed dark staining in wounded cotyledons confined to the periphery of the wound site in PmBIPPro1-1 and PmBiPPro1-3 plants (Figure 43A, B). PmBiPPro1-5 plants also showed staining around the wound site but expression levels appeared much less than that of the other two constructs (Figure 43C). Unwounded cotyledons showed no similar staining pattern. These results were consistent for each of three seedlings tested per PmBiPPro1 construct.

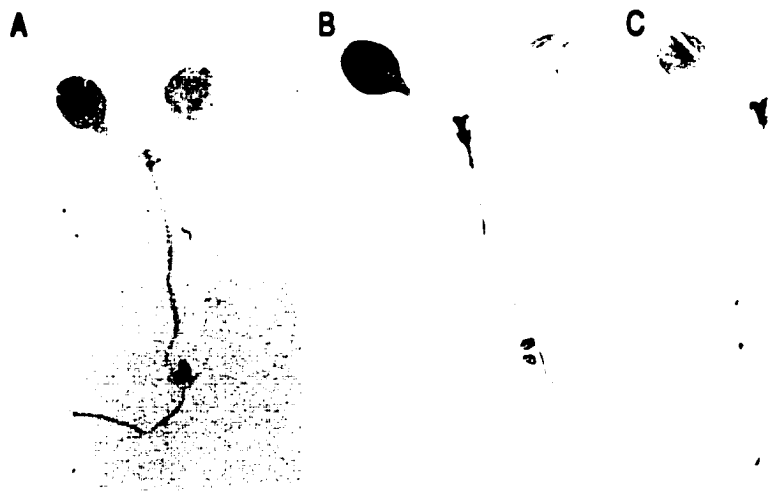

Figure 43. GUS expression in wounded cotyledons of PmBiPPro1 transgenic *Arabidopsis* seedlings

The left cotyledon of PmBiPPro1-1 (A), PmBiPPro1-3 (B), and PmBiPPro1-5 (C) 18-day-old transgenic *Arabidopsis* seedlings was wounded by pinching with forceps. Histochemical GUS staining was performed 18 h after wounding as described in Materials and Methods.

To exclude the possibility that increased staining surrounding the wound site was a result of unequal GUS substrate penetration, *In vitro* GUS assays were conducted on total protein extracts of wounded and control cotyledons of PmBiPPro1-1 plants. The results of an 18 h endpoint reaction using p-

nitrophenyl ß-D-glucuronide as substrate indicated that wounded cotyledons had higher GUS activity than control cotyledons in all three seedlings tested (Figure 44).

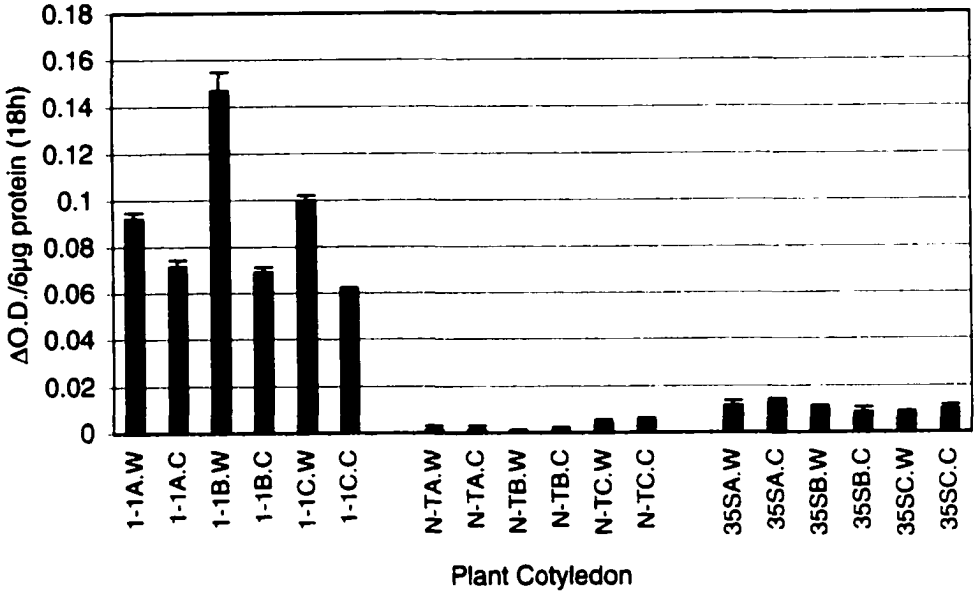

Figure 44. Effect of wounding on *In vitro* GUS activity in *Arabidopsis* cotyledons

GUS activity was measured in wounded (.W) and control (.C) cotyledons from each of three (A-C) 21-day-old PmBiPPro1-1 (1-1), non-transformed (N-T), and CaMV35S (35S) *Arabidopsis* plants. GUS activity was measured using 6 µg of total protein extracted 18 h after wounding (pinching with forceps). GUS activity was measured as a change in absorbance after 18 h. Results represent the average and standard deviation of three trials for each cotyledon extract.

CHAPTER 4: DISCUSSION

This research describes the cDNA cloning, characterization and expression of BiP in developing Douglas-fir seeds and seedlings, regulation in response to various treatments, the association of PmBiP with other proteins, and the isolation of the PmBiP promoter and its expression in *Arabidopsis*. To date, PmBiP is the only reported BiP sequence from a gymnosperm.

PmBiP is encoded by a single gene but other alleles exist

Restriction digestion and Southern hybridization showed that PmBiP is encoded by a single gene. This result is consistent with the observation that the PmBiP antiserum detected only a single isoform after 2D SDS-PAGE and western blotting of proteins isolated from various stages of seedling development. The appearance of 2-3 other weakly hybridizing bands following low stringency wash suggests that other PmBiP family members with lower sequence similarity to PmBiP may exist in the Douglas-fir genome. It is most likely that these weakly hybridizing bands represent other HSP70 family members as the full length PmBiP cDNA was used as a probe. Screening of the cDNA library also produced 3 other cDNAs containing a full-length open reading frame. The cDNA library was made with a population of individuals from an open pollinated seed lot, and the isolation of other cDNAs nearly identical to the sequence reported here suggests that multiple BiP alleles exist in Douglas-fir. The majority of differences were located in the 5' and 3' untranslated regions and those in the coding region were always in the 3rd base of the codon causing no change in the amino acid sequence (Table 1). BiP is

encoded by a multigene family in tobacco (Denecke et al., 1991), soybean (Figueiredo et al., 1997; Kalinski et al., 1995), *Arabidopsis* (Koizumi and Sano, 1997), and maize (Wrobel et al., 1997) whereas the spinach BiP homologue is encoded by a single gene (Anderson et al., 1994a).

PmBiP deduced amino acid sequence is highly conserved

The deduced amino acid sequence of PmBiP is similar to other BiPs characterized but is most similar to angiosperm BiPs suggesting conserved functions between these two plant groups. This is particularly evident in the conservation of sites involved in ATP binding and in regions involved in binding and release of peptide substrates. Significant sequence divergence is evident among the N-terminal signal sequences where there is little conservation of sequence length or identity. Such low sequence similarity is commonly observed in signal peptides from a variety of organisms (von Heijne, 1985).

