Identification and characterization of a matrix metalloproteinase (*Pta1-MMP*) expressed during Loblolly pine (*Pinus taeda*) seed development and germination

By

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Dissertation submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements of the degree of

Ph.D.

In Forestry

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March 19, 2009
Danville, VA

Keywords*: Pinus taeda* L., Embryo development, Germination, Extracellular matrix, Proteolysis, Matrix metalloproteinase

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Abstract

Extracellular matrix (ECM) modifications occur during plant growth, development, and in response to environmental stimuli. Key modulators of ECM modification in vertebrates, the extracellular matrix metalloproteinases (MMPs), have also been described in a few plants. Here, we report the identification of Loblolly pine (*Pinus taeda*) *Pta1-MMP* and its characterization during seed development and completion of germination. The *Pta1-MMP* protein has the structural characteristics of other plant MMPs, and a recombinant protein (r*Pta-MMP*) generated by using EST sequences for a seed-expressed MMP exhibits Zn^{2+}-dependent protease activity, and is inhibited by the active site-binding hydroxamate inhibitor GM6001 and EDTA. The *Pta1-MMP* gene is expressed during embryo development, with transcript levels increasing from proembryo to early cotyledonary stage, then declining during late cotyledonary expansion and maturation drying. Protein extracts exhibited similar developmental-stage MMP-like activity. Seed germination was stimulated by GA_3 and inhibited by ABA. The timing of completion of germination was mirrored by the presence of MMP-like protease activity in both water- and GA_3-imbibed embryos. *Pta1-MMP* transcript levels increased in association with completion of germination for both GA_3- and water-treated embryos, in agreement with MMP-like activity. In contrast, by 10 days after imbibition, *Pta1-MMP* transcripts in ABA-treated embryos were at levels similar to the other treatments, although MMP-like activity was not observed. The application of GM6001 during Loblolly pine seed imbibition inhibited completion of germination in a dose-dependent manner. Our results suggest that *Pta1-MMP* is required for ECM modification, facilitating the cell division and expansion required for both embryo development and completion of germination. To our knowledge, this is the first report of an MMP in any gymnosperm and also its involvement in embryo development, completion of germination, and seedling establishment.

Dedication

I dedicate this dissertation to my parents

Ashok and Sunanda Jambhekar,

my wonderful husband

Milind, and

my lovely daughters

Mohana and Gauri

Acknowledgements

First and foremost, I wish to thank my supervisor, Dr Ulrika Egertsdotter, for her scientific guidance and her constant support during my Ph.D. program. I have been inspired by her meticulousness, her attention to detail and the depth of her knowledge. Throughout my research, she taught me to come up with logical solutions to any problem and thanks to her, I have emerged a more confident and stronger person than I was before joining this program. I value her involvement in my work and concern for my welfare.

I would like to thank my co-advisor, Dr Barry Flinn for trusting me with this wonderful project and guiding me through every step of my research. The completion of this research was not possible without his guidance. Although sometimes it was hard to keep up with his fast pace of thoughts and dynamism, but at the same time, these qualities kept me motivated and enthusiastic about my research and helped me to hone my scientific abilities. His dedication and sincerity towards science, and concern for his students are truly inspirational.

I am grateful to Dr. Amy Brunner and Dr. Ian Clark for their precious inputs in my research and their time and effort in serving on my graduate committee. Their encouragement and support have been integral to my personal growth and scientific progress. I also wish to thank Dr Harold Burkhart, ex-department head of Forestry, for his help and advice.

I acknowledge Dr. Fujino, Dr. Chieh-Ting, Rubina Ahsan, Carolina Espinosa and Seonhwah Kim for their willingness to share their scientific knowledge with me. Special thanks to Dr. Javed Iqbal, Dr. Tammy Ferguson and Dr. Tongyun Shen for their help in design and execution of some of the experiments, and to Kelly Merricks for her help in gene cloning. Thanks to the staffs of Forestry department at Virginia Tech and IALR for their help in solving the administration related issues, especially Sue Snow, Julie, Amanda and Nancy for their helpful advice from time to time. I enjoyed the company of Dr. Chunxia Wang, Xiaoyan, Dr. Dan, Dr. Mei, Dr. Li, Yeun-Kyung, Sarah, Katie, Faith, Chris, Gouzhu, Kedong, Song, Kristie, Travis and Brandon and I thank them for their help and support.

I wish to thank my room-mate Priti Parikh for making my life fun and easier during my stay at Backsburg. I was away from my family and whenever I missed them, I always found a great friend in her. Also, thanks to Alejandra for making my stay at Danville memorable. Special thanks to Sukhwinder and Kavita for helping me in times of need.

I must make a mention of my parents in law. They took care of my family in my absence and their steadfast faith in my capability has always spurred me to go ahead, especially in difficult times. Thanks to my sister in law and her family for their encouragement. Also, thanks to my friend Bhavani for believing in me and encouraging me. I have no words to express my gratitude for my parents, my husband, my lovely daughters Mohana and Gauri, my wonderful sister Preeti Vajandar and her family without whose love, patience, support and encouragement throughout, I couldn't have come this far.

Finally, I acknowledge the several friends and well wishers, whom I have not mentioned above but whose best wishes have always encouraged me.

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I

Introduction and Justification

Amongst gymnosperms, conifers form an important part of the economy in many parts of the world. In the United States, the forest products industry employs about 1.7 million people directly, approximately 1.1% of the US workforce, and is responsible for creating an additional four million related jobs in distribution, sales, and manufacturing. Coniferous softwood species make up the majority of trees harvested to supply the American forest product industry. In the U.S., Loblolly pine is the primary commercial species in the southern conifer forests covering 13.4 million ha., 45% of commercial forestland, with over 1.5 billion Loblolly pine seedlings planted each year (Cairney *et al.*, 2006) and hence is a major driver of the national economy (Fig. 1), while Douglas-fir [*PseudoTsuga menziesii* (Mirb.) Franco] in the Pacific Northwest is the most important commercial species. In Europe, Norway spruce (*Picea abies* L. Karst.) is probably the most important coniferous species (Pullman *et al.*, 2003a). In tropical countries, four conifer genera feature significantly: *Araucaria, Cunninghamia, Cupressus* and *Pinus*. The pines dominate tropical conifer plantations and within this genus two species are more important than all others: *Pinus caribaea* in the lowland humid tropics, and *P. patula* in the cooler highland tropics and subtropics (Evans, 1992).

Given the commercial importance of conifers, it is highly desirable to mass propagate the genetically superior varieties for production of elite trees in clonal plantations. Somatic embryogenesis is the only available clonal propagation method suitable for large scale, commercial propagation of conifer species. In addition, somatic embryogenesis offers an *in vitro* experimental system for studying embryogenesis (Misra, 1994). Somatic embryogenesis refers to

the initiation of somatic embryos from previously differentiated somatic cells, in conifers typically residing inside the zygotic embryo. Unlike cells of other eukaryotes, plant cells display totipotency, that is, have the capacity to form a new plant. This characteristic relies on reprogramming of gene expression and is associated with structural changes, which in the case of somatic embryogenesis, leads to the development of a plant from somatic cells, through a path similar to what occurs in zygotic embryos.

Production of commercially important trees by *in vitro* propagation has great potential but is currently hampered by the poor response of many genotypes to *in vitro* cultivation as well as difficulties in plantlet regeneration (FAO Forest Genetic Resources Working Paper 59E 2004). As mass propagation of genetically superior trees in clonal plantations is essential to secure sufficient supply of wood in the future, the issues surrounding efficiency of somatic embryogenesis of forest species are of prime importance worldwide.

Loblolly pine (Fig. 2), also known as Arkansas pine, North Carolina pine or old field pine, is characterized by three needles per fascicle. It is considered to be relatively recalcitrant to somatic embryogenesis (Cairney *et al.*, 2000). An understanding of gene expression associated with somatic embryo development events, and the possibility to develop expression markers to monitor developmental progress, would allow us to follow conifer embryogenesis more closely and gain some insight into the metabolic states of zygotic and somatic embryos. Characterization of the genes involved in embryo development, their program of expression, and relating these to physiological events, is a promising strategy for improving the responsiveness of recalcitrant genotypes to somatic embryogenesis protocols. This may not be an easy task, given the daunting

size of its genome, the haploid DNA content being about 20,000 Mbp, which is 160 times that of *Arabidopsis*.

Most of the studies in somatic embryogenesis research have focused on modification and development of tissue culture media based on measurements of the composition of conifer seeds (Tautorus *et al.* 1991, Lara *et al.* in preparation). The results obtained from such media vary depending upon the genotype being cultured. Due to the dearth of knowledge about molecular events in embryo growth and development, the progress of somatic embryogenesis is limited. Several studies have supplied data related to the measurements of trace elements metabolite pools, hormone levels and osmolarity at different stages of development (Teasdale *et al.*, 1986; Feirer, 1995; Kapik *et al.*, 1995; Pullman, 1997), but they provide limited information about cell composition, and details of molecular activities remain obscure. Hence studies directed towards understanding the molecular mechanisms underlying embryo development are required in order to improve somatic embryogenesis protocols to an efficiency level suitable for large scale commercial applications.

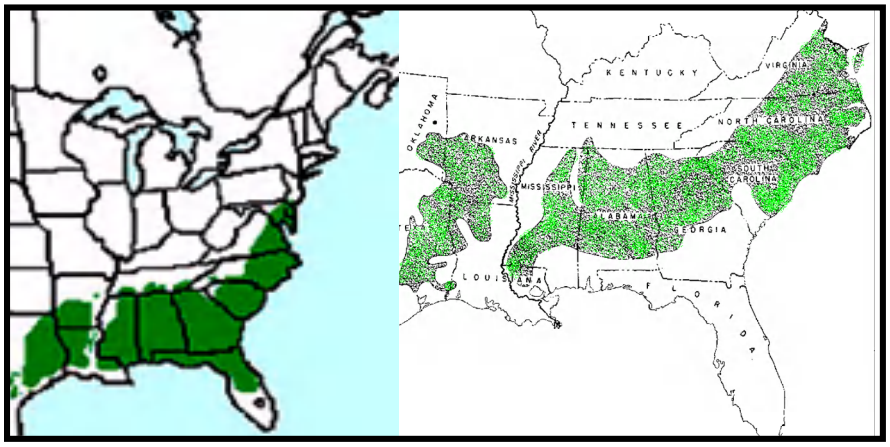
Hypothesis and Objectives

The hypothesis of this study is that proteolytic modifiers of the extracellular matrix, known as matrix metalloproteinases or MMPs, are expressed in seed tissues of Loblolly pine and play a role in seed development. The characterization of these proteases, and any role that they play in seed development, may ultimately lead the identification of molecules, or the development of additional methods, to support the development of enhanced somatic embryogenesis protocols for Loblolly pine, as well as other plant species.

The Objectives.

Five different objectives were laid out to test the above hypothesis. They include:

- Objective 1 – The identification of an embryo/seed-expressed MMP clone.
- Objective 2 – The generation of recombinant embryo/seed-expressed MMP protein and its biochemical characterization.
- Objective 3 – The characterization of MMP gene expression during Loblolly pine embryo/seed development and germination.
- Objective 4 – The characterization of MMP-like protease activity during Loblolly pine embryo/seed development and germination.
- Objective 5- The determination of the impact of MMP inhibitor treatments on seed biochemistry and physiology.



a b

Figure 1: Range of Loblolly pine in the US. a: Natural range of *Pinus taeda* in the United Sates (Source: Virginia Tech Forestry Department); b: Range of *Pinus taeda* commercial plantation in the US.

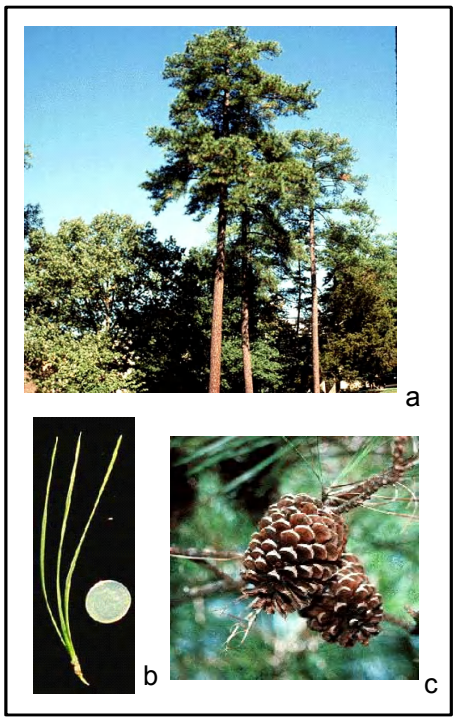


Figure 2: Loblolly pine; a: Loblolly pine tree; b: A whorl of Loblolly pine needles with transverse section of a needle on the right; c: Mature cones of Loblolly pine (Source: Virginia Tech Forestry Department)

II

Literature Review

1 Fertilization and embryo development in Gymnosperms

1.1 Fertilization in Gymnosperms

The female gametophyte contains several archegonia, where the egg cells originate and develop. The gametophyte itself is surrounded by layers of sporangia and integument; all of these elements comprise an ovule, which is found on the surface of a female cone. Fertilization occurs when pollen grains (male gametophytes) are carried by wind to the open end of an ovule, which contains eggs, or female gametophyte. In gymnosperms like *Pinus*, where archegonia occur singly, the neck cells degenerate and the pollen tube penetrates the egg and releases male cells or nuclei inside the archegonia. Thus both male cells/nuclei enter the same archegonium. On entering the egg cytoplasm, the male gamete usually looses some of its cytoplasmic part and only a part of it moves down the egg cytoplasm to the egg nucleus. In *Pinus* only one of the male nuclei enters the egg cytoplasm and moves towards the egg nucleus, while the other remains at the apex of the egg (Singh, 1978).

The nucleoplasms of male and female nuclei form neocytoplasm around the nuclei of the two nucleate proembryo and becomes fully organized as the two proembryonal nuclei descend towards the base of the archegonium. The zygote in most gymnosperms is a fusion nucleus surrounded by neocytoplasm within the archegonial sac (Romberger *et al.*, 1993). The two nuclei

fuse and form a continuous surface. Polyspermy, where more than one egg is fertilized, is a common phenomenon in gymnosperms.

1.2 Embryo development in Gymnosperms

Embryo development, in gymnosperms occurs entirely within the female megagametophyte, which serves as the stored food source for the developing embryo (Owens, 1985).

The important difference between embryogeny in angiosperms and gymnosperms is believed to be the free nuclear phase in gymnosperm embryogeny, prior to cell wall formation. In angiosperms, the division of the zygote is usually followed by wall formation. The process of embryo development in gymnosperms can be divided into three phase: (a) Proembryony, (b) Early Embryony, and (c) Late Embryony (Singh, 1978).

Proembryony involves the developmental stages before elongation of the suspensor and varies between different taxa of gymnosperms. In conifers, there is a "basal plan" for proembryony. In members of *Pinaceae*, the two nuclei, enveloped by dense neocytoplasm move towards the base of the archegonium where further synchronous mitosis takes place (Singh, 1978). The nuclei divide to give rise to eight nuclei arranged in two layers of four each. Fig. 3 illustrates the various stages of development in a conifer embryo. Wall formation results in two tiers, the primary upper (pU) tier, whose cells remain open on the upper side and the lower (pE) tier of primary embryonal cells; each tier comprising four cells. The two tiers go through an internal division to form four tiers out of which the lowest two comprise the embryonal cells (E) followed by the suspensor tier (S) and the upper tier (U) [Fig. 3 (A)]. The cells of the suspensor

tier undergo an abortive meristematic activity and give rise to rosette embryo (R) and hence the tier is termed as dysfunctional suspensor (dS). The upper cells of the embryonal tier elongate to function as suspensor (Es) and the lower four form the embryonal mass (EM), as seen in Fig. 3 (B).

