IDENTIFICATION, ISOLATION, EXPRESSION ANALYSIS AND MOLECULAR CHARACTERIZATION OF NINE GENES KEY TO LATE EMBRYOGENESIS IN LOBLOLLY PINE

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IDENTIFICATION, ISOLATION, EXPRESSION ANALYSIS AND MOLECULAR CHARACTERIZATION OF NINE GENES KEY TO LATE EMBRYOGENESIS IN LOBLOLLY PINE

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To my husband, Shawn, my mom and my children Natasha, Sydney and Branden. I give my deepest expression of love and appreciation to all of you for the encouragement you have given and the sacrifices you have made during this graduate program.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABA	Abscisic Acid
ABI3	ABSCISIC ACID INSENSITIVE 3
ABI4	ABSCISIC ACID INSENSITIVE 4
ABI5	ABSCISIC ACID INSENSITIVE 5
AGPs	Arabinogalactans
AP2	APETELA2
BDL	BODENLOS
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
bZIP	Leucine Zipper
DPBF	Dc3-Promoter Binding Factor
EM	Embryonic Mass
ESM	Embryo Suspensor Mass
FMg	Female Megagametophyte
FUS3	FUSCA 3
GA	Gibberellins
HBT	HOBBIT
Kilobase	Kb
LEA	Late Embryo Abundant
LEC1	LEAFY COTYLEDON 1

LEC2	LEAFY COTYLEDON 2
MP	MONOPTEROS
NCBI	National Center for Biotechnology Information
NUP	Nested Universal Primer
ORF	Open Reading Frame
PGHs	Plant Growth Hormones
QC	Quiescent Center
RACE	Rapid Amplification of cDNA Ends
RAM	Root Apical Meristem
RM	Root Meristem
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAM	Shoot Apical Meristem
SCR	SCARECROW
SE	Somatic Embryogenesis
SHR	SHORT ROOT
UPM	Universal Primer Mix
VP1	VIVIPAROUS 1
WOL	WOODENLEG

SUMMARY

Although somatic embryogenesis in pine is a promising technique that will allow for the mass production of high quality trees, it is not without its issues. The success of somatic embryogenesis is dependent on the embryo genotype. Even the best performing genotypes fail to develop past stage 9.1 and the quality of embryos can be poor [1]. A primary roadblock for somatic embryogenesis is root formation and germination. Many conifers produce somatic embryos that appear to be capable of germination and plant establishment, but do not fully mature. Pullman et al have demonstrated that early somatic embryo development (stages 1 – 6) occur normally. Most somatic embryos are able to develop to stages 7 -8, with only a small percentage being able to develop to stage 9.1 [1]. The work described in this thesis is based upon the theory that a basic understanding of the molecular events occurring during zygotic embryogenesis is required to fully understand how and why only a very small percentage of somatic embryos develop past the late embryogeny phase of embryogenesis [1]. The strategy of this work was to identify pine orthologs of genes essential to embryogenesis in angiosperms. We first identified genes that have been demonstrated to be required for late embryonic development in the model plant system *Arabidopsis thaliana*.

We identified pine orthologs of three transcription factors known to mediate ABA responses in *Arabidopsis thaliana*. Three putative ABA responsive genes were identified in the Pine EST databases established from mature plants. These genes were subsequently isolated and cloned from Loblolly pine embryos. These isolated clones were sequenced and analyzed to reveal significant homology to the known *Arabidopsis*

ABA responsive genes ABI3, ABI4, and ABI5. . Expression analyses of all three genes were completed throughout mid to late embryogenesis in Loblolly pine. These expression analyses were compared to reported data of ABA accumulation, as well as, expression of other ABA responsive genes during the same stages of embryogenesis.

Six putative root development genes were identified in the Pine EST databases established from mature plants. These genes were subsequently isolated and cloned from Loblolly pine embryos. These isolated clones were sequenced and analyzed to reveal significant homology to the known *Arabidopsis* root development genes WOODENLEG, SHORT ROOT, SCARECROW, HOBBIT, BODENLOS, and MONOPTEROS. Full-length cDNAs were isolated and cloned for WOODENLEG, SHORT ROOT, SCARECROW and BODENLOS. Sequence analysis was completed for all isolated clones. Putative conserved domains were found within the deduced amino acid sequences. Expression analyses of all six genes were completed throughout mid to late embryogenesis in Loblolly pine.

Finally, we have isolated novel SHR isoforms in loblolly pine that are each missing essential motifs within the GRAS domain, the only functional recognized domain within the protein. These mRNAs are differentially expressed and are translated into protein. This study illustrates differences between angiosperm and gymnosperm gene expression patterns.

Chapter 1

INTRODUCTION

Forests are Essential to the World Economy

Forests are vital to the world, and are used for a variety of purposes: shelter, timber for construction, raw material for paper and pulp production, anti-cancer drugs, and more recently, as an energy source [2-5]. They are also indispensable to the world economy and human communities everywhere. Furthermore, forests play a crucial role in protection of the environment. A single tree can absorb more than 10 pounds of carbon dioxide each year. In the United States, forests and forest products store enough carbon each year to offset approximately 10% of the nation's carbon dioxide emissions. Two-thirds of the drinking water in the United States comes from forests [6].

One–third of the United States is forested and sixty percent of this forestland is privately owned. These privately owned forests provide 91% of the wood harvested in the United States. There is a declining amount of natural forests nationwide due to the pressures of human development and the increasing demand for wood and wood products [7]. More than 80% of the world's forests are currently affected by human activity. The demand for energy used for synthesis of fuels and bioresources is expected to increase by more than 50% by the year 2025 [5]. If consumption continues at its current rate, there will soon be a shortage of wood and natural forests.

Traditionally, it has been cheaper to harvest trees from the wild than to plant for harvest. This is done by clear-cutting forests with little regard for both environmental and

economical consequences [2]. Development of alternative methods of producing trees for the wood industry is critical. The primary solution has been to grow trees in a systematic and comprehensive manner [7].

Tree plantations allow the growth of fast growing elite genotypes. In the past, these plantations have somewhat reduced the pressures on wild forests. Although clonal plantations have offered increased productivity, they supply only 12% of the world's wood consumption. However, most of the trees used for commercial forestry are wild and tend to come from simple seed collections [4, 8].

There are many existing problems with clonal forestry. Large areas of land are required to be dedicated to a single crop, trees. The crop then only realizes its value every few decades, and can be lost to a variety of natural catastrophes: storms, diseases, and fire. In addition, the scale of planting is highly inadequate. Forest trees have longer generation times, self-incompatibility mechanisms require a large amount of space. Moreover, forest research and tree improvement schemes are time consuming and expensive, and are poorly funded even in comparison to other fields of plant research. These attributions make trees more difficult to work with than other plants [2, 3, 8].

There is a need to overcome the current limitations, and to accelerate tree-breeding programs. Clonal propagation of superior genotypes is a promising method that can be used to overcome some of the current limitations, and to accelerate tree-breeding programs [9]. The advantages of clonal propagation include:

- Dependable production of the same genotypes
- Capture of larger genetic gains than possible with traditional tree breeding techniques

- The ability to rapidly deploy new genotypes in response to changes in environmental conditions and breeding goals
- The ability to manage genetic diversity and genetic gain in plantation forestry

In the past, clonal propagation and clonal forestry has been practiced with some hardwood species such as poplar [10]. Using clonal propagation to produce a collection of desirable physical and chemical traits should result in an at least a doubling of biomass production [5]. Targets for manipulation include [5] (Figure 1.1):

- Increased photosynthetic rates and efficiency
- Pest and disease resistance
- Drought and cold tolerance
- Floral sterility
- Regulated dormancy
- Delayed leaf senescence
- Greater carbon allocation to stem diameter vs. height growth
- Less extensive root system to maximize aboveground biomass
- Increased nitrogen acquisition
- Increased and more readily processable cellulose

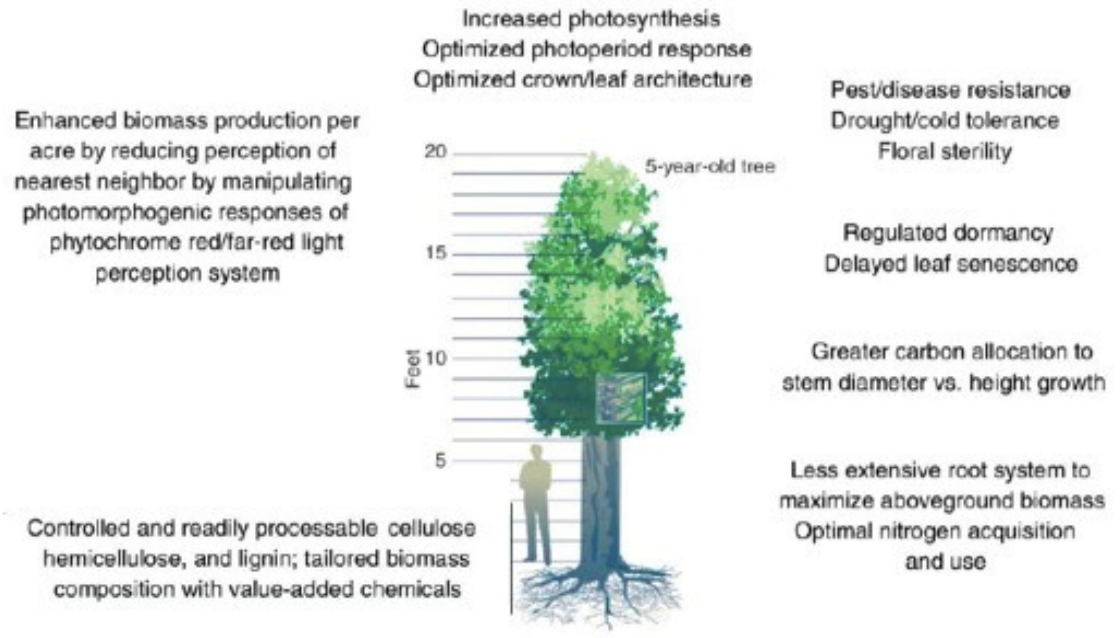


Figure 1.1 Overview of traits that can lead to a doubling of forestry biomass produced [5].

Loblolly Pine is a Major Economic Crop in the United States

The primary organism used in this study is the Loblolly pine (*Pinus taeda L.*). Pines are extremely versatile and can be used to produce wood, fiber and energy [5, 11]. Pine trees are one of the most valuable of all commercial trees because they can be grown across a wide range of soil types and geographies [11]. Pine is the second most important agricultural crop in US after corn and is grown from New Jersey to Texas (Figure 1.2). A number of pines are native to the United States; however, Loblolly pine is most

commonly used for commercial purposes [12]. Loblolly pine is one of the major timber species in the US with 1.5 billion seedlings planted each year. It provides 1.7 million jobs with the annual payroll of $51 billion. [11].

Pines also host a varied wildlife, including bobwhite quail, white tail deer, wild turkey and squirrels. The pine seeds provide food, and the trees provide shelter to local wildlife [3, 11]. This also occurs in commercial plantations where the trees will eventually be harvested. Moreover, lands managed as tree plantation farms provide a more diverse habitat than traditional agricultural crops [12].

Wood from pine has been traditionally used for pulp, paper, furniture, plywood, boxes and pallets. Pines can also provide water quality improvement, land reclamation and rehabilitation, agro forestry, aesthetics, recreational opportunities, places for wildlife as well as food for the cattle [11, 13]. Recently, the use of pine as a source of biomass for biomaterial and bioenergy production has also being investigated [5]. Pine is also able to grow rapidly in a variety of conditions and has a relatively short rotation age of 25 years. Pine predominates in the southeastern United States on 45 percent of available commercial land. It is for over 110, 000 jobs and $30 billion in per year in revenues [11]. There are also about 400 million loblolly pine seedlings planted each year across Asia, Africa and South America [11].

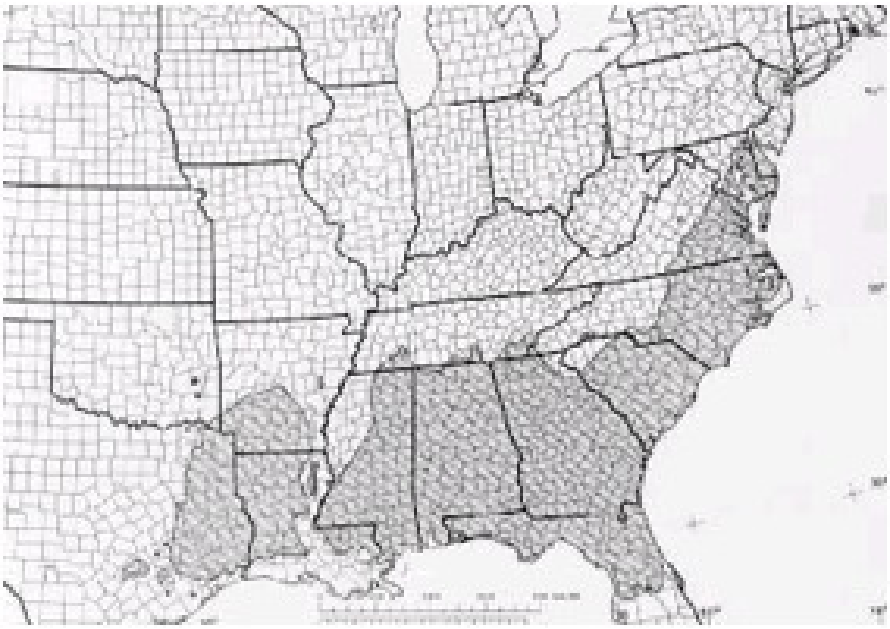


Figure 1.2 Loblolly pine grows over a wide geographic range [11].
Loblolly pine grows in the shaded region in the map shown above. It is the major timber species in the United States and grows from southern New Jersey to central Florida and eastern Texas.

Embryogenesis in Plants

This review highlights the principle features of embryogenesis in plants. It then reviews more specifically embryogenesis in loblolly pine. It establishes a basis for our decision to study gene expression during late embryogenesis in Loblolly pine.

Embryogenesis is the period of development and cellular differentiation of the zygote after the fusion of gametes. A diversity of body plans and physiology are produced during embryogenesis. Plant embryogenesis is composed of two major phases: a morphogenic phase and a maturation phase. In the morphogenic phase the overall body plan is established. The body is divided into 3 primary tissues: outer protoderm, inner

ground tissue, and central procambium. The maturation phase involves accumulation of storage proteins and acquisition of tolerance to desiccation. A simple mature embryo displays an apical-basal axis of polarity and a radial pattern of concentric layers perpendicular to the apical basal axis. The primary shoot meristem is present at the top end of the axis of polarity and the primary root meristem is at the bottom [14].

Differences in embryogeny among plants have been used to divide plants into 2 groups: angiosperms and gymnosperms. The seeds of angiosperms are enclosed within an ovule. In angiosperms, fertilization occurs as a double fertilization event; one haploid sperm cell joins with the haploid egg cell to form the zygote. A separate sperm cell joins with a diploid cell to form a progenitor cell of triploid endosperm [14].

On the other hand, gymnosperms have naked seeds that develop outside the ovary and only require a single fertilization to form the zygote. The zygote will then proceed through embryogenesis in a haploid megagametophyte derived from the mother. Embryo morphogenesis and cell division patterns in angiosperms have been described in detail using the model plant *Arabidopsis* (Figure 1.3) [14, 15].

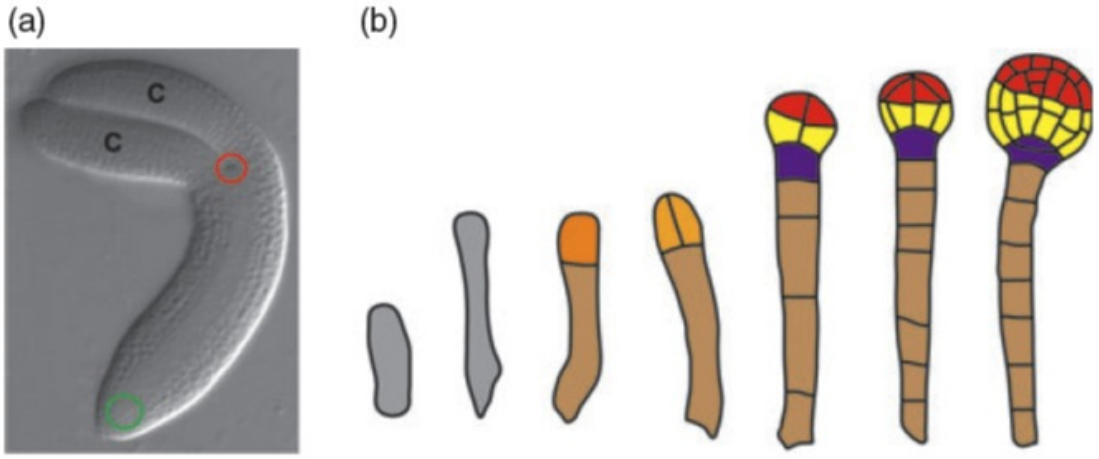


Figure 1.3 Embryogenesis in *Arabidopsis thaliana* [14].
Shown in (a) is a bent cotyledon stage embryo showing the two cotyledons. (b) Depicts development from the elongated zygote in gray to giving rise to an apical (in orange) and basal cell (in brown). The apical cell then undergoes a series of divisions resulting in an upper tier (in red) and a lower tier (in yellow) of the embryo proper. The basal cells will undergo a series of cell divisions to form a suspensor. The uppermost suspensor cell (in purple) is called the hypophysis and contributes to the root pole formation.

During embryogenesis in *Arabidopsis thaliana* (Figure 1.3 and Figure 1.4), the first embryonic division is asymmetric. It establishes a smaller apical cell and a larger basal cell. Thereafter, the cell divisions in the apical region produce the embryo proper. The basal cell divisions give rise to the suspensor and hypophysis. The outer protoderm is formed during the globular stage. (Figure 1.4) [15-17].

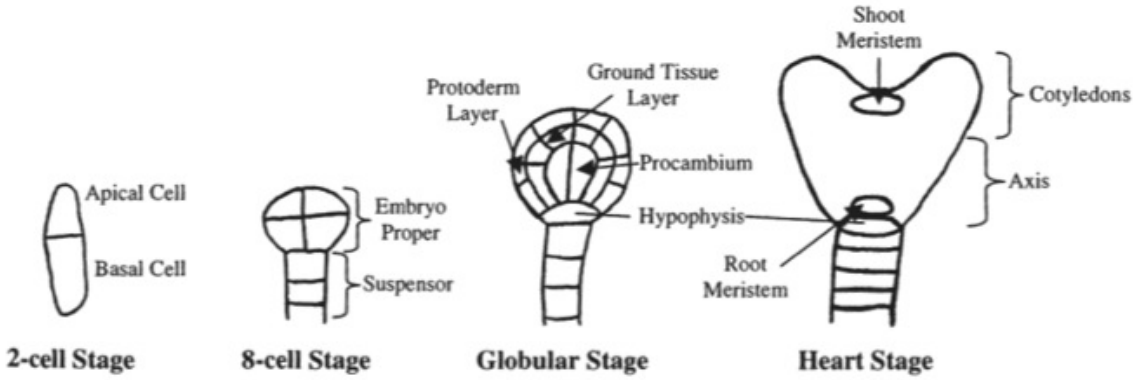


Figure 1.4 Early Stages of Embryogenesis in *Arabidopsis thaliana [15, 17].*
During embryogenesis in *Arabidopsis thaliana* formation of the embryo can be seen clearly. Regular cell division patterns can be seen. There are fewer cell divisions seen during embryogenesis in *Arabidopsis* than pine.

Embryogenesis results in the formation of two organ systems: the axis and the cotyledons. During the heart stage (Figure 1.4) the shoot meristem, root meristem, and cotyledons are formed. Cotyledons are terminally differentiated. They will synthesize and store the nutrients necessary for the embryo upon germination. The axis contains the shoot and root meristems. These structures will eventually form the leaves, stems and roots. The last stage of embryogenesis in *Arabidopsis thaliana* is called the torpedo stage [15, 17].

Loblolly Pine Embryogenesis

Loblolly pine embryogenesis is the subject of this study. The following is a description of Loblolly pine embryogenesis in zygotic embryos. Loblolly pine embryogenesis takes placed within ovules [15, 17]. The fertilization process begins with airborne pollen grains being captured at the micropyle. The pollen may be held for months or years depending on the Pinus species. Typically, in the month of June after the

pollen capture, Loblolly pine pollen tubes growing from pollen grain penetrate the nucellus and eventually breach the neck cells. Once the pollen tube has penetrated the neck cells, it bursts. This allows the male gamete to move into the archegonial cytoplasm. Zygotic embryo development will then occur, beginning with the fusion of at least one of the haploid egg cells with a haploid sperm cell to form a diploid zygote. The process of embryogenesis typically takes 10 weeks from the point of gametic fusion, and will thus be completed by mid July. However, zygotic Loblolly pine embryos may continue enlargement and maturation through the end of August or later depending on the region and climate. [17-19].

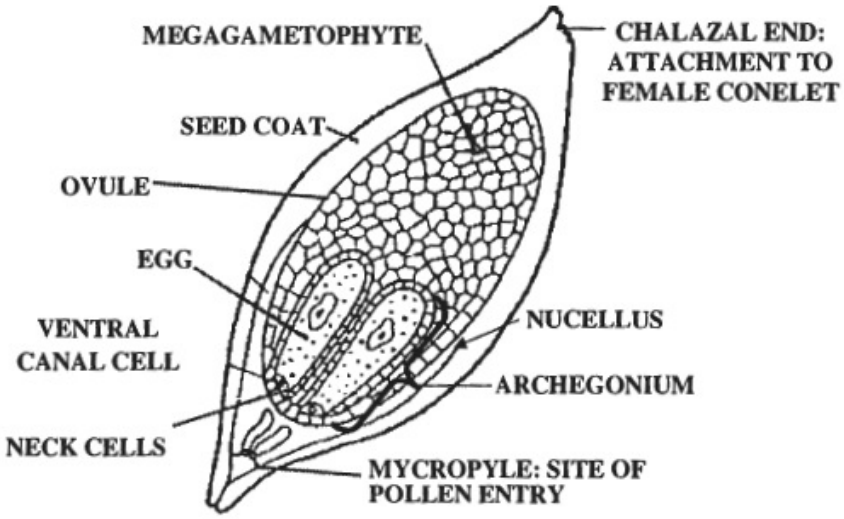


Figure 1.5 Cross-Sectioned Loblolly pine Seed before Fertilization [19].
There are two eggs present within this drawing and each can be fertilized, resulting in both dominant and subordinate embryos**.**

The patterns of cell division in the development of the axis and the cotyledons of conifer embryos have been described. Gymnosperm early embryogeny is similar to the angiosperm development through the globular stage. Late gymnosperm embryogeny is similar to the heart and torpedo stages of angiosperm embryogenesis. Webb and Pullman have described an embryo stage classification system for Loblolly pine (Figure 1.6)., as described below. The method of classification was an integral part of this work because it was used to separate the embryos used for genetic analysis. Please refer to Figure 1.6 throughout this description [17-19].

Proembryogeny

The first stage of embryogenesis in pine begins with proembryony (Figure 1.6). Fertilization and suspensor elongation occurs during this stage of development. The newly formed diploid nucleus undergoes two rounds of free-nuclear divisions immediately following fertilization. These divisions result in the formation of four nuclei. Dense cytoplasm, called neocytoplasm, surrounds the newly formed nuclei. The nuclei and cytoplasm then migrate to the chalazal end of the archegonium. At this location, the nuclei align to form one tier. Eventually, a four celled proembryo will form after multiple rounds of cell division. The distal cell tier comprises the origin of the embryonal mass, while the next tier will elongate and eventually function as an embryo suspensor. The third tier from the top is called the rosette layer, but has no known discrete function. The fourth tier of cells remains contiguous with the egg cytoplasm and will absorb the nutrients stored in the egg cytoplasm [17-19].

Early Embryogeny

Early embryogeny lasts from the point at which suspensor elongation occurs until root meristem establishment. The steps of early embryogeny include: (1) proliferation of suspensor cells, (2) cleavage polyembryogeny, (3) formation of a distinct embryonal mass, and (4) protoderm establishment. In Loblolly pine, the embryonal mass is derived from a single distal cell that will become the dominant zygote over the course of cleavage polyembryony. Primary and secondary suspensor cells and embryonal tubes divide and elongate resulting in a lengthening of the suspensor. The lengthening of the suspensor causes the embryonal mass to be pushed deep into the central portion of the female megagametophyte [19].

Polyembryony

One distinctive characteristic of loblolly pine embryogenesis is the presence of more than one developing embryonic mass inside of the ovule. There are two types of polyembryony: simple and cleavage. Simple polyembryony is the formation of multiple embryos by fertilization of many eggs residing in a single ovule. This results in genetically different embryos within the same ovule. In contrast, cleavage embryogeny is a regular and organized phenomenon whereby cleavage of the embryonal mass and attached suspensors give rise to four separate embryos per fertilized archegonium. In most cases, regardless of the type of polyembryony, one embryo will become more vigorous while the others degenerate. As a result, it is extremely rare for one ovule to yield more than one mature embryo [19].

Late Embryogeny

The final stage of embryogenesis, late embryogeny, is the primary focus of this investigation. The development during late embryogeny consists of cellular differentiation and development of the embryo to prepare for seed dormancy. Late embryogeny begins as cells adjacent to the potential root cap cells begin to divide vertically. This signals the beginning of root initials formation and serves as a demarcation for the shoot apex that forms distal to the root initial. A mature embryo will have a cortex, root cap, epidermis, two polar meristems, and a procambium that is present as a ring in the hypocotyl that extends into each of the five to seven cotyledons [17-19].

Pine- *Arabidopsis* Embryos Develop Similarly but have several Distinct Features

Embryogenesis, the process of growth and differentiation is an important period of plant development because during this phase the meristem and shoot-root body pattern of a plant are specified. Traditionally, plant embryogenesis is perceived as being composed of two phases: a morphogenic phase and a maturation phase. After maturation, the embryo undergoes desiccation and dormancy for a period until conditions are favorable [16]. Based on the differences in the process of embryogenesis, the plant kingdom is divided into angiosperms and gymnosperms.

There are distinct differences between angiosperm and gymnosperm embryo development. Comparisons of angiosperm and gymnosperm embryogenesis are shown in (Figure 1.7). Angiosperms undergo double fertilization to produce a diploid embryo by fertilization of the haploid egg cell with one sperm and a triploid endosperm by fertilization with the second sperm of two polar nuclei. Angiosperm seeds are fully enclosed in the ovary of the mother plant [20-22].

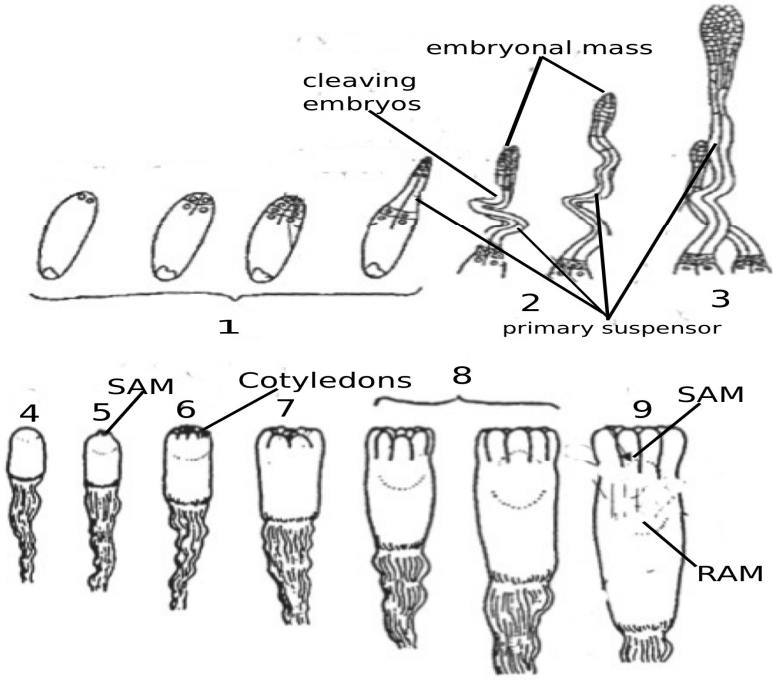


Figure 1.6 Loblolly pine Embryogenesis [19]**.**
Stage 1 is equivalent to the proembryo stage. Stages 2, 3 and 4 are called early embryogeny. In stage 2, the suspensor elongates, the embryo is still at the micropylar end of the ovule. Cleavage embryony is observable at this stage. In stage 3, the suspensor thickens and elongates. The embryonal mass becomes opaque during stage 4. It also lengthens and develops a dome shaped apex. Late embryo includes stages 5 – 9. The shoot apical meristem (SAM) becomes visible during stage 5. By stage 6, cotyledonary primordia become visible below the SAM. In stage 8, though the cotyledons are still visible from above, the cotyledons begin to extend beyond the SAM. By stage 9, the cotyledons have extended even more beyond the SAM, and enclose the SAM. See text for details.

Gymnosperms generally undergo one or more single fertilization events to produce a diploid embryo leaving a haploid female gametophyte. The female gametophyte is the endosperm-equivalent nutritive tissue surrounding the embryo. The

first asymmetric division of a fertilized angiosperm egg cell produces a smaller apical cell and a larger basal cell which give rise to the embryo proper and the suspensor tissue plus some root tissue, respectively [23]. Gymnosperms undergo several nuclear divisions without cytokinesis to enter a free-nuclear phase after fertilization, followed by cellularization to form an eight-celled proembryo arranged in two tiers, which subsequently undergo division to form four tiers. The four cells towards the micropylar end give rise to the embryo proper and the next layer of cells produce the suspensor. Formation of multiple embryos is common for gymnosperms but uncommon in most angiosperms. Eventually only one embryo is produced per seed and the others die by programmed cell death [24]. Polyembryony occurs by fertilization of the egg cells within one or more archegonia by different pollen grains to produce zygotes that have different genotypes within the same seed, or by multiplication of immature embryos. The former is known as simple embryony and the latter is cleavage polyembryony [25].

Angiosperm and gymnosperm embryos also have morphological differences. *Arabidopsis* has a conspicuous embryo with heart, torpedo and bent cotyledonary stage while pine embryos are multicotyledonary with 6 to 8 cotyledons, which enclose the shoot apical meristem (SAM) [18, 26]. The mature pine embryo is a multicotyledonary embryo with cortex, epidermis, root cap, shoot and root meristem and hypocotyl tissues. Cellular differentiation in the embryonic mass (EM) and development of the embryo in preparation for seed dormancy occurs during late embryogeny [18].

Such large and distinct differences in the process of embryogenesis in angiosperms and gymnosperms very likely arises from either unique genes being expressed in each plant type, differential regulation of a collection of genes controlling

embryonic development or both [27-29]. Recently, Chanderbali et al., demonstrated an evolutionary conservation of the genetic programs controlling the development of floral organs in angiosperms and gymnosperm reproductive cones [30]. Likewise, one can expect there to be similarities in the genetic programs controlling embryogenesis in both angiosperms and gymnosperms. The idea that the genetic programs during embryogenesis will be similar in both angiosperms and gymnosperms forms the basis of this thesis. More answers may be gathered from further research into the genes being expressed through embryogenesis in both angiosperms and gymnosperms.

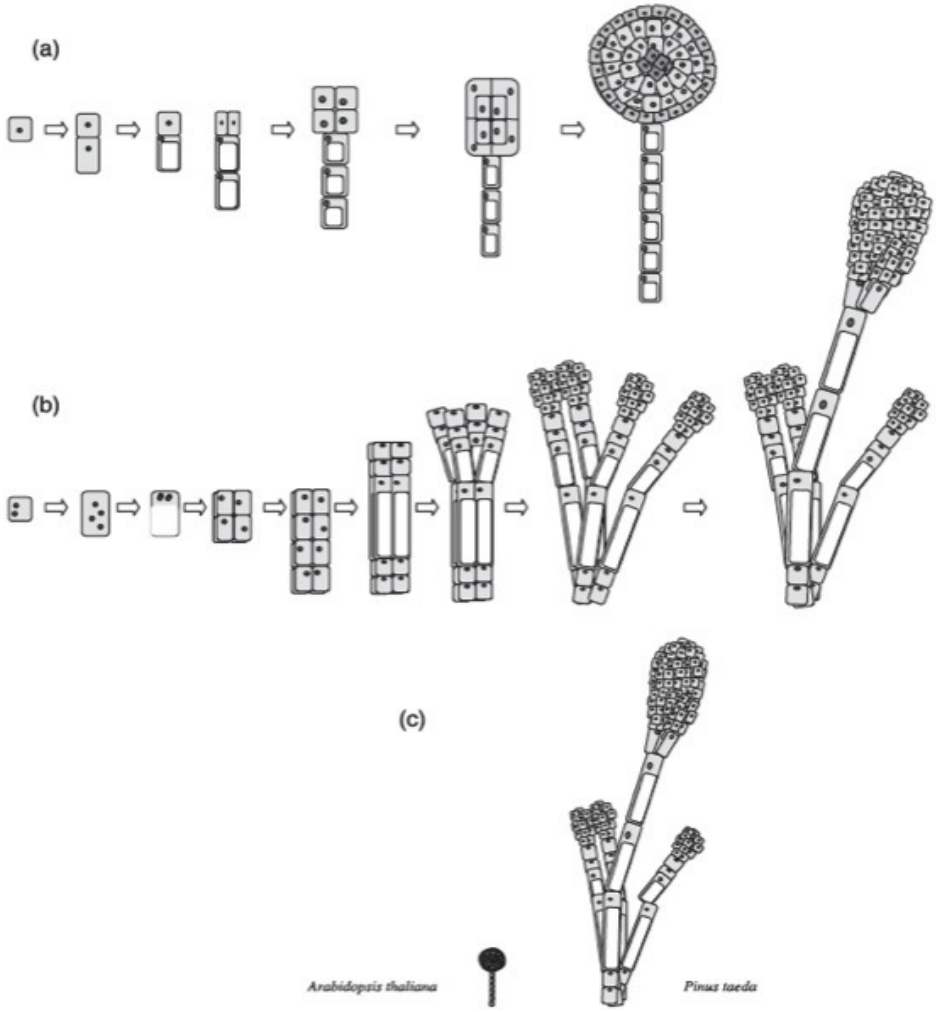


Figure 1.7 Early events of embryogenesis in both Pine and *Arabidopsis* [27, 31, 32]. The early events during embryogenesis in both (a) *Arabidopsis thaliana* and (b) *Pinus taeda* (b) zygotes undergo several rounds of nuclear doubling without cytokinesis. They then enter a free nuclear phase. The free nuclear phase is followed by a cellularization event that produces two tiers of cells and an eight celled proembryo. The tiers will divide to form four tiers. The first two tiers will form the embryo proper. Cells of the third and fourth tiers will form the embryonic suspensor. (c) Represents the relative size of the *Arabidopsis* globular embryo and the developing Loblolly pine embryo.

