Isolation, Characterization and Ectopic Expression of the Douglas-fir Embryo-specific Gene, *LEAFY COTYLEDON1*

by

Mariana Vetrici
B.Sc. Biochemistry, University of Calgary, 1997

A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

Supervisory Committee

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Abstract

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Douglas-fir (*Pseudotsuga menziesii*) is an economically important softwood that is clonally propagated for reforestation purposes by somatic embryogenesis. The molecular basis of embryogenesis in conifers is largely unknown and this prevents progress in somatic embryogenesis protocols. In angiosperms, the *LEAFY COTYLEDON1* (*LEC1*) gene, encoding the HAP3 subunit of the eukaryotic CCAAT box-binding factor, is important in embryo formation, and necessary for somatic embryogenesis.

A candidate gene strategy was employed to isolate the Douglas-fir *LEC1* homologue, *PmLEC1*, *via* the polymerase chain reaction (PCR) with degenerate primers based on the *Arabidopsis* conserved domain, and the full-length cDNA sequence was obtained by rapid amplification of cDNA ends-PCR (RACE-PCR). The putative protein sequence shared high sequence identity with *Arabidopsis* LEC1. Northern analysis and quantitative real-time PCR indicate that this is an embryo-specific gene, expressed with the highest abundance during early embryogenesis. Antibodies were raised against a synthetic 18-amino acid PmLEC1 peptide, and in contrast to mRNA expression, Western blotting shows that PmLEC1 protein expression persists until the seedling stage.

To gain insight into modulation of *PmLEC1* expression and its inducibility in mature tissues, stress and hormone treatments were performed on mature seed and the promoter sequence was isolated by genome walking. Sorbitol, mannitol and 2,4-epibrassinolide were found to significantly up-regulate *PmLEC1* expression. The *PmLEC1* promoter contains a 5' UTR intron with numerous enhancer elements, and factors that bind to these elements mediate responses to auxin, UV light and developmental cues, osmotic stress, biotic stress, and tissue culture. Some of the regulatory elements are binding sites for seed-specific transcription factors that are well known from angiosperms, providing new evidence that AGL15, ABI3 and VP1 proteins have a direct role on *LEC1* expression.

In investigating the embryogenic capacity of *PmLEC1*, ectopic expression of *PmLEC1* in the embryo lethal *Arabidopsis lec1-1* null mutant complemented the mutation and permitted the production of viable, desiccation tolerant seeds. In addition, transgenic seedlings produced embryo-like structures from vegetative organs and expressed seed-specific genes. In wild type plants, ectopic expression of *PmLEC1* resulted in a bushy phenotype but expression of seed-specific genes was not observed.

Taken together, these results show that *PmLEC1* is an embryo-specific gene with an essential role throughout embryogenesis, and PmLEC1 expression may be induced in mature seeds by stress and hormone treatments. Because mature seeds show only trace amounts of *PmLEC1* transcripts and Douglas-fir somatic embryogenesis can only be induced from immature embryos, this information provides useful insight into initiation of embryogenesis from vegetative tissues. The identification of binding sites for transcription factors known from angiosperms in the promoter region of PmLEC1 has

revealed the identity of several genes which are expected to play pivotal roles in conifer embryogenesis.

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List of Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
ABI3	*ABSCISIC ACID INSENSITIVE3*
AGL15	*AGAMOUS-LIKE15*
AGO	*ARGONAUTE*
AGP	**arabinogalactan protein**
AMV	alfalfa mosaic virus
ASC	ascorbic acid
At	*Arabidopsis thaliana*
BAP	benzyl aminopurine
BBM	*BABY BOOM*
bp	**base pair**
Brassinol	2,4-epibrassinolide
BSA	**bovine serum albumin**
BSO	DL-buthionine-[S,R]-sulfoximine
CaMV	cauliflower mosaic virus
CBF	CCAAT box-binding factor
cDNA	complementary deoxyribonucleic acid
Col	Columbia, ecotype of *Arabidopsis*
Ct	**threshold cycle**
CTAB	cetyltrimethylammonium bromide
DEPC-H_2O	diethyl pyrocarbonate-treated water
DNA	deoxyribonucleic acid
EDTA	**ethylenediaminetetraacetic acid**
ELISA	**enzyme linked immunosorbent assay**
ESM	embryogenic suspensor masses
EST	expressed sequence tag
EtBr	ethidium bromide
EtSH	2-mercaptoethanol
FUS3	*FUSCA3*
GA	gibberellic acid
GER	*GERMIN*
GSH	glutathione
GSP	gene specific primer
GSSG	oxidized glutathione
HAP	heme activated protein
HSP	heat shock protein
Inr	initiator sequence
kDa	**kiloDalton**
KLH	keyhole limpet hemocyanin
KNOX	*KNOTTED-like homeobox genes*
L1L	*LEAFY COTYLEDON1-LIKE*
LCO	lipo-chito-oligosaccharide
LEA	late embryogenesis abundant protein

LEC1	*LEAFY COTYLEDON1*
LEC2	*LEAFY COTYLEDON2*
MADS	**MCM1-AGAMOUS-DEFICIENS-SRF family of transcription factors**
MS	Murashige & Skoog plant medium
MT	**metallothionein**
MUSCLE	Multiple sequence comparison by log expectation
MYB1	*MyeloBlastosis virus* oncogene
NF-Y	nuclear factor Y
nt	nucleotide
Os	*Oryza sativa* (rice)
Pa	*Picea abies* (Norway spruce)
PAGE	**polyacrylamide gel electrophoresis**
PCD	programmed cell death
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEM	**proembryogenic mass**
Pg	*Picea glauca* (white spruce)
PGR	plant growth regulator
PKL	*PICKLE*
PR	pathogenesis-related proteins
Pm	*Pseudotsuga menziesii* (Douglas-fir)
QPCR	quantitative real-time PCR
RACE-PCR	rapid amplification of cDNA ends-PCR
RAM	root apical meristem
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription-PCR
SAM	shoot apical meristem
SDS	sodium dodecyl sulfate
SE	somatic embryogenesis/somatic embryos
TE	Tris-EDTA
Tnp	*turnip* mutant
UTR	untranslated region
VAL	*VP1/ABI3-LIKE*
VP1	*VIVIPAROUS1*
Ws	Wassilewskija, ecotype of *Arabidopsis*
WUS	*WUSCHEL*
ZE	zygotic embryogenesis

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Dedication

This dissertation is dedicated to the spirit of my mother. My mother's traits of optimism and perseverance were heritable to my generation, and I found them indispensable in the quest for scientific knowledge and experimental results.

1. Conifer Somatic Embryogenesis: the Road from Medium Manipulation to Candidate Gene Characterization

1.1 Introduction

In higher plants, zygotic embryogenesis (ZE) is not only the time when a fertilized ovule develops into a multicellular embryo but also an advantageous method of survival by life cycle interruption during unfavourable conditions. During morphogenesis, or the early part of embryogenesis, the zygote divides asymmetrically into the apical and basal cells, which lead to the formation of the embryo proper and the suspensor, respectively. Further cell division and differentiation lead to the establishment of the body plan of the juvenile form, consisting of cotyledons (embryonic leaves), hypocotyls (embryonic stem) and radicle (embryonic root). The early embryo is defined by the axis of polarity, the meristems (shoot apical and root apical) and the radial pattern of tissue layers (epidermis, ground tissue and vascular tissue). During the maturation phase, further embryo growth and the cell cycle are arrested while the embryo expands, differentiates and accumulates storage compounds, such as, proteins, carbohydrates and lipids. Late maturation is characterized by metabolic quiescence and desiccation tolerance. Plant embryogenesis is unique in that fertilization is not strictly required and many species produce embryos asexually. The production of asexually derived embryos may be achieved *in vitro* from a variety of somatic and gametophytic tissues by the addition of hormones or stress treatments and this process closely parallels zygotic development, physiology and molecular events. Somatic embryogenesis produces ample tissues for the study of embryogenesis and provides a method for cloning superior species, as in reforestation

applications where planting a mix of superior genotypes leads to rapid gains in productivity. Research over the last 30 years has focused on improving somatic embryogenesis *via* medium manipulation to produce robust embryos, and the isolation and characterization of embryo-specific genes to better control the induction process.

The present research examines Douglas-fir *LEAFY COTYLEDON1* (*PmLEC1*), an embryo-specific gene expected to be essential to normal zygotic embryogenesis and induction of somatic embryogenesis. PmLEC1 RNA expression and protein accumulation were determined in embryonic and vegetative Douglas-fir tissues. In order to gain insight into induction of conifer embryogenesis, the effect of stress and hormone treatments on *PmLEC1* gene expression in mature seeds was assessed by QPCR, and the *PmLEC1* promoter sequence was isolated and surveyed for regulatory elements. Functional analysis of PmLEC1 was achieved *via* ectopic expression in *Arabidopsis* wild type and lec1-1 mutant backgrounds. Taken together, these results reveal the identity of genes involved in conifer early embryogenesis, provide potentially relevant information for somatic embryogenesis induction and contribute new insights into the hierarchy of genes responsible for plant embryogenesis.

1.2 The Promise of Somatic Embryogenesis (SE)

The increased demand for forest products and rapid deterioration of forests pressures government and industry alike to find effective methods for forest regeneration (Minocha & Jain, 2000). Because forest trees need pest and disease resistance, cold and drought tolerance, and a capacity to grow faster in shorter periods of time, a basic strategy for tree improvement is to capture existing genetic gains through clonal

propagation (Gupta et al., 1991). Macropropagation, the rooting of stem cuttings, is not possible with many species and is labour intensive. In micropropagation, tissue explants are cultured in media and given the appropriate stimuli to induce callus formation (Minocha & Jain, 2000). Two processes can be used to generate new plants from the callus. Roots and shoots are regenerated by the addition of hormones *via* organogenesis. Alternatively, in somatic embryogenesis (SE), embryo formation is induced from the callus by the addition of a set of plant growth regulators.

SE is potentially the most efficient method for producing superior trees with desired characteristics because differentiated cells from high value trees are used to initiate and produce thousands of identical embryos, and rapid genetic gains are possible (Gupta et al., 1996). In Douglas-fir, mature somatic embryos can be produced in 4 months. This high productivity is in contrast to zygotic embryogenesis where the fusion of one sperm cell with one egg cell produces a diploid embryo, the entire reproductive cycle lasts 17 months and good seed crops occur approximately every 5 years (Allen and Owens, 1972). At this time, conifer SE can only be induced from juvenile tissues such as megagametophytes, excised zygotic embryos or seedling, and by the time that the superiority of a tree is determined, embryogenic tissues may not be available anymore. The common practice for acquiring high quality zygotic embryos for SE induction is to harvest developing seed after high value trees are pollinated with gametes from other elite genotypes. However, the embryos produced *via* fertilization may be inferior to the superior parents and this necessitates cryopreservation of new hybrids and lengthy field-testing, which may take up to 20 years. Once new hybrids are tested, the stock can be retrieved from storage and planting a large number of elite but diverse high value trees

may be employed to increase forest productivity (Pullman et al., 2005). Gaining the ability to induce SE from mature conifer explants would enable us to clone superior trees directly and would represent a major time and financial saving because the testing and hybrid characterization period would be eliminated.

In conifers, SE is normally induced from immature tissues, followed by proliferation of proembryogenic masses, embryo maturation, embryo germination and conversion to plantlets (Stasolla & Yeung, 2003). Conversion refers to the development of functional root and shoot systems while germination refers to the emergence of an embryonic root (Stasolla & Yeung, 2003). In brief, the basic sequence of SE is: induction, proliferation, maturation, germination and conversion.

The present challenges of somatic embryogenesis in all gymnosperm species, encompassing low initiation rates, low culture survival, culture decline leading to low or no embryo production, the inability of somatic embryos to reach maturity, low conversion rates and reduced growth of somatic plants, prevent mass commercialization of somatic embryogenesis in conifers (Pullman et al., 2005). As a result, much of present-day research has focused on the rational design of growth media in order to produce high quality embryos that will convert successfully (Stasolla et al., 2002). An alternative strategy however, would be to try to improve somatic embryogenesis *via* the identification of genes and proteins that are responsible for quality embryo formation. Yet, we do not understand the structural or molecular events linking the unorganized proliferation of embryogenic tissue with the organized embryonic development phase in conifers (Stasolla & Yeung, 2003). The early stages of SE are known to be critical for the completion of the entire process (Stasolla & Yeung, 2003). This is also seen in

angiosperms, where mutations that affect early embryonic phases result in irreversible developmental arrest or in abnormal embryos that are incapable of producing viable plantlets. Thus, progress in conifer SE will depend on a more thorough understanding of genetic and molecular events during all stages of embryogenesis.

1.3 Rationale for Studying the Molecular Biology of Conifer Embryogenesis

The divergence of gymnosperms (Cycads, Gingko, conifers, gnetophytes) from angiosperms (flowering plants) 300 million years ago and the fact that conifer embryogenesis is not well understood at the molecular level make investigations worthwhile, especially with respect to efforts at reforestation and increased productivity of lumber, pulp and paper industries. The difference in reproductive biology is one reason why we cannot fully rely on knowledge extrapolated from angiosperm SE. In angiosperms, a double fertilization event produces the triploid endosperm, or nutritive tissue, which surrounds the diploid embryo. The embryo is the result of a separate fertilization. In gymnosperms, the diploid embryo is surrounded by a haploid, female megagametophyte (Figure 1). More than one fertilization event may occur within the gymnosperm ovule since multiple archegonia are present, but by the time a seed reaches full maturity, a single dominant embryo is usually produced (Figure 1) (Allen and Owens, 1972). Interestingly, evaluation of the effect of parent genotype on pine somatic embryogenesis revealed that the maternal effect was most pronounced at initiation and that only the maternal genotype had a significant effect on the number of mature embryos produced (Niskanen et al., 2004). Another unusual feature of gymnosperm embryogenesis is that the zygotic nucleus undergoes several divisions without cytokinesis

to form a syncytium (Figure 1). This is followed by cellularization resulting in an eight-celled proembryo with 2 tiers of 4 cells each (Figure 1). In the past, small structures and limited tissues made molecular work difficult and labour-intensive, but this is not an issue anymore due to advances in technology and the availability of unlimited amounts of tissues form SE cultures.

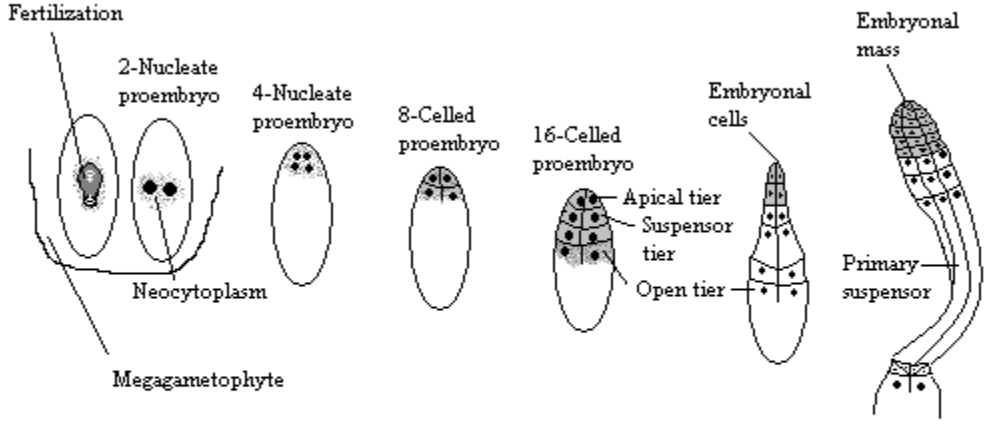


Figure 1. Douglas-fir embryogenesis. Adapted from Allen & Owens, 1972.

Schematic representation of archegonia within megagametophyte, fertilization, cytoplasmic inheritance, nuclear divisions without cytokinesis, the proembryo, and the embryonal mass and suspensor of the early embryo.

SE produces ample amounts of embryos at each developmental stage and this facilitates investigations into molecular, physiological and structural aspects of

embryogenesis. SE reflects zygotic embryogenesis in that somatic embryos proceed through stages of similar morphology and physiology, even though the culture environment replaces the nutritive seed tissue. Classical gymnosperm embryology is characterized by 3 phases: proembryogeny, encompassing the stages prior to the elongation of the suspensor (Figure 1); early embryogeny, including stages after suspensor elongation and before establishment of a root meristem (Figure 1); and, late embryogeny, representing the establishment of the root and shoot meristem and final development of the embryo until maturity (Filonova et al., 2000). Disappointingly, conifer SE protocols are not as efficacious as in flowering plants.

In angiosperms, somatic embryogenesis is easily induced from any tissue of any age by stress and/or hormone treatments (Mordhorst et al., 1998; Ikeda-Iwai et al., 2002; Hecht et al., 2003; Raghavan, 2004). In conifers however, only juvenile tissues have the embryogenic capacity necessary for successful regeneration (Stasolla et al., 2002; Gupta and Timmis, 2005). By understanding both systems, complementary knowledge in somatic embryogenesis may lead to optimization of protocols in both angiosperms and gymnosperms. For example, conifer embryogenesis extends over a longer period of time (months), and the activity of genes during specific stages can be more clearly delineated to specific stages. This information can be related back to angiosperms where developmental stages may only take a few days and the distinction between stages is not as clear. Conversely, the ease of working with angiosperms and widespread genome sequencing, can augment our understanding of conifer embryogenesis *via* the identification of genes or protein activities that should also exist in conifers due to common ancestry.

1.4 Overview of Douglas-fir Somatic Embryogenesis

Douglas-fir (*Pseudotsuga menziesii*) is the most important commercial species in the Pacific Northwest (Pullman et al., 2005), and is a valuable softwood in international markets. While it originated in the Pacific Northwest, it has found use as an alternative forest system in New Zealand, Australia and Europe (Ledgard et al., 2005; Curt et al., 2001; Kaiser, 1999). Douglas-fir produces a first rank timber known for its superior strength-to-weight ratio, resistance to warping, high ratings for extreme fibre stress in bending, and performance against wind, storms and earthquakes. Overcutting of North American forests has resulted in significant shortfalls of Douglas-fir supply to traditional markets and less than 18% of old crop Douglas-fir remains (Ledgard et al., 1994). This number includes the reductions in cutting due to conservation pressure. Years of reforestation in British Columbia have shown that second-growth Douglas-fir has mature characteristics and dimensions that cannot be surpassed by faster-growing softwoods in other areas of the world (Weyerhaeuser.com). Habitat protection and the appeal of new-growth lumber area strong incentives for reforestation with Douglas-fir. A brief review will focus on the improvements that are needed and the progress to date on conifer SE.

In Douglas-fir, immature embryos are induced to multiply by cleavage polyembryony and form embryo suspensor masses, or embryogenic callus, by introducing to the medium a combination of the cytokinins kinetin and benzyl aminopurine, and the auxin 2,4-dichlorophenoxyacetic acid (Gupta et al., 1996). To induce embryo development and inhibit cleavage polyembryony, the auxins and cytokinins are removed from the medium and abscisic acid (ABA) is introduced. This represents the unique role of ABA in Douglas-fir SE because the proembryos form in tight clumps or clusters that must first be

singulated (separated) before proceeding to the next stage of development (Gupta and Pullman, 1991). ABA permits singulation and continued growth of individual embryos and also plays a role in storage compound accumulation and embryo maturation (Gupta et al., 1991). In other conifer species, the SE process includes an incubation period in hormone-free medium prior to ABA treatment, and it is at this time that somatic embryos first appear.

Mature Douglas-fir embryos are obtained by transfer of the singulated embryos to medium containing polyethylene glycol (PEG), ABA, gibberellic acid (GA), and activated charcoal (Gupta et al., 1991). While the described method of SE is productive, more than 50% of Douglas-fir SE cultures discontinue growth after six months and significant losses occur from culture initiation to somatic seedling establishment (Gupta et al., 1996). Because the causes of loss are not known, molecular markers and an understanding of the embryogenic process at the molecular level is urgently needed.

Another drawback of present protocols is that conifer SE can only be induced from immature tissues, and by the time the superiority of the tree is confirmed, embryogenic tissues may not be available (Gupta et al., 1991; Timmis, 1998; Stasolla et al., 2002). It is not yet possible to induce SE from vegetative, mature cells such as those found in stems or needles. SE from mature tissues would result in the production of somatic seedlings with known genetic characteristics. This would eliminate the lengthy field-testing component of developing elite genotypes.

Past work has clearly elucidated the morphological characteristics of Douglas-fir development, from male and female cone development to fertilization, embryo formation and maturation (Allen and Owens, 1972). Morphology is easily correlated with stages of

embryo development, and even if an environmental disturbance occurs (i.e., a frost) and development is delayed, the exact stage of the embryos can be ascertained based on morphology. SE may be facilitated by the identification of genes and proteins whose expression is strongly correlated with the acquisition and maintenance of embryogenic potential (Misra, 1994). Consequently, the molecular characterization of these genes will help us understand the developmental processes that lead to the formation of zygotic and somatic embryos. This will help improve SE protocols (Misra, 1994), and will lead to novel strategies for induction of somatic embryogenesis.

1.5 Present State of Conifer Somatic Embryogenesis: Early Embryogenesis, Induction and Proliferation

Induction and proliferation is achieved from juvenile tissue incubated in darkness in the presence of auxin and cytokinin for 4-6 weeks, at which time embryogenic tissue becomes visible (Stasolla et al., 2002). Most frequently, immature zygotic embryos at the pre-cotyledonary stage are used for initiation, but mature embryos and embryos attached to megagametophytic tissue may also be employed. Exogenous auxin and cytokinin promote proliferation but inhibit somatic embryo formation. In Norway spruce and other conifers, withdrawal of the plant growth regulators (PGRs) triggers the transition of proembryonal masses to early somatic embryos, and this can be accomplished within 7 days in PGR-free medium (Filonova et al., 2000b). Late embryogeny and maturation are achieved by application of ABA to the medium (Filonova et al., 2000b), but these are dependent on successful and vigorous initiation and maintenance of embryogenic potential. At this time, there are no known molecular

markers of early embryogenesis and no genes that can be manipulated for improved performance. Much insight has been gained by thorough investigations into embryogenic and non-embryogenic cell lines, endogenous factors that promote embryogenesis, and structural events and physiological processes characterizing somatic embryogenesis proper. In addition, manipulation of medium composition for robust somatic embryo production has been the trend since the 1970s.

1.5.1 Embryogenic Cell Lines: Lipo-chito-oligosaccharides, Chitinases and Arabinogalactan Proteins Direct Proembryogenic Mass Proliferation and Embryo Formation

As early as 1995, it was recognized that some proliferating cultures had low embryogenic success and despite weekly doubling in mass, mature somatic embryos were rarely produced. After extensive work with Norway spruce somatic embryos, Egertsdotter and von Arnold (1995, 1998) found that embryogenic lines could be classified into two types. Group A lines produced mature embryos in response to ABA and contained somatic embryos with densely packed cells that were clearly separated from the suspensor region. Group B embryogenic lines did not easily form somatic embryos, and the few embryos that formed were characterized by small embryonic regions without well-defined suspensors. Group B embryos appeared to be developmentally blocked (Egertsdotter et al., 1993). In 1995, Egertsdotter and von Arnold noted that extracellular proteins extracted from either A lines or mature Norway spruce seeds, induced group B embryos to develop beyond their blockage and converted them into group A embryos. The contributing factors were arabinogalactan proteins (AGPs), and these were present at high levels in extracellular proteins extracts. AGPs are

signal molecules that promote cell aggregation *via* cell adhesion of the embryogenic cells, and this aggregation and the associated cell-to-cell communication affect responsiveness to ABA (Stasolla and Yeung, 2003).

A similar conversion of group B to group A cell lines occurred after either a chitinase or a nod factor was included in the medium of B cultures (Egertsdotter and von Arnold, 1998; Dyachok et al., 2002). Nod factors are lipo-chito-oligosaccharide (LCO) signal molecules, which stimulate proembryogenic mass growth and early embryo development by suppressing cell death, and are absent in non-embryogenic cell lines (Dyachok et al., 2002). Chitinases were found to be pertinent to early embryogenesis in both gymnosperms and angiosperms. Extracellular chitinases also rescue somatic embryo development of mutant angiosperm lines or stimulate spruce early somatic embryo development *in vitro* (Stasolla and Yeung, 2003). Further, chitinases are under developmental control and were shown to be highly expressed during early embryogenesis and down-regulated during late embryogenesis. Inhibition of chitinase activity resulted in increased LCO expression, which stimulated proembryogenic mass (PEM) proliferation and inhibited embryo development (Dyachok et al., 2002). It was suggested that chitinases degrade LCOs, but both chitinases and LCOs appear to have similar effects on proliferation and early embryo development (Dyachok et al., 2002).

In summary, LCOs and chitinases ensure embryogenic success by promoting proliferation and early embryo formation, while AGPs function in cellular aggregation and mature embryo formation. Despite this information, very little was understood about the process of embryo generation from proembryonal suspensor masses. The observation of embryo formation from PEMs led to three hypotheses: 1) cleavage polyembryony

resulting from multiplication of embryonic heads; 2) formation of somatic embryos by asymmetrical divisions of single cells; and 3) somatic embryo formation by divisions of meristematic cells within the suspensor (Stasolla & Yeung, 2003). Meristematic cells, or undifferentiated cells found in zones of the plant where growth can take place, are the plant analogues to animal stem cells. To understand how the unorganized tissue of proliferating masses becomes organized embryonic tissue, structural events were investigated.

1.5.2 Structural Events in PEMs and the Role of ABA

At the structural level, Filonova et al. (2000a) elucidated the process of somatic embryo development from proembryogenic masses in Norway spruce (Figure 2). Proembryogenic masses (PEM) are cell aggregates composed of densely cytoplasmic cells and highly vacuolated cells. The cell aggregates, or PEM, evolve through 3 stages of increasing complexity (PEM I, PEM II, PEM III) and are distinguished by cellular morphology and developmental pattern (Figure 2). Group A embryogenic cell lines follow this pathway.

In the presence of auxin and cytokinin, PEM I develop into PEM II and then PEM III (Figure 2). When the growth regulators auxin and cytokinin are removed from the medium, embryo formation occurs only from PEM III, and ABA induces further development (Filonova et al., 2000a) (Figure2). At the same time, ABA causes both PEM I and PEM II to die. A single PEM III aggregate transdifferentiates into several somatic embryos (Filonova et al., 2000a). Transdifferentiation, the transformation of a

non-stem cell into a different type of cell, is a common but unique mechanism in plants (Demura et al., 2002).

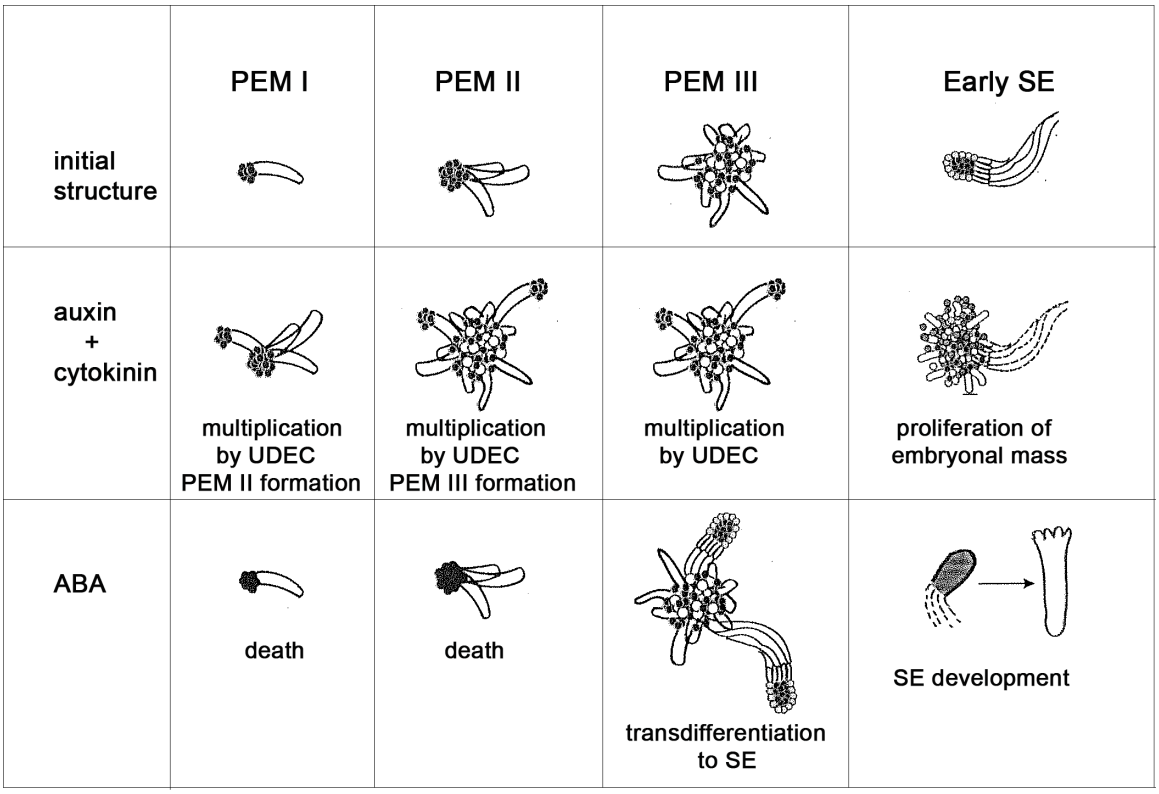


Figure 2. PEM to embryo transition. Adapted from Filonova et al., 2000a.

Development of proembryogenic masses into early somatic embryos and responses of PEMs and early somatic embryos to hormones. Auxin and cytokinin represent proliferation medium, and ABA represents maturation medium. UDEC, unequal division of embryogenic cells with dense cytoplasm.

This work clearly showed how proembryogenic masses become somatic embryos at the cellular level, and confirmed that early somatic embryos subjected to auxin and cytokinin reinitiated the embryogenic process through proliferation of embryonal masses. Because early somatic embryos are equivalent to the pre-cotyledonary stage from which SE is

usually induced, this partially explains the embryogenic success of this stage of zygotic embryos and suggests that something about this stage is conducive to initiation. Moreover, the requirement for both cell types in SE induction was demonstrated when neither the densely cytoplasmic cells nor vacuolated cells on their own could develop into somatic embryos (Filonova et al., 2000a). Control lines had starch as the major storage product. Few-celled aggregates such as PEM I easily developed further and passed through all the stages. Filonova et al. (2000a) concluded that intercellular communication was important for acquisition and realization of embryogenic competence. Finally, this study showed that PEMs I arise by unequal division of embryogenic cells from more developed PEMs and that somatic embryos continue to form by either cleavage polyembryony or re-iteration of the PEM process. Thus, the possibility exists that in earlier descriptions of somatic embryogenesis, PEM I could have been mistaken for true somatic embryo formation (Filonova et al., 2000a). Micrographs of Douglas-fir early pro-embryos in maintenance culture and late pro-embryos at the end of the ABA singulation stage (Taber et al., 1998) resemble PEM II and PEM III, respectively, suggesting that the same process occurs in other conifers. A discovery concurrent with somatic embryo production was that it was accompanied by programmed cell death (PCD).

1.5.3 PCD and Cellular Acidification are Necessary for the Emergence of Somatic Embryos from Pro-embryogenic Masses

Strong cellular acidification mediates the PEM to embryo transition (Bozhkov et al., 2002; Filonova et al., 2000b), and changes in pH commonly characterize developmental

transitions (Fehér et al., 2003). Somatic embryo formation is accompanied by massive cell death, with associated nuclear DNA fragmentation and autophagic degradation of the cytoplasm via autolytic vacuolation. In this type of autolytic degradation, vacuoles accumulate a large spectrum of organic and inorganic acids and acidic hydrolases, and the secretion of these acids and/or vacuolar collapse lead to acidification of the extracellular matrix, or the incubation medium. In the presence of auxin and cytokinin, unbuffered embryogenic cultures maintain a stable extracellular pH of ~5.3 (Bozhkov et al., 2002). After embryogenic cultures are transferred to PGR-free medium, a continuous acidification of the culture occurs until the pH is just below 4 (Bozhkov et al., 2002). When proliferating embryogenic cells were subcultured into medium that had been buffered at either high (5.8) or low (4.5) pH in order to prevent PCD, the yield of cotyledonary embryos was very low (Bozhkov et al., 2002). In fact, this yield was even lower than in treatments where PGRs were still present. Thus, the PEM to somatic embryo transition is a critical developmental switch that determines the yield and quality of mature somatic embryos and consequent plant production, with pH deviations being detrimental (Bozhkov et al., 2002).

With regards to SE performance, the number of somatic embryos produced is directly correlated with the number of cells undergoing PCD (Filonova et al., 2000b). Buffering of the medium to prevent acidification inhibits PCD and abrogates the stimulatory effect of plant growth regulator withdrawal (Bozhkov et al., 2002). Two waves of PCD take place during SE: the first wave results in degradation of PEMs when somatic embryos form, while the second wave degrades the terminally differentiated cells of the suspensor during early embryogeny (Filonova et al., 2000b). In summary, signal molecules such as

AGPs and LCOs promote embryogenesis, somatic embryos develop from PEMs composed of cytoplasmic cells and vacuolated cells, and PCD is an essential part of somatic embryogenesis. The productivity of cell cultures has often been increased through the addition of novel compounds to the culture medium.

1.5.4 Osmotic Potential Enhances Somatic Embryogenesis

Gupta and Grob (1995) found that an increase in the osmolarity of the medium via the introduction of mannitol during proliferation increases the number of somatic embryos produced in *Pinus taeda* and *Pseudotsuga menziesii*. The osmotic environment is important for proper organization of embryonal heads. Often, manipulation of phytohormones *in vitro* may achieve a similar result.

1.5.5 Gibberellic Acid Inhibitors Promote Initiation of Somatic Embryogenesis

Gibberellins are responsible for plant cell expansion and elongation, but their exact role in embryogenesis is not understood. Initiation efficiencies of loblolly pine, slash pine, Douglas-fir and Norway spruce were improved through the use of paclobutrazol, a gibberellic acid inhibitor (Pullman et al., 2005). Addition of paclobutrazol to the initiation medium increased somatic embryo growth, yet differential responses were noted. High initiators responded less than recalcitrant seed sources, some of which had doubled in initiation rates after treatment. Douglas-fir single somatic embryos placed on initiation medium containing paclobutrazol grew into embryogenic masses more rapidly

and these were 29 % larger than control. Other GA inhibitors, fluorprimidol, diaminozide, chloromequat-Cl, were equally effective in increasing initiation. Common side effects of GA inhibitors include alterations in endogenous sterols, carotenoids, cytokinins, ABA, and brassinosteroids. Increased cytokinins or reduced ethylene synthesis may be contributing factors to the observed effect of GA inhibitors. Moreover, presoaking megagametophytes in ABA resulted in further increases in initiation, which implies that a different or separate stimulatory mechanism was activated because ABA is a known GA antagonist. Recent work with angiosperms suggests that paclobutrazol promotes the embryogenic state by derepression of critical regulators of embryogenesis (Suzuki et al., 2007).

1.6 Somatic Embryo Maturation

Embryo maturation begins with the formation of somatic embryos and ends with an imposed desiccation period that is necessary before germination can occur. Morphological and physiological maturity is obligatory for complete maturation because well-formed somatic embryos do not guarantee satisfactory post-embryonic performance (Stasolla and Yeung, 2003).

Maturation is achieved on solid media and it is assessed visually based on similarity to the zygotic embryo pattern (Gupta et al., 1996). The important aspects of maturation are the development of shoot and root apical meristems and the accumulation of storage products. Meristems are localized regions of cell division where new cells arise in a predictable pattern. Initial cells within apical meristems leave one cell in the meristem

upon division. It appears that the shoot apical meristem (SAM) is dependent on genotype and some lines consistently produce organized shoot poles while others produce disorganized SAMs (Stasolla and Yeung, 2003). In addition, somatic embryo SAM initials are less defined and smaller that those seen in zygotic embryos (Stasolla and Yeung, 2003). Although root apical meristems (RAM) of somatic embryos closely correspond to zygotic counterparts, white spruce somatic embryos generally have fewer and less well defined RAM initials (Stasolla & Yeung, 2003). One goal of maturation protocols is to produce somatic embryos of similar morphology to zygotic embryos. Utilizing an adequate carbon source, and regulating the culture environment so that it imitates *in ovulo* conditions as much as possible may achieve this. Refinements as subtle as redox state also improve yields. The quality and germination efficiency of mature somatic embryos is correlated to the abundance of storage product transcripts in late embryogenesis (Dong and Dunstan, 2000).

1.6.1 Sucrose Is the Optimal Carbon Source

Sucrose is known to be the best carbon source for SE induction, maintenance and maturation (Minocha and Jain, 2000; Iraqi and Tremblay, 2001). This topic was addressed in order to identify factors that could improve the embryogenic success of different species. Iraqi and Tremblay (2001) demonstrated that during the maintenance phase and the first week of maturation, carbohydrate metabolism was primarily directed to sustaining growth, and carbon source did not have any visible effects. However, in the period spanning the second to the fifth week of maturation, carbohydrate metabolism was

found to be oriented to storage product accumulation and embryo development. Replacement of sucrose by equimolar concentrations of its monomers, glucose and fructose, resulted in a reduction of the number of embryos produced as well as their germination capacity (Iraqi and Tremblay, 2001). In addition, the starch and protein content in embryogenic tissues was also significantly reduced. The protein content in black spruce somatic embryos at week 5 of maturation was reduced from 100 mg/g dry weight on 6% sucrose, to 38 mg/g dry weight on 3.16% each of glucose and fructose (Iraqi and Tremblay, 2001). In white spruce the protein content was 82 mg/g dry weight on 6% sucrose but only 37 mg/g dry weight on glucose and fructose at 3.16% each (Iraqi and Tremblay, 2001). Starch deposits decreased in a similar fashion. Thus, on sucrose-containing medium, the hexoses produced by cleavage of sucrose could induce the glucose and fructose phosphorylation that is necessary for entering other metabolic pathways (Iraqi and Tremblay, 2001). This supported the hypothesis that the glucose and fructose generated by invertase-mediated cleavage of sucrose could initiate changes in gene expression, and linked invertases to the control of protein and starch synthesis (Iraqi and Tremblay, 2001).

In 2005, Iraqi and Tremblay discovered that the activity of an apoplastic invertase was directly correlated to the number of somatic embryos produced. In plants, invertase and sucrose synthase cleave the glycosidic bond of sucrose. Invertases produce hexoses for cellular metabolism, and control growth, cell differentiation and embryo development. Iraqi and Tremblay (2005) concluded that in spruce, optimal somatic embryo development results from a combination of sucrose utilization from the medium, high apoplastic invertase activity within embryogenic tissues and the release of an active

invertase into the medium. More recently, it was shown that in the presence of sucrose but not so much in other carbohydrates, LEC1, a critical regulator of angiosperm embryogenesis described in Section 1.12.3, promotes cell division and embryonic differentiation (Casson and Lindsey, 2006).

1.6.2 The Effect of Ethylene, ABA and PEG on Somatic Embryogenesis

Sucrose and its associated enzymes are only one element of the maturation process. The gaseous environment in SE culture is very different than the nutritive tissue of the seed, which surrounds the zygotic embryo. In SE culture, embryos are directly exposed to air on solid medium, and ethylene accumulates as a consequence of metabolism. At maturation, the gaseous environment results in poor embryo quality and low conversion frequencies (Stasolla and Yeung, 2003). Recent efforts have focused on reproducing the ovular environment *via* hormones and osmolarity. Modern features of optimized maturation protocols include control of ethylene biosynthesis, stage-specific ABA levels, and the imposition of osmoticum or drying treatments to simulate desiccation (Stasolla and Yeung, 2003).

Ethylene, the gaseous plant-growth-regulator, is present in low amounts in the developing seed. In culture vessels, ethylene increases over time and studies show that treatments, which increase ethylene synthesis, decrease the number and quality of somatic embryos produced. Ethylene accumulation within somatic embryos disrupts the architecture of the shoot apical meristem by creating air spaces and disorganization of tissues. These are detrimental to post-embryonic performance. Ethylene may be

controlled by shortening the maturation period and time in culture, or by inhibiting ethylene synthesis (Stasolla & Yeung, 2003). In contrast to ethylene, ABA and PEG promote the production of robust embryos *in vitro*.

In both angiosperms and gymnosperms, seed ABA levels are low during early embryogenesis; this is followed by an increase during embryonic growth, and a decrease at the onset of desiccation. Dunstan et al. (1998) found that seeds responded to ABA treatment within 6 hours and a cascade of gene expression was triggered by 24 hours. In the absence of ABA, somatic embryos did not elongate but developed highly vacuolated cells, while ABA-treated embryos were cylindrical in shape and contained densely cytoplasmic cells (Gutmann et al., 1996). Although its role in embryo development is not clear, ABA is important because evidence points to its effect on multiple pathways during embryogenesis (Stasolla and Yeung, 2003). ABA reduces nucleotide biosynthesis *via* salvage pathways, restricts embryo proliferation and stimulates embryo growth. It is believed that ABA acts as a physiological signal which induces changes in the redox state. Cell lines with high embryogenic potential exhibit increased levels of reduced ascorbic acid, and application of oxidized glutathione was shown to increase the quality of somatic embryos produced in white spruce (Stasolla and Yeung, 2003). Nitrogen metabolism is activated upon application of ABA resulting in accumulation of Glu, Gln and Arg (Stasolla and Yeung, 2003). The increased activity of the glutamine synthase/glutamate synthase pathway is necessary for storage protein accumulation. Finally, ABA treatment increases spermidine synthesis in *Pinus radiata* and *Picea glauca* somatic embryos (Minocha et al., 1999; Kong et al., 1998). Embryo growth was correlated to polyamine levels when embryogenic cell lines of white spruce showed

higher spermidine levels than non-embryogenic lines (Santanen and Simola, 1992; Stasolla and Yeung, 2003).

Culture conditions intended to duplicate the very low osmotic potential of the seed environment, include the use of compounds such as PEG and dextran (Stasolla and Yeung, 2003). Previously, sugars and salts were used to increase water stress in culture but these molecules can also cross the plasma membrane and lead to plasmolysis. Further, high levels of salts and sugars can be toxic (Stasolla and Yeung, 2003). Kong and Yeung (1995) identified silver nitrate as an additional strategy to enhance maturation. $AgNO_3$ is known to inhibit ethylene responsiveness in plants and to enhance the production and regeneration of embryogenic callus in *Zea mays*. $AgNO_3$ may serve as a stress agent due to the Ag^+ metal ion. Without ABA, spruce proembryos do not develop into cotyledonary embryos but turn brown and degenerate. Yet, Kong and Yeung (1995) found that a combination of 100 μM $AgNO_3$ and 40 g/l PEG was the optimum treatment for cotyledonary embryo formation in the absence of ABA. This is significant because it means that PEG may stimulate endogenous ABA production. In addition, PEG has been shown to up-regulate lipid and protein storage in developing embryos, to inhibit precocious germination and to increase the number of cotyledonary embryos by 3-fold in spruce (Attree et al., 1991; Misra et al., 1993; Kong and Yeung, 1995). However, some species of conifers do not respond well to PEG but fare better in the presence of plasmolysing agents (Stasolla and Yeung, 2003), thus, specific maturation media are diverse. Despite their morphological maturity at the end of the ABA/PEG treatment, somatic embryos are not physiologically mature and they require a desiccation treatment

prior to conversion to viable plantlets. Embryo quality is also important to successful conversion.