Conifers arose approximately 300 million years ago during the Carboniferous period, whereas the angiosperms arose approximately 140 million years ago during the Lower Cretaceous period. The distinct phylogenetic grouping of PmBiP separate from the angiosperm BiP sequences shows that angiosperm BiPs have significantly diverged from their gymnosperm ancestors despite major areas of sequence conservation. Moreover, the phylogenetic analysis of BiP amino acid sequences showed a separation of the monocots and dicots within the angiosperms suggesting that BiP may be a useful phylogenetic marker.

The high sequence similarity and conservation of ATP and peptide binding sites with other BiPs together with the subcellular localization suggests that PmBiP is the Douglas-fir BiP homologue. This identification is further confirmed by the ability of PmBiP to bind to denatured gelatin in an ATP dependant manner.

3-D models show the relative positions of highly conserved residues

Tc visualize the relative position of conserved residuess, 3-D models of PmBiP ATP and peptide binding domains were constructed using the comparative modeling approach (Guex and Peitsch, 1997; Peitsch, 1996). This approach provides a reliable, inexpensive, and easily accessible means to model proteins for which a suitable experimentally determined template exists (>30% amino acid sequence identity). Models derived from templates sharing less than 70% are considered useful for the design of site-directed mutagenesis experiments and templates sharing greater than 70% identity are likely useful for the optimization of drug specificity with closely related enzyme variants (Guex and Peitsch, 1997). The PmBiP ATP and peptide binding domains share 70 % and 40 % sequence identity respectively with their respective templates. Thus, the models of the PmBiP ATP and peptide binding domains are deemed sufficiently accurate to visualize the relative position of highly conserved residues within their respective domains.

The 3-D models of PmBiP showed that the relative positions of highly conserved sites, such as those involved in ATP binding or forming hydrogen bond contacts with peptide substrates, are conserved with their model templates. The presence of a calmodulin binding site in the N-terminal ATP

binding domain is interesting since calmodulin has not been found in the ER. This site has been conserved in plant and animal BiP amino acid sequences (data not shown). The position of this site in the tertiary structure shows that it is surface exposed and may interact with ER proteins containing calcium binding EF-hand motifs, such as those of the CREC protein family (Honore and Vorum, 2000).

PmBiP contains a novel ER retention signal sequence

The PmBiP cDNA C-terminal tetrapeptide HEEL is different from the commonly found HDEL/KDEL of mammalian, yeast and plant BiP sequences. Other variations of the tetrapeptide sequence have been reported. *Trypanosoma brucei* BiP contains MDDL (Bangs et al., 1993) and *Echinococcus multilocularis* contains KEEL at its carboxy terminus (GENBANK Accession No. M63604). The enrichment of PmBiP in the soluble microsomal fraction suggests HEEL functions as an ER retention signal in the Douglas-fir endomembrane system. Further, sucrose gradient fractionation showed that PmBiP was enriched in fractions consistent with the density of ER in other plant species and immunolocalization showed a distinct reticular staining pattern consistent with ER localization. PmBiP is also localized to the nuclear envelope which is continuous with the ER. To determine if HEEL is present in other conifer ER proteins, a search of conifer EST databases was conducted but failed to locate sequences containing C-terminal amino acids of ER proteins.

Immunolocalization provides further insight to PmBiP function

Both immunolocalization and subcellular fractionation has confirmed that PmBiP is localized to the ER and nuclear envelope. At the onset of mitosis, intense staining became associated with the nuclear envelope that underwent significant rearrangement as cell division proceeded. During eukaryotic cell division the nuclear envelope and RER undergo fragmentation at prometaphase following chromatin condensation (Warren, 1993). The nuclear envelope is released from the nuclear lamina and forms large fragments surrounding the chromatin. Fragmentation continues with some fragments aligning with the mitotic spindle while others move to the spindle poles. The RER loses its ribosomes and fragments into flattened cisternal membranes continuous with the nuclear envelope outer membrane and tubulo-reticular structures near the periphery of the cell. As cell division continues the RER forms smaller vesicles and reassembly of the RER and nuclear envelope occur during telophase when the new cell wall is deposited between the daughter cells. The staining pattern observed in Douglas-fir root tip cells during mitosis is consistent with that described above and may reflect an important role for PmBiP during nuclear envelope and ER membrane fragmentation/reformation. The study of yeast karyogamy has provided an important insight into the role of BiP in membrane fusion.

Karyogamy is the fusion of two haploid yeast nuclei during mating in *Saccharomyces cerevisiae* and is genetically separable into a nuclear congression phase and membrane fusion phase (Kurihara et al., 1994; Latterich and Schekman, 1994). Mutation in the yeast BiP gene KAR2 results in mating cells being defective in the nuclear fusion step of karyogamy and inhibits homotypic fusion of ER membranes (Latterich and Schekman, 1994; Ng and Walter, 1996).

The integral membrane proteins Sec63p, Sec71p, and Sec72p are also involved in the membrane fusion step of karyogamy (Ng and Walter, 1996). Sec63p, Sec71p, and Sec72p form part of a multi-subunit complex that mediates protein translocation across the ER membrane in yeast and provide a topological link between BiP in the ER/nuclear envelope lumen and the cytoplasmic face where initial fusion events occur. Sec63p likely mediates this interaction as it has been shown to recruit BiP to the translocon pore and stimulate BiP ATPase activity via its DnaJ domain (Corsi and Schekman, 1997; Misselwitz et al., 1999). The action of BiP in membrane fusion may also be mediated through another ER DnaJ homologue, JEM1p, as mutants are similarly defective in the membrane fusion step of karyogamy (Nishikawa and Endo, 1997).

BiP genes in eukaryotic cells are highly conserved and it is likely that BiP in plants and other organisms perform a similar function during fragmentation/fusion of nuclear and ER membranes. Further research is needed to clarify the role of PmBiP during cell division in Douglas-fir and determine if such a role is conserved for BiP in higher plants.

PmBiP is developmentally and seasonally regulated

The deposition of seed storage proteins and other storage reserves in developing Douglas-fir seeds begins shortly after fertilization, during embryogenesis, and decreases as seeds mature (Misra, 1994; Owens et al., 1993). PmBiP mRNA and protein levels were highest in early seed development stages prior to embryo formation and the deposition of seed storage proteins. This result was unexpected as BiP is known to assist in the folding and processing of newly synthesized polypeptides and has been implicated in the

folding and assembly of plant storage proteins (D'Amico et al., 1992; Li et al., 1993; Pedrazzini et al., 1994). Similar amounts of PmBiP mRNA were observed in both the MG and embryo during embryogenesis when both tissues synthesize and accumulate storage proteins. Further research is needed to elucidate the molecular events underlying the relatively high expression observed during early seed development (pre-embryogenesis) and to examine the role of PmBiP in the deposition of legumin-like and 2S albumin seed storage proteins in Douglas-fir seeds (Chatthai and Misra, 1998; Leal and Misra, 1993b; Leal and Misra, 1993c).