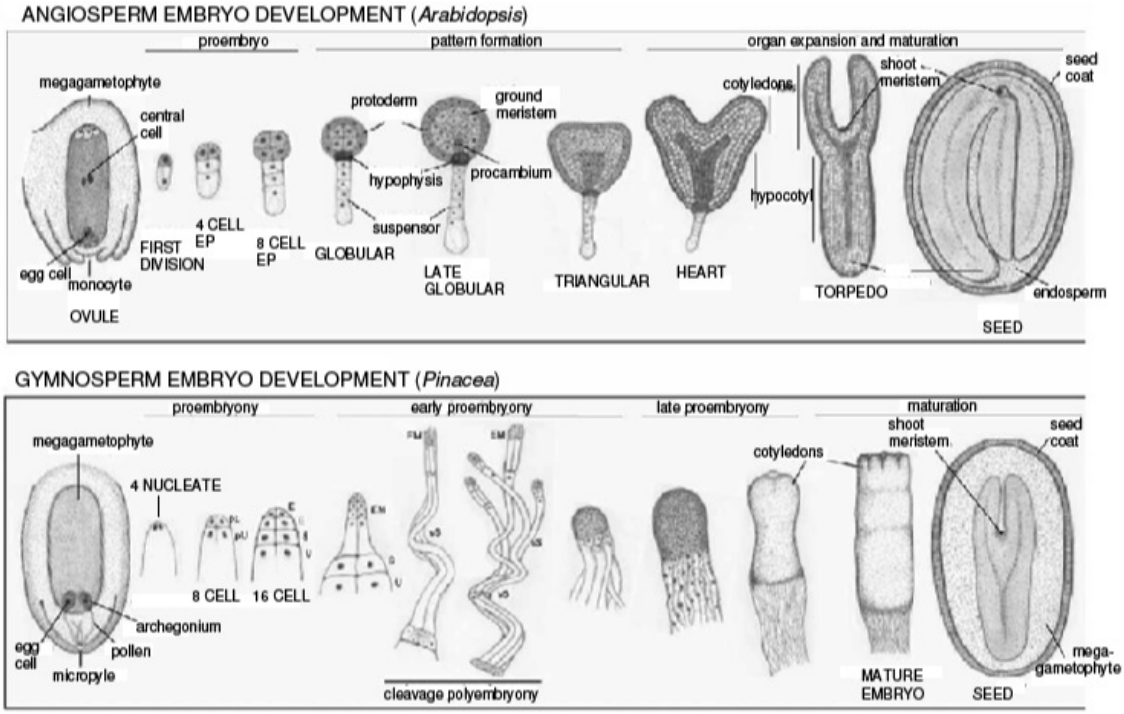


Figure 1.8 Embryogenesis in Angiosperms and Gymnosperms [33].
Embryogenesis in angiosperms and gymnosperms are generally similar, however, they have very distinct differences. Gymnosperms undergo single fertilizations to produce a diploid embryo and haploid megagametophyte (FMg). Angiosperms undergo double fertilizations to produce diploid embryos and triploid endosperm. In gymnosperms, the FMg develop before fertilization, however, the endosperms of angiosperms develop after fertilization. In gymnosperms the polyembryony exists during early stage embryogenesis, however, in angiosperms a single embryo develops. During later stages, morphological differences between Loblolly pine and *Arabidopsis thaliana* are seen. The gymnosperm embryo's SAM will be completely enclosed by growing cotyledons. While the *Arabidopsis* embryo proceeds through discernable stages of development: heart, torpedo, and bent cotyledonary.

Loblolly Pine Somatic Embryogenesis

The increasing demand of forest products and decreasing availability of land-base for producing trees are huge roadblocks for the forest product industries. There is a need to

propagate large number of pine trees, and clonal propagation techniques are expected to play a significant role in providing genetically superior pine trees [20, 34].

Clonal plantations can be established from both organogenesis and embryogenesis. In the 1960s, the first plantlets from forest tree tissue culture were produced by organogenesis using adventitious shoots [35]. Organogenesis includes the use of:

- Outgrowths and division of seedling shoots
- Callus and cell cultures to regenerate adventitious shoots and complete plants
- Cultured cotyledons and entire embryos to induce adventitious buds
- Cultured shoot tips to induce adventitious and axillary buds.

Stem cuttings, leaf cuttings, root cuttings, root suckers, root-stem cuttings, grafting and air-layering are some other methods of forest tree clonal propagation [35]. Loblolly pine can be clonally propagated in its juvenile state, however, they become difficult to clone once they reach maturity. Somatic embryogenesis offers an alternative to this roadblock [36].

Somatic embryogenesis (SE) is the process through which embryo formation in seeds can be mimicked in the lab. It was first developed for carrot propagation in 1958 [37]. Somatic embryogenesis for Loblolly pine was first reported 20 years ago [38-40]. The first propagation of a conifer (*Picea abies*) through somatic embryogenesis was accomplished in 1985. Since that time, SE has been used for many commercially important coniferous species, representing the genera Larix, Picea, and Pinus [10, 41-43].

Loblolly Pine Somatic Embryogenesis Procedure

SE is the formation of an embryo from a cell other than a gamete or the product of gametic fusion. This process is in sharp contrast to zygotic embryogenesis, which is the result of the fusion of male and female gametes. The process of somatic embryogenesis in Loblolly pine consists of four main steps (Figure 1.9): (1) culture initiation, (2) culture maintenance, (3) embryo maturation, and (4) embryo germination. Each step has a different culture medium associated with it. The compositions of the media are constantly being modified to improve embryo development.

The initiation process induces an explant to produce the embryonal suspensor mass (ESM). The embryonal suspensor mass is often referred to as embryogenic tissue. All subsequent somatic embryos are derived from this explant. Conifer explants used in the initiation process generally must be juvenile tissues. Typically, precotyledonary and cotyledonary embryos and seedling explants from 7 to 30 day old seedlings are used [32]. However, there is limited information regarding the frequency of embryo regeneration from mature tissues [44-46].

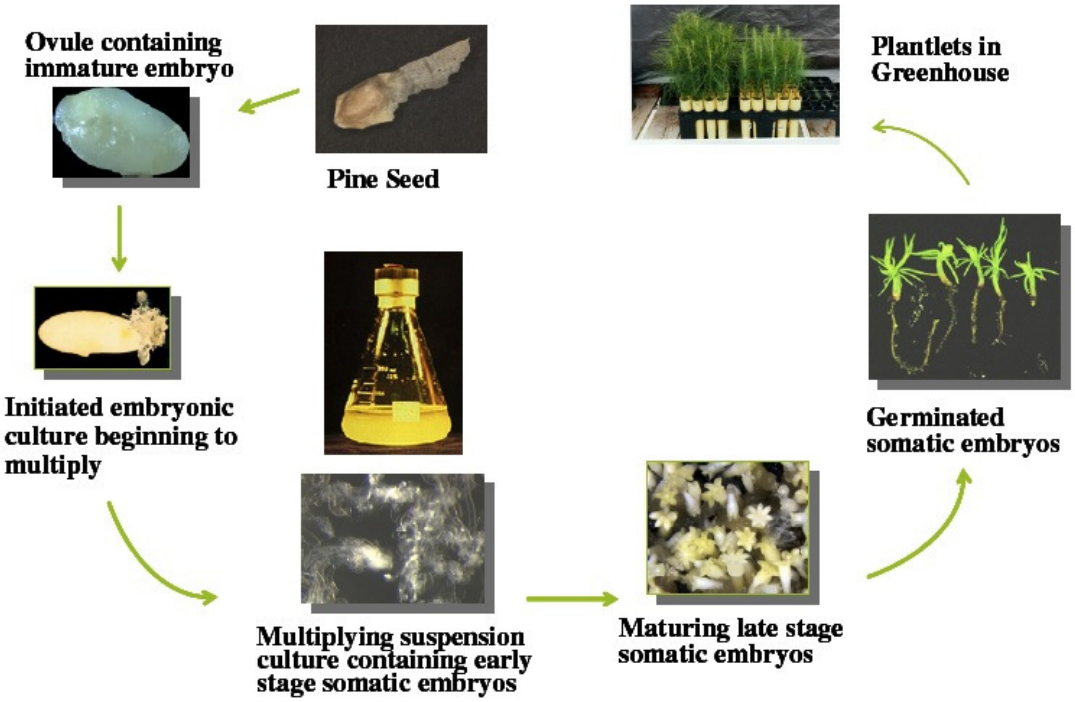


Figure 1.9 Somatic Embryogenesis in Loblolly Pine [47].
In this process, pine seeds are extracted from cone and are cracked to reveal the ovule containing an embryo, which is then placed onto an initiation medium. Later, the embryo is transferred to multiplication and maturation medium. After germination, the resulting plantlets are transferred into soil.

Typically, an embryo around stage 2 must be used as an explant for loblolly pine systems (Figure 1.10) [48]. The loblolly pine embryo suspensor mass (ESM) originates from the meristematic regions and attached suspensors [49]. It is composed of both proliferating cells and somatic embryos. To promote the initiation process, an intact ovule containing a dominant immature zygotic embryo and the subordinate embryos is

placed on an initiation medium. The composition of this initiation medium is designed to promote growth and extrusion of the subordinate embryos. The process of initiation is defined by the ability to form somatic embryos from extruded subordinate zygotic embryos. Common components present in the initiation medium are: inorganic nutrients, vitamins, amino acids, a carbon source, an osmoticant (myo-inositol), a gelling agent, and plant growth regulators (cytokinin or naphthalene acetic acid). Auxin and cytokinin are required for somatic embryogenesis initiation. The ratio of auxin to cytokinin and the concentration of plant growth regulators are important. The auxin and cytokinin cause promotion of cell elongation and cytokinesis, however, it is not known how these plant growth regulators control initiation of the ESM [2, 49].

After the initiation process is complete, the ESM is transferred to maintenance medium where the goal is to promote embryo multiplication. Maintenance media can be both liquid or semi-solid. However, the embryos in semi-solid media experience higher multiplications rates. In addition, there are decreased upkeep requirements and the embryos are more easily inspected in this state. The composition of maintenance medium is very similar to that of initiation medium. However, maintenance medium does have lower concentrations of auxin and cytokinin compared to initiation medium. The ratio of auxin to cytokinin is also reduced. Cells from maintenance medium are sub cultured on a weekly basis and aliquots from semi solid cultures are moved to maturation medium [39].

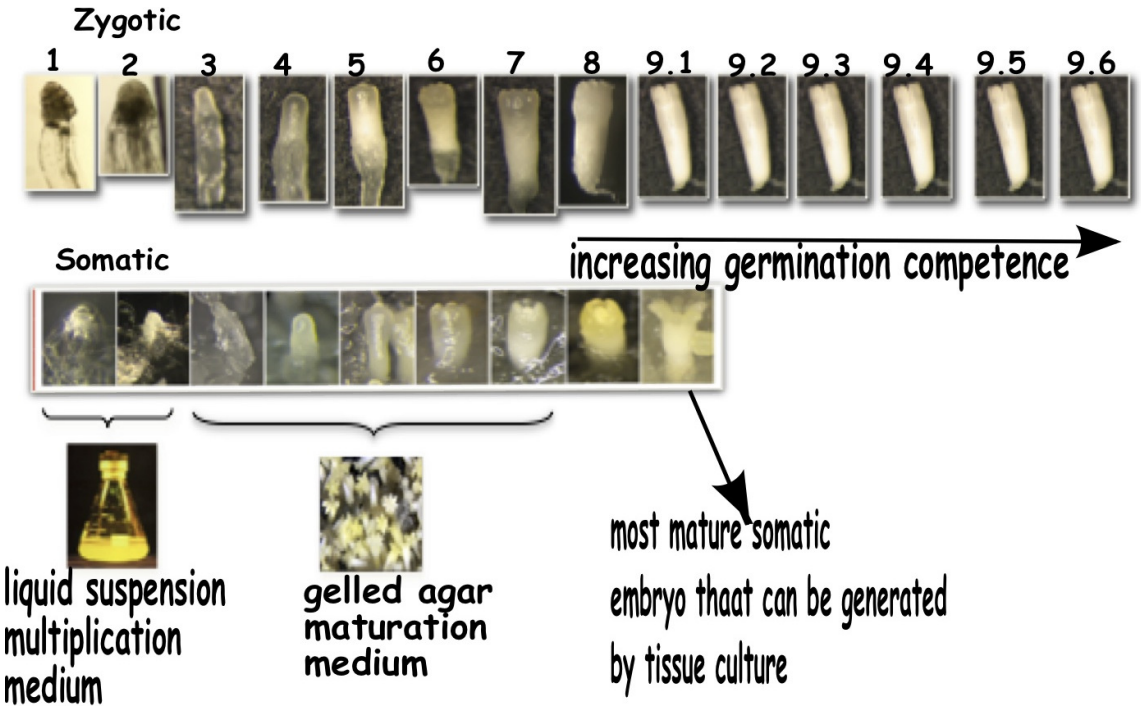


Figure 1.10 Zygotic and Somatic embryos develop analogously [27].
Pine zygotic embryos have been staged from 1 to 9.12 based on morphology. Stages 9.1 -9.12 are morphologically similar, but are biochemically different. Somatic embryos (shown on bottom) do not fully mature. Even the best genotypes do not develop past stage 9.1.

The maturation phase allows the embryo to develop from a stage 2 or 3 to a mature stage 9 embryo. This phase is completed on a semi-solid medium with the growth period lasting 3 to 6 weeks. A primary change in the composition of this media is the removal of auxins and cytokinins and a switch to abscisic acid (ABA) as the major plant growth regulator, along with an increase in the osmolarity of the medium [8, 50].

No growth regulators were included in the maturation medium described in initial reports of somatic embryogenesis in loblolly pine [51]. A significant improvement in the

efficiency of somatic embryogenesis has been achieved by the inclusion of ABA in the maturation medium [39]. ABA in plants typically is involved in mediating plant responses to drought, salt, and cold stresses. In somatic embryogenesis, the presence of ABA inhibits cleavage polyembryony and suppresses precocious germination. Expression of ABA responsive genes occurs in parallel with accumulation of storage proteins, lipids, starches, and late embryogenesis abundant (LEA) proteins. The LEA proteins are believed to be associated with desiccation tolerance [8, 50, 52].

Along with increased ABA concentration present within the maturation media, there is also an increase in the osmolarity of the medium. The increased osmolarity enhances the effects of ABA. Some commonly added osmoticants include:

- Easily metabolized simple sugars (sucrose)
- Poorly metabolized osmoticants
- Sorbitol
- Inositol
- Polyethylene glycol

Osmoticants of a larger molecular size, such as poly ethylene glycol, appear to work better than smaller sugars. This is because the smaller molecules are more easily able to traverse the plant plasma membrane and reduce the osmotic gradient. The addition of activated carbon to the medium has reportedly increased maturation efficiency [53]. The presence of activated carbon results in:

- More genotypes forming cotyledonary embryos
- Greater yields of cotyledonary embryos
- Improved desiccation tolerance

•Greater germination percentages

The activated carbon is thought to act in one of two ways. First, it may adsorb residual auxin and cytokinin carried over from maintenance culture and thereby suppresses cleavage polyembryony. Secondly, it may also adsorb excess ABA. This makes less ABA available to the embryo during the later stages of embryogenesis [53].

Germination involves root development and growth, as well as the development in embryos of the ability to begin synthesizing their own food. Placing mature embryos on a basal medium that has reduced osmotic levels and lacks growth regulators induces germination. The embryo remains on the germination medium for six to eight weeks. During this period, the plants are typically a 16-hour light/8 hour dark photoperiod [1, 3, 4, 8, 28, 29, 39, 54].

Benefits Somatic Embryogenesis in Loblolly Pine

The process of somatic embryogenesis is rapid, and has the capability of producing thousands of high quality embryos that are genetically identical. The process not only has the capability of mass producing genetically improved trees but also has the capabilities of providing an in vitro regeneration system that is amenable to genetic transformation. This ability of somatic embryogenesis to capture large gains via clonal propagation has been recognized by both foresters and scientists as an integral component of a clonal forestry program, along with selection, progeny testing and controlled breeding. In fact, somatic embryogenesis is preferred over rooted cuttings and micropropagation because it has the potential to be the cheapest method per unit propagule. It can be scaled up with both synthetic seeds and bioreactors. Somatic embryogenesis also enables embryonic masses (EM) to be more easily stored through

cryogenics, thereby allowing indefinite storage, and subsequent repetitive retrieval of specific genotypes. This is advantageous because unproven genotypes can be maintained in cryogenic storage while they are simultaneously tested in the field [3, 4, 34].

The benefits of somatic embryogenesis coupled with the economic importance of Loblolly pine have resulted in a significant amount of research and effort concentrated on developing loblolly pine somatic embryogenesis. It is expected that these techniques will become a major part of forest tree improvement programs. Recent breakthroughs in somatic embryogenesis processes for Loblolly pines provide further evidence that widespread clonal forestry will become a reality in the near future [3, 4, 34].

Obstacles in Somatic Embryogenesis of Loblolly pine

Although the process of somatic embryogenesis in pines is extremely promising, researchers working in this area face many challenges. The success of somatic embryogenesis is very dependent on the genotype of the embryo. The somatic embryos do not develop fully. Even the best performing genotypes fail to develop past stage 9.1 (Figure 1.10), and the quality of embryos can be poor. There are also low initiation rates, low culture survival, low maturation rates and low germination, as well as low or no embryo production. Other challenges include: (1) defining and managing somaclonal variation in somatic embryos, (2) defining and managing the effect of multiple somatic embryo genotypes within an embryogenic culture, and (3) optimizing transformation methods [28, 52, 55-57]. One major and often cited shortcoming of somatic embryogenesis is the necessity of using unproved genotypes for culture initiation under the present technology. This necessitates time consuming field trials to assess clone

performance before somatic embryo derived plants can be used in reforestation. Three main areas require improvement:

- Maximization of the number of genotypes that initiate somatic cultures
- Maximization system yields from initiated cultures, and
- Minimization of the costs of converting cloned embryos to plants

Initiation and maintenance culture establishment is key to the success of somatic embryogenesis. Reports show that low initiation rates limit the number of genotypes that can be propagated through somatic embryogenesis [56]. This leads to the underrepresentation of certain genotypes that may have elite characteristics, and lowers the genotypic variation of the population produced by somatic embryogenesis [34]. Poor performance of maintenance cultures reduces the yield and quality of cotyledonary embryos on maturation medium. These deficiencies in the process of initiation and maintenance have later effects on embryo germination and plantlet establishment [1, 58, 59]. This limits the overall efficiency of the system due to resources being exhausted when attempts are made to germinate low quality embryos whose fate was predetermined by sub optimal initiation and maintenance conditions. Therefore, improvements in initiation and maintenance are essential to improved embryo quality [1, 28, 47, 53, 54, 56, 58, 59].

Molecular Genetics of Embryogenesis in Loblolly pine

Pine is a leader in new initiation concepts and media analyses in tissue culture techniques, however, genetics and genetic engineering in pine lags behind the availability of biotechnology in other plant species [36]. Recently, more attention has been paid to analysis of genetic, biochemical and molecular control of embryogenesis as a means to

understanding and improving somatic embryogenesis. As stated earlier, initiation is a major obstacle in somatic embryogenesis. Previous data has shown that seeds from specific mother trees initiate more easily [60]. Molecular markers have been found that distinguish embryogenic from non-embryogenic conifer cell suspension cultures. Examples include extracellular arabinogalactans (AGPs) and chitinases in somatic embryo cultures of *Picea abies* and glycosylated peptides in *Pinus caribaea,* both of which have been correlated with embryogenic capacity [1, 27-29, 54].

A basic understanding of the molecular events occurring during zygotic embryogenesis is necessary to develop further refinements in somatic embryogenesis systems for pine. We can look to *Arabidopsis* and other model angiosperms to suggest what molecular events might occur during embryogenesis in Loblolly pine. This is because there are many orthologous genes in angiosperms that appear to be conserved in gymnosperms. Cairney et al. have reported that out of 108-angiosperm embryogenesis related genes; homologs for 83 of the genes were present in an EST collection from loblolly pine embryos [29]. Trends in gene expression are also conserved. Most discoveries of embryogenesis related genes and their expression patterns have been made with the *Arabidopsis thaliana* model system. Consequently, it is necessary to review important advances in the understanding of molecular events in angiosperm embryogenesis [27-29].

Arabidopsis thaliana is most often used for molecular genetic studies because it is small with a completely sequenced genome, has a short life cycle, large seed set and is amenable to both forward and reverse genetic analysis. Reports have shown that large numbers of genes are expressed during embryogenesis. It has been estimated that

anywhere from 12,000 to 34,000 genes are expressed during embryogenesis in soybean, cotton and wheat embryos [61-63]. There is also evidence that changes occur in gene expression programs throughout embryogenesis [28]. For example, some genes may be expressed during early embryogenesis and not during late stage embryogenesis [27-29].

The functions of many of these genes expressed during embryogenesis have been determined using various molecular biology techniques, including knockout or knockdown mutations, alterations in gene expression, protein tagging, and activity profiling. The loblolly pine genome is huge and complex at over 2 X 10^{10} bp [64]. This is about 100 times larger than the fully sequenced genome of *Arabidopsis thaliana* and 20 times larger than the fully sequenced genome of *Populus trichocarpa* [65]. It is estimated that over 99% of the pine genome is composed of either or repetitive sequences [65]. Mutation studies using pines are extremely difficult because of the large amount of gene redundancy within the genome [65]. Many current gene expression studies in Loblolly pine focus on embryo maturation, desiccation and germination [27].

Control of Seed Dormancy and Germination

In higher plants, germination does not occur while the seed is still attached to the mother plant. However, embryos are capable of germination at this time if a late stage embryo is removed from the mother plant. This process is called early or precocious germination [66]. Precocious germination results in deviant and overly grown cotyledons, poor hypocotyl development, low root emergence, and little to no storage protein accumulation within the seed. This typically results in death [67].

High ABA concentrations suppress precocious germination [68].

The embryonic development process in plants is highly complex, and highly regulated. One major class of regulators is plant growth hormones (PGHs). Minute quantities of these hormones are capable of precise regulation of embryonic development. These PGHs drive many developmental processes, such as: cell division, cell expansion, and elongation. They promote responses to the environment. Examples include temperature, light intensity, day length, soil moisture, humidity, nutritional factors, and genetic regulation of germination, transpiration, and protein synthesis. PGHs allow communication between different parts of the plants by acting as chemical messengers [68].

ABA Responsive Genes

Lopez-Molina and Mongrand first reported ABA's ability to delay both germination and early growth in *Arabidopsis* [69]. They discovered that *Arabidopsis* seeds would not germinate after a period of exposure to the hormone [69]. Although ABA controls dormancy in seeds, it has also been reported to control a number of genes, including a group called ABA responsive genes. These elements are believed to confer hormone responsiveness as well as stage-and tissue-specificity. Currently only six classes of transcription factors have been demonstrated to be essential for ABA- or seed-specific gene expression in *Arabidopsis thaliana*, including: ABI3/VP1, ABI4, ABI5, LEC1, LEC2, and FUS3 [70].

Table 1.1 Three ABA responsive genes of interest to this investigation

Gene	Mutation Selection/Screen	Phenotype	Gene Product	References
abi3	ABA-resistant germination	Produces many defects in seed maturation; there are also vegetative effects seen on plastid differentiation	B3 domain transcription factor	[71, 72]
abi4	ABA-resistant germination	Sugar and salt resistant germination and seedling growth	APETALA2 domain transcription factor	[73-76]
abi5	ABA-resistant germination	Stunted growth, defective stomatal regulation; male sterility	bZIP domain transcription factor	[69, 74, 77]

Development and maturation are largely controlled by the coordinated action of transcription factors. The transcription factors ABI3, ABI4 and ABI5 are the subject of this study. ABI3, ABI4, and ABI5 act in the same seed-specific signaling pathway (Figure 1.11). Mutants in each of these genes exhibit defects in seed sensitivity to ABA and accumulation of Late Embryogenesis Abundant (LEA) transcripts. However, they displayed normal vegetative growth. It has recently been reported that ABI3, ABI4 and ABI5 also have functions outside of modulating seed sensitivity [78]. It has been reported by Pullman et al. (2007) that during zygotic embryogenesis in Loblolly pine, increases in expression of three ABA responsive LEA-like genes coincided with stage-specific peaks of endogenous ABA accumulation in the embryo [52, 79]. ABI3, ABI4, and ABI5 will be reviewed here since they are the subjects of this study.

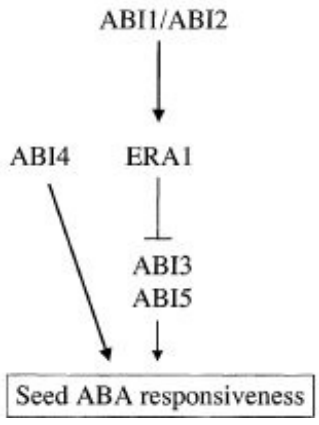


Figure 1.11 Genetic interactions between ABA-responsive genes ABI3, ABI4 and ABI5 [80]

The ABI3 Gene Product is Essential for Correct Completion of Seed Maturation

ABI3 is a major regulator of the transition from seed maturation through germination into an early seedling. Expression of ABI3 is required for entry into the maturation phase of development and for proper germination, and is essential to the process of embryogenesis [66]. Loss in function of the ABI3 gene results in an embryo that is unresponsive to the hormone ABA [81].

abi3 mutations radically alter cell states during embryogenesis. The seeds of the abi3 mutants remain green, fail to establish desiccation tolerance, and will germinate at a stage when wild type seeds will not normally germinate (Figure 2.2). ABI3 may also be involved in plastid development, bud dormancy, and the genes involved in light regulation. Interestingly, abi3 mutant embryos develop normally until 8 days after fertilization, but the embryo desiccates by 16 – 18 days after fertilization [66]. Wild type plants undergo growth arrest after germination. abi3 mutants are unable to initiate the arrest and continue activity within the meristem.

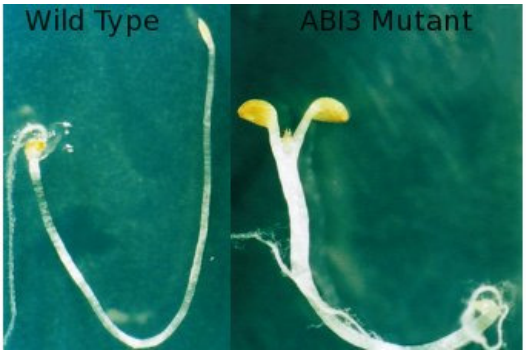


Figure 1.12 ABI3 mutant *Arabidopsis thaliana* plant [82].
ABI3 mutants continue germination without entering the normal period of seed dormancy.

ABI3 causes activity within the apical meristems to become repressed in seeds and completely arrested in seedlings. Its activity represses outgrowth of axillary meristems as well [83]. ABI3 and VIVIPAROUS1 (VP1) are orthologous genes form *Arabidopsis* and maize. They encode transcription factors of the B3 domain family. They contain four conserved domains: an acidic activation domain (A1) and 3 basic domains (B1 – B3) (Figure 1.13). The B1 domain modulates protein-protein interactions with another transcription factor, ABI5. Both the B2 and B3 domains are capable of binding DNA [84]. The B3 domain is unique among plants and specific to gene families [85].

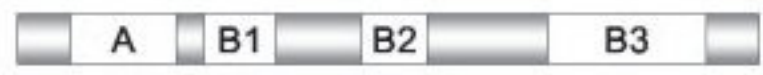


Figure 1.13 Diagram of ABI3 Domain Structure [86].
ABI3/VP1 and their orthologs contain four conserved domains. : An acidic activation domain (A) and three basic domains (B1, B2 and B3). They activate the transcription of ABA promoters in vivo.

ABI4

ABI4 is an essential protein that serves to separate functions during embryogenesis. It encodes an AP2/ERF transcription factor that plays an important role in seed development and germination. It is also essential for lipid mobilization from the embryo, chloroplast retrograde signaling, and glucose responses. ABI4 serves as a transcriptional repressor of sugar-regulated genes. ABI4 is an essential activator in the process of

signaling and sugar responses. The abi4 gene encodes a putative DNA binding and protein interaction domain. ABI4 belong to the family of APETALA 2 (AP2) transcription factors (Figure 1.14) [78, 87, 88].

Figure 1.14 Diagram of ABI4 domain structure [87].
The ABI4 gene contains one known domain, APETELA2.

ABI4 mutants exhibit defects in ABA sensitivity in seeds, and seed specific gene expression, however, they exhibit normal vegetative growth [70]. ABI4 is required to regulate many genes controlled by both ABA and sugars [78, 87].

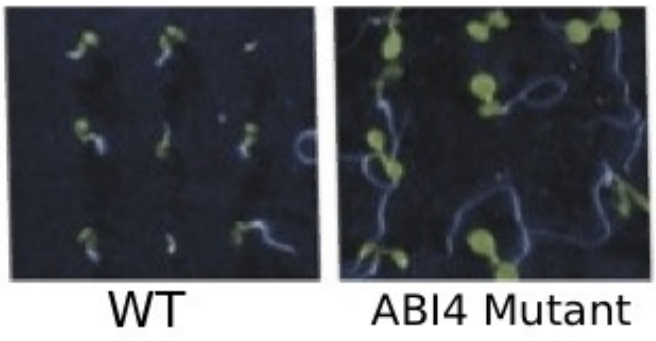


Figure 1.15 ABI4 mutants in *Arabidopsis* [89].
The plants containing the ABI4 mutation are no longer responsive to ABA cues for arrest of germination. WT= Wild type

ABI5

ABI5 encodes a basic leucine zipper transcription factor. It contains putative DNA binding and protein interaction domain [70]. ABI5 is a member of the Dc3-promoter binding factor family (DPBF) subfamily. The domain structure of the ABI5 protein includes the basic leucine zipper (bZIP) domain and a proline rich region (Figure 1.16) [77]. The transcription factor, ABI4, has been shown to bind to ABI5 both in vitro and in vivo. ABI5 acts downstream of ABI3 to reactivate late embryogenesis programs and to arrest growth of germinating embryos.

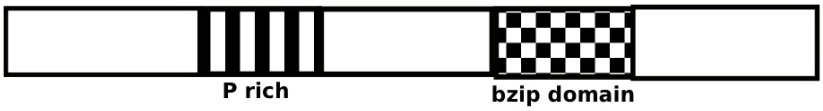


Figure 1.16 Diagram of ABI5 domain structure [77].
The ABI5 protein is a transcription factor, and contains a proline rich (P rich) region and a basic leucine zipper (bZIP) domain.

ABI5 is needed to induce an ABA dependent growth arrest. Wild type *Arabidopsis* can enter an ABA dependent growth arrest after breakage of seed dormancy but prior to autotrophic growth. The abi5-4 mutant lacks ABI5, and is unable to execute this arrest [90]. Arrested embryos germinate and are viable but inactive, and osmotolerant as long as ABA is present. This action is reportedly an adaptive mechanism that leads to increased in the survival of drought stressed *Arabidopsis* plants. Yeast two-hybrid assays have shown that ABI3 directly interacts with ABI5 [84]. There are two conserved charged domains in the amino terminal half of ABI5 required for interaction with ABI3.

Figure 1.17 ABI5 *Arabidopsis thaliana* mutants are nonresponsive to ABA germination arrest cues [91].

Root Development

Plant roots play a vital role in water and mineral acquisition, and are essential for plant growth and development. The stronger and bigger the root, the more the root is able to transport water and nutrients to the plants. This can result in huge rewards in terms of the quality of trees produced, their proper development and survival [92-94].

Plant roots carry out an assortment of biological functions. Roots are plant organs tailored to obtain water and nutrients from their surroundings [93]. They keep the plant upright and are important storage organs. The roots are also able to produce growth regulators and secondary metabolites. The formation of the root during embryogenesis is a result of events in a precise developmental pathway [95].

Root development and pattern formation is typically divided into three steps. First, neighboring cells deliver positional signals. These signals promote differentiation of undifferentiated cells. Next, the fate of these cells is fixed through the expression of

transcription factors and other proteins. Finally, the activation of transcription factors leads to orientated cell division, cell specification and cell elongation [94].

Root formation begins with the root apical meristem (RAM) being formed by cells in the apical and basal tiers in developing embryos. All future root cells can be traced back to these initial cells from the meristematic region of plants [95]. They are able to generate specific tissues through regulated cell divisions [93]. Once germination begins, the initial cells from the RAM divide and provide new cells. These cells then enter the expansion zone. Here they slowly continue to grow and divide. As they expand, they will be pushed into the maturation zone. While in the maturation zone they finally begin to differentiate into root cells [95].

The mature *Arabidopsis* root contains four concentric layers. Organized from the outside to the inside they are composed of the following cell layers: an epidermis, cortex, endodermis and stele. The stele is composed of the vasculature and the pericycle. The cells in each of the layers can be traced back to the initials or stem cells located in the root meristem (RM) (Figure 1.18) [96].

The study of mutants affecting normal developmental patterns allowed the discovery of genes involved in root patterning. Some of these genes encode transcription factors or transcription regulators [97]. These transcription factors and transcriptional regulators regulate cascades of genes that can lead to important morphological, physiological, and/or metabolic changes [95].

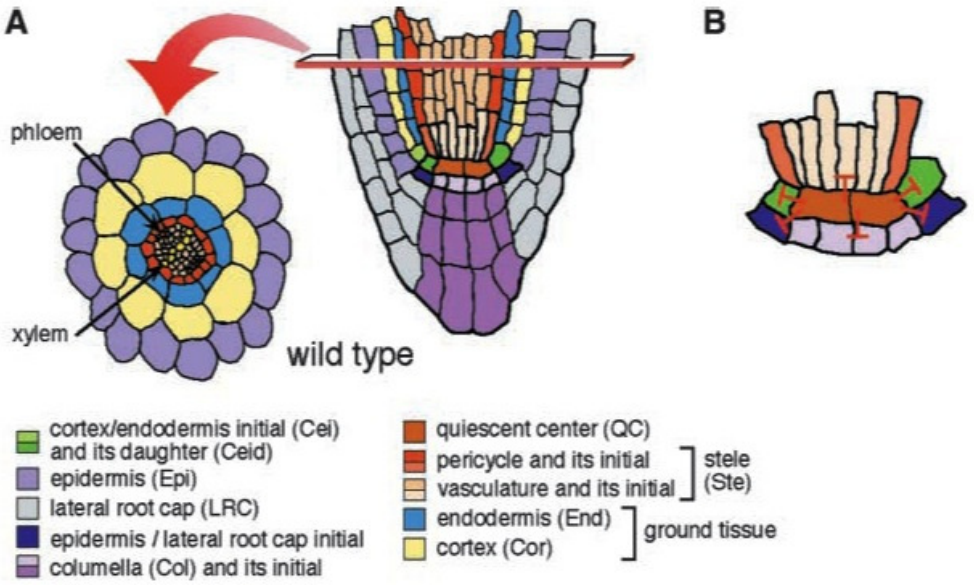


Figure 1.18 Scheme of *Arabidopsis* root structure. Adapted from Nakajima and Benfey, 2002 [96, 98].
(A) Wild type root [98]. Cell types are shown in the key at the bottom. (B) QC cells function as an organizing center of the root meristem by inhibiting the differentiation of surrounding initial cells (stem cells) [99, 100]

In *Arabidopsis thaliana*, mutation of specific genes causes dramatic alterations in postembryonic root development. A number of these genes, including SHORT ROOT, MONOPTEROS, WOODENLEG, BODENLOS, HOBBIT, and SCARECROW, are involved in pattern formation, cell division and cell expansion of the root (Table 1.2) [97, 101-103].

Table 1.2 Six key root genes that control root development in *Arabidopsis thaliana*.
Adapted from Cairney and Pullman, 2007 [27].