In early embryony, several distinct phenomena such as elongation of suspensor, cleavage of embryonal mass to initiate polyembryony, formation of young embryonal mass, proliferation of suspensor cells and formation of rosette embryos takes place. The genus *Pinus* consists of an embryonal suspensor where the suspensor is formed by simultaneously elongating cells (Es1 and Es2) of the upper cells of the earlier mentioned embryonal tier. This suspensor elongates (Es3) while carrying the embryonal cells at its tip. The presence of more than one embryo is a common phenomenon in gymnosperms and is due to fertilization of more than one egg and the development of multiple zygotes (simple polyembryony). In *Pinus*, a single zygote forms multiple embryos by splitting of the cells of embryonal tier [Fig. 3 (C)]. Interestingly, in *Pinus*, there is no indication in the beginning as to which embryo will persist in the end and hence the phenomenon is called indeterminate cleavage polyembryony. Cleavage polyembryony is a common phenomenon in *Pinus*, *Tsuga*, *Cedrus* and *Libocedrus* and can be recognized by counting the number of embryos produced from one zygote (Buchholz, 1926). The transition from early embryogeny to late embryogeny takes place when the embryonal mass is thrust through the archegonial wall into the corrosion cavity in the megagametophyte, by vigorous elongation of the suspensor system (Gifford and Foster, 1988).

Late embryogeny refers to 'post globular embryo' development, which is essentially histogenesis and organogenesis in the developing embryo. The root and shoot apical meristems are delineated and the plant axis is established (Spurr, 1949). The cells of the proximal region which do not form suspensor are forerunners of the root cap, and the distal region forms the hypocotyl, shoot apex and cotyledons. The core of this region forms the root organization center (ROC). The cells of this region will form the central column or the "columnella" of the root cap and the central cylinder of the root-hypocotyl axis [Fig. 3 (D)]. A group of cells forms the pith, which is usually the first to develop in the hypocotyl/shoot axis. The cells surrounding the pith divide longitudinally to form procambium. As these changes take place, the embryonal axis goes through remarkable elongation. During further differentiation of the young embryo, the embryonal cortex originates from peripheral regions of the hypocotyls/shoot axis. The procambium differentiation in continuation with that in the hypocotyl, extends into the cotyledons. The cells of the apex slowly grow to form a conical mound of tissue, the epicotyl.

The mature embryo of most gymnosperms is well developed and shows good internal differentiation into two polar meristems, epidermis, cortex, epicambium and pith [Fig. 3 (E)]. The hypocotyl is elongated in conifers and shows secretory cells in the pith and cortex. Resin ducts have been reported in the hypocotyls of *Pinus*. These resin ducts develop during germination. A procambial strand is present in each cotyledon of a pine embryo (Singh, 1978).

1.3 Female megagametophyte

The development of the megagametophyte, in gymnosperms, is completed prior to fertilization and the gametophyte is haploid, unlike in angiosperms (Misra, 1994).

In *Pinus sylvestris*, the innermost region has no starch or only simple grains; the middle zone has starch masses, while the cells of the peripheral zone have dense contents but no solid storage material. Later the whole megagametophyte becomes packed uniformly. The accumulated food reserves seem to be utilized at the time of seed germination. Thus the female megagametophyte in gymnosperms serves the dual purpose of bearing gametes and nourishing the embryo (Singh, 1978).

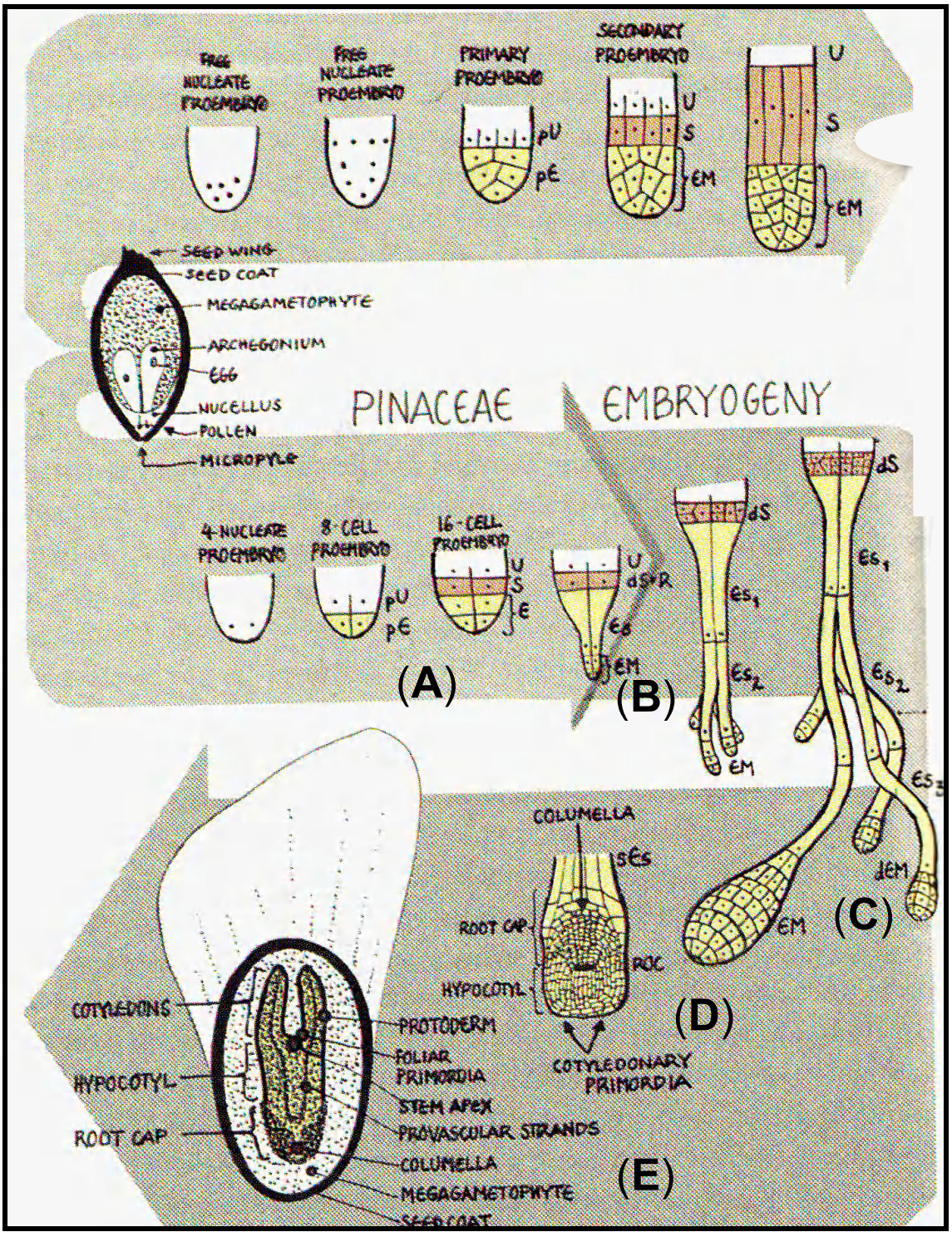


Figure 3: Schematic overview of gymnosperm embryo development : pU, primary upper tier, pE, primary embryonal tier, U, upper tier, S, suspensor tier, EM embryonal mass, dS, dysfunctional suspensor tier (also known as rosette tier, Es Embryonal suspensor tier, dEM degenerating embryo mass, ROC , root organization center, sEs, secondary embryonal suspensor cells (Adapted from Egertsdotter,1996).

1.4 The process of Gymnosperm embryogenesis differs from Angiosperms

The processes of fertilization and embryogenesis in gymnosperms and angiosperms differ as a consequence of both plant anatomy and cell biology. In angiosperms, embryo development follows a double fertilization event. *Arabidopsis* embryogenesis is treated as the representative for dicotyledonous embryo development and hence, is described here. The zygote, formed as a result of fertilization [Fig. 4 (A)], divides into two asymmetric cells, the apical smaller cell forms the embryo proper and the basal larger cell leads to formation of suspensor [Fig. 4 (B)] (Lindsey, 1993). The apical cell divides to form an eight-cell structure called the octant stage, in which the cells of the embryo proper are divided into two tiers, out of which the upper is destined to form the cotyledons and the shoot apex, while the lower will develop into the hypocotyl. The boundary between the two tiers is termed the "O" boundary [Fig. 4 (C)] (Tykarska, 1976). The distal cell of the suspensor, hypophysis ultimately forms the root of the seedling. The first sign of differentiation appears with the 16-cell stage, at which protoderm is formed, which eventually constitutes the seedling epidermis. This is followed by the globular stage [Fig. 4 (D], where the ground meristem and procambium initiate. This is followed by triangular stage [Fig. 4 (E)] and heart stage [Fig. 4 (F)], by which the three fundamental tissues of the seedling, the epidermis, ground tissue, and vascular tissue, have been established (Mayer *et al.*, 1991). Furthermore, by torpedo stage [Fig. 4 (G)], clearly defined cotyledons and hypocotyl are visible. The cotyledons eventually fold back to mark what is called the "walking stick" stage [Fig. 4 (H)]. This is followed by organ maturation, expansion and storage protein accumulation. The only differentiation process at this stage is the formation of the shoot meristem. Further, the embryo reaches its maximum size, becomes dehydrated and all the metabolic activities cease [Fig. 4 (I)].

The main differences between gymnosperm and angiosperm embryogenesis are presented in Table 1.

The different pathways of embryogenesis in pine and angiosperms could arise from the activity of a number of genes unique to each plant (gene content differences), the different regulation of a common set of ‘‘plant embryogenesis” genes (gene regulation differences) or some measure of each (Cairney *et al.*, 2006). The development of the plant embryo comprises two fundamental processes (Lindsey, 1993): first, the establishment of precise spatial organization of the component cells derived from a single fertilized egg cell (pattern formation) and second, the generation of cellular diversity within the developing embryo (cytodifferentiation). Both processes are tightly coordinated to create recognizable morphological structure, many features of which are characteristic of embryogeny within the given species. The whole process of embryogenesis in gymnosperms and angiosperms involves a number of physiological and biochemical processes taking place within and outside the cell. The extracellular matrix (ECM) plays an important role in all the developmental processes of an organism, and can be expected to change during cell division and cell expansion, key aspects of embryogenesis. The ECM will be discussed in detail in a later section.

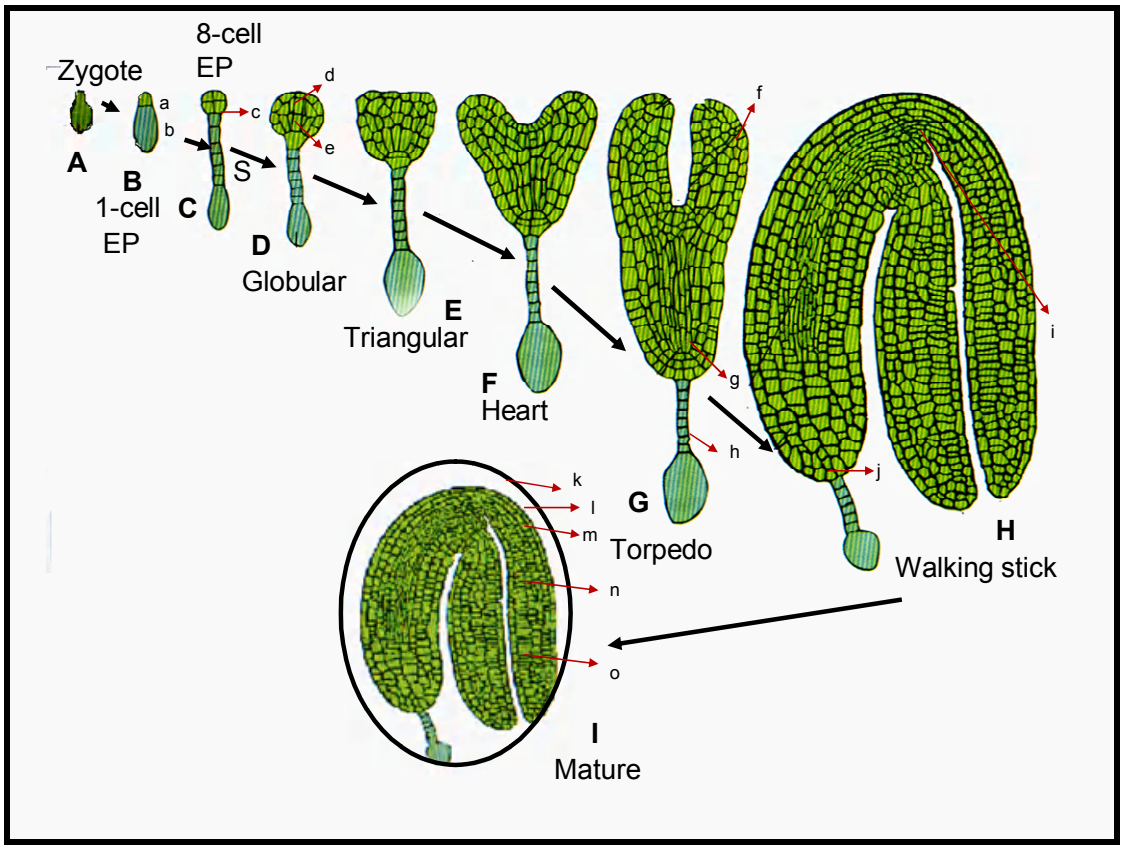


Figure 4: Schematic overview of angiosperm embryo development exemplified by *Arabidopsis thaliana.* A: apical cell, B: basal cell, EP: embryo proper, S: suspensor; a: apical cell; b: basal cell; c: "O" boundary; d: ground meristem; e: protoderm; f: cotyledons; g: hypocotyl; h: suspensor; i: shoot meristem; j: root meristem; k: seed coat; l: endosperm; m: protoderm; n: ground meristem; o: procambium.

Table 1: The main differences between Angiosperm and Gymnosperm embryogenesis.

	Angiosperms	**Gymnosperms**
1.	Embryos develop after a double fertilization event with one sperm nucleus fusing with the haploid egg nucleus to form a diploid zygote and the second sperm fusing with the diploid endosperm nucleus to create a triploid nucleus.	Embryos arise from a single fertilization within the ovule that creates a diploid embryo.
2.	Endosperm, the equivalent tissue of female megagametophyte in gymnosperms, does not develop until after fertilization has occurred.	Embryos develop within the maternally derived female gametophyte, the formation of which begins and is more than halfway complete prior to fertilization.
3.	Asymmetric division of the zygote to form two cells differing in cytoplasmic content: a smaller apical that is destined to form the embryo proper, and a larger, basal cell which will form the suspensor and contribute to the formation of the root.	First few rounds of division in most gymnosperms are free-nuclear to form a syncitium.
4.	In most angiosperms, a single embryo develops within the endosperm.	Polyembryony is common especially in conifers.
5.	The stages of embryo development comprise of morphology changes conspicuously, passing through ‘‘heart’’, ‘‘torpedo’’, and ‘‘bent cotyledon’’ stages before producing a dicotyledonous (or monocotyledonous, in monocots) seedling upon germination in angiosperms.	Alterations in embryo morphology, in general, are more subtle, with the formation of a dome, which is then ringed with a 6–8 cotyledonary primordial (in *Pinus*) which grow to form cotyledons that ultimately enclose the stem apical meristem.