Douglas-fir seeds require a cool, moist 3-5 week stratification treatment to break dormancy and obtain a reasonably high level of germination. Stratification initiates the synthesis of new mRNA and protein necessary for germination and seedling growth (Taylor and Davies, 1995; Taylor et al., 1993). Following stratification PmBiP mRNA amounts began to increase and accumulated to high levels during germination and early seedling growth. BiP has been shown to assist in the cotranslational translocation and folding of newly synthesized polypeptides through the ER membrane and has been suggested to be involved in the deposition of new cell wall proteins in actively dividing plant cells (Denecke et al., 1991; Hendershot, 1990; Knittler and Haas, 1992; Nguyen et al., 1991; Vogel et al., 1990). The increased PmBiP synthesis in Douglas-fir seedlings is likely due to increased levels of protein traffic through the ER necessary for the formation of new cell walls. Immunolocalization in Douglas-fir root tip cells using the PmBiP antiserum supports this idea as increased staining was observed in telophase cells near the newly forming cell wall.

Increasing amounts of PmBiP transcript in developing seedlings correlated with increased growth and storage protein mobilization in 4-day and 14-day old seedlings of different sizes. Higher transcript amounts in 4-day seedlings of various sizes versus 14-day seedlings of various sizes may be due to genotypic differences in germinability between the seed which is under strong maternal control in Douglas-fir (El-Kassaby et al., 1992). Lower germinability in 14-day seeds correlated with reduced amounts of PmBiP transcript and reduced storage protein mobilization. The molecular basis of this genetic predisposition may be directly linked biochemical pathways controlling the expression of PmBiP.

Seasonal variation of PmBiP protein in the needles of 1-yr-old Douglas-fir seedlings suggests PmBiP plays a role in the adaptation of seedlings to low temperature. Elevated amounts of PmBiP correlated with decreased monthly average temperatures. Increased PmBiP mRNA and protein amounts in cold treated seedlings and ESM culture further suggests control of PmBiP expression by low temperature. Whether this trend is the result of increased synthesis or increased stability of PmBiP mRNA and protein at low temperature is unknown. BiP is up-regulated by cold treatment in spinach and has been shown to have an increased association with non-native proteins upon exposure of plants to low, non-freezing temperatures (Anderson et al., 1994a; Guy et al., 1998). Exposure of plants to low temperature has been shown to stimulate the production of extracellular proteins believed to be necessary for over-wintering (Antikainen and Griffith, 1997; Griffith et al., 1992; Griffith et al., 1997; van Cleve et al., 1991). Increased synthesis of such proteins and an accumulation of misfolded intermediates may lead to an increase in the

production of BiP to maintain normal protein traffic through the secretory pathway and allow plants to adapt to low temperatures. Further work is necessary to clarify what role, if any, PmBiP plays in the adaptation of Douglas-fir seedlings to seasonal variations in temperature.

PmBiP expression is modulated by different treatments

The expression of PmBiP is modulated both positively and negatively by various treatments in 14-day-old seedlings. The reduction in PmBiP mRNA amounts in response to desiccation is similar to that observed in spinach (Anderson et al., 1994a) but different to that observed in soybean. BiP protein amounts increased in soybean leaves and cell cultures exposed to desiccation conditions (Figueiredo et al., 1997). The plant hormone ABA is known to be an important signaling molecule in the conveyance of dehydration stress (Busk and Pages, 1998). Plants exposed to dehydration stress synthesize ABA in the root that is then translocated to other regions of the plant to signal the closing of stomata and induction of dehydration response genes. During seed development, the level of ABA increases and is known to affect the expression of many genes during mid-late embryogenesis (Misra, 1994). ABA is also a potent inhibitor of germination. The effect of ABA on PmBiP expression appears to be inductive after a 24 h treatment but repressive after 48 and 96 h suggesting the existence of a feedback regulatory mechanism(s). The initial effect of ABA may be to prime the cell for subsequent dehydrative stress requiring changes in protein synthesis and ER physiology; following this adaptive change, expression is curtailed. The differential response to ABA and NaCl following 24 h of treatment suggests that ABA does not mediate the

repressive effect caused by salt stress during the initial stages of the response. Whether desiccation or ABA influences PmBiP gene expression during seed development is unknown.

PmBiP mRNA and protein amounts were relatively unaffected by heat shock (3h, 40°C). Contrary to this, transcripts of the Douglas-fir LMW HSPs PM18.2 A and PM18.3B were induced in response to the same heat treatment in 4-day-old seedlings (Kaukinen et al., 1996). Spinach BiP mRNA amounts were greatly reduced in leaves following a 2 h, 37°C treatment (Anderson et al., 1994a) but *Arabidopsis* BiP mRNA increases in response to heat treatment (37°C) within 30-60 min (Koizumi, 1996). BiP protein amounts increased following heat shock treatment (1h, 37°C) in soybean cotyledon cell cultures (Figueiredo et al., 1997) whereas no change occurred in barley aleurone layers treated with 40°C for 3 h (Jones and Bush, 1991). Variation in heat shock response between species may be due to differences in the length of heat treatment, the temperature used or the fact that some studies only examined either transcript or protein amounts. It is also possible that each species has evolved a different response pathway to heat stress that may or may not include BiP induction.

PmBiP associates with other proteins *In vivo*

To identify potential substrate proteins of PmBiP, CoIp was conducted on various Douglas-fir tissues. Unfortunately, substrate proteins associated with PmBiP in developing Douglas-fir seedlings could not be identified to lend support to the idea that increased PmBiP synthesis results from increased secretory protein traffic. ESM culture proved useful system for this analysis due to the rapid growth of culture and relative tissue simplicity. ESM Proteins

were associated with PmBiP in an ATP dependent manner but present in very small amounts and detectable only by silver staining. Although PmBiP mRNA and protein increased in response to cold and tunicamycin, the type and quantity of proteins associated with PmBiP did not change. The identity of associated proteins remains unknown as the minute quantities isolated precluded N-terminal sequencing via Edman degradation. Identification of silver stained proteins in polyacrylamide gels using mass spectrometry is becoming widely used and may provide a possible alternative for the identification of these proteins (Shevchenko et al., 1996a; Shevchenko et al., 1996b; Shevchenko et al., 1996c). BiP is known to form ATP sensitive complexes with calreticulin in tobacco (Crofts et al., 1998; Denecke et al., 1995). However, the apparent molecular mass of the associated proteins did not resemble that of calreticulin in Douglas-fir (see Figure 13). It remains possible that the proteins detected in untreated culture do not represent substrate proteins.

Studies that examined the association of BiP with proteins in plants and mammalian cells employed radioactivity for detection, even when using a single cell type (Guy et al., 1998; Hendershot, 1990; Pedrazzini et al., 1994). Further, the association of BiP with newly synthesized proteins is believed to be transient for most native proteins (Hebert et al., 1995). Substrate proteins that show a more stable association with BiP are mutated in such a way that they are unable to attain a proper folded conformation (Pedrazzini et al., 1997; Pedrazzini et al., 1994). More prominent BiP associations occur in pre-B cells that produce heavy IgG chains but no light chains (Hendershot, 1990). BiP remains associated with the heavy chains until light chains are present to allow

the assembly and secretion of mature antibody molecules. However, radioactive labeling was also necessary to observe such associations.