Gene Name	Genbank Accession	Protein Function	Mutant phenotype	Reference
BODENLOS (BDL)	NM_179258	AUX/IAA protein	Fails to initiate root meristem during embryogenesis.	[101]
MONOPTEROS (MP)	NM_101840	Auxin Response factor, transcription factor. Required for patterning of the basal region of the embryo.	Fails to initiate root meristem during embryogenesis and no root formation	[104]
SCARECROW (SCR)	NM_115282	GRAS family transcription factor that regulates the radial organization of the root, and is required for cortical morphogenesis.	Asymmetric division produces a single layer within the root with both cortex and endodermis attributes. No individual cortex layer forms.	[105]
SHORT ROOT (SHR)	NM_119928	GRAS family transcription factor that regulates the radial organization of the root	No endodermis in the root system. A single layer of cortex attributes develops.	[106]
WOODENLEG (WOL)	NM_201667	Encodes a two-component signal transducer. Needed for stele cell proliferation.	Too few vascular cells form within the root. All of these differentiate into xylem.	[107]
HOBBIT (HBT)	AJ487669	CDC27 homolog	Improper cell division and differentiation of the root. The quiescent center does not form	[108]

Comparison of Gene Expression in Zygotic and Somatic Embryos

Advancement in somatic embryogenesis techniques would be helped by advancement in our knowledge of the molecular events during embryogenesis and development in the loblolly pine [27-29, 55]. Cairney et al (2000) made comparisons of global gene expression between somatic and zygotic embryos. Differential display was used to clone over 500 cDNAs whose transcript abundance appeared to be developmentally regulated during embryogenesis [28]. The cDNAs were spotted on membrane arrays and hybridized against total cDNA probes from zygotic and somatic embryos of analogous stages to produce transcript profiles and identify differences in gene activity [55]. The results allowed for inferences to be made about developmental pathways involved [28]. The data suggested alterations to the somatic embryogenesis process that could cause a development to occur more similar to zygotic embryogenesis [28, 29].

Dissertation Objectives

The work described in this thesis is based upon the idea that a basic understanding of the molecular events occurring during zygotic embryogenesis is required to fully understand how and why only a very small percentage of somatic embryos develop past the late embryogeny phase of embryogenesis [1]. The strategy of this work was to identify pine orthologs of genes essential to embryogenesis in angiosperms. We first identified genes that have been demonstrated to be required for late embryonic development in the model plant system *Arabidopsis thaliana*. This investigation originated from the express sequence tags generated by Cairney et al. as discussed in section 1.7 [29].

However, instead of studying changes in global gene expression throughout pine embryogenesis, this research attempts to identify, analyze and characterize individual groups of genes that exhibit developmental regulation throughout pine embryogenesis. By identifying which genes are expressed during embryogenesis and determining at which embryonic stages they are active, we can draw conclusions about the activation of specific developmental pathways. In addition, the appearance of certain mRNAs can act as molecular markers, indicating the progress of an embryo through development. By comparing the gene expression patterns of zygotic and somatic embryos, differences and similarities in their genetic programs can be determined [28].

Root Development

A primary roadblock for somatic embryogenesis is root formation and germination. Many conifers produce somatic embryos that appear to be capable of germination and plant establishment, but do not fully mature. Pullman et al have demonstrated that early somatic embryo development (stages 1 – 6) occur normally. Most somatic embryos are able to develop to stages 7 -8, with only a small percentage being able to develop to stage 9.1 [1]. This results in slow germination and slowed growth of the surviving somatic embryos [1]. This research partly focuses on a search to identify genes that control root formation, and therefore have a huge impact on the ability of the embryo to germinate and produce viable plants.

ABA Responsive Genes

Previous data has shown that inclusion of ABA into the media of developing somatic embryos promotes their development. Pullman et al demonstrated the addition of ABA to the medium of developing somatic embryos increases the total percentage of embryos

able to complete the initiation stage [54]. It has also been reported by Vales et al (2007) that increases in abundance of three Late Embryonic Abundant (LEA) genes correlates with increases in ABA levels inside the embryo during development. It was also reported that an elevation of ABA during the mid maturation period of development in somatic embryos caused increases in successful germination and resulted in gene expression trends more similar to those seen in zygotic embryos [17, 52]. This thesis also investigates the hypothesis that the expression of the hormone ABA correlates with the expression of other key ABA responsive genes that function during late embryogenesis.

Differences in Angiosperm and Gymnosperm Gene Expression

Approximately 85% of the genes expressed during embryogenesis in pine and *Arabidopsis* show strong sequence similarity [27, 29]. It seems, therefore, unlikely that differences in embryo development arise from a set of unique embryo-specific gymnosperm genes. That minor sequence differences result in different protein interactions is certain but the contribution of such novel interactions is difficult to assess. The objectives of this dissertation include:

Objective 1: The identification, isolation and characterization of the ABI3, ABI4, and ABI5 genes from Loblolly pine. We will isolate the cDNAs from pine embryos, complete sequence analysis, gene expression analysis, and correlate ABA levels throughout embryogenesis with gene expression levels.

Objective 2: The isolation and characterization of a suite of genes controlling root development: SHORT-ROOT (SHR), SCARECROW (SCR), HOBBIT (HBT), WOODENLEG (WOL), MONOPTEROS (MP), and BODENLOS (BDL). We will isolate the cDNAs from pine embryos, complete sequence analysis, gene expression

analysis, and a comparison of germination data with the gene activities throughout development.

Objective: 3: Illustration of differences in Pine and *Arabidopsis thaliana* programs of gene expression by comparing differences in both mRNA expression and protein expression between the two.

Chapter 2

ISOLATION AND EXPRESSION ANALYSIS OF ABA INSENSITIVE GENES DURING EMBRYOGENESIS IN LOBLOLLY PINE

Summary

Although the hormone ABA has been demonstrated to be required for proper development of somatic embryos in conifers [52-54], very little has been demonstrated about the effects of ABA accumulation on gene expression during embryogenesis in conifers. In this study, we have attempted to identify pine orthologs of three transcription factors known to mediate ABA responses in *Arabidopsis thaliana*. Three putative ABA responsive genes were identified in the Pine EST databases established from mature plants. These genes were subsequently isolated and cloned from Loblolly pine embryos. These isolated clones were sequenced and analyzed to reveal significant homology to the known *Arabidopsis* ABA responsive genes ABI3, ABI4, and ABI5. Full-length cDNAs were isolated and cloned for ABI4. Sequence analysis was completed for all isolated clones. Putative conserved domains were found within the deduced amino acid sequences. Expression analyses of all three genes were completed throughout mid to late embryogenesis in Loblolly pine. These expression analyses were compared to reported data for ABA accumulation, as well as, expression of other ABA responsive genes, during the same stages of embryogenesis. This data provides novel information that can

be compared with somatic embryos to determine differences between zygotic and somatic embryogenesis.

Introduction

Seed dormancy occurs when germination of a healthy seed is blocked under favorable conditions for further development and growth. The process of seed dormancy exists within all higher plants no matter in what climate they live [109]. A mature embryo remains dormant until it senses the soil is ready to sustain seedling growth, at which point, it breaks through the seed coat to continue development [90]. Mechanical restriction by the seed coat can contribute to prevent early germination of the plant [110]. Since the timing of germination is critical, there are multiple control mechanisms to prevent premature or "precocious" germination and development. Other mechanisms controlling seed dormancy and germination include environmental cues, such as,- temperature, light, nitrates, as well as, hormones [109, 111].

During dormancy there is typically a high ratio of the hormone abscisic acid (ABA) to gibberellins (GA) within the seed. During this period, the seed will have high sensitivity to ABA and low sensitivity to GA. To leave the state of dormancy the seed must reverse the hormone concentration to a low ABA: GA ratio. It must also increase its sensitivity to GA while lowering its ABA sensitivity [110, 112]. The high ABA concentration is required for both the initiation and maintenance of dormancy in embryos [113-117]. Embryos with inconsistent ABA production are reportedly unable to enter a dormant state. In contrast, embryos with increased ABA concentrations exhibit enhanced

seed dormancy and delayed germination [67, 70, 118]. Mutations in genes that control responses to the hormone abscisic acid (ABA) remove this blocking mechanism, and promote early germination and development of plants that are not ready to sustain life [85]. Mutations in the ABA responsive *Arabidopsis thaliana* genes, ABI3, ABI4, and ABI5, cause reduced sensitivity to the hormone ABA and premature germination [70, 77, 85, 87, 119].

ABA has been reported to play a vital role in promoting maturation in both zygotic and somatic Loblolly pine embryos [120]. Early stage conifer somatic embryos require the addition of 20- 50 μM exogenous ABA in order to develop into cotyledonary stage embryos [52, 57, 120]. In Loblolly pine, the concentration of ABA in zygotic embryos was found to increase during the formation of mature zygotic embryos [52, 79]. There was an initial peak in ABA concentration between stages five and seven. This was followed by a second peak in stage 9.4 [52, 68, 79]. Vales et al. (2007) were able to elevate exogenous ABA during the mid maturation period in zygotic embryos. Additions of ABA during this specific period of development resulted in increased germination and more zygotic-like gene expression in the somatic embryos. The changes in ABA concentration caused an increase from 22 % to 66% in the amount of embryos able to develop to the cotyledonary stage [52].

LEA Gene Expression as it Correlates with ABA Expression in Loblolly Pine

Until recently, not much was known concerning how ABA levels in Loblolly pine embryos affects gene expression. In the model plant *Arabidopsis thaliana*, the expression of Late Embryo Abundant (LEA) genes is controlled by the coordinated actions of transcription factors responding to ABA levels [70]. Three LEA genes

isolated from pine were named LPZ 202, LPZ 216 and LPS 094 [52]. These LEA genes were found to be inducible by the hormone ABA during development in Loblolly pine zygotic embryos [52]. All three genes exhibited similar expression patterns during normal embryo development. There was an increase in expression around stage 5. Their expression levels remained high until stage 9.1, then decreased and remained low from stages 9.2 to 9.4. Their expression level increased during late embryogenesis from stages 9.5 -9.10 (Figure 2.1). The pattern of expression of these isolated LEA genes correlated well with ABA expression in Loblolly pine embryos [52, 68, 79].

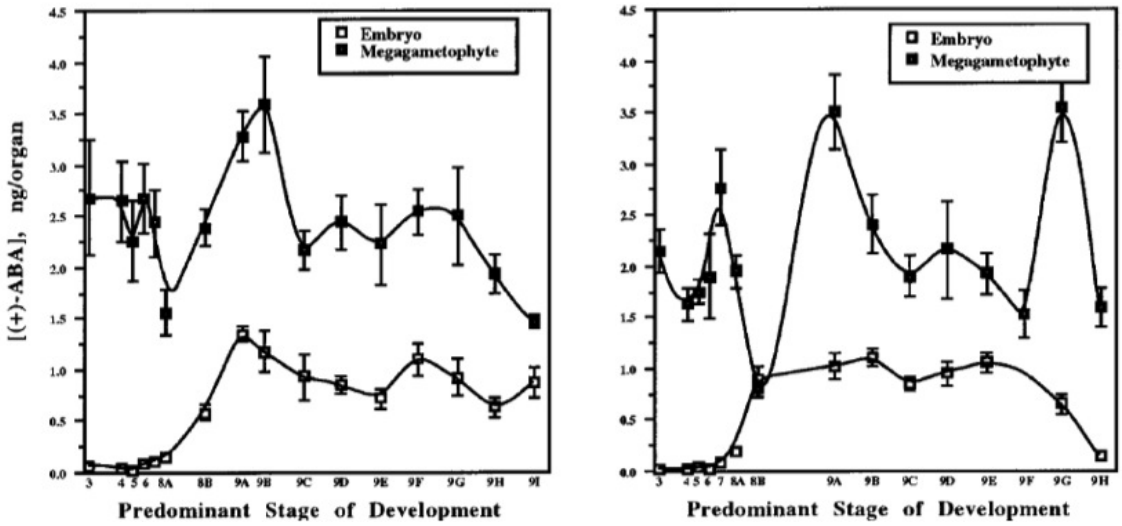


Figure 2.1 **ABA content of Loblolly embryos and megagametophytes [52, 79].**
ABA content in two different Loblolly pine genotypes was determined. Shown above are Tree UC (left) and WV (right) from stage 3 to cone ripening. There is a biphasic pattern of expression seen within the embryo.

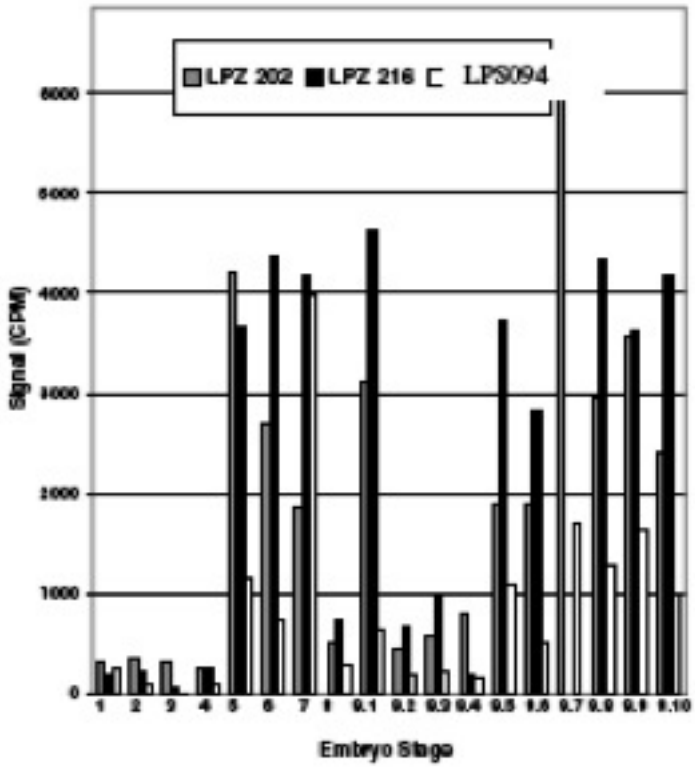


Figure 2.2 LEA gene Expression throughout Embryogenesis [52].
Steady –state mRNA levels of three loblolly pine LEA genes (LPZ202, LPZ216, and LPS094) in zygotic embryos from stages 1 to 9.10.

Plant growth regulators are now added to maturation media of Loblolly pine somatic embryos in an attempt to mimic the natural environment of the developing zygotic embryos [17]. Positive physiological effects from the addition of ABA include: inhibition of cleavage polyembryony and suppression of early germination. Expression of ABA during the maturation phase has also been correlated with the accumulation of storage proteins, lipids, starches and some LEA proteins related to desiccation tolerance [52].

ABA has been demonstrated to be important to the proper development and maturation of somatic embryos, and the genes ABI3, ABI4 and ABI5 are essential to proper responses to ABA accumulation in *Arabidopsis thaliana* [72, 77, 78, 85, 87, 90]. Although the expression of the ABA responsive genes, ABI3, ABI4 and ABI5 has not been characterized in Loblolly pine embryos thus far, our hypothesis is that each of these genes should be expressed in pine embryos, and their expression levels should follow a similar biphasic pattern of expression seen in both ABA expression and LEA gene expression in Loblolly pine embryos. To test this hypothesis we first identified these genes in a pine EST database. We then cloned ABI3, ABI4 and ABI5 cDNAs from Loblolly pine embryos. We completed sequence analyses of these ABA insensitive genes, comparing them to their *Arabidopsis* orthologs. We then assayed their levels of expression in zygotic Loblolly pine embryos throughout development. The knowledge gained from profiling these genes in Loblolly pine embryos will assist in developing improved techniques for somatic embryogenesis.

Materials and Methods

Database Search

Basic Local Alignment Search Tool (BLAST) searches were performed to find homology between available plant ABI3, ABI4, and ABI5 gene sequences and the pine EST sequences available at http://www.fungen.org, http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine, and http://dendrome.ucdavis.edu/adept/. Several sequences were identified (Table 2.6). PCR primers were designed against various regions using IDT PRIMERQUEST

(http://www.idtdna.com/Scitools/Applications/Primerquest/) program. The primers were ordered from Integrated DNA Technologies (San Diego, CA).

Plants and Embryo Tissue

Loblolly pine embryos and female megagametophytes are available in Dr. Pullman's laboratory at the Institute of Paper Science at Georgia Institute of Technology. Seeds were isolated from pinecones harvested weekly from open-pollinated loblolly pine mother tree 7-56. Cone collection took place from 7/01/04 until 10/15/04 in a commercial pine orchard in Lyons, Georgia, US.

Megagametophytes were removed from the seeds. The megagametophytes were dissected under the microscope to excise the whole embryos, which were then flash frozen in liquid nitrogen. Embryos were staged according to the staging system developed by Pullman and Webb 1994 [121]. RNA was isolated as described below.

RNA Isolation

Total RNA was isolated from embryos using the RNAeasy Kit (Qiagen) from stages 7 – 9.12 of pine embryo development. Purified RNA was aliquoted and stored at -80° C.

Reverse Transcription and Polymerase Chain Reaction

Reverse transcription is performed on 500ng RNA using a RACE (rapid amplification of cDNA ends, [122]) kit purchased from Clontech, (Mountainview, CA) to synthesize first-strand cDNA. The resulting first-strand cDNA contains adaptors ligated to the 5' end of the cDNA. Two gene-specific primers were designed within the 3' UTR. 5` RACE reactions were performed using the universal primer mix as a forward primer

and Advantage2 polymerase mix from Clontech(Mountainview, CA) [122]. All primers are listed in Table 2.1, Table 2.2, Table 2.3, Table 2.4, and Table 2.5. The total volume of the pcr mixture was 50µl containing 2.5µM of each dNTPs, 1X PCR buffer, 1 unit of advantage Taq polymerase, 1 µM of the gene-specific primer and 1µM of universal primer mix (UPM) or the nested universal primer mix (NUP). PCR amplification took place under the following conditions: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, T_m of primer for 30 seconds, and 72°C for 2 minute; followed by 72°C for 10 minutes and 4°C hold. PCR reactions were performed using an Eppendorf (Westbury, NY) thermocycler, model 5331 gradient master cycler.

DNA Purification, Cloning and Sequencing

The PCR products were seperated on a 1.5% agarose gel to verify size. The PCR products were gel purified using a QIAquick PCR purification kit from Qiagen (Valencia, CA) and the resulting cDNA fragments were cloned into a pGEM-T Easy vector system from Promega© (Madison, WI). The product sizes were confirmed by running colony PCR [123]. Selected colonies were grown in bacterial culture, and plasmids were purified using Promega's Wizards SV miniprep kit. The purified plasmids were then sequenced and analyzed.

Real-Time PCR

Primer Design

Real-time PCR was used to study PtABI3, PtABI4 and PtABI5 gene expression in different stages of Loblolly pine embryos [124]. Primers for real-time pcr were designed

with IDT PRIMERQUEST software (http://www.idtdna.com/Scitools/Applications/Primerquest/). The primers were designed so that the pcr products were less than 150 base pairs in length. The designed primers are listed in Table 2.1, Table 2.2 and Table 2.5

Reverse Transcription

Total RNA was freshly isolated using the RNAeasy kit from Qiagen. Isolated RNA was quantified using a UV spectrophotometer, and the measurements were verified using a ribogreen RNA quantification system with a flourometer. RNA quantification was completed using standard RNA from the Ribogreen RNA assay kit (Invitrogen, Camarillo, CA) and a RNA standard curve [124]. Reverse transcription was completed using Applied Biosystems Taqman Reverse Transcription Reagents kit (Foster City, CA) and 400ng of RNA was used for each RT reaction.

Polymerase Chain Reaction

Real-time PCR primers from Integrated DNA Technologies were diluted to 100 µM concentrations in RNAse-and DNAse-free water. Stock solutions were stored at -20°C. Working solutions were diluted to 45 µm final concentration. Equal amounts of the forward and reverse primers were mixed for the real time PCR reactions. ABI Prism micro amp optical tubes and caps were used to set up the PCR reaction. The Sybergreen® master mix from Applied Biosystems (Foster City, CA) was used for the PCR reactions. The mix used for the reaction consisted of: (1) 2 µl cDNA from RT reaction (2) 7 µl water, (3) 10.0 µl SYBR® green, and (4) 1.0 µl primer mix. Reactions were performed in triplicate. The tubes were placed in the PCR rack, and covered with

foil due to the light sensitivity of Sybergreen. 18S RNA was used to normalize the reaction. The traditional real-time pcr program was used: (1) 15s at 94 °C and (2) 1 minute at 60°C for 40 cycles [125]. Amplification was followed by a thermal denaturing step to verify the specificity of the amplification. The products were cloned and sequenced using the same protocol discussed above.

Comparison of RNA expression of RNA expression at different stages was based on the comparative C_t method ($\Delta\Delta C_T$). The relative mRNA expression is quantified using the following formula: $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_{T\ sample} - C_{T\ reference\ RNA}) - (C_{T\ calibrator} - C_{T\ reference\ RNA})$ [126]. Stage 7 tissues were used as a calibrator. This calibration allows the comparison of all samples to a single standard [126].

Bioinformatics

Orthologous proteins were found by searching NCBI's database with the blastx tool. Open reading frames and deduced amino acid sequences were determined by using NCBI's ORF finder program. ClustalW and Boxshade programs from The Biology Workbench (http://workbench.sdsc.edu/) were used to produce alignments and view the alignments [127, 128].

Table 2.1 Primers designed for PtABI3 gene amplification.

Name	Tm	Sequence	Binding site/ Use
PtABI3F	58.6 ° C	CGAGTCAGGCGGTTGGAGTA	Used for real time pcr. Will create a product of 66 bp
PtABI3R	60.4 ° C	CCGACACCGAAGATGAGGA	Used for real time pcr. Will create a product of 66 bp
abi3racef1	61.2 ° C	CACCATAACAGGTCCGTGGAGGAGAGTC	Forward primer; used for 3' RACE
abi3racef2	60.7 ° C	CGTTCCCAAGGTGTATGTAGGCGTTC	Froward inner primer used for nested pcr in 3' RACE

Table 2.2 Real time PCR primes designed for PtABI4 gene amplification.

Primer Name	**Tm**	**Primer Sequence 5'-3'**	**Binding Location and Use**
ABI4SYBF	58.2° C	CAGACAGCCCGGAAAACG	Forward primer used for real time PCR; The product size is 60 bp.
ABI4SYBR	60.5° C	GCCTGCTCGGCTGTAGCA	Reverse primer used for real time PCR. The product size is 60 bp.

Table 2.3 Reverse primers designed for PtABI4 gene amplification.

Primer Name	Tm	Primer Sequence 5'-3'	Binding Location and Use
ABT7c	62.7 ° C	ATCATCTCTCGATTCTCCCTCGGT	Reverse primer binds at 817bp. Used for 5' RACE
AB45RACE	69.5 ° C	AATTGCAGCCCTTCTTTGACGCAGGCCC	REVERSE PRIMER 1055 bp Used for 5' RACE
AB45RACE2	67.9 ° C	TCGGTCTCGGGCAACGTGCTGCTTT	Reverse primer binds at 950 bp. Used for 5' RACE
AB4T7	62.7 ° C	AGGATTCCCATCATCTCACCTTGG	Reverse primer binds at 1160 bp. Used for 5' RACE
AB4T7B	62.7 ° C	GTGAATTGCAGCCCTTCTTTGACG	Reverse primer binds at 1058 bp. Used for 5' RACE
ABI4R3	62.3 ° C	CGGTCCGTGAATTGCAGCCC	Reverse primer binds at 1218 bp. Used for 5' RACE
ABI4R1	56.2 ° C	TGGAAAGCATGTACAATTTGTGAAG	Reverse primer binds at 1518 bp. Used to isolate complete 3' end.
ABI4R2	59.7 ° C	TCCCATCATCTCACCTTGGCA	Reverse primer binds at 1309 bp

Table 2.4 Forward Primers designed for PtABI4 gene amplification.

Primer Name	Tm	Primer Sequence 5'-3'	Binding Location and Use
AB4T3A	62.7 ° C	TGGTTGGGATCAGTCCAAATCCTC	Forward primer binds at 445 bp. Used for 3' RACE.
AB4T3B	62.7 ° C	CGGAAGTACTGCATCGTGAATCCT	Forward primer binds at 765 bp. Used for 3' RACE
AB4T3A	62.7 ° C	TGGTTGGGATCAGTCCAAATCCTC	Forward primer binds at 445 bp. Used for 3' RACE.
AB4T3B	62.7 ° C	CGGAAGTACTGCATCGTGAATCCT	Forward primer binds at 765 bp. Used for 3' RACE
ABI4L1	64.5 ° C	GCGGGGGAAGGGAGGAAGGA	Forward primer binds at 1bp. Used for 3' RACE
ABI4L2	61.9 ° C	CGGGGGAAGGGAGGAAGGAA	Forward primer binds at 2 bp. USED for 3' RACE
ABI4L3	63.8 ° C	CACACCTGCACCGCCCTCCA	Forward primer binds at 198 bp. Used for 3' RACE

Table 2.5 Primers designed for PtABI5 gene amplification.

Name	Tm	Sequence	Binding Site/ Use
PtABI5F	65.2 ° C	TGGTGCTGGATTGGGTGG	Used for real time PCR; Will create a 65 bp product.
PTABI5R	60.1 ° C	TAGTAGGCGATCCTGCTCCG	Used for real time PCR; Will create a 65 bp product.
ab55'race2	57.6 ° C	AGATCCTGGAGCAAAGCCCACAGAGCAAT	Reverse primer. Used in 5' race nested reaction
ab53'race2	65.4 ° C	TTTCTGCCATTTCCGCCGGTTGGTTGCTT	Forward primer. Used in 3' race nested reaction.
ab53'race1	63.7 ° C	ATTGCTGTGGCTTTGCTCCAGGATCT	Forward primer. Used in 3' Race first reaction.

Results

ABI3, ABI4 and ABI5 –like Genes are Expressed in Loblolly Pine and Contain Highly Conserved Domains

To identify the ABA responsive genes, ABI3, ABI4, and ABI5 in Loblolly pine, publically available pine EST databases were searched using the *Arabidopsis* gene sequences as queries. Clones were identified in the Pine EST databases located at http://www.fungen.org, http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine, and http://dendrome.ucdavis.edu/adept/ (Table 2.6).

Table 2.6 Pine expressed sequenced tags identified with sequence similarity to ABA responsive genes in *Arabidopsis*. These ESTs can be located at http://www.fungen.org, and http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine

Gene	Pine EST identified
ABI3	TC142582 TC103460
ABI4	TC115273 TC56384 TC62108 TC81088
ABI5	RTDS1_15_C02_A015 FLD1_42_D07.g1_A029

Identification, Isolation and Sequence Analysis of PtABI3

Pine EST clones TC142582 and TC103460 were identified by searching the pine EST database using AtABI3 gene as query sequence. The ABI3-like sequence derived from ESTs was 979 nucleotides in length. Because the sequence was isolated from mature Loblolly pine tissues, primers were designed to determine if the gene was expressed in pine embryos during development and to verify sequence information. Designed primer sequences are located in (Table 2.1). We completed 3' RACE PCR reactions, and a fragment of the expected size was amplified using RNA isolated from Loblolly pine embryos derived from mother tree 7-56 as template. The amplified fragment was cloned and sequenced. The isolated sequence was 1232 nucleotides in length. The sequences were analyzed by using the blastx program on the NCBI website to find sequence similarity with known proteins from other plants. The highest match was found with the Picea abies VIVIPAROUS 1 protein (VP1), and ABI3 ortholog, with a score of 490, and an E-value of $9e^{-137}$. The best match received with Arabidopsis thaliana was the ABI3 protein with a score of 246, and an E-value of $4\ e^{-46}$ (Table 2.7).

The deduced amino acid sequence of PtABI3 was determined and aligned with both the AtABI3 and PaABI3 proteins (Figure 2.5). The B3 domain belonging to the plant super family of transcription factors was identified within the deduced amino acid sequence (Figure 2.6).

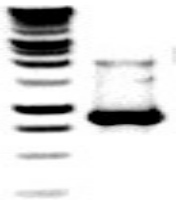


Figure 2.3 Isolation of PtABI3-like cDNA from Loblolly pine zygotic embryos.
RT-PCR was completed using RNA from mother tree 7-56. The PCR products were loaded onto a 1.5% gel, and ran alongside a 1-kilobase ladder from Promega®. The band migrated to approximately 1,000 base pairs when compared to the Promega® DNA marker.

Table 2.7 Blast results of pine ABA Insensitive-like genes.
Isolated pine sequences were used to query against the NCBI database using the blastx program. The results show the deduced amino acid sequence of isolated clones has similarities with ABA responsive genes in other plants. The asterisk (*) signifies the full-length sequence of the PtABI4 cDNA isolated, including the 3' and 5' UTR.

Clone	Best Match	Score	E-value	% Similarity	% Identity	Organism matched
PtABI3	Viviparous 1	490	9e-137	84%	79%	*Picea abies*
	ABA Insensitive 3	246	4e-65	68%	61%	*Arabidopsis thaliana*
PtABi4*	ABA Insensitive 4	135	1 e-34	60%	69%	*Arabidopsis thaliana*
PtABI5	ABA Insensitive 5	149	2 e-36	70%	59%	*Arabidopsis thaliana*

Figure 2.4 Deduced amino acid sequence and ORF of PtABI3
The analysis was completed using NCBI's ORF FINDER program.

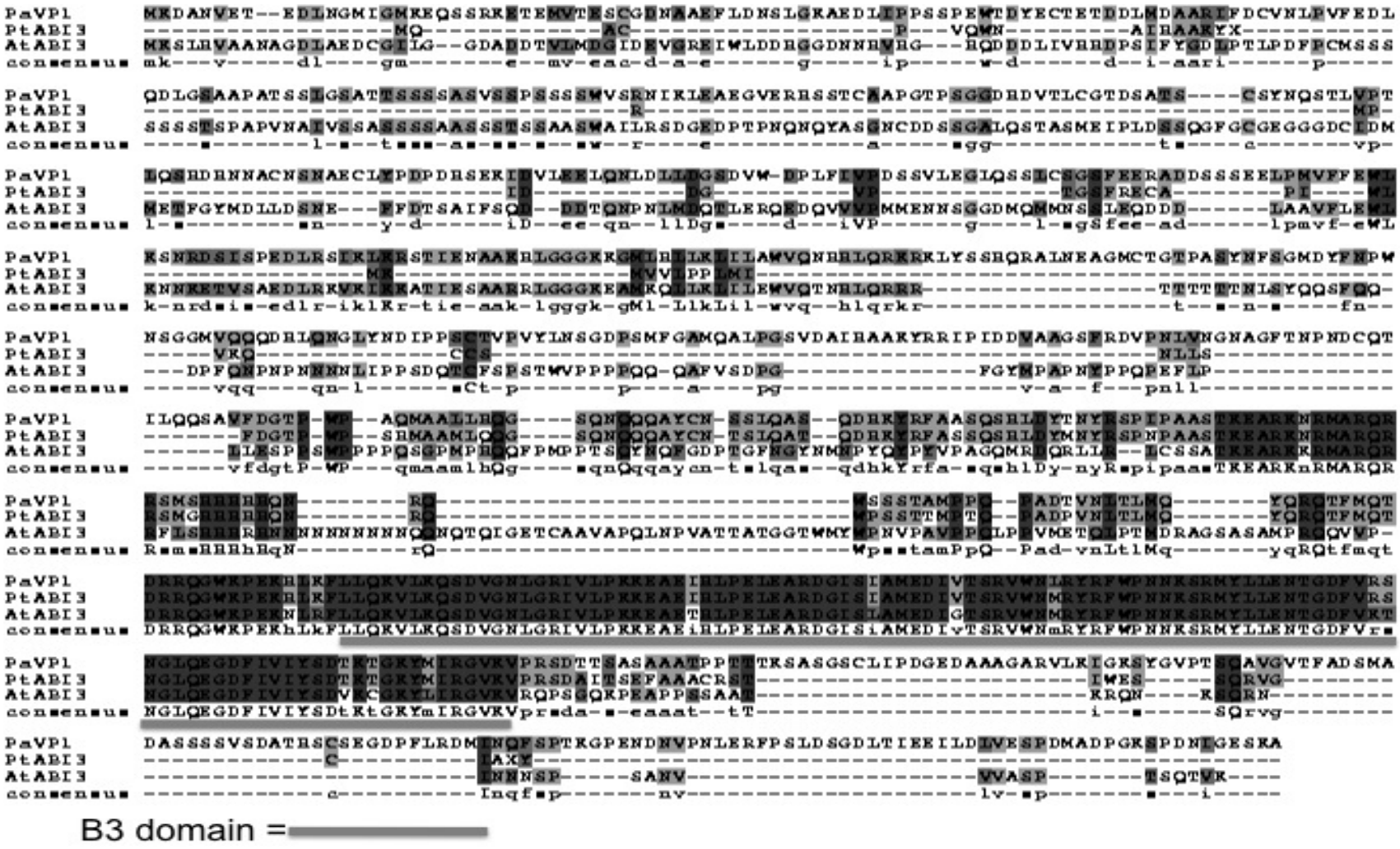


Figure 2.5 Alignment of the pine ABI3-like deduced amino acid sequence against Norway spruce VP1 and *Arabidopsis* ABI3.

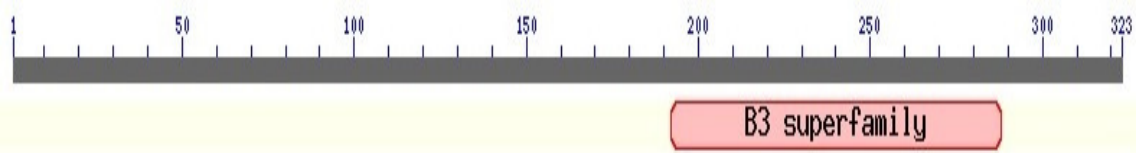


Figure 2.6 PtABI3-like deduced amino acid sequence contains the highly conserved B3 domain.

Identification, Isolation and Sequence Analysis of PtABI4

A BLAST search of the PINE EST databases with the *Arabidopsis* ABI4 sequence identified pine clones TC81088, TC115273, TC56384, and TC62108. These sequences were assembled to produce PtABI4. Primers were designed in an attempt to isolate the PtABI4 mRNA in pine embryos. A fragment of the desired length was isolated using RT-PCR. The product was cloned and sequenced. The cloned sequence was used to complete 5' RACE in an attempt to determine the 5' UTR of the mRNA. The PCR product from the 5' RACE was cloned and sequenced. Finally, 3' RACE was used to generate cDNA clones containing the 5'UTR, protein coding sequence and the 3' UTR (Figure 2.7). The complete cDNA was 1,518 nucleotides long. The open reading frame was 1,013 nucleotides long (Figure 2.9). A list of the primers used for both 5' and 3' PCR are available in Table 2.3 and Table 2.4. Six full-length clones were independently isolated and sequenced. The sequences attained were compared and determined to be virtually identical. The predicted protein shows strong sequence conservation within the AP2 domain (Figure 2.8). Alignments between the deduced amino acid sequence of PtABI4 and AtABI4 show that they are highly similar, especially within the AP2 domain (Figure 2.10).

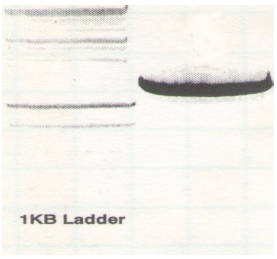


Figure 2.7 PCR amplification of PtABI4-like cDNA from zygotic embryos.
RT-PCR was completed using RNA from mother tree 7-56. The pcr product migrated to approximately 1.5 kilobases on a 1.5% gel.

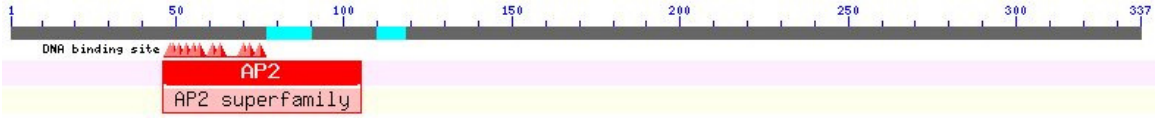


Figure 2.8 PtABI4 contains the conserved AP2 domain.
The PtABI4 deduced amino acid sequence contains the APETALA 2 conserved domain. This was determined using NCBI's conserved domain database.