1.5 Seed dormancy and germination

Seed dormancy is a temporary failure or block of a viable seed to complete germination under physical conditions that normally favor germination (Bewley, 1997). Germination commences with the uptake of water by imbibition of the dry seed, followed by embryo expansion. This usually culminates in rupture of the covering layers and emergence of the radicle, generally considered as the completion of the germination process. Radicle protrusion at the completion of seed germination depends on embryo growth driven by water uptake. Uptake of water by a seed is triphasic, with a rapid initial uptake (phase I, i.e. imbibition) followed by a plateau phase (phase II). A further increase in water uptake (phase III) occurs only when germination is completed, as the embryo axis elongates and breaks through its covering structures (Bewley, 1997). The imbibition of water brings about a number of physiological and biochemical changes within the seed. It brings about the mobilization of the stored reserves, increased availability of oxygen to the cells and many such changes within the cells and their environment. The transition from embryo development to germination necessitates fundamental changes in the control of gene expression within a seed. At some stage, the expression of genes coding for embryo development-related proteins has to be switched off and the genes coding for germination-related enzymes, the initiation of axial growth, germination and subsequent reserve mobilization must be switched on. Figure 5 illustrates an overview of the changes taking place during the process of seed germination. The role of various growth regulators on dormancy and germination has been studied using both zygotic and somatic embryo systems. Overexpression of genes for ABA biosynthesis can increase seed ABA content and enhance seed dormancy or delay germination (Thompson *et al.*, 2000). In studies with the ABA-deficient tomato mutant, ABA was shown to

be essential for the induction of dormancy and inhibition of precocious germination (Groot *et al.*, 1992). ABA is not only a positive regulator of dormancy induction; it also inhibits seed germination, has a role during after-ripening and has been proposed to be a positive regulator of dormancy maintenance. ABA inhibits embryo growth potential and endosperm cap weakening during coffee seed germination (da Silva *et al.*, 2004). Karssen and Lacka (1986) have suggested that ABA induces dormancy during maturation, and GAs play a key role in dormancy release and in the promotion of germination. Ethylene is implicated in the promotion of germination of non-dormant seeds of many species (Lashbrook *et al.*, 1998), and seems to counteract the inhibitory effects of ABA on seed germination by interfering with ABA signaling. A proteomic approach was used to analyze mechanisms of dormancy breaking in beech (*Fagus sylvatica* L.) seeds and the participation of abscisic and gibberellic acids (ABA and GA) in this process. After imbibition in water, ABA, or GA_3 solutions, beechnuts were subjected to cold stratification, which breaks their dormancy. ABA delayed, whereas GA_3 promoted seed dormancy breaking. The breaking of seed dormancy involves proteins associated with many processes, beginning with hormone signal initiation, through signal transduction, transcription, protein synthesis, energy metabolism, storage materials, and ending with the cell cycle (Pawlowski, 2007).

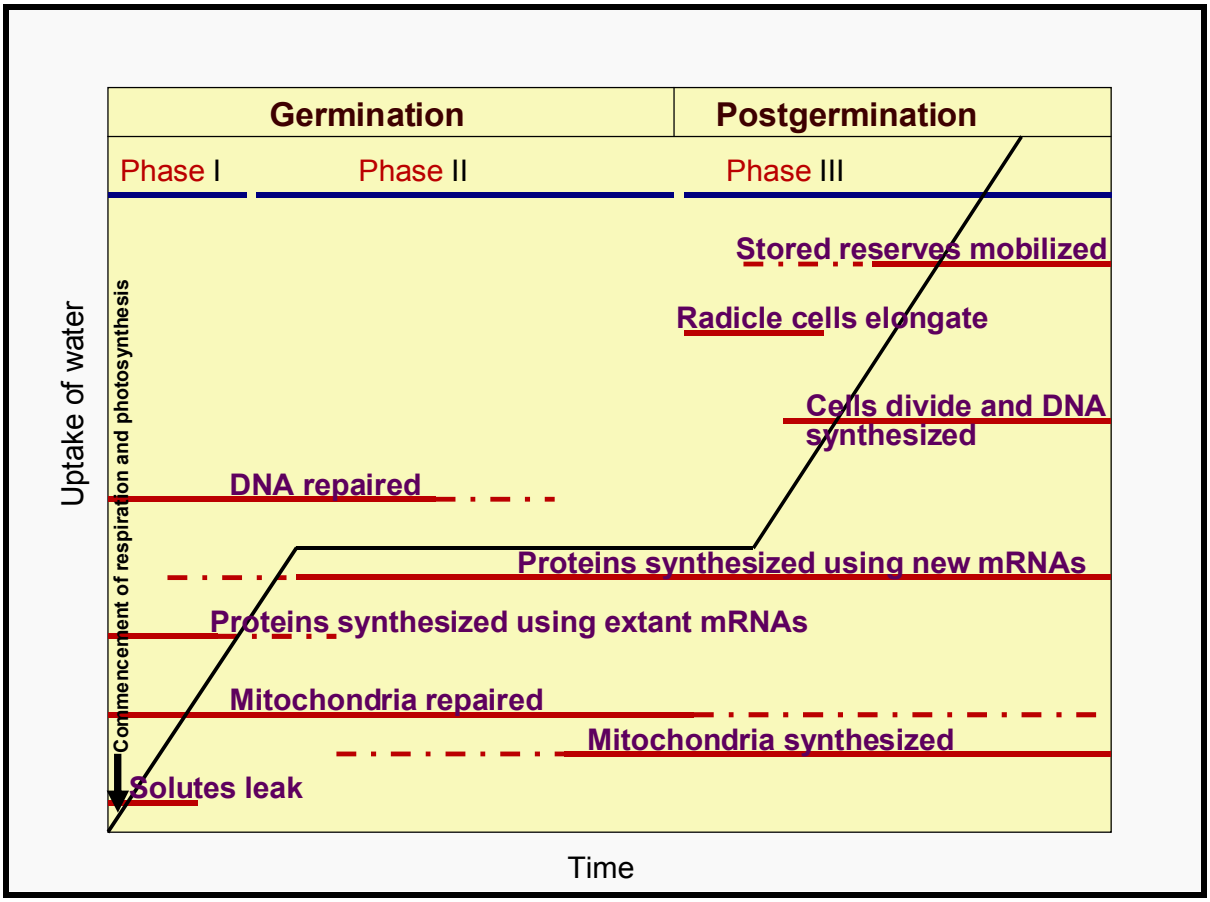


Figure 5: Time course of major events associated with germination and subsequent Post-germination growth (Bewley, 1997). Intact lines represent peak period of the event and the green arrow represents commencement of physiological processes such as respiration and protein synthesis.

2 Somatic embryogenesis

Plants possess the ability to produce morphologically and developmentally normal embryos and, indeed, whole plants from undifferentiated somatic cells in culture, through the process of somatic embryogenesis. Somatic embryogenesis was first reported in carrot (Steward *et al.*, 1958) and in conifers with *Picea abies* (Hakman *et al.*, 1985), with the first report of somatic embryogenesis in *Pinus taeda* by Gupta and Durzan (1987). Somatic embryogenesis represents a suitable model system for investigating factors affecting embryo growth (Stasolla *et al.*, 2004). A large number of embryos at a defined stage of development can easily be obtained through this process. In addition, the chemical environment can be easily manipulated in order to study the embryogenic process, and to investigate the basic mechanisms that underlie the formation of somatic and zygotic embryos.

2.1 Somatic embryogenesis in Angiosperms

For a long time, somatic embryogenesis has been studied in cultures of carrot (Komamine *et al.*, 1990) and alfalfa (Dudits *et al.*, 1991). Somatic embryos are induced from cultured callus cells by a relatively simple manipulation of the culturing conditions. In carrot, this generally involves (1) the establishment of a callus cell line from small hypocotyl pieces cut from sterilely germinated individual seeds, (2) the selection of an embryogenic subpopulation of the cultured cells through sieving or gradient fractionation, (3) the removal of auxin from the culture medium, and (4) the dilution of the cells to a relatively low density (Zimmerman, 1993). Suspension cultures are often described as unorganized, subcellular populations that retain features

associated with specific differentiated cell types. The term 'embryogenic cell' would be limited to cells which have achieved the transition from a somatic cell to a stage where no further external stimuli are required to produce a somatic embryo (Komamine *et al.*, 1990). Cells able to undergo embryo development generally appear as proembryogenic masses (PEM) composed of dense cytoplasmic small cells (Halperin, 1966). Somatic embryogenesis, in angiosperms, has been achieved from various explants, such as stems, hypocotyl, root, leaf inflorescence, pedicel, floral bud, excised embryo, anther filament and nucellus.

2.2 Somatic embryogenesis in Gymnosperms

Cloning of trees using somatic embryogenesis (SE) in conjunction with cryopreservation could have major impact on tree breeding and commercial plantation forestry. The main advantages of clonal forestry include: consistent production of the same genotype over time, the capture of larger genetic gains compared to conventional tree breeding, flexibility to rapidly deploy suitable clones given the changing breeding purposes and/or environmental conditions, and the ability to manage genetic diversity and genetic gain in plantation forestry (Park *et al.*, 2002). Given the economic importance of conifers, they represent very desirable candidates for clonal propagation. In conifers, somatic embryogenesis generally comprises three stages; induction, proliferation and development, and maturation. In the first phase, embryogenic cultures can be initiated from several explant types, such as immature and mature zygotic embryos, young seedlings, and megagametophytes (Gupta *et al.*, 1991). Most often, an organized mass of compact embryonal cells associated with long suspensors comparable to early stages of zygotic embryo development is formed on the explants, and is referred to as an embryonal mass or stage

1 somatic embryo (von Arnold and Hakman, 1988), embryonal suspensor mass (Gupta *et al.*, 1991), or proembryo and embryonic calli (Tautorus *et al.*, 1991). During proliferation, these embryonal masses undergo cleavage polyembryony and the development of the embryonal mass to form bipolar embryos with cotyledons, hypocotyl and radicle is then induced by modification of the culture medium (Misra, 1994). Maintenance of the embryogenic potential through proliferation of proembryogenic masses (PEMs) occurs in the presence of the exogenously supplied plant growth regulators (PGRs), auxin and cytokinin. Late embryogeny requires absicic acid (ABA) (Stasolla *et al.*, 2004) and possibly increased osmotic concentration (Attree *et al.*, 1991). A critical step during overall embryogenic process is trans-differentiation of PEMs into somatic embryos and requires the execution of several physiological events, including programmed cell death (Filinova *et al.*, 2000).

The various *Pinus* species are regarded as relatively recalcitrant with respect to the initiation of somatic embryogenesis. The immature zygotic embryo is usually the most responsive tissue when used as the explant source (Laine and David, 1990) and mature seed explants yield a lower frequency of initiation (Bozhkov *et al.*, 1998). An efficient somatic embryogenic system has been established in *Pinus radiata,* which does not require exogenous hormones to stimulate embryo development (Smith, 1996).

3 Regulation of plant embryo development

A plant's progression through embryogenesis requires the expression of a complex array of genes, some of which are specific to discrete phases of embryo development. Their expression is in turn, co-coordinately controlled in response to an array of developmental signals. Thus certain

post-transcriptional and translational regulations are also involved in the whole process. Several biological molecules are involved in various functions in a concerted manner to accomplish the process of embryo development. These molecules could include certain hormones (such as GAs, Brassinosteroids, Cytokinins) or they could be signalling peptides (such as AGPs and Phytosulfokines), which initiate the downstream reactions in order to stimulate genes required for embryogenesis. In addition to these, there are certain extracellular proteases which are believed to generate signalling peptides on the cell surface. Hormones also play a vital role in regulation of embryo and seed development.

3.1 Hormonal control of embryo and seed development

Signal transduction pathways, mediated by environmental and hormonal signals, regulate gene expression in seeds. The plant hormones abscisic acid (ABA), gibberellins (GA), ethylene, brassinosteroids (BR), auxins, cytokinins and other signaling molecules have profound effects on plant development at vanishingly low concentrations (Kucera *et al.*, 2005). They are chemical messengers for the communication between cells, tissues and organs of higher plants. Plant hormones are extremely important for the regulation of seed dormancy and germination (Finkelstein, 2004). The relationship between the role of growth regulators and seed development is mostly evident from the content of growth regulators present at different stages. GAs appear to be involved in many aspects of seed development, including fertilization, embryo growth, assimilate uptake, fruit growth and the prevention of seed abortion, in tomato, pea and several species of the *Brassicaceae* (Kucera *et al.*, 2005). Studies in pea and *Phaseolus* suggest that the highest concentration of biologically active GAs occur during the maximum growth of

the developing embryo, and in *Phaseolus*, the suspensor of the developing embryo was found to be a good source of GAs (Bewley, 1997).

Cytokinins are known to promote cytokinesis in certain plant tissues and it is likely that they have the same role in embryogenesis. The initial stages of embryo development involve cell division and multiplication and thus it is also the period where cytokinins are found in highest concentration. Cytokinins are present in developing seeds and accumulate predominantly in the liquid endosperm (Emery *et al.*, 2000). They may have roles in embryogenesis, in embryonic pattern formation, in the early period of grain filling of cereals, and in enhancing sink strength. In gymnosperms, the first few divisions are free nuclear, which is later followed by wall formation. It can be stipulated thus, that this is the stage of development where cytokinins will have an important role to play in gymnosperms.

Auxins seem to play a major role in embryogenesis, providing positional information for the coordination of correct cellular patterning from the globular stage onwards (Teale *et al.*, 2005). In *P. taeda* megagametophytes, IAA showed stable levels during the initial developmental stages of embryos, increasing significantly only in the cotyledonary stage followed by a posterior reduction in the mature seed (Silveira, 2004). Tryptophan (which is a precursor of Auxin) biosynthesis is important only in the initial phase of seed development, during the establishment of embryo polarity (Michalczuk *et al.* 1992).

ABA is associated with arrest of embryo growth. The ABA content is higher in young non-germinable seeds than in older ones. In Pine, the highest level of ABA was observed in the globular stage, followed by a continuous reduction until stabilization in the pre-cotyledonary stage (Silveira, 2004). Kapik *et al.* (1995) suggested that *P. taeda* zygotic embryos may import

ABA from the megagametophyte especially during early development, when the water content is high and these tissues are in close contact with each other.

Somatic embryogenesis has been widely used as a model system to study the hormonal regulation of embryogenesis. Levels of endogenous hormones in cultured explants are considered to be the most important factor for the determination of tissue specific embryogenic potential (Jime´nez, 2001a). Divergent hormonal requirements for SE show that sensitivity of the cultured cells/tissue to embryogenesis-inducing signals, including hormones, is more decisive for induction of the embryogenic pathway (Feher, 2003). There are reports where GAs stimulated SE, and GA inhibitors repressed SE (Biddington *et al.* 1992; Rudus *et al.* 2002), while there are also opposite reports where GA inhibitors improved SE (Pullman *et al.*, 2005). Higher levels of endogenous auxins were characteristic of embryogenic versus nonembryogenic cultures of carrot (Jime´nez and, 2001a), maize (Jime´nez and Bagnerth, 2001b), and *Prunus* sp. (Michalczuk and Druart, 1999); but in oil palm (Besse *et al.*, 1992) and carrot (Michalczuk *et al.*, 1992), on the contrary, the cultures did not have elevated auxin contents. Likewise, endogenous ABA concentrations in embryogenic culture lines were found to be higher (Jime´nez and Bagnerth, 2001a) or lower (Ivanova *et al.*, 1994) in comparison to non-embryogenic ones. ABA is believed to inhibit precocious germination and is known to be an important factor, besides osmoticum, for the maturation of seeds of many angiosperm species (Kermode, 1990; Hetherington and Quatrano, 1991). In *Dactylis glomerata* (Wenck *et al.*, 1988) and *Medicago arborea* (Pintos *et al.*, 2002), a higher concentration of endogenous cytokinin was found to be unfavorable for inducing SE while mutants with an elevated level of endogenous cytokinin displayed increased embryogenic potential in callus culture derived from seedlings (von Recklinghausen *et al.*,

2000). Brassinosteroids (BR) and GA interact with light in regulating elongation of shoots and photomorphogenesis of seedlings by apparently independent pathways (Szekeres, 2003). GA and BR act in parallel to promote cell elongation and germination and to counteract the inhibitory action of ABA. Brassinolides have been reported to improve somatic embryogenesis in conifers and rice (Pullman *et al.*, 2003b).