The PmBiP promoter contains a variety of cis-acting regulatory elements

Since nothing is known about the cis- and trans-acting factors responsible for the transcriptional control of BiP in plants, the promoter region of PmBiP was isolated and characterized. Although the TSS remains to be experimentally confirmed, the 3' distal start site identified by NNPP is favored due to its proximity to the 5' end of PmBiP cDNA sequence and that this region does not contain any overlapping cis-elements.

Scanning of PmBiPPro1 against the PLACE identified a variety of cis-elements that are arbitrarily grouped according to the type of gene they are found in. Group A elements are found in storage protein genes and involved in regulating seed-specific expression during embryogenesis and seed development. The number and variety of elements belonging to this group is significant and suggests involvement in PmBiP transcriptional regulation during seed development. The SEF3, SEF4, and RY repeat cis-elements are located in the promoter region of the α' subunit gene of soybean ß-conglycinin storage protein and have been implicated in controlling seed-specific expression (Allen et al., 1989; Fujiwara and Beachy, 1994; Lessard et al., 1991). The RY element is found in the promoter of other seed storage protein genes and is thought to act as a seed-specific enhancer but represses expression in leaves when fused to a -90 CaMV 35S minimal promoter (Ezcurra et al., 1999; Fujiwara and Beachy, 1994; Lelievre et al., 1992). The $(CA)_n$ element is involved in the transcriptional regulation of the napin storage protein gene of *Brassica*

napus and is functionally similar to the RY repeat as it enhances seed specific transcription and represses expression in other tissues but functions synergistically with an E-box/ABRE-like element (Ellerstrom et al., 1996). Although several E-box elements were identified in PmBiPPro1, one E-box (1799-1804) is spaced 9 bp upstream from a $(CA)_n$ element (1814-1820), similar to the 10 bp spacing in the napin gene and five of the 9 intervening nucleotides showed an identical alignment to the 10 bp spacer (data not shown). Another synergistic element set, two RY elements surrounding a G-box motif, was located approximately 42 bp downstream from the $(CA)_n$ element in the napin gene (Ezcurra et al., 1999). Although both E-box/$(CA)_n$ and G-box/RY/G-box elements were necessary for high-level seed specific expression, each individual set could activate seed specific expression at a much reduced level. Similar spacing between the RY repeats and the G-box was not observed in PmBiPPro1.

Group B elements are found in a variety of genes regulated by light (Terzaghi and Cashmore, 1995). BiP gene expression in plants has not been shown to be directly affected by light but diurnal variations in BiP mRNA amounts in mature soybean leaves have been observed (Kalinski et al., 1995). Both I-box and G-box motifs are required for the light regulated expression of the *Arabidopsis* rbcS-1A gene (Donald and Cashmore, 1990). Although these elements are present in PmBiPPro1, the relative position of each element does not closely resemble that observed in the promoter of the rbcS-1A gene. Other common elements involved in light regulation, such as the GT1 consensus site (Terzaghi and Cashmore, 1995), are also present in PmBiPPro1. The presence

of numerous light regulated cis-elements suggests that light may influence the transcriptional activity of PmBiP and warrants further investigation.

Group C elements are commonly found in the promoters of phenylpropanoid biosynthesis enzymes such as PAL and CHS and interact with the Myb family of transcriptional activators. Plant Myb proteins are related to the mammalian c-Myb and v-Myb transcription factors and contain a DNA binding domain and transcriptional activation domain (Jin and Martin, 1999; Martin and Paz-Ares, 1997). PAL and CHS are major enzymes in the phenylpropanoid pathway and are involved in the synthesis of cell wall precursors and secondary metabolites involved in pathogen defense and UV protection. The maize P gene encodes a plant Myb transcriptional activator that stimulates transcription of several flavonoid biosynthesis genes specifying red pigmentation in corn kernel pericarp and floral organs (Grotewold et al., 1994). The petunia Myb.Ph3 gene is a transcriptional activator of the CHSJ gene in tobacco protoplasts and homologues of this protein may regulate transcription of CHS in other plant species (Solano et al., 1995). Myb26 is another transcriptional activator that recognizes cis-elements present in phenylpropanoid biosynthesis genes of pea (Uimari and Strommer, 1997). Myb305 of snapdragon is very similar to Myb26 in both the transactivation and DNA binding domains and has been shown to bind to the same DNA sequence. Myb305 transcriptionally activates the PAL2 gene but requires a G-box motif located approximately 50 bp downstream for high-level activation (Sablowski et al., 1994). Myb305 has been implicated in the transcriptional activation of other phenlypropanoid genes such as CHS and has been suggested to play a role in the activation of phenylpropanoid genes in response

to UV, wounding and pathogen attack (Sablowski et al., 1994). This suggestion remains to be investigated as do the involvement of other Myb proteins in the transcriptional activation of phenylpropanoid genes in response to UV, wounding and pathogens. Recently discovered Myb genes in *Arabidoipsis* have been shown to be induced by wounding and pathogen attack (Bender and Fink, 1998; Kirik et al., 1998). PmBiP mRNA has been shown to increase in response to UV treatment in young Douglas-fir seedlings (A.M. Schmidt, unpublished results) and increased expression in actively dividing tissues necessary for new cell wall synthesis suggests this group of cis-elements may play a role in PmBiP transcriptional activation.

Since PmBiP Protein amounts showed seasonal variation and PmBiP mRNA increased in response to cold treatment, it is of interest to note the presence of low-temperature-response elements (LTRE) in PmBiPPro1. LTRE are located in the promoter regions of low temperature responsive genes such as *cor15a* (Baker et al., 1994) and barley lipid transferase (Dunn et al., 1998). The C-repeat (CRT) was identified by Baker et al. (1994) as containing a CCGAC core element that conferred cold responsive gene expression to the promoter of the *Arabidopsis cor15a* gene. The CRT was repeated twice and noted in the promoters of other cold responsive genes such as *cor15B* and *cor78* (also known as *rd29A* and *lti78* respectively). The dehydration response element (DRE) also contains a CCGAC core element and was found to activate transcription in response to cold and dehydration from the promoter of the *Arabidopsis RD29A* gene (Yamaguchi-Shinozaki and Shinozaki, 1994). The response to cold or dehydration is mediated through the DRE/CRT via interactions with different transcription factors (Shinozaki and Yamaguchi-Shinozaki, 2000). Cold

signaling is mediated through the CBF1/DREB1 transcription factor and dehydration response is mediated through the DREB2 transcription factor (Liu et al., 1998; Stockinger et al., 1997). The presence of two CCGAC core elements in PmBiPPro1 suggests that low temperature and dehydration may be controlled at the level of transcription by these elements. However, PmBiP mRNA decreased in response to desiccation indicating a different regulatory system may control expression during this stress.

The induction of PmBiP mRNA in response to tunicamycin treatment in ESM culture shows that Douglas-fir contains a UPR pathway. The UPR is an intracellular signaling pathway that leads from the ER to nucleus and increases the transcription of genes encoding ER resident proteins in response to misfolded protein accumulation (Sidrauski et al., 1998). Signaling is mediated through an ER transmembrane serine/threonine kinase, Ern1p/Ire1p (Cox et al., 1993; Mori et al., 1993) and a basic-leucine zipper transcription factor, Ern4p/Hac1p (Cox and Walter, 1996; Mori et al., 1996). The UPRE-like sequence in PmBiPPro1 may be involved in the transcriptional activation of PmBiP by the UPR in response to the accumulation of misfolded proteins in the ER.