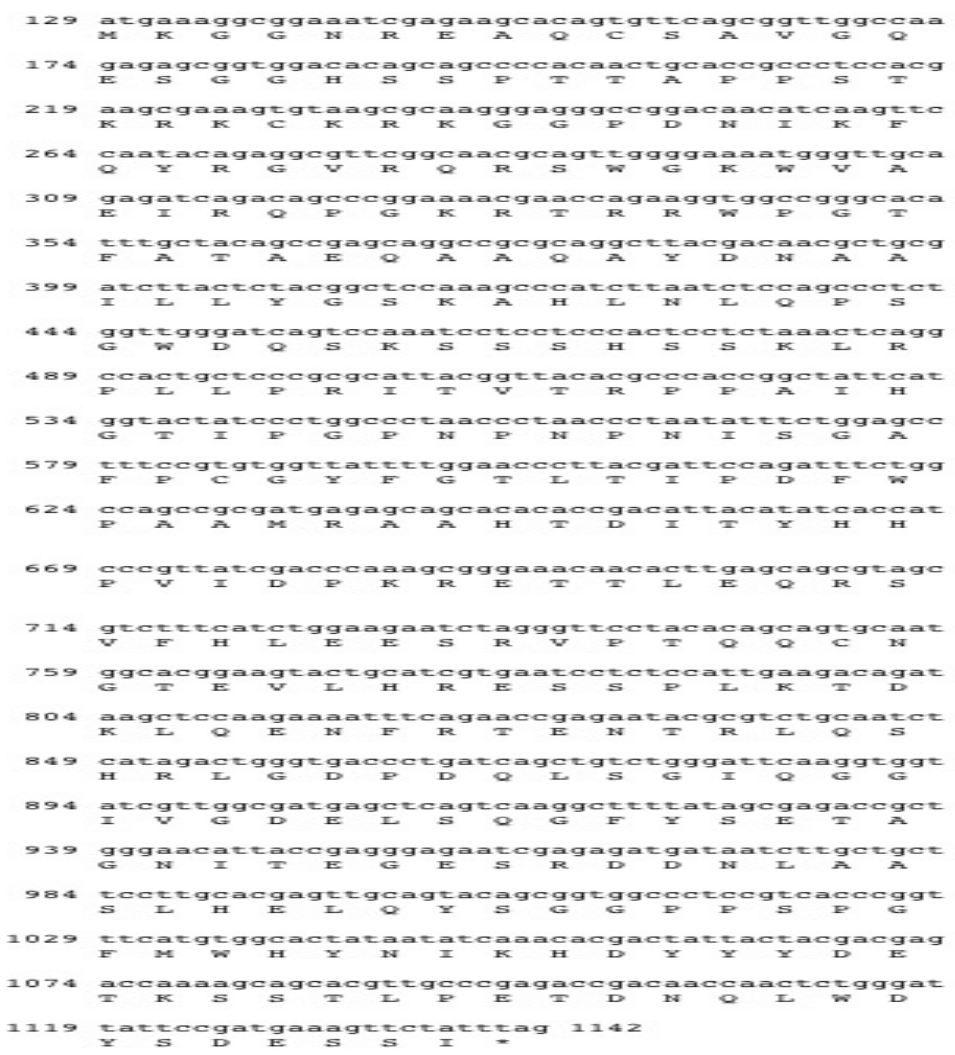


Figure 2.9 PtABI4 open reading frame and deduced amino acid sequence. The open reading frame contains 1013 nucleotides.

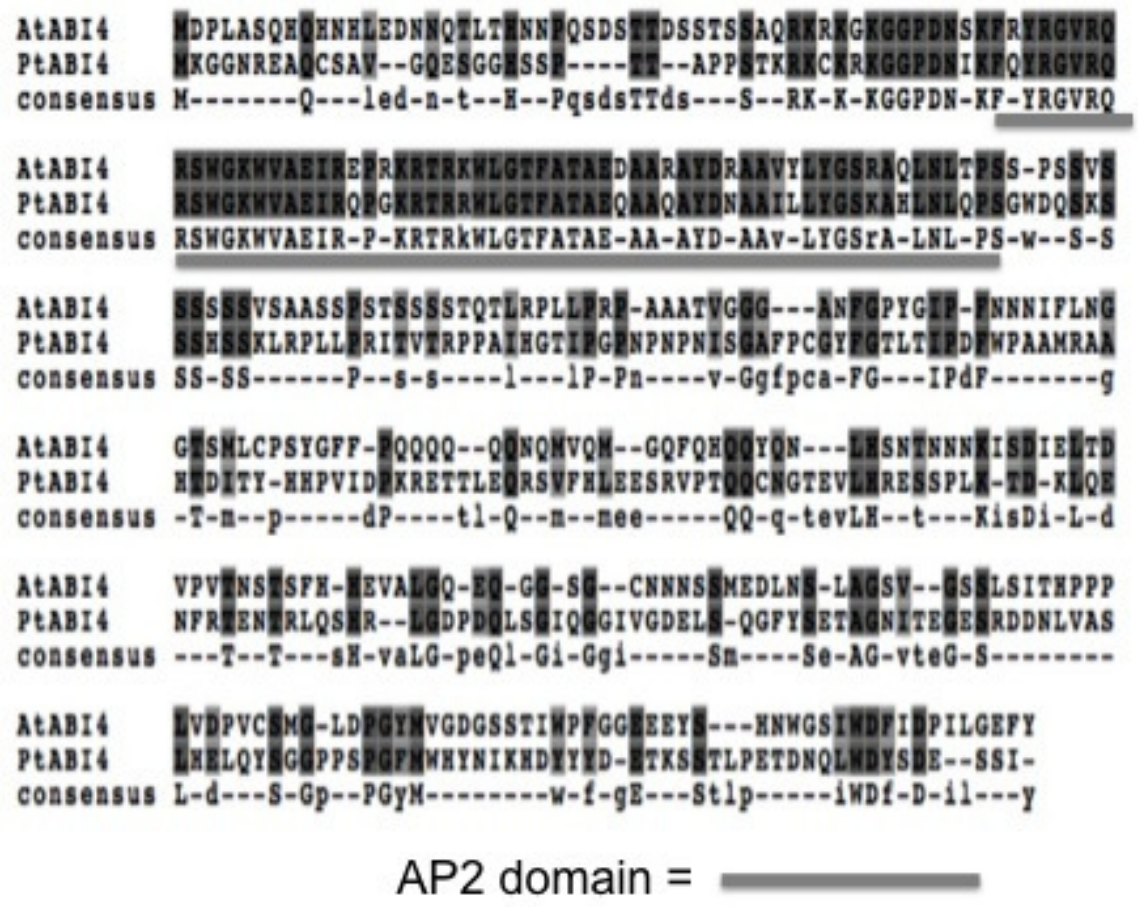


Figure 2.10 The deduced amino acid sequence of PtABI4 is similar to AtABI4.
Alignment of the deduced amino acid sequence of the PtABI4-like clone with the protein sequence of ABI4 from *Arabidopsis thaliana*. The sequences are similar, especially within the conserved AP2 domain.

Identification, Isolation and Sequence Analysis of PtABI5

We identified EST sequences RTDS1_15_C02_A015 andFLD1_42_D07.g1_A029 by searching the pine EST database using the AtABI5 mRNA a query sequence in the blastx program. The identified ESTs were isolated from drought stressed Loblolly pine roots of mature plants (Table 2.6). These sequences were assembled to produce PtABI5, and then

translated to an amino acid sequence. The identified EST was 417 nucleotides in length. Primers were designed to determine if, in fact, ABI5 was expressed in loblolly pine embryos and to verify the sequence. A 3' RACE PCR was completed using RNA isolated from Loblolly pine embryos and primers designed from the EST previously identified. The primer sequence is listed in Table 2.5. The fragment was observed in Loblolly pine embryos (Figure 2.11). Sequencing of the PtABI5 clone confirmed it was identical to the previously discussed EST from the UGA EST database. This PtABI5 clone was 1295 nucleotides in length. Sequence analysis confirmed the presence of a bZIP domain within the deduced amino acid sequence.

The NCBI blastx program was used to search for sequence similarity with PtABI5. The highest match was found with ABI5 in Arabidopsis thaliana, with a score of 149 and an E-value of 2 e-36. There were 70% similar amino acid residues and 59% identical amino acid residues found within the deduced amino acid sequence as compared to the Arabidopsis sequence (Table 2.7). The predicted protein shows strong sequence similarity within the bZIP domain (Figure 2.12 and Figure 2.13).

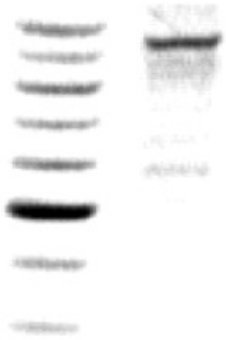


Figure 2.11 Isolation of PtABI5-like cDNA via RT-PCR.
A RT-PCR was completed using RNA isolated from all stages of Loblolly pine embryos from mother tree 7-56. Product is seen (on right) above 1kb. It was cloned and sequenced. A 100 bp DNA marker from Promega is shown in the left lane.

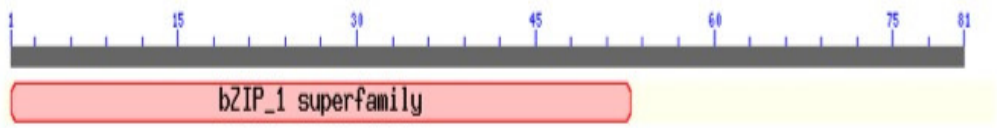


Figure 2.12 PtABI5-like clones contain the bZIP-conserved domain.
The deduced amino acid sequence of PtABI5 contains the conserved leucine zipper domain contained with transcription factors. This was determined using NCBI's conserved domains and protein classification software.

Figure 2.13 PtABI5 is highly similar to AtABI5.
Shown above is an alignment of the deduced amino acid sequence of PtABI5 with the ABI5 protein from *Arabidopsis thaliana*. These proteins are highly similar both inside and outside of the conserved leucine zipper domain.

Differential Expression of ABI3, ABI4 and ABI5 during embryogenesis

We determined the expression pattern of these ABA insensitive genes throughout late embryo development because the expression of ABI3, ABI4 and ABI5 in *Arabidopsis thaliana* has such significant effects on the potential of the plants for growth and development during late embryogenesis and germination [80, 87, 129]. To determine the expression patterns of these genes during embryogenesis, quantitative PCR was performed using primers specific for each gene. Primer locations are shown in Table 2.1, Table 2.2 and Table 2.5. Expression during tissue stages 7 – 9.12 was observed throughout these experiments. 18S rRNA expression was used to normalize all data.

Fold differences in expression were determined by comparing all stages to expression in stage 7.

ABI3 expression appeared to be stable from stages 7 -9.3. A nearly fifty-fold increase in ABI3 expression was observed in stage 9.4 with a sharp decrease seen in stage 9.5. This was followed by a 300-fold increase in ABI3 expression seen in stage 9.6 followed by a drastic decrease. The expression of ABI3 increased by nearly 100 fold in stage 9.12 (Figure 2.14).

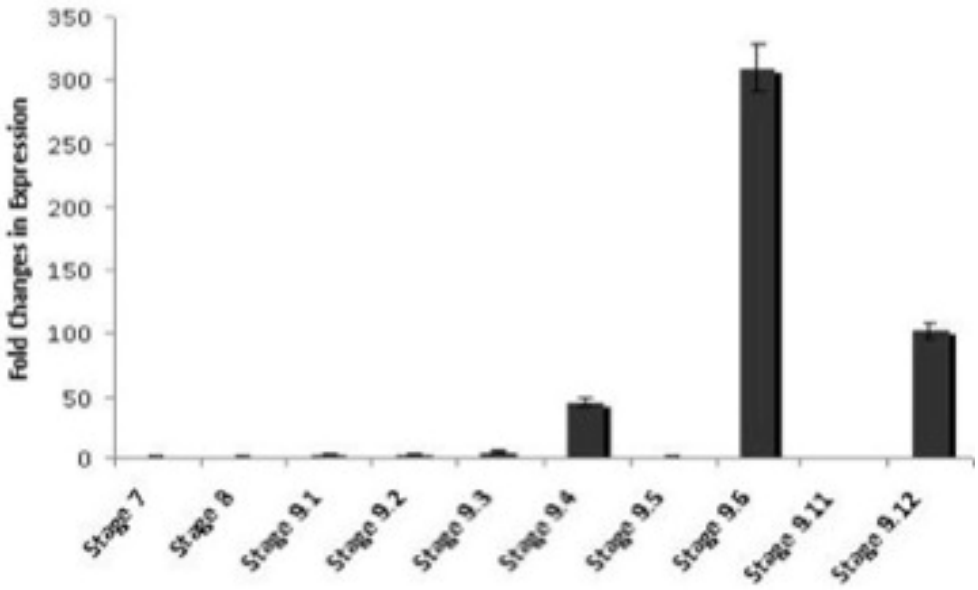


Figure 2.14 Expression of PtABI3-like in Loblolly pine zygotic embryos.
Quantitative PCR was completed on RNA isolated from embryos collected from mother tree 7-56 in 2003. Expression of PtABI3-like mRNA is constant and stable throughout late embryogeny except for three stages. Three peaks in expression are seen at stages 9.4, 9.6 and 9.12. Standard error bars are shown for each data point containing three replicates.

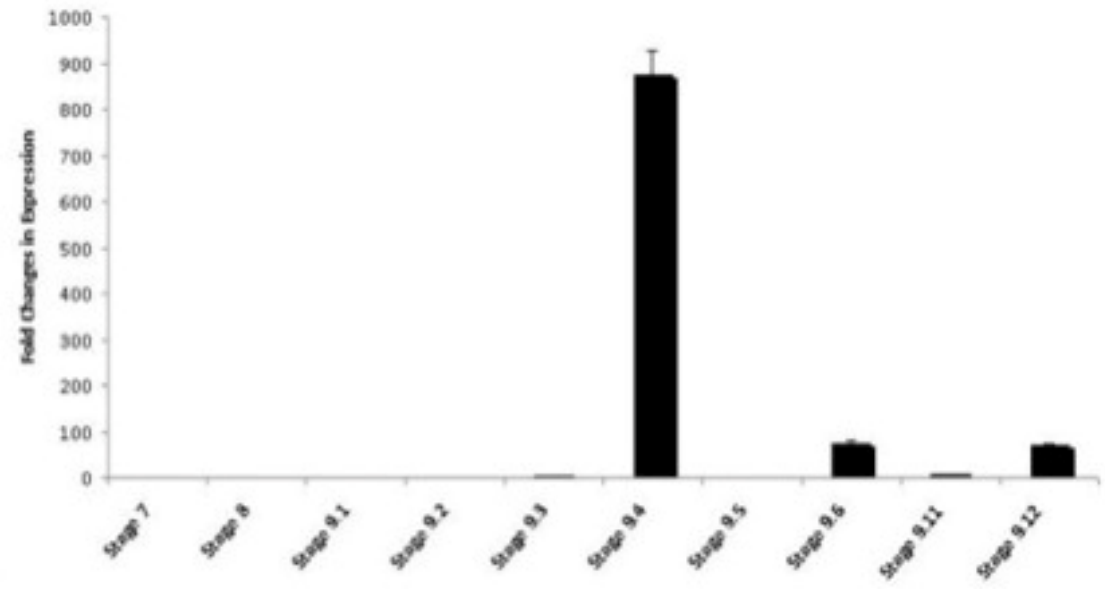


Figure 2.15 Expression of PtABI4-like mRNA in Loblolly pine zygotic embryos. Quantitative PCR was completed on RNA isolated from embryos collected from mother tree 7-56 in 2003. Expression of PtABI4-like mRNA is constant and stable from stages 7 – 9.3. There is a drastic increase in expression seen in stage 9.4 followed by a dramatic and immediate increase in stage 9.5. Two more small changes (less than 50-fold) are seen in stages 9.6 and 9.12. Standard error bars are shown for each data point containing three replicates.

ABI4 expression also appeared to be stable from stages 7 – 9.3. There was a 900fold increase seen in expression of ABI4 in stage 9.4. ABI4 expression then falls drastically back to zero in stage 9.5. A similar pattern to ABI3 expression is followed where we se an increase in ABI4 expression in stage 9.5 followed by decrease then another increase in expression in stage 9.12. However, these changes in expression are in much smaller increments than seen with ABI3 expression (Figure 2.15).

ABI5 expression appeared to be stable from stages 7 – 9.5. This is followed by a sharp 500-fold increase in ABI5 expression at stage 9.6. Its expression level then drops, followed by a rapid, greater than 100-fold increase in stage 9.12 (Figure 2.16).

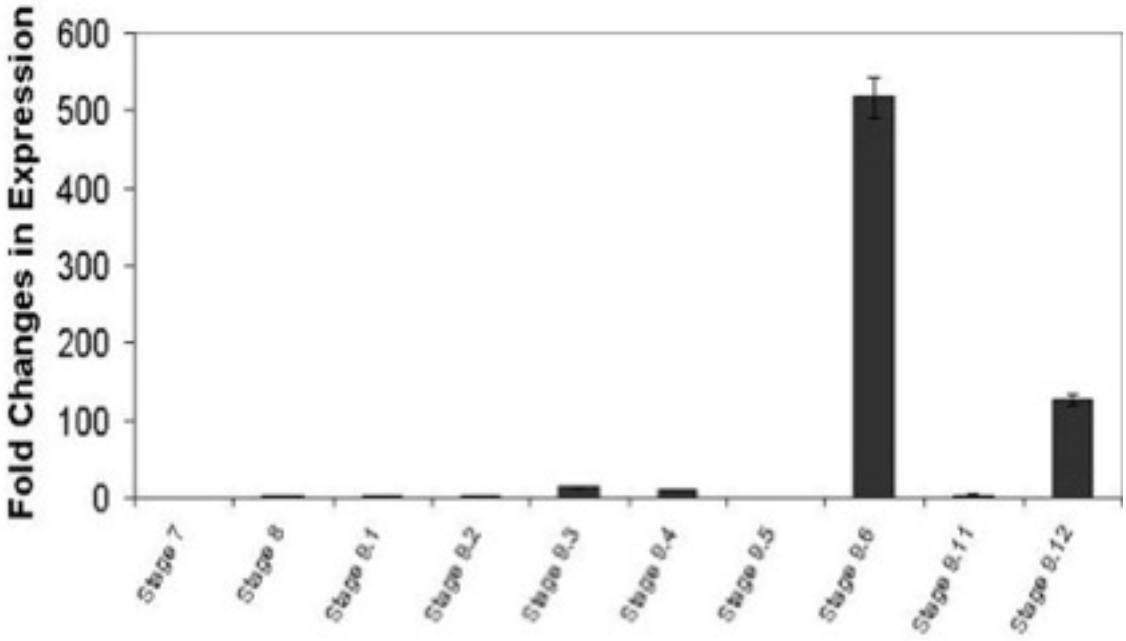


Figure 2.16 Expression of PtABI5 in Loblolly pine zygotic embryos
Quantitative PCR was completed on RNA isolated from embryos collected from mother tree 7-56 in 2003. PtABI5-like Expression is stable and constant from stages 7 -9.5. There is an initial peak in expression seen in stage 9.6 followed by a decrease in expression. There is another increase in expression seen in stage 9.12. Standard error bars are shown for each data point containing three replicates.

Discussion

While the mechanisms of ABA signaling have been thoroughly reported in *Arabidopsis*, there is not a significant amount of information on ABA signaling in the agricultural plant, Loblolly pine [77, 78, 87]. More specifically, there is lack of information on how ABA signaling affects embryogenesis in Loblolly pine. Moreover, there is not yet a significant amount of genetic evidence indicating how expression of ABA responsive genes relates to ABA accumulation in pines. Here we identified and isolated three ABA insensitive genes, PtABI3, PtABI4, and PtABI5 that are expressed in loblolly pine zygotic embryos and correlate their expression with ABA expression in pine. Blast searches confirm high sequence similarity between these genes and their *Arabidopsis* orthologs.

The isolated PtABI3 cDNA contains the B3 domain of transcription factors present in both its homologues AtABI3 and VP1 of Norway spruce. The B3 domain is a DNA binding domain found exclusively in transcription factors in higher plants [66, 71, 83, 130]. The deduced amino acid sequence of the isolated PtABI4 cDNA contains the conserved AP2 domain. The AP2 domain is present in the AtABI4 gene, belongs to a family of transcription factors, and has shown to be required for DNA binding [78, 87]. The deduced amino acid sequence of the isolated PtABI5 cDNA contains the conserved bZIP domain. The bZIP domain has been demonstrated to be used for both DNA binding and dimerizaton of proteins [69, 77, 84]. The data from the sequence analysis suggests that all three isolated Loblolly pine cDNAs may also serve as transcription factors when expressed in Loblolly pine embryos.

Expression analysis of these genes in Loblolly pine zygotic embryos has provided evidence of an initial increase of gene expression during late stage 9.4 for both PtABI3 and PtABI4. However, PtABI4-like displayed a more dramatic increase in gene expression, more than 800 fold changes in gene expression, while PtABI3 mRNA displayed a change of only 50 fold between stages 9.3 and 9.4.

A significant increase in expression of PtABI5-like mRNA is not seen until stage 9.6. There is also a more dramatic increase seen in PtABI3-like mRNA seen during stage 9.6 as well. The initial increase in expression also correlates well with the increase in ABA accumulation during stage 9.4 during Loblolly pine embryogenesis (Figure 2.1) [52, 68, 79]. Each gene ABI3, ABI4, and ABI5 show a significant increase in expression during stage 9.12. There is a 100-fold difference in expression seen in with the ABI3 and ABI5 mRNAs, while there is only a 50-fold increase in expression seen in ABI4 mRNA during stage 9.12.

The overall expression pattern of both PtABI3 and PtABI5 are extremely similar. The expression of both transcripts is greatest in stages 9.6 and 9.12, while PtABI4 is expressed greatest in stage 9.4. This expression data correlates well with what has been reported about expression of ABA responsive genes in the model plant system, *Arabidopsis thaliana*. Interaction between ABI3 and ABI5 has been demonstrated in *Arabidopsis[84]*. It has also been noted in *Arabidopsis* that ABI3 is initially expressed earlier than ABI5. Lopez-Molina et al., have reported evidence of ABI5 acting downstream of ABI3 [90]. There is a significant increase in PtABI3 expression seed in stage 9.4; however, PtABI5 expression does not increase until stage 9.6. This expression

data provides evidence that PtABI3 and PtABI5 may play similar roles in pine as they do in *Arabidopsis*.

On the other hand, the expression pattern of these genes does not coincide with what has been reported regarding the expression pattern of other ABA responsive genes in pine, the LEA genes. Vales et al., have shown three LEA genes expressed in Loblolly pine to have a very different pattern of expression. Their expression is high until the end of stage 9.1. This is followed by a significant decrease from stages 9.2 to 9.4. There is an increase in expression during stage 9.5 (Figure 2.2). This lasts throughout the remainder of late embryogenesis [52].

Interestingly, what is seen in this study is a three-phase pattern of expression during late embryogenesis. This may be because there are a multiple factors that may play a role in changes of expression of the ABA responsive genes. For example, other transcription factors may possibly play a role in expression of ABI3, ABI4 and ABI5. Arroyo et al., have demonstrated that both ABI4 and ABI5 are regulated by glucose, osmotic stress and ABA in *Arabidopsis* [78]. Both ABI4 and ABI4 play a role in sugar sensing in *Arabidopsis* [78, 131, 132]. Likewise, PtABI3, PtABI4 and PtABI5 are most probably accepting multiple cues that affect their expression. An oscillating pattern of sugar accumulation during late embryogenesis was recently reported in Loblolly pine [133]. A increase in osmotic potential of the megagametophytes of Loblolly pine embryos has also been reported between stages 9.5 and 9.7 [134]. While the initial increase in the expression of the mRNAs appears to be in response to the increase in ABA expression, the second peak seen at stage 9.6 may be a result of other developmental cues.

In *Arabidopsis*, ABA responsive genes collectively act to inhibit germination and arrest precocious growth of newly germinated *Arabidopsis* plants. This investigation gives a more comprehensive understanding of how ABA responsive genes are expressed during embryogenesis. Previous studies are only able to give a limited view of the gene expression patterns during embryogenesis due primarily to the difficulty of attaining tissue of discrete stages. This increased understanding of the timing of gene expression and the function of the proteins expressed provide additional insights into the biochemistry and physiology of the Loblolly pine embryonic system. It is possible that these drastic changes in gene expression are a requirement for the embryos to continue on the proper developmental pathway.

Chapter 3

ISOLATION AND EXPRESSION ANALYSIS OF SIX KEY GENES CONTROLLING ROOT DEVELOPMENT DURING EMBRYOGENESIS IN LOBLOLLY PINE

Summary

Clonal propagation of Loblolly pine holds great promise in providing enough material to meet the current industrial demands for wood materials [1]. However, one of the major shortfalls with somatic embryogenesis is many embryos do not have the ability to develop and maintain a viable root system [1]. Pullman et al (2003) have reported that early embryogenesis occurs normally in Loblolly pine somatic embryos, and provides evidence of difficulties with the somatic embryos proceeding through late embryogenesis [1]. The same study provided evidence that somatic embryos develop only to maximum embryo stages 7-9.1. In that study, the most advanced somatic embryos appeared to germinate at the same rate as stages 7-8 zygotic embryos [1]. Our study has focused on identifying, isolating and characterizing genes that control root development in pine because of the issues reported with the formation of root and germination competence during Loblolly pine somatic embryogenesis. Six putative root development genes were identified in the Pine EST databases established from mature plants. These genes were subsequently isolated and cloned in Loblolly pine embryos. These isolated clones were sequenced and analyzed to reveal significant homology to the known *Arabidopsis* root

development genes WOODENLEG, SHORT ROOT, SCARECROW, HOBBIT, BODENLOS, and MONOPTEROS. Full-length cDNAs were isolated and cloned for WOODENLEG, SHORT ROOT, SCARECROW and BODENLOS. Sequence analysis was completed for all isolated clones. Putative conserved domains were found within the deduced amino acid sequences. Expression analyses of all six genes were completed throughout mid to late embryogenesis in Loblolly pine. These expression analyses were compared to reported germination data for Loblolly pine somatic embryos. Expression of these genes coincides with root development and germination of Loblolly pine embryos. This data provides novel information that can be compared with somatic embryos to determine deviations between zygotic and somatic embryogenesis.

Introduction

The current demands for wood and wood products far exceed that which forests can supply [3]. Somatic embryogenesis is a promising method whereby conifers can be massed produced [135]. The use of somatic embryogenesis in forestry will ensure large numbers of the highest quality of trees available for commercial use [136]. However, somatic embryogenesis is not without its problems. Mass production of conifers through somatic embryogenesis is still not yet employed for industrial use worldwide. The quality of somatic embryos is typically determined by the ability of the embryos to form functional root and shoot systems [135]. Issues with root formation and germination allow only a small percentage of the total embryos initiated to convert into plantlets [54, 135]. We have chosen to focus our work on the molecular genetics of root development

in Loblolly pine embryos because of the reported difficulties with root formation and subsequent germination.

The study of mutants affecting normal developmental patterns allowed the discovery of genes involved in formation and patterning of the root. Many of recently discovered genes encode transcription factors or transcription regulators [97]. These transcription factors and transcriptional regulators regulate cascades of genes that can lead to important morphological, physiological, and metabolic changes [95].

In the model plant, *Arabidopsis thaliana*, mutation of specific genes causes dramatic alterations in postembryonic root development. Several of these genes, SHORT ROOT, MONOPTEROS, WOODENLEG, BODENLOS, HOBBIT, and SCARECROW, are involved in pattern formation, cell division and cell expansion of the root [97, 101-103].

Specification of root quiescent center and stem cell functions is controlled by two transcription factors: SHORT-ROOT (SHR) and SCARECROW (SCR). Both SHR and SCR act as key regulators of radial root patterning and stem cell maintenance [106, 137, 138]. This radial patterning of the root is formed during embryogenesis. Both SHR and SCR are members of the GRAS family of putative transcription factors. This family of transcription factors contains several specific motifs: homopolymeric repeat regions, leucine heptad repeats, and putative nuclear localization signals. Nuclear localization of both SHR and SCR has been demonstrated in the model plant *Arabidopsis thaliana [106]*.

MONOPTEROS and BODENLOS are involved in identical developmental pathways. Their expression is mediated by the plant hormone, auxin. Mutations in both

genes fail to initiate the root meristem in early embryogenesis. MP and BDL mutations cause horizontally divisions in the apical daughter cell within the zygote. This results in an embryo with too many embryonic cells, a double octant, pre-embryo. The mutants fail to specify the quiescent center and have root systems that are extremely reduced in size. MONOPTEROS is a transcription factor belonging to the auxin response factor (ARF) family of transcription factors [101].

This family of transcription factors works to activate auxin responsive target genes[104, 139]. BDL encodes indole -3-acetic acid (IAA)12. It is the first IAA protein found to participate in embryogenesis. ARF proteins interact with IAA proteins. Most IAA proteins repress auxin response gene activation [140]. They tend to be short-lived, and have the AUX/IAA domain.

BDL mutants are insensitive to 2,4-D, an auxin analog. In *Arabidopsis*, the two genes are co expressed during early embryogenesis, and BODENLOS is believed to inhibit the action of MONOPTEROS in root meristem initiation [92, 141]. The same mutant phenotype is expressed in MONOPTEROS and BODENLOS double mutants[101].

WOODENLEG encodes a two-component signal transducer, and is required for asymmetric cell divisions during vasculature tissue morphogenesis [142]. Mutations in WOL affect the radial organization of the root [107]. The vascular systems of WOL mutant seedlings contain fewer cells when compared to the wild type. All of the vascular cells differentiate into xylem components.

HOBBIT is required for cell division and cell differentiation in meristems [102, 143]. Mutations in the HOBBIT gene prevent postembryonic cell division and

differentiation of the quiescent center (QC) [102]. The earliest defects are misalignment of the plane of cell division in a progenitor cell of distal root cell types [102]. The HOBBIT mutants appear to form a root meristem, but it does not function when seedlings germinate, these mutants are also unable to form lateral roots [143].

A deeper understanding of the gene expression in zygotic embryos is necessary before we can determine how gene expression in somatic embryos is aberrant. In this work, we describe the identification, isolation and characterization of six cDNA clones that are homologous to genes known to control root development in *Arabidopsis thaliana*. These genes are differentially expressed during late embryogenesis in Loblolly pine. To our knowledge, this is the first report of the isolation and analyses of sequence and expression of these genes during zygotic embryogenesis in Loblolly pine.

Materials and Methods

Database Search

Blast searches [144, 145] were performed to find homology between available plant SHORT ROOT, SCARECROW, MONOPTEROS, BODENLOS, WOODENLEG, ROOT MERISTEMLESS and HOBBIT gene sequences and the pine EST sequences available at http://www.fungen.org, http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine, and http://dendrome.ucdavis.edu/adept/ . Several sequences were identified. PCR primers were designed against various regions (CHART) using IDT PRIMERQUEST (http://www.idtdna.com/Scitools/Applications/Primerquest/) program. The primers were

ordered from Integrated DNA Technologies (San Diego, CA).

Plants and Embryo Tissue

Loblolly pine embryos and female megagametophytes are available in Dr. Pullman's laboratory at the Institute of Paper Science at Georgia Institute of Technology. Seeds were isolated from pine cones harvested weekly from open-pollinated loblolly pine mother tree 7-56. Cone collection took place from 7/01/04 until 10/15/04 in a commercial pine orchard in Lyons, Georgia, US.

Ovules were removed from the seeds. The ovules were dissected under microscope to excise the whole embryos, which were then flash frozen. Embryos were staged according to the staging system developed by Pullman and Webb 1994.

RNA Isolation

Total RNA was isolated from pooled stages 7 – 9.12 of pine embryo development using the RNEASY kit (Qiagen Hilden, Germany) and following the manufacturer's instructions. Purified RNA was aliquoted and stored at -80° C.

Reverse Transcription and Polymerase Chain Reaction

Reverse transcription was performed using 500ng RNA using RACE (rapid amplification of cDNA ends) kit purchased from Clontech© (Mountainview, CA) to synthesize first strand cDNA. 500-bp segment is generated based on which new primers are designed and using RACE experiment, 5` end of the cDNA is isolated [122]. The resulting first strand cDNA contains adaptors ligated to the 5' end of the cDNA. Two gene specific primers were designed within the 3' UTR and using universal primer mix as forward primer from RACE kit and Advantage2 polymerase mix from promega 5` RACE reaction is

performed. The total volume of the pcr mixture was 50µl volume containing 2.5µM of each dNTPs, 1X PCR buffer, 1 unit of advantage Taq polymerase, 1 µM of the gene specific primer and, 1µM of universal primer mix (UPM) or the nested universal primer mix (NUP) to perform the RACE pcr. The pcr was completed with the following conditions: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, Tm of primer for 30 seconds, and 72°C for 2 minute; followed by 72°C for 10 minutes and 4°C hold. The PCRs were performed on an eppendorf thermocycler (Westbury, NY). All primer sequences are listed in Table 3.1,Table 3.2, Table 3.3, Table 3.4, Table 3.5 and Table 3.6.

DNA purification, Cloning and Sequencing

The pcr products were run on a 1.5% agarose gel to verify size. The PCR products are gel purified using QIAquick from Qiagen (Valencia, CA) pcr purification kit and the resulting cDNA fragments are cloned into a pGEM-T Easy vector system from promega (Madison, WI). The product sizes are confirmed by running colony PCR, selected colonies are grown in bacterial culture, and plasmids are purified using Promega's Wizards SV miniprep kit. The purified plasmids were then sequenced and analyzed.

Real Time PCR

Primer Design

Primers for real time pcr were designed with IDT PRIMERQUEST software (http://www.idtdna.com/Scitools/Applications/Primerquest/). The primers were designed so that the pcr products were less than 300 bp in length.

Reverse Transcription

Total RNA was freshly isolated using the RNAeasy kit from Qiagen. Isolated RNA was quantified using a UV spectrophotometer, and the measurements were verified using a ribogreen RNA quantification system with a flourometer. RNA quantification was completed using standard RNA from the Ribogreen RNA assay kit (Invitrogen, Camarillo, CA) and a RNA standard curve [124]. Reverse transcription was completed using Applied Biosystems Taqman Reverse Transcription Reagents kit (Foster City, CA) and 400ng of RNA was used for each RT reaction.

Polymerase Chain Reaction

Real-time PCR primers from Integrated DNA Technologies were diluted to 100 µM concentrations in RNAse-and DNAse-free water. Stock solutions were stored at -20°C. Working solutions were diluted to 45 µm final concentration. Equal amounts of the forward and reverse primers were mixed for the real time PCR reactions. ABI Prism micro amp optical tubes and caps were used to set up the PCR reaction. The Sybergreen® master mix from Applied Biosystems (Foster City, CA) was used for the PCR reactions. The mix used for the reaction consisted of: (1) 2 µl cDNA from RT reaction (2) 7 µl water, (3) 10.0 µl SYBR® green, and (4) 1.0 µl primer mix. Reactions were performed in triplicate. The tubes were placed in the PCR rack, and covered with foil due to the light sensitivity of Sybergreen. 18S RNA was used to normalize the reaction. The traditional real-time pcr program was used: (1) 15s at 94 °C and (2) 1 minute at 60°C for 40 cycles [125]. Amplification was followed by a thermal denaturing step to verify the specificity of the amplification. The products were cloned and sequenced using the same protocol discussed above.