Seed development and germination are characterized by cross talk between the various hormones which interact at several levels in their signaling pathways. ABA and GA act at different times and sites during seed life. GA biosynthesis in developing seeds of many species leads to the accumulation and storage of either bioinactive GA precursors or bioactive GA. GA20-oxidases (GA-20ox) and GA3-oxidases (GA-3ox) are key enzymes that catalyze the final steps in the synthesis of bioactive GA, whereas GA2-oxidases (GA-2ox) are responsible for GA deactivation (Sunb *et al.*, 2004). DELLA proteins are transcription factor that inhibit GA responses and thus are considered to be negative regulators of GA signal transduction (Lee *et al.*, 2002). GA signaling requires degradation of DELLA proteins through the 26S proteosome pathway mediated by F box proteins such as *Sly1* (McGinnis *et al.*, 2003). A similar mechanism is observed in auxin signaling where the F box protein *TIR1* promotes degradation of Aux/IAA transcriptional repressors (Dharmasiri *et al.*, 2005). Ethylene is considered to be a promoter of seed germination and cross talk between ethylene and GA during the transition from seed dormancy to germination has been postulated from the hormonal regulation of a GA20-oxidase in *Fagus*s *sylvatica* (Calvo *et al.*, 2004). ABA inhibiton of seed germination in *Arabidopsis* has been found to be reversed by ACC which is a precursor of ethylene (Ghassemian *et al.*, 2000). Brassinosteroids (BR) and GA interact with light in regulating elongation with shoot and photomorphic genesis of seedlings by apparently independent pathways. BR treatment rescues

the germination phenotype of the severe GA biosynthesis mutants and seed germination of BR deficient mutants is more strongly inhibited by ABA as compared to wild type (Kucera *et al.*, 2005).

3.2 Genetic regulation and protein expression during embryo development in plants

Embryo gene expression during development is critical for establishing the polarity and morphological pattern of the plant (Raghavan, 1986). Most of the gene expression studies during embryogenesis have been directed towards studying regulation of seed storage protein genes (Higgins, 1984; Flinn *et al.*, 1993), and identifying genes responsible for controlling pattern formation and morphogenesis during early development (Meinke, 1986). Crouch (1982) reported that gene sets active within cotyledon cells of zygotic embryos are also active in analogous cells during somatic embryogenesis. Although most genes that are expressed during the later phases of embryogenesis are also active during the post-germination phase, some genes have been identified as either specific to embryos or expressed at a much higher levels in embryos than at other periods of development (Dure *et al.*, 1981). Fig. 6 shows embryo-specific transcript sets expressed at different periods of seed development and are believed to be responding to different developmental signals. Seed protein genes, in particular, are highly regulated embryo specific genes. In general, seed proteins are regulated temporally during embryogenesis; they are either expressed exclusively during embryogenesis (Walling *et al.*, 1986) or at a very low level in mature plant organ systems (Goldberg *et al.*, 1983). Seed protein gene expression is regulated spatially within the embryogenic organ system and is localized with specific cells in the embryo (Goldberg *et al.*, 1989). Some examples of genes active at various stages are discussed in this section.

The *LEAFY COTYLEDON (LEC)* genes define a small class of regulators that function during both morphogenesis, as well as maturation phases of embryo development. Mutant analysis of these genes reveal that LEC genes are responsible for maintaining suspensor cell fate and specifying cotyledon identity during early phases of development and during later stages they are required for initiation and/or maintenance of maturation and the repression of precocious germination (Meinke, 1992; Harada, 2001).

The apical-basal axis of the embryo comprises several pattern elements including the cotyledons, shoot apical meristem, hypocotyl, root and root apical meristem. Genes involved in establishing these have been identified using various mutants and include *SHOOTMERISTEMLESS (STM)* and *WUSCHEL (WUS*) which play critical roles in formation of shoot apical meristems (Barton and Poethig, 1993; Laux *et al.*, 1997). Recently, activities of both the *PINOID (PID)* and *ENHANCER OF PINOID (ENP*) genes have been shown to be required for the initiation of cotyledon development (Treml *et al.*, 2005). *MONOPTEROS (MP*), and auxin responsive factor and the Aux –IAA protein *BODENLOS (BDL*) (Hamann *et al.*, 1999; 2002) are required for the formation of the hypophysis cell of the embryo, which is a precursor for the root apical meristem in angiosperms. *SHORT_ROOT (SHR*) and *SCARECROW (SCR*) transcription factors specify the patterning of the ground tissue systems of embryo (Benfey *et al.*, 1993; and Scheres *et al.*, 1995).

The genes involved in the maturation phases of embryogenesis are mostly the ABA responsive genes. ABA has been shown to influence the accumulation of embryo specific mRNAs such as storage protein mRNAs and LEA (Late Embryogenesis Abundant) mRNAs (Galau *et al.*, 1986). LEA genes have been found to be developmentally regulated and reach maximal expression in

mature cotyledonary embryos (Galau *et al.*, 1986), including those of white spruce (Dong and Dunstan, 1996). Three Loblolly pine cDNAs (LPZ 202, LPZ 216 and LPS 094) were earlier used as indicators of developmental stage of seeds in Loblolly pine (Vales *et al.*, 2007). Auxin response factors are involved in signal transduction and polarity in embryogenesis (Sauter and Lindsey, 2000). At the molecular level, the embryo-specific conifer genes identified are predominantly homologous to angiosperm seed storage protein and LEA genes (Dunstan *et al.*, 1998). This suggests that genes central to embryogenesis will exhibit a high degree of conservation with their angiosperm cousins, despite the passage of approximately 300 million years since the conifers diverged from the ancestral line that gave rise to the angiosperms (Stewart and Rothwell, 1993).

Somatic embryogenesis has been used as a model system to study the genes functional during embryogenesis. Studies in carrot somatic embryogenesis have shown that some embryogenesis related genes (LEA genes, *SERK*, *AGL15*, *BBM, LEC1*, *FUS3* and *ABI3*) were expressed in the same manner in both zygotic and somatic embryos (reviewed by Ikeda *et al.*, 2006). Mature spruce somatic embryos contain all of the major storage protein classes found in zygotic embryo, i.e., vicilin, legumins and albumin (Flinn *et al.*, 1993). However, in some plants the accumulation of storage proteins is not observed during somatic embryo development and this feature has been used to distinguish between somatic and zygotic embryos (Morcillo *et al.*, 2001). Several lines of evidence show that ABA plays a role in suppression of precocious germination and the appearance of germination specific enzymes in cultured embryos (Finkelstein *et al.*, 1985).

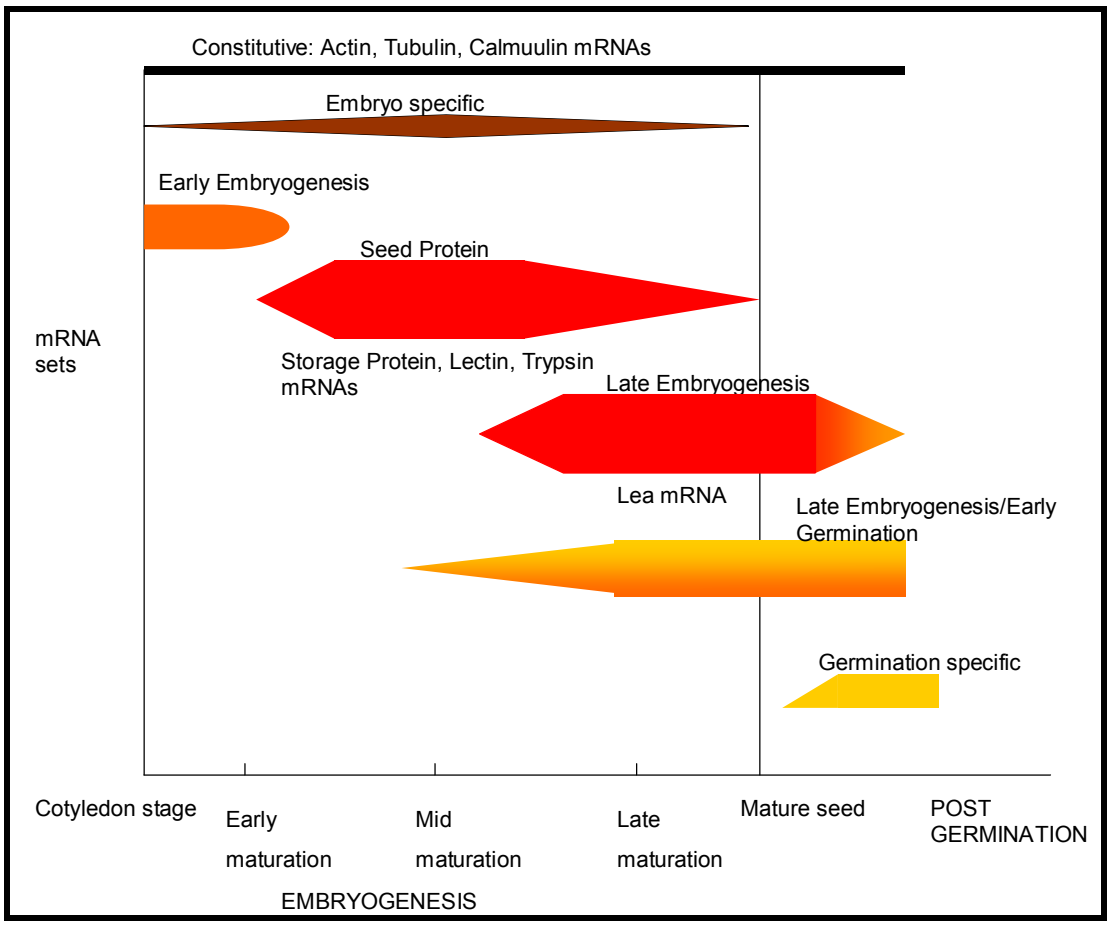


Figure 6: Regulation of prevalent mRNA sequence sets during seed development and germination. The thickness of each line represents the relative prevalence of each mRNA set. Tapering of each line shows periods when the mRNA set accumulates or decays (Adapted from Goldberg *et al*., 1989).

3.3 Genomics as a tool for studying embryo development in Gymnosperms

In gymnosperms, an understanding of the molecular regulation of embryogenesis is limited. However, advances in conifer somatic embryogenesis now allow a more detailed investigation of gymnosperm embryogenesis. In Norway spruce, somatic embryo production is well characterized, as are the growth and morphology of distinct cell lines (Bozhkov and von Arnold, 1998; Filonova *et al.*, 2000). A seed has to achieve successful embryo development and germination, and it has to integrate embryo and seedling development with the environment. Genetic analysis provides the opportunity to perform the dissection of complex developmental processes. Genome level research in angiosperm embryo development is quite advanced while this is not the case in gymnosperms, mainly due to their large genome sizes. Lately, there has been interest in understanding gymnosperms at the genomic level, partly owing to their economic importance. At present, the whole genome sequencing project is underway for *Pinus taeda*. The success of this project will provide a better understanding of genes that are functional at various levels and aspects of Pine growth and development, and provide a model for other gymnosperms. At present EST databases are available for major Gymnosperm genera such as *Pinus* and *Picea*. In a comparative analysis of proteins implicated in embryogenesis in angiosperms and Loblolly pine embryo EST sequences, putative homologs for 83 embryogenesis-related proteins were found (Cairney *et al.*, 2006). An enormous amount of information relating to various plant genomes is available publicly. However, it is very important to exploit these resources to develop an understanding of the role of various genes during the course of plant development.

3.4 ECM modification and the importance for regulation of growth and development

The plant extracellular matrix (ECM) is complex and diverse, and is involved in cell–cell communication in a wide range of developmental, reproductive and pathogenic processes. Characterization of integral ECM components will lead to improved understanding of their roles in signaling. Cell-cell signaling is crucial for co-ordination of a wide range of activities in plants, and can occur via a number of different routes. It is also apparent that the plant extracellular matrix (ECM) plays a variety of roles in cell-cell communication in addition to its structural cell wall functions. It acts as a conduit for signals, as a source of signaling molecules and as the extracellular domain of plasma membrane receptors. Cell fate decisions during plant development are largely determined by cellular position. Signaling via the ECM has been shown to underlie fate decisions during the patterning of the shoot apical meristem (Brownlee, 2002). Various aspects of growth and development ultimately involve modifications to the ECM, including processes such as stigma development, pollen tube growth, root growth and *Rhizobium*-legume symbiosis (Flinn, 2008). Certain ECM molecules such as cell surface-exposed AGPs display a diverse array of carbohydrate epitopes, possibly associated with a signaling function in the control of cell proliferation and morphogenesis (Seifert and Roberts, 2007). This often correlates with the formation of anatomical patterns and appears to be developmentally regulated. The plant cell wall is emerging as a dynamic and functionally important entity in plant developmental processes, with functions that include mechanical support, a medium for nutrient transport and external stimuli transduction and the first line of defense against pathogens and other biotic and abiotic stresses. The cell wall provides a structural and functional continuum to the whole plant body, the apoplast. This provides a medium for interaction between external and internal environment and stimuli.

The main components of the cell wall represent carbohydrates, such as cellulose, hemicellulose and pectin, which provide structural rigidity and mechanical support to the plant cell. However, the dynamic nature of the ECM can be attributed to the proteins present in the ECM. Proteins represent only 10% of the total cell wall component, but functionally they are the extremely important. They are involved in modifications of wall structures, cell wall components, signaling and interactions with plasma membrane proteins at the cell surface. Fig. 7 illustrates the major types of components found in the plant cell wall.

The structural proteins include hydroxyproline-rich proteins, glycine-rich proteins and proline-rich proteins, which interact with structural carbohydrate components of the ECM. In order for the cell to grow, expansion is required, which is facilitated by cell wall loosening. One of the major components responsible for cell wall loosening is the expansin family, with 36 expansins identified in *Arabidopsis* (Sampedro and Cosgrove, 2005). Expansins are secreted proteins found in relatively low abundance. They have been shown to directly bind to cellulose and break the non-covalent bonds between cellulose and matrix polysaccharides (McQueen-Mason and Cosgrove, 1995).

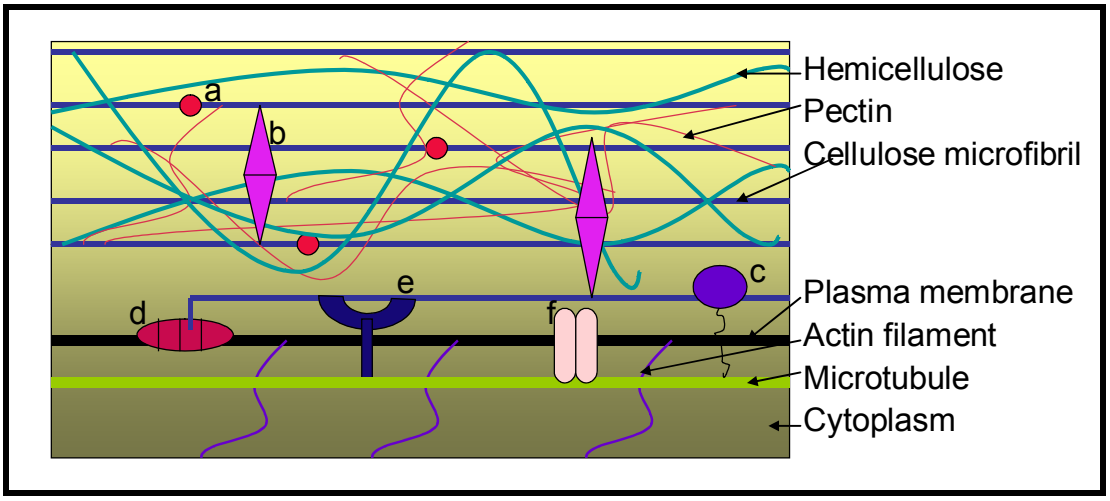


Figure 7: Model of the cell wall–plasma membrane–cytoskeleton continuum illustrating the main polysaccharide and protein components. The wall consists of cellulose microfibrils, cross-linked by hemicelluloses, and embedded in a pectin matrix as well as numerous protein components such as (a) expansins, (b) extensins and (c) glycosylphosphatidylinositol (GPI)-anchored proteins which are heavily glycosylated and associated with the extensive polysaccharide network. Various plasma membrane proteins such as the (d) cellulose synthase complex, (e) receptor kinases, (f) ion channels (Adapted from Humphrey *et al.*, 2007).