1.6.3 Low Glutathione Redox State Increases Quality of Mature Somatic Embryos

Somatic embryo yield and quality may be improved by maturation in the presence of a low glutathione redox state (Belmonte and Stasolla, 2006) and redox status and glutathione content of cells is important for reactivation of cell division (Fehér et al., 2003). A high glutathione redox state occurs during the proliferative stage of somatic embryogenesis, but in later stages of development, the glutathione (GSH) pool shifts toward its oxidized form, GSSG, resulting in a lower redox state. Glutathione promotes proliferation by enabling tissues to produce ATP *via* purine salvage pathways. A low glutathione redox state, [GSH]/[(GSSG + GSH)], is known to increase the number and quality of white spruce somatic embryos. Embryo quality is based on structural organization and is characterized by well-organized apical meristems, storage product deposition, the presence of 4 or more cotyledons, and high conversion rates. Belmonte & Stasolla (2006), demonstrated that culturing proliferative embryogenic tissue in the presence of GSH for 7 days, prior to transfer to maturation medium supplemented with GSSG, increased the number of embryos with 4 or more cotyledons from 24 to 66 per 100 mg of tissue, and the conversion frequency was increased from 16% to 61%. However, the cost of GSH and GSSG is prohibitive and the step-wise process is labour-intensive. DL-buthionine-[S,R]-sulfoximine (BSO) inhibits *de novo* synthesis of reduced glutathione. The application of 0.01 mM BSO to white spruce maturation medium,

almost tripled the production of embryos in 2 different cell lines (Belmonte and Stasolla, 2006). Anatomical studies revealed that BSO eliminated intercellular spaces in the meristems, resulting in subapical domains that were composed of tightly packed cells instead of elongated cells, and protein accumulation was favoured over starch accumulation (Belmonte and Stasolla, 2006). In addition, BSO promoted the formation of larger root apical meristems with more initials, and BSO treatment improved the conversion frequency from 31% to 64% (Belmonte and Stasolla, 2006). The complexity of embryo maturation is discouraging if we are to continue striving to improve SE protocols through rational design of media. The sequence determination of differentially expressed transcripts could have led to the identification of transcription factors or pertinent genes which have a greater role in embryogenesis and in turn, these would provide better tools for increasing productivity. Molecular markers and critical regulatory genes would help us understand what is going on in the overall scheme of embryogenesis.

1.6.4 Late Embryogenesis Abundant Proteins (LEA), Heat Shock Proteins (HSP) and Pathogenesis-related Proteins (PR) Are Highly Expressed During Late Maturation and May Serve as Molecular Markers

A second reprogramming of gene expression takes place during embryo maturation, resulting in the transcriptional up-regulation of storage proteins, late embryogenesis abundant (LEA) proteins, heat-shock proteins (HSPs), metallothionein-like proteins (MT-like) and pathogenesis-related (PR) proteins (Dong and Dunstan, 2000; Stasolla and Yeung, 2002). High quality embryos are characterized by an abundance of storage products and stress-related proteins (Dong and Dunstan, 2000). Legumins, vicilins and

albumins are storage proteins, which contribute to the robustness of the embryo by providing a nitrogen supply during seed germination and seedling development (Dong and Dunstan, 2000). LEA proteins protect the embryo from damage during desiccation and prevent precocious germination (Dong and Dunstan, 2000). Transcripts of storage proteins and LEA proteins are abundant during conifer embryo maturation but they disappear immediately after the embryos are transferred to germination medium (Dong and Dunstan, 2000). Furthermore, LEA proteins are indicators of the desiccation phase (Dong and Dunstan, 2000).

HSPs protect against desiccation and stress during plant development, while MT-like proteins probably function in metal homeostasis (Dong and Dunstan 2000), but the pathogenesis-related (PR) proteins do not have a distinct function during embryogenesis.

PR proteins are induced by pathogens and abiotic stress, but are also are developmentally regulated (Liu and Ekramoddoullah, 2006). They function in cell wall rigidification, signal transduction and antimicrobial activity (Liu and Ekramoddoullah, 2006). The paradoxical character of PR proteins is illustrated by the following facts: 1) over-expression does not consistently enhance pathogen resistance; 2) they have additional activities such as, ribonuclease, cryoprotection and cytokinin-binding; and, 3) constitutive expression of PR proteins in some species enhances germination under saline conditions (Liu and Ekramoddoullah, 2006). This multitude of functions and peak of expression during embryo maturation suggest a more global role during embryogenesis, and this remains to be determined.

Liu and Ekramoddoullah (2006) suggested that “PR proteins with RNase activity may help to protect plants during programmed cell death around infection sites or to act

directly on the pathogens," but PR proteins could also be protecting the embryo from PCD in the surrounding megagametophyte. During zygotic seed development, the megagametophyte undergoes slow, programmed cell death, a process that allows it to function as carbon and nitrogen repository that the embryo can use upon germination (Filonova et al., 2002). In addition, PR protein may protect the embryo during desiccation and an extended period in dormancy. Because chitinases and glucanases are also considered PR-like proteins and chitinases have been shown to facilitate the completion of carrot somatic embryo development (Dong and Dunstan, 2000), it is conceivable that PR proteins also have a signaling role as was discussed earlier for AGPs, LCOs and chitinases.

1.7 Desiccation

Desiccation is the naturally occurring loss of water during the final stages of seed development and it represents the transition that terminates the developmental program (Stasolla and Yeung, 2003). The drying period is known to result in a decrease of ABA levels and an increase in nucleotide levels and nucleotide biosynthesis enzymes, which are necessary for growth and cell division at the beginning of germination (Stasolla and Yeung, 2003). There are two methods for achieving desiccation *in vitro*: partial drying, involving a gradual and limited loss of water, and full drying, employing saturated salt solutions to cause a rapid and dramatic water loss (Stasolla and Yeung, 2003). Conversion frequency increases as a result of drying (Stasolla and Yeung, 2003).

1.8 Germination and conversion: Ascorbic Acid Helps Poor Converters

Conversion of somatic embryos to viable plantlets, or the development of a functional root and shoot, is typically less likely than germination (the emergence of an embryonic root), and the post-embryonic performance of white spruce somatic embryos was seen to be dependent on the regenerative ability of the apical meristems (Stasolla and Yeung, 2006). Previous studies with spruce identified ascorbic acid as a positive elicitor of cell division and meristemoid formation in mature embryos that had failed to form shoots due to large intercellular air spaces at the shoot pole (Stasolla and Yeung, 2003). This was confirmed at the structural level, where vascular connections were established between the new shoots and the hypocotyls of germinating embryos, leading to the formation of viable plantlets. Ascorbic acid (ASC) is believed to influence pyrimidine nucleotide synthesis (Stasolla and Yeung, 2003).

Stasolla and Yeung (2006) found that cell lines showing poor conversion were re-activated by ASC treatment, indicating a role for ASC-regulated pyrimidine metabolism in germinating somatic embryos. Unfortunately, this type of enhancement was not achieved with cell lines that already exhibited high conversion frequencies (ASC had no effect at all), but at least a method is provided for inducing conversion in embryos that are not likely to convert.

Conifer somatic embryogenesis has benefited from many technical advances over the last 10 years. The diversity of manipulations necessary for proper embryo formation and conversion does suggest that several regulatory genes, perhaps under the control of a master switch, orchestrate embryogenesis. The identification of such genes and the elucidation of their roles in embryogenesis may lead to exponential improvements in

somatic embryogenesis. The role of medium components, with positive effects on embryogenesis, and the genes that are known to function during embryogenesis provide clues about how these master genes may be regulated and their potential downstream targets. With recent advances in molecular technology, it is finally possible to identify genes involved in embryogenesis, to determine gene expression profiles during each stage, as well as to establish a pathway for the whole process. The following section describes advances in the molecular biology of conifer embryogenesis.

1.9 Expressed Sequence Tags (ESTs), cDNAs and Microarrays Provide Insight into Conifer Embryogenesis

During the last 5 years, high throughput analyses led to the identification of markers that are specific to stages of embryogenesis. Early work with proteomics and microarrays showed that housekeeping genes are modulated during various stages of embryogenesis. Proteome analysis of white spruce SE stages failed to identify novel embryo-specific proteins, but regulation of proteins involved in stress response and metabolism was observed (Lippert et al., 2005). A problem with initial studies was that proteins or cDNAs obtained from needles or embryogenic cell lines did not include samples or representatives of the very early stages of embryogenesis. Therefore, the genes that are responsible for initiation or are expressed during initiation could not be identified. Work with microarrays has become more productive as researchers started to ask more specific questions.

Van Zyl et al. (2003) discovered a global gene expression pattern during gymnosperm embryo development by studying early embryogenesis. Norway spruce cDNAs

hybridized against pine and spruce ESTs on microarrays resulted in the identification of 107 genes that were regulated in an up-down-up pattern during the sequential stages of proembryogeny (PEMs in proliferation medium), early embryogeny (proembryos cultured in the absence of PGRs), and late embryogeny (embryos treated with ABA). This pattern was not observed in a developmentally arrested cell line, strongly suggesting that this fluctuation in gene expression is necessary for the differentiation of somatic embryos from pro-embryogenic masses. Another 35 genes were found to be differentially expressed during embryo development, and by comparison with a developmentally arrested cell line, 22 of the 35 genes were directly associated with embryo pattern formation and therefore can serve as molecular markers of early embryogenesis (van Zyl et al., 2003). Eighteen of these 22 genes displayed the up-down-up signature. These 18 genes grouped into 2 subcategories: 1) post-translational modification, protein turnover, chaperones, and, 2) translation.

Stasolla et al. (2003) used microarrays to study the effects of PEG on embryo-related gene expression in spruce. RNA was isolated from maturing white spruce embryos treated with or without PEG, and hybridized against 2178 cDNAs from loblolly pine. The categories of genes whose expression was affected by PEG treatment included: establishment of embryo body plan, stress response mechanisms, carbohydrate metabolism and nitrogen metabolism. Several auxin- and ABA-responsive genes showed differential expression under PEG treatment and this suggested that PEG might affect initial developmental stages by altering responsiveness of tissues to growth regulators.

PEG treatment induced responsiveness in homologues of the following angiosperm genes that function in the shoot apical meristem formation: *ZWILLE*, *ARGONAUTE*,

CLAVATA1, *FIDDLEHEAD* and *KNOTTED*-like (Stasolla et al, 2003). In *Arabidopsis*, mutations in *ARGONAUTE* and *SHOOT MERISTEMLESS*, result in meristem abortion. In addition, two genes homologous to the angiosperm *SCARECROW,* which is involved in root apical meristem formation, were up-regulated by PEG. Therefore PEG could have a role in the radial growth pattern of embryonic roots, which are paramount to successful conversion at germination.

Further, Stasolla et al. (2003) described the effects of PEG on gene expression. Superoxide dismutase and glutathione peroxidase were up-regulated, indicating increased protection against reactive oxygen species. Up-regulation of HSP and LEA protein transcripts points to the protective effect of PEG against severe dehydration. Transcripts of enzymes involved in sucrose catabolism were down-regulated and this was attributed to the inhibitory effect of PEG on precocious germination. Glutamine and glutamic acid synthases were up-regulated by PEG. These enzymes are part of the nitrogen assimilation and polyamine synthesis pathways and this is pertinent because storage products were preferentially accumulated in PEG-treated somatic embryos. In conclusion, this work highlighted genes or metabolites that may be used to improve somatic embryo quality through genetic engineering or media manipulation.

Van Zyl et al. (2002) and Stasolla et al. (2003) have identified 200 genes that were differentially expressed during the early stages of embryogenesis. The main conclusions from these efforts are that a reprogramming of gene expression is required for the initiation of embryogenesis, but not for its continuation, and the formation of a proper apical meristem in immature somatic embryos is critical to embryogenic success (Stasolla et al. 2003). The genes involved in establishment of apical poles, *ZWILLE*,

SCARECROW, *SHOOT MERISTEMLESS,* are expected to be potential molecular markers for screening the embryogenic potential of cell lines (Stasolla et al., 2003).

Recently, a major undertaking involving ESTs from 4 pine cDNA libraries revealed that 83 of 108 genes involved in angiosperm embryogenesis had homologues in pine (Cairney et al., 2006). The libraries were constructed of embryogenic tissues, and un-normalized, normalized and subtracted libraries were utilized in order to reduce high redundancy and to increase the efficiency of gene discovery. The list of pine homologues of angiosperm genes involved in embryogenesis included *ARGONAUTE1* (*AGO1*), *BABY BOOM* (*BBM*), *CLAVATA1* (*CLV1*), *CLAVATA2*, *LEAFY COTYLEDON1* (*LEC1*), *PICKLE*, *ROOT MERISTEMLESS1*, *SCARECROW*, *SOMATIC EMBRYOGENESIS RECEPTOR KINASE3*, *WUSCHEL* (*WUS*), *WUS-like*, *WUS-related*. Some of these are known central transcriptional regulators of angiosperm embryogenesis. In *Arabidopsis*, *WUSCHEL* encodes a homeodomain transcription factor that regulates pluripotent stem cell induction and maintenance in conjunction with CLAVATA proteins, but WUS overexpression induced somatic embryo formation (Verdeil et al., 2007). The spatio-temporal expression of these transcription factors is regulated by chromatin remodeling factors, and some of those identified included *chromomethylase*, *Curly Leaf*, *DDM1*, *FAS2*, *FIE*, *N-acetyltransferase*, *ISW2*, *MSI1*. However, some of the well-known transcriptional regulators that were missing from the list were there LEAFY COTYLEDON2 (*LEC2*), *FUSCA3* (*FUS3*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*) and others. A unique finding was the identification of 25 sequences with no known function, which could serve as starting points for further investigations. The results of microarray

experiments have provided a fertile area of research into the isolation and characterization of these genes in various conifer species.

1.10 Novel Conifer Genes Show the Importance of Embryo Pattern Formation and Embryo Quality During Embryogenesis

Many of the genes identified from microarray experiments had functions related to the morphogenetic processes that occur during plant embryogenesis, when the basic body plan of the plant is established. The root and shoot axis of polarity give rise to organ systems and the radial organization of the primary tissue layers: protoderm, ground tissue and procambium. The epidermis is derived from the protoderm; the cortex and the endodermis are derived from the ground tissue, while the pericycle and vascular tissues are derived from the procambium.

1.10.1 Chitinases

The role of chitinases is to hydrolyze AGPs and to stimulate embryo and seed development (Wiweger et al., 2003). The Norway spruce *chitinase*, *Chia4-Pa*, was expressed at low levels in proliferating embryogenic cultures but expression increased significantly after withdrawal of plant growth regulators (Wiweger et al., 2003).

1.10.2 *ARGONAUTE* Transcription Factors

A member of the *ARGONAUTE* family of transcription factors, *PgAGO* was identified in white spruce and proven to be responsible for shoot and root apical meristem differentiation or embryo development (Tahir et al., 2006). Argonaute (AGO) proteins function in RNA interference or post-transcriptional gene silencing in plants, as well as regulation of stem cell identity and cell fate decision (Tahir et al., 2006). *PgAGO* transcripts were localized in the cells of the shoot and root meristems from early embryogenesis until embryo maturation. RNA-mediated suppression of *PgAGO* limited embryo production and caused severe abnormalities in the few embryos that did progress through development: stunted growth, lateral expansion, lack of cotyledons, absence of root meristem cores, and poorly organized apical meristems. In *Arabidopsis*, two members of the AGO family, AGO1 and ZWILLE, maintain an undifferentiated pool of cells in the shoot apical meristem during late embryogenesis. It is these cells that have the ability to differentiate into new tissues and organs during post-embryonic development. The importance of this function is realized in mature *zwille* embryos whose disrupted meristems contain stem cells that are capable of differentiation but are unable to produce new organs. Also, expression of *SHOOT MERISTEM-LESS* is reduced in these embryos. The effect of AGO protein on conifer root meristem is a novel finding that differentiates gymnosperm AGO proteins from angiosperms.

1.10.3 *Germins*

Germins and germin-like proteins are apoplastic (site of cell-cell communication, outside plasma membrane) proteins believed to be important in cell wall metabolism,

during stress responses and in developmental processes (Mathieu et al., 2006). *GER1* was identified in *Pinus caribea*, *Pinus radiata* and *Larix x marschlinsii* Coaz (Mathieu et al., 2006). Work with *LmGER1* has defined its importance during embryogenesis. LmGER1 has superoxide dismutase activity necessary for cell wall remodeling during development. It is expressed in the suspensor and in the region between the embryonal head and the suspensor in embryonal masses. Expression persists in developing cotyledons and procambium of mature embryos but is never seen in the apical meristems. At germination, expression is observed in the cotyledons but not in the roots or hypocotyls, and continues until germination when expression takes place in the central vein and stomata of euphylls. RNA silencing of this gene reduced the embryogenic potential of the cell lines, and arrested embryo development at the globular stage. The few embryos that were able to continue development had an irregularly shaped round head and a swollen root cap (Mathieu et al., 2006).

1.10.4 *Picea abies Homeobox* Genes

Two distinct genes involved in radial pattern formation were identified during Norway spruce somatic embryogenesis (Ignuoff et al., 2003). The *Picea abies Homeobox* genes (*PaHB1* and *PaHB2*) function in cell differentiation and patterning. *PaHB1* expression is ubiquitous in embryogenic cultures but becomes protoderm-specific during somatic embryo development. On the other hand, *PaHB2* expressed throughout early somatic embryos before any tissue differentiation, becomes localized in the outer cortical layer

and root cap of mature embryos, a subepidermal/epidermal specification. These genes are considered potential markers of radial pattern formation (Ignuoff et al., 2003).

1.10.5 *VIVIPAROUS1/ABSCISIC ACID INSENSITIVE3* (*VP1/ABI3*)

The angiosperm *VP1/ABI3* gene family controls expression of embryo maturation genes, the acquisition of desiccation tolerance and dormancy. ABI3 proteins act as intermediates regulating ABA responsiveness during seed development, including genes involved in reserve deposition, acquisition of desiccation and dormancy induction (Zeng et al., 2003). In yellow cedar, *Chamaecyparis nootkatensis*, the ABI3 homologue was found to be synthesized strictly in seeds and it was not detected in roots or leaves (Zeng et al., 2003). Ectopic expression of CnABI3 activated vicilin and napin storage gene promoters in leaves and other tissues, with ABA having a synergistic effect (Zeng et al., 2003). Further, a homologue of the angiosperm *VP1*/*ABI3* gene family, *Pavp1*, was isolated and characterized in *Picea abies* (Norway spruce) (Footitt et al., 2003). *Pavp1* was expressed in proliferating embryogenic cultures, expression decreased during withdrawal of PGRs and then increased again in response to ABA treatment. *Pavp1* expression was detectable during desiccation and germination conditions.

1.10.6 *KNOTTED-like Homeobox* Genes

Angiosperm *KNOTTED*-like homeobox (*KNOX*) genes are a group of transcription factors involved in plant growth and development. Class I *KNOX* genes regulate cell

specification and pattern formation, have a meristem-specific expression profile, and *SHOOT MERISTEMLESS* also belongs to this class (Rosin et al., 2000). A class I homeobox gene *HBK3*, was identified in Norway spruce (Belmonte et al., 2007). Transgenic Norway spruce lines expressing sense and antisense *HBK3* were generated. Embryo development was arrested in the embryogenic lines expressing antisense HBK3 (Belmonte et al., 2007). In contrast, lines over-expressing HBK3 were characterized by large embryogenic heads and enlarged suspensor-like tails, when compared to control lines (Belmonte et al., 2007). Further development and differentiation led to embryos with SAMs that were larger than control and accumulated both protein and starch in subapical cells, with protein being the major storage product, as opposed to control lines which had starch as the major storage product (Belmonte et al., 2007). The higher amount of protein is indicative of higher quality embryos. When HBK3 was expressed in *Arabidopsis*, the SAM of the transgenic embryos was always larger than wild type (Belmonte et al., 2007). Immature somatic embryos with larger embryonic heads are expected to be more responsive to ABA and to produce more cotyledonary embryos (Belmonte et al., 2007). KNOX I overexpression led to decreased GA synthesis (Belmonte et al., 2007), and since GA signaling suppresses embryogenesis, this could be the mechanism by which embryo formation was bolstered. On the other hand, phenotypic aberrations were observed in plants ectopically expressing HBK3 (Belmonte et al., 2007), and further research will be necessary before this gene will find applicability in improving the quality of mature embryos.

1.11 Future Initiatives for Conifer SE Research

Work over the last 20 years has focused on designing optimal maturation media to improve somatic embryogenesis because it was thought that treatments provided during embryo development would lead to successful regeneration (Stasolla and Yeung, 2003). The recent surge in genetic information is finally providing a much-needed molecular framework on which to base the efficacy of these treatments. Several of the genes identified *via* high throughput techniques (*ZWL*, *AGO*, *CLV1*, *FDL*, *KNOX*) appear to be involved in responses to medium manipulations that were deduced empirically, and the establishment of correlations between *in vitro* treatments and gene expression will elucidate the role of these genes in embryogenesis. The lack of embryo stage-specific molecular markers has been a disadvantage to assessing the quality and embryogenic capacity of somatic embryos. To this end, the tissue- and stage-specificity of genes such as *WUS*, *LEC1*, *PKL*, *SCR*, *STM*, *ZWL* can be exploited, and transcript profiling will be necessary to realize their utility in conifer embryogenesis.

Because events leading to the initiation of somatic embryogenesis have an impact on yield and quality of mature somatic embryos (Stasolla and Yeung, 2003; Eggertsdotter and von Arnold, 2001), efforts must be directed at optimizing early embryogenesis in order to improve the success rate of somatic seedling establishment. Furthermore, instead of focusing on separate stages, effectors and genes should be studied during the entire process and with determination of activating or deactivating effects during each stage. Interactions among genes may lead to unexpected consequences due to synergistic effects.

Induction of embryogenesis and the maintenance of embryogenic potential remain the greatest challenges of conifer SE. The strict requirement of juvenile explants for induction of conifer somatic embryogenesis suggests that we are still unable to re-direct developmental programs *via* culture conditions (Stasolla and Yeung, 2003). Because angiosperm embryogenesis has been studied more widely and there are parallels between angiosperm and gymnosperm SE *in vitro*, a solution to unlocking the secrets to induction is to identify and study embryo regulatory homologues in conifers.

1.12 Recent Progress in the Molecular Biology of Angiosperm Embryogenesis

Numerous genes with key regulatory roles in angiosperm embryogenesis have been characterized. The behaviour of these genes in response to induction and culture conditions will ultimately enable us to have more control over the process of somatic embryogenesis. Transcription factors such as *LEC1*, *LEC2* and *BBM* not only regulate embryogenesis but are also capable of inducing somatic embryogenesis when expressed ectopically in mature tissues. *LEC1* functions during early and late embryogenesis, and it is a key regulator in the network of transcription factors that interact during embryogenesis. Isolation and analysis of gymnosperm *LEC1* and gaining insight into its regulation will be a first step in unravelling the molecular biology of conifer embryogenesis.

1.12.1 Exogenous Compounds Induce Embryogenesis in Angiosperms

In angiosperms, SE is induced by exogenous hormones and culture-related stress (Dong and Dunstan, 2000). A variety of simple and widely used methods exist for inducing somatic embryogenesis from mature tissues using either stress or hormonal treatments (Ikeda-Iwai et al., 2003; Hecht et al., 2003; Boutilier et al., 2002). In tissue culture ethylene may act as either a promoter or inhibitor of regeneration depending upon species (Minocha and Jain, 2000). ABA, sugars, PEG stimulate embryo development and maturation, while auxins and cytokinins play critical roles in regeneration (Minocha and Jain, 2000), but the genes that are responsive to these treatments or how their activity is modulated remain largely unknown.

Insight into gene responses to exogenous compounds may be gained by studying the effect of these compounds on gene expression. Ikeda-Iwai et al. (2002, 2003) used osmotic stress, salinity and heavy metals to induce somatic embryogenesis in *Arabidopsis* and carrot. Their work demonstrated that the expression of embryo-specific genes, *LEC1*, *FUS3* and *ABI3*, was activated in the somatic embryos produced, but not in the under-lying callus.

1.12.2 Ectopic Expression of *LEC1*, *LEC2* and *BBM* Transcription Factors Induces Embryogenesis from Mature Tissues

The Arabidopsis *LEC1* gene was identified from an embryo defective deletion mutant whose embryonic leaves (cotyledons) resembled mature leaves. *LEC2*, belonging to a different transcription factor family, also induced SE and caused embryo-like seedlings to proliferate and produce callus, and cotyledon-like and leaf-like organs (Stone et al.,

2001). Both *LEC1* and *LEC2* act early in embryogenesis to specify suspensor cell fate and cotyledon identity, and are needed in late embryogenesis for acquisition of desiccation tolerance and expression of maturation-specific genes (Stone et al., 2001). It is suggested that these transcription factors activate genes that establish embryogenic competence while obviating the need for hormonal treatments. *BABY BOOM* (*BBM*) was identified by subtractive hybridization of genes upregulated during induction of embryo development from immature pollen grains in canola (Boutilier et al., 2002). Ectopic over-expression of BBM resulted in the spontaneous formation of somatic embryos on post-germinative organs, and in some cases, caused a reiteration of the embryo-forming process so that new embryos formed on the cotyledons of pre-existing embryos. BBM expression was also shown to stimulate pathways responsible for cell division and differentiation. Extensive work with angiosperm *LEC1* has broadened the embryogenesis knowledge base and pinpointed other genes that impact embryogenesis.

1.12.3 *Arabidopsis LEC1*, a Transcription Factor Subunit Essential to Embryogenesis

LEAFY COTYLEDON1 (*LEC1*) is a eukaryotic transcription factor homologue that was identified in *Arabidopsis* (Meinke, 1992; West et al., 1994; Lotan et al., 1998) and the first gene shown to induce somatic embryogenesis. Expression studies have shown that *LEC1* acts during both early and late embryogenesis but *LEC1* mRNA levels are highest during early embryogenesis (Lotan et al., 1998). *LEC1* accumulation is seen in both the embryo proper and suspensor of the early pro-embryo (Lotan et al., 1998).

The function was deduced from the phenotypes of mutants. *LEC1* is believed to suppress the embryogenic potential of the suspensor in early embryogenesis, specify embryonic organ identity, inhibit premature germination, activate some maturation specific genes and impart desiccation tolerance to the seed (Lotan et al., 1998). Inhibition of vegetative development or premature germination was inferred from the observation that mutant embryos exhibit a greatly enlarged SAM as well as premature activation of the SAM and root apex (Meinke et al., 1994; Lotan et al., 1998). The presence of trichomes on the cotyledons of mutant embryos represents the loss of identity of embryonic organs (Meinke 1992; Lotan et al., 1998). The suspensors of mutant embryos exhibited additional cell divisions not normally seen in wild type suspensors, and further analysis *via* a double mutant (*lec1 fus3*) demonstrated continued proliferation of the suspensor cells and the formation of embryos (Lotan et al., 1998). Whereas wild type embryos are filled with protein and lipid bodies but very little starch, *lec1* embryos contained starch as the main storage product, a few lipid bodies and no protein bodies (Meinke et al., 1994) and this is further supported by gene expression analysis of maturation specific genes (West et al., 1994). In effect, *LEC1* regulates embryonic processes by activating transcription of genes required for normal *Arabidopsis* development.

The capacity of LEC1 to induce embryonic programs in vegetative cells was demonstrated by ectopic expression of a *35S::LEC1* cDNA clone into the *Arabidopsis lec1-1* null mutant. Null mutant seed are not viable because they cannot tolerate desiccation and dry up and die during maturation. Transgenic *35S::LEC1* seeds (T_1) were able to tolerate desiccation and germinate but 75% of the T1 seedlings had abnormal

embryo-like morphologies (Lotan et al., 1998). The rest of the T1 seedlings grew vegetatively and 9 out of 10 produced seed (T2) (Lotan et al., 1998). Although the majority of T2 seeds exhibited the Lec1$^-$ phenotype (desiccation intolerance) and were not viable after desiccation, the presence of the *35S::LEC1* transgene was confirmed by PCR, indicating that transgene silencing had occurred (Lotan et al., 1998). The rare, desiccation-tolerant T2 seed germinated into seedlings that had embryonic morphological characteristics and expressed genes that are usually active only in developing seeds; intriguingly, embryo-like structures appeared on the leaves of T2 seedlings (Lotan et al., 1998). In brief, post-embryonic expression of the *LEC1* gene induces expression of embryo-specific genes and formation of embryo-like structures, (Lotan et al., 1998).

Bioinformatic analyses show that LEC1 is homologous with the HAP3 subunit of the eukaryotic CCAAT box-binding factor (CBF). CBFs are conserved eukaryotic oligomeric transcription factors containing 3 non-identical subunits (HAP2, HAP3, HAP5) that interact to form a complex that binds the CCAAT DNA motif (Lee et al., 2003). Mammalian CBFs act to enhance transcription rates while yeast CBFs activate genes involved in mitochondrial respiration (Kwong et al., 2003). Further, a mammalian CBF was shown to regulate transcription of genes required for cell proliferation and viability (Kahle et al., 2005). The HAP3 proteins consist of an N-terminal A domain, a central B domain, and a C-terminal C domain (Lee et al., 2003). The B domain is required for DNA binding and interaction with other CCAAT binding subunits and this domain is conserved across eukaryotic species (Lee et al., 2003). The B domain was shown to be critical for LEC1 function by domain swapping experiments, and mutagenesis experiments identified the Asp-55 residue as necessary for LEC1 function

(Lee et al., 2003). Contrary to yeast and mammals, which have a single gene for each HAP subunit, plants have HAP gene families (Kwong et al., 2003). While *Arabidopsis HAP2* and *HAP5* expression was observed ubiquitously in a wide range of tissues, *HAP3* expression appeared to be under developmental and/or environmental control (Edwards et al., 1998). In *Arabidopsis*, the HAP2 family contains 6 members while the HAP5 family contains 8 members and this allows for 48 possible combinations with LEC1. The *Arabidopsis* HAP3 family is made up of 10 members, which are further classified as LEC1-type and non-LEC1-type. *LEC1* and *LEC1-LIKE* (*L1L*) make up the LEC1-type class of HAP3 subunits based on shared conserved residues that are not present in the 8 other subunits, their requirement in normal embryo development; and, the possibility that they have a common evolutionary origin due to the fact that yeast and mammalian HAP3 subunits are more similar to the non-LEC1-type HAP3 subunits (Kwong et al., 2003). The non-LEC1-type subunits cannot substitute for LEC1 and are expressed at high levels in non-embryonic tissues.

The 2 LEC1-type subunits are differentiated by several characteristics. Although *LEC1* and *LEC1-LIKE* share significant sequence homology, their functions and expression patterns are different. *LEC1* is an embryonic regulator that is only expressed in embryonic tissues. In *Arabidopsis*, the *LEC1* gene is 836 bp in length, with an open reading frame at position 40–666, encoding a protein of 208 amino acids (The *Arabidopsis* Information Resource (TAIR)). *LEC1* contains a single exon and 5' and 3' untranslated regions (TAIR, 2003). The *L1L* gene is 1330 bp in length, with an open reading frame at position 1–915, an intron at position 68–277, and a 3' untranslated region (TAIR, 2003). The L1L protein consists of 234 amino acids (TAIR, 2003). L1L

may also be an embryonic regulator but its expression peaks at a later embryogenic stage than LEC1 and low amounts of L1L are present in all vegetative organs. Ectopic expression of L1L suppresses the *lec1* mutation, but it does not induce somatic embryogenesis (Kwong et al., 2003). Although embryo-regulatory genes have been characterized much remains to be determined about the molecular events responsible for embryogenesis. The initial observations of lec mutants and double mutants of *lec1*, *lec2*, *fus3*, suggested a complex network of regulatory factors as opposed to a linear pathway for embryogenesis (Meinke, 1992).

1.12.4 LEC1 has a Prominent Role in the Network of Transcription Factors which Interact During Embryogenesis

Until recently, LEC1, LEC2, FUS3, PKL, ABI3, and the hormones gibberellic acid (GA) and ABA were considered the main regulators of embryogenesis and their interactions were crudely defined. The *LEC* gene products, LEC1, LEC2 and FUS3, are the only known regulators that are required for normal development during both the morphogenesis and the maturation phases (Caruba et al., 2004). LEC and ABI3 transcription factors have complementary regulatory roles, thus they act synergistically to control multiple events during seed development (Caruba et al., 2004). While overexpression of LEC2 and FUS3 causes expression of embryonic traits in vegetative tissues, this effect is not as strong as LEC1 overexpression (Suzuki et al., 2007). The major function of LEC2, FUS3 and ABI3 is activation of the genes involved in accumulation of storage proteins and lipid reserves (Suzuki et al., 2007).

PKL, a CHD3 domain chromatin factor, in conjunction with gibberellic acid controls *LEC1* and *LEC2* expression (Rider et al., 2003). Kagaya et al. (2005) suggest that LEC1 regulates expression of *LEC2*. Further, it is proposed that LEC1 controls seed storage protein accumulation by acting on *ABI3* and *FUS3* in the presence of ABA (Kagaya et al., 2005). Each of these in turn, regulates other processes or genes that are essential to normal embryogenesis. Several researchers have sought to establish how these genes interact to control embryogenesis, and the role of hormones.

ABA establishes dormancy during seed development while GA breaks dormancy and induces germination. In addition, GA is also produced during earlier stages of embryogenesis.

Trichome formation is a GA-dependent process on leaves, and because trichome formation was observed on *lec* embryos, the state of the GA biosynthetic pathway was investigated in *lec* mutants (Caruba et al., 2004). The AtGA3ox2 enzyme, which catalyzes the final step in GA biosynthesis, was up-regulated in the *lec2* and *fus3* mutants (Caruba et al., 2004). Further, it was shown that the FUS3 protein binds directly to the 2 RY targets present in the promoter of *AtGA3ox2* (Caruba et al., 2004). The absence of LEC2 and FUS3 leads to an increase in GA expression and consequent trichome formation on cotyledons; therefore, one function of these genes is to reduce GA levels during embryogenesis.

A further refinement of the embryogenesis model proposes that embryo development is regulated by the network of transcription factors composed of HAP3 genes (LEC1 and LEC1-LIKE), and the B3 domain genes (LEC2, FUS3 and ABI3) (Suzuki et al., 2007). The B3 domain is a plant-specific DNA-binding motif that is necessary for the regulation

of genes during seed maturation (Caruba et al., 2004). *LEC1* expression is initiated prior to LEC2, FUS3 and ABI3, and Kagaya et al. (2005) gave proof that LEC1 also regulates the expression of these genes. LEC2, FUS3 and ABI3 activate downstream genes when their B3 domain binds to the Sph/RY cis-element(s) within promoters. The Sph/RY promoter element is also a target of *VP1*/*ABI3-like* (*VAL*) genes. The binding of VAL proteins to Sph consensus sequences affects the entire process of embryogenesis (Suzuki et al., 2007).

1.12.5 VAL Proteins Repress Expression of Embryo-specific Genes

VAL genes encode B3 proteins that are associated with chromatin factors (Suzuki et al., 2007). The presence of 3 *VAL* genes, *VAL1*, *VAL2*, *VAL3*, was confirmed in *Arabidopsis* and VAL factors act as local repressors of the HAP3/B3 network. Suzuki et al. (2007) generated single, double and triple *val* mutants in *Arabidopsis*. The single mutants lacked discernible morphological phenotypes, while double and triple mutants were similar in phenotype. The *val1 val2* double mutant produced seedlings that survived up to 30 days on medium without ever producing leaf primordia. This indicates lack of a functional meristem. The seedlings had stunted, club-like roots, and developed cell proliferations with embryonic characteristics including callus formation in shoot and root regions, formation of embryo-like structures at apical meristem regions and at the margins of cotyledons. It was concluded that VAL genes are functionally redundant and are required for the development and maintenance of a functional apical meristem. They function to repress embryo development in post-embryonic tissues. It is interesting to

note that the phenotype resulting from *VAL* suppression strongly resembles the constitutively expressed *LEC1* phenotype.

To obtain a global picture of gene expression changes, Suzuki et al. (2007), used microarray analysis of *Arabidopsis* wild type and mutant seedlings. The known regulators of seed development were strongly up-regulated (> 4-fold) in the double mutant, especially *LEC1*, *L1L*, *ABI3*, and *FUS3*. However, LEC2 expression was not affected. These findings were reproduced by RT-PCR analyses. Consistent with FUS3 and ABI3 deregulation, 2S albumin and cruciferin, the downstream targets of B3 factors, were also up-regulated in the double mutants. This confirmed that the failure to repress LEC1-related and ABI3/FUS3 transcription factors during seed germination caused the embryonic seedling phenotype.

Because VAL proteins also contain B3 domains, it was presumed that they might interact with downstream genes directly, or indirectly via derepression of the B3 activators (Suzuki et al., 2007). The promoters and/or the first introns, of the up-regulated genes, showed an enrichment of the Sph/RY motif. Of the genes that showed $\geq$ 4-fold increase in expression, 54% contained at least one Sph/RY consensus motif. *LEC1*, *L1L*, *FUS3*, *ABI3*, *VAL1* and *VAL2* had the Sph/RY sequence, but *LEC2* did not (Suzuki et al., 2007). Additional support for the role of Sph/RY in the promoter of *LEC1* is that *LEC1* is ectopically expressed in the *Arabidopsis turnip* mutant, where the Sph/RY sequence is deleted. Constitutive expression of *LEC1* in this mutant leads to an up-regulation of early and late embryonic genes and results in the turnip phenotype, in which the embryonic stem is replaced by a swollen structure (Casson & Lindsey, 2006).

Further insights into embryogenesis, *PKL* expression was not affected in the double mutants, indicating that *VAL* is downstream of *PKL*. However, *PKL-like*, also a CHD3 chromatin remodeling gene was up-regulated in the double mutant. *AtGA3ox1*, a key GA synthesis gene expressed during germination, was down-regulated by 10-fold relative to wild type in the val double mutant.

To test the effect of gibberellic acid on VAL, *val1* mutant seeds were germinated on medium containing paclobutrazol, a GA synthesis inhibitor, at a concentration (5 nM) that was well below that for inhibiting germination. Approximately 23% of the seedlings exhibited strong embryonic transformations, including embryogenic callus on cotyledons and leaf margins, ectopic embryo formation on leaves, partial or complete transformation of leaves to cotyledon-like organs. Since single mutants lacked discernible phenotypes, this result illustrates that GA signaling down-regulates VAL repression of embryogenesis.

The hierarchy of gene regulators may be summarized as: PKL > VAL > LEC1 and L1L > LEC2/ABI3/FUS3. Inhibition of GA is useful for initiation of SE, while ABA is necessary for maturation.

It is evident that multiple pathways operate during embryogenesis. This complexity is reminiscent of the modularity of the cellular interactome described by Lage et al. (2007) when they put forth the idea that a phenome-interactome network of protein complexes is responsible for human genetic disorders. Lage et al (2007) explain that numerous genes exert their functions as part of protein complexes, such as metabolic networks, and that defects in several proteins, acting alone or in combination, cause overlapping clinical manifestations. This concept can be applied to plant embryogenesis where we saw that

single mutants of *lec1*, *lec2* or *fus3* manifest leafy cotyledons and ectopic expression of LEC1, LEC2 or BBM manifested as spontaneous somatic embryogenesis. The evidence for an embryogenic network has been discussed. Combined with the knowledge that plants have a proclivity for gene families, the existence of several embryogenic networks may be inferred. However, embryogenesis and vegetative development do not occur simultaneously and the transition between these 2 modes of development implies that a definitive change in gene expression takes place.

After the discovery of *LEC1*, others have theorized that embryogenesis is the default pathway for plant growth, and they predicted that ectopic expression of other genes would lead to somatic embryogenesis. If we think in terms of interactive complexes then a mutation in one or several genes may affect the entire cellular interactome so that one of the modes of growth is completely blocked and the other becomes the default. Thus embryogenesis is not a default but it may become a default. However, the more likely explanation is that embryogenesis is a condition or a syndrome, which results from an overabundance of embryo regulatory genes and a scarcity of genes that promote vegetative development. In terms of the interactome, even a slight disturbance could be consequential in upsetting the balance so that one "condition" is favoured over the other. This is supported by the observation that embryogenesis is most easily initiated from juvenile tissues, such as immature embryos, which already express a substantial complement of embryo-specific genes. Treatment with one hormone or one stress-inducing chemical is enough to upset the developmental sequence so that embryogenesis resumes. Nevertheless, the genes that have been identified and characterized have shaped our understanding of plant embryogenesis, but the gaps in knowledge signify that much

molecular work is still needed. To date, similar genes have not been characterized in conifers and this work is fundamentally important to a proper understanding of conifer SE and ZE.

1.13 Research hypotheses and objectives

1. In angiosperms, the *LEC1* gene functions during early and late embryogenesis, is involved in proper embryo formation, and its ectopic expression induces embryonic programs. *LEC1* is necessary for initiation of somatic embryogenesis and its ectopic expression in some cases leads to the formation of somatic embryos from mature tissues. Because conifer embryogenesis is poorly understood at the molecular level, the identification and characterization of a *LEC1* gene in conifers has the potential to provide insight into genetic interactions necessary for embryo development and initiation of SE. *LEC1* may serve as a molecular marker to assess the embryogenic potential of SE cultures. *LEC1* is a plant homologue of the eukaryotic CCAAT box-binding transcription factor. Therefore, it is expected that *LEC1* is present in conifers due to the common ancestry of plants and it will have significant sequence identity to *Arabidopsis LEC1*. Such a gene should be called *PmLEC1* if it is specifically expressed in embryonic tissues and it has the ability to induce embryogenesis in the *lec1-1* null mutant, or *PmLEC1-LIKE* if it is expressed in vegetative tissues and lacks embryogenic capacity. This will be confirmed by analyzing *PmLEC1* steady state

transcript levels and protein profiles in embryonic and vegetative tissues and during the stages of embryogenesis.

2. Presently, conifer embryogenesis is induced from juvenile tissues incubated in the dark in the presence of plant growth regulators, such as auxins. A series of hormonal treatments are necessary for further embryo development and maturation. In addition, some of the medium components may also serve as chemical stressors: PEG, sucrose, L-Gln, myo-inositol. Studies with angiosperms have shown that somatic embryogenesis is induced in tissues subjected to the auxin 2,4-D following exposure to stresses such as salinity, osmotica and heavy metals. Based on the work of Ikeda-Iwai et al. (2002), who showed that sorbitol, mannitol, sucrose, salinity and heavy metals led to the formation of embryogenic callus, the spontaneous formation of somatic embryos directly from the callus, and that the somatic embryos expressed LEC1, it is expected that such treatments will up-regulate *LEC1* expression in mature Douglas-fir seeds. Because auxins and cytokinins (2,4-D/BAP) are required for induction of conifer SE while ABA/GA are required for proper embryo maturation, we predict that 2,4-D/BAP will have a strongly inductive effect, while ABA/GA should have a lesser inductive effect. Brassinosteroids are a new class of plant growth regulators that improve SE initiation rates in conifers and rice and this treatment should strongly up-regulate PmLEC1 expression (Pullman et al., 2003). Alternatively, isolation and analysis of the promoter sequence will also provide insight into the induction of PmLEC1. The identification of modulators of *LEC1* expression in mature

conifer seeds will provide useful information for inducing *LEC1* expression and consequently embryogenesis in other vegetative tissues such as needles.

3. Ectopic expression of *AtLEC1* rescued the *Arabidopsis lec1-1* null mutant, led to the spontaneous formation of embryo-like structures from vegetative tissues of second-generation transgenic plants and concomitantly activated embryonic programs, as confirmed by the presence of seed-specific transcripts in abnormal seedlings. Ectopic expression of Douglas-fir *LEC1* in the *Arabidopsis* null mutant is expected to rescue the mutant and lead to the appearance of abnormal embryo-like transgenic seedlings in the first and second transgenic generations and the spontaneous formation of somatic embryos in the second generation. The seed-specific genes, *oleosin* and *cruciferin*, will be expressed in embryo-like seedlings. If *LEC1* is truly a master regulator of plant embryogenesis then ectopic expression of *PmLEC1* in wild type background should also cause abnormal seedling morphology, formation embryo-like structures and expression of seed-specific genes.

2. Douglas-fir *LEAFY COTYLEDON1* (*PmLEC1*) Is an Embryo-specific Gene Whose Expression in Mature Tissues is Up-regulated by Brassinosteroid and Osmotic Stress

2.1 INTRODUCTION

The importance of reforestation efforts and the need for robust forests cannot be overstated. The demand for pulp, paper and lumber surpasses natural resources, while the capacity of forests to absorb emissions and provide time for transition to new energy sources benefits the environment. SE, the asexual production of embryos from differentiated tissues, is the most promising method for propagating high value trees. SE ensures the consistent and unlimited production of quality embryos at any time of the year. This is a major economic benefit because conifers tend to have lengthy reproductive cycles lasting from 1 to 2 years, seed production is limited and uncertain, and germination efficiencies can be as low as 40%. In species such as Douglas-fir, good seed crops occur approximately every 5 years (Allen and Owens, 1972), placing severe strains on reforestation initiatives. A further advantage of SE is that forests made up of mixtures of elite genotypes will acquire genetic gains much faster than could occur by natural selection (Pullman et al., 2003).

The success of SE is inconsistent, and quite often, superior genotypes are recalcitrant to induction of somatic embryogenesis. At this time there are no molecular indicators of embryogenic potential or gene expression profiles, which could help researchers to overcome the recalcitrance. Part of the problem is that very little is known about the genes and the molecular events responsible for embryogenesis in conifers. On the other

hand, much is known about optimal medium composition, hormone cocktails capable of initiating somatic embryogenesis and methods to induce the later stages of development.