Comparison of RNA expression of RNA expression at different stages was based on the comparative C_t method ($\Delta\Delta C_T$). The relative mRNA expression is quantified using the following formula: $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_{T\ sample} - C_{T\ reference\ RNA}) - (C_{T\ calibrator} - C_{T\ reference\ RNA})$ [126]. Stage 7 tissues were used as a calibrator. This calibration allows the comparison of all samples to a single standard [126, 146].

Bioinformatics

Orthologous proteins were found by searching NCBI's database with the blastx tool. Open reading frames and deduced amino acid sequences were determined by using NCBI's ORF finder program. ClustalW and Boxshade programs from The Biology Workbench (http://workbench.sdsc.edu/) were used to produce alignments and view the alignments [127, 128]. The TargetP online tool (http://www.cbs.dtu.dk/services/TargetP/) was used to identify putative nuclear localization sequences [147].

Table 3.1 Primers designed for PtHBT gene amplification.

Name	Tm	Sequence	Binding Site/ Use
hbtsybf	58 ° C	GGGAGCTGATGCAAACAATCCA	Forward primer used for real time PCR
hbtsybr	61 ° C	TGGCTTCAAATCCAAAGCAAGCCC	Reverse primer used for real time PCR
hbt5'race1	63° C	TGCAACTTCCTTTAGCAACTCCAGCTCC	Reverse primer. Used in 5' RACE reaction
hbt5'race2	63 ° C	TACTTCACGTCCTCCACGACTCCACAA	Reverse primer. Used in 5' RACE nested reaction
hbt3'race1	59 ° C	CAGAAGGCAAACGTGTTAGTGAGC	Forward primer. Used in 3' RACE first reaction.
hbt3'race2	64 ° C	AGGAGCTGGAGTTGCTAAAGGAAGTTGCAC	Forward primer. Used in 3' RACE nested reaction.

Table 3.2 Primes designed for PtBDL gene amplification.

Name	Tm	Sequence	Binding Site/ Use
bdnsybf	62 ° C	GCTGAATCAGGCATCTGGATCGCTTT	Forward primer used for real time PCR
bdnsybr	60 ° C	AGGTGAACATGGATGGTGTGCCTA	Reverse primer used for real time PCR
bdn5'race1	64° C	AGTCGGCATGAAAGCCGCTAACAACA	Reverse primer. Used in 5' RACE reaction
bdn5'race2	63 ° C	TTGGCACCAACATGCCCTCTTTCCAT	Reverse primer. Used in 5' RACE nested reaction
bdn3'race1	66 ° C	TATGTTTCAACGGCCCAACAATGGCAACGG	Forward primer. Used in 3' RACE first reaction.
bdn3'race2	65 ° C	ACTGCATTGGAGTGGGATGGATTCGAGCTT	Forward primer. Used in 3' RACE nested reaction.

Table 3.3 Primers designed for PtMP gene amplification.

Name	Tm	Sequence	Binding Site/ Use
mpsybf	60 ° C	AGGAGGCATGCTGATGAGTGTCTT	Forward primer used for real time PCR
mpsybr	61 ° C	TCGAAAGATATGTCGGAAGCGCCA	Reverse primer used for real time PCR
mp5'race1	63° C	ATGTCGGAAGCGCCATTCTGTTCCAT	Reverse primer. Used in 5' RACE reaction
mp5'race2	64 ° C	AACTAAACCAAGCAAAGCCGCCCACT	Reverse primer. Used in 5' RACE nested reaction
mp3'race1	64 ° C	AGTGGGCGGCTTTGCTTGGTTTAGTT	Forward primer. Used in 3' RACE reaction.

Table 3.4 Primers designed for PtWOL gene amplification.

Name	Tm	Sequence	Binding Site/ Use
wolsybf	61 ° C	GGTGAAACTCTGGCTGCCAACAAT	Forward primer used for real time PCR
wolsybr	60 ° C	TGAGACATACCCATCCATGCCACA	Reverse primer used for real time PCR
wol5'race1	66° C	ATTGTTGGCAGCCAGAGTTTCACCACCGTT	Reverse primer. Used in 5' RACE reaction
wol5'race2	63 ° C	ATGCTGTCCACCTGTATGCCAAACCT	Reverse primer. Used in 5' RACE nested reaction
wol3'race1	66 ° C	TGGATGTGCAGATGCCAGAAATGGATGGGT	Forward primer. Used in 3' RACE reaction first reaction.
wol3'race2	66° C	AACGGTGGTGAAACTCTGGCTGCCAACAAT	Forward primer. Used in 3' RACE reaction nested reaction.

Table 3.5 Primers designed for PtSCR gene amplification.

Name	Tm	Sequence	Binding Site/ Use
scrsybf	59 ° C	GTGACGAGGTACTGGTGGTAAACT	Forward primer used for real time PCR
SCRSybr	58 ° C	GGCTATCCTGAACCACAGTCTCAT	Reverse primer used for real time PCR
scr5race1	65° C	TCCCTGCCACTGTTTGTAAGTTTCTGGCCT	Reverse primer. Used in 5' RACE reaction
scr5race2	66° C	ATCCAGCAAATTCCGCAACCTGCCCA	Reverse primer. Used in 5' RACE nested reaction
scr3race1	60 ° C	GTCTGGCAAATCGTCCTTGTGGT	Forward primer. Used in 3' RACE reaction first reaction.
scr3race2	64° C	TTCTGCCAAGGCAAGCATCCGACTTT	Forward primer. Used in 3' RACE reaction nested reaction.

Table 3.6 Primers designed for PtSHR amplification.

Name	Tm	Sequence	Binding Site/ Use
shrsybf	60.4° C	TGGAGAAATTTGCACGGCTCATGG	Forward primer used for real time PCR
shrsybr	60.2 ° C	TTGTTAACACGTTGCAGGCTGTGG	Reverse primer used for real time PCR
shrcpf1	65° C	CTCTGAGGGAAGTGCCCACCTAGC	Reverse primer. Used in 5' RACE reaction
shr5'race1	62.9° C	AACACGTTGCAGGCTGTGGATGCAGTTGAT	Reverse primer. Used in 5' RACE nested reaction
shrcpr1	60 ° C	GGCGCACTGCTCCTTCCAGGTCA	Forward primer. Used in 3' RACE reaction first reaction.
shr3'race1	65.8° C	TGGACAACCTTTGGTCATGTTGCTGCCA	Forward primer. Used in 3' RACE reaction nested reaction.

Results

Isolation of Root Genes cDNAs from Loblolly Pine

Despite differences in embryogenesis between pine and *Arabidopsis*, the genes controlling their development are extremely similar [29]. The goal of this study was to determine if genes known to control root formation during embryonic development in *Arabidopsis thaliana* are also expressed in loblolly pine embryos. To explore this hypothesis we selected six genes that are involved in root development in *Arabidopsis*: SHORT-ROOT, SCARECROW, HOBBIT, MONOPTEROS, WOODENLEG and BODENLOS. We used the *Arabidopsis* gene sequence as a query sequence to search the pine EST database using blastx. A cut off E-value was set to e^{-15} to ensure high homology between the matches. Homologous sequences in the pine EST database were identified (Table 3.7). 3' RACE reactions were completed in an effort to verify sequence information and confirm the expression in embryos.

Table 3.7 Putative root development genes identified in the loblolly pine EST database.

Arabidopsis gene	Pine EST identified
SHORT ROOT	RTDR2_19_F11.g1_A021
WOODENLEG	RTWW1_2_C12.g1.A015
SCARECROW	RTBOR1_11_B08.b1_A029 RTWW3_24_F02.g1_A022 RTAL1_2_B12.g1_A029
HOBBIT	NXLV_16_B02.g1_A017
MONOPTEROS	RTDR1_15_D11.b1_A015 RTDR1_15_D11.g1_A015
BODENLOS	NXCI_27_F08.g1_A016 NDL1_31_F11.g1_A029

Isolation and sequence analysis of a MONOPTEROS-like cDNA (PtMP) in Pine embryos

3' RACE was completed for PtMP using RNA from pine embryos (Tree 7-56) (Figure 3.1). Primers were designed from the previously identified EST (Table 3.7). The amplified product was cloned and sequenced. The sequenced clone was 1,008 nucleotides in length. The NCBI blastx program was completed using the sequenced

pine clone as a query sequence. The pine MONPTEROS-like clone had a best match with the MONOPTEROS protein found in *Arabidopsis*. The percent amino acid similarity between the two was 86% and the percent amino acid identity was 75% (Table 3.8). Alignments show the deduced amino acid sequence of the isolated cDNA to be similar to the MONOPTEROS protein in *Arabidopsis thaliana* (Figure 3.2).

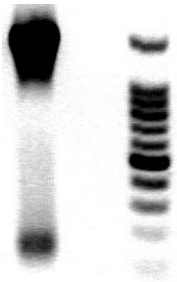


Figure 3.1 Amplification of pine MONOPTEROS-like cDNA by RT PCR
Shown above is a 1.5% gel. The PCR product was loaded into the left lane. A Promega 100 base pair marker was loaded into the right lane. The PCR has migrated to approximately 1,500 base pairs when compared to the DNA marker.

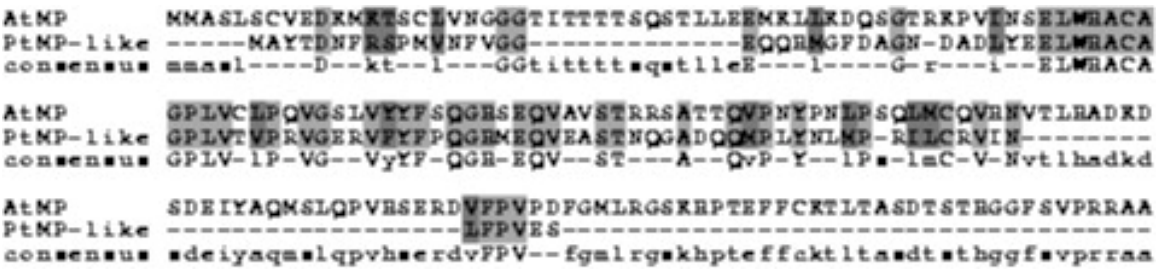


Figure 3.2 Amino acid sequence alignment of PtMP with the MONOPTEROS protein from *Arabidopsis thaliana (Accession number* AF037229).

Isolation and sequence analysis of a HOBBIT-like cDNA (PtHBT) in Pine embryos

Using forward primers designed from the identified pine EST (Table 3.7), 3' RACE was completed to amplify the pine HBT-like cDNA (PtHBT) (Figure 3.3). The PCR product was cloned and sequenced. The product was 785 base pairs in length. Using the blastx program from NCBI the pine HOBBIT-like (PtHBT) clone was compared against a database of plant proteins. It had a best match with the *Arabidopsis thaliana* HOBBIT protein. The score was 115 and the E-value was $7e\text{-}^{26}$. The percent similarity between the PtHBT deduced amino acid sequence and the *Arabidopsis* HBT protein was 82%. The percent identity was 68% (Table 3.8). Alignments of the deduced amino acid sequence of the PtHBT-like cDNA and the *Arabidopsis* HOBBIT protein illustrate the similarity of the two proteins.

Table 3.8 BLASTX Results show high similarity between the isolated pine clones and their *Arabidopsis* counterparts.

The isolated pine sequences were used as query sequences in the NCBI blastx program. The table below provides the Score, E-value percent amino acid similarity and identity, as well as the organism with the best match. The asterisk (*) marks the full-length cDNA being isolated.

Pine Clone	Score	E-value	% Similarity	% Identity	Organism with Best Hit
*PtSHR** *(SHORT ROOT)*	1021	0	99 %	99%	*Monterey pine*
*PtSCR** *SCARECROW*	1312	0	87%	85%	*Monterey pine*
PtBDL *BODENLOS **	152	e^{-35}	53%	47%	*Arabidopsis thaliana*
PtMP *MONOPTEROS*	114	e^{-25}	86%	75%	*Arabidopsis thaliana*
*PtWOL** *WOODENLEG*	244	e^{-63}	66%	52%	*Arabidopsis thaliana*
*PtHBT** *HOBBIT*	115	$7e^{-26}$	82%	68%	*Arabidopsis thaliana*

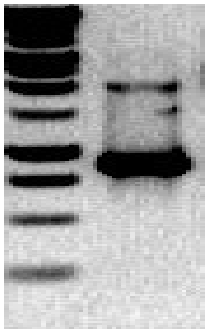


Figure 3.3 PtHBT-like cDNA amplified by RT-PCR
Show above is RT-PCR product run on a 1.5% agarose gel. On left- 1,000 bp DNA ladder from Promega; Right: PCR product approximately 750 base pairs in length.

```
AtHBT        MAFLINPSSSVIMSYLGTSLHALKRSEEALEIMEQAIVADRRNPLPMYQRANILVCLERI
PtHBT-like   ------------------------------------GADANNPLPIYQRANVLVSNERY
consensus    maflinpsssvimsylgtslhalkrseealeimeqai-AD--NPLPmYQRANiLV--ER-

AtHBT        DEALEVLEELKEYAPSESSVYALMGRIYKRRNMHDKAMLHFGLALDMKPPATDVAAIKAA
PtHBT-like   NNALEELELLKEVAPRESSVYSLIGKIYKRINMPERAMYHFGLALDLRPSTADVAIIKSA
consensus    --ALE-LE-LKE-AP-ESSVY-LmGrIYKR-NM-dkAM-HFGLALDmRP---DVA-IK-A

AtHBT        MERLHVPDEIDESP
PtHBT-like   IERLHVPDDIEDHL
consensus    mERLHVPDeIde--
```

Figure 3.4 Alignment of deduced amino acid sequence from PtHBT cDNA with the HOBBIT protein from *Arabidopsis thaliana* (AtHBT), accession number NM_179663.

Isolation and sequence analysis of a full length SHORT ROOT-like cDNA (PtSHR) in Pine Embryos

The pine SHR-like cDNA was isolated from pine embryos by completing a 3' RACE PCR using primers designed from the pine SHR-like EST (Table 3.7). Blastx was completed using the isolated pine cDNA as a query sequence. The Pine SHORT ROOT-

like clone had a best match with the SHORT ROOT protein in Monterey pine. Both the percentage of similarity and identity found was 99%. The full-length sequence of the PtSHR cDNA was isolated by completing 5' RACE with primers in the 3' UTR (Figure 3.5). Both the 5' and 3' UTRs were isolated. The open reading frame and corresponding amino acid sequence was deduced using the NCBI ORF finder program (Figure 3.6). The full-length cDNA was 2,442 base pairs in length. The 5' UTR was 731 base pairs in length and the isolated 3' UTR was 255 base pairs in length. The deduced amino acid sequence of PtSHR-like cDNA was nearly identical to SHR protein found in Monterey pine (Figure 3.8). As expected, the GRAS domain was present within the deduced amino acid sequence. The deduced amino acid sequence also contained a putative nuclear localization sequence, LPSRRDKSRQ, identified using the TargetP online tool (http://www.cbs.dtu.dk/services/TargetP/) [147]. This provides additional evidence that PtSHR-like gene functions as a transcription factor in loblolly pine as it does in other plants.

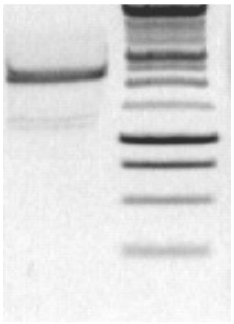


Figure 3.5 RT-PCR of SHR-like cDNA from pine embryos
PCR products run on a 1.5% agarose gel. Right: 1,000 base pair DNA ladder from Promega. On left: PCR product at that has run to the 3,000 base pair marker.

There was also a high degree of similarity found between PtSHR-like deduced amino acid sequence and the SHORT ROOT protein in *Arabidopsis thaliana*, especially within the GRAS domain. The percent amino acid similarity was 64% and the percent amino acid identity was 48%. The analyzed PtSHR-like protein was also highly similar to other SHR-like proteins found in multiple plants species, particularly within the GRAS domain (Figure 3.10).

```
732  atggatagattgttcacctccagattagcagattatcaatccgaa
     M  D  R  L  F  T  S  R  L  A  D  Y  Q  S  E
777  caccctctgtcatgcttcaattctaacaaaaattgtgaagatgca
     H  P  L  S  C  F  N  S  N  K  N  C  E  D  A
822  Gacacccctagacacatagacagctacaacaaccatctcagccag
     D  T  P  R  H  I  D S Y N N H L S Q
867  tttgttctcccatccagaagggataaatcaaggcaatgcaattct
     F  V  L  P  S  R  R  D  K  S  R  Q  C  N  S
912  ttcatggaggacgaagacttctcattcaagcaattccctcctttc
     F  M  E  D  E  D  F  S  F  K  Q  F  P  P  F
957  aaagaaatgttcaacaacaatcagaccggaaagggtaccacggga
     K  E  M  F  N  N  N  Q  T  G  K  G  T  T  G
1002 cctaaccaatttagcggcactagggctacttctagcagatcaagt
     P  N  Q  F  S  G  T  R  A  T  S  S  R  S  S
1047 gaagtaccagaccccagcttgttttctgaactgaaccctaatttt
     E  V  P  D  P  S  L  F  S  E  L  N  P  N  F
1092 ccagaggagtttggaccagcaggatcacgtcgatgggcttcaaat
     P  E  E  F  G  P  A  G  S  R  R  W  A  S  N
1137 cttctgctagaatgtgccagagcaatagcagagaatgaaaaaagc
     L  L  L  E  C  A  R  A  I  A  E  N  E  K  S
1182 cgaacccaacatctgctatggatgctaaatgaactatcatctccc
     R  T  Q  H  L  L  W  M  L  N  E  L  S  S  P
1227 tatggggattgtgagcagaaattggcctcgtacttcttgcaggcc
     Y  G  D  C  E  Q  K  L  A  S  Y  F  L  Q  A
1272 ttttttttgcaaaataacagatactggtcctcgttgctacaccaca
     F  F  C  K  I  T  D  T  G  P  R  C  Y  T  T
1317 ctttgttcagctgctgaaaaaacatactcatttgattccacaaga
     L  C  S  A  A  E  K  T  Y  S  F  D  S  T  R
1362 aaaatgatcttgaaattccaggaatcaagcccatggacaaccttt
     K  M  I  L  K  F  Q  E  S  S  P  W  T  T  F
1407 ggtcatgttgctgccaatggagcaatcctggagtcctttgaggga
     G  H  V  A  A  N  G  A  I  L  E  S  F  E  G
1452 gaaatgaagctgcacatagttgacctgagcaacacttttttgcaca
     E  M  K  L  H  I  V  D  L  S  N  T  F  C  T
1497 cagtggcctactttgttagaggccttggccaccagaagtgatgac
     Q  W  P  T  L  L  E  A  L  A  T  R  S  D  D
1542 acccctcatcttaggttgactactgtagtcaccagcaaggaagcc
     T  P  H  L  R  L  T  T  V  V  T  S  K  E  A
1587 acagcaatgaaggtcatgaaggaaatagggcaaagaatggagaaa
     T  A  M  K  V  M  K  E  I  G  Q  R  M  E  K
1632 tttgcacggctcatgggggttccatttgaattcagtgttattcat
     F  A  R  L  M  G  V  P  F  E  F  S  V  I  H
1677 caacaacatctccacaagctcaacgttggtgcccttaaaatcaga
     Q  Q  H  L  H  K  L  N  V  G  A  L  K  I  R
1722 ccagatgaagctctggccatcaactgcatccacagcctgcaacgt
     P  D  E  A  L  A  I  N  C  I  H  S  L  Q  R
1767 gttatcaagaatggaagggattccatattgtccacgttctacagc
     V  I  K  N  G  R  D  S  I  L  S  T  F  Y  S
1812 atgaaccccaagatagttactgttgtagaggacgaagtagacctg
     M  N  P  K  I  V  T  V  V  E  D  E  V  D  L
1857 actcatgaagattttggtgattgttttagtgaatgccttcgtttt
     T  H  E  D  F  G  D  C  F  S  E  C  L  R  F
1902 ttttccttgtttttttgattctctagaggagagcttctccagaacc
     F  S  L  F  F  D  S  L  E  E  S  F  S  R  T
1947 agcaacgagagattaatgcttgaaagaaccagtgcaaggagcatt
     S  N  E  R  L  M  L  E  R  T  S  A  R  S  I
1992 gtgaacatattggcctgtgaggattctgaagtttatgagcgcagg
     V  N  I  L  A  C  E  D  S  E  V  Y  E  R  R
2037 gaaaagggtgcacagtgggcttggaggctcaaggaggcaggattc
     E  K  G  A  Q  W  A  W  R  L  K  E  A  G  F
2082 atacatgctgcattcagtgatgatgttgttgatgatgttagggct
     I  H  A  A  F  S  D  D  V  V  D  D  V  R  A
2127 cttctcaagagatacaaggagggttggggtcactgtagcaattca
     L  L  K  R  Y  K  E  G  W  G  H  C  S  N  S
2172 gatgggcttttcttgacctggaaggagcagtgcgccatttgggct
     D  G  L  F  L  T  W  K  E  Q  C  A  I  W  A
2217 tctgcctggaagccatgcttgtaa 2240
     S  A  W  K  P  C  L  *
```

Figure 3.6 ORF Nucleotide sequence and deduced amino acid sequence of the loblolly pine PtSHR cDNA. The asterisk (*) marks the putative stop codon.

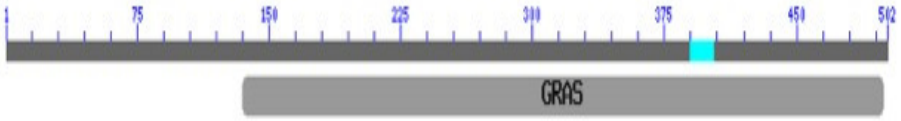


Figure 3.7 PtSHR-like deduced amino acid sequence contains the GRAS domain found in plant transcription factors

```
PrSHR       MDRLFTSRLADYQSEHPLSCFNSNRNCEDADTPRHIDSYNNHLSQFVLPSRRDRSRQCNS
PtSHR-like  MDRLFTSRLADYQSEHPLSCFNSNRNCEDADTPRHIDSYNNHLSQFVLPSRRDRSRQCNS
consensus   MDRLFTSRLADYQSEHPLSCFNSNRNCEDADTPRHIDSYNNHLSQFVLPSRRDRSRQCNS

PrSHR       FMEDEDFSFRQFLPFREMFNSNQTGRGTTGPNQFSGTRATSSRSSEVPDPSLFSELNPSF
PtSHR-like  FMEDEDFSFRQFPPFREMFNNNQTGRGTTGPNQFSGTRATSSRSSEVPDPSLFSELNPNF
consensus   FMEDEDFSFRQF-PFREMFN-NQTGRGTTGPNQFSGTRATSSRSSEVPDPSLFSELNP-F

PrSHR       PEEFGPAGSRRWASNLLLECARAIAENERSRTQHLLWMLNELSSPYGDCEQRLASYFLQA
PtSHR-like  PEEFGPAGSRRWASNLLLECARAIAENERSRTQHLLWMLNELSSPYGDCEQRLASYFLQA
consensus   PEEFGPAGSRRWASNLLLECARAIAENERSRTQHLLWMLNELSSPYGDCEQRLASYFLQA

PrSHR       FFCRITDTGPRCYTTLCSAAERTYSPDSTRRMILRFQESSPWTTFGHVAANGAILESFEG
PtSHR-like  FFCRITDTGPRCYTTLCSAAERTYSPDSTRRMILRFQESSPWTTFGHVAANGAILESFEG
consensus   FFCRITDTGPRCYTTLCSAAERTYSPDSTRRMILRFQESSPWTTFGHVAANGAILESFEG

PrSHR       EMRLHIVDLSNTFCTQWPTLLEALATRSDDTPHLRLTTVVTNREATAMKVMREIGQRMER
PtSHR-like  EMRLHIVDLSNTFCTQWPTLLEALATRSDDTPHLRLTTVVTSREATAMKVMREIGQRMER
consensus   EMRLHIVDLSNTFCTQWPTLLEALATRSDDTPHLRLTTVVT-REATAMKVMREIGQRMER

PrSHR       FARLMGVPFEFSVIHQHHLHRLNVGALRIRPDEALAINCIHSLQRVTRNGRDSILSTFYS
PtSHR-like  FARLMGVPFEFSVIHQQHLHRLNVGALRIRPDEALAINCIHSLQRVIRNGRDSILSTFYS
consensus   FARLMGVPFEFSVIHQ-HLHRLNVGALRIRPDEALAINCIHSLQRV-RNGRDSILSTFYS

PrSHR       MNPRIVTVVEDEVDLTHEDFGDCFSECLRFFSLFFDSLEESFSRTSNERLMLERTSARSI
PtSHR-like  MNPRIVTVVEDEVDLTHEDFGDCFSECLRFFSLFFDSLEESFSRTSNERLMLERTSARSI
consensus   MNPRIVTVVEDEVDLTHEDFGDCFSECLRFFSLFFDSLEESFSRTSNERLMLERTSARSI

PrSHR       VNILACEDSEVYERRERGAQWAWRLREAGFIHAAFSDDVVDDVRALLRRYREGWGHCSNS
PtSHR-like  VNILACEDSEVYERRERGAQWAWRLREAGFIHAAFSDDVVDDVRALLRRYREGWGHCSNS
consensus   VNILACEDSEVYERRERGAQWAWRLREAGFIHAAFSDDVVDDVRALLRRYREGWGHCSNS

PrSHR       DGLFLTWREQCAIWASAWRPCL
PtSHR-like  DGLFLTWREQCAIWASAWRPCL
consensus   DGLFLTWREQCAIWASAWRPCL
```

Figure 3.8 SHR-like proteins found in Loblolly and Monterey pines are almost identical

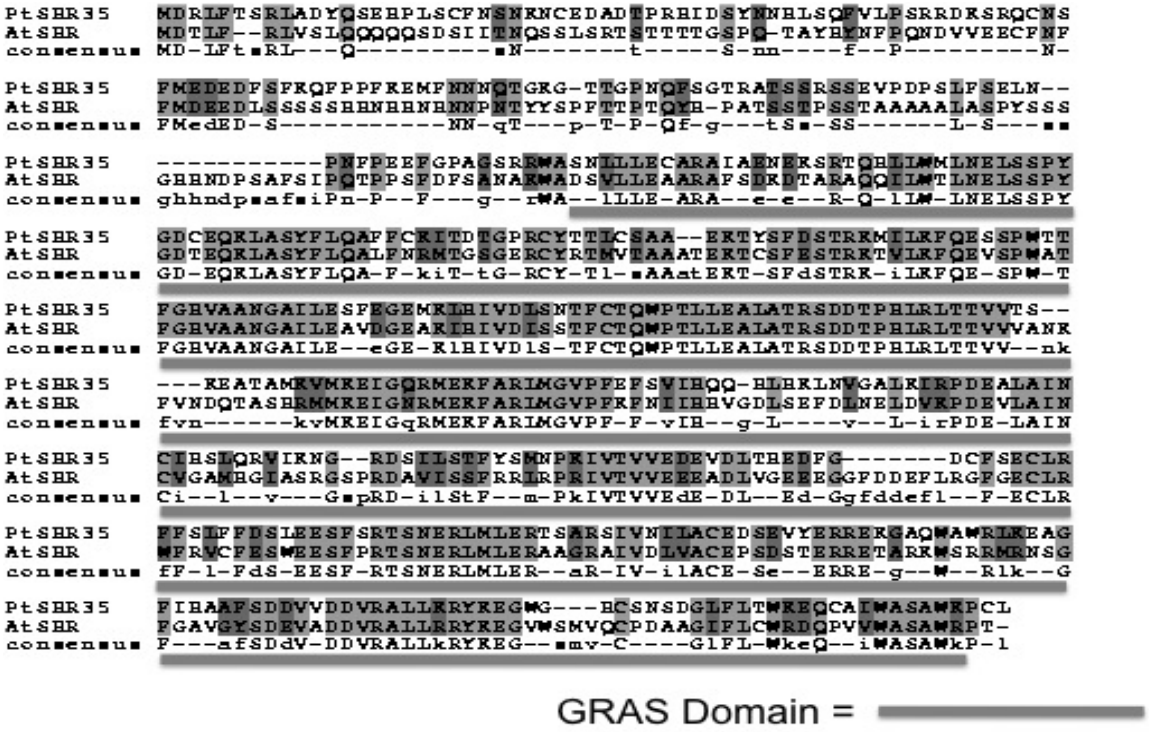


Figure 3.9 Amino acid alignment of the loblolly pine PtSHR cDNA with the *Arabidopsis* SHR protein (AtSHR), accession number

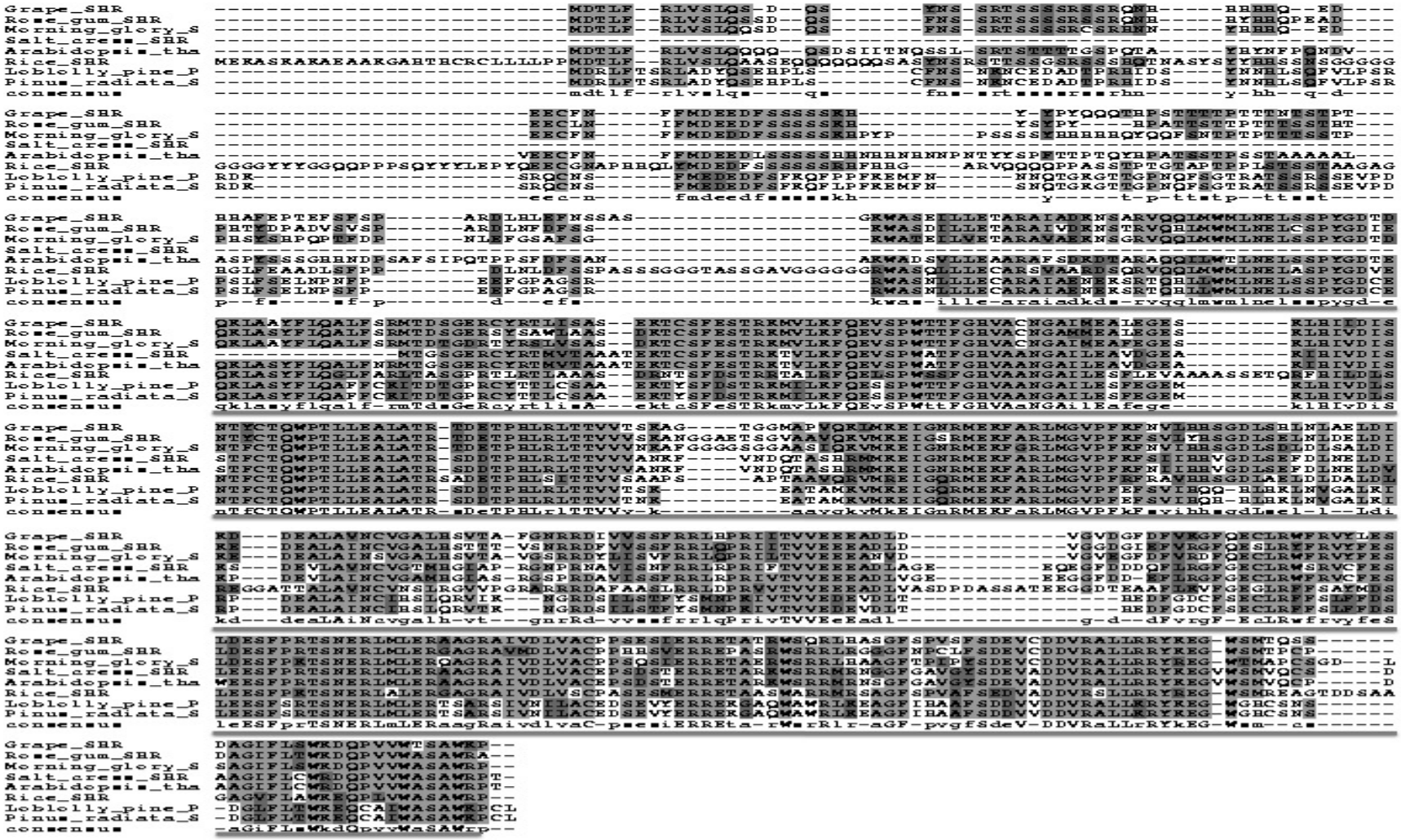


Figure 3.10 Alignment of the deduced amino acid sequence of PtSHR-like cDNA against putative SHR proteins from other plants.

Isolation and Sequence Analysis of a SCARECROW-like cDNA (PtSCR) in Pine Embryos

Using RACE (Clontech) technologies, the full-length cDNA was isolated, cloned and sequenced by designing primers based upon the sequences of the SCR-like clones in the Pine EST database. The total cDNA was 3,215 base pairs in length (Figure 3.11). Similarities between the isolated cDNA and other plant proteins was determined using the blastx program on the NCBI website. The Pine SCARECROW-like clone had a best match with the SCARECROW-like protein found in *Monterey pine*. The score from the blastx program comparing the two proteins was 1312 and the E-value was zero. The percent of amino acid similarity was 87%, and the percent identity was 85%. The top hit with a non-gymnosperm plant was the Scarecrow protein found in *Lily longiforum*. The percent amino acid similarity was 67 % and the percent of amino acid identity was 51% (Table 3.8). Using the NCBI ORF finder program, the open reading frame was determined to ne 2,204 base pairs in length. The 5' UTR was 342 base pairs in length, and the 3' UTR was 668 base pairs in length (Figure 3.13). The deduced amino acid sequence contains the GRAS domain (Figure 3.12). The deduced amino acid sequence shows a high degree of similarity with both the close relative Monterey pine and the lily plant (Figure 3.14 and Figure 3.15). The presence of the GRAS domain in the deduced amino acid sequence of the PtSCR-like clone supports the theory that PtSCR also functions as a transcription factor in pine.

Figure 3.11 Pine SCR-like cDNA amplified by RT PCR.
Shown above are PCR products run on a 1.5% agarose gel. A Promega 1,000 base pair ladder is shown on the left. The PCR is run in the right lane. There is a PCR product above the 3,000 base pair marker.

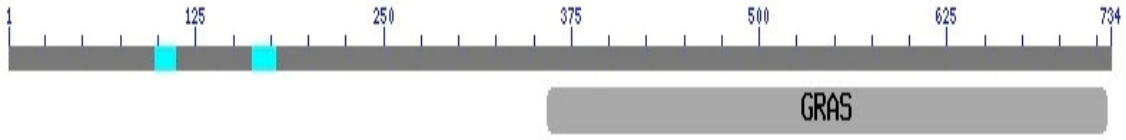


Figure 3.12 The GRAS domain is present within the deduced amino acid sequence the PtSCR-like cDNA.