In addition to expansins, there are xyloglucan hydrolases which function by cleaving and re-grafting xyloglucans. Wall loosening is followed by separation of microfibrils allowing deposition of new wall material and cell elongation. In order to maintain the cell shape, microfibrils need to be laid down in a proper orientation and this is facilitated by a GPI-anchored protein, COBRA (Brady *et al.*, 2007), which has been proposed to regulate the orientation of cell expansion by influencing the way in which cellulose microfibrils are laid down. The following events involve extensive cross-linking between polysaccharide and protein components, resulting in rigidification of the wall. Cell wall-located peroxidases can oxidize various substrates within the cell wall, forming cross-links between cell wall polymers and proteins (Passardi *et al.*, 2004). Extensins are characterized by a series of Ser(Hyp) 4 that become arabinosylated, and undergo specific glycosylation, hydroxylation, crosslinking with other cell wall components and in some cases attachment of glycosylphosphatidylinositol (GPI) anchors. These proteins play a major role in cell wall rigidification (Borner *et al.*, 2002).

The involvement of the cell wall in stress response is exhibited by a mutation of cellulose synthase enzyme that has shown an increase lignin and pectin deposition and also an increase in ethylene and jasmonic acid responsive genes. As a result, the plant in general becomes more tolerant to drought and salt stresses (Caño-Delgado *et al.*, 2003).

Mechanical stimuli reception is another function involving the cell wall. An increase in cytosolic Ca^{+} deposition has been found to occur in response to mechanical stimuli, such as wind, touch and gravity. In animals, integrins serve as a link between the external mechanical stimuli and the internal environment through extracellular proteins that interact with them. These proteins have a

characteristic RGD containing motif and are thought to interact directly with integrins. Currently there are no known true integrins but some integrin-like proteins have been found in plants, and RGD peptides have been shown to induce responses in plants (Hynes, 2002).

Apart from structural proteins, there are certain receptor proteins and signal peptides which contribute to signal transduction through cell wall. For example, wall-associated kinases (WAKs) which are positioned at the plasma membrane with an external domain embedded within the wall and an intracellular kinase domain (He *et al.*, 1996). Like the WAKs themselves, most of the WAK-like proteins (WAKLs) are predicted to encode a transmembrane protein with a cytoplasmic Ser/Thr kinase domain and an extracellular region with similarity to vertebrate epidermal growth factor (EGF)-like domains (Verica and He, 2002). WAKs have been proposed to sense cell wall expansion through their attachment to pectin, and signal via influencing the activity of vacuolar invertase, thus controlling turgor during cell expansion. The *Arabidopsis* glycine-rich protein 3 (*AtGRP3*) could be the ligand for the WAK1 receptor, and would provide evidence for involvement in pathogenesis-related processes. Lectins are proteins that bind to but do not alter carbohydrates, and a large family of receptor kinases have been identified which contain an extracellular lectin-like domain, the lectin receptor kinases (*LecRKs)* (Herve *et al.*, 1999). Eight of these were receptor kinases, four of which contain a lectin-like extracellular domain, which was shown to be responsible for protein–protein interactions in binding to the RGD motif as well as having potential for binding carbohydrates. The leucine-rich extensin (LRX) gene family encodes 11 chimeric proteins consisting of an N-terminal leucine-rich repeat (LRR) domain and a C-terminal extensin-like domain which are insolubilized in the cell wall (Rubinstein *et al.*, 1995; Baumberger *et al.*, 2001). LRR domains are important for protein–

protein interactions and, in plants, are commonly found in signaling proteins such as LRR-receptor kinases, nucleotide binding site (NBS)-LRR proteins, and GTPase activating proteins.

Many cell wall proteins are attached to the outer face of the plasma membrane by virtue of their GPI anchors, which are added to the protein post-translationally. As the GPI anchors may be cleaved by phospolipase C or D, it is possible for GPI-anchored proteins (GAPs) to be liberated from the plasma membrane into the cell walls. GPI- anchored proteins interact with the wall matrix as well as with internal cytoplasmic proteins, and the actin and tubulin cytoskeleton (Humphrey *et al.*, 2007).

3.4.1 Regulation of embryo development by extracellular proteins

It has been suggested that proteolytic extracellular molecules in plant cells are involved in the degradation of extracellular molecules, to generate signaling substances regulating pathogen responses, and developmental processes. Typically, the functions assigned to plant proteases include: a) the removal of abnormal/misfolded, modified, and mistargeted proteins; b) the supply of amino acids needed to make new proteins; c) the maturation of zymogens and peptide hormones through limited cleavages; d) the control of metabolism and homeostasis by reducing the abundance of key enzymes and regulatory proteins; and e) the cleavage of targeting signals from proteins prior to their final integration into organelles (Palma *et al.* 2002). These are the characteristic features of any given developmental process. Embryo development and germination are two fundamental plant developmental processes of a plant's life, and understanding how they are regulated could lead to advances in plant improvement. Most of the studies involving extracellular proteins expressed during embryogenesis have been carried out in

somatic embryogenesis system, due to the ease of manipulating the environment of the developing embryo. The media in which somatic embryos develop mimics the megagametophyte/endosperm which serves as the nourishing tissue for the developing embryo. During somatic embryogenesis, extracellular proteins are secreted into the culture medium in both angiosperms (de Vries *et al.*, 1988) and gymnosperms (Egertsdotter *et al.*, 1993). The initiation of somatic embryogenesis results in major changes of the extracellular protein pattern compared with that of intracellular proteins (de Vries *et al.*, 1988). The extracellular protein *EP1* is secreted only by nonembryogenic cells (Van Engelen *et al.*, 1991), whereas the extracellular protein *EP2*, identified as a lipid-transfer protein, is secreted only by embryogenic cells and somatic embryos (Sterk *et al.*, 1991). *EP3*, which was originally purified as a protein capable of rescuing somatic embryos in the mutant carrot cell line ts11 at the nonpermissive temperature (De Jong *et al.*, 1992), is an acidic endochitinase. The chitinases are involved in the generation of signal molecules essential for embryogenesis in *ts11* (De Jong *et al.*, 1993). In spruce embryonic cultures, specific profiles of extracellular arabinogalactan proteins (AGPs) correlate with different developmental stages of somatic embryos, and extracellular AGP species isolated from late stage somatic embryos affect the development of embryos at earlier stages (Egertsdotter and von Arnold, 1995). Some secreted proteins like avematin (Mo *et al.*, 1996), and chitinase 4 (Egertsdotter, unpublished), only appear in embryos at later stages of development and appear to be required for the formation of the mature embryo (Mo *et al.*, 1996). Cell wall proteins have also been implicated in regulating polarity during embryogenesis. Arabinogalactan protein epitopes are known to display developmentally regulated patterns of expression in several plant tissues. A role for AGPs was suggested in plant reproductive development (Pennell *et al.*, 1991), pattern formation in roots (Knox *et al.*, 1991) or maize coleoptiles (Schindler *et al.*, 1995), and

somatic embryogenesis (Pennell *et al.*, 1992). AGPs have been implicated in cell division (Serpe and Nothnagel, 1996), cell expansion (Willats and Knox, 1996) and cell death (Schindler *et al.*, 1995). The JIM8 antibody, which recognizes AGPs, reveals differences between embryo-proper and suspensor, binding only to the cells whose fate is as the suspensor (Pennell *et al.*, 1991). AGPs have been identified as differentially expressed during zygotic embryogenesis, as well as during somatic embryogenesis (Sauter and Lindsey, 2000). Addition of AGPs from an embryogenic cell-line to a non-embryogenic cell line induced embryogenic capacity in those lines (Kreuger and van Holst, 1996). Activation of immature seed AGPs by hydrolytic enzymes like EP3 endochitinases is an important component of embryogenesis (van Hengel *et al.*, 2002). Germins are another class of extracellular proteins that primarily act as oxalate oxidases, and germin-like proteins (GLPs) are mainly superoxide dismutases (SOD). Germin/GLP genes are often expressed during the early growth stages in wheat embryos and callus of pine, *Arabidopsis*, and cotton and also during organ formation in *Arabidopsis*, barley and potato. In gymnosperms, the first GLP was identified by Domon *et al.* (1995), whose corresponding cDNA was reported as *PcGER1* by Neutelings *et al.* (1998). A similar GLP cDNA, *PrGLP* was reported in *Pinus radiata* by Bishop-Hurley *et al.* (2003).

3.4.2 Protein degradation within the plant ECM

Without proteolysis, the cell surface and extracellular matrix would be a rather static environment. Proteases have the unique ability to hydrolyse peptide bonds, irreversibly modifying the function of a substrate protein. Through specific processing, rather than the complete degradation of the substrates, proteases can modify signaling circuits and cell function (Overall and Blobel, 2007). Examples of extracellular proteolytic modifications in cell signaling

include the extracellular serine proteases *LIM9* (a role in microsporogenesis and pollen development - Taylor *et al.* 1997), *ARA12* (a role in cell wall metabolism or precursor protein processing - Hamilton *et al.* 2003), *SDD1* (a regulator of stomatal distribution and density - Berger and Altmann 2000), *BRS1* (involved in the proteolytic processing of a rate-limiting protein in brassinosteroid signaling - Li *et al.* 2001), the extracellular cysteine protease RCR3 (a role in disease resistance - Rooney *et al.* 2005), and the extracellular aspartic proteases *CDR1* (a role in disease resistance - Xia *et al.* 2004) and *Cardosin B* (a proteolytic aid in pistil development, in pollen-pistil interactions, and/or in pathogen protection - Vieira *et al.* 2001). Furthermore, the extracellular serine protease *ALE1 (*a role in embryo epidermal surface formation and cuticle development - Tanaka *et al.* 2001) and the extracellular cysteine protease *DEK1* (helps to maintain and restrict the aleurone cell fate - Lid *et al.* 2002) illustrate the potential for ECM-localized protease activity in the processes of seed and embryo development.

In addition to these, matrix metalloproteinases (MMPs) represent an additional class of extracellular proteinases that have been poorly studied in plants, but are well characterized in mammalian systems. The clustering of microarray data that reveals transcript patterns associated with somatic embryogenesis in soybean, illustrated a set of genes involved in cell wall remodeling, which often occurs in response to stress. These genes are MMPs, which facilitate extracellular matrix degradation in senescing tissue (Delorme *et al.*, 2000) and during tumor invasion (Zhu *et al.*, 2001).

4 Matrix Metallo Proteinases

Matrix metalloproteinases are a family of calcium-dependent, zinc-containing endopeptidases, similar in structure and function (Bode *et al.*, 1999). Most studies have been carried out on

human MMPs, of which there are 23 different forms that are divided into six groups based on their substrate specificity and sequence similarity (Snoek-van Beurden *et al.*, 2005). Each MMP consists of a specific domain sequence with several domain motifs including, the signal peptide, the propeptide domain, the catalytic domain, and the C-terminal hemopexin like domain. However, several MMPs have additional domains such as a transmembrane or cytoplasmic domain (Woessner and Nagase, 2000). They are secreted in a latent form called pro-MMP or zymogens which require activation before they can cleave the extracellular matrix components. Extracellular activation is a complex mechanism, involving the disruption of the interaction between the conserved cysteine of the prodomain and zinc (Zn^{2+}). The cys-Zn^{2+} bond is disrupted when a water molecule binds to the Zn^{2+} ion of the zinc binding domain, which then turns into a catalytic domain, as shown in Fig. 8 (Snoek- van Beurden *et al.*, 2005). This disruption can be achieved *in vitro* by chemicals such as APMA, low pH and heat treatment. Removal of the prodomain can also occur directly through proteolytic cleavage. Pericellular activation of proMMP-2 and proMMP-13 by membrane-type MMPs has also been demonstrated (Lemaitre and D'Armiento, 2006).

Expression of most MMPs is tightly regulated at the transcriptional level. MMP activity in tissues is regulated at multiple levels including conversion from proenzyme to the activated form, and complexing with specific inhibitors. MMPs are also controlled by endogenous inhibitors, the Tissue Inhibitors of Metallo-Proteinases (TIMPs). TIMPs are also secreted proteins, but they may be located at the cell surface in association with membrane-bound MMPs (Baker *et al.*, 2002). There are currently four known TIMPs, and they operate with different inhibition efficiencies against the different MMPs. The four TIMPs are also differentially expressed in tissues, and temporally follow the influx of MMPs (Woessner and Nagase, 2000).

Normal and pathological tissue remodeling is regulated by the balance between MMPs and TIMPs (Bode *et al.*, 1999). Synthetic inhibitors of MMPs such as Ilomastat (GM6001), BB-94 etc., which incorporate a hydroxamate group to block the active site Zn (thus rendering the protein inactive), have also been used commonly in MMP inhibition assays (Alexander *et al.*, 2001, Webber *et al.*, 2002). Fig. 9 shows the crystal structure of GM6001. GM6001 is also known as galardin, and it's IUPAC name is (2R)-N'-hydroxy-N-[(2S)-3-(1H-indol-3-yl)-1-methylamino-1-oxopropan-2-yl]-2-(2-methylpropyl) butanediamide with MW: 388.460720 g/mol and MF: $C_{20}H_{28}N_4O_4$.

The MMPs are involved in a wide range of proteolytic events, in normal and pathological circumstances. Normal processes in which MMPs are implicated include developmental processes such as blastocyte implantation, embryonic development, nerve growth, skeletal , bone growth; reproductive processes such as endometrial cycling, luteolysis, cervical dilation, mammary gland morphogenesis; maintenance related processes such as wound healing, angiogenesis, enamel formation, hair follicle cycle, nerve regeneration, macrophage function, and apoptosis (Woessner and Nagase, 2000).

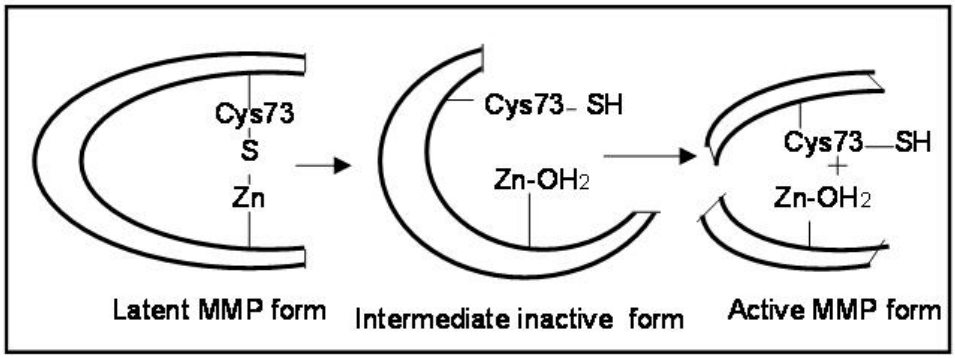


Figure 8: Mechanism of activation of MMPs. The activation of MMPs involves a disruption of the Cys 73-Zn+ bond that results in an intermediate active form, thereafter fully active form results from proteolysis (Adapted from Snoek-van Beurden, 2005).