In Douglas-fir, immature embryos are induced to multiply and form embryo suspensor masses, or embryogenic callus, by subjecting them to a combination of the cytokinins kinetin and benzyl aminopurine, and the auxin 2,4-dichlorophenoxyacetic acid. At the cellular level, this process has been described as cleavage polyembryony (Gupta; Filonova et al., 2000a) and/or unequal divisions of embryogenic cells (Filonova et al., 2000a). To effect embryo development and inhibit the embryogenic proliferative state, the auxins and cytokinins are removed from the medium and replaced with ABA. ABA permits singulation, a process unique to Douglas-fir SE, and continued growth of individual embryos. ABA also plays a role in storage compound accumulation and embryo maturation (Gupta et al., 1991). Mature embryos are obtained by transfer of the singulated embryos to medium containing polyethylene glycol, ABA, GA, and activated charcoal (Gupta et al., 1991). High quality, mature somatic embryos are characterized by high amounts of accumulated storage compounds. Although this method of SE is productive, more that 50% of Douglas-fir SE cultures discontinue growth after six months and significant losses occur from culture initiation to somatic seedling establishment.

A drawback of present protocols is that conifer SE can only be induced from juvenile tissues (megagametophytic tissue, excised zygotic embryos, seedlings) and such embryogenic tissues may not be available by the time that the superiority of a tree is confirmed. It is not yet possible to induce SE from vegetative, mature cells such as those found in roots or needles. A series of processes remain to be elucidated before progress

can be made. The molecular characterization of conifer embryogenesis will help us understand the developmental processes that lead to the formation of zygotic and somatic embryos, and enable us to improve SE protocols (Misra, 1994). The compilation of gene expression profiles characteristic of embryogenesis will elucidate the key genes at different stages of the embryogenic pathway. The establishment of a correlation between manipulation of medium composition and embryogenic gene expression may provide the solution to breaking recalcitrance. A strategy for understanding conifer SE would be to isolate a single gene important in angiosperm SE and then identify other genes with which its products interact.

In the last 10 years, different attempts at isolating genes important to early embryogenesis or induction of embryogenesis in conifers have yielded few results. Differential display techniques, proteomics, microarrays have been hampered by the fact that critical regulators are present in trace amounts and there was no access to very early stages of embryogenesis due to minute amounts of tissue. More recently, high through-put methods involving ESTs and knowledge from angiosperm embryogenesis have started to yield results in the form of homologous gene identification. In addition, over 25 novel and previously uncharacterized embryo-specific sequences have been isolated. There is evidence that some of the well-studied, angiosperm embryo-regulatory genes are present in gymnosperms.

In contrast to gymnosperms, over 100 embryo-specific genes have been identified in angiosperms and several of these are transcription factors, which are critical during early embryogenesis and are capable of inducing somatic embryogenesis when expressed ectopically in mature cells. The *Arabidopsis thaliana SOMATIC EMBRYOGENESIS*

RECEPTOR KINASE1 confers embryogenic competence in culture and increases the efficiency of somatic embryogenesis initiation (Hecht et al., 2001). *BABY BOOM* (*BBM*) encodes a transcription factor that is capable of activating signal transduction pathways, and inducing embryo development from differentiated somatic cells in *Arabidopsis thaliana* and *Brassica napus* (Boutilier et al., 2002). Not only did ectopic over-expression of BBM result in the spontaneous formation of somatic embryos on post-germinative organs, but also in some *35S::BBM* lines, over-expression of BBM caused a reiteration of the embryo-forming process so that new embryos formed on the cotyledons of pre-existing embryos. BBM expression also stimulates pathways responsible for cell division and differentiation. *LEAFY COTYLEDON1* (*LEC1*) is a CCAAT box-binding transcription factor homologue that was identified in *Arabidopsis* (Lotan et al., 1998) and it was the first gene shown to induce somatic embryogenesis. Expression studies have shown that LEC1 acts during both early and late embryogenesis but *LEC1* mRNA levels are highest during early embryogenesis. *LEC1* mRNA accumulation is seen in both the embryo proper and suspensor of the early pro-embryo. It has been proposed that LEC1 regulates embryonic processes by activating transcription of genes required for normal *Arabidopsis* development. LEC1 is believed to suppress the embryogenic potential of the suspensor in early embryogenesis, specify embryonic organ identity, and inhibit premature germination.

It is known that SE is induced in higher plants by exogenous hormones and culture-related stress (Dong & Dunstan, 2000). In tissue culture ethylene may act as either a promoter or inhibitor of regeneration depending upon species (Minocha & Jain, 2000). ABA, sugars and PEG stimulate embryo development and maturation, while auxins and

cytokinins play critical roles in regeneration (Minocha & Jain, 2000). A system for induction of SE in carrot and *Arabidopsis* involves the incubation of shoot-apical-tip explants on phytohormone-free medium containing stress chemicals followed by the transfer of explants to phytohormone-free medium without the stress chemicals (Ikeda-Iwai et al., 2003). Compounds known to induce culture-related stress are frequently utilized in conifer somatic embryogenesis media, yet we do not know which genes are responsive to these treatments and how their activities are modulated.

Insight into gene response to exogenous compounds may be gained by studying the effect of these compounds on gene expression. Ikeda-Iwai et al. (2003), used chemical stressors to induce somatic embryogenesis and demonstrated that the expression of embryo-specific genes was activated. *Arabidopsis* shoot-apical tip explants incubated for several hours on phytohormone-free medium containing a stress-inducing compound such as sorbitol, mannitol, sucrose, NaCl or $CdCl_2$, and transferred to 4.5 μM 2,4-D medium produce a callus and somatic embryos in the region of the SAM after 10-21 days (Ikeda-Iwai et al., 2003). Transfer of the callus to phytohormone-free medium results, after 14 days, in the production of more somatic embryos adjacent to the initial somatic embryos (Ikeda-Iwai et al., 2003). Expression of embryo-specific genes *FUS3* and *ABI3* was observed in the somatic embryos but not in the callus (Ikeda-Iwai et al., 2003). A similar technique for induction of SE involves treatment *Arabidopsis* immature zygotic embryos with 4.5 μM 2,4-D for 10 days to produce primary somatic embryos, which are subsequently incubated on 9.0 μM 2,4-D medium for 8-21 days to produce embryogenic cell clusters and secondary somatic embryos (Ikeda-Iwai et al., 2002). Expression of *LEC1*, *FUS3* and *ABI3* was confirmed in the primary and secondary somatic embryos and

the embryogenic cell clusters (Ikeda-Iwai et al., 2002). These reports demonstrate that SE can be induced by stress and/or hormone treatments and embryo-specific gene expression may be used to assess the outcome of induction.

Because *Arabidopsis LEC1* was shown to be important for the induction of somatic embryogenesis *in vitro* (Lotan et al., 1998; Stone et al., 2001) and induction is one of the greatest challenges with conifers, we sought to isolate and characterize such a gene in Douglas-fir/*Pseudotsuga menziesii*. The pertinence of isolating and characterizing this gene is realized when considering that very little is known about the genes that function during early embryogenesis, and this stage dictates the success of the mature conifer embryos (Stasolla & Yeung, 2003). To gain insight into the *PmLEC1* gene and its regulation we analyzed its sequence as well as its promoter sequence. *PmLEC1* expression was analyzed by QPCR in developing zygotic embryos and mature tissues. The PmLEC1 protein profile was determined. Stress and hormone treatments were performed on mature seed and responses of individual seed were assessed by QPCR.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Somatic embryos were kindly donated by Dr. P. Gupta of Weyerhaeuser. These were maintained in culture as embryogenic suspensor masses (ESM) at the maintenance stage (ESM-2) as described by Gupta et al., 1991. Singulated, stage 3 somatic embryos (ESM-

3) and mature, stage 4 somatic embryos (ESM-4), were induced by sub-culturing in appropriate media (Gupta et al., 1991).

Developing zygotic seeds, vegetative buds, pollen cones and mature needles were collected from open-pollinated trees growing on campus at the University of Victoria. Vegetative buds, pollen cones and mature needles were immediately frozen in liquid nitrogen and stored at –80 ^{o}C. Seeds at various developmental stages were removed from the cones, and the seed coats were detached prior to freezing in liquid nitrogen and stored at –80 ^{o}C.

Mature Douglas-fir seeds were obtained from an open-pollinated seed orchard in Sorrento, BC, Canada. The seeds were imbibed by enclosing them in 1 layer of cheesecloth and placing them in distilled water for 24 hr in a beaker covered with tinfoil and the water was agitated by a magnetic stirrer at low speed. The seed were stratified at 4 ^{o}C for 3 weeks between layers of Whatman 1MM filter paper that was placed on top of sponges, which were soaked in water, with the water level remaining halfway up the thickness of the sponges. To expose seed to germination conditions, Kimpack absorbent cellulose wadding (LPS Industries, New Jersey) were cut to the size of 9 cm Petri dishes; they were soaked with water and covered with 2 layers of filter paper such that the filter paper itself was also moist. The seeds were placed on top of the filter paper, the Petri dishes were covered with their lids and they were sealed with Parafilm. These were placed in a growth chamber with regulated light and temperature conditions. The seeds germinated and they were harvested at pre-determined intervals and frozen in liquid nitrogen and stored at –80 ^{o}C until molecular work was performed. After exposure to germination conditions, germinating seed were collected after 2, 6, 10, 12 and 14 days.

Seedlings were harvested at 1.5 months and 3 months after exposure to germination conditions.

Arabidopsis seeds were surface sterilized and germinated on ½ Murashige and Skoog (MS) media (3 % sucrose, 0.6 % agar, pH 5.75), (Murashige and Skoog, 1962). Seedlings were transferred to pots or magenta boxes, as necessary, and grown.

2.2.2 DNA Isolation

DNA was isolated from somatic embryos by grinding tissues in liquid nitrogen and using the Sigma GenElute Plant Genomic DNA kit according to manufacturer's instructions.

DNA was isolated from mature needles using the modified cetyltrimethylammonium bromide (CTAB) method. Briefly, 2 – 5 g of tissue were ground in liquid nitrogen and the powder was suspended in 20 ml ice-cold CTAB extraction buffer (50 mM Tris HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA), 0.35 M sorbitol, 0.1% BSA, 10 % polyethylene glycol (PEG) 4000, 0.1% Spermine, 0.1% Spermidine) to which 20 µl 100% 2-mercaptoethanol (EtSH) had been freshly added. The solution was filtered through several layers of cheesecloth and 1 layer of Miracloth. Organelles were pelleted at 9800 x g and 4 ^{o}C for 15 min in a Beckmann J2-21 Ultracentrifuge. The pellet was resuspended in 5 ml CTAB wash buffer (50 mM Tris HCl (pH8.0), 25 mM EDTA, 0.35 M sorbitol) with 5 µl EtSH, and the solution was brought to RT. One ml of 5% Sarcosyl was added and the solution was incubated at RT for an additional 10 min, at which time the solution became very viscous. Addition of 0.9 ml 5M NaCl, was followed by

inversion of the tube and this was followed by addition of 0.7 ml of 8.6% CTAB in 0.7 M NaCl and inversion. The solution was incubated at 60 ^{o}C for 15 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the solution was mixed by inversion until an emulsion formed. The phases were separated by centrifugation at 3000 x g for 10 min. The upper aqueous phase was removed to a clean tube and DNA was precipitated by addition of 2 ml 3M NaOAc (pH 5.7) and 15 ml 95% EtOH and incubation on ice 30 min to overnight. The DNA was pelleted at 24000 x g for 20 min. The supernatant was discarded and the pellet was air dried in the fume hood for 15 min. The pellet was dissolved in 300 μl Tris-EDTA (TE) buffer, pH 8.

2.2.3 Isolation of conserved *LEC1* sequence by PCR

Degenerate primers based on the conserved sequence of *AtLEC1*, and a partial cDNA sequence of *Pinus taeda* were designed to isolate a Douglas-fir homologous sequence. The primer sequences employed were 5'-AGAGAGCAAGATAGGTTCATGCC-3' and 5'-CCMARCTTGCTCATAGCCCARAG-3'. The PCR reaction was performed with 0.5 μg DNA in a 50 μl reaction using the QIAGEN Taq PCR Master Mix according to the manufacturer's instructions, in the Perkin Elmer Cetus 480 DNA Thermal Cycler. The thermocycle program consisted of 5 min denaturation at 94 ^{o}C, 40 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 60 ^{o}C for 30 s and extension at 72 ^{o}C for 1 min, followed by 10 min extension at 72 ^{o}C. The products were separated by electrophoresis on a 1 % agarose gel and visualized by ethidium bromide (EtBr) staining.

The single PCR product, which was approximately 200 bp in length, was extracted from the gel using the QIAquick Gel Extraction Kit (QIAGEN). The purified DNA (2 μl) was ligated into the pCR2.1 TOPO vector and transformed into *E. coli* using the TOPO TA cloning kit (Invitrogen) according to the instructions. Plasmid DNA was purified from transformed colonies using QIAprep and the DNA inserts were sequenced at the DNA Sequencing Centre, Centre for Biomedical Research, Biology Department, University of Victoria. Database searches were performed at the National Center for Biotechnology Information using the BLAST network service (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.4 RNA Isolation

RNA was isolated from somatic embryos, developing zygotic seed, imbibed seed and stratified seed by the TRIzol (Invitrogen) method according to the manufacturer's instructions modified for plants. Briefly, at the RNA precipitation stage, 0.25 ml high salt solution (0.8 M Na citrate and 1.2 M NaCl)/1 ml TRIzol was added to the aqueous phase immediately after the isopropanol. All other steps were carried out as per manufacturer's instructions.

RNA isolation from seed exposed to germination conditions, seedlings, vegetative buds and pollen cones was carried out via the modified hot-phenol extraction method (Verwoerd et al., 1989). Up to 100 mg of tissue were ground in liquid nitrogen and the powder was transferred to 1.5 ml Eppendorf tubes containing 0.5 ml hot phenol extraction buffer (100 mM LiCl, 100 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS,

phenol solvent pH 6.7) that was preheated at 85 ^{o}C. The tubes were vortexed to suspend the powder and then they were incubated in an 85 ^{o}C waterbath for 1 min. An aliquot of 0.25 ml chloroform:isoamyl alcohol (24:1) was added to the hot solution and the tube was mixed by vortexing. The samples were centrifuged fir 5 min at maximum speed in a tabletop centrifuge at 4 ^{o}C. The aqueous phase was transferred to a new tube and 1 μl glycogen was added, followed by one volume of 4M LiCl. The RNA was precipitated overnight at –20 ^{o}C. The samples were centrifuged at 4 ^{o}C for 6 min at maximum speed. The pellets were dissolved in 250 μl diethylpyrocarbonate-treated water (DEPC-H_2O). RNA was precipitated by addition of 25 μl 3M NaOAC pH 5.2 and 500 μl ice-cold 100% EtOH, and RNA was precipitated at – 20 ^{o}C for 1 hr. The tubes were centrifuged at 4 ^{o}C and maximum speed for 5 min, the supernatant was discarded and the RNA was washed with 1 ml of 70 % EtOH. The pellets were resuspended in 40 μl DEPC-H_2O.

2.2.5 RT-PCR for isolation of expressed Douglas-fir *LEC1* sequences

RNA isolated from somatic embryos at the maintenance and singulated stages as well as immature zygotic seed was used in separate RT-PCR reactions. The absence of DNA contamination was confirmed by attempting PCR directly with the RNA template. For first strand cDNA synthesis, 5 μg total RNA was incubated with 1 μl oligo $(dT)_{12}$VN and SuperScript II RNase H^- reverse transcriptase (Invitrogen) according to manufacturer's instructions. The Invitrogen Ribonuclease Inhibitor (1 μl at 10U/μl) was utilized to prevent RNA degradation in these reactions.

PCR was performed with 2.5 μl cDNA in 50 μl reactions employing the QIAGEN Master Mix, as described previously.

2.2.6 RACE-PCR for Obtaining Full-length cDNA

In order to isolate the sequences upstream and downstream of the conserved domain, rapid amplification of cDNA ends (RACE) was performed with 1 μg total RNA isolated from singulated somatic embryos, the stage where PmLEC1 expression was shown to be the highest. The SMART RACE cDNA Amplification Kit (BD Biosciences) was utilized according to the manufacturer's instructions. A new set of gene specific primers (GSPs) was designed from the sequence of the conserved domain. GSP1, 5'-CTGACAACGTTCATTGGCCTCACTGG-3', was employed in the 5'-RACE reaction to obtain the upstream sequence, and GSP2, 5'-CCCACCCATGCAAAGATTTCTGATG-3', was employed in the 3'-RACE reaction.

The 5'- and 3'- RACE-PCR products were separated on 1 % agarose gels, excised from the gels, cloned and sequenced, as described above. Once these sequences were obtained, they were confirmed to be LEC1 sequences by TBLASTX searches. Further, a new set of primers was designed based on the start and stop codons of individual RACE-PCR sequences showing the highest identity to the translated AtLEC1 sequence: 5'-ATGATGTCCGAAGTTGGAAGCCCT-3' and 5'-CTTATACTGAGCAT-AGGGATCATA-3'. These new primers were utilized in PCR reactions with genomic Douglas-fir DNA and RT-PCR reactions with RNA isolated from zygotic developing

seed. Both types of products were visualized on agarose gels, extracted from the gels, cloned and sequenced, as described earlier.

2.2.7 Phylogenetic Analysis

The full-length PmLEC1 sequence was queried against the TBLASTX database (Altschul et al., 1990). Of the results obtained, all full-length LEC1 or LEC1-like sequences were aligned with the MUSCLE (Edgar, 2004) program available on the European Bioinformatics Institute (EBI) website at http://www.ebi.ac.uk/Tools. Prior to this, alignments were performed with both Base-by-base (Brodie et al., 2004) and CLUSTALW (Higgins et al., 1994), but the resulting alignments were of low quality, with numerous gaps and single bases scattered throughout. Manual editing was likely to result in much bias. The MUltiple Sequence Comparison by Log Expectation (MUSCLE) program (Edgar 2004) was chosen because it claims to have better accuracy and speed. The parameters for the MUSCLE alignment included FASTA output format, the Output tree was "from second iteration," and the Output order was aligned. No additional adjustments were necessary after the alignment.

Phylogenetic tree construction was achieved by importing the MUSCLE alignment into CLUSTALW, and utilizing the neighbour joining tree option in the Phylogenetic Tree section with default settings. Bootstrapping was also performed in Clustal W with 1000 replicates. The tree was drawn in Treeview (Page, 1996).

Accession numbers for the LEC1 and LEC1-LIKE sequences are: FJ418168 (*Pseudotsuga menziesii LEC1*), AJ879074.1 (*Helianthus annuus LEC1-like*),

AM494833.1 (*Theobroma cacao LEC1-like*), AC155343.1 (*Brassica rapa* genomic sequence), AM428051.2 (*Vitis vinifera* genomic sequence), NM_102046.4 (*Arabidopsis thaliana LEC1* ecotype Columbia), AF036684.1 (*Arabidopsis thaliana LEC1* ecotype Wassilewskija-0), AF533650.1 (Phaseolus coccineus LEC1-like), AB104611.1 (*Daucus carota C-LEC1*), AY264284.1 (*Oryza sativa indica* cultivar *LEC1*), (*Oryza sativa LEC1A*), DO674267.1 (*Kalanchoe daigremontiana LEC1-like*), AF410176.1 (*Zea mays LEC1*), AY138461.1 (*Arabidopsis thaliana LEC1-like*), BT013228.1 (*Lycopersicon escutelum* mRNA), AC155343.1 (*Brassica rapa* subsp. *perkinensis* genomic sequence), AC210129.1 (*Populus trichocarpa* genomic sequence), BT009029.1 (*Triticum aestivum* mRNA), AC151709.9 (*Medicago truncatula* genomic sequence), AW754604 (*Pinus taeda* EST sequence).

2.2.8 Real-time quantitative PCR (QPCR) analyses

Quantification of relative gene expression was achieved by QPCR. Total RNA was obtained by one of the methods described above and treated with Invitrogen Amplification Grade DNase I according to manufacturer's instructions. Briefly, these reactions were carried out on the benchtop for not more than 14 min. DNase I-treated RNA (1 μg) was reverse transcribed using an oligo$(dT)_{12}$VN primer (V = A or C or G, N = A or C or G or T) and Invitrogen SuperScript II RNase H^- Reverse Transcriptase according to manufacturer's instructions. The cDNA was diluted 20-fold prior to QPCR and the reactions were carried out in quadruplicate.

The Invitrogen Platinum SYBR Green qPCR SuperMix-UDG ready-to-use cocktail was obtained and the Stratagene Mx4000 real-time cycler (Stratagene, La Jolla, CA, USA) was utilized. The QPCR reactions were set up as follows: 3.5 µl dH_2O, 7.5 µl Supermix, 1 µl forward primer, 1 µl reverse primer, 1 µl ROX dye diluted 1:10 immediately before use, 2 µl cDNA (20-fold) dilution. The amplification program consisted of 9 min enzyme activation at 94 ^{o}C, and 40 cycles of denaturation (94 ^{o}C for 15 s), annealing (60 ^{o}C for 30 s) and extension (72 ^{o}C for 45 s). Specificity of the reactions was confirmed by gel electrophoresis and the appearance of single amplified DNA products of the expected size for each target gene. The reactions were performed in quadruplicate and the cycle threshold (Ct) data obtained were compared to the expression of the invariant ribosomal protein L8 control gene using the comparative Ct method (ddCt) (http://www.dorak.info/genetics/realtime.html). The resulting gene expression data during development are presented as fold change relative to the pollen cone sample, which showed the lowest level expression. For the stress and hormone treatments, the data are presented as fold change relative to control seed.

SPSS (SPSS Version 12.0, Chicago, IL) was employed for the statistical analyses of the resulting quantitative PCR data. Because all data were non-parametric, the Kruskal-Wallis and the Mann-Whitney U two-tailed tests were used.

2.2.9 Stress and Hormone Treatments

Stratified seeds were placed in Petri dishes on filter paper that had been soaked with the various treatment compounds and incubated in the dark for 24 hours. The treated

seeds were excised from the seed coats and they were immediately placed in liquid nitrogen and stored at – 80 ^{o}C until the time of RNA isolation. The concentrations of the treatment solutions were 0.7 M sorbitol, 0.7 M mannitol, 0.7 M sucrose, 0.3 M NaCl, 0.6 mM $CdCl_2$, 23.75 mM PEG 8000, 10 μM 2,4-epibrassinolide, 50 μM 2,4-D and 20 μM BAP; 7.2 μM GA and 38 μM ABA.

Each treatment group consisted of 5 seeds. RNA was isolated separately from each individual seed. All isolations and all molecular work was performed simultaneously for each treatment group. QPCR and statistical analyses was performed as described above.

2.2.10 Protein Isolation

Tissue was weighed and ground in liquid nitrogen. The powder was suspended in 3 μl extraction buffer per mg tissue, in 1.5 ml Eppendorf tubes. Extraction buffer: 65 mM tris(hydroxymethyl)aminoethane (Tris) (pH 6.8), 1% sodium dodecyl sulfate (SDS), 5% glycerol, 2.5% EtSH. The suspension was set in a heating block preheated to 100 ^{o}C for 5 min. The tubes were immediately frozen in liquid nitrogen for 5 min, and then placed again in the 100 ^{o}C heating block for an additional 5 min. The samples were centrifuged at 16,000 x g and 4 ^{o}C for 25 min. The supernatant, containing total cellular proteins, was removed to a clean, sterile Eppendorf tube. Protein concentration was determined *via* the Bradford assay (Bradford, 1976). A standard curve was prepared with BSA diluted over a range of 2 – 9 μg/ml. To determine protein concentration as accurately as possible and to minimize interference by SDS, 10 μl aliquots of total cellular protein extracts were assayed in 1 ml reactions, thus reducing the SDS concentration to 0.01%.

2.2.11 Antibody Production

A synthetic peptide corresponding to the first 18 amino acids of the putative PmLEC1 protein, with an additional cysteine residue at the C-terminus (MMSEVGSPTSQDSRNSEDC) and coupled to the KLH carrier protein was obtained from GenScript Corporation, New Jersey, USA. Antibody production was performed at Immuno-Precise Antibodies, Ltd, Victoria, BC, Canada. Four Balb/C mice were each immunized with 25 μg of the KLH-coupled peptide, mixed with Freund's complete adjuvant. This was followed by 6 additional immune boosts of 25 μg of peptide-KLH in Freund's incomplete adjuvant, at 3-week intervals.

Various dilutions of the polyclonal mouse serum were tested by enzyme-linked immunosorbent assay (ELISA) against the free peptide. The polyclonal serum from two mice showed a significant response against the peptide, and a dilution factor of 1:1000 was shown to be appropriate.

2.2.12 Western Blotting

Total cellular proteins (20 μg) were suspended in protein sample buffer (12.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 5% 2-mercaptoethanol; 0.1% bromophenol blue) and denatured for 5 min at 100 ^{o}C. Proteins were separated by SDS-PAGE using the Mini-PROTEAN II gel electrophoresis system (BioRad) with a 4% (w/v) polyacrylamide stacking gel (75 V, constant voltage) and a 14% (w/v) polyacrylamide separating gel (120

V, constant voltage). The proteins were stained with Coomassie blue R250 or transferred to PVDF membranes (Amersham Biosciences) by electroblotting at 100 V for 80 min in protein transfer buffer (25 mM Tris, 190 mM Glycine, 20% MeOH, 0.1% SDS).

The membranes were washed twice with PBST (80 mM Na_2HPO_4, 20 mM $NaH_2PO_4 \bullet H_2O$, 100 mM NaCl, 0.05% w/v Tween 20) and then blocked overnight at 4 ^oC in 5% w/v skim milk PBST. The membranes were rinsed with PBST twice and incubated with primary antibody (mouse polyclonal immune serum, diluted 1:1000) for 1 hr at RT on a rotary shaker. The membranes were rinsed twice with PBST and washed 4 times with PBST for durations of 15 min, 5 min, 5 min and 5 min. The membranes were incubated with ImmunoPure Goat Anti-Mouse IgG (H+L), Peroxidase Conjugated (Pierce Biotechnology, cat. # 31430) for 1 hr at 37 ^oC on a rotary shaker, rinsed and washed as above. Immunoreactive bands were visualized by chemiluminescent detection with ECL Plus Western Blotting Detection Reagents (Amersham) according to manufacturer's instructions. Autoradiography film was exposed to the membranes for 15 min in an X-ray film cassette and developed.

2.2.13 Promoter Sequence

The *PmLEC1* promoter sequence was obtained using the Genome Walker Universal Kit (Clontech, Mountain View, California, USA, cat. # 638904). Douglas-fir genomic DNA was assessed for quality and purity on a 0.9% agarose/0.01% ethidium bromide gel. Genomic DNA, 2.5 μg, was digested in 4 separate reactions at 37 ^oC for 2 hr using 80 units of each of *Dra* I, *Eco* RV, *Pvu* II and *Stu* I, to create 4 genomic libraries. The

digested DNA was purified and ligated to Genome Walker Adaptors. The primary PCR reactions were performed with the QIAgen Taq PCR Master Mix and aliquots from each library and a gene specific primer, GSP1, which was based on an internal *PmLEC1* sequence. GSP1 was the same as that utilized in RACE-PCR, 5'-CTGACAACGTTCATTGGCCTCACTGG-3'.

The secondary PCR reaction was performed with the QIAgen Taq PCR Master Mix, aliquots of the primary PCR and a nested primer, GSP2, which was upstream of GSP1 and did not overlap with GSP1. GSP2, 5'-CTTCCAACTTCGGACATCATAC-CTAC-3', also spanned the start codon. In the secondary PCR reaction, only the *Dra* I and *Pvu* II libraries yielded products that were specific, major bands. These 2 products were purified from the gel, cloned and sequenced. The purified DNA (2 μl) was ligated into the pCR2.1 TOPO vector and transformed into *E. coli* using the TOPO TA cloning kit (Invitrogen) according to the instructions. Plasmid DNA was purified from transformed colonies using QIAprep and the DNA inserts were sequenced at the DNA Sequencing Centre, Centre for Biomedical Research, Biology Department, University of Victoria. The sequences were identical except that the *Dra* I sequence was longer.

2.2.14 Promoter and 5' UTR Sequence Analyses

The *Dra* I sequence, FJ418169 (*Pseudotsuga menziesii LEC1* promoter), was analyzed using the SoftBerry NSITE-PL program (http://softberry.com), which recognizes transcription factor binding sites and other consensus regulatory sequences; the Neural Network Prediction Program of the Berkeley Drosophila Genome Project (Reese et al.,

1999); and, the Signal Search program of Plant Cis-Acting Regulatory DNA elements (PLACE) (Higo et al., 1999; Prestridge, 1991).

2.3 RESULTS

2.3.1 Isolation of the Douglas-fir *PmLEC1* cDNA Sequence and Genomic DNA Sequence

To isolate a gene expected to be important to conifer early embryogeny, PCR primers were designed based on the central, conserved domain of Arabidopsis *LEC1*. The *Arabidopsis LEC1* gene is expressed during early embryogenesis and it is necessary for proper embryo formation (Lotan et al., 1998). Once a partial sequence was obtained, a RACE-PCR strategy was employed to obtain the full-length sequence. The 779 bp *PmLEC1* cDNA sequence isolated from *Pseudotsuga menziesii* (Douglas-fir) is shown in Figure 3. This sequence contains 5' and 3' UTRs, no start codons within the 5' UTR, an in-frame stop codon within the 5' UTR, a 5' UTR intron, 2 start codons in tandem and a single, in-frame stop codon. The presence of an in-frame stop codon within the 5'-UTR indicates that this UTR is spliced, and the stop codon may indicate initiator codons (Soccio et al., 2002). The absence of start codons within the 5' UTR is correlated with strong translational efficiency, while multiple ATG triplets in the 5' UTR result in low, basal translation rates (Rogozin et al., 2001).

PCR primers based on the sequences surrounding the start and stop codons were utilized to obtain the genomic PmLEC1 sequence. The genomic DNA sequence was

identical to the cDNA sequence, indicating the absence of introns within the coding sequence of this gene. However, isolation and sequencing of the DNA sequence 1400 bp upstream of the start codon, revealed the presence of a 5' UTR intron (Figure 3). The features of the 5' UTR intron are described in section 2.3.3. The validity of the 5' UTR was confirmed when a query of the promoter sequence against the Neural Network Promoter Prediction program (Reese et al., 1996) identified a transcriptional start site at the beginning of the 5' UTR.

Translation of the *PmLEC1* cDNA sequence results in a protein of 180 amino acids, while the putative AtLEC1 comprises 208 amino acids. Alignment of PmLEC1 with AtLEC1 in MUSCLE (Edgar, 2004) results in 3 gaps of 3, 14 and 10 amino acids in the C terminus, indicating possible deletions. The PmLEC1 protein shows 52% identity and 75% similarity to AtLEC1 (WS ecotype).

The expected size of the PmLEC1 protein is 21 kDa. ScanProsite (DeCastro et al., 2006) analysis of the PmLEC1 protein sequence revealed that it has the potential for the following modifications: 4 phosphorylation sites, 2 N-myristoylation sites and 1 N-glycosylation site. N-myristoylation is an irreversible, co-translational acylation of the alpha-amino group of Gly with myristic acid. In addition, PmLEC1 contains numerous polar residues, 28 acidic amino acids and 22 basic amino acids. These characteristics hint at the possibility that the protein will not migrate at the expected size.

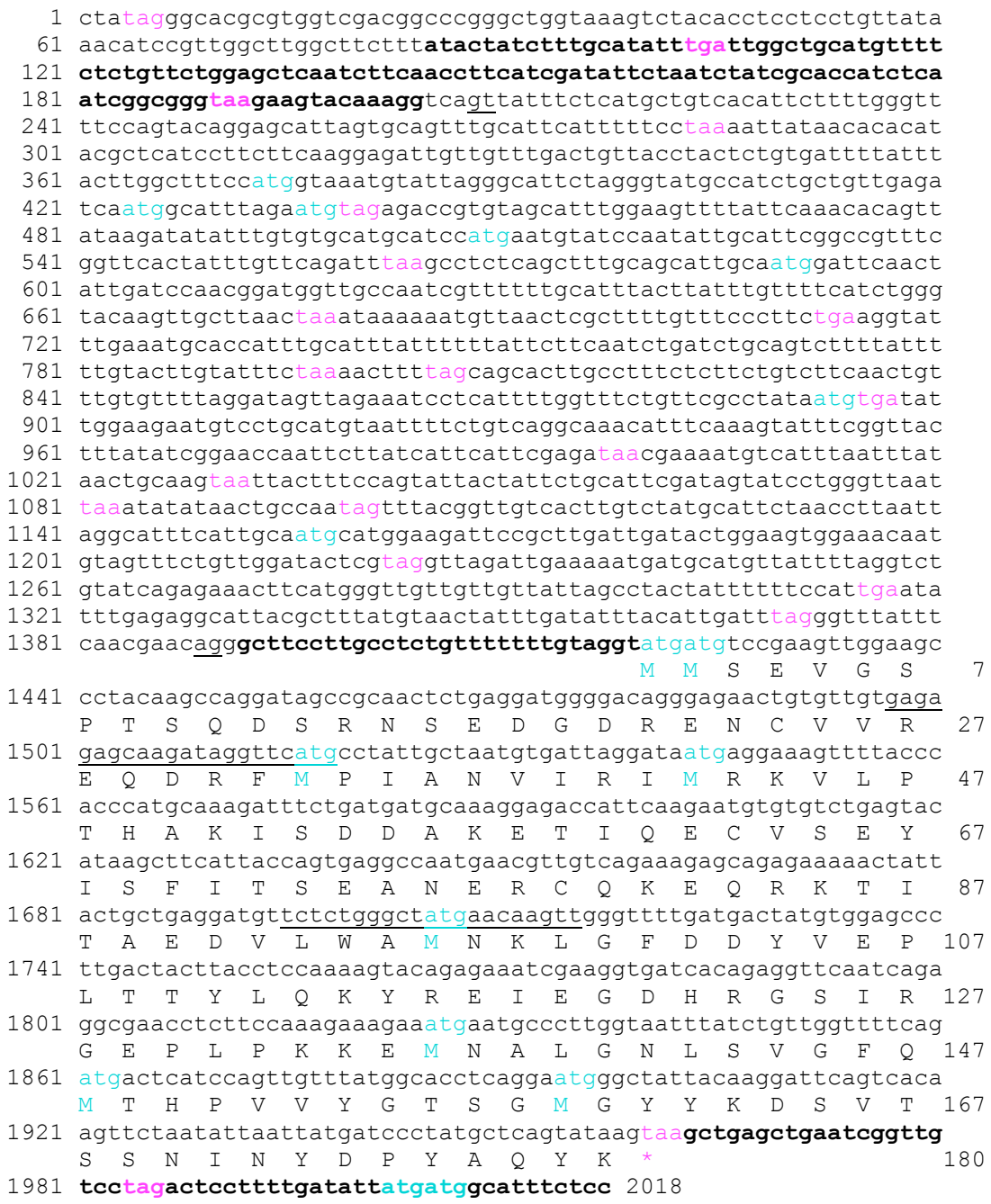


Figure 3. Nucleic acid sequence and translated sequence of PmLEC1.

Nucleic acids are numbered on the left and amino acids are numbered on the right. The 5' UTR intron occurs between nucleotides 206-1393, and is positioned between 2 sections of 5' UTR (in bold). Potential intron splice sites are underlined. The full-length *PmLEC1* cDNA excluding the polyA tail is 779 bp in length, possesses 5' and 3' UTRs shown in bold, 2 start codons in tandem and encodes a polypeptide of 180 amino acids. The underlined sequences within the coding sequences represent the primer sequences utilized in QPCR.

The full-length *PmLEC1* sequence was queried against the TBLASTX database (Altschul et al., 1990). Of the results obtained, all previously characterized full-length LEC1 or LEC1-like sequences, genomic sequences and mRNA sequences that translated to full-length proteins were selected for alignment with the MUSCLE (Edgar, 2004) program available on the European Bioinformatics Institute website. The alignment is shown in Figure 4.

The MUSCLE program (Edgar, 2004) provided the highest quality alignment, with the majority of gaps accommodating the longest sequences, *Triticum* and *Zea*. It is evident from the alignment that the central B domain of the LEC1-type family of proteins is highly conserved across gymnosperms and angiosperms, though not all species contain the 16 characteristic residues identified by Kwong et al., 2003. However, the critical Asp residue necessary for LEC1 function (Kwong et al., 2003) is present in all species. Further the A (N-terminal) and C (C-terminal) domains show substantial conservation among all species analyzed.

2.3.2 Phylogenetic Analyses

Phylogenetic analyses were performed in order to examine the relationship between the putative conifer sequences and the angiosperm LEC1 and LEC1-LIKE sequences. Figure 5 shows the cladogram constructed from the MUSCLE alignment. The conifer sequences were most closely related to each other, and they grouped within the LEC1 clade.

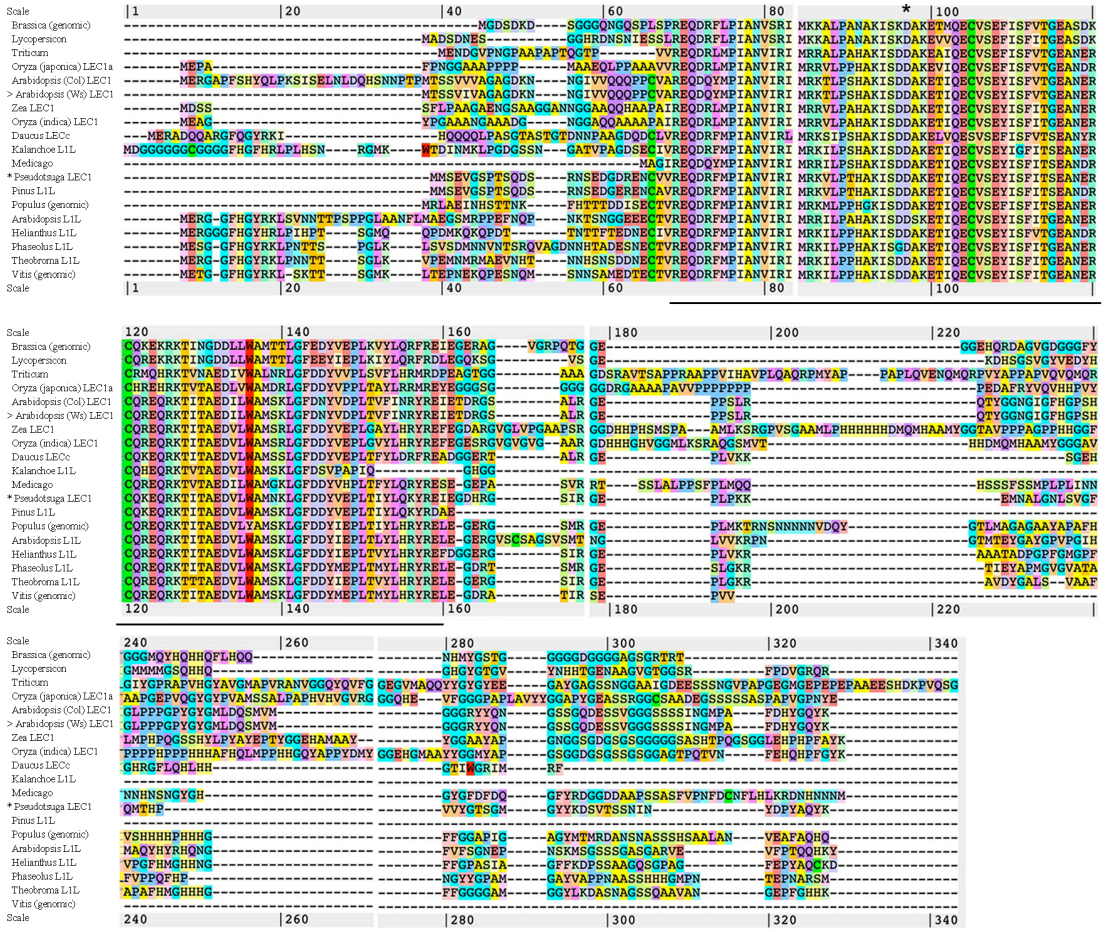


Figure 4. MUSCLE alignment of LEC1 and LEC1-LIKE sequences from plants shows high conservation of the 3 domains.

The A domain is represented by the N-terminal sequences, ending at position 68. The B-domain extends from position 69-158, and is indicated by the black underscore. The C-domain represents the C-terminus. The Asp-55 residue, which is critical for LEC1 function, is indicated by *. *Pseudotsuga LEC1 represents the Douglas-fir sequence. >Arabidopsis (Ws) LEC1 represents the Arabidopsis sequence of the Wassilewskija ecotype.

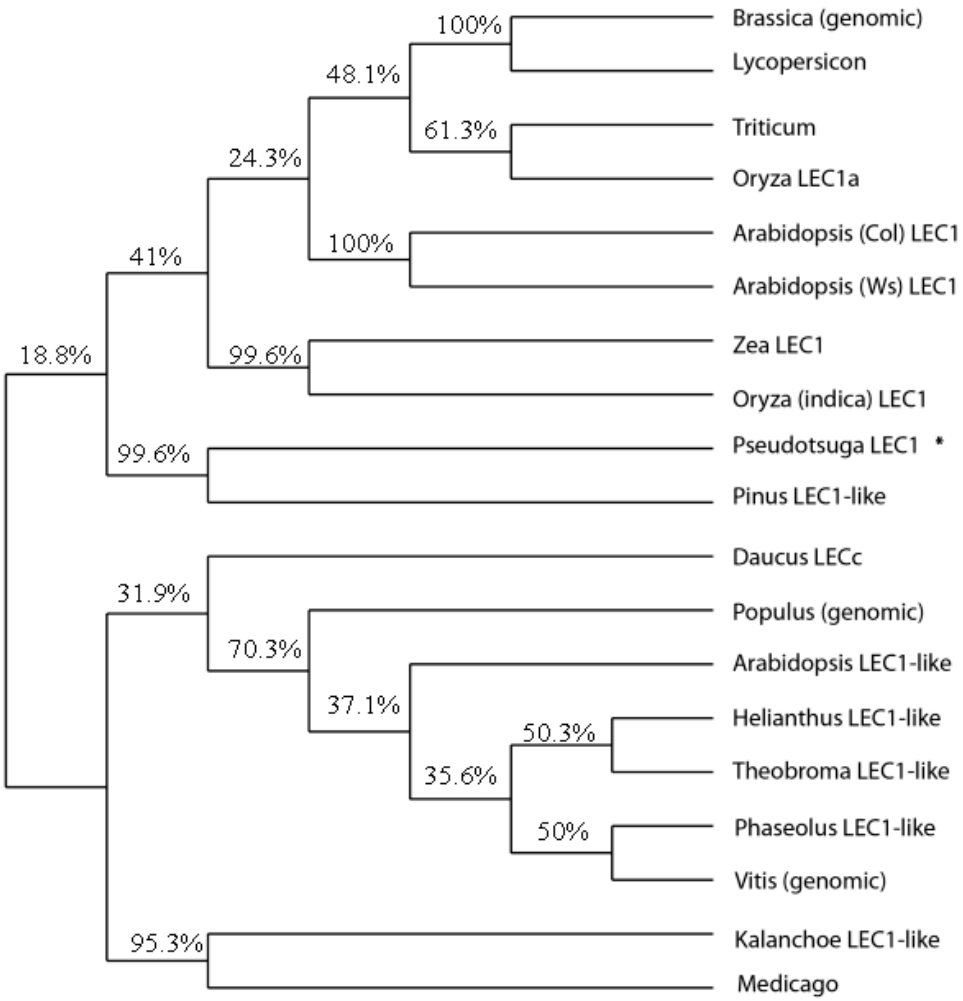


Figure 5. PmLEC1 is more closely related to AtLEC1 than to AtLEC1-LIKE

Evolutionary relationships among LEC1-LIKE sequences deduced from plants were determined *via* the neighbour-joining tree method in Clustal W. Bootstrap values were obtained from 1000 replications and the tree was drawn using TreeView. *, Pseudotsuga LEC1 represents PmLEC1, Arabidopsis (Ws) LEC1 represents AtLEC1, Arabidopsis LEC1-like represents AtLEC1-LIKE.