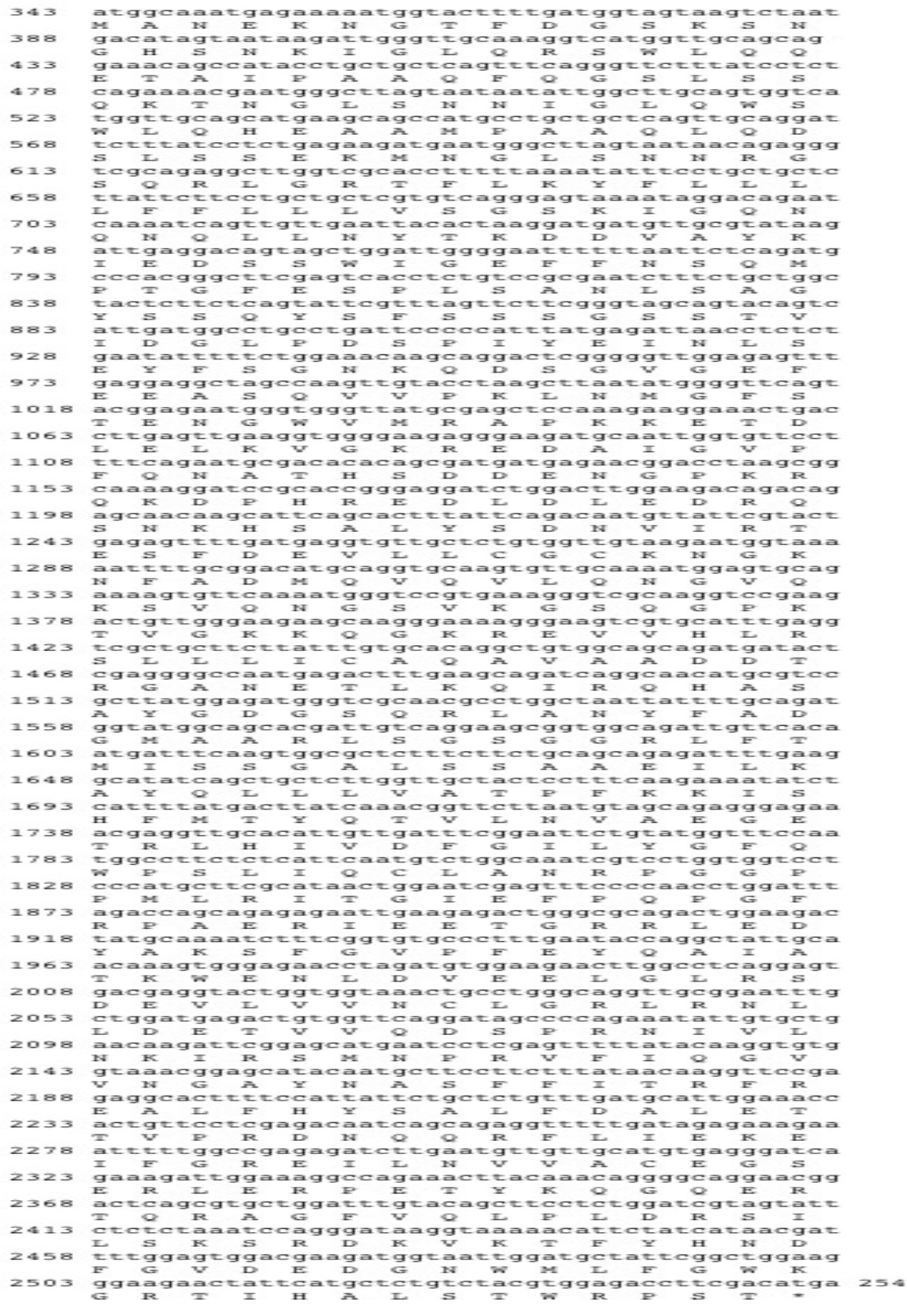


Figure 3.13 PtSCR ORF and deduced amino acid sequence vi a NCBI's ORF finder program.

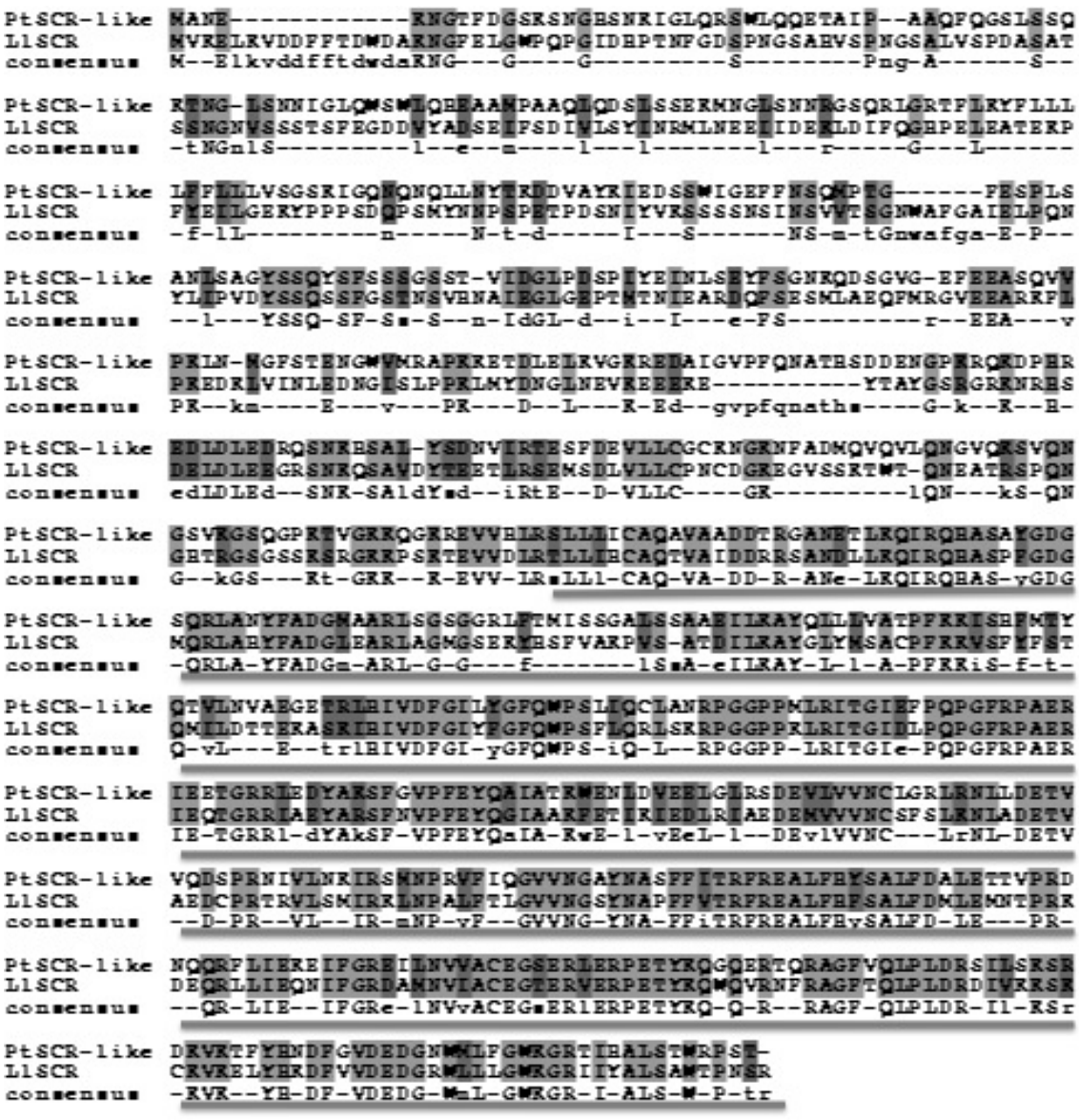


Figure 3.14 PtSCR-like clone is highly similar to the SCR protein found in the Lily. An amino acid alignment of PtSCR against the SCARECROW protein in the Lily. The amino acid sequences are highly similar, especially within the GRAS domain.

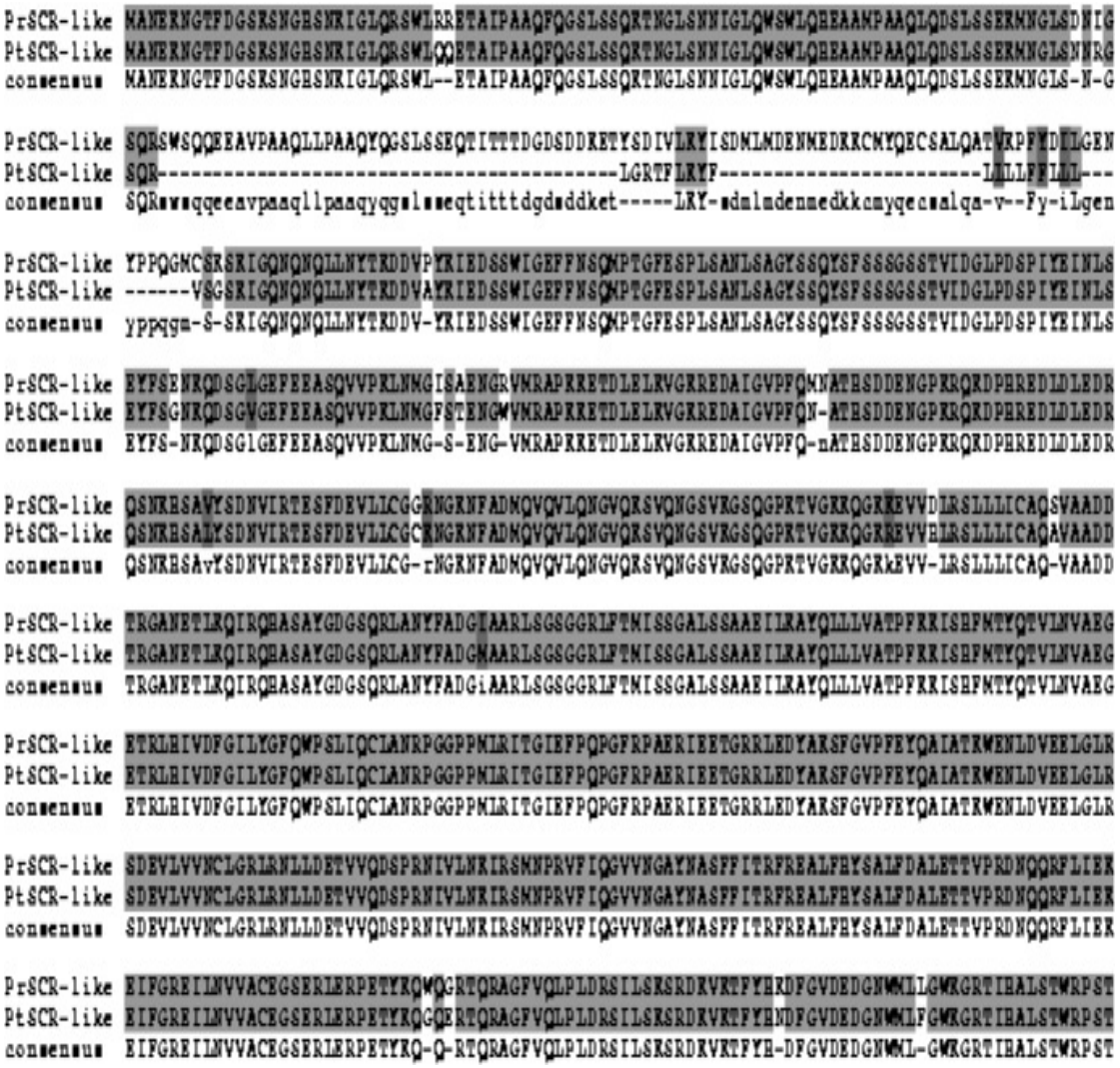


Figure 3.15 PtSCR-like deduced amino acid sequence is almost identical to the SCARECROW-like protein sequence of Monterey pine.
Amino acid alignment of the PtSCR sequence against the SCARECROW protein sequence of Monterey pine.

A full-length pine BDL-like cDNA was isolated from pine embryos using RACE technologies (Figure 3.16). The primer sequence was designed based upon the identified EST (Table 3.7). The amplified product was cloned and sequenced. After running the cDNA sequence in the NCBI blastx program, the pine BODENLOS-like clone had a best

match with the BODENLOS (IAA12) protein found in *Arabidopsis thaliana*. The percent amino acid similarity was 53%, and the percent of amino acid identity was 47%. The deduced amino acid sequence contained the Aux/IAA domain that is also found in the BODENLOS protein in *Arabidopsis (*Figure 3.17*)*. Alignments of the deduced amino acid sequence against the AtBDL show that the two are highly similar, especially within the AUX/IAA domain (Figure 3.18).

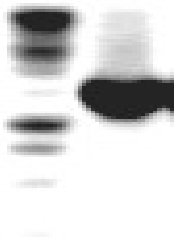


Figure 3.16 Amplification of PtBDL-like cDNA by RT-PCR
The PCR product was run on a 1.5% gel. The Promega 1,000 base pair marker is run in the left lane. The PCR product is run in the right lane. The PCR band has migrated to approximately 1.5 kilobases when compared to the marker.

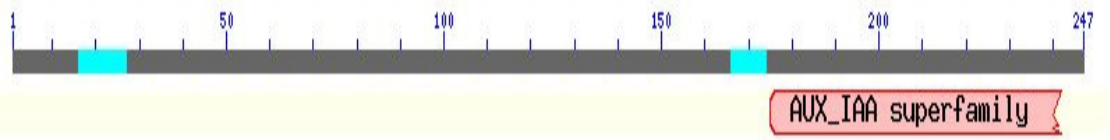


Figure 3.17 PtBDL-like deduced amino acid sequence contains the Auxin-IAA domain

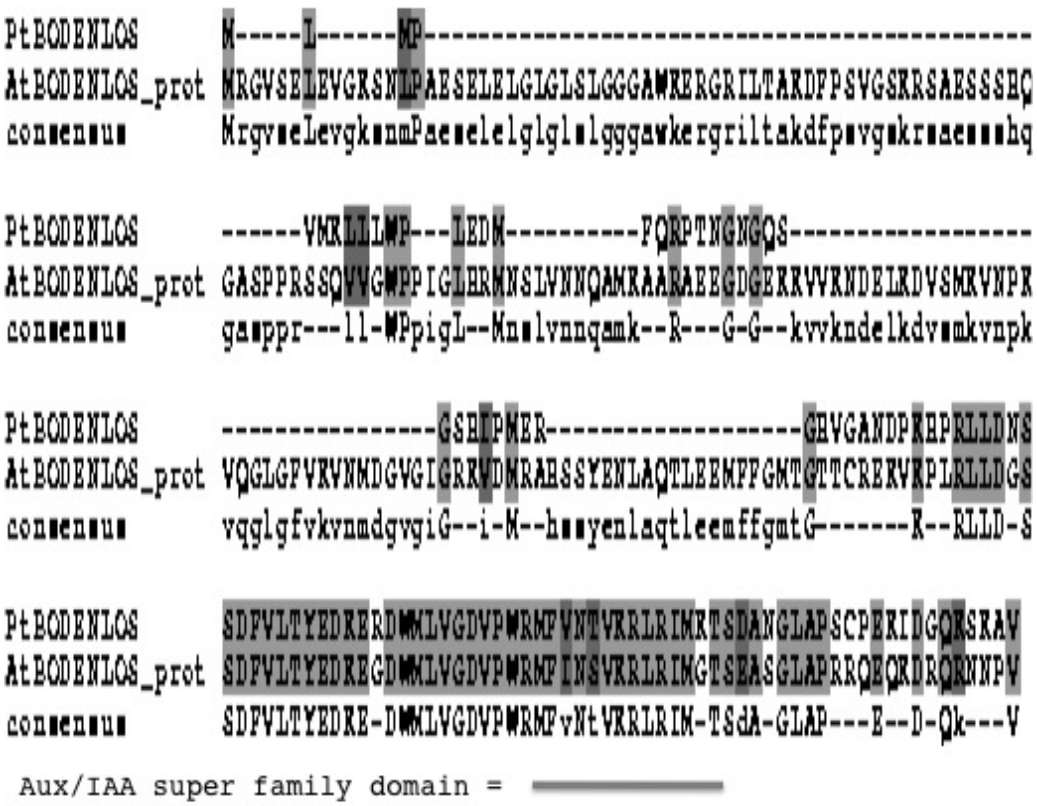


Figure 3.18 Alignment if PtBDL-like deduced amino acid sequence with the *Arabidopsis* BDL protein.

Isolation and Sequence Analysis of a WOODENLEG-like cDNA (PtWOL) in Pine Embryos

Finally, the Pine WOODENLEG-like clone was isolated by first designing primes based upon the WOL-like sequence identified from the pine EST database. The isolated product was cloned and sequenced. To determine its homology with other plant proteins the sequence was used to search against known protein sequences in the NCBI database via the blastx program. It had a best match with the WOODENLEG gene found in *Arabidopsis*. The percent amino acid similarity was 67% and the percent amino acid identity was 54% (Table 3.8). The open reading frame and resulting amino acid sequence

were determined through the ORF finder program on the NCBI website (Figure 3.20). The deduced amino acid sequence contained a histidine kinase domain, histidine kinase – like ATPase domain and the REC receiver domain for signal transduction (Figure 3.21). Sequence alignments show the deduced amino acid sequence of the PtWOL-like cDNA to be highly similar to the protein sequence of WOOODENLEG in *Arabidopsis*, especially within the conserved domains (Figure 3.22).

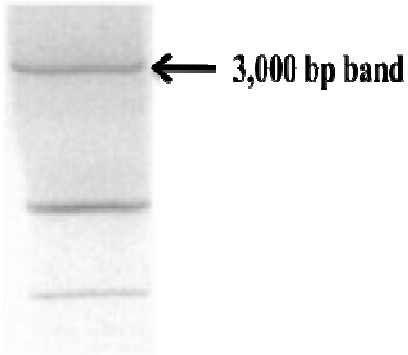


Figure 3.19 Amplified PtWOL-like cDNA via RT-PCR.
The PCR product was run on a 1.5% gel. The top PCR band has migrated to approximately 3 kilobases when compared to the marker.

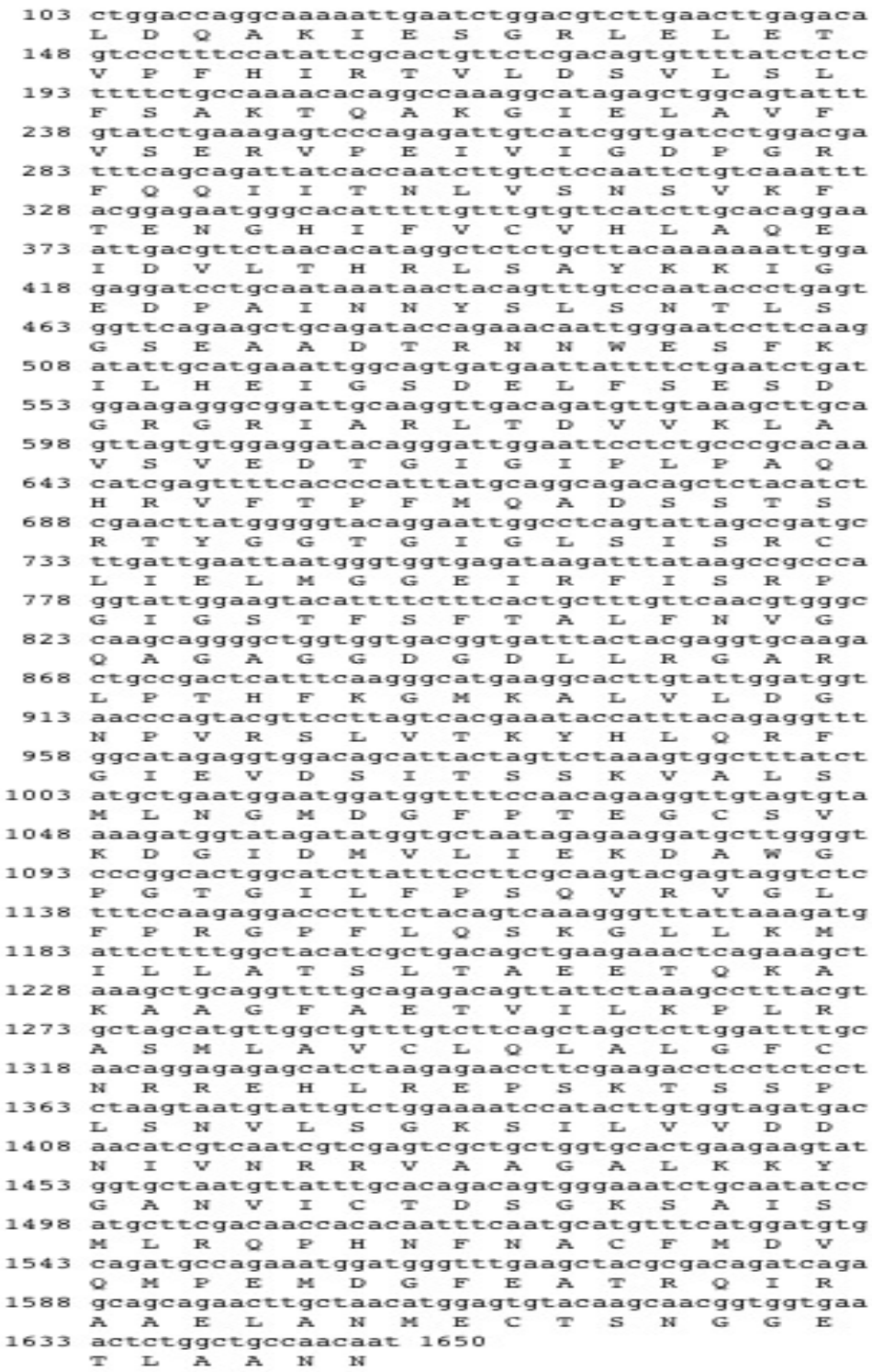


Figure 3.20 PtWOL-like ORF with deduced amino acid sequence

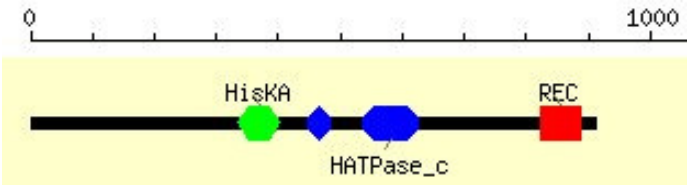


Figure 3.21 PtWOL-like domain structure
The deduced amino acid sequence of PtWOL-like cDNA contains the Histidine Kinase A super family domain. Histidine kinase-like ATPases, a signal receiver domain.

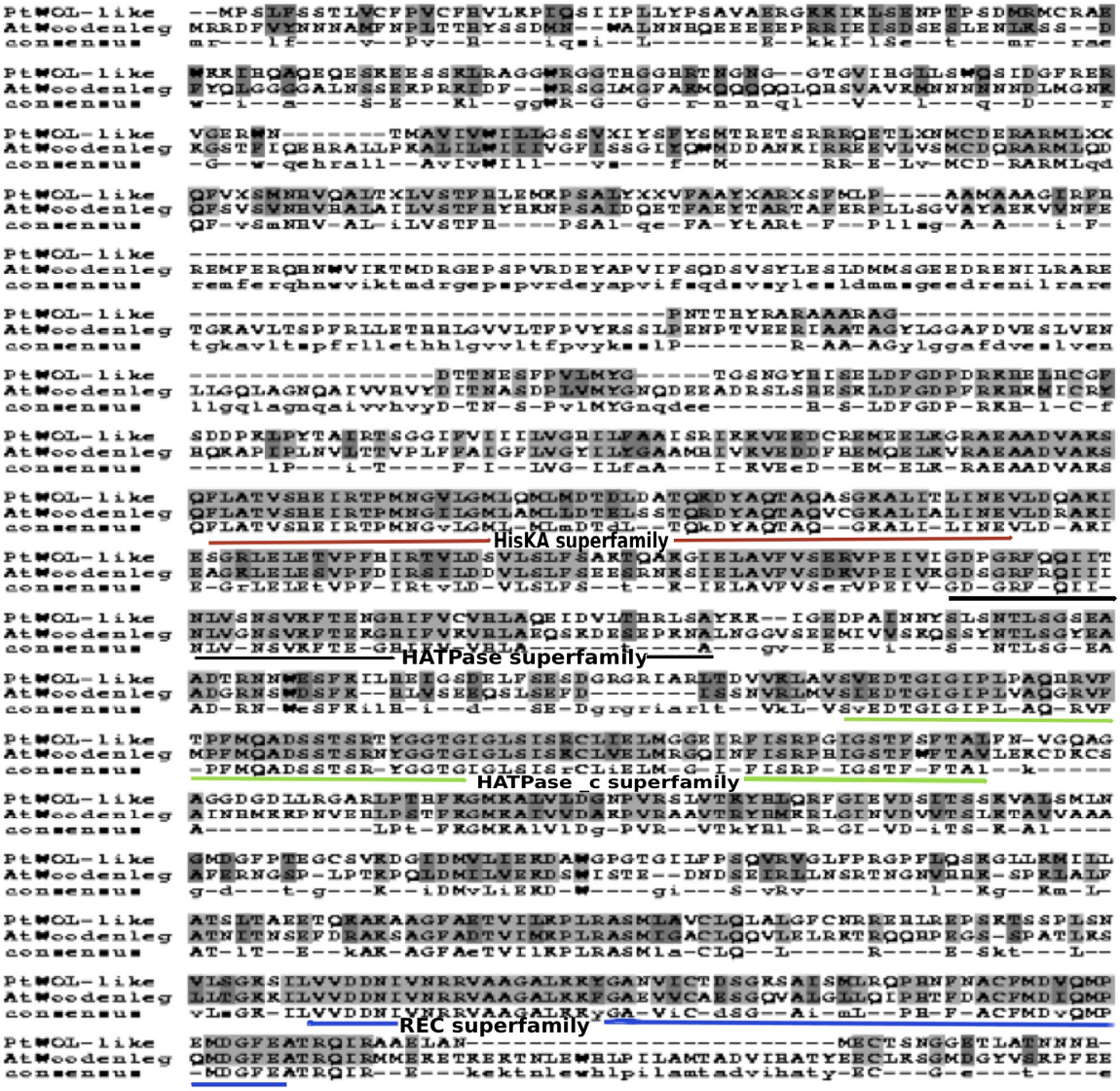


Figure 3.22 PtWOL-like deduced amino acid alignment. Alignment of the PtWol deduced amino acid sequence against the WOODENLEG protein found in *Arabidopsis*, especially within the four conserved domains.

Differential Expression of 6 genes Controlling Root Development during Late Embryogenesis

We studied the expression pattern of these genes controlling root development throughout late embryo development because the lack of proper root development and germination is a significant problem in the somatic embryogenesis process with Loblolly pine embryos. These genes will have a significant effect on the potential of the plants for growth and development during late embryogenesis and germination. To determine the expression patterns of these genes during expression, quantitative PCR was performed using primers specific for each cDNA (PtSHR, PtWOL, PtSCR, PtBDL, PtMP and PtHBT). Expression during tissue stages 7 – 9.6 was observed throughout these experiments. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7.

Expression of PtSHR-like mRNA appears to be stable in all stages tested except for stage 8. There is a two-fold increase in PtSHR-like mRNA expression from stage 7 to stage 8. It's expression then drop back down to a basal level in stage 9.1 (Figure 3.23). A second genotype shows an increase a significant increase in stage 9.1, followed by a significant decrease to a basal level of expression in stage 9.2 (Figure 3.24). Interestingly, the PtSCR-like cDNA follows a similar pattern of expression. There is an almost two-fold increase in PtSCR-like mRNA expression between stages 7 and 8. Its mRNA level then drops back down to a basal level at stage 9.1. This level is lower than that seen in stage 7. PtSCR-like mRNA remains at this level until stage 9.4. There is a gradual increase in .2 fold of stage 7 expressions at stage 9.5. The mRNA level then drops back

down to a basal level in stage 9.6 (Figure 3.25). A second genotype, tree UC, exhibits a similar pattern of expression whereby there is a basal level of expression present, followed by a significant increase in stage 9.1. Its expression level then decreases significantly during stage 9.2.

PtWOL, PtBDL, PtHBT and PtMP mRNAs all experience similar patterns of expression, however the level of changes are extremely different. PtWOL-like mRNA has a basal level of expression in stages 7 and 8 of pine embryonic tissue. There is a rapid increase of over 40 fold in stage 9.1. This is followed a rapid decline to the basal level in stage 9.2. The embryo remains at this basal level until the end of embryogenesis in stage 9.12 (Figure 3.27 and Figure 3.28).

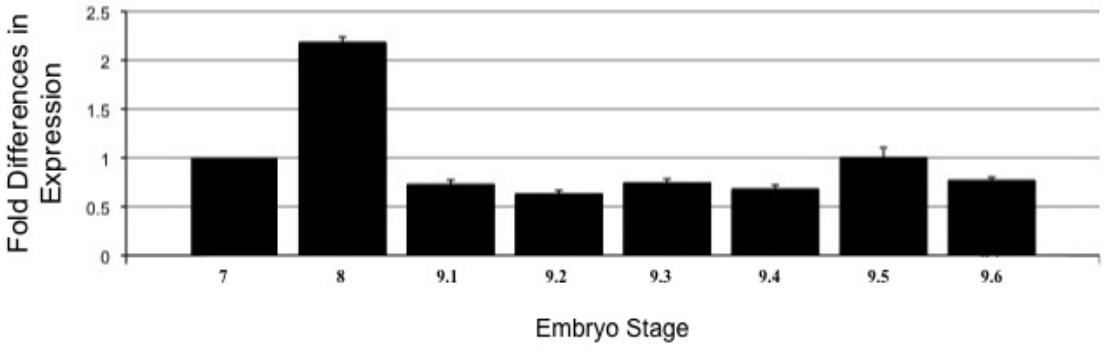


Figure 3.23 Expression of PtSHR mRNA in Loblolly pine zygotic embryos.
Quantitative PCR was completed using RNA from tree 7-56 and primers specific for PtSHR. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

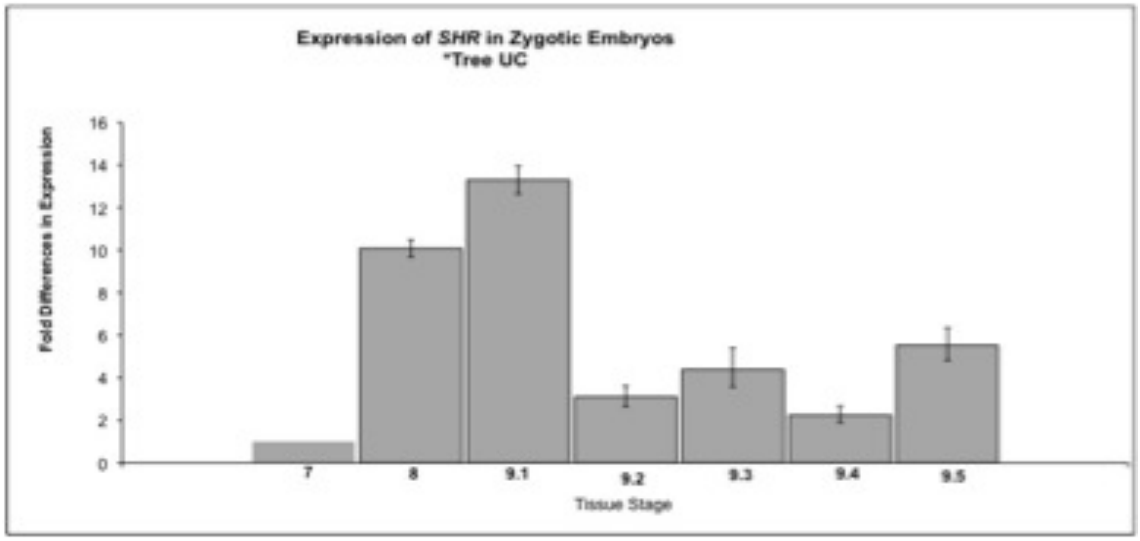


Figure 3.24 Expresson of PtSHR mRNA in Loblolly pine zygotic embryos.
Quantitative PCR was completed using RNA from tree UC and primers specific for PtSHR. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

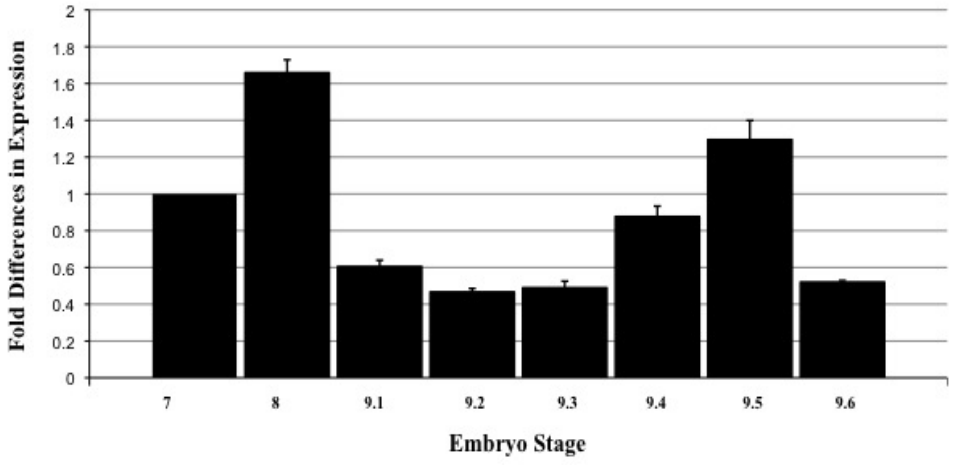


Figure 3.25 Expression of PtSCR-like mRNA in Loblolly pine zygotic embryos
Quantitative PCR was completed using RNA from tree 7-56 and primers specific for PtSCR. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

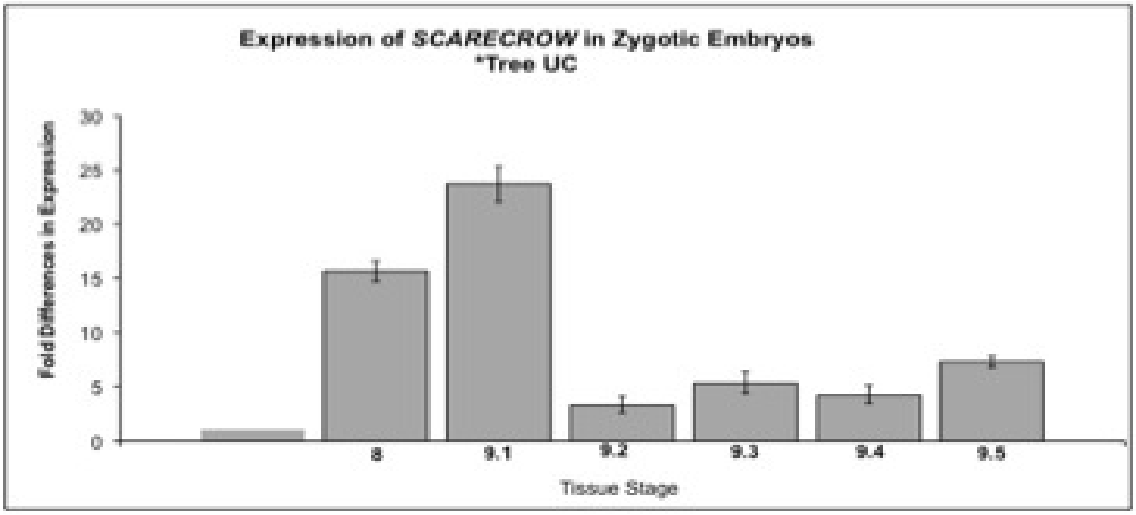


Figure 3.26 Quantitative PCR was completed using RNA from tree UC and primers specific for PtSCR. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

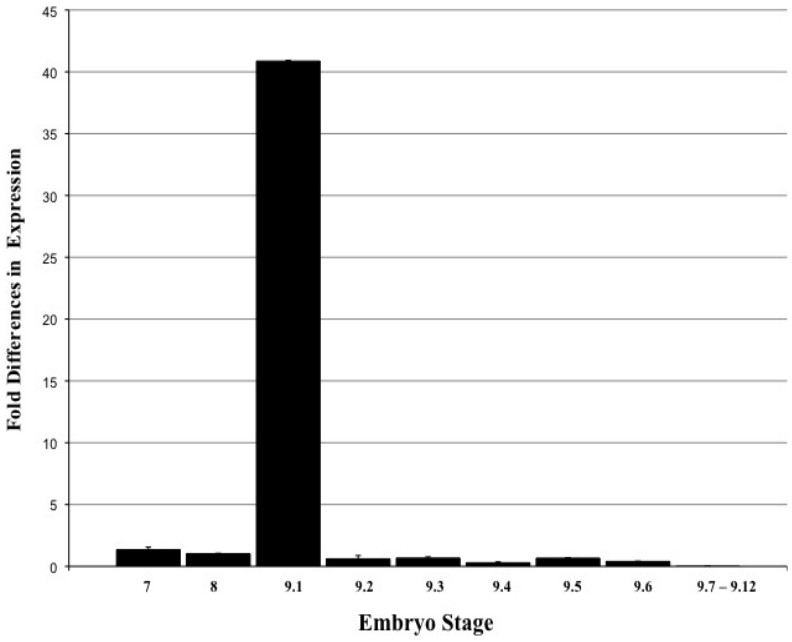


Figure 3.27 Expression of PtWOL - like mRNA in Loblolly pine zygotic embryos
Quantitative PCR was completed using RNA from tree 7-56 and primers specific for PtWOL. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

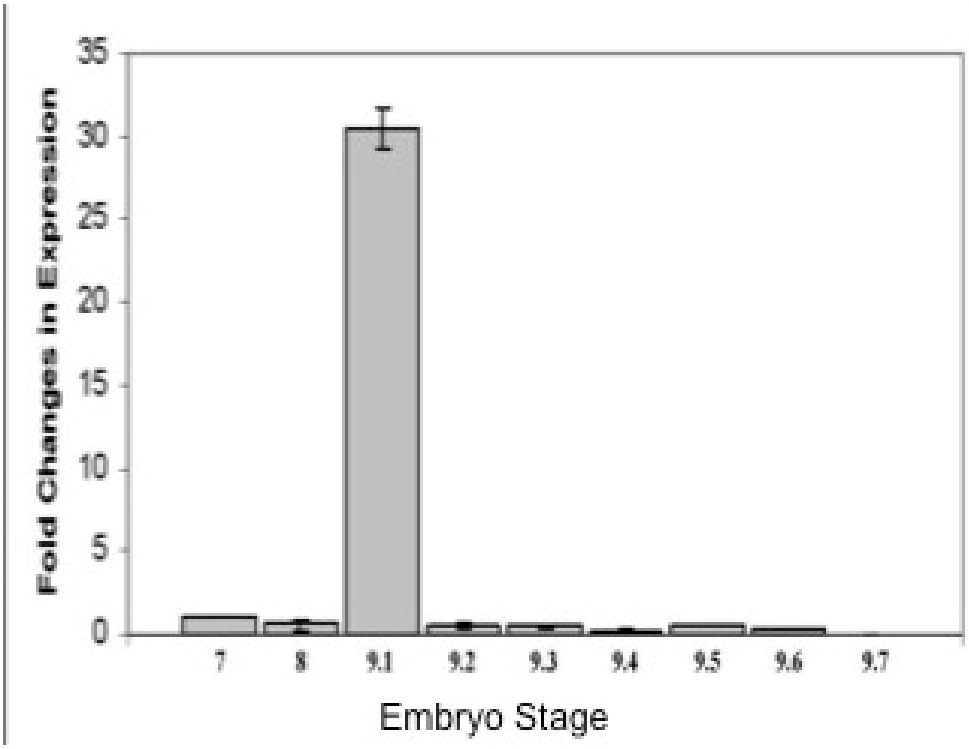


Figure 3.28 Expression of PtWOL - like mRNA in Loblolly pine zygotic embryos. Quantitative PCR was completed using RNA from tree UC and primers specific for PtWOL. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

There is also a basal level of expression of the PtHBT-like mRNA during stages 7 and 8 in pine embryos. There is a rapid increase of over 300-fold exhibited in stage 9.1. In stage 9.2 the level of PtHBT-like mRNA decreases just as rapidly to same basal level seen in stage 7. The mRNA remains at this level until the end of embryogenesis in stage 9.12 (Figure 3.29 and Figure 3.30).