Together, the presence of various cis-element groups suggests that the transcriptional activity of PmBiP can be modulated by a variety of signaling pathways allowing precise control of expression levels tailored for each tissue, developmental stage, or environmental stimuli. It is also possible that the transcriptional regulation of PmBiP is controlled by only a distinct number of cis-elements.

The PmBiP promoter is functional in Douglas-fir

A functional analysis of the PmBiP promoter was carried out using the GUS reporter gene system and particle bombardment in transient expression assays in germinating Douglas-fir embryos. Results showed that PmBiPPro1 is functional in Douglas-fir and that DNA sequence in PmBiPPro1-5 is sufficient to initiate transcription of the GUS reporter gene. Important elements controlling the level of transcription were present in upstream flanking sequence as indicated by increasing foci size in embryos bombarded with PmBiPPro1-3 and PmBiPPro1-1.

The PmBiP promoter is functional in *Arabidopsis*

Gene transfer technology has enabled a number of plant genes to be introduced into heterologous hosts to examine qualitative and quantitative aspects of their expression. The demonstration that most genes retain regulated patterns of expression in a foreign host has permitted the dissection of cis-elements involved in tissue-specific and developmental control.

In vitro GUS assays on PmBiPPro1 transgenic *Arabidopsis* showed that PmBiPPro1 was functional in *Arabidopsis* but deletion of ~2000 bp (PmBiPPro1-5) markedly reduced the level of expression. These results are consistent with the transient expression analysis and further demonstrate that PmBiPPro1-5 (263 bp) contains sufficient information to initiate transcription but that upstream cis-elements are necessary for higher level expression.

Histochemical localization of GUS expression in whole seedlings provided further insight into the tissue specificity of PmBiPPro1. Expression was associated with actively dividing/expanding tissues such as leaf primordia,

newly expanding leaves, and the root elongation zone and likely the result of increased secretory traffic during the deposition of new cell wall proteins. Expression was also observed in the hydathode and is possibly due to the secretion of antimicrobial agents as this tissue is a preferred entry point for the plant pathogen *Xanthomonas campestris* (Hugouvieux et al., 1998). These observations correlate with tobacco expression studies showing higher BiP transcript levels in secretory and actively dividing tissues (Denecke et al., 1991). Expression in these tissues is observed in all three PmBiPPro1 constructs suggesting regulatory elements necessary for expression are located in the 263 bp region of PmBiPPro1-5.

Expression analysis of PmBiPPro1 deletion constructs identified two possible cis-elements controlling expression in young seedlings that were identical to elements controlling the expression of the *Brassica napus* extensin gene (Elliott and Shirsat, 1998). Extensin is a hydroxy-proline-rich cell wall protein secreted as a soluble monomer into the extracellular space where it is quickly insolubilized through cross-linking of adjacent monomers by the action of wall bound peroxidases. Extensin is also induced by wounding and aids in the strengthening of cell walls preventing pathogen infiltration. The first deletion resulted in increased expression levels and expanded tissue specificity in PmBiPPro1-3 plants suggesting the deletion of a negative regulatory element. Examination of the deleted nucleotide sequence revealed an element at position 407 identical to the negative regulatory region (NRR) of the extensin promoter (Elliott and Shirsat, 1998). Deletion of the NRR from the extensin promoter led to expression in virtually all parts of transgenic tobacco plants. Further deletion of PmBiPPro1 caused a significant reduction in expression levels as

observed in PmBiPPro1-5 plants and suggested that element(s) necessary for high level expression had been removed. Examination of the deleted region revealed an element at position 1061 identical to the quantitative activator region (QAR) from the extensin gene promoter (Elliott and Shirsat, 1998). Deletion of the QAR from the extensin promoter caused a ~20 fold reduction in expression. Together these results suggest that high level expression in young 14-day-old seedlings is mediated by the QAR while the NRR may restrict expression in some tissues. Expression in PmBiPPro1-5 plants may involve G-box elements as they are found in the promoters of many plant genes and are necessary for regulation by a variety of environmental signals and physiological cues (Menkens et al., 1995; Terzaghi and Cashmore, 1995; Williams et al., 1992). Further research is required to confirm the involvement of these elements in the transcriptional regulation of PmBiPPro1.

The PmBiP promoter is wound inducible

Wounding of young Douglas-fir seedlings resulted in increased PmBiP mRNA amounts and suggested that the PmBiP promoter is wound inducible. This hypothesis was confirmed in transgenic *Arabidopsis* seedlings containing PmBiPPro1::GUS fusion constructs. BiP mRNA levels in tobacco and soybean increase in response to wounding and treatment with fungal cellular degrading enzymes (Jelitto-Van Dooren et al., 1999; Kalinski et al., 1995). In tobacco, BiP mRNA increased locally and systemically in response to treatment of leaves with cellular degrading enzymes (Jelitto-Van Dooren et al., 1999). Increased transcript amounts were observed prior to the increase of a PR gene transcript ß-1,3-glucanase, an antifungal enzyme targeted to the extracellular space

involved in the degradation of fungal cell walls. Systemic increases in BiP and ß-1,3-glucanase mRNA was found to be independent of SA and the UPR. It was postulated that the prior increase in BiP amounts was necessary to sustain an increase in secretory traffic through the ER resulting from PR, and cell wall protein secretion (Jelitto-Van Dooren et al., 1999). Increased staining of cells surrounding the wound site in PmBiPPro1 transgenic *Arabidopsis* cotyledons supports this idea.

Of all constructs, PmBiPPro1-5 showed the weakest response indicating elements conferring minimal wound inducibility are located within the 263 bp fragment. Examination of this region shows the presence of two G-box (CACGTG) motifs at positions 2021 and 2119. The G-box has been shown to be responsible for the wound induced expression of a horseradish peroxidase gene, *prx*C2 (Kawaoka et al., 1994b). Increased expression of horseradish peroxidase in response to wounding has been suggested to aid in secondary cell wall synthesis for protection against pathogen infiltration (Kawaoka et al., 1994a). A G-box like motif has been implicated in mediating the wound response of a *Brassica napus* extensin gene (*extA*)(Elliott and Shirsat, 1998). Results also showed that elements conferring higher wound inducible expression are present in upstream flanking sequence. Examination of the upstream flanking sequence revealed two more G-boxes at positions 1756 and 1791 suggesting that all four elements are necessary to mediate the higher wound inducible expression observed in PmBiPPro1-1 and PmBiPPro1-3 plants. Further work is needed to confirm the identity of cis-elements conferring both minimal and enhanced wound inducible transcriptional activation of PmBiPPro1.