2.3.3 Promoter Sequence Analyses

To gain insight into the type of signals or medium components that could modulate *PmLEC1* expression and to identify genes that function upstream of PmLEC1, we isolated and analyzed the genomic DNA sequence 1400 bp upstream of the PmLEC1 coding sequence (Figure 6). Prediction programs utilized to locate promoter elements included the SoftBerry NSITE-PL (http://softberry.com), the Neural Network Promoter Prediction Program of the Berkeley Drosophila Genome Project (Reese et al., 1999) to identify the transcriptional start site and TATA box, and the Signal Search program of Plant Cis-Acting Regulatory DNA elements (PLACE) (Higo et al., 1999; Prestridge, 1991). Similar results were obtained with all programs but the outputs were characterized by repetitive sequences representing species-specific variants as well as sequences of diverse species. The SoftBerry Inc. NSITE-PL program recognizes plant regulatory motifs and analyzes regulatory regions and the composition of their functional motifs based on a statistical evaluation of expected number of a nucleotide consensus pattern in a given sequence (http://softberry.com). The search parameters include 0.05 expected mean number, 0.95 statistical significance and 80% homology between known regulatory elements and sequence motifs. The results of the NSITE-PL search are summarized in Table I.

Table I shows the complexity and abundance of regulatory elements that potentially control *PmLEC1* expression. The location of regulatory elements within the promoter and corresponding binding factors are shown in Figure 6. *PmLEC1* has regulatory elements located both upstream and downstream of the transcriptional initiation site (Table I, Figure 4). The initiator sequence was identified *via* a combination of data

obtained with the Neural Network Promoter Prediction Program (Reese et al., 1999) and the 5' UTR sequence. A TATA box was not identified by any of the prediction programs. *PmLEC1* expression appears to be regulated by factors that mediate responses to auxin, abscisic acid, salicylic acid, UV light and developmental cues, osmotic stress, salinity, tissue culture, water stress, wounding and methyl jasmonate. Multiple elements mediating responses to the same cues suggest a combinatorial effect and enhancement of activation. For example, there are 5 elements from which light induced regulation may be directed.

Table 1. Regulatory elements 1400 bp upstream of the *PmLEC1* coding sequence.

Putative cis-acting elements identified using SoftBerry NSITE-PL (http://softberry.com). Locus, location on DNA sequence in bp from initiator sequence (Inr). Gene/Source organism, the gene containing the regulatory element and the original plant from which it was characterized. Regulatory Element and Binding Factor, the name of the regulatory site, the name of the corresponding binding factor in parenthesis, the actual DNA sequence found in Douglas-fir with mismatches from consensus represented by lower case letters, and the DNA strand on which the site is located.

Locus (bp from Inr)	Gene / Source Organism	Regulatory Element and Binding Factor RE(BF)/Sequence/Strand	Function
-79	*H3 Triticum aestivum*	hex3 (ASF1) GcACGCGTGGT (+)	Transcriptional activation in response to salicylic acid and auxin in leaves and protoplasts, but constitutive in roots (Krawczyk et al., 2002).
-72	*HVA1 Hordeum vulgare*	Ce3 (Unknown Nuclear Factor, UNF) ACGCGTGcCCTa (-)	Coupling element, enhances responsiveness to ABA. Necessary for ABRE function (Zhang et al., 2005).
-44	*rbcS2 Lycopersicon escutelum*	C-rich Q (UNF) CaCCTCCTC (+)	Light-induced and/or developmental regulation (Manzara et al., 1993a).
+30	*rab28 Zea mays*	ABRE GRA (ABF) CATGCaGCC (-)	ABA and water stress induction (Busk et al., 1997).
+104	TDC Catharantus roseus	GT-1#Box7 (GT-1) AAgAAGTAcAAA (+)	Induces expression in response to UV light (Ouwerbeck et al., 1999).
+123	*DcPAL1 Daucus carota*	box-L2 (DcMYB1) ACTgACCTTtGT (-)	Transcriptional activation in response to abiotic stresses, i.e., UV light and osmotica (Maeda & Ozeki, 2006)
+453	*FIL Arabidopsis thaliana*	12 bp element (UNF) AACGGccGAATG (-)	Repression of transcription on adaxial side of leaf primordia (Watanabe & Okada, 2003).
+732	*MsPRP2 Medicago sativa*	Alfin1 BS4 (Alfin1) CAAGTGcTGCTa (-)	Root- and callus-specific regulatory element, responsive to salt. Alfin1 is essential for root growth and inducible by salt (Winicov et al., 2004).
+789	*rbcS3 Lycopersicon escutelum*	15 Z (UNF) AAaATGAGGaTTtCT (-)	Cotyledon-specific regulatory element, light-induced or developmentally regulated (Manzara et al., 1993).
+872 +888	*DTA4 Arabidopsis thaliana*	CarG3 (AGL15) TTACTtTATATcGgAA (+) CarG3 (AGL15) TTcCgATATAaAGTAA (-)	AGL15, embryo-specific transcriptional activator, preferentially binds to the CarG3 motif with the longer central AT-rich sequence (Tang

			& Perry, 2003).
+1027	*napA* *Brassica napus*	ABRE BD (ABI3) GtCACTTGTC (+)	ABI3 is a seed-specific transcription factor that mediates response to ABA (Ezcurra et al., 2000).
+1062	*DTA4* *Arabidopsis thaliana*	CarG2 (AGL15) ATGCCTAAtTa (-)	Embryo-specific activation *via* classical MADS box motif. AGL15 is the only MADS box protein found in embryonic tissue (Tang & Perry, 2003).
+1090	*C1* *Zea mays*	Sph-core (VP1) TCCATGCAT (-)	VP1 is a transcriptional activator that mediates ABA response (Suzuki et al., 2005).
+1092	*C1* *Zea mays*	VP1 RE (UNF) CtTCCATGCAT (-)	VP1 is a transcriptional activator that mediates ABA response (Suzuki et al., 2005).
+1134	*LTR-Ttol* *Nicotiana tabacum*	13 bp box (UNF) TcGTAGGTtAGAT (+)	Mediates responsiveness to tissue culture, wounding and methyl jasmonate (Takeda et al., 1999).
+1152	*RbcS-3.6* Lycopersicon escutelum	Box I (GBF) TTTCAATC (-)	Reference could not be found.
+1183	*GapB* *Arabidopsis thaliana*	AE box 1 (AEF) AGAAACTT (+)	Enhances responsiveness to light (Park et al., 1996).
+1283	*A-p40* *Arabidopsis thaliana* (also, *rp L9* *Pisum sativum*)	Inverted telo box (UNF) TTAGGGTTT (+)	Activation of gene expression in root cells and meristematic cells, especially if downstream of Inr (Tremousaygue et al., 1999).
+1293	*A1 EF-1alpha* *Arabidopsis thaliana*	Telo box (UNF) TAAACCCTAA (-)	Activation of gene expression in root cells and meristematic cells, especially if downstream of Inr (Tremousaygue et al., 1999).

When the newly isolated upstream sequence was aligned with the cDNA sequence, a 5' UTR intron became evident (Figure 3), adding another level of complexity to the regulatory function of the promoter region. 5' UTR introns are known to enhance RNA levels as well as protein expression *via* intron mediated enhancement (Chung et al., 2006). During splicing, a complex of proteins are deposited on the mRNA nucleotides at

a position that is 20 - 24 upstream of the exon – exon junction to form exon junction complexes (Hir et al., 2003; Chung et al., 2006). These complexes increase the affinity of mRNA to ribosomes and the resulting enhancement in translation may be as much as a 1000-fold increase in protein accumulation (Nott et al., 2003; Chung et al., 2006). Normally, 5' UTR introns are located 80-300 nt from the beginning of the 5' UTR and 1-40 nt away from the start codon, and are usually longer than introns within coding sequences (Chung et al., 2006). The *PmLEC1* 5' UTR fits the above description, with splice sites located at 123 nt from the beginning of the UTR and 30 nt upstream of the start codon. In addition, the intron is 1176 nt in length and Chung et al. (2006) have noted the possibility that large 5' UTR introns function as spacers within the genome, which provide an AT-rich region between the coding sequence and the genome. AT-rich stimulatory elements (Chung et al., 2006) could also play a role in regulation of *PmLEC1* expression.

Recently, intronic enhancers have been shown to result in synergistic activation of gene transcription while removal of the intron resulted in inactivation of gene expression (Finkbeiner, 2001). The presence of this 5' UTR intron is an unexpected clue to elucidating the regulatory mechanism governing *PmLEC1* expression.

The transcription factors that have well-characterized functions and appear to function upstream of *PmLEC1* are ABF and Ce3, ABI3, VP1, AGL15 and VAL. These are seed-specific transcription factors in angiosperms, and therefore it is expected that orthologues of these genes will be found in conifers. The presence of regulatory elements for these factors in the Douglas-fir *PmLEC1* promoter supports the theory that conifer

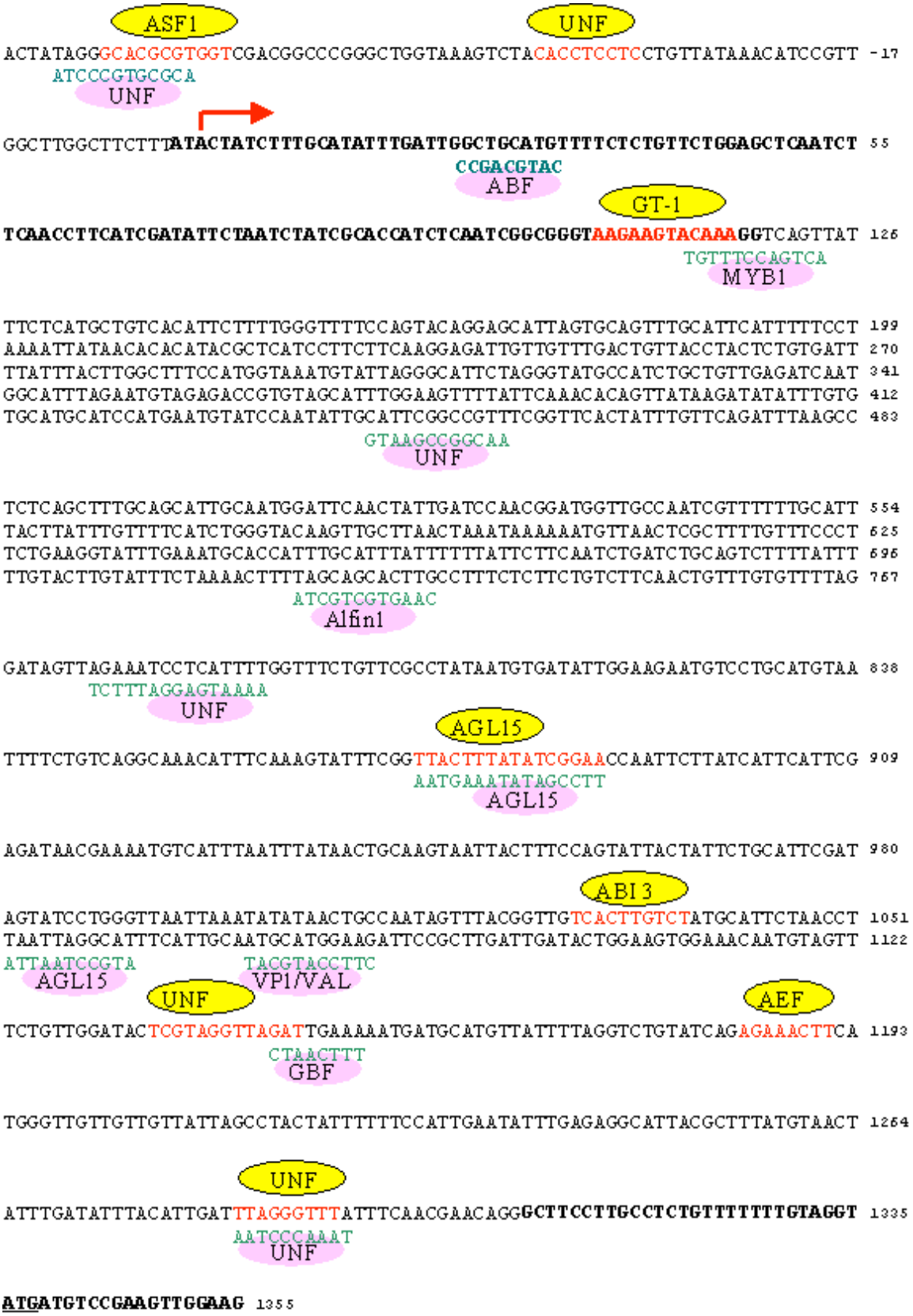

ASF1
UNF
ACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTAAAGTCTACACCTCCTCCTGTTATAAACATCCGTT -17
ATCCCGTGCGCA
UNF
GGCTTGGCTTCTTTATACTATCTTTGCATATTTGATTGGCTGCATGTTTTCTCTGTTCTGGAGCTCAATCT 55
CCGACGTAC
ABF
GT-1
TCAACCTTCATCGATATTCTAATCTATCGCACCATCTCAATCGGCGGGTAAGAAGTACAAAGGTCAGTTAT 126
TGTTTCCAGTCA
MYB1
TTCTCATGCTGTCACATTCTTTTGGGTTTTCCAGTACAGGAGCATTAGTGCAGTTTGCATTCATTTTTCCT 199
AAAATTATAACACACATACGCTCATCCTTCTTCAAGGAGATTGTTGTTTGACTGTTACCTACTCTGTGATT 270
TTATTTACTTGGCTTTCCATGGTAAATGTATTAGGGCATTCTAGGGTATGCCATCTGCTGTTGAGATCAAT 341
GGCATTTAGAATGTAGAGACCGTGTAGCATTTGGAAGTTTTATTCAAACACAGTTATAAGATATATTTGTG 412
TGCATGCATCCATGAATGTATCCAATATTGCATTCGGCCGTTTCGGTTCACTATTTGTTCAGATTTAAGCC 483
GTAAGCCGGCAA
UNF
TCTCAGCTTTGCAGCATTGCAATGGATTCAACTATTGATCCAACGGATGGTTGCCAATCGTTTTTTGCATT 554
TACTTATTTGTTTTCATCTGGGTACAAGTTGCTTAACTAAATAAAAAATGTTAACTCGCTTTTGTTTCCCT 625
TCTGAAGGTATTTGAAATGCACCATTTGCATTTATTTTTTATTCTTCAATCTGATCTGCAGTCTTTTATTT 696
TTGTACTTGTATTTCTAAAACTTTTAGCAGCACTTGCCTTTCTCTTCTGTCTTCAACTGTTTGTGTTTTAG 767
ATCGTCGTGAAC
Alfin1
GATAGTTAGAAATCCTCATTTTGGTTTCTGTTCGCCTATAATGTGATATTGGAAGAATGTCCTGCATGTAA 838
TCTTTAGGAGTAAAA
UNF
AGL15
TTTTCTGTCAGGCAAACATTTCAAAGTATTTCGGTTACTTTATATCGGAACCAATTCTTATCATTCATTCG 909
AATGAAATATAGCCTT
AGL15
AGATAACGAAAATGTCATTTAATTTATAACTGCAAGTAATTACTTTCCAGTATTACTATTCTGCATTCGAT 980
ABI3
AGTATCCTGGGTTAATTAAATATATAACTGCCAATAGTTTACGGTTGTCACTTGTCTATGCATTCTAACCT 1051
TAATTAGGCATTTCATTGCAATGCATGGAAGATTCCGCTTGATTGATACTGGAAGTGGAAACAATGTAGTT 1122
ATTAATCCGTA
AGL15
TACGTACCTTC
VP1/VAL
UNF
AEF
TCTGTTGGATACTCGTAGGTTAGATTGAAAAATGATGCATGTTATTTTAGGTCTGTATCAGAGAAACTTCA 1193
CTAACTTT
GBF
TGGGTTGTTGTTGTTATTAGCCTACTATTTTTTCCATTGAATATTTGAGAGGCATTACGCTTTATGTAACT 1264
UNF
ATTTGATATTTACATTGATTTAGGGTTTATTTCAACGAACAGGGCTTCCTTGCCTCTGTTTTTTTGTAGGT 1335
AATCCCAAAT
UNF
ATGATGTCCGAAGTTGGAAG 1355

Figure 6. The Douglas-fir *PmLEC1* promoter region contains regulatory elements for transcriptional activators known from angiosperms

Transcriptional start site, indicated by the red arrow, determined with the Neural Network Promoter Prediction version 2.2 (Reese, 2001) and the 5'-UTR of the cDNA sequence. The cDNA sequence is represented in bold letters. Promoter elements (sequences in red = elements on coding strand, sequences in green = elements on template strand) and corresponding names of binding factors known from angiosperms. UNF, unknown nuclear factor. The start codon is underlined.

embryogenesis has many parallels with angiosperm embryogenesis, thus candidate gene characterization is likely to lead to rapid advances in SE technology. The body of knowledge that is available for angiosperms may be applied to gymnosperms once the genes are isolated.

2.3.4 *PmLEC1* mRNA is Highly Abundant During Early Embryogenesis

To establish whether *PmLEC1* is an embryo-specific gene, the steady-state transcript levels were analyzed during zygotic seed development and vegetative growth in Douglas-fir. Initial data acquired from Northern analyses (Figure 7) showed that *PmLEC1* is expressed during early embryogenesis in both somatic embryos and zygotic developing seed. A single, strong band of approximately 1.2 kb was observed. The cDNA sequence is 779 bp and this difference in size may be explained by a lengthy polyA tail that was not captured during cloning and sequencing. *PmLEC1* expression was not observed in mature tissues such as, seedling roots, stems or needles. To quantify and confirm these results with a more sensitive method, QPCR was performed with Douglas-fir *L8* ribosomal protein encoding mRNA as the normalizer (Appendices A and B, Figure 8).

The samples utilized in Northern analysis could not be utilized in QPCR since 5 individual samples were required for each stage. Therefore, a new set of samples were collected the following year analyzed by QPCR.

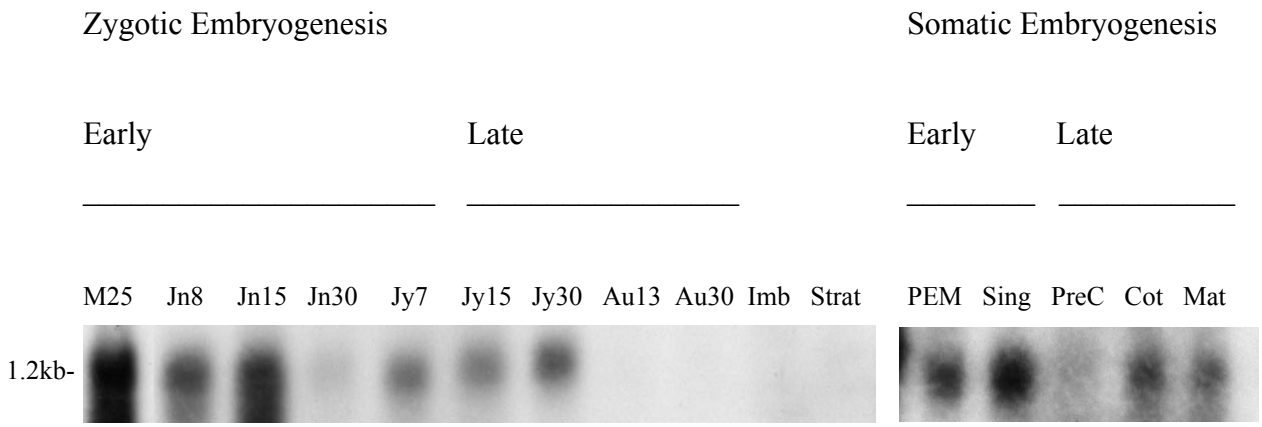


Figure 7. PmLEC1 is expressed during early zygotic and somatic embryogenesis

Total RNA (20μg) was isolated from the indicated stages of embryogenesis by TRIzol (Invitrogen): May 25 (M25), June 8 (Jn8), June 1(Jn15), June 30 (Jn30), July 7 (Jy7), July 15 (Jy15), July 30 (Jy30), August 13 (Au 13), August 30 (Au30), imbibed seed (Imb), stratified seed (Strat), pro-embryogenic masses (PEM), singulated somatic embryos (Sing), pre-cotyledonary somatic embryos (PreC), cotyledonary somatic embryos (Cot), mature somatic embryos (Mat). The RNA was separated by formaldehyde gel electrophoresis and transferred to a Bio-dyne nylon membrane. The blot was hybridized with ^{32}P-labeled PmLEC1 cDNA. A single band was visible at approximately 1.2 kb. This size is larger than the 779 bp cDNA and may be attributed to a longer polyA tail than was captured by cloning and sequencing.

Based on QPCR, the highest expression was observed during early embryogenesis, which encompasses the period between May 24 and July 11 (Figure 8). The unfertilized ovule, represented by May 24, showed the highest expression of *PmLEC1*. Expression remained significantly high until August 14, marking late embryogenesis and the beginning of dormancy, at which time a reduction of two orders of magnitude of *PmLEC1* occurred. In mature tissues, PmLEC1 expression was extremely low or non-detectable. Trace amounts were observed in 1.5-month-old seedlings and the youngest

pollen cones and vegetative buds. No *PmLEC1* expression was observed in the 3-month-old seedlings, some of the 1.5-month-old seedlings, pollen cones >2 mm in diameter, and vegetative buds >3 mm in diameter. This is not evident in Figure 8 because the chart is based on pooled data. However, these results are consistent with the theory that juvenile tissues have embryogenic potential and delineate the approximate size or age of tissues exhibiting *PmLEC1* expression. From August 30 onward, *PmLEC1* expression was 3 orders of magnitude lower than during early embryogenesis. Somatic embryo samples were not analyzed by QPCR.

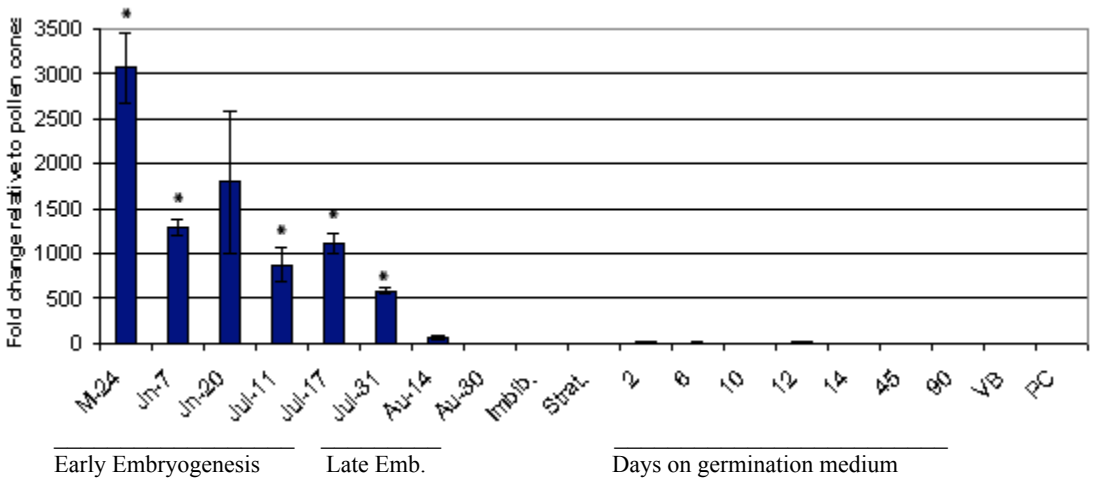


Figure 8. Expression of PmLEC1 in embryonic and vegetative tissues of Douglas-fir

Total RNA was isolated by TRIzol or the hot phenol extraction method, from the indicated developmental stages. VB, vegetative buds; PC, pollen cones. DNaseI treatment and reverse transcription was carried out with 5 samples of 1μg from each stage. The QPCR reactions were carried out in quadruplicate and Douglas-fir ribosomal *L8* transcripts served as the internal control. Expression was determined by quantitative real-time RT-PCR with calculations according to the ΔΔCt method (Dorak, 2006). Relative expression was based on comparison to transcript levels in pollen cones, which showed the lowest expression. Statistical analysis was performed on the pooled groups (n=5) by the Mann-Whitney *U* test. Statistical significance is indicated by * $p<0.05$ and error bars represent standard error of the mean.

2.3.5 PmLEC1 Protein Expression Profile

To further understand the expression of PmLEC1 and to relate transcript levels to protein accumulation, antibodies were raised against a synthetic PmLEC1 peptide, consisting of the first 18 amino acids of the N-terminus and attached to the KLH carrier protein *via* a cysteine residue. Previous to this, a PmLEC1 fusion protein with a histidine tag was cloned for over-expression in *Saccharomyces cerevisiae* but repeated attempts to purify this protein *via* a Ni^{2+} column and to optimize induction conditions yielded very little protein. A bacterial expression system was not attempted due to past work showing very little success with plant protein expression.

Various dilutions of mouse polyclonal immune serum were tested by ELISA against the free peptide as well as Douglas-fir developing seed extracts. The polyclonal serum showed a significant response against both the peptide and the cellular proteins. However, there was a discrepancy between RNA expression and protein expression. Whereas *PmLEC1* transcripts were most abundant during early embryogenesis and almost non-existent during late embryogenesis, protein accumulation peaked during both early and late embryogenesis (May 24 and Aug. 30) with an increase in intensity during the late stage (Appendix C).

To test the specificity of the antibodies and to determine whether they truly recognized the PmLEC1 antigen, total proteins were isolated from developing Douglas-fir seed, transgenic *Arabidopsis* plants expressing PmLEC1 (wt^{PmLEC1} = wtP, $lec1\text{-}1^{PmLEC1}$ = lecP), transgenic *Arabidopsis* expressing AtLEC1 (lecA) and untransformed plants (wt) (Figure 9A). The reactivity of the polyclonal serum was assessed (Figures 9B, 11, 13) and compared to normal mouse serum (Figure 9C) by Western blotting. In Douglas-fir

tissues, immunoreactive bands were observed at 59 kDA and 36 kDa, while a strong immunoreactive band was observed at 32 kDa in transgenic *Arabidopsis* expressing PmLEC1 (Figures 9B, 11, 13). Very weakly reactive bands were apparent in wild type *Arabidopsis* and in transgenic plants expressing AtLEC1 (Figures 9B and 11). Because the expected size of PmLEC1 is 21 kDa, the discrepancy in size required additional analyses. However, very little background reactivity was seen with normal mouse serum indicating that immunoreactive bands were the result of antibodies recognizing PmLEC1, KLH epitopes or the maleimide linker epitope.

Purified PmLEC1 is not available and peptide inhibition assays with free peptide, peptide bound to KLH and peptide bound to BSA did not yield interpretable results. The ability of the antibodies to recognize PmLEC1 was deduced by comparison with HAP3 proteins purified from other species. The HAP3 subunit is also known as CBF-A or NF-YB. The CCAAT-binding heterotrimeric complex, NF-Y, is composed of HAP2, HAP3 and HAP5. HAP2 migrates to the nucleus rapidly and on its own while HAP3 and HAP5 dimerize prior to migration to the nucleus and their transport is much slower (Kahle et al., 2005).

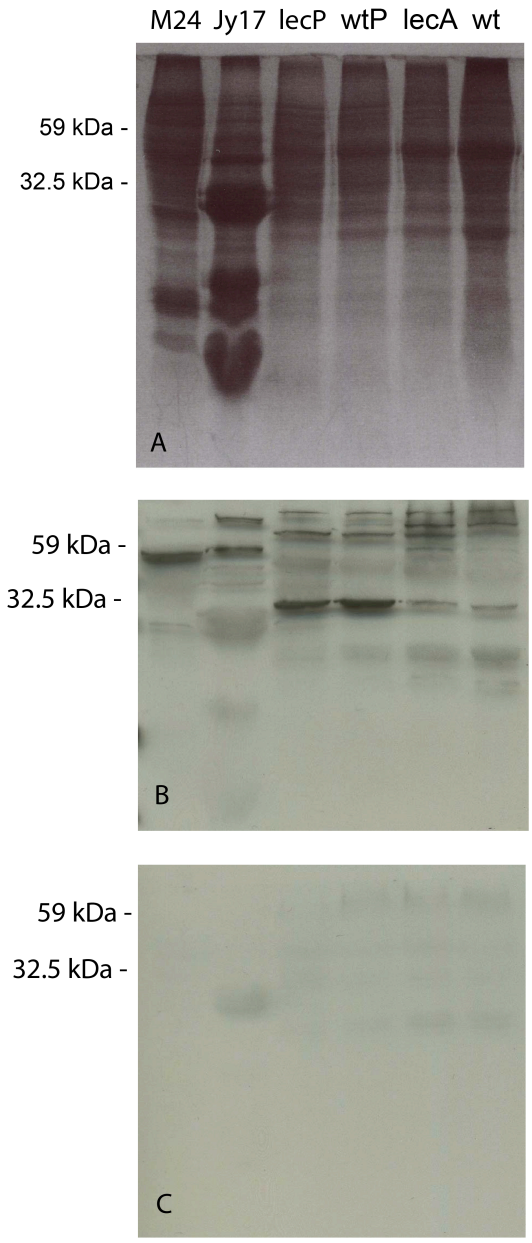


Figure 9. Evaluation of PmLEC1 antiserum by Western blot analysis.

A, Coomassie blue stained gel of total proteins were isolated from developing Douglas-fir seed harvested on May 24 (M24) and July 17 (Jy17); *lec1-1* null mutant expressing PmLEC1 (lecP), wild type expressing PmLEC1 (wtP), lec1-1 null mutant expressing AtLEC1 (lecA), all represent second generation transgenic *Arabidopsis* plants; untransformed wild type *Arabidopsis* (wt). Aliquots of 20 μg were separated on a 14% SDS-PAGE gel and transferred to a PVDF membrane.
B, Western blot analysis of protein extracts shown in A. The PVDF membrane was incubated with polyclonal anti-PmLEC1 serum diluted 1:1000 for 1 h.
C, Western blot analysis of proteins shown in A with pre-immune mouse serum diluted 1:1000 for 1 h.

To further clarify the relevance of the weakly reactive bands seen in the lecA and wt lanes (Figure 9B), the first 18 amino acids of PmLEC1 and AtLEC1 protein sequences were compared and this revealed 5 identical amino acids located in the same position in both peptides (Figure 10). Because this may lead to cross-reactivity of the antiserum with AtLEC1, a band would be expected in wild type *Arabidopsis* but not in the *lec1-1* null mutant, in which the *LEC1* gene is deleted. An additional immunoblot demonstrated that the PmLEC1 antiserum also reacted weakly with a species in the *lec1-1* null mutant (lec in Figure 11), thereby excluding the possibility of cross-reactivity with AtLEC1 (Figure 11). The reactive species is likely the result of non-specific binding of antibodies and it is an unfortunate co-incidence. In Figure 11, the size of this reactive species appears to be slightly larger than PmLEC1. At this time, the intensity of the signal is the most reassuring indicator that PmLEC1 is being detected.

PmLEC1: MMSEV GSPTS QDSRN SED
AtLEC1: MTSSV IVAGA GDKNN GIV

Figure 10. Comparison of PmLEC1 and AtLEC1 N-terminal sequences reveals 5 identical residues.

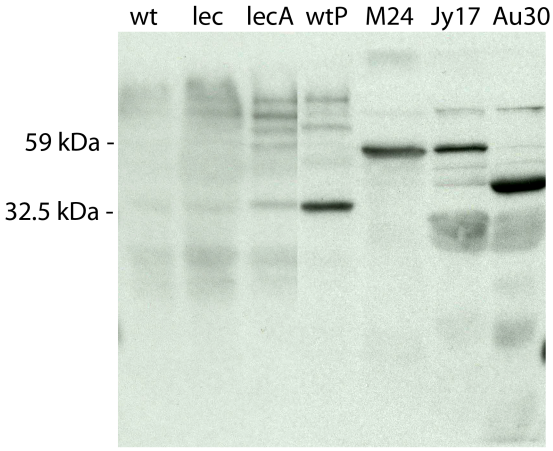


Figure 11. Polyclonal antiserum is specific for PmLEC1 and does not cross-react with AtLEC1.

Total proteins (20 μg) from wild type Arabidopsis (wt), lec1-1 null mutant (lec), second generation transgenic *lec1-1* null mutant plants transformed with *AtLEC1* (lecA), second generation wild type plants transformed with *PmLEC1* (wtP), developing Douglas-fir seed harvested on May 24 (M24), July 17 (Jy17) and August 30 (Au30) were separated by SDS-PAGE and transferred to a PVDF membrane. The PVDF membrane was incubated with polyclonal anti-PmLEC1 serum diluted 1:1000 for 1 h.

In the lanes representing Douglas-fir zygotic development in Figures 9 and 11, the PmLEC1 antiserum reacted with a MW species of 59 kDa (May 24 and July 17 seed) and 36 kDa (Aug. 30 seed). Further insight into the mobility difference may be gained by comparison of the amino acid composition of AtLEC1 and PmLEC1. AtLEC1 contains less charged/polar residues than PmLEC1, 39% and 47.3%, respectively. This difference could be related to the difference in seed environment between the 2 species leading to sequence diversity and/or to variability in post-translational modifications. Because AtLEC1 has a more hydrophobic character there is a possibility that the *Arabidopsis* seed environment is more hydrophobic and that post-translational modifications in

Arabidopsis will reflect this tendency. This is supported by the lower MW reactive species (34 kDa) observed in transgenic *Arabidopsis* expressing PmLEC1.

To better understand the change in apparent molecular size, a closer look at the tissue characteristics of developing Douglas-fir seeds is helpful (Figure 12). Prior to fertilization and during early embryogenesis, or until July 4th in Figure 12, the developing seed is very aqueous. As storage compounds start accumulating, the seed tissue becomes more hydrophobic and desiccated. It is expected that proteins need to be differentially modified in order to remain functional in both environments. A radical change in protein profile is observed between June 20 seed and July 17 seed (Figure 13, top panel). At the same time (July 17) 3 bands of almost equal intensity are visible in the immunoblot, suggesting a transition between the 2 dominant MW forms.

In combination with the possibly even more hydrophobic environment within *Arabidopsis* seeds, it is conceivable that PmLEC1 migrates with the highest MW mobility in the aqueous environment of the ovule (59 kDa), a lower MW mobility in maturing Douglas-fir seed (36 kDa), and the lowest mobility in the transgenic *Arabidopsis* plant (34 kDa). The transition from an aqueous environment to a hydrophobic environment represents a decrease in charge and hydrophilicity and results in a decrease of apparent molecular weight. It is also possible that the PmLEC1 protein is cleaved. The other very high MW but weakly reactive species seen in the lanes representing developing Douglas-fir seed, could be the result of the polyclonal serum binding to random epitopes that resemble KLH or the maleimide linker, but it is very likely that this serum specifically recognizes PmLEC1.

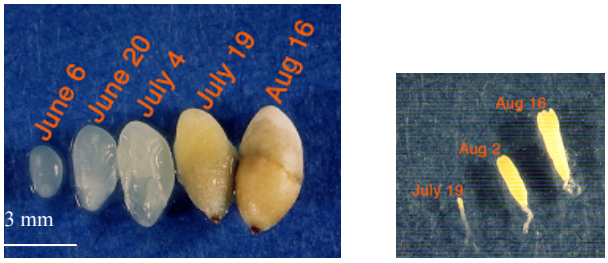


Figure 12. Developing Douglas-fir seed and corresponding embryos show transition from aqueous fertilized ovules to hydrophobic desiccated seed.

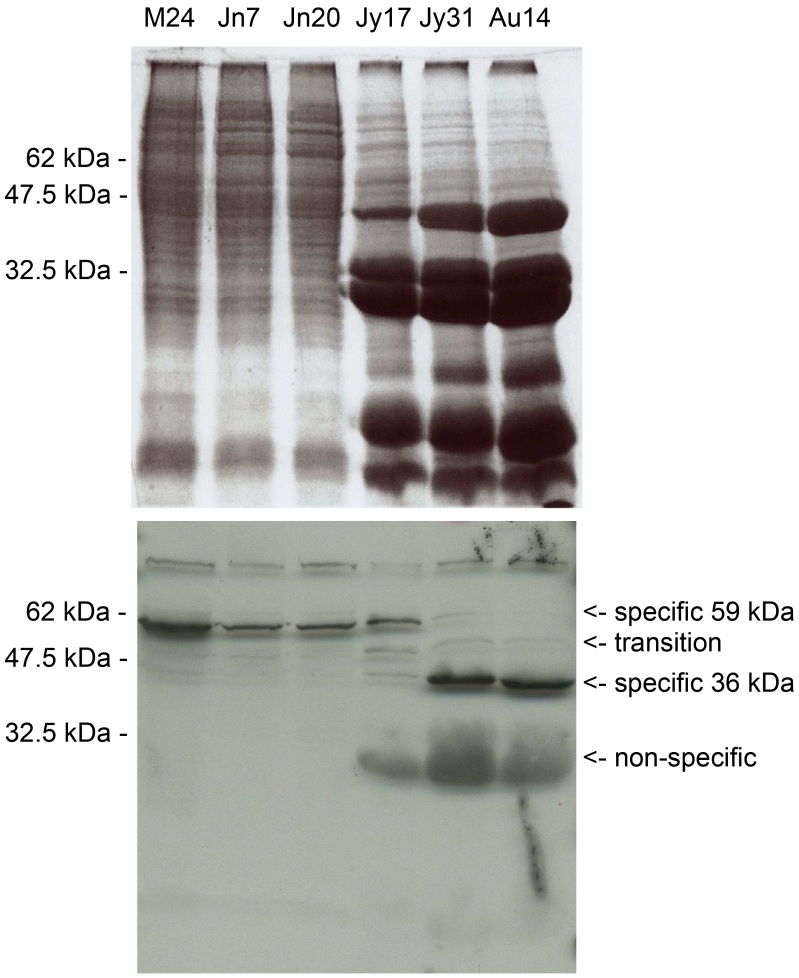


Figure 13. PmLEC1 antiserum detects 2 major molecular species during Douglas-fir zygotic embryogenesis.

Top panel, Coomassie blue stained gel of total proteins isolated from developing Douglas-fir seeds on days indicated. Bottom panel, A 59 kDa form is dominant during early embryogenesis (May 24 – June 20). July 17 represents a transition state. A 36 kDa form is dominant during late embryogenesis (July 31 – August 14).

To further characterize the PmLEC1 protein profile, total proteins were isolated from all stages of zygotic embryogenesis; imbibed, stratified and germinating seed; 1.5- and 3-month-old seedlings, vegetative buds and pollen cones, and Western blotting was carried out as before. During the stages of early zygotic embryo development, the polyclonal immune serum reacted with a higher MW form of 59 kDa (Figure 14). Once again, a transition state was apparent on July 17 (Figure 14). From July 31 until 10 days after exposure to germination conditions, a lower MW form, 36 kDa, was recognized, yet there were no reactive bands in the stratified seed lane (Strat in Figure 14). At 12 and 14 days after exposure to germination conditions, the higher MW form re-appeared. No protein expression was observed in 1.5- and 3-month-old seedlings, vegetative buds or pollen cones (Figure 14).

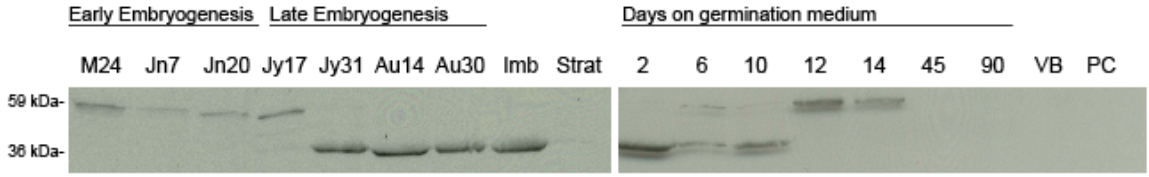


Figure 14. Western blot analysis of PmLEC1 protein during embryonic and vegetative growth

Total cellular proteins were isolated from the indicated stages of development, 20 μg were separated on 14% SDS-polyacrylamide gels and transferred to PVDF membranes. PmLEC1 was detected by incubation with polyclonal antiserum against PmLEC1 synthetic peptide diluted 1:1000, followed by incubation with peroxidase-conjugated anti-mouse IgG diluted 1:100,000, and chemiluminescent detection with the ECL System (Amersham Bioscience). Developing zygotic seed, collected on May 24 (M24), June 7 (Jn7), June 20 (Jn 20), July 17 (Jy17), July 31 (Jy31), August 14 (Au14), and August 30 (Au30). Imb, imbibed seed; Strat, stratified seed. Imbibed, stratified seed were incubated on germination medium and collected at 2, 6, 10, 12, 14, 45 and 90 days. VB, vegetative buds; PC, pollen cones.

2.3.6 *PmLEC1* RNA Expression Is Enhanced by Stress and Hormone Treatments

To assess whether *PmLEC1* expression could be induced in mature, vegetative tissues and to identify potential elicitors of *PmLEC1*, we employed a variety of stress and hormone treatments on mature seeds (Figures15 - 20). Three sets of hormones known to be important in SE protocols were used. SE induction is normally achieved in the presence of 2,4-D/BAP. Embryo development and maturation is promoted by a combination of GA/ABA. Brassinosteroids, the steroidal lactones initially identified in *Brassica*, are a new class of plant growth regulators, which were found to play a role in embryogenesis and demonstrated the ability to improve SE initiation rates in conifers and rice (Pullman et al., 2003). Plasmolysing stress in the form of sorbitol, mannitol and sucrose facilitate the induction of SE in angiosperms and are also medium components in conifer SE protocols. Additional treatments relevant to SE are PEG, a non-plasmolysing stress, salinity and heavy metals (i.e., $CdCl_2$).

The Douglas-fir heat shock protein *HSP18.1A* was utilized as a calibrator of the treatments because it was shown to be responsive to hormonal and heavy metal stress treatments (Kaukinen et al., 1996). Unexpectedly, *HSP18.1A* was only effective in some of the cases showing a discrepancy between results of Northern analyses and QPCR. For example, $CdCl_2$ was expected to result in a low but significant HSP response, yet in this work the response was pronounced. The GA/ABA combination was expected to show a strong response but this was not observed. This was the first time that such work was attempted, the low number of replicates complicates analysis and further assessment of this calibrator is necessary.

In this work, each treatment group consisted of 5 individual seed and transcript levels were measured by quantitative real-time PCR. When the results were analyzed by group, the differences were not statistically significant even though a response to treatment was evident. For this reason, the individual seed responses were analyzed. Seed that did not show a response compared to baseline seed were removed from the analysis under the assumption that approximately 40% were not viable and would not be expected to respond. This manipulation is supported by the fact that seeds drawn from the same batch as those for stress and hormone treatment, germinated and developed into seedlings at a rate of ~ 60%. Therefore, ~ 40% of seed were not viable.

Figure 16 shows that stratified seed incubated for 24 h on filter paper soaked with hormone solutions resulted in an up-regulation of *PmLEC1* only in the presence of the brassinosteroid 2,4-epibrassinolide (10 µM) (Brassinol, Figure 16). Surprisingly, the combinations of 2,4-D/BAP (110 mg/l 2,4-D and 45 mg/l BAP) and GA/ABA (2.5 mg/l GA and 10 mg/l ABA) had no effect on *PmLEC1* expression, although the change in HSP levels indicated that all treatments were perceived. The plant growth regulators 2,4-D/BAP are known to induce cell division and are necessary for the induction of embryogenesis. However, incubation on induction medium requires 4-6 weeks.

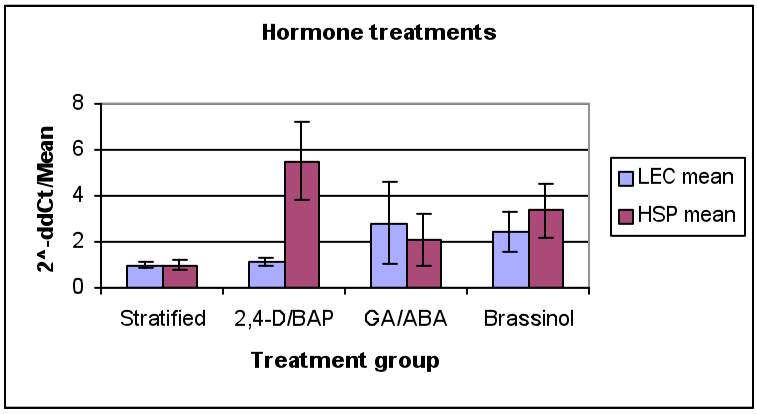


Figure 15. The effect of 24 h hormone treatments on PmLEC1 and HSP18.1A expression. RNA was isolated from Douglas-fir stratified seed (control) and stratified seed incubated for 24 h in the indicated solutions. Each group consisted of 5 seed. *PmLEC1* and *HSP* transcript levels were analyzed by QPCR in quadruplicate reactions. Statistical analyses were done on pooled groups (n=5). None of the treatments were statistically significant with respect to PmLEC1, by the Mann-Whitney U test. The HSP response to 2,4-D/BAP was statistically significant. Error bars represent standard error of the mean.