The PtMP-like mRNA exhibits a basal level of expression during stages 7 and 8 of pine embryogenesis. There is a rapid increase of more than 30 fold during stage 9.1.

This is followed by a significant decrease of the same level during stage 9.2. The expression level of this mRNA remains at this basal level throughout the remainder of embryogenesis (Figure 3.31 and Figure 3.32).

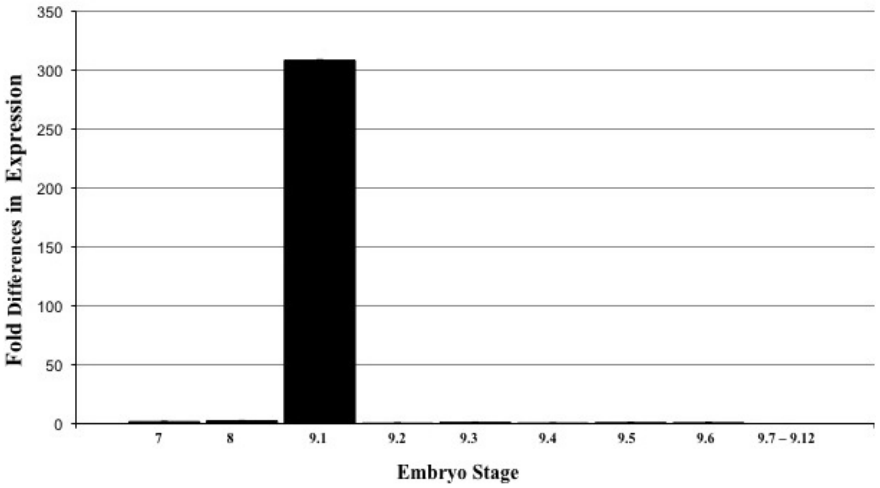


Figure 3.29 Expression of PtHBT-like mRNA in Loblolly pine zygotic embryos
Quantitative PCR was completed using RNA from tree 7-56 and primers specific for PtHBT. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

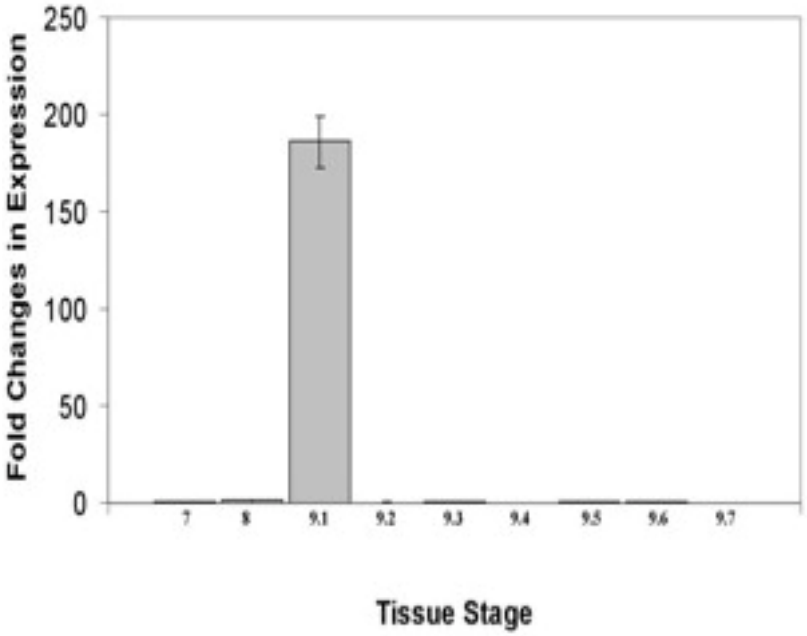


Figure 3.30 Expression of PtHBT-like mRNA in Loblolly pine zygotic embryos
Quantitative PCR was completed using RNA from tree UC and primers specific for PtHBT. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

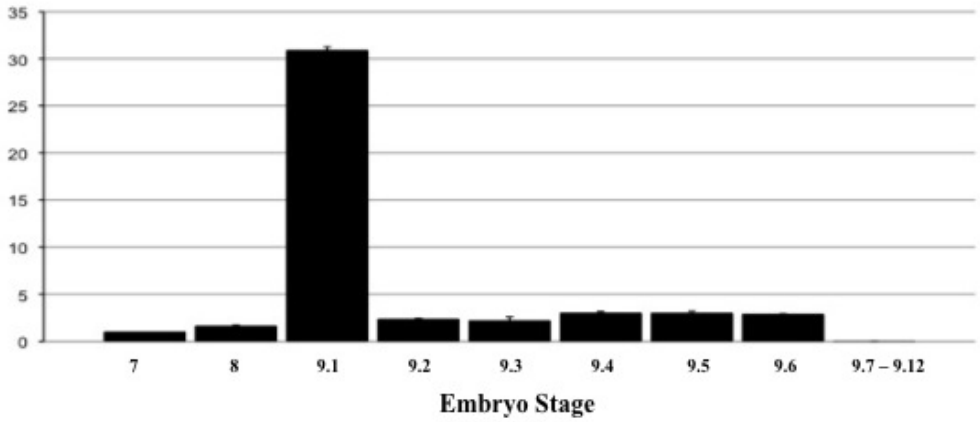


Figure 3.31 Expression of PtMP-like mRNA in Loblolly pine zygotic embryos
Quantitative PCR was completed using RNA from tree 7-56 and primers specific for PtMP. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

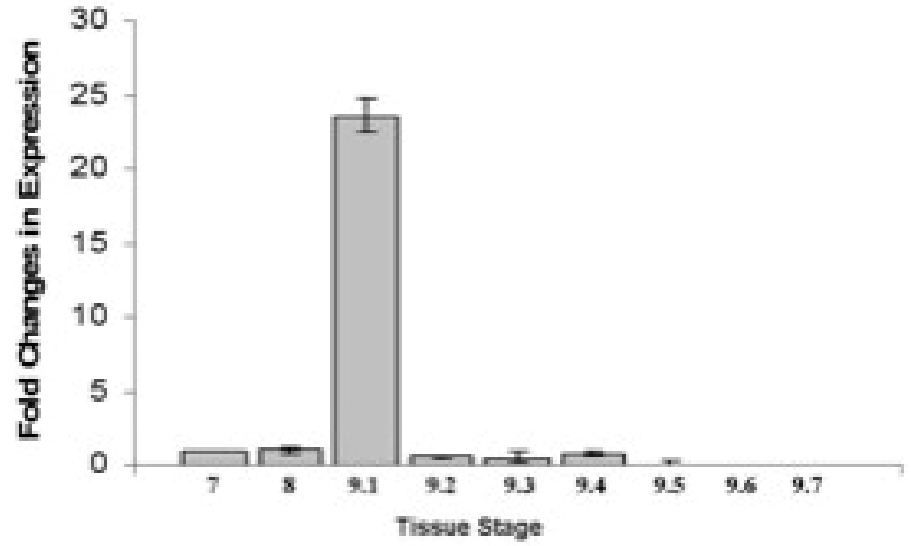


Figure 3.32 Expression of PtMP-like mRNA in Loblolly pine zygotic embryos
Quantitative PCR was completed using RNA from tree UC and primers specific for PtMP. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

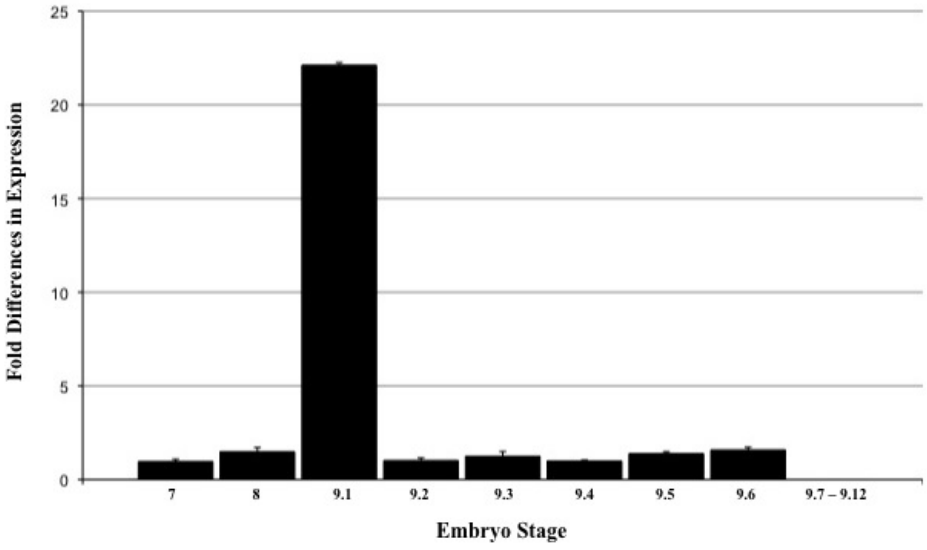


Figure 3.33 Expression of PtBDL-like mRNA in Loblolly pine zygotic embryos
Quantitative PCR was completed using RNA from tree 7-56 and primers specific for PtBDL. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

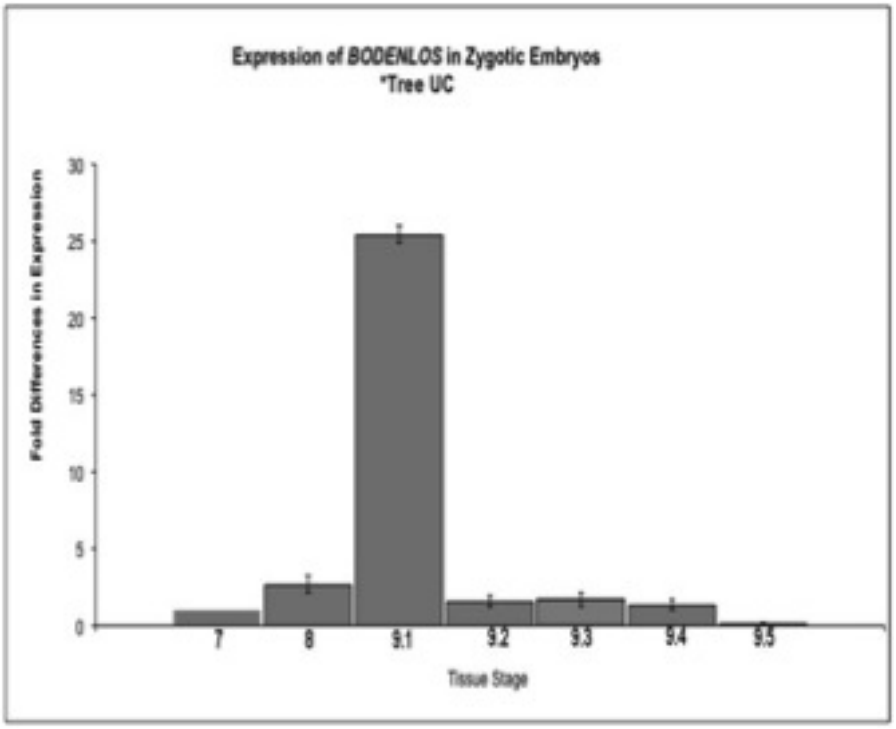


Figure 3.34 Expression of PtBDL-like mRNA in Loblolly pine zygotic embryos
Quantitative PCR was completed using RNA from tree UC and primers specific for PtBDL. 18S rRNA expression was used to normalize all data. Fold differences in expression were determined by comparing all stages to expression in stage 7. Standard error bars are shown for each data point containing three technical replicates.

Lastly, PtBDL-like mRNA is expressed at a basal level in stages 7 and 8. There is a rapid increase of 20 fold during stage 20 followed by a rapid decrease of the same level of expression during stage 9.2. The mRNA remains at this basal level of expression until the end of embryogenesis.

Discussion

Here we report the identification, isolation, sequence and expression analysis of six genes in Loblolly pine: PtWOL, PtSHR, PtSCR, PtHBT and PtBDL. Through sequence analysis, we find these genes to be highly similar to their *Arabidopsis* orthologs: WOODENLEG, SHORT-ROOT, SCARECROW, HOBBIT and BODENLOS.

We were able to isolate the full-length sequence of PtWOL, PtSCR and PtBDL, including the 5' and 3' UTRs. Both PtSHR and PtSCR contain the GRAS domain. This is only identifiable functional domain present within the *Arabidopsis* SHR and SCAR orthologs [138, 148]. PtWOL contained contains the receptor domain of histidine kinases, the REC receiver domain, as well as, the HATPase c domain used to relay the signal. [142]. PtBDL contained the IAA super family domain present within the deduced amino acid sequence.

Expression analysis during late embryogenesis shows that both PtSCR and PtSHR exhibit a similar pattern of expression. There is an almost two-fold increase in both their expression during stage 8(Figure 3.23, Figure 3.24 ,Figure 3.25 and Figure 3.26). It is logical for SHR and SCR to increase their expression first during stage 8 because they control differentiation of the cells around the root meristem to begin formation of the layers that will eventually form the root. The expression of PtSCR and PtSHR correlates well with current data concerning SHR and SCR in *Arabidopsis*. SHR activates expression of SCR and SCR is required for SHR function [105, 106, 138, 149-152]. It is reasonable to see them being expressed at the similar points in development at almost the same intensity. All of the other mRNAs analyzed exhibit similar patterns of expression. They all show a basal level of expression until stage 9.1 where there is a significant increase in expression. Their expression drops significantly following stage 9.1. This PtMP mRNA and PtBDL expression data correlates with reports of BDL and MP being co expressed in the model plant, *Arabidopsis thaliana* [101].

Our data provides evidence that these root development genes are differentially expressed during pine embryogenesis. The pattern of expression of these genes

coincides with root formation seen in zygotic embryos. Root development is seen in a small percentage of plants in stage 8 with more being present in stage 9.1 in zygotic embryos (Figure 3.35. A significant amount of germination is not seen until the embryos have bypassed stage 9.1 where these root development genes are expressed. Pullman et al (2003) suggested the presence of a developmental transition that occurs between stages 8 and 9.1 that allows increased competence for germination [1]. This data provides molecular evidence in support of Pullman's physiological data of the developmental transition occurring between stages 8 and 9.1.

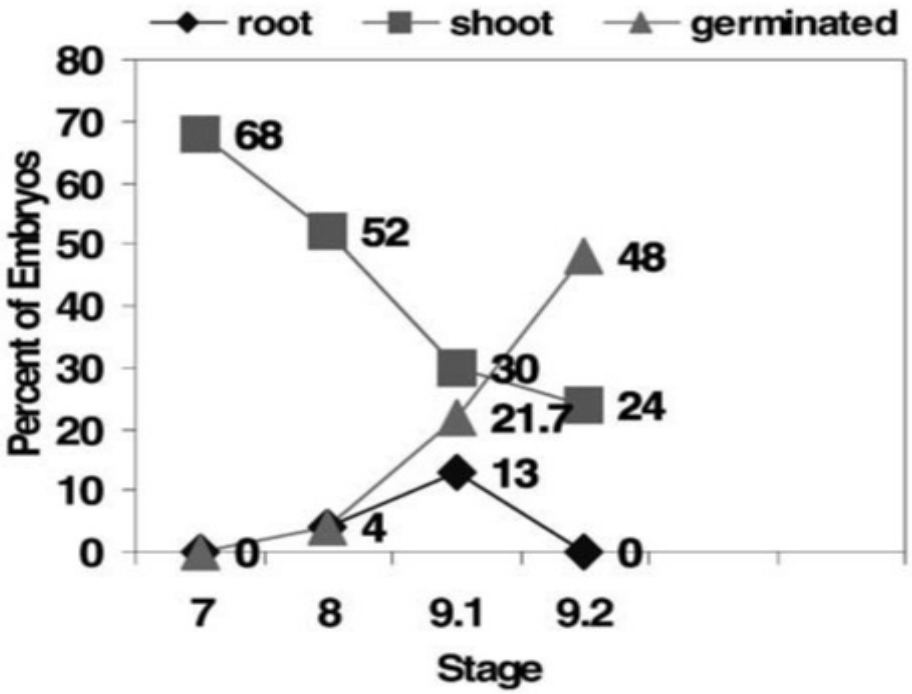


Figure 3.35 Germination of Loblolly pine embryos (stages 7 - 9.2) isolated at specific stages of development [1].

Although there have been a few reports in both angiosperms and gymnosperms analyzing the expression of these genes in different plant organs, this is the first time, a profile of root development gene expression throughout embryogenesis has been reported. The greatest barrier to attaining this type of information in angiosperms is the size of their typical embryo size. However, loblolly pine embryos are large enough to dissect and use at discrete embryonic stages. Our study provides novel information that may serve in establishing molecular markers to ensure embryos have reached a level of competence before attempting full-scale germination trials.

Chapter 4

THE DISCOVERY AND CHARACTERIZATION OF NOVEL *SHORT ROOT* ISOFORMS IN LOBLOLLY PINE EMBRYOS

Summary

In this study, we have isolated several cDNA of multiple lengths. When sequenced, and aligned with the sequence of the predominant mRNA, these clones resembled the products of alternative mRNA splicing, in that regions of sequence that were non-contiguous in the SHR gene DNA sequence, were adjacent in the mRNA clones. Different mRNA sequences thus, presumably, different splices were observed. The sequences that flanked the 'exons' did not conform to the standard GU-AG rule, nor did they strongly resemble the consensus for alternative splicing.

Although SHR is present as a single exon gene in the *Arabidopsis* genome, we have cloned multiple isoforms of PtSHR cDNAs, which lacked sequences from within the open reading frame. When aligned, multiple isoforms were missing portions of the open reading frame, however, all sequences present matched the longest version with 100% similarity. We show that SHR is also present in loblolly pine as a single exon gene. We provide evidence that the SHR isoforms are translated into protein. We were unable to find similar isoforms expressed in *Arabidopsis thaliana* and poplar. This study provides novel information that may begin to explain the differences in embryogenesis in loblolly pine and *Arabidopsis thaliana*.

Introduction

Embryogenesis in Loblolly Pine vs. *Arabidopsis*

Embryogenesis plays a significant role in the life cycle of plants. This development process in both angiosperms and gymnosperms has been extensively studied, and this knowledge has the ability to have significant effects on agriculture and forestry practices [1]. Though this process is broadly similar in angiosperms and gymnosperms, there are major differences between the two. During early embryogenesis in pine, multiple embryos develop before one dominates and ultimately becomes a single embryo with 6 – 8 cotyledons [32, 33, 49, 153]. In *Arabidopsis, thaliana* the zygote divides asymmetrically and produces a smaller apical cell, the embryo. In addition, a larger basal cell is produced and will form the suspensor. The embryo then proceeds through a variety of stages featuring many shape changes. It eventually forms a dicotyledonous structure [153-155]. Even with these drastic differences in development, 75.6% of the embryogenesis related *Arabidopsis thaliana* genes are also expressed during embryogenesis in Loblolly pine [29]. It has been hypothesized that the distinguishing features of embryogenesis in pine are a result of differences in the timing, localization, or level of expression of specific regulatory proteins [29]. In this study, we illustrate one specific deviation in Pine and *Arabidopsis* programs of gene expression during embryogenesis.

The ability to domesticate a wide range of crops such as wheat, corn and tomato has been attributed to changes in the expression of only a few transcription factors [156]. Since formation of the embryonic root is key for successful somatic embryogenesis, we

chose to focus this study on the transcription factor SHORT ROOT (SHR), which is a principal regulator of root formation in plants [96, 106]. In *Arabidopsis thaliana*, this transcription factor controls the formation and maintenance of the root [97, 106].

It is a vital element in the developmental pathway controlling both the specification of the root stem cell niche, and the differentiation potential of a subset of stem cells in the *Arabidopsis* root. The root stem cells are located in specialized structure called the root apical meristem (RAM). In the RAM of *Arabidopsis*, the stem cells surround a group of slowly dividing cells, called the quiescent center (QC). The QC acts as a signaling center that has the ability to position and maintain the stem cell niche. Standard divisions of initial cells generate the radial symmetry of the root. Inside of the root are concentric layers of epidermis, cortex, endodermis, and pericycle surrounding a central vascular cylinder [98, 157].

In SHR mutants, there is a progressive disorganization of the QC, a loss of stem cell activity and a cessation of root growth. There is also a perturbation of the radial pattern of the root and only a single layer within the root [97, 106]. SHR is critical to development of *Arabidopsis*, as it regulates both the specification of the RAM and the differentiation potential of a subset of cells within the RAM.

Transcription factors are regulatory proteins that control the level of transcription of a subset of target genes. SHR is a member of the GRAS family of transcription factors. Members of the GRAS family of proteins are unique to plants and have been isolated in *Arabidopsis*, corn, poplar, rice and barley. GRAS proteins are typically composed of 400-770 amino acid residues and exhibit sequence homology to each other at their c-termini. The distinguishing domains of the GRAS proteins are two leucine rich

regions flanking a VHIID motif. The VHIID motif is present in all members of the GRAS family, however, only the histidine and aspartic acid are absolutely conserved. PFYRE, RVER and a c-terminal SAW motifs are also present. The N-terminal sequence is of the GRAS family of proteins is highly divergent ([138, 148]). Gras proteins contain features present in many transcriptional regulatory proteins. Several GRAS proteins contain homopolymeric stretches of amino acid residues that can be found in several other transcription factors and co activators. Leucine heptad repeats are found in other transcription factors, such as bZIP proteins, and are important for protein-protein interactions [148].

Several members of the GRAS family of proteins contain canonical nuclear localization sequence. Both SHORT ROOT and SCARECROW have been shown to accumulate in the nucleus. GRAS proteins have parallels in their domain structure to STAT proteins. STAT proteins have been shown to bind DNA. In vitro and In vivo assays established that some GRAS proteins contain transactivation domains and may act as transcriptional activators or co-activators [148]. Some direct targets of SHR include: Scarecrow (SCR), Magpie (MGP), Nutcracker (NUC), and SNEEZY (SNE). SCR is another GRAS protein expressed in the endodermis of roots. MGP and NUC are closely related C2H2 zinc finger transcription factors. SNE is an F box protein thought to play a role in gibberellin signaling. It has been confirmed that the promoter of these genes is bound by SHR [151]. Four loss of function SHR mutants have been identified [106]. In shr-1, there is a deletion of 50bp toward the 3' end of the open reading frame (Figure 4.1). This results in a frame shift and a premature termination of translation of the putative amino acid sequence. In shr-2, there is a deletion of 10 bp followed by an

insertion 431bp toward the 5' end of the open reading frame. This causes a premature termination of the translated protein. Previous analysis of both shr-1, shr-2, shr-3 and shr-4 illustrated the need for a functioning SHR protein for proper radial patterning of the root. All four alleles exhibit severely reduced root growth and darker cotyledons [106].

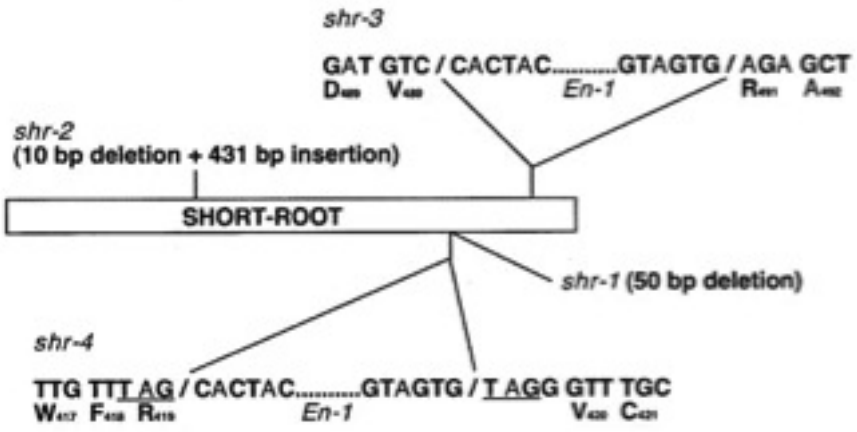


Figure 4.1 ***Arabidopsis thaliana*** **SHR mutants [106].**
Shr-2 has a 421 bp insertion and shr-1 has a 50 bp deletion. Both are transcribed but result in a truncated non-functional proteins being produced.

This study focuses on the transcription factor, SHORT-ROOT (SHR), which has been isolated and characterized in the plant *Arabidopsis thaliana*. SHR is essential to the specification of both the root quiescent center and stem cells within the root meristem of the plant. Previous data describes SHR as a single exon gene present in only one copy in the *Arabidopsis* genome [97, 106, 138, 158]. Two known forms of SHR have been

isolated, and the only difference in these two forms is in their 3' UTR. This difference in the 3' UTR is a result of the use of alternative polyadenylation sites; as a result, their coding sequences are identical [106].

In contrast, multiple forms of this gene have been isolated in pine, although this gene has been characterized as a single copy, intronless gene. The hypothesis of this study is although the isolated Pine SHORT ROOT shows significant homology to the SHR in *Arabidopsis thaliana* we should expect to also see significant differences in their expression to account for differences in development.

Materials and Methods

Obtaining Plants and Embryo Tissue

Pine embryos were isolated from pines cones harvested weekly from open pollinated the open pollinated loblolly pine mother tree 7-56. Cone collection took place from 7/1/04 until 10/15/04 in Lyons, Georgia, at a commercial pine orchard. Pine seeds were extracted from the pine cones. The ovules were removed from the seed, and dissected to remove the pine embryo. The embryos were then staged based on morphology and flash frozen in liquid nitrogen to preserve for later use (Pullman and Webb, 1994). *Arabidopsis* tissue and RNA were obtained from Dr. Joe Nairn (UGA), Dr. John Ohlrogge (Michigan State University), and Dr. Phillip Benfey (Duke University).

RNA Isolation

Total RNA was isolated using the Tri Reagent from pine embryos. The flash frozen tissue from pine embryos was powdered with a mortar and pestle, followed by the addition of 1000 µl of TRI reagent. The sample is then stored for 5 minutes at room temperature, followed by the addition of 100 µl of 1-bromo-3-chloropropane (BCP, ACROS). The mixture is then vigorously shaken for 15 seconds, and then stored at room temperature for 15 minutes. It is then centrifuged for 15 minutes ate 14,000 rpm at 4 degrees Celsius. The aqueous phase is then collected into an RNAse free 1.5ml eppendorf tube. 250 µl of isopropanol and 250 µl of high salt precipitation buffer (0.8 M sodium citrate and 1.2 M NaCl) are added and stored for 10 minutes at room temperature. The sample is then centrifuged for 10 minutes. The supernatant is poured out and the pellet is washed with 1.0ml 75% ethanol, centrifuged for 5 minutes, and air dried under the fume hood for 5 minutes. After drying, the pellet is resuspended in 30ul of molecular grade water. The mixture is incubated at 55 degrees Celsius for 10 minutes then stored at -80° C.

cDNA Synthesis

Reverse transcription is performed using 500ng of total RNA from each embryo stage. The Clontech kit Rapid Amplification of cDNA Ends (RACE) was used. The first strand of cDNA was used as a template for pcr reactions. Advantage 2 Taq Polymerase from Clontech was used to complete all pcr Reactions. The PCR conditions used were 94 degrees Celsius for 1.5 minutes, 35 cycles of 94 degrees Celsius for 30 seconds, melting temperature of the primer for 30 seconds and 72 degrees Celsius for 2.5 minutes followed by 72 degrees for 10 minutes.

Western Blotting

Embryos from all stages were homogenized separately. Then sample buffer (10%, glycerol, 2% SDS, 5%Bmercaptoethanol was add at a ratio of 1ml sample buffer for every .2g of tissue. The samples were boiled at 95 degrees Celsius, and then centrifuged at 14000 rpm for 5 minutes. The resulting supernatant is the protein sample for western blotting. The samples were loaded on a 4-10% gel from Invitrogen (Carlsbad, CA) and run at 200V for an hour. The proteins were then transferred to a nitrocellulose membrane overnight at 20V in a cold room. The membrane was blotted using a blocking solution (.4% BSA, 3% milk). It was incubated with both the primary and secondary antibodies for one hour each, with rinses in between.

DNA Purification, Cloning and Sequencing

The pcr products are gel purified using a Qiaquick DNA purification kit from Qiagen. The purified cDNA fragments are inserted into a pGEM T-Easy vector from Promega. E. coli JM109 cells are transformed with the altered pGEM T Easy vector. The product sizes are confirmed by running colony PCR, and selected colonies are growing bacteria culture. The plasmids are purified, sequenced and analyze.

Results and Discussion

Isolation of a SHORT-ROOT –like cDNA from Loblolly Pine.

Despite differences between pine and *Arabidopsis* embryogenesis, the genes controlling their development have very similar sequences [29]. Using the sequence of

the *Arabidopsis* SHORT ROOT protein, we have isolated a PtSHR-like cDNA (PtSHR) in pine by first identifying an SHR-like sequence in the Pine EST database (http://fungen.org:8080/blast/blast_Pine.html). Homologous sequences were found, however, they were only partial clones beginning at the 3' untranslated region (Table 3.1). Primers were designed to isolate the entire pine cDNA sequence using 5' RACE PCR (Clontech) (Figure 4.2). The isolated cDNAs were cloned and sequenced. Blastx was completed using PtSHR sequence as query. The results revealed a high match with the SHORT ROOT protein in *Arabidopsis* (AtSHR) (Table 4.1). The full-length sequence was 2,442 nucleotides in length. I determined the 5' UTR to be 731 base pairs in length and the 3' UTR to be 255 base pairs in length using the ORF finder tool on the NCBI website (Figure 3.13). The GRAS only domain was the only domain present within the deduced amino acid sequence. The PtSHR deduced amino acid sequence was compared to the AtSHR amino acid sequence by an alignment. The sequences were highly similar within the GRAS domain (Table 4.1).

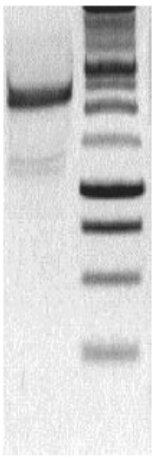


Figure 4.2 Isolation of the SHR-like cDNA in pine
PCR products run on a 1.5% agarose gel. Right: 1,000 base pair DNA ladder from Promega. On left: PCR product at that has run to the 3,000 base pair marker.

Table 4.1 Results from BLASTX Analysis of the NCBI database using the PtSHR amnin acid sequence as query.
The nucleotide sequence from the isolated clone (PtSHR) was searched against the proteins within the *Arabidopsis thaliana* database. The sequence of the GRAS domain within the PtSHR clone was also searched against the same database.

Clone	**Score**	**E- value**	**% Similarity**	**% Identity**	**Organism matched**
PtSHR	**439**	**e-121**	**64%**	**48%**	***Arabidopsis thaliana***
PtSHR Gras Domain only	**452**	**2e-127**	**74%**	**56%**	***Arabidopsis thaliana***

SHORT-ROOT is differentially expressed

To determine the expression pattern of PtSHR, we completed real time PCR using Sybergreen and two gene specific primers in a unique region of the 3' UTR of the PtSHR cDNA with stages 7 – 9.6 tissues. Expression of PtSHR peaked at stage 8 (Figure 3.23). The peak of expression in this gene coincides with the time of initial root formation within the pine embryo [1]. The expression data also correlates with PtSCR expression (Figure 3.25).

Isolation of Novel Multiple Isoforms of SHORT-ROOT in Loblolly Pine.

Because at least two forms of SHR have been previously characterized in *Arabidopsis* [106], we attempted to isolate the gene at each stage of development to determine if there are also two forms expressed in pine. While attempting to isolate the full-length PtSHR clone, several smaller sized bands were identified. This was of great interest because no other isoforms of SHORT ROOT have been in reported in *Arabidopsis thaliana* thus far. In *Arabidopsis*, there are typically two forms of SHORT-ROOT expressed, differing only in their polyadenylation sites [106]. However, RT-PCR using two gene specific primers that bind within the 5' and 3' UTRs of PtSHR results in multiple cDNAs being amplified (Figure 4.3).