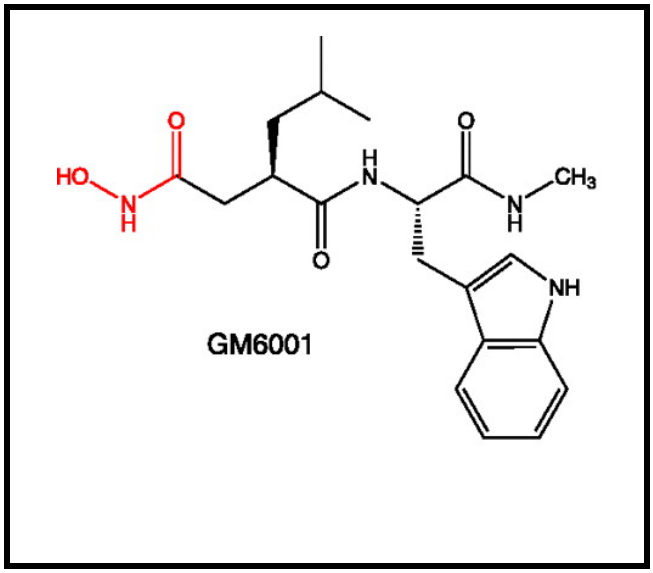


Figure 9: Crystal structure of GM6001 (Balakrishnan *et al*., 2006).

Pathological processes involving MMPs include tumor growth and migration, fibrosis, arthritis, glaucoma, lupus, scleroderma, cirrhosis, multiple sclerosis, aortic aneurysms, infertility, and many more diseases. It is fair to say that a proteolytic event is key to a wide range of biological processes, including tissue remodeling and also modification or release of biological factors (Woessner and Nagase, 2000).

MMP expression has been reported during embryogenesis and developmental processes in various organisms besides humans. Collagenase plays a major role in skeletal morphogenesis during mice embryogenesis (Fini *et al.*, 1998). MMP-21 is expressed transiently in mouse embryogenesis and increased in embryonic neuronal tissues (Marchenko *et al.*, 2003). MMPs have a tight but distinct association with tissue remodeling in different regions in *Xenopus* during embryogenesis (Damjanovski *et al.*, 2000).

Krane (1994) has suggested criteria for proving the role of an MMP in a remodeling process: Remodeling can be blocked with a drug or antibody specific to the MMP, or can be reproduced by over expression of the MMP gene in a transgenic animal. It can also be abolished by deleting the MMP gene. Spontaneous mutations can be identified and the phenotypes characterized. Mutations can be induced in the gene that produces the remodeling process.

4.1 Plant Matrix Metalloproteinases

Plant metalloproteinase activity was first detected in Soybean leaves as Azocollase (Ragster and Chrispeels, 1979). This protein could not be purified until Graham *et al.* (1991) finally obtained and characterized the first plant metalloproteinase *SMEP1* from soybean leaves. However, the roles of MMPs in higher plants remain unclear. It has been speculated that MMPs are involved in

remodeling of plant extracellular matrix in association with plant growth, development, and possibly defense processes (Pak *et al.*, 1997, Maidment *et al.*, 1999). All the plant MMPs reported so far share common structural features with animal MMPs, such as the characteristic cysteine switch PRCGXXD motif (except *GmMMP2*, which has LRCGVPD), the zinc binding catalytic HEIGHXLGLXH domain, followed by a conserved methionine residue of the Met-turn (Fig.10). All the plant MMPs reported so far are intronless (Flinn 2008). Interestingly, all plant MMPs contain an invariant DLE/QS/TV/A sequence on the N-terminal side of the zinc binding region, which is not found in any of the human MMPs and its function is unknown. Massova *et al.*, (1998) have performed a multiple sequence analysis on 64 MMPs that demonstrates that plant MMPs are most closely related to those from invertebrates (sea urchin and nematode worm) (Maidment *et al.*, 1999).

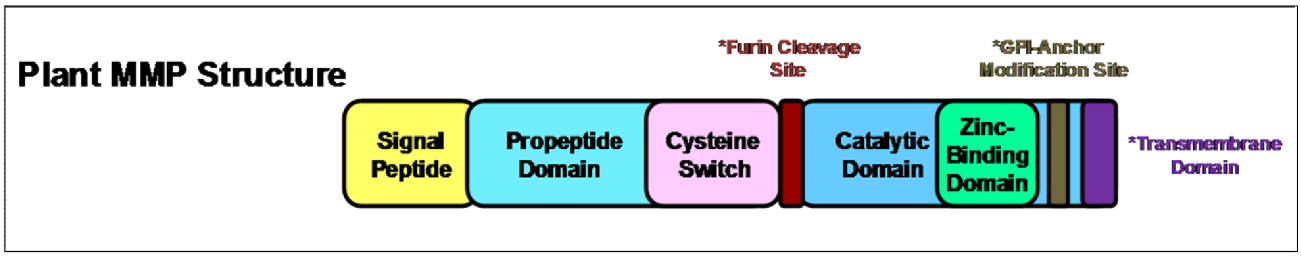


Figure 10: General plant MMP structure showing the color coded relevant domains. Positions of putative furin cleavage sites, GPI modification sites and C-terminal transmembrane domains, when predicted, are indicated (Flinn, 2008).

There are no known substrates of MMPs in plants. However, some molecules in the plant extracellular matrix (ECM) contain similar motifs to those found in mammalian ECM including vitronectin (Sanders *et al.,* 1991), fibronectin (Pellenc *et al.,* 2004) and integrin (Katembe *et al.,* 1997).

The extracellular matrix of cells is involved in cell differentiation and determination of cell fate. Matrix metalloproteinases regulate the composition of the ECM in mammalian cells by acting on the proteins and macromolecules providing the structural framework of the ECM. Plant MMPs are comparatively less studied than the animal MMPs. The available reports of plant MMPs, their expression and putative functions are briefly described below.

Five different MMPs (*At1-MMP* to *At5-MMP*) have been reported in *Arabidopsis thaliana*. The domain structure of these MMPs is similar to mammalian *MMP-7*. The expression study of the five MMP genes showed that each At-MMP had a distinct pattern of expression, suggesting a functionally different role for each. For *At1-MMP*, the expression level was F>R>S>L, (F= flower, R= root, S= stem, L= leaf). For *At2-MMP*, R>F>L>S; for *At3-MMP*, L>R>F>S; for *At4-MMP*, F>S>L>R; for *At5MMP*, L>R>S>F. Myelin basic protein was degraded by active *At1-MMP,* but with a digestion pattern different from that of stromelysin 1 (MMP-3). Human *TIMP-1* and *-2* were both able to inhibit the activity of *At1-MMP* (Maidment *et al.*, 1999). The function of these MMPs is not yet known. However a study with mutant *At2-MMP* revealed an inhibition of root, shoot and leaf growth, as well as late flowering, faster chlorophyll degradation and earlier senescence (Golldack *et al.*, 2002).

Soybean *SMEP1* was the first clearly demonstrated MMP from a higher plant. A similar enzyme had been reported from green alga, *Chlamydomonas* (Pak *et al.*, 1997). The extracellular localization of *SMEP1* in mature leaves suggests a role in tissue remodeling during leaf expansion. Another MMP from soybean, *GmMMP2* was reported to be activated in response to pathogenic infections in soybean. Differential display experiments using RNA samples prepared from etiolated soybean hypocotyls infected with the fungus *Phytophthora sojae* or mock inoculated with water revealed a cDNA similar to *SMEP1* and members of mammalian MMPs. Southern hybridization revealed that there is only a single copy of the gene and sequencing of the genomic DNA revealed that the gene does not contain any introns. There was a rapid accumulation of *GmMMP2* in response to wounding, however no correlation was found with the senescence process. However, it may function in mediating the activities of plant-derived enzymes involved in fungal cell wall degradation and help in releasing pathogen growth inhibiting substances (Liu *et al.*, 2001).

Cucumber *Cs1-MMP* was reported to be expressed at the boundary of senescence and programmed cell death. It has a collagenase-like activity that can cleave synthetic peptides and type-I collagen, a major component of animal ECM. *Cs1-MMP* activity is completely inhibited by a hydroxamate-based inhibitor that binds at the active site of MMPs in a stereospecific manner. The *Cs1-MMP* gene is expressed *de novo* at the end stage of developmental senescence, prior to the appearance of DNA laddering in cucumber cotyledons, leaf discs and male flowers. As the steady-state level of *Cs1-MMP* mRNA peaks late in senescence and the pro-enzyme must undergo maturation and activation, the protease is probably not involved in nutrient

remobilization during senescence but may be involved in elimination of remnants or releasing signal molecules during this process (Delorme *et al.*, 2000).

A recent study in *Medicago truncatula* revealed the presence of an MMP-like gene, *MtMMPL1* (Combier *et al*. 2007). This protein has an E to Q substitution in the zinc binding motif. This mutation in mammalian MMPs has been known to have dramatically inhibited the mammalian MMP proteolytic activity (Roswell *et al.*, 2002). Several legume MMPs contain this substitution. The transcripts of *MtMMPL1* were specifically associated with rhizobial infection, strongly induced in young root nodules and detectable by day three after inoculation, but were not found in any other tissue. A strong association of their presence with nodule tissues associated with infection thread formation and development was established by *in situ* hybridization. This indicates a possible role of these proteins in aiding the development of the infection thread cell wall, or a role in regulating the number of infecting bacteria (Combier *et al.*, 2007).

Conserved regions corresponding to different metalloprotease domains and sequence motifs were identified in the sugarcane expressed sequence tag (SUCEST) DNA library. At least four classes of sugarcane metalloproteinase have been identified; i.e., matrix metalloproteases, zincins, inverzincins, and ATP-dependent metalloproteases (Ramos and Selistre-de-Araujo, 2001). Amongst lower plants, a gamete lytic enzyme (GLE) of *Chlamydomonas reinhardtii* is a zinc metalloprotease and mediates digestion of the cell walls of the two mating-type gametes during mating as a necessary prelude to cell fusion, and is highly homologous to those in mammalian collagenase. A putative calcium binding site was present in the near C-terminal region of the mature GLE. Both propolypeptide and mature polypeptide had potential sites for asparagine-

linked glycosylation, and the Arg-(Pro) 3 and Arg-(Pro) 2 motifs, which are known to exist in hydroxyproline-rich glycoproteins of the Chlamydomonas cell wall (Kinoshita *et al.*, 1992).

4.2 Confirmation of plant MMP activity

MMP activity has been studied in plants by using either substrates or inhibitors. One or both of the substrates, myelin basic protein and azocoll, were used to confirm protease activity of *At1-MMP* (Maidment *et al.*, 1999), recombinant soybean *GmMMP2* (Liu *et al.*, 2001), and *SMEP1* (Graham *et al.*, 1991). The activity of cucumber *Cs1MMP* (Delorme *et al.* 2000,) and *At1-MMP* were also confirmed by using fluorescence labeled short amino acid peptides containing the scissile Gly-Leu/Ile bond of collagen. In *Cs1-MMP* protease activity could be confirmed using gelatin zymography, while gelatin was not successful in *At1-MMP* activity confirmation.

Inhibition studies using chelators like EDTA, EGTA and 1,10-phenanthroline were shown to inhibit activity of MMPs in plant. In addition, *TIMP-1* and *TIMP-2* could inhibit the activity of *At1-MMP*, while the activity of *Cs1-MMP* was only partially inhibited by *TIMP-1* and not at all by *TIMP-2, -3, or -4*. The recombinant proteins showed more proteolytic activity when synthesized without the prodomain (Delorme *et al.*, 2000; Liu *et al.*, 2001), or when lacking the prodomain (Liu *et al.*, 2001; Maidment *et al.*, 1999). *At1-MMP* could also be activated using 4-aminophenyl mercuric acetate (APMA) (Maidment *et al.*, 1999).

5 Modulation of Plant Embryogenesis – A Role for MMPs?

In a study involving an *Arabidopsis* mutant (*At2-mmp*-1) carrying a tDNA insertion in *At2-MMP*, neither germination nor development of plants was modified in comparison to the wild type in the juvenile rosette stage. Starting with the onset of shoots, growth of roots, leaves, and shoots was inhibited compared with the wild type, and the plants were characterized by late flowering. Besides the flowering, *At2-mmp*-1 plants showed fast degradation of chlorophyll in leaves and early senescence. These results demonstrate the involvement of *At2-MMP* in plant growth, morphogenesis, and development with particular relevance for flowering and senescence (Golldack *et al.*, 2002).

A virtual analysis of expression of various *Arabidopsis* MMPs using Genevestigator (https://www.genevestigator.ethz.ch/, Zimmerman *et al.*, 2004) indicates varying expression of various *Arabidopsis* MMPs at different stages of development (Fig. 11). Of all the At-MMPs, *At5-MMP* is expressed across all the developmental stages and tissues/organs. *At1-MMP* and *At4-MMP* are primarily expressed from young flower to mature silique/seed, with maximal expression during mature silique/seed stage. *At4-MMP* is also highly expressed during seed germination. *At2-MMP* is primarily expressed in young and developing rosette stages, in young flower and mature silique/seed. *At3-MMP* was least expressed over the various developmental stages (Flinn, 2008).

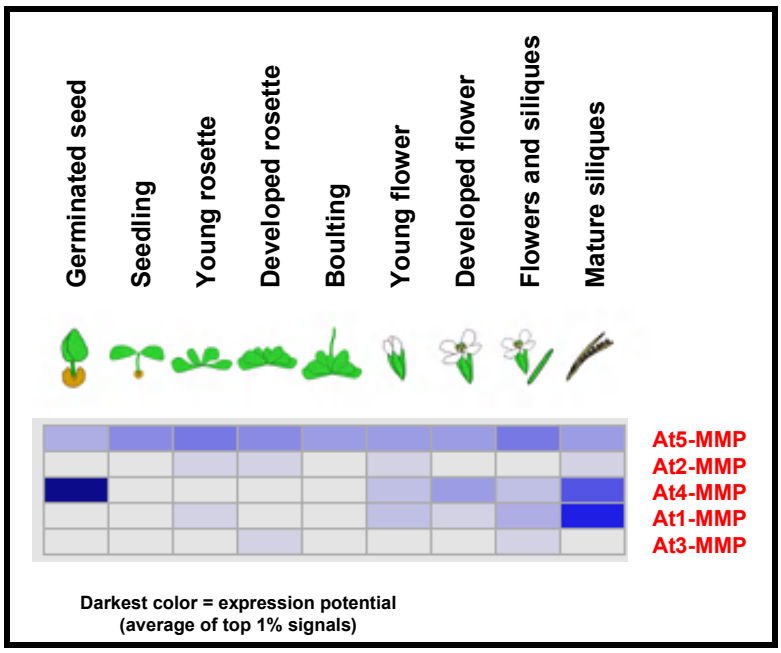


Figure 11: Genevestigator analysis of MMP gene expression during *Arabidopsis* development. Darker shades of blue represent stronger expression (Flinn, 2008).

None of the reported plant MMP studies have focused on studying their role in embryo development while these proteases stand out as very strong candidates as modulators of extracellular modulations required for proper embryo development, given their roles in mammalian embryo development (Alexander *et al.*, 1996). Furthermore, given all of the previous studies in which secreted molecules in the extracellular environment have modulated embryo development (Section 3.4.1), it is not beyond reason that ECM-modifying proteases, like the MMPs, could contribute a role in seed and embryo development. Hence, the primary aim of this research is to study the expression and MMP-like activity at various stages of seed development in Loblolly pine, as well as during the transition to germination.

III

Materials and Methods

1 Database search

A BLAST search was performed to find homology between available plant MMP sequences and the pine EST sequences available on www.fungen.org. Several EST clones were identified which assembled into a single contig (STRR1_76_H11 - shoot tip cDNA library, RTCNT1_5_D08 - root cDNA library, STRS1_39_H03 - stem cDNA library), and the clones obtained from the University of Georgia. The EST sequences were translated, and PCR primers designed against the two conserved regions (cysteine switch and zinc-binding catalytic domain (Table 1) with the help of the IDT PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/) program. Primers were obtained from Integrated DNA Technologies (San Diego, CA).