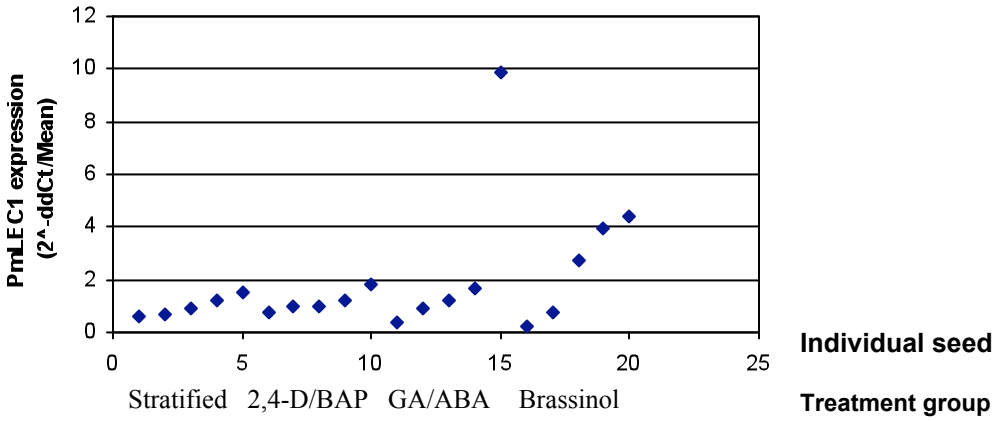


Figure 16. Individual responses of seed to 24 h hormone treatments indicate that the brassinosteroid 2,4-epibrasssinolide up-regulates PmLEC1 expression in stratified seed

Each diamond represents an individual seed from the QPCR analyses in Figure 15. The batch of seed showed 60% germination and therefore the 2 lowest-responding seed were removed per treatment group prior to statistical analysis. When the Mann Whitney U test was performed with the 3 top responders from each treatment group (m=3) and 3 control seed (n=3), 2,4-epibrassinolide was statistically significant. brassinosteroid 2,4-Brassinolide (10 µM) (Brassinol).

Of the plasmolysing osmotic stresses in the form of carbohydrates, 0.7 M mannitol and 0.7 M sorbitol up-regulated *PmLEC1* expression in stratified seed while 0.7 M sucrose did not have a significant effect. The stress treatments, 0.6 mM $CdCl_2$ (heavy metal), 190 g/l PEG 8000 (non-plasmolysing osmotic) were perceived as stresses by the seed. However, only 0.3 M NaCl treatment shows potential for direct induction of *PmLEC1* expression. These results are consistent with the information obtained from the promoter/5' UTR intron sequence and regulatory elements (Alfin1 BS4).

When statistical analysis (Mann Whitney U test) were carried out with responding seed only, the results were significant for 2,4-epibrassinolide, sorbitol and mannitol. In the NaCl group, there were only 2 responders and this is below the minimum number required for statistical significance. However, from a biological perspective, the response is very strong and worthy of further investigation.

Although *PmLEC1* expression was not up-regulated in response to the other treatments, these conditions should not be excluded from further investigations with longer incubation times or with combinations of stresses and hormones. Judging by the variety and multitude of promoter elements, it is likely that *PmLEC1* expression may be modulated by different elicitors as a function of developmental stage. It is possible that during early development, *PmLEC1* is induced by auxins and abiotic stresses while later in development, signal molecules and brassinosteroids play an important role. Sorbitol, mannitol and NaCl are all abiotic stresses and the present work suggests that they will be useful in inducing *LEC1* expression in mature tissues. Also, it would be interesting to see the response of PmLEC1 to AGPs because the addition of AGPs to media led to the

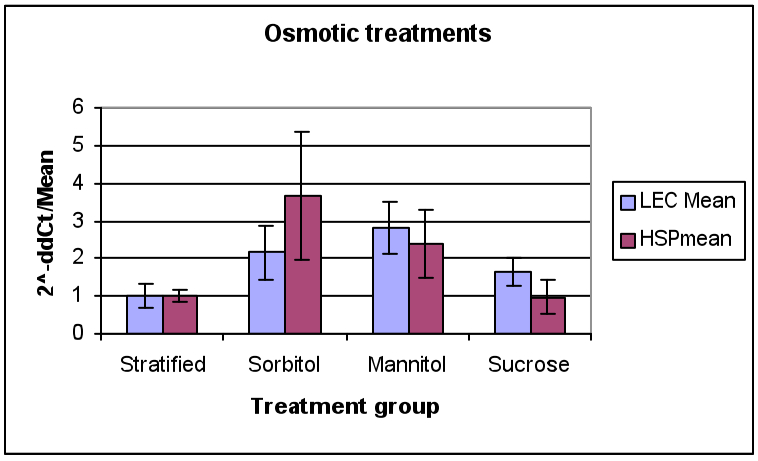


Figure 17. The effect of 24 h osmotic treatments on *PmLEC1* and *HSP18.1A* expression

RNA was isolated from Douglas-fir stratified seed (control) and stratified seed incubated for 24 h in the indicated solutions. Each group consisted of 5 seed. *PmLEC1* and *HSP* transcript levels were analyzed by QPCR in quadruplicate reactions. Statistical analyses were done on pooled groups (n=5, except in sucrose n=4). None of the treatments were statistically significant with respect to *PmLEC1* or *HSP*, by the Mann-Whitney U test. Error bars represent standard error of the mean.

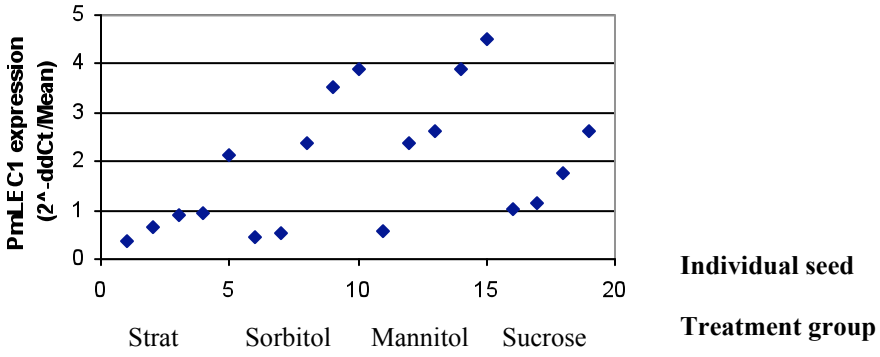


Figure 18. Individual responses of seed to 24 h osmotic treatments indicate that sorbitol and mannitol up-regulate *PmLEC1* expression in stratified seed

Each diamond represents an individual seed from the QPCR analyses in Figure 17. The batch of seed showed 60% germination and non-responding seed, 2 per treatment group were removed from the analysis, except in sucrose where 1 was removed. When the Mann Whitney U test was performed with the 3 top responders from each treatment group (m=3) and 3 control seed (n=3), the responses to sorbitol and mannitol were statistically significant.

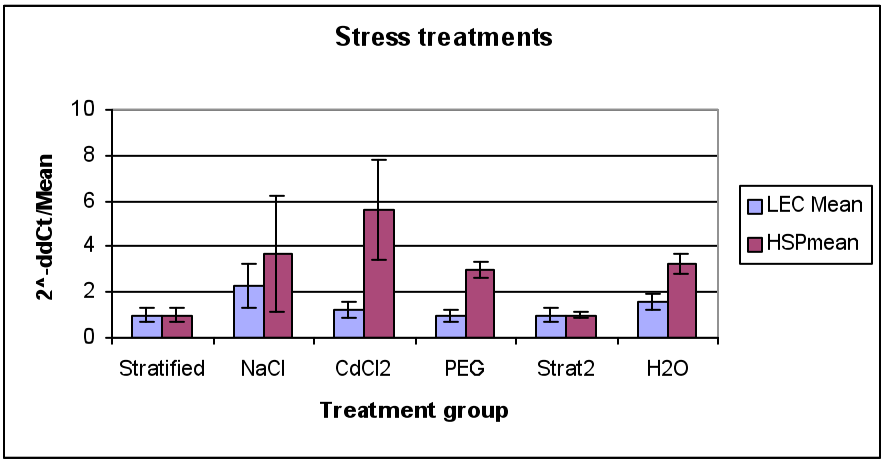


Figure 19. The effect of 24 h stress treatments on PmLEC1 and HSP18.1A expression

RNA was isolated from Douglas-fir stratified seed (control) and stratified seed incubated for 24 h in the indicated solutions. Each group consisted of 5 seed. *PmLEC1* and *HSP* transcript levels were analyzed by QPCR in quadruplicate reactions. Statistical analyses were done on pooled groups (n=5). None of the treatments were statistically significant with respect *to PmLEC1*, by the Mann-Whitney U test. The *HSP* responses to $CdCl_2$, PEG and H_2O were statistically significant. Error bars represent standard error of the mean.

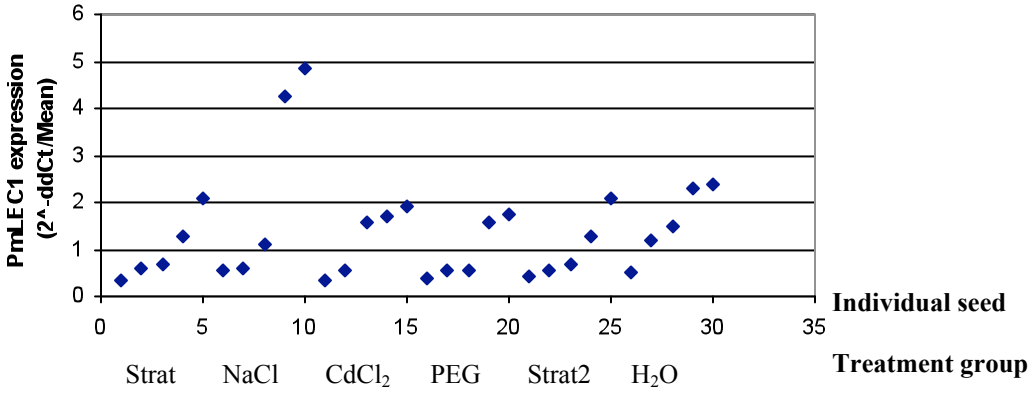


Figure 20. Individual responses of seed to 24 h stress treatments indicate that NaCl may up-regulate PmLEC1 expression in stratified seed

Each diamond represents an individual seed from the QPCR analyses in Figure 19. The batch of seed showed 60% germination and non-responding seed, 2 per treatment group were removed from the analysis. When the Mann Whitney U test was performed with the 3 top responders from each treatment group (m=3) and 3 control seed (n=3), none were statistically significant.

production of robust somatic embryos and one of the functions of LEC1 is proper embryo formation.

2.4 DISCUSSION

In *Arabidopsis* and a select few angiosperms, the *LEC1* gene was shown to be critical for proper embryo development (Lotan et al., 1998; Yazawa et al., 2003; Fambrini et al., 2006; Alemanno et al., 2007). The identification of such a gene in conifers is a step toward understanding and controlling embryogenesis at the molecular level because very little is known about the genes responsible for embryogenesis in conifers. At this time, few embryo-specific genes have been characterized in conifers, and *LEC1* is a promising candidate gene because it functions both during early and late embryogenesis. Presently, somatic embryogenesis in conifers may be induced from immature zygotic embryos and a few other juvenile tissues. The Douglas-fir *LEC1* gene was isolated, its promoter sequence and expression profiles were analyzed in order to gain insight into its function, potential for regulation and induction in mature embryos. This information opens up our understanding of conifer embryogenesis and may be further extrapolated to initiate embryogenesis from vegetative tissues.

Alignment shows that the central B domain is conserved across angiosperms and gymnosperms and that there is a high degree of conservation in the plant LEC1-type family. Both conifer sequences grouped with the angiosperm LEC1 clade, and not LEC1-LIKE, suggesting functional similarity to AtLEC1. The difference between these

genes in *Arabidopsis* is that *LEC1* is strictly expressed in embryonic tissues while *LEC1-LIKE* is expressed at low levels in all tissues. *AtLEC1* was shown to induce embryogenesis when expressed ectopically in the *lec1-1* null mutant while *AtLEC1-LIKE* only rescued the mutant but embryogenesis was not observed.

The putative Douglas-fir protein sequence is 28 amino acids shorter than the putative *Arabidopsis* LEC1 (Ws ecotype). In the MUSCLE alignment, this difference is observed as 3 gaps within the C-terminal domain. For the partial pine sequence that is available, only 6 amino acids differ from Douglas-fir and it is likely that this protein will have the same function. The sequence information from Douglas-fir and loblolly pine should facilitate the isolation of LEC1 genes in other conifers.

It appears that LEC1 is a well-conserved embryo-regulatory gene that is seen in a wide range of plants, from conifers to cactuses (*Kalanchoe daigremontiana* in Figures 2 and 3). The high degree of conservation suggests that its function is specialized and necessary, with divergence likely being fatal.

2.4.1 Insights from Promoter/5' UTR Intron Sequence: Synergy, Embryogenic Pathway and Immediate Regulators of *PmLEC1*

The promoter of *PmLEC1* holds important implications for plant embryogenesis and how a master regulator of embryogenesis may be activated. Work in the past has focused on identification of master regulatory genes, which could be modulated to induce embryogenesis. However, in light of the *PmLEC1* promoter sequence, it now appears that multiple genes and signals act in concert to establish embryogenesis, and the integration of multiple signals for synergistic activation may be the only mechanism to

induce SE at any time and in any tissue. The relevance of the master regulatory genes is that embryogenesis cannot proceed in their absence, and by understanding the regulation of PmLEC1, advances can be made in the initiation and optimization of SE protocols. Further, the key steps and genes necessary for the entire process of embryogenesis may be deduced from the numerous and diverse *PmLEC1* promoter elements. Lastly, the promoter sequence provides compelling evidence to postulate that *PmLEC1* expression is regulated by AGL15, VAL and VP1.

The large number of elements mediating responses to a variety of signals implies that this is a strategy used for coupling brief stimuli to long-term adaptive responses (Finkbeiner 2001), such as the induction of embryogenesis in response to stress and hormone treatments. Both direct and indirect embryogenesis occur after several weeks on induction medium (Stasolla et al., 2002; Ikeda-Iwai et al., 2003). Multiple regulatory sites on introns lead to synergistic activation rather than additive activation, and a new theory in signal transduction is that specific biological responses may be achieved combinatorially (Finkbeiner 2001). Finkbeiner predicts that combinatorial responses result when 2 signaling pathways converge on 2 or more transcription factors that cooperate. This is applicable to the promoter/5' UTR intron of *PmLEC1*. The multiple pathways that converge on this sequence are biotic and abiotic stress responses, light induction, developmental cues and hormone (ABA and auxin) signaling. Work with genetic crosses in angiosperms has shown that LEC1/FUS3, LEC1/ABI3 and FUS3/ABI3 interact synergistically during seed development and maturation (Lotan et al., 1998; Parcy et al., 1997; Vicient et al., 2000; Kagaya et al., 2005), and the identification of regulatory elements in the *PmLEC1* promoter explains these results at the molecular

level. This cooperation should also be present during the initiation of embryogenesis. Hence, multiple signals are likely to be necessary for induction of SE and this may be achieved *in vitro* by a complement of signals, as opposed to a single medium manipulation or elicitor.

Because PmLEC1 is a critical regulator that acts during both early and late embryogenesis and the promoter contains binding sites for multiple signals as well as for transcription factors that act during early, middle and late stages of embryogenesis, the promoter sequence could help to decipher the regulatory processes that control embryogenesis and lead to the establishment of the plant embryogenic pathway. The upstream sequence of Douglas-fir *LEC1* suggests that transcription factors like AGL15, VAL, MYB1, VP1 and ABI3 as well as diffusible cues regulate *LEC1* expression. This new information does not abrogate what is already known but provides a molecular basis for some of the genetic interactions that are necessary for normal embryo development. AGL15 is expressed very early in embryogenesis, while VAL is expressed throughout the plant life cycle. MYB1 activates transcription of genes in response to the dilution effect, UV-B irradiation and elicitor treatments but it also has developmental roles (Maeda and Ozeki, 2006). MYB genes are known to control proliferation and differentiation of several cell types. Because VP1 and ABI3 are seed-specific transcriptional activators and their transcripts are most abundant during middle and late embryogenesis (Kurup et al., 2000; Suzuki et al., 2007), these proteins may strongly influence *PmLEC1* expression during those time points. It is expected that not all transcription factors bind at the same time and that the modularity of the transcriptional complex will result in differential effects. Further work capturing the complexes bound to the promoter at each stage of

embryogenesis could identify the genes responsible for molecular and developmental events. Additional determination of the genes activated at the respective stages *via* microarray experiments will lead to a comprehensive erudition of plant embryogenesis.

The regulation of *LEC1* is central for SE initiation and optimization of SE protocols because the Arabidopsis *LEC1* gene is necessary for somatic embryogenesis (Gaj et al., 2005). Further, it is possible that the combined effect of LEC1, L1L, FUS3, ABI3, cruciferin and 2S storage protein are necessary for somatic embryogenesis. When Ikeda-Iwai et al. (2003) induced embryogenesis from various mature explants of Arabidopsis, they showed that *LEC1*, *ABI3* and *FUS3* were expressed in the somatic embryos but not in the underlying callus. In previous work, they found that these same genes were also expressed in embryogenic cell clusters (Ikeda-Iwai et al., 2002). These proteins could influence each other's expression by positive feedback mechanisms, i.e., LEC1 induces ABI3 expression, which maintains *LEC1* expression.

Until recently, the only factors known to influence *LEC1* expression were PKL and LEC2 (Rider et al., 2003; Santos-Mendoza et al., 2008). PKL is a chromatin-remodeling factor functioning upstream of *LEC1* to suppress its expression (Ogas et al., 1999), while LEC2, a B3 transcription factor, was shown to induce *LEC1* expression when expressed ectopically (Santos Mendoza et al., 2005), although *in vivo*, *LEC1* expression precedes *LEC2*. The current understanding of embryogenesis is that LEC1 functions upstream of LEC2, FUS3, ABI3/VP1 and LEC1-LIKE and rapidly activates *ABI3* and *FUS3* expression, while the *LEC2* transcript is induced more slowly (Kagaya et al., 2005). Cruciferin and 2S storage protein genes are downstream targets of ABI3 and FUS3. LEC1 activates the expression of seed storage protein genes, but this starts to happen

even before embryogenesis is induced (Kagaya et al., 2005). The discovery that VAL proteins suppress the *LEC1* and the *FUS3/ABI3* networks (Suzuki et al., 2007) has brought further insight into regulation of embryogenesis. Analysis of the *PmLEC1* promoter sequence combined with the present knowledge of embryo-regulatory transcription factors suggests that AGL15, VP1 and ABI3 induce *LEC1* expression, while VAL proteins suppress it. This broadens our understanding of plant embryo-regulatory interactions and shifts the knowledge to earlier points of embryogenesis.

VAL proteins bind the Sph/RY consensus sequence, CATGCA, which is found in the promoter regions of angiosperm *LEC1*, *ABI3*, *FUS3*, *L1L*, and the suppressive effect of these proteins became evident in *val* double mutants (*val1val2*) in which these 4 embryo-specific genes are strongly up-regulated (Suzuki et al., 2007). Germinated mutant seeds (*val1val2*) were not capable of developing into normal seedlings but resulted in cell proliferations with embryonic characteristics: callus formation from shoot and root, and embryo-like structures at apical meristems and cotyledon margins (Suzuki et al., 2007). In these embryo-like seedlings, *cruciferin* and *2S* storage protein genes were also up-regulated but *PKL* expression remained the same as in wild type, and therefore, *VAL* genes do not act upstream of PKL, but they repress LEC1, ABI3, FUS3 and L1L directly (Suzuki et al., 2007). The Sph/RY element is also present in the *PmLEC1* promoter (Figure 6) showing that its regulation in conifers follows a similar mechanism to angiosperms.

While VAL proteins repress embryogenesis, VP1 activates expression from the same regulatory element (Suzuki et al., 2005). In *Arabidopsis*, expression of the 3 *VAL* genes is observed throughout the plant life cycle and VAL proteins function in chromatin

regulation, leading to further insight about how *LEC1* expression is activated. If VAL suppresses *LEC1* expression and VAL is expressed at all times, then other factors that bind the same regulatory element and promote *LEC1* expression must either have stronger affinity or higher abundance at the time of *LEC1* induction. In angiosperm embryogenesis, *VP1* (maize) and *ABI3* (*Arabidopsis*) are homologous proteins whose expression is characteristic of mid and late embryogenesis and are known to mediate responses to abscisic acid (Parcy et al., 1997) however VP1 can also trans-activate expression in the absence of ABA (Hattori et al., 1992). Closer examination reveals that ABI3 transcripts are induced as early as 2 d after pollination (Kurup et al., 2000), while VP1 expression is observed as early as 10 d after pollination. This places greater consequence on low-level expression of VP1 and ABI3 and their effects on LEC1. Separate response elements for both VP1 and ABI3 are present in the *PmLEC1* promoter, and if expression of these genes in conifers also occurs shortly after pollination then VP1 derepresses VAL suppression and ABI3 also influences PmLEC1 expression during early stages of embryogenesis.

A novel conclusion from promoter analysis is that AGL15 plays a role in the initial appearance of *LEC1* as well as its abundant expression during early embryogenesis. In *Brassica*, maize and *Arabidopsis*, AGL15 is the only MADS box protein that preferentially accumulates in developing embryos and associated maternal tissues, as well as in embryos of apomictic (derived from unfertilized ovules), somatic and microspore (derived from immature pollen grains) origin (Perry et al., 1996; Harding et al., 2003). MADS box proteins are a family of transcription factors with important roles in developmental processes. AGL15 accumulates very early in embryogenesis and

persists until desiccation, when its expression is greatly reduced. After germination, AGL15 is expressed in the shoot apical meristem and at the bases of lateral organs, but this expression is 10-fold lower than in embryonic tissues (Harding et al., 2003). Ectopic expression of AGL15 promotes and supports somatic embryo production as evidenced by post-germinative somatic embryo development from the SAM of transgenic plants and the enhanced production of secondary embryos from zygotic embryos cultured on medium free of growth regulators (Harding et al., 2003). This effect may have been mediated by LEC1. Of all the transcription factors identified in the *PmLEC1* promoter, AGL15 is the only one known to be present during early embryogenesis and earlier, and this could be the most significant factor responsible for *LEC1* induction. The high abundance of AGL15 before embryogenesis, the 3 AGL15 binding sites present in the promoter of *PmLEC1* (Figure 6) and the enhanced activation when 2 or more such elements are present, support this hypothesis. Figure 21 integrates the information regarding the role of LEC1 in embryogenesis.

The involvement of a MADS box protein in embryogenesis offers prospects for other members of this family. Although no *cis* elements through which LEC1 acts have been identified (Kagaya et al., 2005), it is known that LEC1 encodes the HAP3 subunit of a CCAAT box-binding transcription factor, which is also known as NF-YB. However, 25% of eukaryotic promoters contain CCAAT boxes (Masiero et al., 2002), and thus it is expected that LEC1 would be capable of activating a substantial number of a plant's genes and consequently have a less specific function than embryo formation. Recently, it was shown that NF-YB subunits of rice and mouse interact with a specific MADS box protein, OsMADS18, resulting in a heterotrimeric complex, which does not bind the

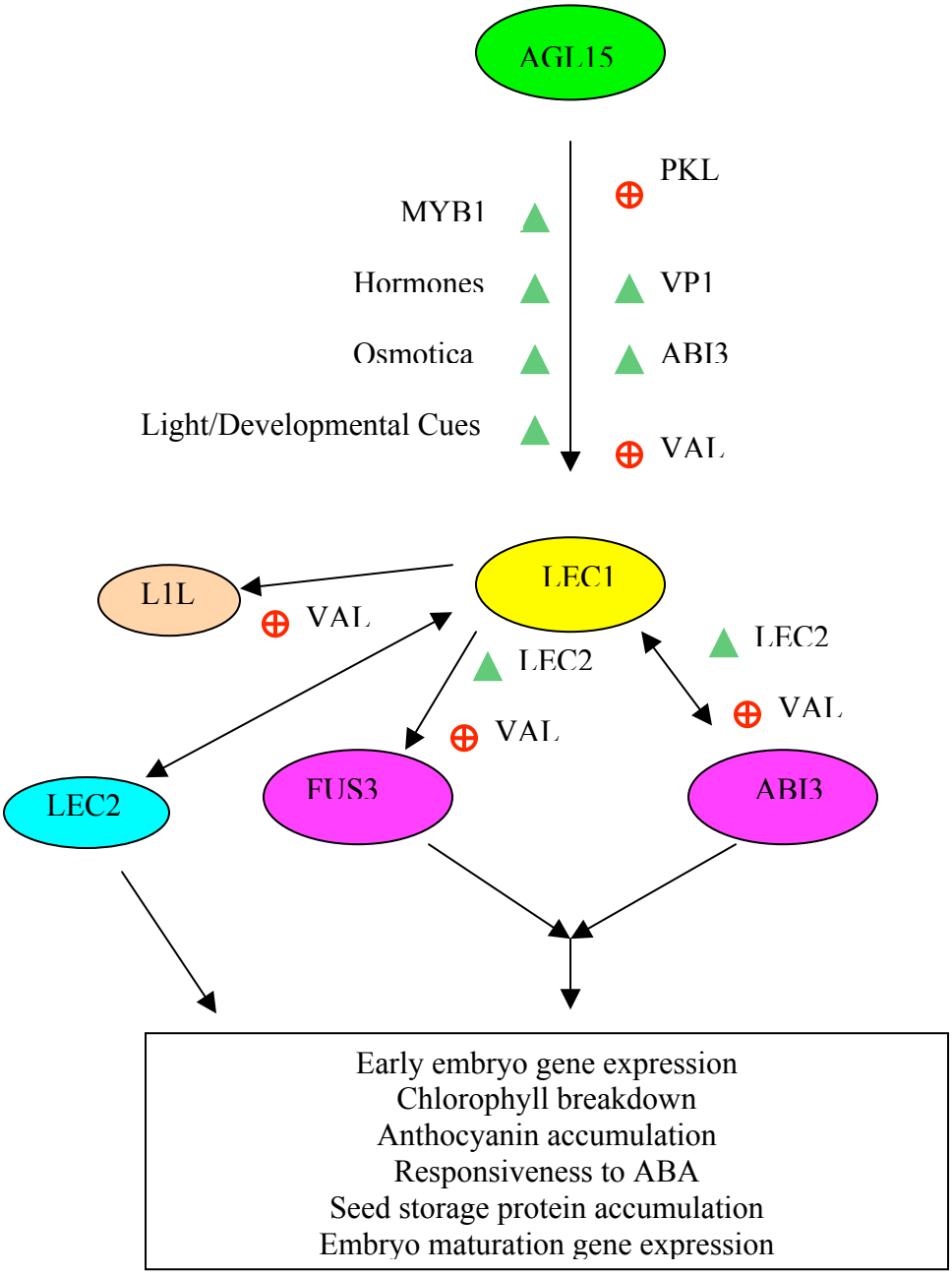


Figure 21. Model of signaling pathway responsible for plant embryogenesis.

classical CCAAT box but could exert its action *via* a different mechanism (Masiero et al., 2002). MADS18 may interact with LEC1 during embryogenesis to activate a set of genes that are required for proper embryo formation. The interaction between the MADS box protein and NF-YB is *via* the histone-fold motif (Masiero et al., 2002), which is also present in LEC1-LIKE and could explain why LEC1-LIKE rescues the *lec1-1* null mutant. Further work testing the interaction of PmLEC1 with OsMADS-18 will provide insight into its function.

2.4.2 *PmLEC1* Expression Is Characteristic of Early Embryogenesis

High expression of *PmLEC1* during embryogenesis, its absence in mature, vegetative tissues and the fact that it is a transcription factor emphasizes its role in proper embryo formation and prevention of precocious germination. The presence of trace amounts of *PmLEC1* transcripts and protein in germinating seedlings (Figures 8 and 9) is not surprising. Previous work in *Arabidopsis* has relied upon Northern analyses but QPCR is a more sensitive technique that allows detection of extremely low transcript levels. Moreover, Casson and Lindsey (2006) observed low levels of *LEC1* transcripts in germinating wild-type seedlings of the *Arabidopsis* C24 ecotype.

The presence of LEC1 in juvenile tissues links the embryogenic potential of this tissue to a molecular cause. LEC1 is important for embryogenesis and up-regulation of its expression may lead to somatic embryogenesis. Malabadi and van Staden (2005) showed that vegetative buds of pine could be used for induction of somatic

embryogenesis. A problem with this work is that it was not always successful. However, in QPCR analysis, *PmLEC1* transcripts were only observed in the youngest of vegetative buds, possibly the only window for inducing somatic embryogenesis. Thus, the presence of *PmLEC1* in juvenile tissues may indicate their predisposition to SE induction.

PmLEC1 may serve as a molecular marker of embryogenesis, and further investigation into PmLEC1 expression in embryogenic and non-embryogenic cell lines is necessary in order to clearly establish the levels that are characteristic of each stage. Ikeda-Iwai et al. (2002) differentiated embryogenic cell clusters from non-embryogenic callus during establishment of a reproducible system for SE induction and found that *AtLEC1* was expressed only in the embryogenic cell clusters. Consequently, analysis of protein accumulation will be useful in fully understanding the function of LEC1. The difference in apparent MW may provide insight into post-translational regulation, i.e., PmLEC1 protein accumulation begins in early embryogenesis, and it is modified to an inactive form until specific cues cause its activation during seedling development. Alternatively, a post-translationally modified form enables PmLEC1 to retain its activity in desiccated seed. In *Arabidopsis*, AtLEC1 is known to inhibit precocious germination and perhaps this is why PmLEC1 persists into later stages of development. There could be additional functions that have not yet been characterized. This is the first time that an antibody has been raised against a LEC1 protein and its applications to conifer SE remain to be determined.

2.4.3 HAP3 proteins migrate with a greater than expected mobility

The rat HAP3 gene encodes a putative polypeptide of 207 amino acids with an expected molecular weight of 25 kDA (Vuorio et al., 1990). However, *in vitro* transcription and translation of the cDNA clone results in a protein that migrates with an abnormal mobility of 32 kDa (Vuorio et al., 1990). Purification of this subunit *via* DNA affinity chromatography results in a fraction containing 2 species of 32 and 32.5 kDa, both of which are active in CCAAT DNA binding (Vuorio et al, 1990). Mouse HAP3 is also a 207 amino acid protein but it migrates at 36 kDa (Gilthorpe et al., 2002), therefore a protein with a predicted size of 25 kDa may have a mobility between 32 – 36 kDa.

HAP3 and HAP5 dimerize in order to be transported to the nucleus (Vuorio et al., 1990) but under SDS-PAGE conditions NF-Y subunits always separate (Vuorio et al., 1990; Sinha et al., 1995; Gilthorpe et al., 2002; Kahle et al., 2005). The HAP5 subunit migrates with a mobility of 40 kDa and if HAP3 and HAP5 were in a complex, the expected mobility of migration would be greater than 70 kDa. Based on this discussion, it is unlikely that PmLEC1 is in a complex with other HAP subunits during SDS-PAGE, but very likely that PmLEC1 is variably modified at different stages of embryogenesis.

2.4.4 *PmLEC1* Expression May Be Modulated in Mature Tissues

At this time, evidence from the promoter sequence implies that other factors also need to be up-regulated or induced along with *PmLEC1* in order to achieve somatic

embryogenesis from mature tissues. However, a spike in PmLEC1 expression my trigger complex formation and induction of somatic embryogenesis under some conditions. In stratified seed, PmLEC1 expression is very low but after 24 hr, expression was up-regulated by 2,4-epibrassinolide, osmoticum, in the form of sorbitol and mannitol, and salinity. The presence of a promoter element for MYB1, which activates transcription in response to osmotic stress, supports this finding. Further, longer incubation periods may be necessary for up-regulation of *PmLEC1* and subsequent initiation of SE.

Brassinosteroids are newly identified phytohormones that are gaining importance in induction of embryogenesis. Our results support this and suggest that brassinosteroids regulate *PmLEC1* expression. A plant promoter element responsive to brassinosteroids has not been identified yet.

The single seed, which displayed an extreme response to the GA/ABA treatment (Figure 11), represents a "strong initiator." The high level of PmLEC1 expression is reminiscent of late embryogenesis and further expression analysis of this seed would yield potentially useful information. For example, are conifer equivalents of genes such as *AGL15*, *LEC2*, *seed storage proteins* and *FUS3* also up-regulated when compared to other seed in this treatment group? Had this been an embryogenic culture, the culture before treatment could have served as a control and the results would have had a greater impact.

In the NaCl group only 2 seeds appeared to be responders, and this is below the minimum number required for statistical analysis ($m \geq 3$). When compared to the mannitol group, which contained 4 responders, the average germination rate is still maintained between these two groups and indicates that this happened by chance. The

response of the two seeds is very strong and worthy of further investigation, with a higher number of seeds.

Although the initial modulation results are encouraging, in retrospect this type of experiment is best carried out with embryogenic cultures or cell lines. Assessing the response of 5 embryogenic versus 5 non-embryogenic cell lines would standardize the results across the different genotypes and strengthen the analysis.

Expression profiling and knowledge of the genes that are essential to conifer embryogenesis will enable us to have more control over somatic embryogenesis because medium manipulations can be verified against physiological responses, we can strive to restore gene expression that is characteristic of embryogenic cultures, and aberrant cultures will be identified and discarded faster. Also, because PmLEC1 responded to several single treatments, it is anticipated that multiple or combined treatments will activate embryo-specific genes in mature tissues and lead to SE.

In closing, PmLEC1 is one of the first early embryo regulatory gene characterized in conifers. The promoter sequence of PmLEC1 provides clues about the identity of other genes involved in conifer embryogenesis and potentially provides a map of key genes necessary for the entire embryogenic pathway. The activation of PmLEC1 in mature tissues may lead to improvement of SE protocols and possible induction of SE from vegetative conifer explants. The development of an anti-PmLEC1 antibody will provide a simple and rapid method for classifying cell lines as embryogenic or non-embryogenic once stage-specific PmLEC1 expression levels are confirmed.

3. Douglas-fir *LEC1* rescues the *Arabidopsis lec1-1* null mutant and has a role in inducing embryogenic programs

3.1 INTRODUCTION

The spontaneous production of embryos is the ultimate goal of any somatic embryogenesis initiative, even more so when it comes to the production of superior somatic embryos for reforestation with conifers. The recalcitrance of coniferous species to induction of somatic embryogenesis and the paucity of knowledge regarding the molecular events responsible for gymnosperm embryogenesis (Cairney et al., 1999) are a driving force toward the identification and characterization of embryo-regulatory genes.

The discovery of the *Arabidopsis LEAFY COTYLEDON1* (*AtLEC1*) gene (Lotan et al., 1998) started a trend in the search for genes, which could induce somatic embryogenesis. This led to the characterization of genes with embryogenic capabilities, *LEC2, BBM, WUS, LEC1-LIKE*, (Stone et al, 2001; Boutilier et al., 2002; Zuo et al., 2002a; Kwong et al., 2003), and the isolation of *LEC1* and *LEC1-LIKE* genes in several plant species. The striking feature of AtLEC1 is that its ectopic expression induces embryonic programs and spontaneous formation of somatic embryos from vegetative tissues (Lotan et al., 1998). The *AtLEC1* transgene rescued the *lec1-1* null mutant by enabling it to produce viable, desiccation-tolerant seed (Lotan et al., 1998). In addition, abnormal, embryo-like morphological characteristics, observed in both T1 and T2 seedlings, indicated that embryonic programs had been activated in vegetative tissues. This was confirmed by the presence of seed-specific transcripts (*oleosin, cruciferin, 2S storage proteins*) in the embryo-like seedlings. Although some argue that constitutive expression of *AtLEC1* is

not likely to become a strategy for induction of somatic embryogenesis due to the severe phenotype and rare occurrence of embryos in transgenic plants (Zuo et al., 2002b), *AtLEC1* is necessary for proper zygotic embryo formation and maturation (West et al., 1994; Lotan et al., 1998) and induction of somatic embryogenesis *in vitro* (Gaj et al., 2005). Wild-type *Arabidopsis* immature zygotic embryos showed a high efficiency of somatic embryo induction in the presence of the auxin, 2,4-dichlorophenoxyacetic acid, while *lec1* mutants were prone to callus formation that rarely led to the development of somatic embryos (Gaj et al., 2005). Further investigations into the function of *LEC1* genes will provide critical information for improving protocols.

The angiosperm *LEC1*-type gene family has two members, *LEC1* and *LEC1-LIKE*, as described by Kwong et al. (2003) working with *Arabidopsis*. The *AtLEC1* gene shows strict embryo-specific expression, rescues the *lec1-1* null mutant and can induce somatic embryogenesis in the *lec1-1* null mutant. While *AtLEC1-LIKE* also rescues the *lec1-1* null mutant and is highly expressed during embryogenesis, it also shows low-level expression in vegetative tissues, and ectopic expression does not lead to somatic embryogenesis in the *lec1-1* null mutant. Although it is conceivable that both genes exist in all plants, the presence of both genes in any other plant has not been confirmed thus far. Analyses of recently characterized *LEC1* in carrot, and *LEC1-LIKE* in sunflower and cocoa tree (Yazawa et al., 2004; Fambrini et al., 2006; Alemanno et al., 2007) have clearly shown that expression of these genes is characteristic of embryogenesis, but much of this work has focused on RNA localization. Presently, there is no evidence that ectopic expression of carrot LEC1 induces somatic embryogenesis. Sunflower LEC1-LIKE may be involved in switching cell fate and leading to embryogenic competence

(Fambrini et al, 2006). Ectopic expression of the *Theobroma cacao* LEC1-LIKE did not result in the appearance of somatic embryos on transgenic seedlings (Alemanno et al., 2007). There is no clear way to classify a gene as *LEC1* or *LEC1-LIKE* based on its sequence or expression pattern, but this can be resolved by determining whether or not ectopic expression leads to somatic embryogenesis.

We previously showed that Douglas-fir *LEC1* (*PmLEC1*) is an embryo-specific gene whose expression may be modulated by stress and hormone treatments (Chapter 2). At the genomic level, *PmLEC1* has a 5'UTR intron that contains numerous binding sites for transcription factors, and the intersection of multiple pathways on this intron points to its role as a master regulatory gene. In order to determine the function of PmLEC1 and its downstream effects, we transformed *Arabidopsis* wild type and the *lec1-1* null mutant with *PmLEC1* under control of the CaMV double 35S promoter with the AMV transcriptional enhancer (35S-35S-AMV). *PmLEC1* rescued the *Arabidopsis lec1-1* null mutant, resulted in the production of T_2 seedlings with both normal and embryo-like characteristics, and expression of embryo-specific genes was limited to the embryo-like seedlings. The appearance of somatic embryos on vegetative tissues was confirmed. Ectopic expression of *PmLEC1* in wild-type *Arabidopsis* resulted in bushy T_2 plants, which initially started out as a callus-like mass. Taken together, these results indicate that similarly to AtLEC1, PmLEC1 rescues the *lec1-1* null mutant, and ectopic expression induces embryonic programs as well as the formation of embryo-like structures. However, it is apparent that LEC1 must interact with other seed-specific factors to induce the embryo-like phenotype. Ectopic expression of *PmLEC1* in wild type plants resulted in *PmLEC1* RNA expression in vegetative tissues that had surpassed

the seedling stage but embryonic programs were not induced and embryo-like structures were not observed.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Seed stocks for Arabidopsis WS-2 (CS2360) and *lec1-1* WS-2 (CS8101) were obtained from the Arabidopsis Biological Resource Center, Columbus, Ohio.

3.2.2 Generation of the *lec1-1* Null Mutant

Heterozygous seed (*LEC1/lec1*) were germinated and grown in soil. The plants self-pollinated, flowered and produced seeds. Immature seed were removed from green siliques, surface sterilized and plated on 1/2 MS medium (Murashige and Skoog, 1962) (Sigma-Aldrich modified MS basal salts, catalogue # M8280). Immature wild type or heterozygous seed do not germinate while *lec1-1* null mutant seed germinate. The seeds that germinated were grown into plantlets and the genotype was confirmed by removing small amounts of tissue, isolating DNA, and PCR amplification of *AtLEC1*. The plants that did not yield a PCR product with primers based on the *AtLEC1* sequence were utilized for transformation. The integrity of genomic DNA was confirmed on 1% agarose gels prior to amplification. DNA isolated from wild type Arabidopsis was utilized in

positive control reactions, while no template control reactions confirmed the absence of contamination in the primer solutions and the reaction mixture.

3.2.3 Construction of Expression Cassettes

Two expression cassettes were constructed, one containing the *PmLEC1* gene and one containing the *AtLEC1* gene. PCR amplification of *PmLEC1* and *AtLEC1* coding sequences was carried out with a pair of primers designed to incorporate the *Xba* I and *Bam* HI recognition sites at 5'- and 3'-ends of each gene respectively. The PCR product was digested with restriction enzymes and the *PmLEC1* (or *AtLEC1*) gene was directionally inserted between the *Xba* I and *Bam* HI sites of the pBI221 vector containing the 35S-35S-AMV promoter and the NOS terminator, and the neomycin phosphotransferase II (NPT II) gene for Kanamycin resistance. The recombinant vector was propagated in *E. coli* and the integrity of the insert was confirmed by sequencing. The vector was transformed into *Agrobacterium tumefaciens* strain M90 as described by Datla et al. (1993). The trans-conjugants were selected and the presence of the insert was confirmed by restriction analyses.

3.2.4 *Agrobacterium*-mediated Plant Transformation

The floral dip method described by Clough and Bent (1998) was used to transform *lec1-1* null mutant plants and wild type plants. Transgenic plants were selected on Kanamycin media and transgene integration was confirmed by PCR.

3.2.5 DNA Isolation

DNA was isolated from transgenic plants by grinding in liquid nitrogen and using the Sigma GenElute Plant Genomic DNA kit according to manufacturer's instructions.

3.2.6 PCR

PCR reactions were performed with 0.5 μg DNA in a 50 μl reaction using the Taq PCR Master Mix (QIAGEN, California, USA) containing Taq DNA polymerase, according to the manufacturer's instructions, in the Perkin Elmer Cetus 480 DNA Thermal Cycler. The products were separated by electrophoresis on a 1 % agarose gel and visualized by ethidium bromide staining.

For *AtLEC1* amplification, the primers utilized were 5'-ATGACCAGCTCAGTC-ATAG-3' and 5'-TCACTTATACTGACCATAATG-3', which generate a 624 bp product. The thermocycle program consisted of 5 min denaturation at 94 ^{o}C, 40 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 51 ^{o}C for 30 s and extension at 72 ^{o}C for 1 min, followed by 10 minutes of extension at 72 ^{o}C.

PmLEC1 was amplified using the primers 5'-ATGATGTCCGAAGTTGGAAGC-CCT-3' and 5'-CTTATACTGAGCATAGGGATCATA-3', which generate a 540 bp product. The thermocycle program consisted of 5 min denaturation at 94 ^{o}C, 40 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 61 ^{o}C for 30 s and extension at 72 ^{o}C for 1 min, followed by 10 minutes of extension at 72 ^{o}C.

Cruciferin was amplified using primers 5'-ATGGTGCTTCCTAAATACAAG-3' and 5'-TTAAGCCTCGACAATCTCCT-3', which generate a 382 bp product. The thermocycle program consisted of 5 min denaturation at 94 ^{o}C, 40 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 53 ^{o}C for 30 s and extension at 72 ^{o}C for 1 min, followed by 10 minutes of extension at 72 ^{o}C.

Oleosin was amplified using primers 5'-ATGGCCGATACAGCTAGAGG-3' and 5'-AGAGAAAACGGTTATAGCGGC-3', which generate a 321 bp product. The thermocycle program consisted of 5 min denaturation at 94 ^{o}C, 40 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 58 ^{o}C for 30 s and extension at 72 ^{o}C for 1 min, followed by 10 minutes of extension at 72 ^{o}C.

Wild type Arabidopsis DNA was employed as a positive control for *AtLEC1*, *cruciferin* and *oleosin* amplifications. No template negative controls were performed for all reactions.

3.2.7 RNA Isolation and Gene Expression Analyses by RT-PCR

Tissue used for RNA isolation excluded flowers, seeds and siliques because the presence of seed of any stage could show expression of seed storage proteins, cruciferin and oleosin. RNA was isolated by TRIzol method according to the manufacturer's instructions modified for plants. RNA isolated from all samples was treated with amplification grade DNase I (Invitrogen) according to the manufacturer's instructions.