CHAPTER 5: CONCLUSIONS AND FUTURE STUDIES

The isolation of a set of cDNAs differentially expressed during germination and early seedling growth in Douglas-fir (Tranbarger and Misra, 1995) initiated several studies to determine the role of these genes in development (Kaukinen et al., 1996; Tranbarger et al., 2000; Tranbarger and Misra, 1996). The results presented in this dissertation provide further insight into the role of PmBiP in Douglas-fir. BiP genes are highly conserved between angiosperms and gymnosperms indicative of common mechanisms of translocation and folding of newly synthesized polypeptides. Increased growth and development of young seedlings correlated with the mobilization of storage proteins and increased PmBiP expression. Increased PmBiP expression likely facilitates the translocation and folding of newly synthesized proteins necessary for growth and development. Such proteins may include cell wall synthesis and structural proteins as indicated by immunolocalization of PmBiP near regions of cell wall deposition. Unfortunately, CoIp analysis of PmBiP in germinating and developing seedlings was unable to identify potential substrate proteins.

The association of PmBiP expression with actively dividing and secretory tissues in transgenic *Arabidopsis* correlates with the accepted function of PmBiP in the translocation and folding of newly synthesized polypeptides. These results suggest that PmBiPPro1 transgenic *Arabidopsis* represent a good model system to study transcriptional control of the Douglas-fir BiP promoter.

A future direction of the study of the developmentally regulated genes in Douglas-fir is to isolate and characterize cis-elements controlling their expression and elucidating signal transduction pathways invoked during

germination (Tranbarger, 1998). The isolation and characterization of the PmBiP promoter represents the forefront of knowledge toward this end. Although some details of PmBiP transcriptional regulation have emerged, further research is necessary to fully elucidate the cis- and trans-acting factors involved.

Further Characterization of PmBiPPro1

Determination of the transcriptional start site

The NNPP analysis of PmBiPPro1 identified two potential TSSs. Primer extension using the Promega Primer Extension System failed to determine the true TSS in three separate attempts with two different primers. The most probable reason for this failure is inefficient primer annealing resulting from secondary structures in PmBiP mRNA, as the buffers employed in the Promega kit contained no RNA denaturants. An alternative protocol employs 80% formamide in the primer annealing buffer (Sambrook et al., 1989) and has proven useful for determining the TSS of Douglas-fir metallothionein-like and 2S storage protein genes (Chatthai, 1999). S1 nuclease mapping may also prove useful in determining the TSS (Sambrook et al., 1989).

Identification of functional cis-elements

To confirm the role of NRR, QAR, and G-box motifs in the transcriptional control of PmBiPPro1 and to identify other potential functional cis-elements a variety of techniques can be applied. DNA footprinting and gel mobility shift assays can be used to determine if identified cis-elements interact with nuclear proteins isolated from different tissues or developmental stages. It would be of

great interest to compare DNA footprints generated using nuclear extracts from Douglas-fir and *Arabidopsis* isolated from similar stages of development. Such information would prove useful in determining the degree of evolutionary conservation of developmental pathways. Specific elements could then be examined by gain of function analysis where they are fused to a truncated -90 or -45 CaMV 35S promoter and examined for developmental and tissue specific activity. This would be particularly helpful in the identification of wound inducible elements as the CaMV 35S promoter is not wound inducible (Figure 43).

Identification of PmBiPPro1 trans-acting factors

The PmBiP cDNA was isolated from a cDNA library prepared from mRNA isolated from 4-6 day old seedlings. PmBiP mRNA showed a significant increase during this period of seedling development and was most likely due to an increase in the transcriptional activity of the PmBiP promoter. Increased transcriptional activity results from the binding of trans-acting factors to cis-elements contained in the promoter region. Thus, it is likely that trans-acting factors responsible for activation of PmBiP transcription are represented in the cDNA library. To identify transacting factors involved in the transcriptional activation of PmBiP, the cDNA library can be screened as an expression library using radioactively labeled DNA fragments of PmBiPPro1 as probe. This approach has been used to identify trans-acting factors involved in the regulation of a variety of genes (Abe et al., 1997; Baranowskij et al., 1994). Alternatively, application of the modified yeast one-hybrid system could prove

useful as it has successfully isolated a number of trans-acting factors (Kim et al., 1997; Liu et al., 1998; Stockinger et al., 1997).

Expression of PmBiPPro1 constructs in Tobacco, Potato, Poplar, and Douglas-fir

The current results of PmBiP promoter characterization are readily applicable to the design and construction of genetically modified plants. The developmental and tissue-specific expression pattern combined with the wound-inducible nature exhibited by PmBiPPro1 make it a worthy prospect for the expression of heterologous proteins involved in pathogen and pest defense. The associated high level expression in secretory tissues may also provide for the ability to produce large amounts of heterologous proteins in the secretions of some plant species.

A desirable feature of promoters used for transgenics research is that they maintain the desired expression characteristics across several species. Towards this end, PmBiPPro1, transgenic tobacco and potato were created using PmBiPPro1::GUS fusion constructs as described above. Both species are currently utilized routinely in our laboratory for the creation of transgenic plants expressing heterologous proteins under the control of a variety of promoters. Transformation protocols, tissue culture, growth, and testing have been optimized to facilitate rapid analysis of transgenic constructs. To determine if PmBiPPro1 is functional and retains similar expression characteristics in other species, transgenic Tobacco and Potato plants were created containing each PmBiPro1 construct.

Results of this analysis to date show that all three PmBiPPro1 constructs are functional in young plantlets of regenerated transgenic tobacco and potato (Figure 45). At this early stage of analysis, tissues within the young plants are not fully discernable.

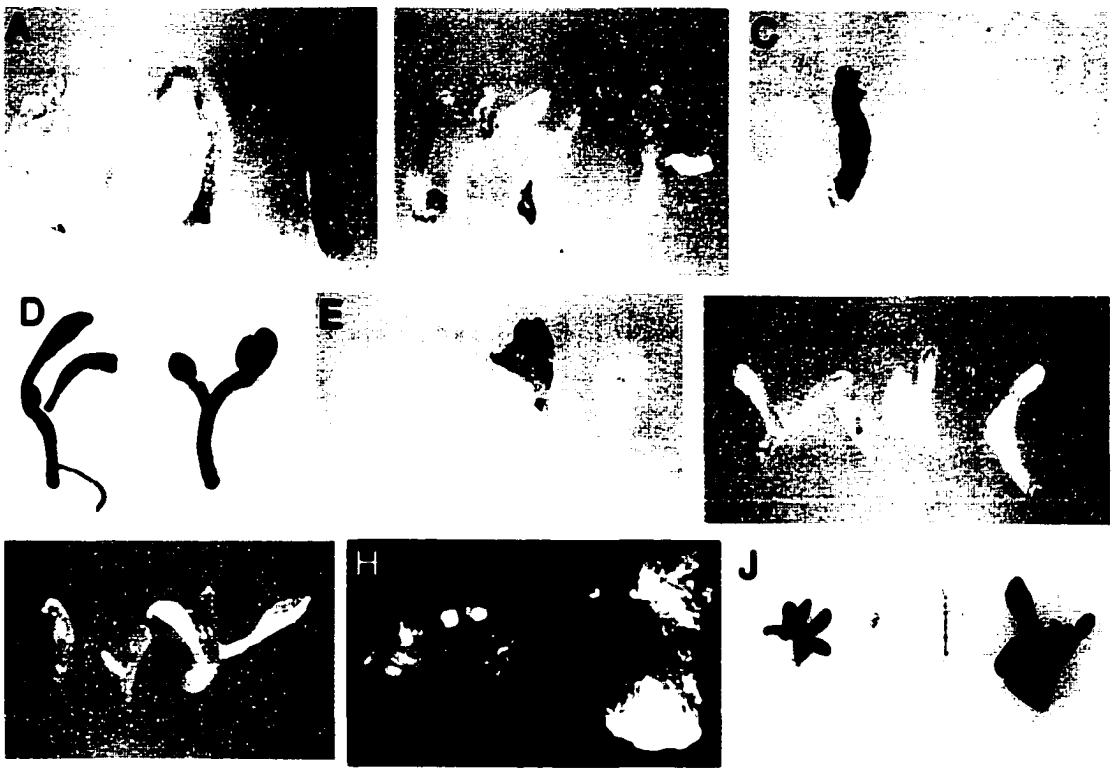

Figure 45. PmBiPPro1 constructs and CaMV35S expression in potato and tobacco.