2 Plant material and pre-treatments

Two sets of plant material were used in this study. The first set comprised zygotic embryos and megagametophytes at various stages of development. Seed cones were collected from July 2008 through to the end of August 2008, from the Virginia Tech Department of Forestry Reynold's Homestead field site. Cones were stored at 4^0C until tissue dissection, which was done within a week of receipt. Seeds were collected from the cones, with embryo and megagametophyte removal by dissection. Embryos were classified according to their developmental stage based on morphological criteria established by Buchholz and Stiemert (1945) and stored at -80^0C until

required. Embryos with suspensors and small embryonal heads were obtained in very small amounts. Immature zygotic embryos were separated at different stages of development representing proembryos (Stage 1), globular embryos (Stage 2), early cotyledonary embryos with small cotyledonary nubs (Stage 3), larger cotyledonary embryos in which the cotyledons have overgrown the shoot apex (Stage 4), late cotyledonary embryos during the maturation drying stage (Stage 5), and mature, dry seed embryos (DE). Megagametophytes were collected at the same time from seeds at the developmental stages described above, as well as mature, dry seed megagametophytes (DM).

The second set of plant material comprised *Pinus taeda* mature dry seeds which were obtained from the Virginia Department of Forestry. These were soaked for three nights in pre-treatment growth regulator solutions (GA_3, BA, NAA, 2, 4-D or ABA) at various molar concentrations (10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}, 10^{-8}, 10^{-9}), or in water alone as a control. The seeds were removed from the pre-treatment solutions by filtration on filter paper and the seeds transferred onto wetted filter paper (with water or the appropriate growth regulator solution). Fifty seeds were placed on each plate in triplicate, in order to determine the impact of each growth regulator solution on germination. After determining the appropriate growth regulator concentrations to use, 120 seeds were used for each optimum treatment and treated as mentioned above. The same number of seeds was harvested from each treatment and at each of the time points: day 0, day 1, day 2, day 3 and when the radicle emerged, and the zygotic embryos were separated from the megagametophyte. The experiment was repeated twice.

3 Reverse transcription PCR

Total RNA was isolated using the RNAeasy kit (Qiagen) from embryo and megagametophytes of mature pine seeds, various seedling tissues (cotyledons, hypocotyls and radicles), and also from mature tree needles. Each RNA sample (2.5 µg) was converted to cDNA using the SMART ^{TM} PCR cDNA Synthesis kit (Clontech). Reverse transcription PCR was performed with the above mentioned cDNA samples as templates and primers (Table 2) designed against conserved regions using the following PCR conditions: cycle 1 (1x) - 94^0C for 2 mins; cycle 2 (35x) - 94^0C for 45 sec, a temperature gradient ranging from 58^0C to 65^0C for 45 sec, 72^0C for 1 min; and cycle 3 (1x) - 720C for 1 min. Products were separated on a 1% agarose (Sigma) gel in Tris borate-EDTA. The PCR products thus obtained were cloned into the pGEM-T Easy vector (Promega) and sequenced on a Beckman CEQ8800 following instrument protocols.

4 Expression, purification and N-terminal sequencing of *Pta-MMP*

cDNA for *Pta-MMP* was amplified by RT-PCR from *the EST clone* STRS1_39_H03 using primers (Table 2) designed against this EST sequence . Using these primers and the high fidelity *Pfu* polymerase (Stratagene), the sequence was amplified, flanked by a 5' *Bam*HI site and a 3' *Nde1* site. This product was then sub-cloned into pET15b (Novagen) using standard techniques, and the reading frame verified by sequencing. The plasmid was then transformed into *E. coli* BL21(DE3) (Novagen) for expression. A 200 ml culture of cells was grown overnight in LB containing 100 mg/ml ampicillin at 37 °C, with protein induction using 1 mM IPTG. The pellet was ground in mortar and pestle and resuspended in TALON bead (Clontech) 1X

equilibrium/wash buffer containing 6M guanidine HCl. Insoluble material was pelleted by centrifugation at 10,000 x *g* at 4 °C for 20 min. The supernatant was collected and dialyzed overnight against 6M urea. An aliquot of 20ul from the sample was analyzed by SDS-PAGE to confirm expression of desired protein. The His-tagged recombinant protein was then affinity purified using TALON beads and manufacturer's instructions. Refolding of the purified protein was carried out by serial dialysis with decreasing concentrations of urea (3M, 1.5M, .3M) in 10 mM Tris-HCl (7.5) and finally refolded overnight by final dialysis against 150 ml of 20 mM Tris-H_2SO4 pH 7.5, 5 mM CaSO4, 100 mM Na_2SO4, 1 mM ZnSO4, 10% glycerol, 0.05% Brij 35, 0.02% NaN3 and 1% Protease Inhibitor Cocktail Mix III (Calbiochem) at 6^0C with stirring, followed by aliquoting, freezing in liquid N_2, and storage at -80^0C. Protein quantity was estimated by the modified method of Ghosh *et al*. (1998). Purified fractions were analyzed by SDS-PAGE, such that in addition to the urea-denatured and 6^0C refolded samples, 6^0C refolded samples were also incubated overnight at 37^0C to allow for complete autocatalytic activation to occur. For N-terminal sequencing, the final refolded protein sample (o/n at 37^oC) was separated by SDS-PAGE and blotted onto PVDF membrane (Pierce) using a 10 mM MES (pH 6) + 20% methanol transfer buffer. Following transfer, the membrane was washed several times with distilled water, stained (0.02% Coomassie Brilliant blue in 40% methanol, 5% acetic acid) for 30 seconds, destained (40% methanol, 5% acetic acid) for 1 minute, then given several final distilled water rinses. After membrane drying, the protein band was excised and sent to the Tufts University Core Facility (http://www.tucf.com/hplc-ms-f.html) for N-terminal sequencing using an ABI 494 Protein Sequencer.

Squence used for generation of recombinant protein (Positions of primers is highlighted, underlined sequence indicates cyteine switch motif)

AGTTCAGGACTGACGAACGCTGCAAGGCGACGATGCAGCTGCGAACTGAAGCGTACTCGTC
GCTCGATACCTCAGCGCGCAGAACAACGTGACCGAGACTCGACGAGTCGTGGAGAGCGCGG
TGCGGACATACCAGAAAAACTTCGGGCTCAACGTTACCGGGGTCCTGGACGAGACACGATT
TCGCAGCTCATGGTGCCGCGGTGCGGCCGCGAGGACATCATCAACGGGAGCTCCGCCATGC
GGGGACGTGGTCTGTACACGTTCTTCCCGGGGAGCCCCCGCTGGGGACCCGACAAGAGGGT
GCTCAGTTACGCCTTCTCGCCGGACCACGAGGTGCTATCGGAGATCAGCCTGGCAGAGCTCA
GCACTGTGGTCGGGCGCGCCTTCAAGCGCTGGGCCGACGTGATCCCCATAACGTTCACCGAG
AGCTCCGATTATTCCTCCGCCGATATCAAGGTCGGGTTCTACAGCGGCGACCACGGCGACGG
GCACCCCTTCGACGGACCCCTCGGGACCCTCGCCCATTCCTTCTCCCCGCCCGACGGCCGCTT
CCACCTGGACGCCGCCGAGTCGTGGACCGTCGACCTCTCGTCCGACTCAGCTGCCACAGCCA
TCGACCTGGAGTCCATCGCCACCCACGAGATCGGCCACCTGCTCGGCCTCGGCCACACGACC
GAGAAAGCCGCCGTCATGTACCCCAGCATCGCGCCGCGAACCAGAAAAGTCGACCTCGTCC
TTGATGACGTGGATGGCGTCCAGTATTTGTACGGCGCCAATCCCAACTATAACGCCTCTGCC
GTCCTCGCCCAGAACGAGACCAGCTCTGCAACTGCCATACAACTCCCCCCTGGAGCTTCTTT
GCTCCCAATTCTTTTCTTCATTTCGTCTTTGCTGCCATTAATTCTGTGA

5 Expression studies

Total RNA was extracted from the pools of zygotic embryos and megagametophyte from all the samples mentioned earlier, according to manufacturer's instructions (RNAeasy, Qiagen). The RNA samples were then treated with DNAse (DNA-free, Ambion) to remove genomic DNA. The RNA quantity and quality was estimated with a NanoDrop spectrophotometer (ND-1000 V3.2.1). The RNA samples were also visually observed by Agarose-Formaldehyde Electrophoresis. Each RNA sample (2.5 µg) was converted to cDNA using iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. Relative transcript abundance of MMP genes was analyzed by real time PCR using real-time PCR primers, designed using the IDT primer quest program against the sequence proximal to the 3' UTR (sequences shown in Table 2) and SYBR Green detection (iScript One-Step RT-PCR Kit with SYBR Green - Bio-Rad). All of the PCR products were sequenced to confirm the desired

product. The reactions were carried out using a Bio-Rad iQ5 Multicolor Real Time Detection system using the default program. The transcript levels were normalized to Tubulin which was used as endogenous control. Each sample was subjected to three technical replicates and three biological replicate. Relative expression was analysed by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using Dry embryo/ Dry megagametophyte as calibrators whose samples were included in each reaction set of embryo/ megagametophyte analysis respectively.

6 Metalloprotease activity

Protein was extracted from the above mentioned plant materials using Tris-HCl buffer (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 5 mM $CaCl_2$, 100 µM $ZnCl_2$, 0.025% Brij 35, 0.02% NaN_3) and stored at -20^{o}C until used. Protein quantity was estimated by the modified method of Ghosh et al. (1998). Degradation of myelin basic protein (MBP) was assayed using bovine MBP (Sigma) at a final concentration of 0.25 mg/ml in 100 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 0.05% Brij 35, 0.02% NaN_3 and 1% Protease Inhibitor Cocktail Mix III (Calbiochem) A total of 1.5 µg of each protein extract was incubated with MBP in a 15 µl reaction volume for 20 h at 37 °C, and products were analyzed by SDS-PAGE to visualize MBP degradation, using 12% separating/5% stacking gels with Bio-Rad reagents and protocols. The gels were stained with Imperial protein stain (PIERCE) for 45 mins and then destained overnight. Inhibition of protease activity was determined by addition of the general MMP inhibitor GM6001 at a concentration of 500 µM. DMSO was used as the solvent control for GM6001 at the same level (20%) as used in the GM6001 assays. Similar assays were performed to test the activity of recombinant *Pta1-MMP* (r*Pta1-MMP*) by incubating MBP with r*Pta1-MMP* (final concentration 128 nM) in presence or absence of additional 100µM $ZnCl_2$, instead of total protein extract. Inhibition of protease

activity was determined by the addition of EDTA at a concentration ranging from 100 μM to 1mM and GM6001 at a concentration ranging from 50 μM to 500 μM.

7 Inhibition of MMP activity in germinating seeds by imbibition in GM6001

Mature Loblolly pine seeds were treated with various concentrations of GM6001 (5μM GM6001, 50 μM GM6001 and 500 μM GM6001). Water and DMSO (20% final concentration) were used for control treatments. The seeds were soaked in treatment solutions for 3 nights and then placed on wet filter paper saturated with treatment solution (an equal volume of DMSO was used for each treatment and the volume was adjusted according to the highest concentration of GM6001). Germination of seeds was monitored daily. Seedling lengths from water-imbibed, DMSO-imbibed, 5μM GM6001- and 500μM GM6001-imbibed treatments were measured on the 19^{th} day. Embryos were collected from 20 randomly selected seeds for each of the treatments, and protein extracts prepared as described earlier. MBP degradations assays were performed as described earlier using these protein extracts.

8 Cloning of full length genomic clone

To confirm that the sequence obtained from the EST clones was seed-expressed, a cDNA library was prepared from mature zygotic embryos, using the CloneMiner cDNA Library Construction Kit (Stratagene) following the supplied instructions. This was screened for the presence of a matrix metalloproteinase clone by PCR reactions using primers designed in various regions of the EST contig, including the 3' UTR (Table 2). PCR conditions used were: cycle 1 (1x) - 94^0C

for 2 mins; cycle 2 (35x) - 94^{0}C for 45 sec, 58^{0}C for 45 sec, 72^{0}C for 1 min; cycle 3 (1x) - 72^{0}C for 5 min. The PCR products were separated on a 1xTAE/1 % agarose gel (Sigma) and cloned into pGEM-T Easy vector and sequenced. As a full length cDNA clone was not obtained from this library screen, genomic DNA was extracted from mature needles using the Qiagen DNAeasy kit according to manufacturer's instructions. In order to obtain full length sequence using information from the existing clones, the GenomeWalker Universal Kit (Clontech) was used according to the manufacturer's instructions. Gene specific primers (GSPs) were designed in order to amplify the 5' end, with primer sequences shown in Table 2. Primary and nested PCR reactions were performed with these GSPs and the forward primers for the adaptor sequence provided with the kit. The PCR conditions were as follows: cycle 1 (7x) - 94^{0}C for 25 sec, then 72^{0}C for 3 mins; cycle 2 (32x) - 94^{0}C for 25 sec, then 67^{0}C for 3 min; cycle 3 (1x) - 67^{0}C for 7 min. The PCR products were cloned into pGEM-T Easy easy vector (Promega) and sequenced as above. Following the identification of the 5' end of the MMP sequence, the complete full length open reading frame was amplified from genomic DNA using primers designed to 5' UTR and 3' UTR sequences. The correct 1170 bp product was obtained, digested with EcoRV (New England Biolabs) to release the predicted fragment sizes, and the fragments sequenced for confirmation of identity.

Table 2: Primers used for isolation and characterization of *Pta-MMP*.

	Primer	**Sequence**	**Annealing temp. (^{0}C)**	**PCR Product size**
For revererse transcription PCR	PtMMP1 PtMMP2	5'CATACCAGAAAAACTTCGGGCTCA 3' 5'GGCGATGGACTCCAGGTCGA 3'	55	507
For Genome Walker	Primer 1 Reverse primer Primer 2 Nested Reverse primer GSP1 GSP2	5' AAGAACGTGTACAGACCACGTCCCCC 3' 5'GAGGAATAATCGGAGCTCTCGGTGA 3' 5' GGCGGAGCTCCCGTTGATGATGTCCTCGC 3' 5' CCGCGGCACCATGAGCTGCGAAATCGTGTC 3'	Gradient from 56 to 63 60.3	978 bp 952 bp
For library screening	Conserved region Forward Reverse 3'UTR Forward Reverse	 5'CGGGCTCAACGTTACCGGGGTCCTGGACG 3' 5'GGCGATGGACTCCAGGTCGATGGCT 3' 5'CGCGAACCAGAAAAGTCGAC 3' 5'GGTAAAGTAAGGGGAAAGTGCAG 3'	 65 55	 491 bp 223 bp
Primers for recombinant protein generation	Forward Reverse	5' AACCCCCATATGAGTTCAGGACTGACG 3' 5'CCCCGGATCCTCACAGAATTAATGGCA 3'	58	931 bp
Real time primers	GSP Forward Reverse Endogenous control Forward Reverse	 5' TGACGTGGATGGCGTCCAGTATTT 3' 5' AGAATTGGGAGCAAAGAAGCTCCA 3' 5' ACAGCTGATCTCGGGCAAAGAAGA 3' 5' TGCAGTTATCGGCTAGCTTCCTCA 3'	58	 132 bp 119 bp

IV

Results

1 Identification and cloning of a full length Loblolly pine MMP sequence

In an attempt to identify MMPs expressed during conifer embryogenesis, publicly available ESTs were screened using the cysteine switch and catalytic domains identified in other plant MMPs. EST clones showing similarity to plant MMP-like proteins were identified in the pine EST database (www.fungen.org). The STRR1_76_H1 (stem cDNA library), RTCNT1_5_D08 (root cDNA library), and STRS1_39_H03 (shoot tip cDNA library) sequences were aligned using clustal W (Fig. 12), and displayed almost complete identity throughout the putative coding region and the 3' UTR, except at the extreme 5' end and the extreme 3' end. These ESTs, when translated, displayed the two domains described above, characteristic for MMPs.