For first strand cDNA synthesis, 1 μg total RNA was incubated with 1 μl oligo $(dT)_{12}VN$ (V = A or C or G; N = A or C or G or T) and SuperScript II RNase H^- reverse

transcriptase in 20 μl reactions (Invitrogen) according to manufacturer's instructions. The Invitrogen Ribonuclease Inhibitor (1 μl at 10U/μl) was utilized to prevent RNA degradation in these reactions.

PCR was performed with 2 μl cDNA in 25 μl reactions employing the QIAGEN Master Mix, as described above.

3.3 RESULTS

To characterize the function of Douglas-fir LEC1, the *PmLEC1* coding sequence was inserted into a vector under control of a promoter consisting of the cauliflower mosaic virus (CaMV) duplicated enhancer in combination with the alfalfa mosaic virus (AMV) untranslated leader sequence (35S-35S-AMV). This promoter/leader sequence has the highest expression potential in plants (Datla et al., 1993). Wild type *Arabidopsis* and the *lec1-1* null mutant plants were transformed with *PmLEC1 via* the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). Additional plants were transformed with the coding sequence of *AtLEC1* under control of the 35S-35S-AMV promoter/leader, to serve as controls and to investigate differences between *AtLEC1* and *PmLEC1*. The PmLEC1 and AtLEC1 expression cassettes are shown in Figure 22. The focus was on the second transgenic generation, T2, because that was where others (Lotan et al., 1998) working with *AtLEC1*, observed the spontaneous formation of somatic embryos. Plant morphology and seed-specific gene expression were analyzed.

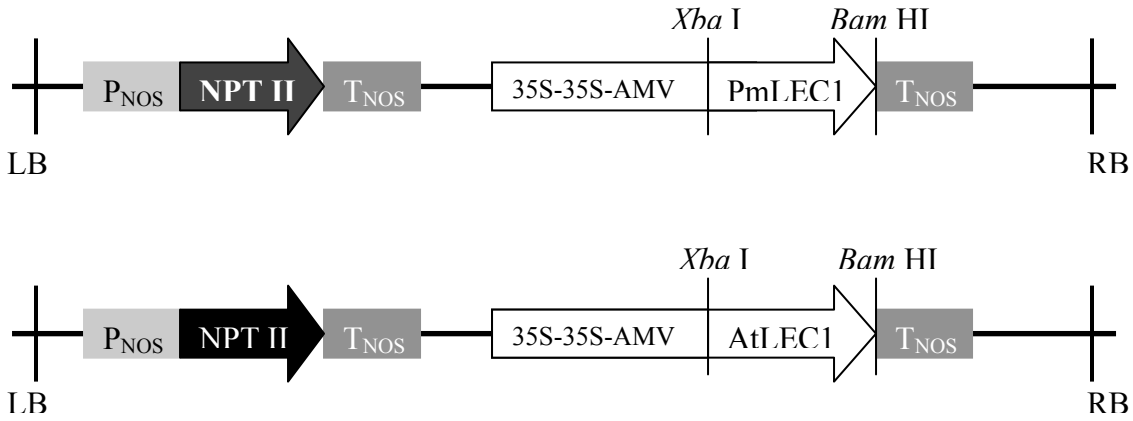


Figure 22. The *LEC1* expression cassettes utilized in *Agrobacterium*-mediated plant transformation.

RB and LB, right and left border regions, respectively, of the Ti plasmid. P_{NOS}, promoter and T_{NOS}, terminator of the nopaline synthase gene. NPT II, neomycin phosphotransferase II conferring Kanamycin resistance. 35S-35S-AMV, cauliflower mosaic virus duplicated 35S enhancer and alfalfa mosaic virus RNA4 leader sequence. PmLEC1, coding sequence of the Douglas-fir *LEC1* gene. AtLEC1, coding sequence of the Arabidopsis *LEC1* gene.

3.3.1 *PmLEC1* rescues the *lec1-1* null mutant, inhibits vegetative development and leads to the formation of embryo-like structures in abnormal T1 seedlings

Arabidopsis lec1-1 null mutant plants transformed with *PmLEC1* produced viable, desiccation-tolerant T1 seed with a transformation efficiency of 2% (18/1000), indicating that the *PmLEC1* transgene complemented the null mutation. However, 4 distinct outcomes were observed post-germination: 1) normal vegetative development, 2) stunted development or fasciation, 3) callus formation and 4) death. Approximately 25% (47/200) of the T1 seed that germinated produced seedlings with normal morphology (Figure 23). Of the remaining seed, 50% (102/200) produced seedlings that started vegetative growth but were blocked before flowering could take place (Figure 24). These seedlings either had multiple, dark green, cotyledon-like organs (Figure 24A) or thick

stems with stunted lateral organs and fleshy leaves (Figure 24B), with the twisted hooked stem representing extreme fasciation. Fasciation is an abnormal flattening or compression of stems or leaf stalks resulting from abnormal development of the meristem as a band-like structure (Mordhorst et al., 1998). The final 25% (51/200) developed to the point of 2 white, fleshy leaves and died (Figure 24C), indicating that development could not proceed further. Some of the developmentally arrested seedlings formed normal roots but instead of forming shoots, a mass of white, callus-like tissue proliferated from the region of the shoot apical meristem (Figure 25A). Retrospectively these images show evidence of somatic embryos (Figure 25C). Because these seedlings germinated on selective medium (1/2 MS – Kanamycin) free of growth hormones, the abnormal morphology was due to the presence of the transgene. Some of these masses were maintained on ½ MS – Kanamycin medium for over one year, with sub-culturing every 4 months. The callus grew and multiplied indefinitely and after 1 year, shoots became visible and these developed into plants (Figure 25B). The decline of embryogenic potential with prolonged sub-culturing has been reported previously (Mordhorst et al., 1998), and perhaps this phenomenon finally permitted vegetative growth.

Although these percentages are reminiscent of Mendelian inheritance, these phenotypes are the result of direct transformation of cells by *Agrobacterium tumefaciens* at the time of fertilization. This does not exclude gametes from being transformed and producing plants that are homozygous for the transgene, however due to the constitutive nature of the promoter, both homozygous and heterozygous plants may show similarly high transgene expression. In nature, both homozygous and heterozygous *LEC1* plants exhibit wild type phenotypes. All plants must have contained the transgene in order to be

selected, therefore, all plants were *LEC1/LEC1* or *LEC1/lec1*. The T1 plants that developed normally were grown to full maturity and all produced mature, desiccated seeds of heterozygous phenotype, i.e., each silique contained seeds that were either golden-brown and globular, or dark and shrunken. Homozygous *LEC1* plants produce only golden-brown, globular seeds. Thus, the 25 % of plants that developed normally could be classified as heterozygous due to the absence of transgenic plants that produced only golden-brown, globular seeds. The remaining 75 % of plants may have been homozygous for the gene and their severe phenotype could be correlated to excessive transgene expression. However, multiple copies of the transgene are expected to lead to transgene silencing and the opposite is observed. Further testing will be necessary to determine the genotype of these plants.

The inhibition in development seen with 75% of the T1 seedlings is in line with activation of seed maturation programs as a result of transgene expression: in the plant life cycle, vegetative development and morphogenesis are normally arrested during seed maturation (Lotan et al., 1998). Thus, when seed maturation programs are activated, normal vegetative development will not take place. This is substantiated by a majority of seedlings with embryonic characteristics of cotyledon-like organs, fasciation and formation of embryogenic masses (Figure 24, A and B). Also, the seedlings that germinated but did not proceed beyond the two-leaf stage and appeared to die after 1 month on growth medium may be the result of developmental arrest (Figure 24C).

The 25% of seedlings exhibiting normal vegetative development were likely the result of post-transcriptional silencing, which is reset at meiosis (Fu et al., 2000; De Wilde et

al., 2000) and explains the reactivation of the gene in T2 seeds that were desiccation tolerant.

Transgene silencing is frequently observed in plants and can occur when the transgene is inserted in heterochromatin, which is not transcriptionally active, or alternatively, it may be a homology dependent process mediated by RNA interference, where initially high expression is followed by low or no expression (Fu et al., 2000; De Wilde et al., 2000;). The components of the RNA interference pathway also function in maintaining the structure and organization of the genome, thus activation of this pathway can cause pre-transcriptional gene regulation by inducing heterochromatin formation (Holmquist and Ashley, 2006; Grishok et al., 2005). Often there is a correlation between transgene copy number and the severity of silencing, as multiple gene copies become a target for methylation (Fu et al., 2000; De Wilde et al., 2000). The transcriptional silencing that results when the promoter region becomes hypermethylated interferes with transcription or alters chromatin structure is heritably stable (Fu et al., 2000). Transgene reactivation has been observed in instances where methylation occurs in the coding region. Previously methylated regions were demethylated within the same plant after a 3-week interval in a stable, single-copy transgene in rice, suggesting developmentally regulated silencing (Fu et al., 2000). Further, transgene reactivation in the second generation occurred as a result of the coding sequence being hemimethylated and the progeny receiving the non-methylated strand (Fu et al., 2000). Evidence exists that transgene silencing may arise *de novo* in any generation, and different silencing effects may occur simultaneously and independently in adjacent, heterologous transgenes (Fu et al., 2000).

The presence of *PmLEC1* and the absence of *AtLEC1* genes in transgenic plants (T1) were confirmed by PCR. Twenty-four T1 plants showing normal vegetative growth were transferred to soil for growth in the greenhouse and production of T2 seed. We were interested to see whether ectopic expression of PmLEC1 led to the production of somatic embryos in the second generation.

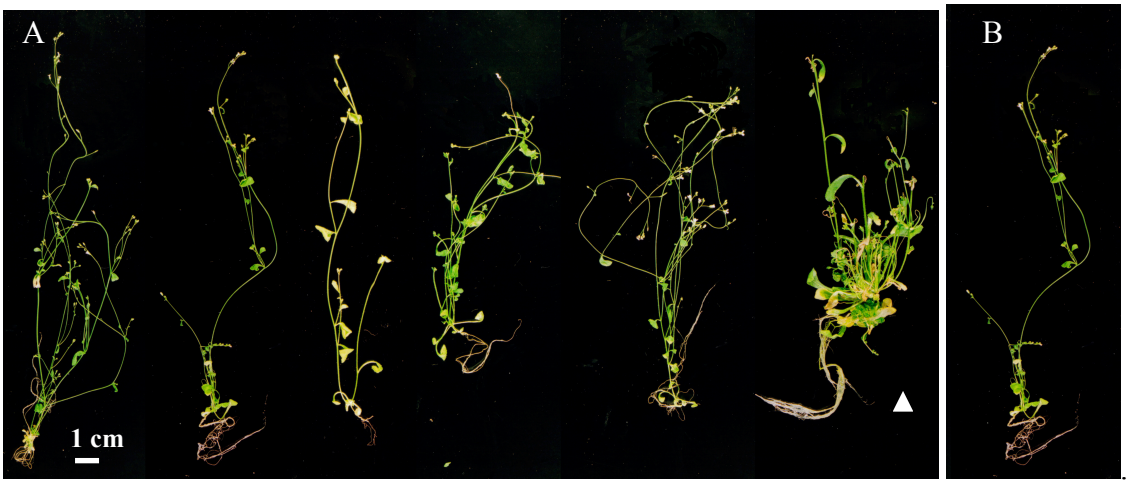


Figure 23. Transgenic T1 *lec1-1* plants transformed with *PmLEC1* showing normal development

A, T1 plants. B, wild type plant. Transgenic plants show normal phenotype, except plant 6 has a more bushy character, arrowhead. Bar = 1 cm

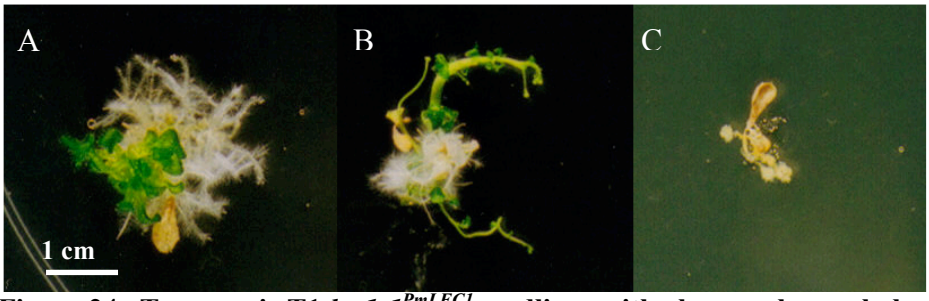


Figure 24. Transgenic T1 *lec1-1*^{PmLEC1} seedlings with abnormal morphology

A, Seedling with multiple cotyledon-like organs. Hairy roots are due to carbenicillin and cefotaxime antibiotics present in the medium. B, plant with stunted lateral organs. C, seedling that turned white and died.

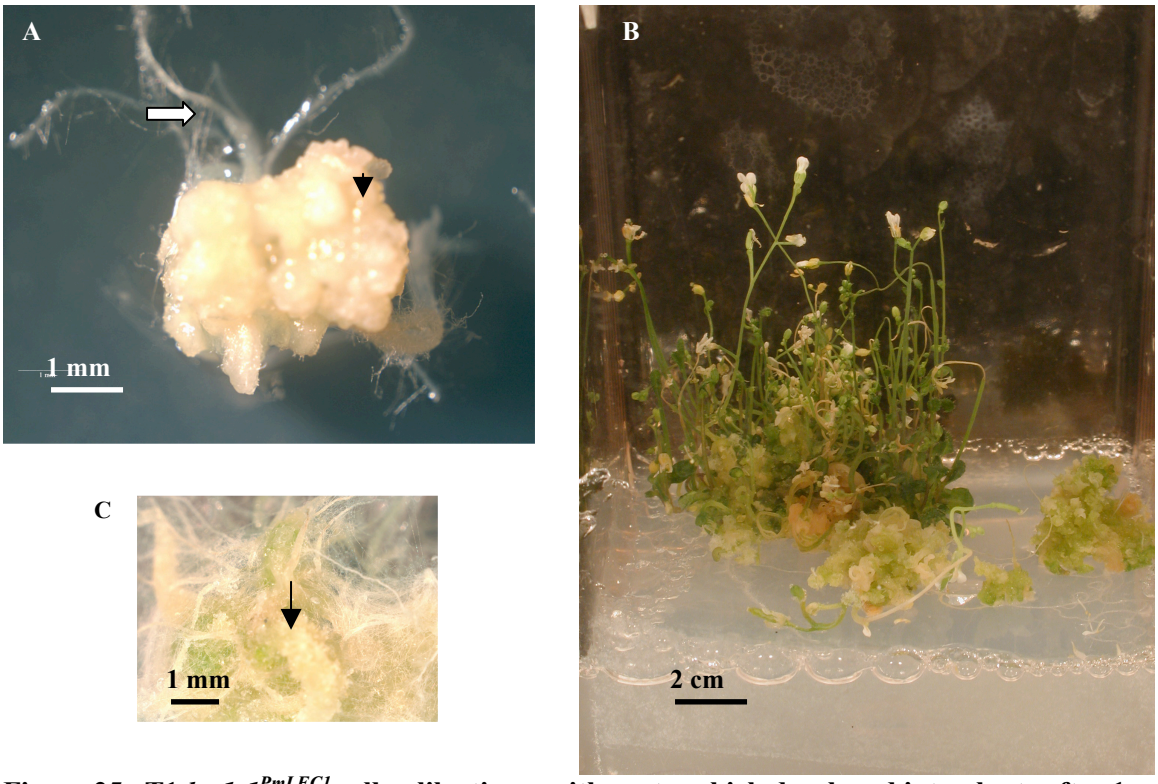


Figure 25. T1 *lec1-1*^{PmLEC1} callus-like tissue with roots, which developed into plants after 1 year of sub-culturing

A, Seedling with normal roots, white arrow, and mass of callus-like tissue, black arrow. B, callus that multiplied on ½ MS medium and gave rise to normal plants. C, Evidence of globular stage somatic embryos, arrow.

3.3.2 The control transformation of *lec1-1* null mutant plants with *AtLEC1* also resulted in inhibition of vegetative growth in T1 seedlings.

The T1 generation of the *lec1-1* null mutant transformed with *AtLEC1* was very similar to that transformed with *PmLEC1*. T1 desiccation-tolerant seed were obtained at a rate of 2% (16/1000). Twenty-five percent of transgenic plants exhibited normal vegetative growth (Figure 26), and the rest were arrested at various stages of development (Figure 27). This is similar to what was reported by Lotan et al. (1998) when the lec1-1 null

mutant was transformed with a 35S::AtLEC1 construct and 10 of 43 plants exhibited vegetative growth. As with the *PmLEC1* transgene, some abnormal seedlings with roots and callus were also observed, some of which showed evidence of embryo-like structures (Figure 28). The callus multiplied on antibiotic medium for over 1 year and then shoots appeared (data not shown).

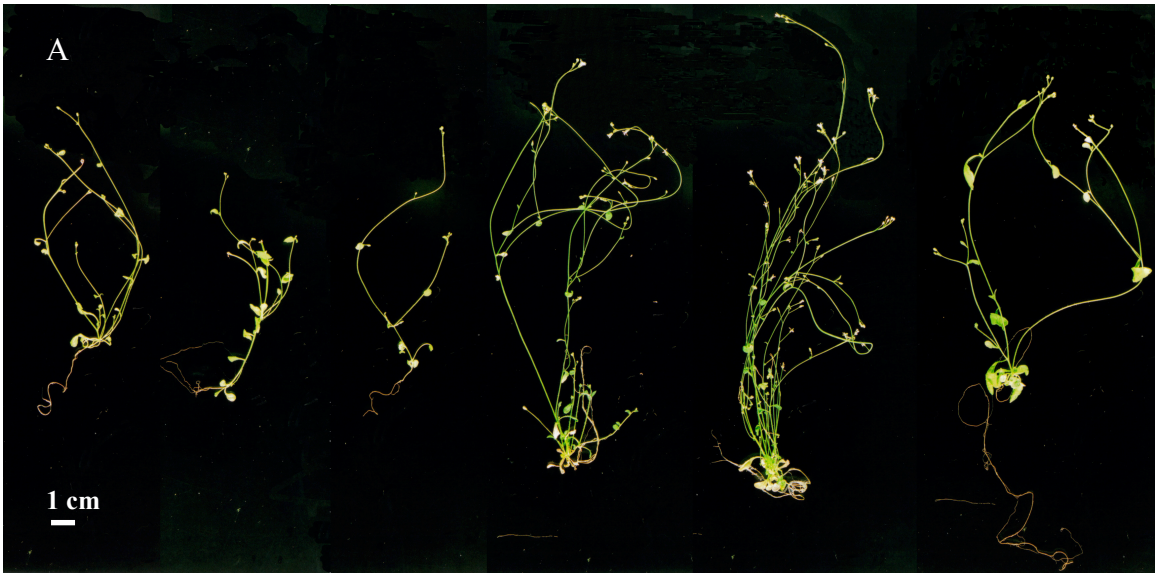


Figure 26. Transgenic T1 *lec1-1*^{AtLEC1} plants showing normal vegetative growth

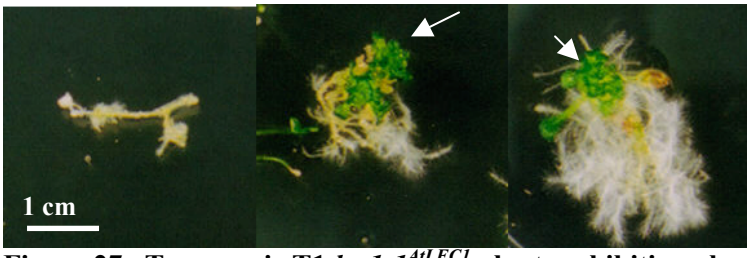


Figure 27. Transgenic T1 *lec1-1*^{AtLEC1} plants exhibiting abnormal morphology

A, seedling that did not develop further and died. B, C seedlings with multiple cotyledon-like organs, indicated by arrows.

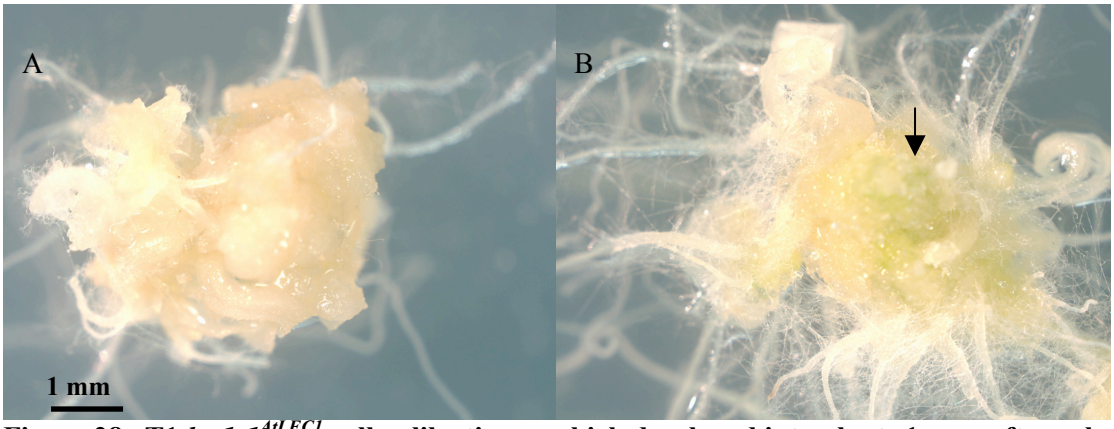


Figure 28. T1 *lec1-1*^{AtLEC1} callus-like tissue, which developed into plants 1 year after sub-culturing

A, callus with smooth surface. B, callus with globular-stage embryo-like structures, black arrow.

3.3.3 Transformation of wild type plants with either *PmLEC1* or *AtLEC1* did not have a visible effect on T1 plant morphology

The first generations transgenic seedlings obtained from transformations of wild type plants with either *PmLEC1* or *AtLEC1* did not show any morphological differences when compared to wild type. Selection on antibiotic medium also resulted in 2% (4/200) transformation efficiency (Figure 29). Plants were transferred to soil in the greenhouse. All transgenic plants produced seed.

Figure 29. Selection of T1 generation of wild type plants transformed with *PmLEC1*
Because all wild type seed had the potential to germinate and kanamycin prevents root formation in plants, only plants expressing neomycin phosphotransferase were able to grow and turn green.

3.3.4 Ectopic expression of *PmLEC1* in a *lec1-1* null mutant background induces embryonic programs and somatic embryo formation in second-generation transgenic seedlings

Out of 24 T1 plants grown in the greenhouse, 14 were sterile and did not produce progeny, while 10 produced seed. The desiccated seed from 6 lines were capable of germination on selective media. This is similar to the work of Lotan et al. (1998) who found that the T2 seed from the majority of plants could not tolerate desiccation even though the presence of the transgene was confirmed by PCR. Transgene silencing was the inferred mechanism of inactivation.

The T2 *lec1-1*^{PmLEC1} seed germinated at a rate of ~25% (13/50), however, only ~10% developed beyond the cotyledon stage. Many seeds also showed evidence of seed coat cracking but reached a standstill. Some seedlings stopped growth shortly after germination. The seedlings from 5 of the 6 lines that germinated resembled masses of

embryo-like structures beyond germination (Figure 30). These structures arose from the cotyledons or the shoot apical meristem region. Numerous somatic embryos at the late globular stage were visible and they grew recurrently, indicating that an embryogenic switch was activated. Recurrent somatic embryogenesis is characterized by the spontaneous appearance of somatic embryos, including complete or partly fused embryo axes and fused cotyledons, and is differentiated from the mode of embryogenesis in which proembryogenic masses (PEM) are propagated (Mordhorst et al., 1998). Luo and Koop (1997) observed similar structures when SE was induced *via* 2,4-D treatment of heart stage zygotic embryos of *Arabidopsis*. The recurrent embryo pattern shown in Figures 30 and 31 strongly resembled the somatic embryo induction pattern of *primordia timing* and *clavata* mutants, which show an enhanced embryogenic phenotype characterized by increased SAM size, embryogenic cluster formation from seedlings treated with 2,4-D, maintenance of embryogenic capacity for 2 years in sub-culture, polycotyly, and at the plant level, increased number of rosette leaves, fasciation and increased number of side shoots (Mordhorst et al., 1998). Green, organized structures were also observed, which were reminiscent of the green embryogenic clusters described by Mordhorst et al. (1998).

Only one of the 6 transgenic lines (T2 *lec1-1*^{PmLEC1}) produced plants that were capable of wild type vegetative growth (Figure 32A). Again, this singularity can be attributed to the strength of the promoter and the propensity of LEC1 to maintain embryogenesis while suppressing vegetative development. The wide range of phenotypes exhibited by this line made it an interesting case study for determining the involvement of PmLEC1 in the phenotype and its effects at the level of transcription. The presence of the transgene

and the absence of *AtLEC1* in the genome were confirmed by PCR (Figure 32B). RNA was isolated from leaves, stems, callus-like growth and roots, but no flowers or siliques

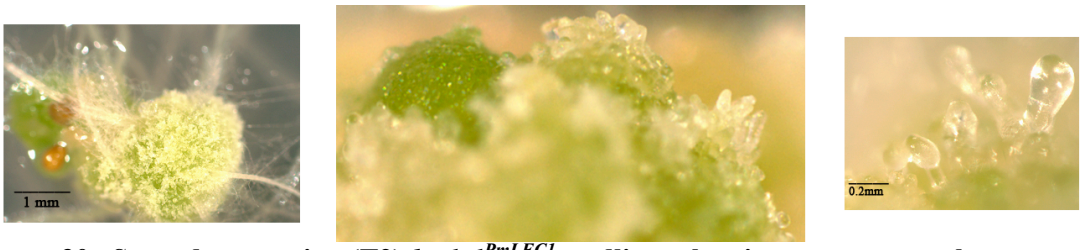


Figure 30. Second generation (T2) *lec1-1*^{PmLEC1} seedlings showing recurrent embryogenesis.

Second generation (T2) *lec1-1*^{PmLEC1} seedlings. Numerous embryo-like structures arose from the cotyledons. Embryo-like structures and green, globular proembryos. Globular structure of embryo-proper and suspensor-like extension (far right).

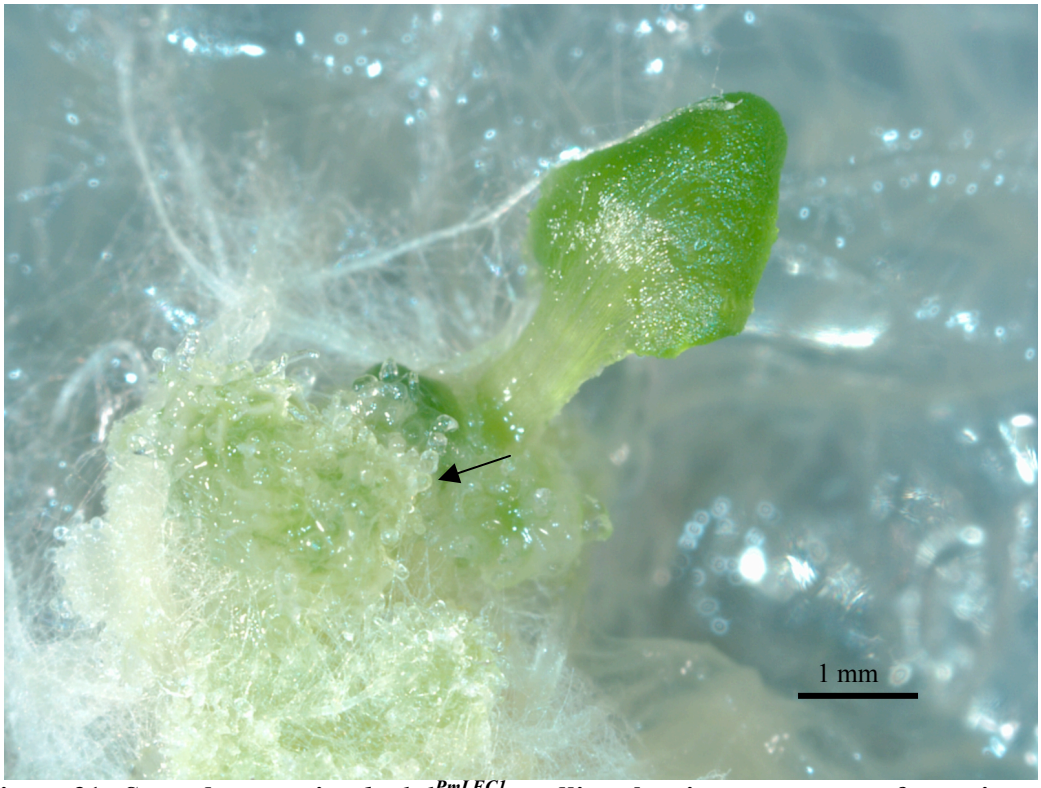


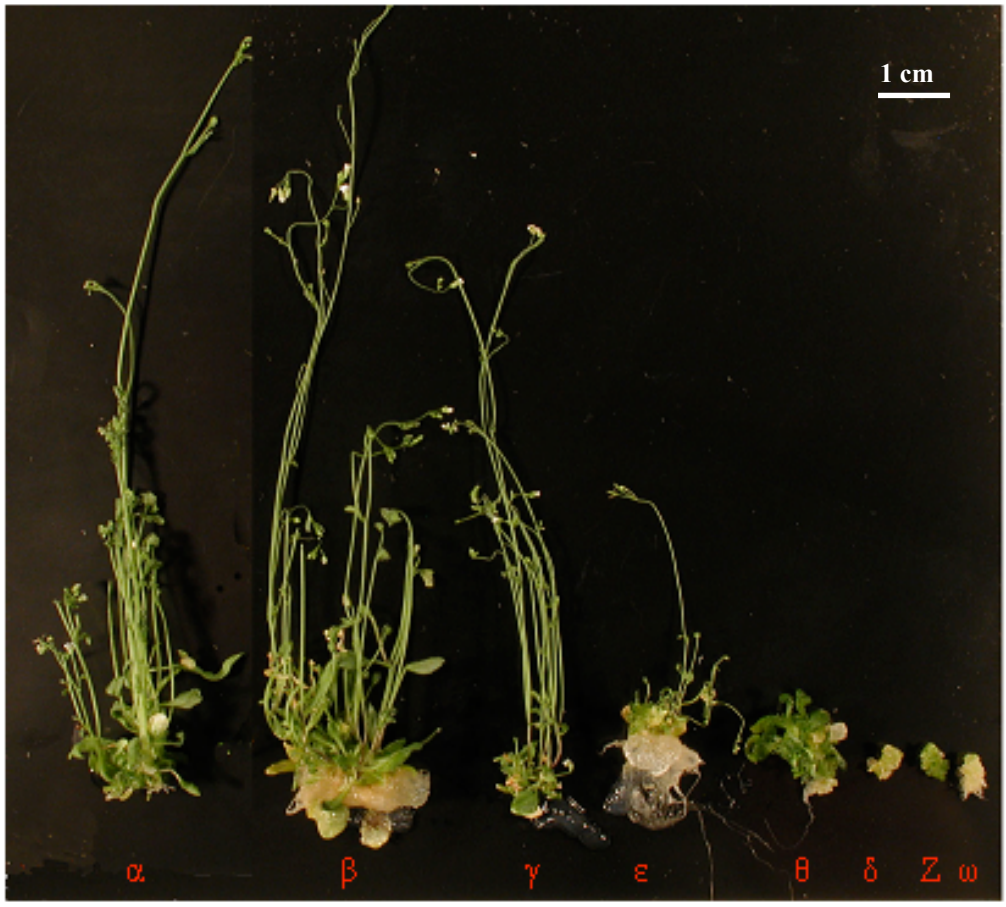
Figure 31. Second generation *lec1-1*^{PmLEC1} seedling showing spontaneous formation of embryo-like structures.

since they could contain developing seed, which express embryo-specific genes and this could confound the results. Plants α and β did not possess any callus-like tissue. The RNA was treated with DNase, and RT-PCR was carried out to assess the expression of the transgene, *PmLEC1*, and embryo-specific genes, *oleosin* and *cruciferin* (Figure 32C). At the genomic level, all seedlings contained the transgene and lacked native *AtLEC1*. All transgenic seedlings accumulated *PmLEC1* transcripts to varying degrees, while neither wild type nor untransformed *lec1-1* null mutant plants expressed *LEC1* genes. Transgenic seedlings did not express *AtLEC1*. Transgenic seedlings with the greatest embryo-like character (ε, θ, δ, Z, ω) expressed *oleosin* and *cruciferin* while those showing normal plant development (α, β) did not express these seed-specific genes (Figure 32, A and C). Plant γ was unusual in that it started out as a callus-like mass followed by normal development (Figure 32A). Both callus and vegetative tissue were utilized in RNA isolation and the high level of *oleosin* expression combined with the low level of *cruciferin* expression and normal vegetative character may represent a transition state in which embryonic programs are being shut down and vegetative growth begins (Figure 32C, lane γ). Wild type and *lec1-1* null mutant plants also did not express *oleosin* and *cruciferin*. The presence of seed-specific transcripts indicates activation of embryonic programs. The accumulation of seed-specific transcripts is normally seen only in developing seed, not after germination. It is clear that the expression of the seed-specific genes caused the embryo-like character of the seedlings. The fact that plants showing normal vegetative development expressed *PmLEC1* but no seed-specific genes indicates that PmLEC1 is not the only factor necessary for induction of seed-specific genes and the embryo-like character. Taken together, our results show that the transgene

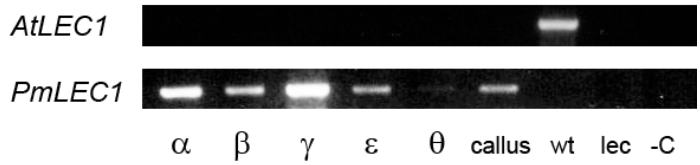
was heritable to the second generation and led to the expression of seed-specific genes and the formation of embryo-like structures on post-embryonic tissues.

Segregation of the transgene in the T2 generation may be a consideration, however, the embryo-like phenotype does not appear to be dosage dependent since *PmLEC1* transgene expression was not proportional to abnormal phenotype. In some cases, comparable levels of transgene expression had opposite results. The PCR part of the experiment was repeated twice for the *PmLEC1 transgene* and the results were identical. Despite showing approximately the same level of transgene expression, plants β, θ and Z showed varying degrees of embryonic program activation, which is based on *cruciferin* and *oleosin* expression (Figure 32C). Plant β did not express *oleosin* or *cruciferin* and showed the greatest amount of normal development. Plant θ, resembling an embryo-like seedling expressed a higher level of *oleosin* and a lower level of *cruciferin*. Plant Z resembled an embryo-like callus and expression of *oleosin* and *cruciferin* was approximately equal. Both plant δ and plant ω exhibited the embryo-like callus phenotype and *oleosin* and *cruciferin* levels were approximately the same in both plants. However, *PmLEC1* expression in plant ω was significantly higher than in plant δ and this did not have a corresponding effect on the observed phenotype. Furthermore, plants θ and ω both showed the higher level of *PmLEC1* transgene expression but cruciferin expression was lower in plant θ. In effect, the observed phenotypes may be better correlated to post-transcriptional gene silencing, and this may be assessed through the level of embryo-specific gene expression. In support of this conclusion, it is expected that 2 or more copies of the transgene would result in stronger silencing of the transgene. Absolute silencing of the transgene after transformation but before completion of seed

A


1 cm
α
β
γ
ε
θ
δ
Z
ω

B


AtLEC1
PmLEC1
α β γ ε θ callus wt lec -C

C

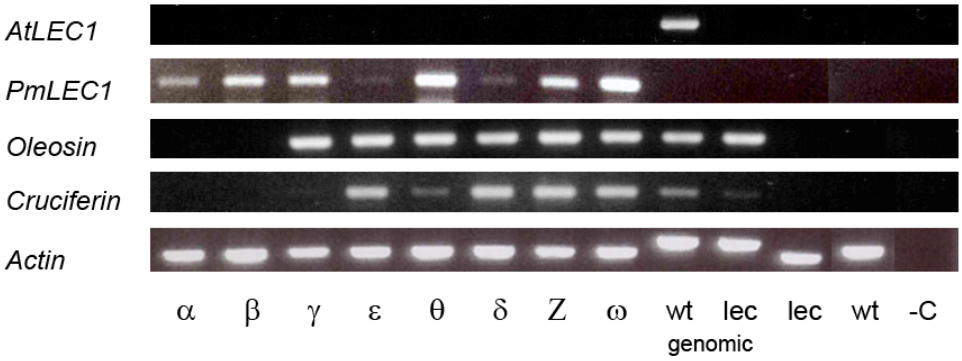

AtLEC1
PmLEC1
Oleosin
Cruciferin
Actin
α β γ ε θ δ Z ω wt lec lec wt -C
genomic

Figure 32. T2 generation of *lec1-1* plants transformed with *PmLEC1* expressed seed-specific genes in seedlings with abnormal morphology

A, The wide range of phenotypes resulting from one transgenic line.

B, Confirmation of genotype and integration of the transgene. α, β, γ, ε, θ represent plants shown in A. Callus, callus-like growth similar to δ, Z, ω. wt, wild type DNA serving as positive control. –C, negative control, PCR without DNA template. Top panel, amplification of *AtLEC1* with 0.02 μg of genomic DNA. Bottom panel, amplification of *PmLEC1* with 0.02 μg of genomic DNA.

C, gene expression analysis. RT-PCR with 0.1 μg DNase I-treated RNA. Letters indicate plants shown in A. wt, wild type plant. lec, lec1-1 null mutant plant. –C, negative control, reverse transcription without RNA followed by PCR.

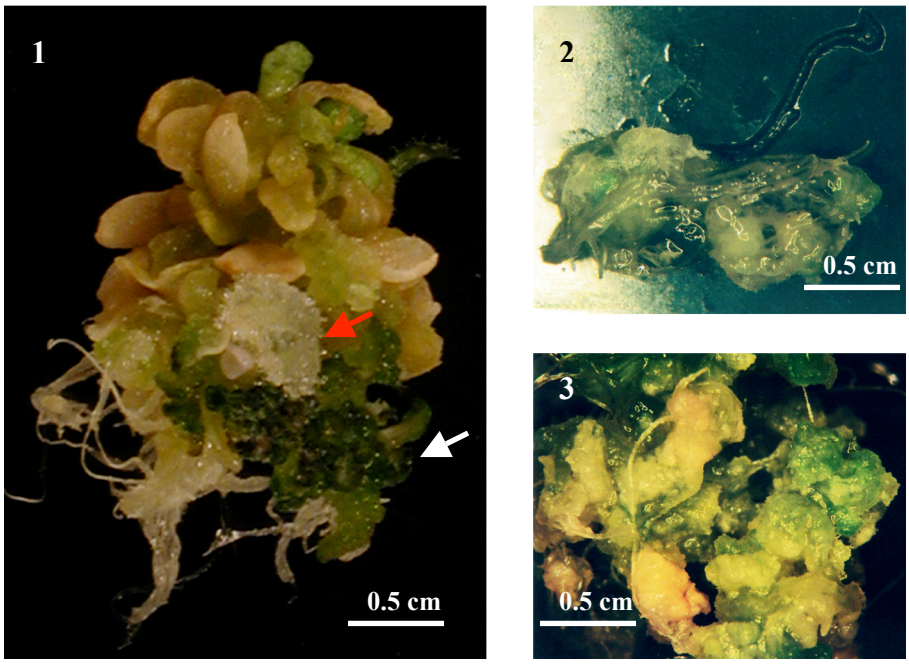
maturation would re-establish the *lec1-1* phenotype and produce non-viable seeds, which would not germinate on any medium, and therefore, this type of plants would not be available for investigation. This may explain the decreasing number of viable transgenic plants obtained in the T2 generation.

3.3.5 Ectopic expression of AtLEC1 in the *lec1-1* null mutant arrests vegetative development but does not guarantee activation of embryonic programs

Of the 24 T1 plants grown on soil, 15 were sterile and did not produce progeny, while 9 flowered and produced seed. Only 3 lines produced seed that were capable of germination, and less than 1% grew vegetatively. Most of the T2 seedlings did not escape the embryo-like character, i.e., normal leaves and flowers never developed (Figure 33A). The presence of the transgene was confirmed by PCR. RT-PCR was performed as described above, to analyze expression of seed-specific genes (Figure 33B). Plant 1 was most unique: a stack of fleshy, leaf-like structures with a section of dark-green cotyledon-

like structures. Surprisingly, a mass of recurrent embryo-like structures was generated from one of the fleshy leaves (Figure 33A, plant 1).

A



B

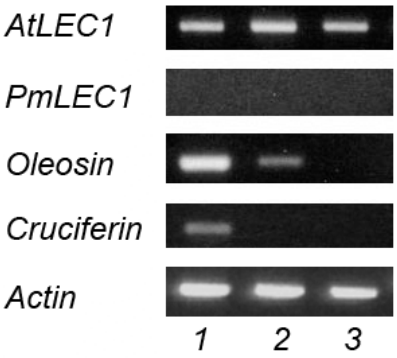


Figure 33. T2 generation of *lec1-1* plants transformed with *AtLEC1*

A, Range of phenotypes from one transgenic line, producing plants 1, 2, and 3. Red arrow, mass of embryo-like structures. White arrow, dark green cotyledon-like structures. B, gene expression analysis by RT-PCR with 0.1 μg DNase I-treated RNA. Lane 1, plant 1 from panel A. Lane 2, plant 2 from panel A. Lane 3, plant 3 from panel A.

As expected, ectopic expression of AtLEC1 resulted in abnormal, embryo-like (Figure 32A, plant 1) and callus-like seedlings (Figure 32A, plant 2 and plant 3), activation of embryonic programs (Figure 32B) and the appearance of embryo-like structures (Figure 32A, plant 1). However, the inconsistency of seed-specific gene expression (Figure 32B) may indicate that some of these callus-like seedlings were not embryogenic as a result of transgene silencing. Thus, expression of AtLEC1 arrested normal vegetative development but did not always induce somatic embryogenesis.

3.3.6 Ectopic expression of PmLEC1 in a wild type background reduces apical dominance but does not activate embryonic programs

If ectopic expression of PmLEC1 results in embryogenesis, then new protocols may be developed to induce PmLEC1 expression *via* medium manipulations or transient ectopic expression *via* particle bombardment. We wished to gain insight into the outcomes of over-expressing LEC1 in wild type plants for the purpose of inducing SE in recalcitrant species. T2 seed from wild type plants transformed with *PmLEC1* were germinated on selective media and 25% possessed Kanamycin resistance. Initially, the seedlings started out as a green callus-like mass but normal vegetative growth resumed within 2-3 weeks. These plants produced leaves and flowers and resembled wild-type plants for the most part, except that they had numerous bolts, which caused them to be bushier (Figure 32A). Only vegetative tissues, stems and leaves, were utilized for molecular analyses. All plants contained endogenous *AtLEC1* and the transgene, *PmLEC1* (Figure 32B). While

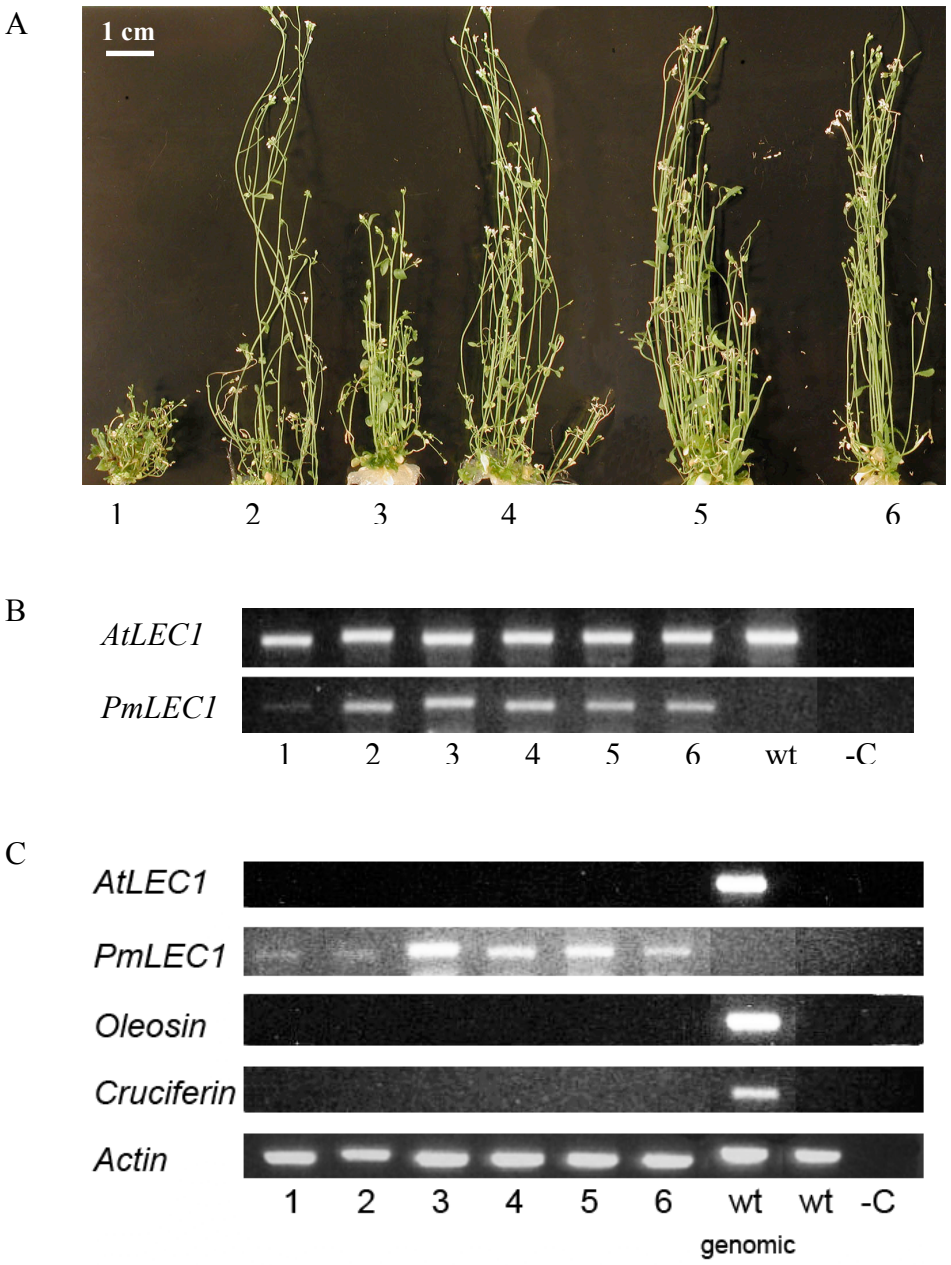


Figure 34. T2 generation of wild type plants transformed with PmLEC1 exhibited a bushy phenotype but did not express embryo-specific genes.