Histochemical GUS staining of plantlets was conducted as described in Materials and Methods. Samples include potato (A-D) PmBiPPro1-1, PmBiPPro1-3, PmBiPPro1-5, and CaMV35S respectively or tobacco (E-J) PmBiPPro1-1, PmBiPPro1-3, PmBiPPro 1-5 (G and H (callus)), and CaMV35S respectively. Expression was examined in 3-4 independent transformants.

Further analysis is required in mature plants to determine tissue-specific and developmental patterns of expression. Further, plant constructs will also be tested for wound response. Ideally, this analysis should be conducted

following the generation of T2 seeds for statistical considerations as it is important to distinguish between aberrant expression patterns resulting from somaclonal variation or gene silencing from that of the promoter's natural expression pattern.

The creation of PmBiPPro1::GUS transgenic poplar is currently in progress to examine the expression of the Douglas-fir BiP promoter in other economically important tree species. Histochemical localization of GUS expression in leaves of transgenic poplar indicates that PmBiPPro1 is functional (Dmytro Yevtushenko, personal communication). Transformation of conifer species has been limited by the slow and difficult propagation of explant tissues, poor selection efficiency and low transformation frequency. However, stable transformation of somatic embryos and establishment of transgenic plants of several conifer species has recently been achieved (Levee et al., 1997; Wenck et al., 1999). Results of this work may be readily applicable to the creation of PmBiPPro1::GUS transgenic Douglas-fir and other conifer species.

Creation and analysis of transgenic *Arabidopsis* over-expressing PmBiP

Seasonal variation of PmBiP protein in needles of over-wintering Douglas-fir seedlings and increased BiP mRNA in seedlings treated with cold suggested a role for PmBiP in cold acclimation. To determine whether the over-expression of PmBiP can increase the cold hardiness of plants, PmBiP was expressed in *Arabidopsis* under the control of the "Superpromoter" (Ni et al., 1995). The "Superpromoter" is a chimeric construct consisting of a trimeric fusion of the nopaline synthase gene from *Agrobacterium tumefaciens* and is reported to

express heterologous genes at high levels. T_2 generation seeds have been obtained from 18 independent lines (Table 3).

pSBiP #	Stratification (Days)	Growth Chamber (Days)	#Resistant Plants (Green)	#Susceptible Plants (Yellow)	# Chimeric Plants (Green/Yellow)	Segregation Ratio
1	4	14	31	9		3.4 : 1
2	4	14	28	12		2.3 : 1
3	3	14	30	10		3.0 : 1
4	3	14	29	11		2.6 : 1
5	4	14	8	10	21	1.0 : 1.25 : 2.6
6	4	14	28	11		2.5 : 1
7	4	14	26	13		1.5 : 1
8	4	14	30	10		3.0 : 1
9	4	14	35	5		7.0 : 1
10	4	14	33	5		6.6 : 1
11	3	14	25	15		1.7 : 1
12	3	14	0	26	14	0.0 : 1.9 : 1
13	4	14	20	20		1.0 : 1
14	4	14	25	15		1.7 : 1
15	N/A	N/A				
16	4	14	30	10		3.0 : 1
17	N/A	N/A				
18	4	14	0	2	38	0.0 :1 : 19
19	4	14	16	19	5	3.2 : 3.8 :1
20	4	14	28	12		2.3 : 1

Table 3. Segregation ratios of PmBiP transgene in *Arabidopsis* T_2 Plants

Selected T_1 transgenic *Arabidopsis* plants were grown in soil as described in Materials and Methods and allowed to set seed. Collected seeds were grown on selective media and resultant seedlings were scored for kanamycin resistance. Segregation ratios were calculated based on the ratio of tolerant (green) to susceptible (yellow) seedlings. Some T_2 lines also displayed an intermediate (Chimeric) phenotype in which some plants had both green and yellow areas.

Transgenic lines showing a 3:1 ratio indicate the presence of a single transgene loci. These plants can be further selected to obtain homozygous plants containing a single transgene per haploid genome. The next step in this analysis requires the confirmation of PmBiP expression in these plants. This can be

accomplished using SDS-PAGE and western blotting of plant protein extracts as the PmBiP peptide antiserum does not cross-react with the *Arabidopsis* BiP homologue (A.M. Schmidt, personal communication). Positive lines can then be examined for proper ER targeting of PmBiP through western blotting of soluble and microsomal protein fractions. Transgenic lines showing variations in the amount of PmBiP expression can be examined for differences in freezing tolerance using electrolyte leakage tests. Correlation can be made between the amount of PmBiP production and the degree of freezing tolerance.

A role for PmBiP in membrane fusion

To study the role of PmBiP in cell division it may be necessary to develop a population of synchronously dividing cells from which to isolate purified ER and nuclear membranes. It is possible that homotypic fusion of ER and nuclear membranes is restricted to certain periods of the cell cycle. Aphidcolin has been used to establish synchronously dividing populations of tobacco BY-2 cells (Nishimura et al., 1991; Okushima et al., 2000) and wheat HY-1 cells (Minami et al., 2000; Ohtsubo et al., 1993) and may be applicable to the establishment of synchronously dividing ESM culture. A wide variety of chemical agents are available to develop populations of synchronously dividing cells and to arrest cells at particular points in the cell cycle (Planchaisa et al., 2000).

An assay measuring ER membrane fusion has been developed in yeast but relies on membranes derived from glucosidase I mutants for the detection of fusion competence (Latterich and Schekman, 1994). Alternatively, reconstituted ER microsomes could be prepared as described elsewhere

(Hamman et al., 1998; Nicchitta and Blobel, 1993) and separated into two different sets. One set of microsomes could contain the ß-glucuronidase enzyme while the other set contains the enzyme substrate (e.g. 4-MUG). Fusion of membranes could be observed by measuring the change in florescence over time following addition of each microsome set. Further, microsomes can be selectively depleted of PmBiP through immunoprecipitation of luminal contents prior to reconstitution and examined for changes in membrane fusion.