The shorter length cDNAs were cloned and sequenced. After analyzing the sequences, we concluded that all isoforms were derived from PtSHR. The nucleotide sequences were identical, with the only exception being they were missing various portions of the cDNA sequence. These isoforms appear to be alternative splicing products (Figure 4.4). There were seven different types of isoforms isolated. One isoform had no

stop codon and appeared truncated. This product was most likely created by the amplification cDNA with only the forward primer. There were six isoforms that contained a 5'UTR, start codon, stop codon, and 3'UTR. These six isoforms were 100% identical in sequence within the common regions (Figure 4.4). PtSHR clone SHR2_8 was the only deviation to the observation of 100% similarity. There was an insertion of 109 nucleotides within its 5' UTR. The isolated clones contained both canonical splicing donor and acceptor sites GT and AG, however, they do no exist as pairs. Non-canonical splicing sites were also present. We were able to identify both canonical and non-canonical splice sites using NCBI's Spidey splicing tool (Figure 4.4).

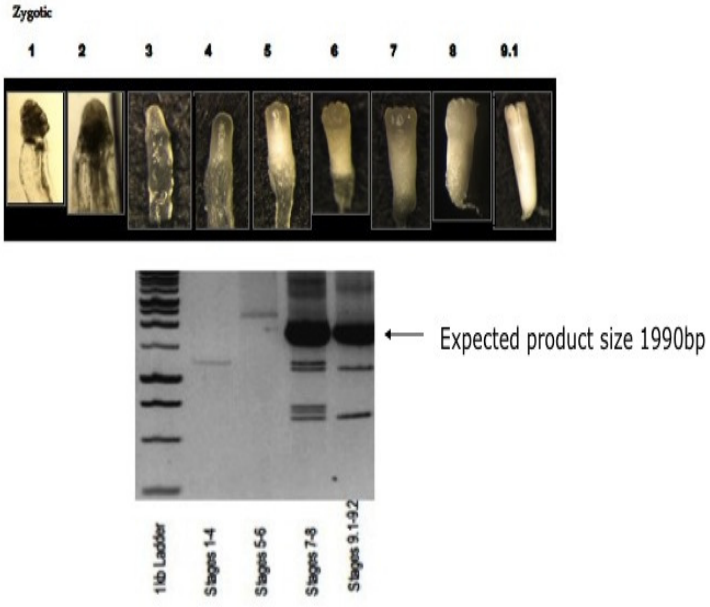


Figure 4.3 RT-PCR analysis of PtSHR cDNAs
RT-PCR was completed using two gene specific primers and RNA isolated from the 7-56 mother tree. The expected product size was 1,990 nucleotides in length. This product did not appear until stage 7-8. Unexpected smaller and larger sized products are seen in earlier stages. The relative embryo stages being compared are shown above [121].

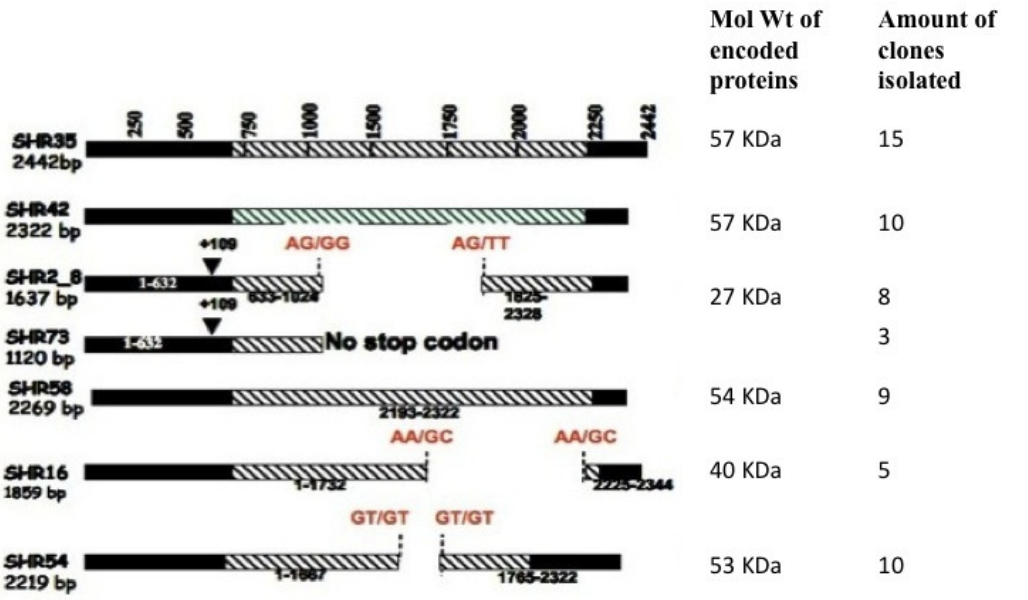


Figure 4.4 Diagram of novel PtSHR forms
RT-PCR products from Figure 4.3 were cloned and sequenced. These forms are 100% identical in sequence; however, specific pieces are missing from some clones as noted. Noted in red are the dinucleotides sequences preceding and following the splice sites. Also noted on the right of the diagram are the size of the putative protein in kilo Daltons, and the amount of clones sequenced.

PtSHR is Present as a Single Exon Gene in Pine

We attempted to isolate the genomic sequence of PtSHR in an attempt to further characterize SHR in pine. This is of interest because the novel PtSHR isoforms isolated appear to be a result of alternative splicing. This phenomenon has not been previously reported in AtSHR analysis. Interestingly, AtSHR is present within the *Arabidopsis thaliana* genome as a single-exon gene. To determine if the same is true of pine we sought to isolate PtSHR's genomic sequences using two gene specific primers within the 5' and 3' UTR regions. The forward primer binds at six base pairs (bp) and the reverse

primer binds at 2286 base pairs. If PtSHR is present as a single exon gene in pine, only one band of the full-length 2280 base pairs is expected. The products were run on a 1.5% agarose gel to verify size (Figure 4.5). There was a single band of approximately 2280 base pairs present in the sample. The PCR product was cloned, sequenced, and compared against the previously isolated cDNAs. Twenty-two independently isolated clones were sequenced and analyzed. The sequences of the twenty-two clones were virtually identical, with a minimum of zero to a maximum of 10 nucleotide differences over their length.

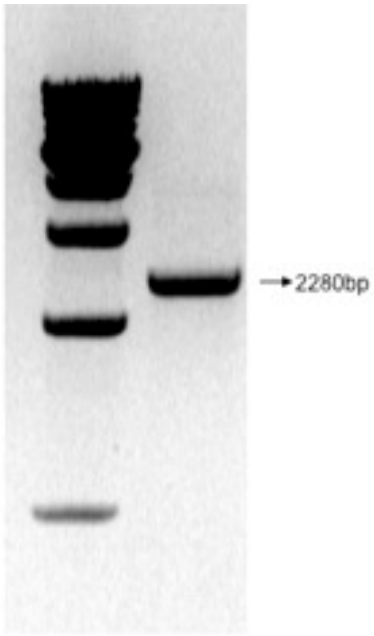


Figure 4.5 A PCR using loblolly pine genomic DNA from mother tree 7-56 as template was completed with two gene specific primers.
One prime binds in the 5' UTR and the second primer binds in the 3'UTR. Forward primer binds at 6bp. The reverse primer binds at 2286 bp. Shown on the right: PCR products on a 1.5% gel. A 1,000 base pair DNA marker from Promega is shown in the left lane and the PCR product is in the right lane. There is a product of approximately 2,280 nucleotides present.

The sequences of the isolated genomic clones were identical to the longest form of PtSHR (SHR35). This data provides evidence of PtSHR being present within the Loblolly pine genome as a single exon gene.

The Multiple PtSHR isoforms are Missing Motifs Essential to the GRAS Domain.

The GRAS domain consists of two leucine rich regions, a VHIID motif, a PFYRE motif and a saw motif (Figure 4.6)[148]. An alignment of the putative amino acid sequences of the pine isoforms illustrates that each of the isolated PTSHR isoforms are missing essential portions of the GRAS domain (Figure 4.7 and Figure 4.8). However, all isolated isoforms contain putative nuclear localization sequences (Figure 4.7). The isolated clone shr2-8 is missing the entire GRAS domain. Clone SHR-54 is missing portions of both the second leucine rich and PFYRE motifs. SHR-16 is missing portions of the second leucine rich, PFYRE, and SAW motifs (Figure 4.7 and Figure 4.8).

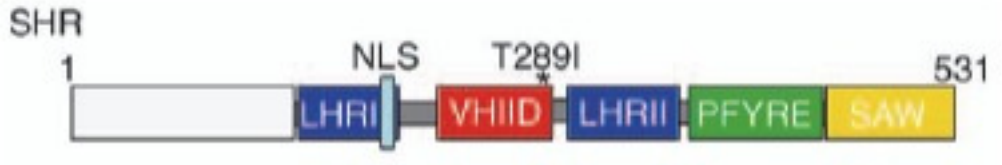


Figure 4.6 Structure of *Arabidopsis thaliana* SHORT ROOT protein [159].
The GRAS domain is the only conserved domain present in the protein sequence. However, the GRAS domain contains five conserved motifs: two leucine rich motifs (blue), a VHIID motif (red), a PFYRE motif (green), and a SAW motif (yellow).

The missing sub domains suggest that these isoforms could play important roles as SHR regulators. The leucine rich consensus motif has been demonstrated to mediate protein-protein interactions [148, 160]. They may be able to still bind DNA, but unable to pair with other proteins essential to carrying out its function. Alternatively, it could act as a dominant negative regulator. The isoforms would be able to enter the nucleus because all of the isoforms contain a nuclear localization sequence. However, if they are missing the DNA binding domain they will not be able to bind the DNA in order to initiate transcription of downstream target genes.

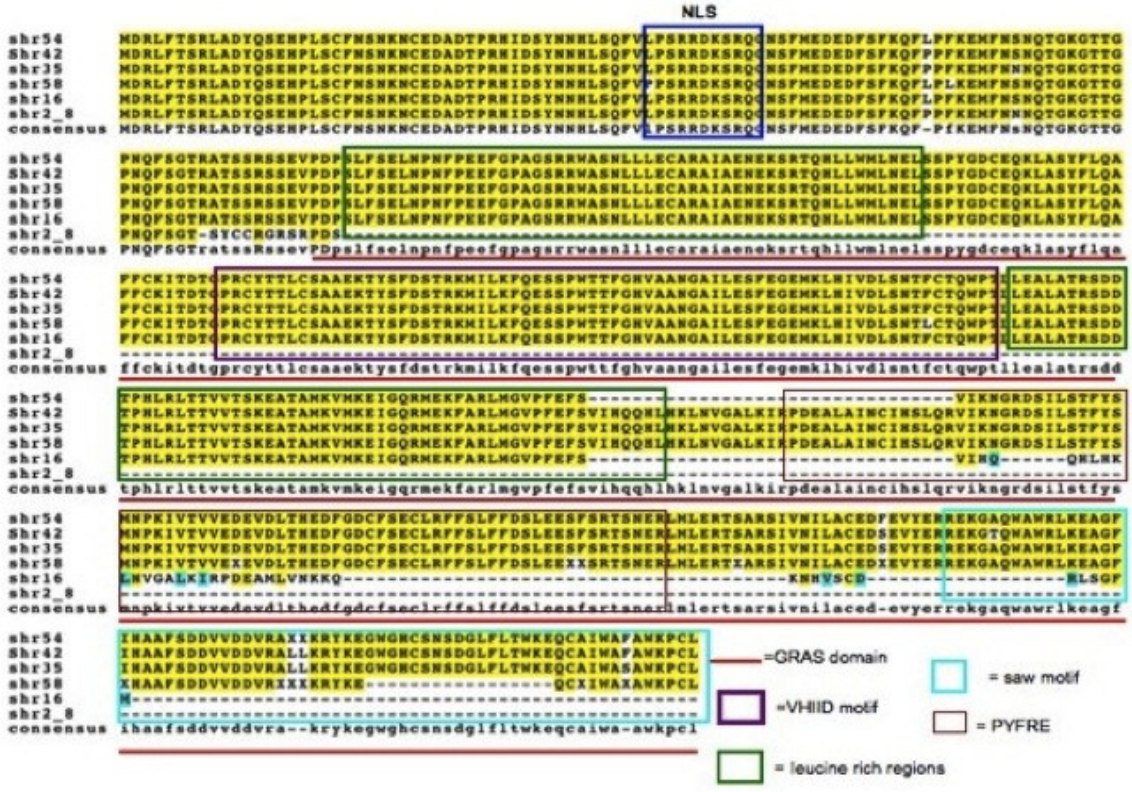


Figure 4.7 PtSHR-like isoforms are missing essential parts of the GRAS domain
The deduced amino acid sequences of each isolated isoforms are aligned again one another. The GRAS domain is underlined and each motif within the GRAS domain is illustrated.

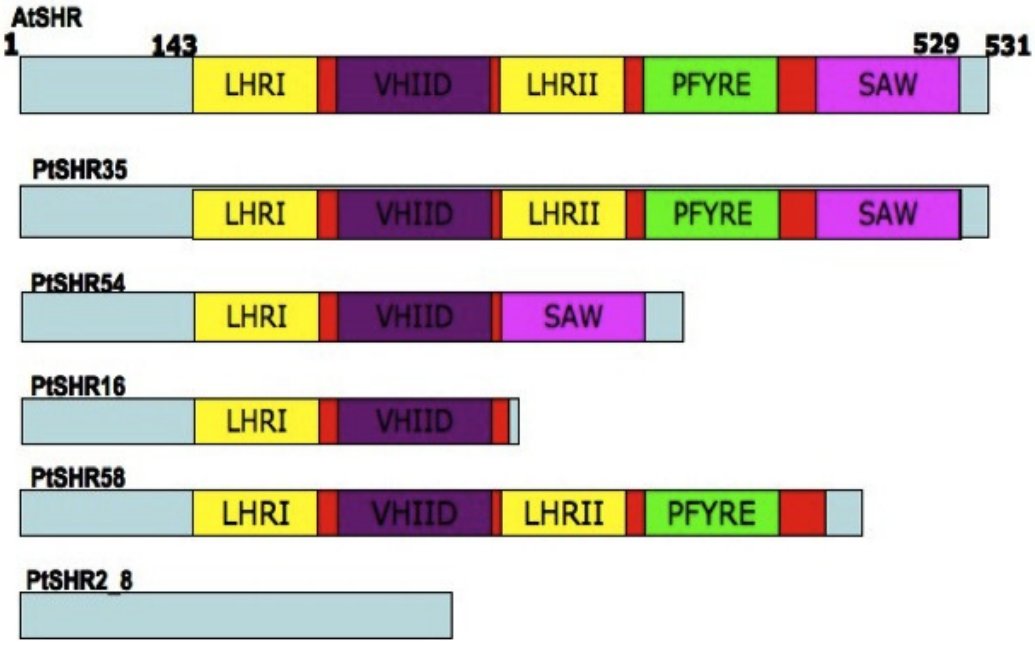


Figure 4.8 Diagram of missing motifs within each isoform
The deduced amino acid sequence of each isoforms is missing specific motifs within the GRAS domain. SHR2_8 is missing the entire GRAS domain. Each of the PtSHR isoforms is compared to AtSHR (shown at very top).

Table 4.2 Results from blastx using sequences of the SHORT ROOT isoforms as the query sequence.
This table illustrates the comparison via the NCBI blastx program of the PtSHR isoforms against the *Arabidopsis* protein database. The top hit for all isoforms was AtSHR. The score, e-value, percent similarity, percent identity and the presence/absence of the required domains are shown.

Clone	Score	e-value	% Similarity	% Identity	LEUCINE RICH I Motif	Leucine Rich II Motif	VHIID Motif	PFYRE Motif	SAW Motif	Nuclear Localization Sequence
PtSHR 35	455	6.00E-127	67	51	+	+	+	+	+	+
PtSHR 54	379	5.00E-104	62	47	+	-	+	-	+	+
PtSHR 16	290	3.00E-77	66	51	+	-	+	-	-	+
PtSHR 42	455	1.00E-126	67	51	+	+	+	+	+	+
PtSHR 58	455	2.00E-126	67	51	+	+	+	+	-	+
PtSHR 2_8	151	1.00E-35	70	52	-	-	-	-	-	-

The PtSHR Isoforms have very Specific Trends in Development

As shown in the RT PCR, the PTSHR isoforms are differentially expressed throughout zygotic development (Figure 4.9). This data demonstrates that SHR2_8 mRNA, the isoforms lacking a GRAS domain, is expressed more during mid embryogenesis (stages 5-6), and its expression decreases as development progresses.

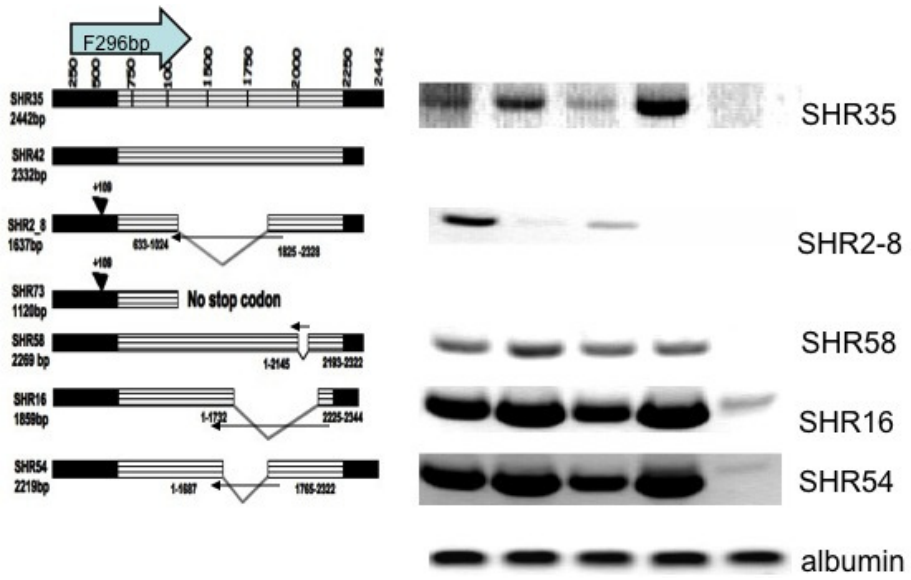


Figure 4.9 Semi quantitative RT-PCR for PtSHR isoforms
RNA from mother tree 7-56 was used to complete RT PCR for PtSHR isoforms. A common forward primer binding at 296 bp in the 5' UTR was used and primers specific for each isoforms were used as reverse primers. Albumin was used as a control.

Westerns were completed to determine if these mRNA are translated into proteins or if they are merely artifacts. These westerns were probed using polyclonal antibodies raised against the *Arabidopsis* SHR. The western show the appropriate size bands for each of the alternate forms of SHR cDNAs isolate in Pine (Figure 4.11). Furthermore, the western also provides evidence that these forms are differentially expressed. The expression and sizes somewhat correlate with what we see in the cDNA size and expression levels in the RT PCR.

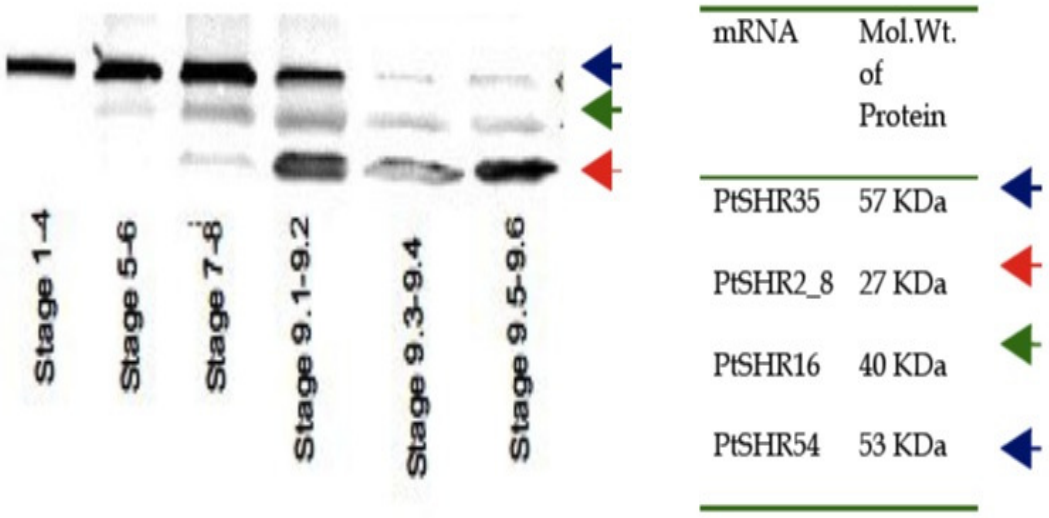


Figure 4.11 Western Blot of SHR isoform expression
Total proteins were isolated from stages 1 -9.6. A western was completed using polyclonal antibodies designed against AtSHR. The expected sized products are denoted by the colored arrows.

Alternative SHORT-ROOT forms are also Expressed in other Plants.

The homologs of 40% of alternatively spliced *Arabidopsis* genes in rice are also alternatively spliced [161]. This conservation of alternative splicing across monocots and dicots suggests that the process is important. Finding SHR spliced in number of different plant species would give more evidence that this splicing is real and not due to an error.

We used one gene specific primer in a 3' RACE reaction to determine if multiple isoforms were also expressed in *Arabidopsis*. Multiple forms of SHR were also found in *Arabidopsis* (Figure 4.13 and Figure 4.15).

We used two-gene specific primers specific for SHORT ROOT within the open reading frame in the poplar plant. We completed RT-PCR using mRNA from poplar. We found multiple forms of SHR present in poplar as well (Figure 4.15). However, the SHR isoforms isolated in both *Arabidopsis* and Poplar are not comparable in size or sequence. Primers need to be isolated in the 5' and 3' UTR of the poplar plant to gain more specific sequence information.

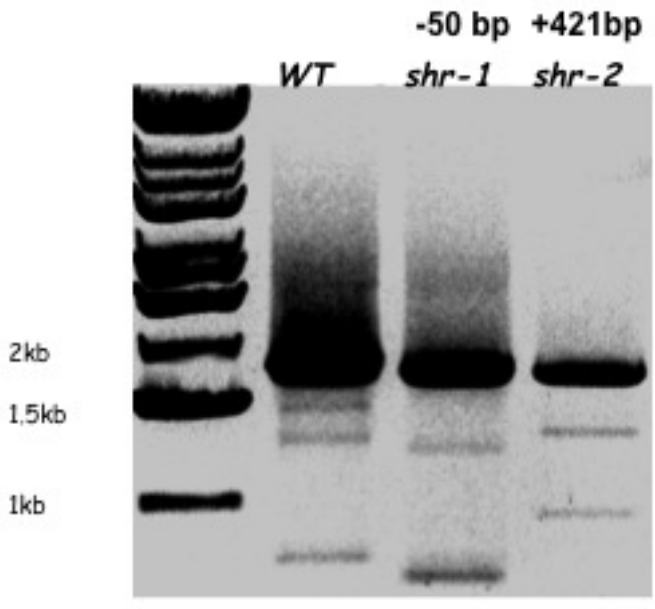


Figure 4.12 AtSHR RT-PCR

RT PCR (3' RACE) with *Arabidopsis* Wild type (WT), shr-1 mutant and shr-2 mutant. The full length characterized SHR is seen in the 1.5% gel shown above, however smaller bands of differential sizes are also present.

Figure 4.13 Alternate version of AtSHR isolated.
The PCR products from WT *Arabidopsis* PCR from **Figure 4.12** was cloned and sequenced. Two types of clones were found: a full length and a shortened clone. Sequence analysis demonstrates that the shorter clone is missing the GRAS domain.

Figure 4.14 AtSHR Western Blot
A western blot was completed using wild type (wt) Arabidopsis tissue and tissue from the SHR mutants (shr-1 and shr-2). The western was probed with polyclonal antibodies raised against AtSHR

Polyclonal antibodies for AtSHR were used to determine if multiple SHR proteins are produced. However, only a single band of lower than expected size was seen in each of the samples.

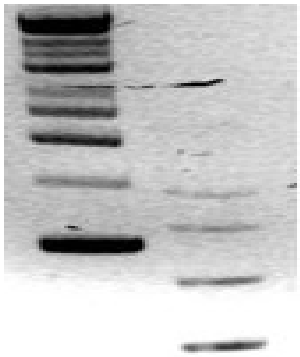


Figure 4.15 Poplar SHR RT-PCR
RT-PCR with Poplar cDNA was used with two gene-specific primers for Poplar SHR to determine if any uncharacterized forms may be present. The full-length size is seen in the 1.5% gel, however smaller sizes are also present.

Discussion

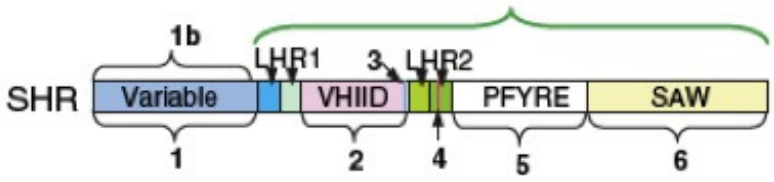
Here we report the identification and isolation of six different isoforms of SHORT ROOT in Loblolly pine. Four of these isoforms have not been reported in any other plant species. The first two isoforms, SHR35 and SHR42, differ only in polyadenylation sites. The presence of two isoforms differing only in polyadenylation sites has been previously reported in *Arabidopsis thaliana* [106]. However, each of the remainder of the isoforms

are missing essential motifs within the GRAS domain present within their deduced amino acid sequence (Figure 4.4). PtSHR2_8 is missing the complete GRAS domain, although it still contains the putative nuclear localization sequence. PtSHR54 is missing both a leucine rich motif and the PFYRE motif. PtSHR16 is missing both a leucine rich, PFYRE and SAW motifs. PtSHR58 is missing the SAW motif. All isoforms except the PtSH2_8 contain the VHIID motif. It has previously been reported in *Arabidopsis* that the VHIID motif is required for AtSHR activity [162]. In SLENDER RICE the first leucine rich motif has been shown to be required for homodimerization [163]. If this also holds true for PtSHR, all isoforms except SHR2_8 are capable of homodimerization.

Western analysis reveals that these isoforms are translated into protein and are differentially expressed throughout embryogenesis. However, the function of these putative proteins remains an unknown. Although not naturally occurring, Gallagher et al. (2009) prepared deletion constructs, each missing one of the essential GRAS motifs. This experiment was completed in an effort to determine which motifs were required for both activity and movement of the protein. The constructs were expressed directly in the endodermis under the control of the SCARECROW promoter along with a green fluorescent protein (GFP) fusion (Figure 4.16). The SAW motif appeared to be required for stability of the SHR protein. No GFP was detected when in any line expressing the construct with SAW deletion. Deletion of either VHIID or PFYRE motifs resulted in loss of movement and activity of the SHR protein. Mutation of the second Leucine rich motif resulted in loss of movement, but not activity [138]. Although the reported experiments by Gallagher et al. suggest no role for the PtSHR isoforms in development,

more information may be gained by over expressing these isoforms in a gymnosperm system.

(a)



(b)

ID	GFP	Accumulation		Protein moves	Active
		Nuc	Cyt		
SHR (unmodified)	Yes	Yes	Yes	Yes	Yes
SHRΔNta [-1] *1-162*	No				
SHRΔNTb [-1b] *1-139*	No				
SHRΔSAW [-6] *451-531*	No				
SHRΔVHIID [-2] *222-294*	**Yes**	**No**	**Yes**	**No**	**N/D**
SHRΔPFYRE [-5] *350-454*	**Yes**	**No**	**Yes**	**No**	**No**
SHR T289 [3] *289*	**Yes**	**No**	**Yes**	**No**	**No**
SHRΔLNELDV [-4] *341-346*	**Yes**	**No**	**Yes**	**No**	**Yes**

Figure 4.16 AtSHR deletion constructs [138].
In (a) diagram of domains deleted in AtSHR to analyze to SHR movement and activity. Shown in (b) is the summary of results of deletion analysis.

This study provides evidence of deviations in gene expression between gymnosperms and angiosperms. The novel isoforms found in pine have not been reported elsewhere. However, other recent studies have also reported novel isoforms of other transcription factors and enzymes in pine due to alternative splicing [164, 165]. Sheth (2008) isolated novel KNOX cDNAs in pine lacking a homeodomain. Production

of this KNOX cDNA lacking a homeodomain was demonstrated to be a result of alternative splicing through genomic sequence analysis [164]. Zhu (2008) isolated a novel ceramide kinase in loblolly pine embryos. It was significantly shorter than the main form and was determined to be a result of alternative splicing. Through functional assays, the novel isoform was able to perform the same catalytic functions as its *Arabidopsis* counterpart, however, with lower efficiency [165]. Therefore, the identification of novel SHR isoforms is not an isolated occurrence. In fact, functional characterization in pine may provide evidence that the differences in expression of SHR forms in pine may cause some differences in development seen in pine.

Chapter 5
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Three putative ABA genes were identified and isolated in Loblolly pine. The genes were cloned, sequenced and analyzed to reveal significant homology to the *Arabidopsis* ABA genes ABI3, ABI4 and ABI5. Full-length cDNAs were isolated and cloned for PtABI4. Blast searches confirm high sequence similarity between these genes and their pine orthologs. Putative conserved domains were found within each of the deduced amino acid sequences. By sequence similarity, PtABI3 belongs to the B3 plant super family of transcription factors. The B3 domain is a DNA binding domain found exclusively within plant transcription factors [83, 130]. PtABI4 possesses conserved regions of the APETELA2 domain belonging to a family of transcription factors. The AP2 domain is required for DNA binding in ABI4 [78, 87]. PtABI5 contains the bZIP domain present in plant transcription factors. The presence of these DNA binding sites and their high degree of similarity with their *Arabidopsis* orthologs provide evidence that the PtABI3, PtABI4 and PtABI5 may function as transcription factors in pine.

Expression analysis of these genes in Loblolly pine zygotic embryos has also been completed. The expression pattern of these genes does not correlate with reports regarding the expression pattern of ABA and other ABA responsive genes in Loblolly pine [52, 79]. Instead of the reported biphasic pattern of expression seen with both LEA

genes and ABA accumulation, a triphasic pattern of expression during late embryogenesis is seen. This is most likely due to other factors that may play a role in ABA signaling. Both ABI4 and ABI5 have been demonstrated to play significant roles in sugar sensing in addition their role in ABA response [78]. Arroyo et al have shown that the addition of glucose to media caused an increase in expression of both ABI4 and ABI5 in *Arabidopsis* seedlings [78]. It is most likely that PtABI3, PtABI4 and PtABI5 are receiving multiple regulation cues as well.

Six genes determined to be involved in root development by sequence analysis were identified and their expression profiled. The genes were cloned, sequenced and analyzed to reveal significant similarities with their *Arabidopsis* counterparts: MONOPTEROS, HOBBIT, SCARECROW, SHORT ROOT, BODENLOS, and WOODENLEG. Full-length cDNAs were isolated for SCARECROW, SHORT ROOT, BODENLOS ad WOODENLEG. Conserved domains are present in their deduced amino acid sequences. Expression of these genes throughout late embryogenesis is differentially regulated. Their expression provides evidence of a developmental transition occurring between stages 8 and 9.1 in zygotic embryos.

Finally, six novel isoforms of SHORT ROOT have been isolated and characterized in Loblolly pine. Each of these isoforms is missing required sub domains within the GRAS domain. These sub domains are required for protein –protein binding and DNA binding [148] . Analysis of both their mRNA and protein expression was completed. Alternate forms were isolated in both *Arabidopsis* and poplar plants. However, the isolated forms in *Arabidopsis* and poplar were not similar to those found in pine.

Future Directions

This study focused on the identification, isolation and initial molecular characterization of these genes key to late embryogenesis. The next logical step is to verify their function in Loblolly pine. There are many approaches available to determine function, however, many techniques are difficult to complete due to the recalcitrant nature of gymnosperms [135]. One simple approach to avoid difficulties in pine is to make transgenic *Arabidopsis* plants over-expressing all genes. Not only is *Arabidopsis* easy to complete transformations, but there is also a wide variety of mutants available.

It is also necessary to complete expression analysis in somatic embryos to compare expression data. However, this will be difficult due to the inability of somatic embryos to develop past stage 9.1 [1]. Initially, in order to test for deviations from the zygotic system it may be necessary to complete expression analysis of lower embryo stages. Over expression of these genes in somatic embryos is one method of testing for the physiological affects of these genes in somatic embryos. However, constitutively over expressing these genes in pine may prove to be fatal. To overcome this issue one can use an inducible promoter. Nopaline synthase is a frequently used inducible promoter. It is able to be induced by auxin [13]. Using the inducible promote we can induce the genes at different stages of development to determine the effects they may have on physiology as well as expression of other genes. Changes in global gene expression can be determined using microarrays.

An alternative to the inducible promoter is the use of the cauliflower mosaic virus 35S promoter. This will cause over expression of the gene in all plant tissues. *Agrobacterium tumefaciens* can then be transformed with the construct. The resulting

Agrobacterium strain can be transformed into *Arabidopsis* by floral dip method [166]. Transformants can be selected on kanamycin and the phenotypes of plants grown can be studied.

Although more difficult, these genes can also be over expressed in loblolly pine embryos. Over expressing these key genes in pine, embryos may produce abnormal embryos that fail to complete development. The large sizes of loblolly pine embryo facilitate molecular studies in pine which are difficult in *Arabidopsis* embryos [31, 32]. Transformation of mature pine zygotic embryos using *Agrobacterium* [167] and particle bombardment [168] have been successfully used and reported.

To show loss of function phenotypes, antisense or siRNA constructs can be created and used against the pine gene's mRNA. The antisense construct is normally placed downstream from an ubiquitin promoter. The sense orientation of the gene is typically used as a control. Oligonucleotides in sense and antisense orientation in hairpin structure can be purchased. A vector construct can be transformed into pine using particle gene gun or the *Agrobacterium* floral dip method. Transformants can be selected, their physiology studied or molecular changes studied using microarrays.

Interactions can also be studied using in-vitro pull down assays or yeast two hybrid assays can be used to determine the interacting proteins. Pull-down assays have been used successfully to determine interaction of proteins in rice [169].

Application of Technology

Trial and error techniques are often used to improve SE techniques [170]. Other approaches to assist with improvement of SE techniques include the study of the biochemistry, physiology and genetics of zygotic embryos during development [34, 171]. Genetic analysis is a promising alternative to traditional methods because changes during embryogenesis are often difficult to observe visually [170].

This work identifies target genes that may potentially serve as that can potentially be used to index the health and development of somatic embryos. The use of these genes as markers of health can prevent unnecessary expenses and manpower wasted on the production of embryos that do not have the potential to be converted to high quality plantlets [28].

A possible strategy to be employed is to profile the gene expression of candidate genotypes for somatic embryogenesis. Hundreds of genotypes can be tested simultaneously using microarray technologies. Expression changes throughout embryogenesis can be compared to our reports of gene expression during zygotic embryogenesis. Quantitative PCR can be used to verify patterns of expression once initial selections have been made with microarray data. Other techniques can also be used as diagnostic tools, including northern blots, real time PCR and differential display to assay for specifically only these nine genes. The selection of genotypes that exhibit a similar pattern of expression as zygotic embryos will more likely produce healthy embryos. Genotypes with the predicted patterns of expression can be selected for embryo production. These genes can therefore be used as diagnostic tools to evaluate tree genotypes and predict phenotypes.

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