A, T2 progeny from one transgenic line.
B, confirmation of genotype and integration of the transgene. Numbers represent plants shown in A. wt, wild type DNA serving as positive control. –C, negative control, PCR without DNA template. Top panel, amplification of *AtLEC1* with 0.02 μg of genomic DNA. Bottom panel, amplification of *PmLEC1* with 0.02 μg of genomic DNA.
C, gene expression analysis. RT-PCR with 0.1 μg DNase I-treated RNA. Numbers indicate plants shown in A. wt, wild type plant. –C, negative control, reverse transcription without RNA followed by PCR.

all plants expressed the transgene (*PmLEC1*), none expressed *AtLEC1* nor the seed-specific genes oleosin and cruciferin, and no embryo-like morphology was observed (Figure 32C). It is very likely that *PmLEC1* expression led to the bushy character and callus-like mass shortly after germination, but embryonic programs were clearly not activated in the vegetative tissues. In *Arabidopsis*, AtLEC1 is known to prevent precocious germination during embryogenesis, and this may also disrupt apical dominance. Mordhorst et al. (1998) reported a bushy phenotype in the *Arabidopsis primordia timing* mutant, which has an enhanced embryogenic phenotype. The bushy phenotype was attributed to reduced apical dominance resulting in an excessive number of side shoots.

3.3.7 PmLEC1 protein accumulation in transgenic plants correlates with embryo-like character and suppression of vegetative development

Antibodies were raised against a synthetic peptide corresponding to the N-terminus of PmLEC1. Mouse polyclonal immune serum was tested as described. Western analysis was performed to determine the relationship between PmLEC1 accumulation and the observed phenotypes. An antibody capable of detecting PmLEC1 expression will find applications in assessment of embryogenic status of cell cultures.

Total proteins were extracted from the T2 *lec1-1*^{PmLEC1} plants shown in Figure 32 and the T2 *wt*^{PmLEC1} plants shown in Figure 34. To determine the relationship between PmLEC1 protein expression and transgenic plant phenotype, Western blotting was performed as described previously and the results are shown in Figure 35. Strong

immunoreactive bands were observed at ~32.5 kDa in transformed *lec1-1* seedlings showing the greatest embryo-like character and in transformed wild type plants showing reduced apical dominance.

Although all plants expressed *PmLEC1* transcripts to varying degrees (Figures 32 and 34), protein accumulation did not correspond to RNA level. Wild type transformants 1 and 2 (Figure 35B) for example, both showed very low *PmLEC1* gene expression (Figure 34C), however phenotypically, plant 1 showed severe loss of apical dominance and high PmLEC1 protein accumulation (Figure 35B). It is evident that PmLEC1 protein accumulation is the best indicator of phenotypic effects, however, further Western analyses will provide required information. For example, PmLEC1 protein expression may have been higher when the germinating seedlings were a callus-like mass. An incremental analysis of PmLEC1 expression from the time of germination to plant maturation would better show the molecular basis of the phenotype and how PmLEC1 accumulation influences the phenotype. Since all wild type transformants exhibit a bushy phenotype, it is conceivable that PmLEC1 protein expression was higher when the seedlings were younger. Anti-AtLEC1 antibodies are not presently available and none of the transgenic plants showed *AtLEC1* RNA expression.

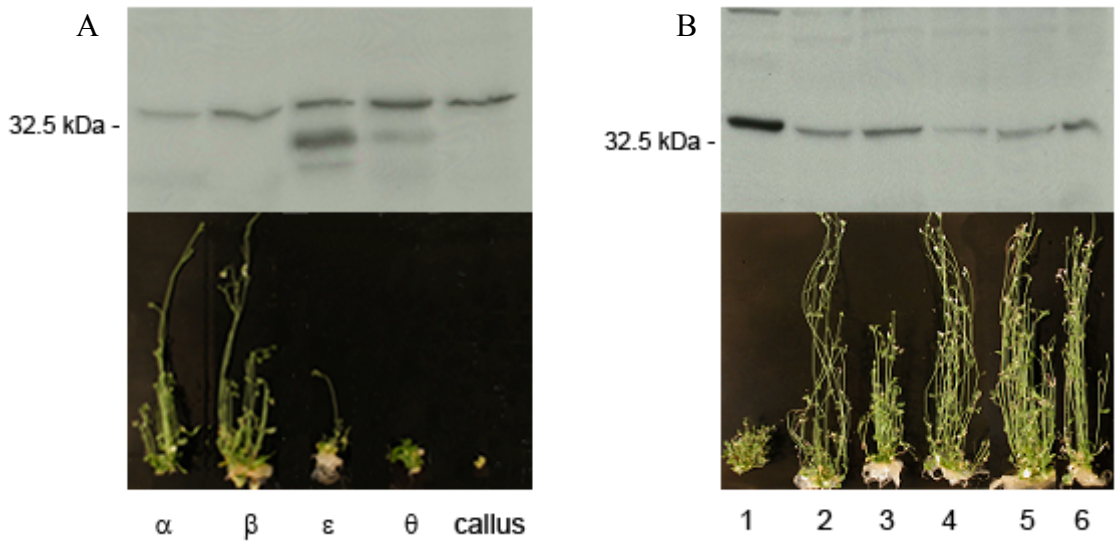


Figure 35. Western blot analysis of T2 plants ectopically expressing PmLEC1.

Total proteins (20 µg) from A, second generation transgenic *lec1-1* null mutant plants transformed with *PmLEC1* and B, second generation wild type plants transformed with *PmLEC1*, were separated by SDS-PAGE and transferred to a PVDF membrane. The PVDF membrane was incubated with polyclonal anti-PmLEC1 serum diluted 1:1000 for 1 h. A, callus represents a callus-like seedling from the *lec1-1*^{PmLEC1} T2 line.

3.4 DISCUSSION

3.4.1 Douglas-fir *PmLEC1* is a functional homologue of *Arabidopsis LEC1*

By complementation analysis we have shown that *PmLEC1* functions in a similar manner to *AtLEC1*. When ectopically expressed in the *lec1-1* null mutant, both genes are capable of inducing embryonic programs and somatic embryogenesis. In the T2 generation, both genes result in seedlings with abnormal morphology, representing an intermediate state between embryonic and vegetative development. These results mirror the work of Lotan et al. 1998. One difference however, is that in the T2 generation *AtLEC1* transformants never escaped the embryo-like character and never produced

progeny. This could be due to the fact that the 35S-35S-AMV promoter is very strong and *AtLEC1* has a more exact function in *Arabidopsis*, while *PmLEC1*, being non-species-specific, is leaky. *AtLEC1* induces embryo maturation programs and suppresses vegetative development (Lotan et al., 1998), and therefore, strong, constitutive *AtLEC1* expression prevented normal vegetative development. At the amino acid level, PmLEC1 shows only 56% identity to AtLEC1 and its interaction with other transcription factor subunits in *Arabidopsis* is not expected to be as strong or specific.

Another notable difference is the timing of somatic embryo generation. In *PmLEC1* transformants, somatic embryo formation started from the cotyledons shortly after root formation. However, in *AtLEC1* transformants, somatic embryogenesis took place after multiple cotyledon-like organs had formed. Again, this difference can be attributed to the primary amino acid sequence and the expectation that AtLEC1 and PmLEC1 have slightly different modes of action.

Ectopic expression of PmLEC1 in wild type plants did not have the same dramatic effect as in the *lec1-1* null mutant. The T1 generation was no different from the wild type and even though the transgene was expressed, it did not result in phenotypic differences. In the T2 generation however, transgenic plants were bushier than wild type and a callus-like mass formed from the cotyledons shortly after germination. *PmLEC1* transcripts accumulated in the seedlings but the seed-specific RNAs, oleosin and cruciferin, did not. The presence of the callus-like masses suggest that within the germinating seed, PmLEC1 interacted with other factors that were present and stimulated embryonic programs. The appearance of shoots, the growth of plants and the absence of seed-specific RNAs indicate that endogenous AtLEC1 suppressed the embryonic programs. The only

difference between wild type and *lec1-1* null mutant plants is the presence of *AtLEC1* and its intact promoter sequence in the wild type genome. One way to explain the bushiness of the wt^{PmLEC1} T2 plants is that multiple fused somatic embryos formed from the callus-like masses, and these germinated and gave rise to plants. In their work with Arabidopsis *LEC1*, Lotan et al. (1998) observed structures resembling fused wild-type embryos on the leaves of transgenic plants by scanning electron microscopy.

3.4.2 PmLEC1 may Function Prior to Embryogenesis

The callus-like mass formation in the T2 generation of wild type plants but not T1, shows that the transgene was more likely to have a phenotypic effect on the second generation, and opens the possibility that LEC1 has activity prior to flowering and fertilization and its interaction with genes at that time has significant long-term effects. There are 2 reasons why we claim this. First, in Douglas-fir PmLEC1 RNA and protein expression are observed in the ovule. Secondly, it is not clear that *AtLEC1* is not expressed in flower tissues (Kwong et al., 2003) due to poor quality Northern blotting. In addition, Cassey and Lindsey (2006) working with the *turnip* mutant of *Arabidopsis*, show evidence that *AtLEC1* RNA is expressed in control seedlings at very low levels, and this is in contrast to findings of Lotan et al. (1998).

At the time of floral dip, developmental events have already taken place but LEC1 was not present in T1 ovules. However, for the T2 generation, LEC1 was present throughout development and available for interaction with other transcription factor subunits and

activating transcription of a specific set of genes. Comparison of the *lec1-1*^{PmLEC1} T1 (Figure 24) and T2 (Figure 30) generations, proves this point. It appears that when LEC1 was not present before embryogenesis, as in T1, which was mostly characterized by developmental arrest (Figure 24), its embryogenic effect was not as strong as in the T2 generation, where recurrent somatic embryogenesis was observed (Figure 30).

3.4.3 LEC1 cannot induce *de novo* embryogenesis by itself

Some of the second-generation *lec1-1* plants containing the *PmLEC1* transgene exhibited normal vegetative development (α and β in Figure 32A), as did all wild type T2 plants containing the transgene (Figure 34). While all these "normally growing" plants expressed the transgene, the absence of *cruciferin* and *oleosin* transcripts (Figures 32C and 34C) indicated that seed maturation programs were not induced. Moreover, all the transformants with a wild type background started from a mass of callus-like cells, which eventually led to shoot and root formation, instead of following the normal developmental pathway, consisting of germination followed by shoot growth.

The expression of *PmLEC1* in wild type T2 plants shows that transgene silencing did not occur, and that the transgene by itself could not induce embryogenesis, stressing the need for additional factors that are present in seed tissue. The fact that the transgenic plants with the greatest amount of normal plant morphology expressed *PmLEC1* but not *oleosin* and *cruciferin* transcripts, combined with the fact that ectopic expression of PmLEC1 in mature and germinating seed resulted in callus-like masses, embryo-like seedlings and spontaneous formation of somatic embryos, suggest that the interaction of

LEC1 with other factors that are present in the seed leads to the embryo-like character and embryogenesis, and that LEC1 genes cannot start *de novo* embryogenesis on their own.

The first reason for this conclusion is that *lec1-1* T2 generation similar amounts of both PmLEC1 RNA expression and protein accumulation were observed in both abnormal embryo-like seedlings and plants with normal appearance. To monitor embryonic program induction, RT-PCR was performed to assess *PmLEC1*, *oleosin* and *cruciferin* transcript levels. Some embryo-like seedlings expressed lower levels of *PmLEC1* but *oleosin* and *cruciferin* expression was not proportional to *PmLEC1* levels. In plant ε of the T2 generation of *lec1-1*^{PmLEC1}, *PmLEC1* expression was low, while *cruciferin* and *oleosin* expression were high (Figure 32C). Conversely, in plant θ, *PmLEC1* expression was high but *cruciferin* expression was very low (Figure 32C). Overall, PmLEC1 protein accumulation does appear to be higher in *lec1-1* T2 plants that are expressing both oleosin and cruciferin (Figures 32, 35). The *wt*^{PmLEC1} T2 plants also showed varying degrees of *PmLEC1* expression and while this was comparable to the *lec1-1*^{PmLEC1} T2 seedlings in some cases (plant θ in Figure 32C, plant 3 in Figure 34C), none of the wild type plants expressed *cruciferin* and *oleosin* (Figure 34C). Also, plant 1 of the *wt*^{PmLEC1} T2 generation accumulated an extreme amount of PmLEC1 protein and exhibited a severe phenotype, however *cruciferin* and *oleosin* were not expressed in this plant (Figures 34, 35). Hence, neither a high abundance of *LEC1* transcripts nor excessive LEC1 protein levels are sufficient for induction of embryonic programs.

A second reason to conclude that PmLEC1 interacts with seed factors, before or during embryogenesis, to induce embryonic programs is that the wild type transformants started

out as masses, implying that PmLEC1 interacted with other factors to start the callus-forming process. Then something occurred to suddenly stop callus proliferation and enable vegetative growth. Endogenous AtLEC1, which was also present, could have mediated this stoppage in wild type plants. The conclusion that we draw here is that at a certain stage, AtLEC1 or its promoter sequence directs the suppression of the other factors necessary for induction of embryonic programs. This explains the switch from callus-like growth to normal vegetative growth. The native untranslated regions of the *AtLEC1* upstream sequence may have a role in this switch. The presence of endogenous AtLEC1 enabled plants to escape the callus-like growth and enter normal vegetative growth, signifying that perhaps LEC1 has a role in directing vegetative growth.

3.4.4 The 5' UTR intron could be a transposable element that coordinates expression of embryogenesis-related genes

Recently, it was shown that in the *turnip* (*tnp*) mutant of *Arabidopsis*, the promoter region of *AtLEC1* is deleted and results in a gain-of-function mutation for AtLEC1 (Casson and Lindsey, 2006). Not only is *AtLEC1* upregulated in this mutant, but *P1P5K*, which is adjacent and downstream of *AtLEC1*, is also upregulated, and the authors suggest that the *AtLEC1* promoter region contains a coordinating mechanism for the repression of both genes (Cason and Lindsey 2006). Further, Zhang et al. (1996) show evidence that DNA sequences located 1200 bp upstream of the *Arabidopsis isocitrate lyase* gene have a major role in its activation, and that the 3500 bp sequence upstream of the gene are sufficient to specify expression at different developmental stages. This

could be one reason why the *PmLEC1* gene has such a large 5' UTR intron and why it integrates so many signals. The PmLEC1 gene is approximately 800 bp and thus the promoter sequence is within 3500 bp of 2 or 3 genes that it may regulate. This accurately explains the results we observed with the transgenic wild type plants. The expression of factors with the potential to induce embryogenesis is strictly controlled in the presence of endogenous *AtLEC1* promoter and gene.

If the function of the 5' UTR intron is to coordinately regulate genes that LEC1 interacts with at various stages of embryogenesis and then suppress them when embryogenesis is complete, then the energetic expenditure of transcribing an intronic nucleotide sequence that is twice as large as the PmLEC1 coding sequence has merits because it ensures proper plant development. It is likely that more than 2 or 3 genes are regulated by the coordinating mechanism found in the promoter and these genes may be present on different chromosomes.

How can the *PmLEC1* promoter achieve regulation of multiple genes? The 5' UTR intron may have the ability to act as a trans-activator after excision. Transposable elements make up a considerable portion of higher plant genomes (Bundock & Hooykaas, 2005) and a theory regarding introns is that they originated from transposable elements. Once the transcription complex forms on the promoter and the intron is excised, the stability of the complex enables it to interact with other genes and trans-activate them. If this hypothesis was found to be true, it could explain the apparent activation of multiple processes leading to recurrent somatic embryogenesis, as shown in Figure 31.

3.4.4 Future directions

To further understand the function of LEC1 in embryogenesis, it will be necessary to determine which genes are activated and which are suppressed by LEC1. This will most easily be achieved with microarrays. Further, there are indications that LEC1 has different roles during different stages of embryogenesis and each major stage should be analyzed. The interactions of LEC1 with other seed-specific factors must be elucidated to show differential effects PmLEC1 on development.

This research has shown that transgenic plants with normal plant morphology expressed *PmLEC1* but not *oleosin* and *cruciferin*, and ectopic expression of PmLEC1 in mature and/or germinating seed resulted in callus-like masses, embryo-like seedlings and somatic embryos. These findings suggest that the interaction of LEC1 with seed-specific factors leads to leads to embryo-like character and embryogenesis, and that LEC1 expression in vegetative tissues cannot start *de novo* embryogenesis on its own. However, the regulatory sequence upstream of *LEC1* may play a role in the suppression of the factors required for induction of embryogenesis. The idea that seed specific factors enhance or promote embryogenesis is not new. In instances when induction protocols are not consistently successful, the addition of these factors acts like an activating switch and it is conceivable that they act on the promoter sequence.

In conifer and angiosperm embryogenesis, arabinogalactan proteins (AGPs) are considered biological signals, which promote somatic embryogenesis (Filonova et al., 2000; van Hengel et al., 2001). In conifer embryogenesis, it was discovered that non-

embryogenic cultures could be made embryogenic *via* the addition of seed extracts to the culture medium and further work identified AGPs as the necessary components (Egertsdotter and von Arnold, 1995; Filonova et al., 2000). It is possible that AGPs transmit signals that lead to the expression of specific genes that promote embryogenesis.

A few ideas from recent research provide examples of additional factors, which might interact with LEC1. Because LEC1, also known as HAP3 or NF-YB, is a subunit of a transcription factor, it is possible that it interacts with various subunits, not only those that form a CCAAT box-binding complex. Recently, the *Oryza sativa* MADS18 transcription factor was shown to interact with a rice HAP3 subunit, OsNF-YB1, as well as the mouse NF-YB (Masiero et al., 2002). OsMADS18 plays a role in the transition from vegetative growth to flowering (Masiero et al., 2002). Evidence is given for the formation of 2 different complexes: OsMADS18/OsNF-YB1/HsNF-YC and OsMADS18/OsMADS6/OsNF-YB1 (Masiero et al., 2002). Neither of these complexes is capable of CCAAT box-binding and this suggests that NF-YB can act through mechanisms other than the CCAAT box (Masiero et al., 2002). Moreover, in the absence of NF-YA, NF-YB also interacts with TFIID, TATA box-binding complex, some HFM TAF_{II}s.

Finally, the promoter/5' UTR sequence of *PmLEC1* contains many binding sites for diverse transcription factors. Some of these are embryo stage-specific and therefore, can only modulate *PmLEC1* expression during specific stages of embryogenesis. It is expected that AGL15 has an important role in the regulation of PmLEC1 since the 5' UTR region contains 3 AGL15 binding sites. AGL15 is also a MADS box protein and it is expressed at high levels before fertilization until seed desiccation in *Arabidopsis*,

maize and *Brassica* (Perry et al., 1996). After germination, it is expressed at lower levels in the shoot apical meristem (Harding et al., 2003). The AGL15 expression pattern is very similar to *PmLEC1*and *AtLEC1*, suggesting that it has a strong influence on *LEC1* expression. Because MADS box proteins have pivotal roles in critical developmental events (Perry et al., 1996), and ectopic expression of AGL15 maintains and enhances embryogenic potential (Harding et al., 2003), AGL15 could be one of the seed-specific factors which regulates LEC1.

3.4.3 Applications to Biotechnology

The propensity of PmLEC1 to induce embryonic programs in post-embryonic tissues could be a solution to rescuing non-embryogenic conifer cultures, initiating embryogenesis from recalcitrant seed and leading to new somatic embryogenesis protocols. Transient expression of PmLEC1 may be achieved *via* particle bombardment of tissues in culture or individual seed. Once embryogenesis is initiated and cells begin to divide and multiply, transgene ejection is common. The absence of the transgene can be easily confirmed by PCR, and consequently, there will not be a need for deregulation of transgenic trees. Transient expression of PmLEC1 can thus lead to the initiation of somatic embryogenesis and the final production of non-transgenic plants. Many successful protocols exist for the maintenance of proliferation and development of mature somatic embryos. Initiation of embryogenesis in conifers is still the greatest challenge.

A second application to biotechnology is that PmLEC1 could increase the productivity of crops or plants. PmLEC1 over-expression may be employed to increase feed sources

and biomass. The bushy character of wild type plants expressing PmLEC1 could be exploited to produce plants with more leaves or more pulp.

Overall Conclusions and Future Directions

The *PmLEC1* gene isolated from Douglas-fir is an ortholog of *Arabidopsis LEC1*. Its expression peaks in early embryogenesis, it has similar activity to AtLEC1 and its ectopic expression results in activation of embryonic programs and the formation of embryo-like structures. The absence of PmLEC1 transcripts and proteins in mature tissues indicates that this gene is embryo-specific. *PmLEC1* is characteristic of early embryogenesis and one of the first embryo regulatory genes characterized in conifers. Future work producing a monoclonal antibody, examination of protein expression profiles in embryogenic and non-embryogenic cultures as well as a greater range of developmental stages will better delineate PmLEC1 protein expression and may be applied to identification and selection of embryogenic cultures. PmLEC1 expression profiles may also provide a trouble-shooting tool when cell cultures become developmentally blocked. CCAAT DNA binding assays and yeast 2 hybrid assays will identify the other subunits or transcription factors with which PmLEC1 interacts and begin to define the genetic mechanism of proper embryo formation. This may lead to the discovery of other factors that are critical to conifer embryogenesis.

The promoter sequence revealed the identity of several transcription factors with the potential to regulate *PmLEC1* expression. The presence of binding sites for transcription factors already characterized in angiosperms, implies that their genes will be present in conifers and broadens the knowledge base of embryogenesis by pointing to genes that are involved in early embryogenesis and essential to embryogenesis prior to fertilization. The use of cross-linkers to capture the complexes bound to the promoter at each stage of

embryogenesis will provide information about *PmLEC1* developmental regulation, will reveal genes that are critical to stage-specific embryogenic activities and will enable the establishment of relationships between genetic or molecular events and developmental or metabolic events.

In addition, the presence of regulatory elements for hormonal, stress, developmental and light signals suggests that these cues will be instrumental in determining future methods of PmLEC1 induction. The present work shows that 2,4-epibrassinolide, sorbitol, mannitol and NaCl may stimulate *PmLEC1* expression in mature tissues. This was an introductory experiment to identify direct elicitors of *PmLEC1*. However, SE protocols normally involve exposure of tissues to induction conditions for several weeks and induction experiments could be modified to analyze *PmLEC1* levels for longer periods of time and also to transfer tissues from stress-containing media to 2,4-D medium and analyze gene expression at weekly intervals. Concomitant QPCR analysis of genes such as *AGL15*, *CLV*, *PT*, *ABI3*, *VP1*, *WUS*, *AGPs*, *LCOs* and *chitinases* in addition to *oleosin* and *cruciferin* will result in a thorough understanding of tissue responses to the treatments and will possibly identify the exact complement of molecules that could induce SE when present in culture medium.

Furthermore, because none of the compounds tested resulted in drastic up-regulation of PmLEC1, its expression is tightly regulated and it will require more than a simple induction treatment. A combination of treatments compliant to the numerous promoter elements may induce PmLEC1 expression and consequent embryogenesis. Finally, previously unknown response elements remain to be identified within the *PmLEC1* promoter sequence. For example, regulatory elements for brassinosteroids have not been

identified, yet 2,4-epibrassinolide was a strong inducer of *PmLEC1* expression in mature seed.

Functional analysis of PmLEC1 *via* complementation of the *lec1-1* null mutant and overexpression in wild type *Arabidopsis* confirmed that PmLEC1 is critical to embryogenesis and induced embryonic programs, however overexpression results suggest that PmLEC1 must act in concert with other seed factors to induce embryogenesis and that the promoter sequence plays a role in silencing other genes that are necessary for induction of embryogenesis. Ectopic expression of *PmLEC1* rescued the *Arabidopsis lec1-1* null mutant, led to the formation of embryo-like structures and activated embryonic programs. Overexpression of *PmLEC1* in wild type *Arabidopsis* resulted in a bushy phenotype, thus highlighting the role of LEC1 in suppressing apical dominance. Although *PmLEC1* expression was observed in vegetative tissues, the lack of *cruciferin* and *oleosin* expression demonstrated that embryonic programs were not activated. The role of the *PmLEC1* promoter in embryogenesis can be deduced in future transformation experiments with *PmLEC1* under the control of its own promoter and the combined 2x35S-AMV and *PmLEC1* promoter. Additional work with overexpression in wild type *Arabidopsis* and assessment of embryonic gene expression at shorter time intervals will show the stages at which LEC1 overexpression stops inducing expression seed specific genes thus facilitating the discovery of genetic/molecular events that inhibit embryogenesis.

Finally, the combined results of the proposed research will find applications in conifer somatic embryogenesis. Perhaps the best solution for inducing embryogenesis would be transient expression of *PmLEC1 via* particle bombardment of young vegetative buds,

which are then incubated in extracts of unfertilized ovules or gametophytic tissue at stages that are found to be conducive to SE induction in wild type plants. Since the superior qualities of the donor tree are already known and the genotype is constant then the 20 or so years worth of field testing, which is currently necessary for new hybrids induced into somatic embryogenesis, will be eliminated. Transient gene expression frequently leads to transgene ejection and once embryogenesis is initiated and further developmental stages are induced, the absence of the transgene can be confirmed by routine PCR. Somatic seedling may be planted in forests or orchards without the need for deregulation of a genetically modified species. This will lead to gains in superiority of forests and increased biodiversity when rare genotypes are cloned.

Bibliography

Ahuja MR (2000) Genetic engineering in forest trees: state of the art and future perspectives. *In* SM Jain, SC Minocha, eds, Molecular Biology of Woody Plants, Vol. 1, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 31-49

Alemanno L, Devic M, Niemenak N, Sanier C, Guilleminot J, Rio M, Verdeil J-L, Montoro P (2008) Characterization of *leafy cotyledon1-like* during embryogenesis in *Theobroma cacao* L. *Planta* 227:853-866

Allen GS, Owens JN (1972) The life history of Douglas fir. Environment Canada, Canadian Forestry Service, Ottawa

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410

Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, Maclean DJ, Ebert PR, Kazan K (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. *The Plant Cell* 16: 3460-3479

Antoine M, Kiefer P (1998) Functional characterization of transcriptional regulatory elements in the upstream region and intron 1 of the human S6 ribosomal protein gene. *Biochemistry Journal* 336: 327-335

Apuya NR, Yadegari R, Fischer RL, Harada JJ, Goldberg RB (2002) *RASPBERRY3* gene encodes a novel protein important for embryo development. *Plant Physiology* 129**:** 691-705

Argüello-Astroga GR, Herrera-Estrella LR (1996) Ancestral multipartite units in light-responsive plant promoters have structural features correlating with specific phototransduction pathways. *Plant Physiology* 112: 1151-1166.

Attree SM (2003) Increasing levels of growth regulator and/or water stress during embryo development. United States Patent, US 6,627,441 B1

Banno H, Ikeda Y, Niu Q-W, Chua N-H (2001) Overexpression of *Arabidopsis ESR1* induces initiation of shoot regeneration. *The Plant Cell* 13: 2609-2618

Bellorini M, Lee DK, Dantonel JC, Zemzoumi K, Roeder RG, Lazlo T, Mantovani R (1997) CCAAT binding NF-Y-TBP interactions: NF-YB and NF-YC require short domains adjacent to their histone fold motifs for association with TBP basic residues. *Nucleic Acids Research* 25: 2174-2181

Belmonte MF, Donald G, Reid DM, Yeung EC, Stasolla C (2005) Alterations of the glutathione redox state improve apical meristem structure and somatic embryo quality in white spruce (*Picea glauca*). *Journal of Experimental Botany* 419: 2355-2364

Belmonte MF, Macey J, Yeung EC, Stasolla C (2005) The effect of osmoticum on ascorbate and glutathione metabolism during white spruce (*Picea glauca*) somatic embryo development. *Plant Physiology and Biochemistry* 43: 337-346

Belmonte MF, Stasolla C (2006) Applications of DL-buthionine-[S,R]-sulfoximine deplete cellular glutathione and improve white spruce (*Picea glauca*) somatic embryo development. *Plant Cell Reports* 26: 517-523

Belmonte MF, Tahir M, Schroeder D, Stasolla C (2007) Overexpression of *HBK3*, a class I *KNOX* homeobox gene, improves the development of Norway spruce (*Picea abies*) somatic embryos. *Journal of Experimental Botany* 58: 2851-28-61

Boutilier K, Offringa R, Sharma VK, Kieft H, Ouellet T, Zhang L, Hattori J, Liu C-M, van Lammeren AAM, Miki BLA, Custers JBM, van Lookeren Campagne M. (2002) Ectopic expression of *BABY BOOM* triggers a conversion from vegetative to embryonic growth. *The Plant Cell* 14**:** 1737-1749

Bozhkov PV, Filonova LH, von Arnold S (2002) A key developmental switch during Norway spruce somatic embryogenesis is induced by withdrawal of growth regulators and is associated with cell death and extracellular acidification. *Biotechnology and Bioengineering* 77: 658-667

Bozhkov PV, Suarez MF, Filonova LH, Daniel G, Zamyatnin Jr AA, Rodriguez-Nieto S, Zhivotovsky B, Smertenko A (2005) Cysteine protease mcII-Pa executes programmed cell death during plant embryogenesis. *Proceedings of the National Academy of Sciences* 102:14463-14468

Bozhkov PV, von Arnold S (1998) Polyethylene glycol promotes maturation but inhibits further development of *Picea abies* somatic embryos. *Physiologia Plantarum* 104: 211-224

Bradford MM (1976) A rapid and sensitive method for the auantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Analytical Biochemistry* 72: 248-254.

Braybrook SA, Stone SL, Park S, Bui AQ, Le BH, Fischer RL, Goldberg RB, Harada JJ (2006) Genes directly regulated by LEAFY COTYLEDON2 provide insight into the control of embryo maturation and somatic embryogenesis. *Proceedings of the National Academy of Sciences* 103: 3468-3473

Bundock P, Hooykaas P (2005) An Arabidopsis hAT-like transposase is essential for plant development. *Nature* 436: 280-284

Busk PK, Jensen AB, Pagès M (1997) Regulatory elements in vivo in the promoter of the abscisic acid responsive gene *rab17* from maize. *The Plant Journal* 11: 1285-1295

Busk PK, Pagès M (1997) Protein Binding to the Abscisic Acid-Responsive Element is Independent of VIVIPAROUS1 in Vivo. *The Plant Cell* 9: 2261-2270

Cairney J, Xu N, MacKay J, Pullman J (2000) Transcript profiling: a tool to assess the development of conifer embryos. *In Vitro Cellular and Development Biology – Plant* 36:155-162

Cairney J, Zheng L, Cowels A, Hsiao J, Zismann V, Liu J, Oujang S, Thibaud-Nissen F, Hamilton J, Childs K, Pullman GS, Zhang Y, Oh T, Buell CR (2006) Expressed sequence tags from loblolly pine embryos reveal similarities with angiosperm embryogenesis. *Plant Molecular Biology* 62: 485-501

Carrari F, Frankel N, Lijavetzky D, Benech-Arnold R, Sánchez R, Iusem ND (2001) The TATA-less promoter of *VP1*, a plant gene controlling seed germination. *DNA Sequence* 12: 107-114

Casson SA, Lindsey K (2006) The *turnip* mutant of Arabidopsis reveals that *LEAFY COTYLEDON1* expression mediates the effects of auxin and sugars to promote embryogenic cell identity. *Plant Physiology* 142: 526-541

Chatthai M, Kaukinen KH, Tranbarger TJ, Misra S (1997) The isolation of a novel metallothionein-related cDNA expressed in somatic and zygotic embryos of Douglas fir: regulation by ABA, osmoticum, and metal ions. *Plant Molecular Biology* 34: 243-254

Chatthai M, Misra S (1998) Sequence and expression of embryogenesis-specific cDNAs encoding 2S seed storage proteins in *Pseudotsuga menziesii* [Mirb.] Franco. *Planta* 206:138-145

Chatthai M, Misra S (2000) Gene expression during *Pseudotsuga menziesii* embryogenesis: low molecular weight proteins. In SM Jain, SC Minocha, eds, Molecular Biology of Woody Plants, Vol. 1, Kluwer Academic Publishers, Dordresch, The Netherlands, 299-314

Chung BYW, Simons C, Firth AE, Brown CM, Hellens RP (2006) Effect of 5'UTR on gene expression in Arabidopsis thaliana. *BMC Genomics* 7:120

Churbanov A, Rogozin IB, Babenko VN, Ali H, Koonin EV (2005) Evolutionary conservation suggests a regulatory function of AUG triplets in 5'-UTRs of eukaryotic genes. *Nucleic acids research* 33: 5512-5520

Ciavatta VT, Egertsdotter U, Clapman D, von Arnold S, Cairney J (2002) A promoter from the loblolly pine *PtNIP1;1* gene directs expression in an early-embryogenesis and suspensor-specific fashion. *Planta* 215: 694-698

Ciavatta VT, Morillon R, Pullman G, Chrispeels M, Cairney J (2001) An aquaglyceroporin is abundantly expressed early in the development of suspensor and the embryo proper of loblolly pine. *Plant Physiology* 127**:** 1556-1567

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* 16: 735-743

Curaba J, Moritz T, Blervaque R, Parcy F, Raz V, Herzog M, Vachon G (2004) *AtGA3ox2*, a key gene responsible for bioactive gibberellin biosynthesis, is regulated during embryogenesis by *LEAFY COTYLEDON2* and *FUSCA3* in Arabidopsis. *Plant Physiology* 136: 3660-3669

Curt T, Bouchaud M, Agrech G (2001) Predicting site index of Douglas-fir plantations from ecological variables in the Massif Central area of France. *Forest Ecology and Management* 149: 61-74

Datla RSS, Bekkaoui F, Hamerlindl JK, Pilate G, Dunstan DI, Crosby WL (1993) Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. *Plant Science* 94: 139-149

Day R (1995) In situ hybridization histochemistry protocols. In: Molecular Techniques, Canada, 1995

De Wilde C, Van Houdt H, De Buck S, Angenon G, De Jaeger G, Depicker A (2000) Plants as bioreactors for protein production: avoiding the problem of transgene silencing. *Plant Molecular Biology* 43: 347-359

DeCastro E, Sigrist CJA, Gattiker A, Bulliard V, Langendijk-Genevaux PS, Gasteiger E, Bairoch A, Hulo N (2006) ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Research* 34 (Web Server Issue): W362-365

Demura T, Tashiro G, Horiguchi G, Kishimoto N, Kubo M, Matsuoka N,Minmi A, Nagata-Hiwatashi M, Nakamura K, Okamura Y, Sassa N, Suzuki S, Yazaki J, Kikuchi S, Fukuda H (2002) Visualization by comprehensive microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells. *Proceedings of the National Academy of Sciences* 99: 15794-15799

DeVerno LL, Byrne JJ, Pitel JA, Cheliak WM (1989) Constructing genomic libraries: a basic guide. Report PI-X-88, Forestry Canada

Durzan DJ, Gupta PK (1987) Somatic embryogenesis and polyembryogenesis in Douglas-fir cell suspension cultures. *Plant Science* 52: 229-235

Dyachok JV, Wiweger M, Kenne L, von Arnold S (2002) Endogenous Nod-factor-like signal molecules promote early somatic embryo development in Norway spruce. *Plant Physiology* 128: 523-533

Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5:113

Edwards D, Murray JAH, Smith AG (1998) Multiple genes encoding the conserved CCAAT-box transcription factor complex are expressed in Arabidopsis. *Plant Physiology* 117: 1015-1022.

Egertsdotter U, von Arnold S (1995) Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (*Picea abies*). *Physiologia Plantarum* 93: 334-345

Egertsdotter U, von Arnold S (1998) Development of somatic embryos in Norway spruce. *Journal of Experimental Botany* 49: 155-162

Ellinghaus P, Badehorn D, Blumer R, Becker K, Seedorf U (1999) Increased efficiency of arbitrarily primed PCR by prolonged ramp times. *Biotechniques* 26**:** 626-630

Ezcurra I, Wycliffe P, Nehlin L, Ellerström M, Rask L (2000) Transactivation of the *Brassica napus* napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different *cis*-elements: B2 mediates activation through an ABRE, whereas B3 interacts with and RY/G-box. *The Plant Journal* 24: 57-66

Fambrini M, Durante C, Cinini G, Geri C, Giorgetti L, Michelotti V, Salvini M, Pugliesi C (2006) Characterization of *LEAFY COTYLEDON1-LIKE* gene in *Helianthus annuus* and its relationship with zygotic and somatic embryogenesis. *Development Genes and Evolution* 261: 253-264

Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. *Plant Cell, Tissue and Organ Culture* 74: 201-228

Fernando DD, Owens JN, Misra S (2000) Transient gene expression in pine pollen tubes following particle bombardment. *Plant Cell Reports* 19:224-228

Filonova LH, Bozhkov PV, von Arnold S (2000a) Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. *Journal of Experimental Botany* 51: 249-264

Filonova LH, Bozhkov PV, Brukhin VB, Daniel G, Zhivotovsky B, von Arnold S (2000b) Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. *Journal of Cell Science* 113: 4399-4411

Filonova LH, von Arnold S, Daniel G, Bozhkov PV (2002) Programmed cell death eliminates all but one embryo in a polyembryonic plant seed. *Cell Death and Differentiation* 9: 1057-1062

Finkbeiner S (2001) New roles for introns: sites of combinatorial regulation of Ca^{2+}- and cyclic AMP-dependent gene transcription. *Science: signal transduction knowledge environment* 94:PE1

Finkelstein RR, Lynch TJ (2000) Abscisic acid inhibition of radicle emergence but not seedling growth is suppressed by sugars. *Plant Physiology* 122**:** 1179-1186

Finkelstein RR, Gampala SSL, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. *The Plant Cell* Supplement 2002**:** S15-S45

Footitt S, Ingouff M, Clapham D, von Arnold S (2003) Expression of the viviparous 1 (*Pavp1*) and $p34^{cdc2}$ protein kinase (*cdc2Pa*) genes during somatic embryogenesis in Norway spruce (*Picea abies* [L.] Karst). *Journal of Experimental Botany* 54: 1711-1719

Forward BS, Misra S (2000) Characterization and expression of the Douglas-fir luminal binding protein (PmBiP). *Planta* 212**:** 41-51

Forward BS, Tranbarger TJ, Misra S (2001) Characterization of proteinase activity in stratified Douglas-fir seeds. *Tree Physiology* 21: 625-629

Fourre J-L (2000) Somaclonal variation and genetic molecular markers in woody plants. In: SM Jain, SC Minocha, eds, Molecular Biology of Woody Plants, Vol. 1, Kluwer Academic Publishers, Dordresch, The Netherlands, pp 425-449

Fu X, Kohli A, Twyman RM, Christou P (2000) Alternative silencing effects involve distinct types of non-spreading cytosine methylation at a three-gene, single-copy transgenic locus in rice. *Molecular and General Genetics* 263: 106-118

Gaj MD, Zhang S, Harada JJ, Lemaux PG (2005) Leafy cotyledon genes are essential for induction of somatic embryogenesis of Arabidopsis. *Planta* 222: 977-988

Gibson S (2004) Sugar and phytohormone response pathways: navigating a signalling network. *Journal of Experimental Botany* 55: 253-264

Gilthorpe J, Vandromme M, Brend T, Gutman A, Summerbell D, Totty N, Rigby PWJ (2002) Spatially specific expression of *Hoxb4* is dependent on the ubiquitous transcription factor NFY. *Development* 129, 3887-3899

Grant JE, Cooper PA, Dale TM (2004) Transgenic Pinus radiata form Agrobacterium tumefaciens-mediated transformation of cotyledons. *Plant Cell Reports* 22: 894-902

Greenboim-Wainberg Y, Maymon I, Borochov R, Alvarez J, Olszewski N, Ori N, Eshed Y, Weiss D (2005) Cross talk between gibberellin and cytokinin: the Arabidopsis GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. *The Plant Cell* 17: 92-102

Grishok A, Sinskey JL, Sharp PA (2005) Transcriptional silencing of a transgene by RNAi in the soma of *C. elegans*. *Genes & Develop*ment 19: 683-696

Grob JA, Carlson WC, Goodwin JB, Salatas M (1999) Dimensional model of zygotic Douglas-fir embryo development. *International Journal of Plant Sciences* 160: 653-662

Groover AT, Mansfield SD, DiFazio SP, Dupper G, Fontana JR, Millar R, Wang Y (2006) The Populus homeobox gene *ARBORKNOX1* reveals overlapping mechanisms regulating the shoot apical meristem and the vascular cambium. *Plant Molecular Biology* 61: 917-932

Guillet-Claude C, Isabel N, Pelgas B, Bousquet J (2004) The evolutionary implications of *knox-1* gene duplication in conifers: correlated evidence from phylogeny, gene mapping, and analysis of functional divergence. *Molecular Biology and Evolution* 21: 2232-2245

Gupta PK, Timmis R (2005) Mass propagation of conifer trees in liquid cultures-progress towards commercialization. *Plant Cell, Tissue and Organ Culture* 81: 339-346

Gupta PK, Timmis R, Pullman G, Yancey M, Kreitinger M, Carlson W, Carpenter C (1991) Development of an embryogenic system for automated propagation of forest trees. In: Cell Culture and Somatic Cell Genetics of Plants, Vol. 8, pp 75-92

Gupta PK, Timmis R, Timmis KA, Grob JA, Carlson WC, Welty DE (1996) Clonal propagation of conifers via somatic embryogenesis. In: MR Ahuja et al., (eds) Somatic Cell Genetics and Molecular Genetics of Trees, Kluwer Academic Publishers, The Netherlands, pp. 3-9

Gutierrez L, Conejero G, Castelain M, Guenin S, Verdeil J-L, Thomasset B, Van Wuytswinkel O (2006) Identification of new gene expression regulators specifically expressed during plant seed maturation. *Journal of Experimental Botany* 57: 1919-1932

Guzman-Rojas L, Sims JC, Rangel R, Guret C, Sun Y, Alcocer JM, Martinez-Valdez H (2000) PRELI, the human homologue of the avian px19, is expressed by germinal center B lymphocytes. *International Immunology* 12: 607-612

Harada JJ (1999) Signaling in plant embryogenesis. *Current Opinion in Plant Biology* 2: 23-27

Harding EW, Tang W, Nichols KW, Fernandez DE, Perry SE (2003) Expression and maintenance of embryogenic potential is enhanced through constitutive expression of *AGAMOUS-Like 15*. *Plant Physiology* 133: 653-663

Hattori T, Vasil V, Rosenkrans L, Hannah LC, McCarty DR, Vasil IK (1992) The Viviparous-1 gene and abscisic acid activate the C1 regulatory gene for anthocyanin biosynthesis during seed maturation in maize. *Genes & Development* 6: 609-618