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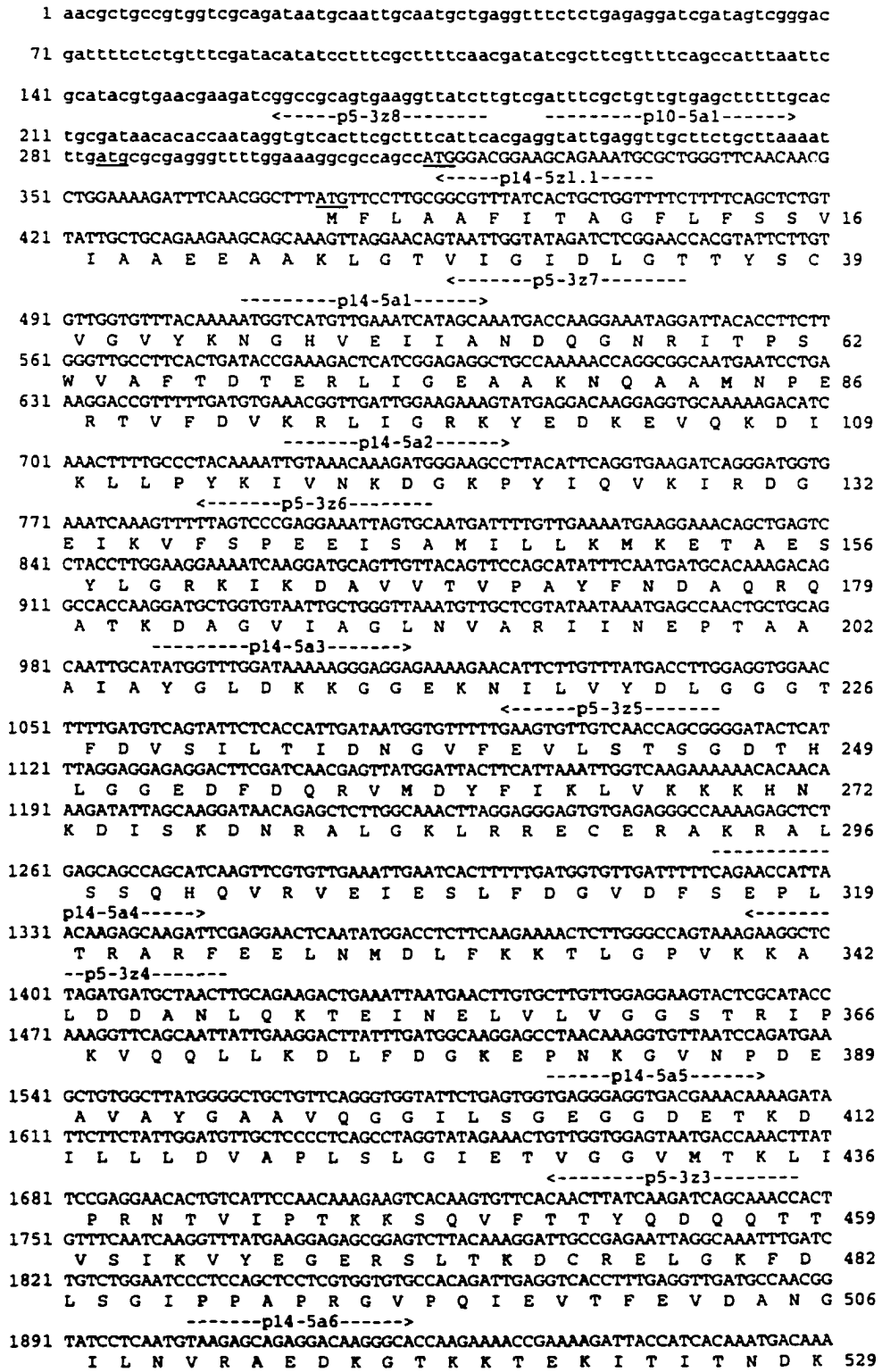
APPENDIX I. Primer Map for PmBiP3 cDNA



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                                                    <-------p5-3z2-------
1961 GGTAGATTGAGCCAGGAAGAAATAGAAAGAATGGTCAAGGAGGCAGAGGAGTTTGCAGAGGAGGATAAGA
      G  R  L  S  Q  E  E  I  E  R  M  V  K  E  A  E  E  F  A  E  E  D  K   552
2031 AAGTGAAGGACAAAATTGATGCGAGGAACAATCTTGAAACATATGTCTACAACATGAAAAGCACCATTAA
     K  V  K  D  K  I  D  A  R  N  N  L  E  T  Y  V  Y  N  M  K  S  T  I  N 576
2101 TGAGAAGGATAAATTGGCAGATAAAATTGATTCCGAAGACAAGGAGAAGATCGAAACTGCTATCAAAGAA
       E  K  D  K  L  A  D  K  I  D  S  E  D  K  E  K  I  E  T  A  I  K  E  599
                    -------p14-5a7----->
2171 GCATTGGAATGGCTTGATGACAACCAGTCGGCTGAGAAGGAGGACTTCGAGGAGAAGTTGAAAGAGGTGG
      A  L  E  W  L  D  D  N  Q  S  A  E  K  E  D  F  E  E  K  L  K  E  V   622
               <--------p5-3z1---------
2241 AAGCTGTATGCAGTCCCATCATCAAGCAAGTATATGAGAAAACTGGAGGAGGATCTTCTGGAGGCGATGA
     E  A  V  C  S  P  I  I  K  Q  V  Y  E  K  T  G  G  G  S  S  G  G  D  D 646
2311 TGAAGACGAGGACTCGCATGAAGAACTCtaagccatttcagtttctgttgaattttagttgtacaaatca
       E  D  E  D  S  H  E  E  L                                             655
                                 -------p14-5a8-------->
2381 cgatgaactaattctacagaagagatctctgagcataatagggtttatgaggatgattggcaacgaacaa
                                   <--------p14-3z1---------
2451 gagattcaactgatgaaagtcaaatgactgtttgttttttctatcaatcagaatgttattttcacagatt
2521 gaaattggcaacgaacaagagattcaactgatgaaagtcaaatgactatttgtttgttttttctatcaat
2591 cagaatgttattttcacagatttttcaatctgtagt
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APPENDIX II. Table of Primers for DNA sequencing of PmBiPPro1

Primers for sequencing are named according to the location and direction of priming. For examples: BP1-3z1 represents PmBiP promoter 1 (BP1), 3 (located at 3'end of PmBiPPro1), z (directed 5', i.e. primes reverse strand), 1 (first primer starting at that end). BP1-5a1 represents PmBiP promoter 1 (BP1), 5 (located at 5'end of PmBiPPro1), a (directed 3', i.e. primes forward strand), and 1 (first primer starting at that end)

Primer DNA sequence (5' to 3')	Primer Name	Length
TTACGAGAGTTCCCTGTAGTCCT	BP1-3z1	23
TCGATTAGAATACCCTCCACAC	BP1-5a1	22
CCATTTCGGCGTAATAGATG	BP1-3z2	20
CCTCCACCCCAAAAATAAAC	BP1-5a2	20
TCAGATAACGAGGAAATACATAAG	BP1-5a3	24
GGACCCATCCTTTTGTTCAG	BP1-3z3	20
ATTGGAATGTTTTAGGTGCC	BP1-3z4	20
CACCTCAAGTTTTCAATAATAATG	BP1-5a4	24