Hecht V, Vielle-Calzada J-P, Hartog MV, Schmidt EDL, Boutilier K, Grossniklaus U, de Vries SC (2001) The Arabidopsis *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1* gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant physiology* 127: 803-816

Heck GR, Perry SE, Nichols KW, Fernandez DE (1995) *AGL15*, a MADS domain protein expressed in developing embryos. *Plant Cell* 7: 1271-1282

Helbing CC, Crump K, Bailey CM, Kohno S, Veldhoen N, Bryan T, Bermudez D, Guillette Jr LJ (2006) Isolation of the alligator (*Alligator mississippiensis*) thyroid hormone receptor α and β transcripts and their responsiveness to thyroid stimulating hormone. *General and Comparative Endocrinology* 149: 141-150

Hieronymus H, Silver PA (2007) A systems view of mRNP biology. *Genes & Development* 18: 2845-2860

Holmquist GP, Ashely T (2006) Chromosome organization and chromatin modification: influence on genome function and evolution. *Cytogenetic and Genome Research* 114: 96-125

Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG, Fraley RT (1989) Leaf disc transformation. Plant Molecular Biology Manual A5: 1-9. Kluwer Academic Publishers, Belgium

Igarashi D, Ishida S, Fukazawa J, Takahashi Y (2001) 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. *The Plant Cell* 13: 2483-2497

Ikeda-Iwai M, Satoh S, Kamada H (2002) Establishment of a reproducible tissue culture system for the induction of *Arabidopsis* somatic embryos. *Journal of Experimental Botany* 53: 1575-1580

Ikeda-Iwai M, Umehara M, Satoh S, Kamada H (2003) Stress-induced somatic embryogenesis in vegetative tissues of *Arabidopsis thaliana*. *The Plant Journal* 34: 107-114

Inguoff M, Farbos I, Wiweger M, von Arnold S (2003) The molecular characterization of *PaHB2*, a homeobox gene of the HD-GL2 family expressed during embryo development in Norway spruce. *Journal of Experimental Botany* 54: 1343-1350

Iraqi D, Le VQ, Lamhamedi MS, Tremblay FM (2005) Sucrose utilization during somatic embryo development in black spruce: involvement of apoplastic invertase in the tissue and of extracellular invertase in the medium. *Journal of Plant Physiology* 162, 115-124

Iraqi D, Tremblay FM (2001) Analysis of carbohydrate metabolism enzymes and cellular contents of sugars and proteins during spruce somatic embryogenesis suggests a regulatory role of exogenous sucrose in embryo development. *Journal of Experimental Botany* 52: 2301-2311

Jenik PD, Barton MK (2005) Surge and destroy: the role of auxin in plant embryogenesis. *Development* 132: 3577-3585

Jürgens G (1995) Axis formation in plant embryogenesis: cues and clues. *Cell* 81: 467-470

Kagale S, Divi UK, Krochko JE, Keller WA, Krishna P (2007) Brassinosteroid confers tolerance in Arabidopsis thaliana and Brassica napus to a range of abiotic stresses. *Planta* 225: 353-364

Kagaya Y, Toyoshima R, Okuda, Usui H, Yamamoto A, Hattori T (2005) *LEAFY COTYLEDON1* controls seed storage protein genes through its regulation of *FUSCA3* and *ABSCISIC ACID INSENSITIVE 3*. *Plant Cell Physiology* **46**: 399-406

Kahle J, Baake M, Doenecke D, Albig W (2005) Subunits of the heterotrimeric transcription factor NF-Y are imported into the nucleus by distinct pathways involving Importin β and Importin 13. *Molecular and Cellular Biology* 25: 5339-5354

Kao C-Y, Cocciolone SM, Vasil IK, McCarty DR (1996) Localization and interaction of the *cis*-acting elements for abscisic acid, *VIVIPAROUS1*, and light activation of the *C1* gene of maize. *The Plant Cell* 8: 1171-1179

Kaukinen KH, Tranbarger TJ, Misra S (1996) Post-germination-induced and hormonally dependent expression of low-molecular-weight heat shock protein genes in Douglas fir. *Plant Molecular Biology* 30: 1115-1128

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science* 236: 1299-1302

Kermode AR, Zeng Y, Hu X, Lauson S, Abrams SR, He X (2007) Ectopic expression of a conifer *Abscisic Acid Insensitive3* transcription factor induces high-level synthesis of recombinant human α-L-iduronidase in transgenic tobacco leaves. *Plant Molecular Biology* 63: 763-776

Kiran K, Ansari SA, Srivastava R, Lodhi N, Chaturvedi CP, Sawant SV, Tuli R (2006) The TATA-box sequence in the basal promoter contributes to determining light-dependent gene expression in plants. *Plant Physiology* 142: 364-376

Kirst M, Johnson AF, Baucom C, Ulrich E, Hubbard K, Staggs R, Paule C, Retzel E, Whetten R, Sederoff R (2003) Apparent homology of expressed genes from wood-forming tissues of loblolly pine (*Pinus taeda* L.) with *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* 100: 7383-7388

Kong L, Yeung EC (1995) Effects of silver nitrate and polyethylene glycol on white spruce (*Picea glauca*) somatic embryo development: enhancing cotyledonary embryo formation and endogenous ABA content. *Physiologia Plantarum* 93: 298-304

Krawczyk S, Thurow C, Niggeweg R, Gatz C (2002) Analysis of the spacing between the two palindromes of *activation sequence-1* with respect to binding to different TGA factors and transcriptional activation potential. *Nucleic Acids Research* 30: 775-781

Krutovskii KV, Vollmer SS, Sorensen FC, Adams WT, Knapp SJ, Strauss SH (1998) RAPD genome maps of Douglas-fir. *The Journal of Heredity* 89: 197-205

Kurup S, Jones HD, Holdsworth MJ (2000) Interactions of the developmental regulator ABI3 with proteins identified from developing *Arabidopsis* seed. *The Plant Journal* 21: 143-155

Kutachi AK, Kadonga JT (2000) The downstream promoter element DPE appears to be as widely used as the TATA Box in *Drosophila* core promoters. *Molecular and Cellular Biology*, 20: 4754-4764

Kwong RW, Bui AQ, Lee H, Kwong LW, Fischer RL, Goldberg RB, Harada JJ (2003) *LEAFY COTYLEDON1-LIKE* defines a class of regulators essential for embryo development. *The Plant Cell* 15: 5-18

Lage K, Karlberg EO, Størling ZM, Ólason PÍ, Pedersen AG, Rigina O, Hinsby AM, Tümer Z, Pociot F, Tommerup N, Moreau Y, Brunak S (2007) A human phenome-interactome network of protein complexes implicated in genetic disorders. *Nature Biotechnology* 25: 309-316

Lam E, Chua N-H (1991) Tetramer of a 21-base pair synthetic element confers seed expression and transcriptional enhancement in response to water stress and abscisic acid. *The Journal of Biological Chemistry* 266: 17131-17135

Le Hir H, Izaurralde E, Maquat LE, Moore MJ (2000) The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. *The EMBO Journal* 19: 6860-6869

Le Hir H, Nott A, Moore MJ (2003) How introns influence and enhance eukaryotic gene expression. *TRENDS in Biochemical Sciences* 28: 215-220

Leal I, Misra S (1993) Molecular cloning and characterization of a legumin-like storage protein cDNA of Douglas fir seeds. *Plant Molecular Biology* 21:709-715

Ledgard N, Knowles L, De La Mare P (2005) Douglas-fir – the current New Zealand scene. New Zealand Journal of Forestry 50: 13-16

Ledgard N, Perley C, Belton M, Edmonds J, Wills B, Bell J, Franklin D (1994) Forestry fact pack. Information pamphlet created by Forest Research, Landcare Research and MAF/MOF, New Zealand.

Lee D-H, Gershenzon N, Gupta M, Ioshikhes IP, Reinberg D, Lewis BA (2005) Functional characterization of core promoter elements: the downstream core element is recognized by TAF1. *Molecular and Cellular Biology* 25: 9674-9686

Lee H, Fischer RL, Goldberg RB, Harada JJ (2003) *Arabidopsis* LEAFY COTYLEDON1 represents a functionally specialized subunit of the CCAAT binding transcription factor. *Proceedings of the National Academy of Sciences USA* 100: 2152-2156

Li Z, Thomas TL (1998) *PEI1*, an embryo-specific zinc finger protein gene required for heart-stage embryo formation in Arabidopsis. *The Plant Cell* 10: 383-398

Liang C, Wang G, Liu L, Ji G, Fang L, Liu Y, Carter K, Webb JS, Dean JFD (2007) ConiferEST: an integrated bioinformatics system for data reprocessing and mining of conifer expressed sequence tags (ESTs). *BMC Genomics* 8: 134

Liu J-J, Ekramoddoullah AKM (2006) The family 10 or plant pathogenesis-related proteins: their structure, regulation, and function in response to biotic and abiotic stresses. *Physiological and Molecular Plant Pathology* 68: 3-13

Lippert D, Zhuang J, Ralph S, Ellis DE, Gilbert M, Olafson R, Ritland K, Ellis B, Douglas CJ, Bohlmann J (2005) Proteome analysis of early somatic embryogenesis in *Picea glauca*. *Proteomics* 5: 461-473

Lotan T, Ohto M, Yee KM, West MAL, Lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ (1998) Arabidopsis *LEAFY COTYLEDON1* is sufficient to induce embryo development in vegetative cells. *Cell* 93: 1195-1205

Luo Y, Koop H-U (1997) Somatic embryogenesis in cultured immature zygotic embryos and leaf protoplasts of *Arabidopsis thaliana* ecotypes. *Planta* 202: 387-396

Maeda K, Ozeki Y (2006) Self-interaction of DcMYB1 proteins involved in stress-inducible *DcPAL1* expression. *Plant Biotechnology* 23: 219-225

Malabadi RB, van Staden J (2005) Somatic embryogenesis from vegetative shoot apices of mature trees of *Pinus patula*. *Tree Physiology* 25: 11-16

Malik MR, Wang F, Dirpaul JM, Zhou N, Polowick PL, Ferrie AMR, Krochko JE (2007) Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. *Plant Physiology* 144: 134-154

Manzara T, Carasco P, Gruissem W (1993) Developmental and organ-specific changes in DNA-protein interactions in the tomato *rbcS1*, *rbcS2* and *rbcS3A* promoter regions. *Plant Molecular Biology* 21: 69-88

Masiero S, Imbriano C, Ravasio F, Favaro R, Pelucchi N, Gorla MS, Mantovani R, Colombo L, Kater MM (2002) Ternary complex formation between MADS-box transcription factors and the histone fold protein NF-YB. *The Journal of Biological Chemistry* 277: 26429-26435

Mathieu M, Lelu-Walter MA, Blervacq AS, David S, Hawkins S, Neutelings G (2006) Germin-like genes are expressed during somatic embryogenesis and early development of conifers. *Plant Molecular Biology* 61: 615-627

Mayer U, Jürgens G (1998) Pattern formation in plant embryogenesis: a reassessment. *Seminars in Cell and Developmental Biology*, 9: 187-193

Meinke DW (1992) A homeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. *Science* 258: 1647-1650

Meinke DW, Franzmann LH, Nickle TC, Yeung EC (1994) *Leafy cotyledon* mutants of *Arabidopsis*. *The Plant Cell* 6: 1049-1064

Misra S (1994) Conifer zygotic embryogenesis, somatic embryogenesis, and seed germination: biochemical and molecular advances. *Seed Science Research* 4: 357-384

Minocha R, Jain SM (2000) Tissue culture in woody plants and its relevance to molecular biology. In: SM Jain, SC Minocha (eds) Molecular Biology of Woody Plants, Vol. 1, Kluwer Academic Publishers, Dordresch, The Netherlands, pp 315-339

Mordhorst AP, Voerman KJ, Hartog MV, Meijer EA, van Went J, Koornneef M, de Vries SC (1998) Somatic embryogenesis in *Arabidopsis thaliana* is facilitated by mutations in genes repressing meristematic cell divisions. *Genetics* 149: 549-563

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497

Miyoshi K, Ito y, Serizawa A, Kurata N (2003) *OsHAP3* genes regulate chloroplast biogenesis in rice. *The Plant Journal* 36: 532-540

Nakamura M, Tsunoda T, Obokata J (2002) Photosynthesis nuclear genes generally lack TATA-boxes: a tobacco photosystem I gene responds to light through an initiator. *The Plant Journal* 29: 1-10

Niskanen A-M, Lu J, Seitz S, Keinonen K, Von Weissenberg K, Pappinen A (2004) Effect of parent genotype on somatic embryogenesis in Scots pine (*Pinus syvestris*). *Tree Physiology* 24: 1259-1265

Nole-Wilson S, Krizek BA (2006) *AINTEGUMENTA* contributes to organ polarity and regulates growth of lateral organs in combination with *YABBY* genes. *Plant Physiology* 141: 977-987

Nott A, Le Hir H, Moore MJ (2004) Splicing enhances translation in mammalian cells: an additional function of the exon junction complex. *Genes & Development*, 18: 210-222

Ogas J, Kaufmann S, Henderson J, Somerville C (1999) *PICKLE* is a CHD3 chromatin remodeling factor that regulates the transition from embryonic to vegetative development in Arabidopsis. *Proceedings of the National Academy of Sciences* 96: 13839-13844

Ogas J, Cheng J-C, Sung, ZR, Somerville C (1997) Cellular differentiation regulated by gibberellin in the *Arabidopsis thaliana pickle* mutant. *Science* 277: 91-94

Oraby HF, Ransom CB, Kravchenko AN, Sticklen MB (2005) Barley *HVA1* gene confers salt tolerance in R3 transgenic oat. *Crop Science* 45: 2218-2227

Osusky M, Zhou G, Osuska L, Hancock RE, Kay WW, Misra S (2000) Transgenic plants expressing cationic peptide chimeras exhibit broad-spectrum resistance to phytopathogens. *Nature Biotechnology* 18: 1162-1166

Owens JN, Morris SJ, Misra S (1992) The ultrastructural, histochemical, and biochemical development of the post-fertilization megagametophyte and the zygotic embryo of *Pseudotsuga menziesii*. *Canadian Journal for Forestry Research* 23: 816-827

Parcy F, Valon C, Kohara A, Miséra S, Giraudat J (1997) The *ABSCISIC ACID-INSENSITIVE3, FUSCA3*, and *LEAFY COTYLEDON1* loci act in concert to control multiple aspects of Arabidopsis seed development. *The Plant Cell* 9: 1265-1277

Park S-C, Kwon H-B. Shih M-C (1996) *Cis*-acting elements essential for light regulation of the nuclear gene encoding the A subunit of chloroplast glyceraldehyde 3-phosphate dehydrogenase in *Arabidopsis thaliana*. *Plant Physiology* 112: 1563-1571

Page RDM (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12: 357-358

Pérez Rodríguez MJ, Suárez MF, Heredia R, Ávila C, Breton D, Trontin J-F, Filonova L, Bozhkov P, von Arnold S, Harvengr L, Cánovas M (2006) Expression patterns of two glutamine synthase genes in zygotic and somatic pine embryos support specific roles in nitrogen metabolism during embryogenesis. *New Phytologist* 169: 35-44

Perry SE, Nichols KW, Fernandez DE (1996) The MADS domain protein AGL15 localizes to the nucleus during early stages of seed development. *The Plant Cell* 8: 1977-1989

Prewein C, Vagner M, Wilhelm E (2004) Changes in water status and proline and abscisic acid concentrations in developing somatic embryos of pedunculate oak (*Quercus robur*) during maturation and germination. *Tree Physiology* 24: 1251-1257

Pullman GS, Gupta PK, Timmis R, Carpenter C, Kreitinger M, Welty E (2005) Improved Norway spruce somatic embryo development through the use of abscisic acid combined with activated carbon. *Plant Cell Reports* 24: 271-279

Pullman GS, Johnson S, Peter G, Cairney J, Xu N (2003) Improving loblolly pine somatic embryo maturation: comparison of somatic and zygotic embryo morphology, germination, and gene expression. *Plant Cell Reports* 21: 747-758

Pullman GS, Mein J, Johnson S, Zhang Y (2005) Gibberellin inhibitors improve embryogenic tissue initiation in conifers. *Plant Cell Reports* 23: 596-605

Pullman GS, Zhang Y, Phan BH (2003) Brassinolide improves embryogenic tissue initiation in conifers and rice. *Plant Cell Reports* 22: 96-104

Raghavan V (2004) Role of 2,4-dichlorophenoxy acetic acid (2,4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. *American Journal of Botany* 91: 1743-1756

Rider Jr SD, Henderson JT, Jerome RE, Edenberg HJ, Romero-Severson J, Ogas J (2003) Coordinate repression of regulators of embryonic identity by *PICKLE* during germination in *Arabidopsis*. The *Plant Journal* 35: 33-43

Rogozin IB, Kochetov AV, Kondrashov FA, Koonin EV, Milanesi L (2001) Presence of ATG triplets in 5' untranslated regions of eukaryotic cDNAs correlates with a 'weak' context of the start codon. *Bioinformatics* 17: 890-900

Rompf R, Kahl G (1997) mRNA differential display in agarose gels. *Biotechniques* 23: 28-32

Sabala I, Elfstrand M, Farbos I, Clapman D, von Arnold S (2000) Tissue specific expression of *Pa18*, a putative lipid transfer protein gene, during embryo development in Norway spruce (*Picea abies*). *Plant Molecular biology* 42: 461-478

Santos-Mendoza M, Dubreucq B, Miquel M, Caboche M, Lepiniec L (2005) LEAFY COTYLEDON 2 activation is sufficient to trigger the accumulation of oil and seed specific mRNAs in *Arabidopsis* leaves. *FEBS Letters* 579: 4666-4670

Santos-Mendoza M, Dubreucq B, Baud S, Parcy F, Caboche M, Lepiniec L (2008) Decipehring gene regulatory networks that control seed development and maturation in *Arabidopsis*. *The Plant Journal* 54: 608-620

Sinha S, Maity SN, Lu J, De Crombrugghe B (1995) Recombinant rat CBF-C, the third subunit of CBF/NFY, allows formation of a protein-DNA complex with CBF-A and CBF-B and with yeast HAP2 and HAP3. *Proceedings of the National Academy of Sciences* 92: 1624-1628

Soccio RE, Adams RM, Romanowski MJ, Sehayek E, Burley SK, Breslow JL (2002) The cholesterol-regulated StarD4 gene encodes a StAR-related lipid transfer protein with two closely related homologues, StarD5 and StarD6. *Proceedings of the National Academy of Sciences* 99: 6943-6948

Stasolla C (2006) Somatic Embryogenesis in *Picea* Suspension Cultures. *Methods in Molecular Biology* 318: 87-99

Stasolla C, Kong L, Yeung EC, Thorpe TA (2002) Maturation of somatic embryos in conifers: morphogenesis, physiology, biochemistry, and molecular biology. *In Vitro Cellular and Developmental Biology-Plant* 38: 93-105

Stasolla C, van Zyl L, Egertsdotter U, Craig D, Liu W, Sederoff RR (2003) The effects of polyethylene glycol on gene expression of developing white spruce somatic embryos. *Plant Physiology* 131: 49-60

Stasolla C, Yeung EC (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. *Plant Cell, Tissue and Organ Culture* 74: 15-35

Stasolla C, Yeung EC (2006) Endogenous ascorbic acid modulates meristem reactivation in white spruce somatic embryos and affects thymidine and uridine metabolism. *Tree Physiology* 26: 1197-1206

Stasolla C, Yeung EC (2007) Cellular ascorbic acid regulates the activity of major peroxidases in the apical poles of germinating white spruce (*Picea glauca*) somatic embryos. *Plant Physiology and Biochemistry* 45: 188-198

Stone SL, Kwong LW, Yee KM, Pelletier J, Lepiniec L, Fischer RL, Goldberg RB, Harada JJ (2001) *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. *Proceedings of the National Academy of Sciences USA* 98: 11806-11811

Sundström J, Engström P (2002) Conifer reproductive development involves B-type MADS-box genes with distinct and different activities in male organ primordia. *The Plant Journal* 31: 161-169

Suzuki M, Ketterling MG, Li Q-B, McCarty DR (2003) Viviparous1 Alters Global Gene Expression Patterns through Regulation of Abscisic Acid Singaling. *Plant Physiology* 132: 1664-1677

Suzuki M, Ketterling MG, McCarty DR (2005) Quantitative statistical analysis of *cis*-regulatory sequences in ABA/VP1- and CBF/DREB1-regulated genes of *Arabidopsis*. *Plant Physiology* 139: 437-447

Suzuki M, Wang HH-Y, McCarty DR (2007) Repression of the *LEAFY COTYLEDON1*/*B3* regulatory network in plant embryo development by *VP1*/*ABSCISIC ACID INSENSITIVE 3-LIKE* B3 genes. *Plant Physiology* 143: 902-911

Taber RP, Zhang C, Hu W-S (1998) Kinetics of Douglas-fir (*Pseudotsuga menziesii*) somatic embryo development. *Canadian Journal of Botany* 76: 863-871

Tahir M, Law DA, Stasolla C (2006) Molecular characterization *PgAGO*, a novel conifer gene of the ARGONAUTE family expressed in apical cells and required for somatic embryo development in spruce. *Tree Physiology* 26: 1257-1270

Tang W, Newton RJ (2003) Genetic transformation of conifers and its application in forest biotechnology. *Plant Cell Reports* 22: 1-15

Tang W, Newton RJ (2004) Increase of polyphenol oxidase and decrease of polyamines correlated with tissue browning in Virginia pine (*Pinus viginiana* Mill.). *Plant Science* 167: 621-628

Tang W, Newton RJ (2005) Plant regeneration from callus cultures derived from mature zygotic embryos in white pine (*Pinus strobus* L.). *Cell Biology and Morphogenesis* 24: 1-9

Tang W, Newton RJ, Charles TM (2006) Plant regeneration through multiple adventitious shoot differentiation from callus cultures of slash pine (*Pinus elliotii*). *Journal of Plant Physiology* 163: 98-101

Tang W, Perry SE (2003) Binding site selection for the plant MADS domain protein AGL15. *The Journal of Biological Chemistry* 278: 28154-28159

Terskikh VV, Feurtado JA, Ren C, Abrams SR, Kermode AR (2005) Water uptake and oil distribution during imbibition of seeds of western white pine (*Pinus monticola* Dougl. ex D. Don) monitored in vivo using magnetic resonance imaging. *Planta* 221: 17-27

Timmis R (1998) Bioprocessing for tree production in the forest industry: conifer somatic embryogenesis. *Biotechnology Progress* 14: 156-166

To A, Valon C, Savino G, Guilleminot J, Devic M, Giraudat J, Parcy F (2006) A network of local and redundant gene regulation governs *Arabidopsis* seed maturation. *The Plant Cell* 18: 1642-1651

Todd CD, Gifford DJ (2003) Loblolly pine arginase responds to arginine in vitro. *Planta* 217: 610-615

Tremousaygue D, Manevski A, Bardet C, Lescure N, Lescure B (1999) Plant interstitial telomere motifs participate in the control of gene expression in root meristems. *The Plant Journal* 20: 553-561

Tsukagoshi H, Saijo T, Shibata D, Morikami A, Nakamura K (2005) Analysis of a sugar response mutant of *Arabidopsis* identified a novel B3 domain protein that functions as an active transcriptional repressor. *Plant Physiology* 138: 675-685

Vahala T, Oxelman B, von Arnold S (2001) Two *APETALA2*-like genes are differentially expressed during development. *Journal of Experimental Botany* 52: 1111-1115

Valluri JV, Treat WJ, Newton RJ, Cobb BG, Soltes EJ (1988) Protein synthesis in slash pine callus cultures exposed to water stress. *Tree Physiology* 4: 181-186

van Frankenhuyzen K, Beardmore T (2004) Current status and environmental impact of transgenic forest trees. *Canadian Journal for Forestry Research* 34: 1163-1180

Van Winkle SC, Pullman GS (2005) Achieving desired plant growth regulator levels in liquid plant tissue culture media that include activated carbon. *Plant Cell Reports* 24: 201-208

van Zyl L, Bozhkov PV, Clapham DH, Sederoff RR, von Arnold S (2003) Up, down and up again is a signature global gene expression pattern at the beginning of gymnosperm embryogenesis. *Gene Expression Patterns* 3: 83-9

Veldhoen N, Boggs A, Walzak K, Helbing CC (2006) Exposure to tetrabromobisphenol-A alters TH-associated gene expression and tadpole metamorphosis in the Pacific tree frog *Pseudacris regilla*. *Aquatic Toxicology* 78: 290-302

Verwoerd TC, Dekker BMM, Hoekema A (1987) A small scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Research* 17: 2362.

Verdeil J-L, Alemanno L, Niemenak N, Tranbarger TJ (2007) Pluripotent versus totipotent plant stem cells: dependence versus autonomy? *TRENDS in Plant Science* 12: 245-252

Verslues PE, Kim Y-S, Zhu J-K (2007) Altered ABA, proline and hydrogen peroxide in an Arabidopsis glutamate:glyoxylate aminotransferase mutant. *Plant Molecular Biology* 64: 205-217

Vicient CM, Bies-Estheve N, Delseny M (2000) Changes in gene expression in the *leafy cotyledon1* (*lec1*) and *fusca3* (*fus3*) mutants of *Arabidopsis thaliana* L. *Journal of Experimental Botany* 51: 995-1003

Vroemen C, de Vries S, Quatrano R (1999) Signalling in plant embryos during the establishment of the polar axis. *Cell & Developmental Biology* 10: 157-164

VuorioT, Maity SN, de Crombrugghe B (1990) Purification and molecular cloning of the "A" chain of a rat heteromeric CCAAT-binding protein. *The Journal of Biological Chemistry* 265: 22480-22486

Watanabe K, Okada K (2003) Two discrete *cis* elements control the abaxial side-specific expression of the *FILAMENTOUS FLOWER* gene in *Arabidopsis*. *The Plant Cell* 15: 2592-2602

Wehmeyer N, Vierling E (2000) The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance. *Plant Physiology* 122: 1099-1108

Wenk AR, Quinn M, Whetten RW, Pullman G, Sederoff R (1999) High-efficiency Agrobacterium-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). *Plant Molecular Biology* 39: 407-416

West MAL, Harada JJ (1993) Embryogenesis in higher plants: An Overview. *The Plant Cel*l 5: 1361-1369

West MAL, Yee KM, Danao J, Zimmerman JL, Fischer RL, Goldberg RB, Harada JJ (1994) *LEAFY COTYLEDON1* is an essential regulator of late embryogenesis and cotyledon identity in Arabidopsis. *The Plant Cell* 6: 1731-1745

Weterings K, Apuya NR, Bi Y, Fischer RL, Harada JJ, Goldberg RB (2001) Regional localization of suspensor mRNAs during early embryo development. *The Plant Cell* 13: 2409-2425

Wilkie GS, Dickson KS, Gray NK (2003) Regulation of mRNA translation by 5'- and 3'-UTR-binding factors. *TRENDS in Biochemical Science* 28: 182-188

Willemsen V, Scheres B (2004) Mechanisms of pattern formation in plant embryogenesis. *Annual Review of Genetics* 38: 587-614

Williams CG, Auckland LD, Reynolds MM, Leach KA (2003) Overdominant lethals as part of the conifer embryo lethal system. *Heredity* 91: 584-592

Williams L, Grigg SP, Xie M, Christensen S, Fletcher JC (2005) Regulation of *Arabidopsis* shoot apical meristem and lateral organ formation by microRNA *miR166g* and its *AtHD-ZIP* target genes. *Development* 132: 3657-3668

Winicov I, Valliyodan B, Xue L, Hoober JK (2004) The *MsPRP2* promoter enables strong heterologous gene expression in a root-specific manner and is enhanced by overexpression of *Alfin 1*. *Planta* 219: 925-935

Wiweger M, Farbos I, Ingouff M, Lagercrantz U, von Arnold S (2003) Expression of *Chia4-Pa* chitinase genes during somatic and zygotic embryo development in Norway spruce (*Picea abies*): similarities and differences between gymnosperm and angiosperm class IV chitinases. *Journal of Experimental Botany* 54: 2691-2699

Xiao W, Brown RC, Lemmon BE, Harada JJ, Goldberg RB, Fischer RL (2006) Regulation of seed size by hypomethylation of maternal and paternal genomes. *Plant Physiology* 142: 1161-1168

Xiao W, Custard KD, Brown RC, Lemmon BE, Harada JJ, Goldberg RB, Fischer RL (2006) DNA methylation is critical for *Arabidopsis* embryogenesis and seed viability. *The Plant Cell* 18: 805-814

Xiao W, Sheen J, Jang J-C (2000) The role of hexokinase in plant sugar signal transduction and growth and development. *Plant Molecular Biology* 44: 451-461

Yamasaki K, Kigawa T, Inoue M, Tateno M, Yamasaki T, Yabuki T, Aoki M, Seki E, Matsuda T, Tomo Y, Hayami N, Tereda T, Shirouzu M, Osanai T, Tanaka A, Seki M, Shinozaki K, Yokoyama S (2004) Solution structure of the B3 DNA binding domain of the *Arabidopsis* cold-responsive transcription factor RAV1. *The Plant Cell* 16: 3448-3459

Yazawa K, Kamada H (2007) Identification and characterization of carrot HAP factors that form a complex with the embryo-specific transcription factor C-LEC1. *Journal of Experimental Botany* 58: 3819-3828

Yazawa K, Takahata K, Kamada H (2004) Isolation of the gene encoding carrot leafy cotyledon1 and expression analysis during somatic and zygotic embryogenesis. *Plant Physiology and Biochemistry* 42: 215-223

Zhang S, Wong L, Meng L, Lemaux PG (2002) Similarity of expression patterns of *knotted1* and *ZmLEC1* during somatic and zygotic embryogenesis in maize (*Zea mays* L.). *Planta* 215: 191-194

Zhang JZ, Santes CM, Engel ML, Gasser CS, Harada JJ (1996) DNA sequences that activate isocitrate lyase gene expression during late embryogenesis and during postgerminative growth. *Plant Physiology* 110: 1069-1079

Zhang W, Ruan J, Ho TD, You Y, Yu T, Quatrano RS (2005) *Cis*-regulatory element based targeted gene finding: genome-wide identification of abscisic acid- and abiotic stress-responsive genes in *Arabidopsis thaliana*. *Bioinformatics* 21: 3073-3081

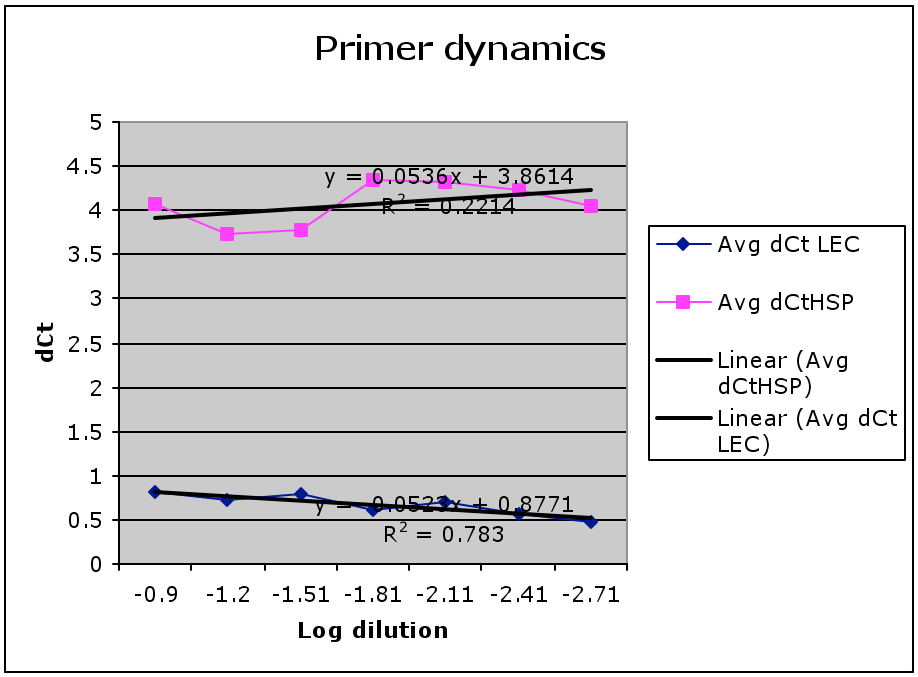
Zuo J, Niu Q, Frugis G, Chua N (2002a) The *WUSCHEL* gene promotes vegetative-to-embryonic transition in *Arabidopsis*. *The Plant Journal* 30: 349-359

Zuo J, Niu Q, Frugis G, Chua N (2002b) Promotion of somatic embryogenesis in plants by *WUSCHEL* gene expression. European patent EP1451301

Zupan J, Muth TR, Draper O, Zambryski P (2000) The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *The Plant Journal 23*: 11-28

Appendix

Appendix A
Comparison of amplification efficiency of LEC1 and HSP primers



Appendix B
QPCR data and relative gene expression analysis of Douglas-fir developmental stages

Stage	Stage code	Sample	Avg L8	Avg LEC	dCt	ddCt	2^-ddCt	Ctrl mean	2^-ddCt/mean	MEAN	SEM
May-24	1	1	21.5	18.96	-2.54	-14.74	27364.16094		3953.43	3060.767	397.070928
May-24	1	2	21.295	19.09	-2.205	-14.405	21693.87271		3134.22		
May-24	1	3	21.195	19.1825	-2.0125	-14.2125	18984.04757		2742.72		
May-24	1	4	22.6175	20.155	-2.4625	-14.6625	25932.97321		3746.66		
May-24	1	5	21.3175	19.9725	-1.345	-13.545	11952.29466		1726.81		
Jun-07	2	1	21.265	20.0925	-1.1725	-13.3725	10605.31609		1532.20	1287.553	95.5589005
Jun-07	2	2	20.9325	19.9575	-0.975	-13.175	9248.476404		1336.17		
Jun-07	2	3	20.975	19.905	-1.07	-13.27	9877.977724		1427.12		
Jun-07	2	4	20.0125	19.44	-0.5725	-12.7725	6996.899237		1010.88		
Jun-07	2	5	19.8575	19.1225	-0.735	-12.935	7831.104042		1131.40		
Jun-20	3	1	26.82	24.225	-2.595	-14.795	28427.5077		4107.06	1792.858	787.997642
Jun-20	3	2	28.3	27.6125	-0.6875	-12.8875	7577.467269		1094.75		
Jun-20	3	3	25.5875	25.7775	0.19	-12.01	4124.489933		595.88		
Jun-20	3	4	23.07	22.055	-1.015	-13.215	9508.486467		1373.74		
Jul-11	4	1	23.86	23.62	-0.24	-12.44	5556.651325		802.80	874.9226	183.165464
Jul-11	4	2	22.325	22.675	0.35	-11.85	3691.521895		533.33		
Jul-11	4	3	22.83	22.8625	0.0325	-12.1675	4600.260998		664.62		
Jul-11	4	4	26.485	25.2675	-1.2175	-13.4175	10941.32622		1580.75		
Jul-11	4	5	22.83	22.6075	-0.2225	-12.4225	5489.655876		793.12		
Jul-17	5	1	23.57	22.53667	-1.033333	-13.23333	9630.088448		1391.31	1105.62	104.74231
Jul-17	5	2	23.3275	22.4075	-0.92	-13.12	8902.531994		1286.19		
Jul-17	5	3	23.37	22.8475	-0.5225	-12.7225	6758.559162		976.44		
Jul-17	5	4	23.67	23.025	-0.645	-12.845	7357.500399		1062.97		
Jul-17	5	5	22.7175	22.4625	-0.255	-12.455	5614.726369		811.19		
Jul-31	6	1	25.2475	25.6775	0.43	-11.77	3492.392517		504.56	582.0951	39.4013956
Jul-31	6	2	26.9675	27.115	0.1475	-12.0525	4247.79963		613.70		
Jul-31	6	3	25.5175	25.5275	0.01	-12.19	4672.56818		675.07		
Jul-31	6	4	25.96	26.04	0.08	-12.12	4451.265997		643.10		
Jul-31	6	5	24.845	25.365	0.52	-11.68	3281.182219		474.05		
14-Aug	7	1	23.085	25.38	2.295	-9.905	958.7427814		138.51	69.75518	25.3234021
14-Aug	7	2	22.98	26.33	3.35	-8.85	461.4402369		66.67		
14-Aug	7	3	24.6225	28.2	3.5775	-8.6225	394.1224171		56.94		
14-Aug	7	4	19.665	24.995	5.33	-6.87	116.9704256		16.90		
30-Aug	8	1	21.6025	29.72	8.1175	-4.0825	16.94162082		2.45	3.734115	1.38377339
30-Aug	8	2	21.485	29.7125	8.2275	-3.9725	15.6979036		2.27		
30-Aug	8	3	25.13	31.345	6.215	-5.985	63.33802601		9.15		
30-Aug	8	4	21.6925	29.38	7.6875	-4.5125	22.82432041		3.30		
30-Aug	8	5	21.4425	30.26	8.8175	-3.3825	10.42879091		1.51		
Imbibed	9	1	20.8275	29.47	8.6425	-3.5575	11.77373373		1.70	3.481022	0.86756629
Imbibed	9	2	20.83	28.02	7.19	-5.01	32.2225776		4.66		
Imbibed	9	3	25.03	33.315	8.285	-3.915	15.08455257		2.18		
Imbibed	9	4	23.425	31.475	8.05	-4.15	17.75311155		2.56		
Imbibed	9	5	22.58	29.3325	6.7525	-5.4475	43.63760445		6.30		
Stratified	10	1	20.3275	29.0125	8.685	-3.515	11.43195312		1.65	1.93344	0.51536601
Stratified	10	2	20.6325	29.4725	8.84	-3.36	10.26740718		1.48		
Stratified	10	3	20.2825	27.77	7.4875	-4.7125	26.21825931		3.79		
Stratified	10	4	19.6125	29.58	9.9675	-2.2325	4.699476317		0.68		
Stratified	10	5	19.655	28.0175	8.3625	-3.8375	14.29560726		2.07		

Stage	Stage code	Sample	Avg L8	Avg LEC	dCt	ddCt	2^-ddCt	Ctrl mean	2^-ddCt/mean	MEAN	SEM
2DAEG	11	1	21.0375	31.00667	9.969167	-2.230833	4.694050404		0.68	12.54155	6.41867688
2DAEG	11	2	22.515	29.01	6.495	-5.705	52.16462927		7.54		
2DAEG	11	3	24.02667	31.57	7.543333	-4.656667	25.22297715		3.64		
2DAEG	11	4	26.54	30.7575	4.2175	-7.9825	252.9134585		36.54		
2DAEG	11	5	20.965	26.535	5.57	-6.63	99.04415959		14.31		
6DAEG	12	1	21.44	30.12	8.68	-3.52	11.47164198		1.66	7.242094	5.21819177
6DAEG	12	2	22.99	31.0425	8.0525	-4.1475	17.72237439		2.56		
6DAEG	12	3	23.57	28.4625	4.8925	-7.3075	158.4078477		22.89		
6DAEG	12	4	20.76	29.27	8.51	-3.69	12.90626815		1.86		
10DAEG	13	1	20.4	28.5025	8.1025	-4.0975	17.11868526		2.47	1.405794	0.53408907
10DAEG	13	2	21.095	30.76	9.665	-2.535	5.795768618		0.84		
10DAEG	13	3	20.605	30.155	9.55	-2.65	6.276672783		0.91		
12DAEG	14	1	20.04	29.67	9.63	-2.57	5.938094283		0.86	16.97294	9.20000273
12DAEG	14	2	22.5425	27.67	5.1275	-7.0725	134.596772		19.45		
12DAEG	14	3	21.855	28.745	6.89	-5.31	39.6706464		5.73		
12DAEG	14	4	26.545	30.2625	3.7175	-8.4825	357.6736431		51.67		
12DAEG	14	5	21.93	28.5	6.57	-5.63	49.52207979		7.15		
14DAEG	15	1	20.62	32.5225	11.9025	-0.2975	1.22901285		0.18	1.559707	1.00297627
14DAEG	15	2	21.0775	28.675	7.5975	-4.6025	24.29352608		3.51		
14DAEG	15	3	24.6425	34.06333	9.420833	-2.779167	6.864557222		0.99		
1.5 mo	16	1	24.0325	33.19667	9.164167	-3.035833	8.20119041		1.18	2.67677	1.88233329
1.5 mo	16	2	26.215	32.575	6.36	-5.84	57.28160454		8.28		
1.5 mo	16	3	22.62	35.1075	12.4875	0.2875	0.819320604		0.12		
1.5 mo	16	4	22.86	32.095	9.235	-2.965	7.808254086		1.13		
3 mo	17	1	24.7575	32.145	7.3875	-4.8125	28.10003457		4.06	2.390652	1.16704633
3 mo	17	2	24.2975	37.4575	13.16	0.96	0.514056913		0.07		
3 mo	17	3	25.485	35.375	9.89	-2.31	4.9588308		0.72		
3 mo	17	4	26.5175	33.69	7.1725	-5.0275	32.61582012		4.71		
veg bud	18	1	22.4375	31.08	8.6425	-3.5575	11.77373373		1.70	0.836433	0.28503995
veg bud	18	2	21.1175	31.8	10.6825	-1.5175	2.862945089		0.41		
veg bud	18	3	26.4275	36.9475	10.52	-1.68	3.20427951		0.46		
veg bud	18	4	24.89	36.16333	11.27333	-0.926667	1.900878955		0.27		
veg bud	18	5	24.4825	33.48	8.9975	-3.2025	9.205524986		1.33		
Pollen	19	1	25.585	34.8975	9.3125	-2.8875	7.39987038	6.921622	1.07	1	0.55291597
Pollen	19	2	27.7525	35.8175	8.065	-4.135	17.56948502		2.54		
Pollen	19	3	20.9575	32.3775	11.42	-0.78	1.717130873		0.25		
Pollen	19	4	20.5025	32.7025	12.2	0	1		0.14		

Appendix C
ELISA: Testing Mouse Anti-PmLEC1 Sera on Protein Extracts (Titrated)

ELISA conditions:
Antigen: 0.5 µg/well Douglas-fir protein extracts titrated in carbonate coating buffer pH 9.6, 100 µl/well, incubated overnight at 4 °C.
Block: 3 % skim milk powder in PBS pH 7.4 for 1 h at RT.
Primary Ab: Mouse anti-PmLEC1 test bleed after 4th boost in PBS-Tween pH 7.4 at 50 µl/well and incubated for 1 h at RT.
Secondary Ab: 1/10000 goat anti-mouse IgG Fc HRP (Pierce cat. # 31328) at 100 µl/well and incubated for 1 h at 37 °C.
Substrate: TMB (BioFx cat. # TMBW-1000-01) at 50 µl/well and stopped with an equal volume of 1 N HCl after 15 min.
Read at 450 nm.

		1/200 Pooled Mouse # 1, 2 + 4				**1/400 Mouse # 3**			
Antigen ↓	Date	**24 May**	**7 Jun**	**20 Jun**	**30 Aug**	**24 May**	**7 Jun**	**20 Jun**	**30 Aug**
Conc. ▼		1	2	3	4	5	6	7	8
5µg/well	A	2.007	0.683	0.406	0.52	0.77	0.347	0.389	0.452
2.5	B	0.641	0.265	0.409	0.686	0.388	0.282	0.355	0.716
1.25	C	0.424	0.236	0.41	0.648	0.292	0.241	0.324	0.729
0.625	D	0.303	0.262	0.407	0.435	0.236	0.221	0.249	0.425
0.313	E	0.351	0.26	0.421	0.404	0.073	0.063	0.064	0.065
0.156	F	0.49	0.263	0.412	0.417	0.07	0.062	0.059	0.063
78 ng	G	0.807	0.213	0.346	0.544	0.067	0.067	0.063	0.07
38 ng	H	0.501	0.125	0.19	0.303	0.063	0.061	0.061	0.063

PBS only, no 1° Ab

		1/400 NMS			
Antigen ↓	Date	**24 May**	**7 Jun**	**20 Jun**	**30 Aug**
Conc. ▼		9	10	11	12
5µg/well	A	0.077	0.087	0.097	0.072
2.5	B	0.078	0.071	0.077	0.083
1.25	C	0.079	0.069	0.085	0.076
0.625	D	0.071	0.067	0.075	0.076
0.313	E	0.069	0.068	0.079	0.071
0.156	F	0.067	0.063	0.071	0.064
78 ng	G	0.067	0.062	0.067	0.069
39 ng	H	0.065	0.068	0.063	0.066