To gauge the expression of this MMP in various Loblolly pine tissues, PCR primers were designed to amplify the region between the cysteine switch and catalytic domain (Table 2). The correct 507 bp fragment was observed in seed tissues (whole embryo, embryonic cotyledons, embryonic hypocotyls, female megagametophyte), and in needles (Fig. 13). However, the fragment was not detected in radicle tissue. Sequencing of this fragment confirmed it as identical to the earlier described ESTs. As the initial PCR primers were directed against conserved domains, additional primers were developed against the 3' UTR region of the ESTs (Fig. 12) and part of the ORF, and were used to amplify the correct 223 bp fragment (Fig. 14) from a whole seed cDNA library, confirming that this particular MMP was expressed in seed tissues.

```
CLUSTAL W (1.83) multiple sequence alignment


_RTCNT          ------------------------------GCGAATAAAACA-ATTTCAACCCAG-AAACA
_STRS           TCTCGGATTACAGCCTGGCGGATTGGACAGAAGATACGCCGTACATCGAATCACCAGGCG
_STRR           ------------------------------------------------------------

_RTCNT          GCTAGCACTG-ATCGAATTCGCACAGGGGGAAGGTTCAGGAACTTGACGAAGCCTGCCAT
_STRS           GTTTAGATTCTATCCTGTAAGCGGCGGGCTGAAGTTCAGGA--CTGACGAACGCTGCAA-
_STRR           ------------------------------------------------------------


_RTCNT          GGCGAACGATGCACGCTTGCCGGACCTGGAGCGCTTACTCGCCGCTTCGTTACTTCAGCG
_STRS           GGCGA-CGATGCAG--CTGC--GAACTGAAGCG--TACTCGTCGCT-CGATACCTCAGCG
_STRR           ---------------------------------------------ACCTGGGCGGATACGTG
                                                             *   *     *  * *

_RTCNT          GCCAGAAACAACGTGACCGAGACTCGACGAG--CGTGGAGAGCGCCGGTGCGGACATACC
_STRS           CGCAGAA-CAACGTGACCGAGACTCGACGAGT-CGTGGAGAGCGC-GGTGCGGACATACC
_STRR           GCCAC---TAGTACTTCAGGACTTCGACGAGGCCGTGGAGAGCGC-GGTGCGGACATACC
                  **     *      * *    ********  ************ **************

_RTCNT          AGAAAA-CTTCGGGCTCAACGTTACCGGGGTCCTGGACGAGGC-ACGATTTCGCAGCTCA
_STRS           AGAAAAACTTCGGGCTCAACGTTACCGGGGTCCTGGACGAGAC-ACGATTTCGCAGCTCA
_STRR           AGAAAAACTTCGGGCTCAACGTTACCGGGGTCCTGGACGAGGCCACGATTTCGCAGCTCA
                ****** ********************************* * ****************

_RTCNT          TGGTGC-GCGGTGCGACCGCGAGGACATCATCAACGGGAGCTCCGCCATGCGGGGACGTG
_STRS           TGGTGCCGCGGTGCGGCCGCGAGGACATCATCAACGGGAGCTCCGCCATGCGGGGACGTG
_STRR           TGGTGCCGCGGTGCGGCCGCGAGGACATCATCAACGGGAGCTCCGCCATGCGGGGACGTG
                ****** ******** ******************************************

_RTCNT          GTCTGTACACGTTCTTCCCGGGGAGCCCCCGCTGGGGACCCGACAAGAGGGTGCTCAGTT
_STRS           GTCTGTACACGTTCTTCCCGGGGAGCCCCCGCTGGGGACCCGACAAGAGGGTGCTCAGTT
_STRR           GTCTGTACACGTTCTTCCCGGGGAGCCC--GCTGGGGACCCGACAAGAGGGTGCTCAGTT
                ***************************  ****************************

_RTCNT          ACGCCTTCTCGCCGGACCACGAGGTGCTGTCGGAGATCAGCCTGGCAGAGCTCAGCACTG
_STRS           ACGCCTTCTCGCCGGACCACGAGGTGCTATCGGAGATCAGCCTGGCAGAGCTCAGCACTG
_STRR           ACGCCTTCTCGCCGGACCACGAGGTGCTGTCGGAGATCAGCCTGGCAGAGCTCAGCACTG
                *************************** ******************************

_RTCNT          TGGTCGGGCGCGCCTTCAAGCGCTGGGCCGACGTGATCCCCATAACGTTCACCGAGAGCT
_STRS           TGGTCGGGCGCGCCTTCAAGCGCTGGGCCGACGTGATCCCCATAACGTTCACCGAGAGCT
_STRR           TGGTCGGGCGCGCCTTCAAGCGCTGGGCCGACGTGATCCCCATAACGTTCACCGAGAGCT
                ************************************************************

_RTCNT          CCGATTATTCCTCCGCCGATATCAAGGTCGGGTTCTACAGCGGCGACCACGGCGACGGGC
_STRS           CCGATTATTCCTCCGCCGATATCAAGGTCGGGTTCTACAGCGGCGACCACGGCGACGGGC
_STRR           CCGATTATTCCTCCGCCGATATCAAGGTCGGGTTCTACAGCGGCGACCACGGCGACGGGC
                ************************************************************

_RTCNT          ACCCCTTCGACGGACCCCTCGGGACCCTCGCCCATTCCTTCTCCCCGCCCGACGGCCGCT
_STRS           ACCCCTTCGACGGACCCCTCGGGACCCTCGCCCATTCCTTCTCCCCGCCCGACGGCCGCT
_STRR           ACCCCTTCGACGGACCCCTCGGGACCCTCGCCCATTCCTTCTCCCCGCCCGACGGCCGCT
                ************************************************************

_RTCNT          TCCACCTGGACGCCGCCGAGTCGTGGACCGTCGACCTCTCGTCCGACTCAGCTGCCACAG
_STRS           TCCACCTGGACGCCGCCGAGTCGTGGACCGTCGACCTCTCGTCCGACTCAGCTGCCACAG
_STRR           TCCACCTGGACGCCGCCGAGTCGTGGACCGTCGACCTCTCGTCCGACTCAGCTGCCACAG
                ************************************************************

_RTCNT          CCATCGACCTGGAGTCCATCGCCACCCACGAGATCGGCCACCTGCTCGGCCTCGGCCACA
_STRS           CCATCGACCTGGAGTCCATCGCCACCCACGAGATCGGCCACCTGCTCGGCCTCGGCCACA
_STRR           CCATCGACCTGGAGTCCATCGCCACCCACGAGATCGGCCACCTGCTCGGCCTCGGCCACA
                ************************************************************


_RTCNT          CGACCGAGAAAGCCGCCGTCATGTACCCTAGCATCGCGCCGCGAACCAGAAAAGTCGACC
_STRS           CGACCGAGAAAGCCGCCGTCATGTACCCCAGCATCGCGCCGCGAACCAGAAAAGTCGACC
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_STRR           CGACCGAGAAAGCCGCCGTCATGTACCCTAGCATCGCGCCGCGAACCAGAAAAGTCGACC
                **************************** ******************************

_RTCNT          TCGTCCTTGATGACGTGGATGGCGTCCAGTATTTGTACGGCGCCAACCCCAACTATAACG
_STRS           TCGTCCTTGATGACGTGGATGGCGTCCAGTATTTGTACGGCGCCAATCCCAACTATAACG
_STRR           TCGTCCTTGATGACGTGGATGGCGTCCAGTATTTGTACGGCGCCAACCCCAACTATAACG
                ********************************************* *************

_RTCNT          CCTCTGCCGTCCTCGCCCAGAACGAGACCAGCTCTGCAACTGCCATACAACTCCCCCCTG
_STRS           CCTCTGCCGTCCTCGCCCAGAACGAGACCAGCTCTGCAACTGCCATACAACTCCCCCCTG
_STRR           CCTCTGCCGTCCTCGCCCAGAACGAGACCAGCTCTGCAACTGCCATACAACTCCCCCCTG
                ************************************************************

_RTCNT          GAGCTTCTCTGCTCCCAATTCTTTTCTTCATTTCGTCTCTGCTGCCATTAATTCTGTGAC
_STRS           GAGCTTCTTTGCTCCCAATTCTTTTCTTCATTTCGTCTTTGCTGCCATTAATTCTGTGAC
_STRR           GAGCTTCTCTGCTCCCAATTCTTTTCTTCATTTCGTCTCTGCTGCCATTAATTCTGTGAC
                ******** **************************** ********************

_RTCNT          TGCACTTTCCCCTTACTTTACCAATTGGTCAATTCATTTGATGCATTTTTCTCTCAAATA
_STRS           TGCACTTTCCCCTTACTTTACCAATTGGTCAATTCATTTGATGCATTTTTCTCTCAAATA
_STRR           TGCACTTTCCCCTTACTTTACCAATTGGTCAATTCATTTGATGCATTTTTCTCTCAA-TA
                ************************************************************ **

_RTCNT          TTATTATTTACCGTACGATTACATTTTTTTTTTTTCTCTCTCTACAGAGGAAATATTGT
_STRS           TTATTATTTACCGTACGATTACATTTTTTTTTTTTCTCTCTCTACAGAGGAAATATTGT
_STRR           TTATTATTTACCGTACGATTACATTACTTTACT---CTCTCTCTACAGAGGAAATATAGT
                ************************  ***  *   ********************** **

_RTCNT          AACTTTCT-GCCACGTGGCATGTAATATTAAATTAAATTATTTTATATTTAATTTCATCC
_STRS           AACTTTCT-GCCACGTGGCATGTAATATTAAATTAAATTATTTTATATTTAA--------
_STRR           AACTTTCTTGCCCCCGCGGCATGTAATATTAAAATAAAATAT---ATATATAAATCATCCC
                ******** *** ** ***************** **** ***   **** ***

_RTCNT          ATTTATCCCACTGCGGATTCCTCACCAGTAAAAAAAAAAAAAAAAAAAACTCGAGAAGTAC
_STRS           ----------------------------AAAAAAAAAAAAAAAAAAAACTCGAGAAGTAC
_STRR           -----------------CTCGTGGCGCGATCGCATCATGGCATAACTGGTTCTGTGGTGG
```

Figure 12: Alignment of the 3 EST sequences; RTCNT1_5_D08, and STRS1_39_H03, STRR1_76_H1**.** The stop codon is shown in red and the 3' UTR which is identical is shown in blue. The primer binding sites for the primers used to amplify the 223 bp 3' UTR fragment are underlined.

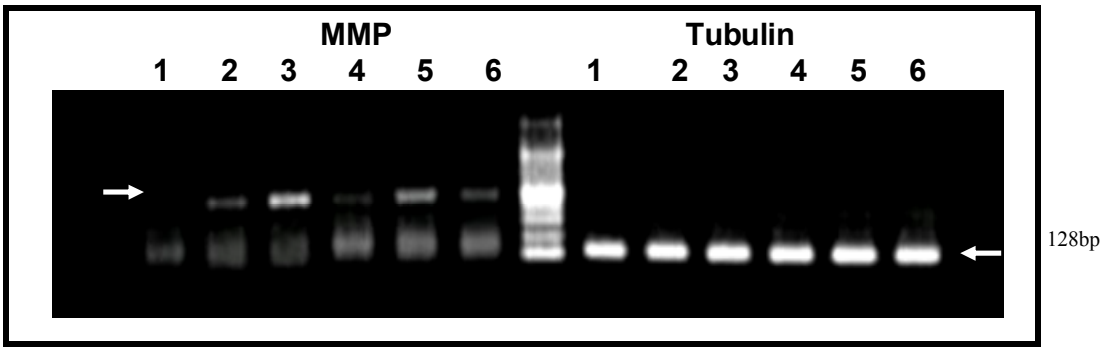

Figure 13: RT-PCR products using pine MMP primers and first strand cDNAs from various pine tissues; 100bp ladder: 1. Radicle; 2. Female megagametophyte; 3. Embryo; 4. Cotyledon; 5. Hypocotyl; 6. Needle. (The smaller bands on the MMP side are primer dimer)

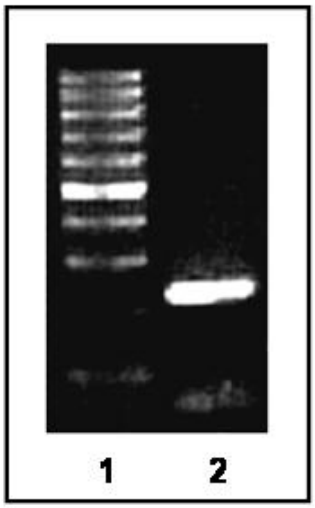

Figure 14: RT-PCR products using first strand cDNA from pine seed, amplified using pine MMP primers; 1. 100bp ladder: 2. 223 bp PCR product.

2 Recombinant *Pta-MMP* expression and protein characterization

The longest EST sequence (STRS1_39_H03) was used to design primers to amplify the region spanning 303 amino acids, including part of the prodomain, the cysteine switch, the catalytic domain and the stop codon. The amplified fragment was cloned in frame into pET15-b and expressed as a His-tagged protein in bacteria. This recombinant hybrid *Pta-MMP* protein (r*Pta-MMP*) was isolated under denaturing conditions, yielding a protein which migrated at approximately 43 kDa (Fig. 15, lane 1) under the SDS-PAGE conditions used. During refolding in the cold and subsequent incubation at 37^{o}C, several processing intermediates were observed (Fig. 15, lane 2), eventually yielding a mature peptide approximately 12 kDa smaller in size (Fig. 15, lane 3). This size modification was due presumably to the autocatalytic activation process that has been observed with other recombinant plant MMPs, in which the propeptide containing the cysteine switch is cleaved off, leaving the catalytically-active mature protein. The N-terminal sequence of the major band observed after refolding at 37^{o}C overnight (Fig. 15, lane 3 – band with asterisk) started with "SSAMRG" (highlighted in pink in Fig. 16) clearly showing that the propeptide domain is cleaved off during refolding, to yield a putative, functional MMP.

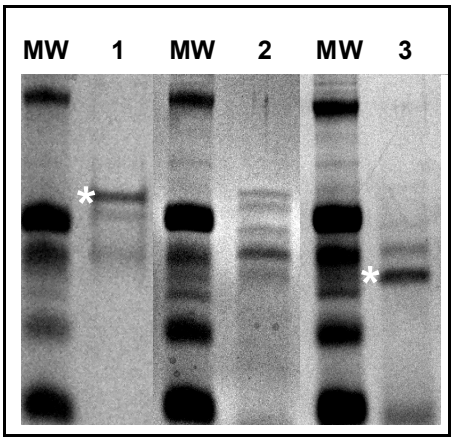

Figure 15: Characterization of recombinant *Pta-MMP* (r*Pta-MMP*). Autocatalytic activation of r*Pta-MMP* during refolding. MW. Molecular weight marker; 1. Purified, denatured r*Pta-MMP* prior to refolding; 2. r*Pta-MMP* after refolding overnight at 6^{o}C; 3. Refolded r*Pta-MMP* after subsequent overnight incubation at 37^{o}C. (The * are the position of recombinant protein band before and after refolding)

M V P R C G R E D I I N G/ S S A M R G R G L Y T F F P G S P R W G P D K R V L S Y A F
S P D H E V L S E I S L A E L S T V V G R A F K R W A D V I P I T F T E S S D Y S S A D I
K V G F Y S G D H G D G H P F D G P L G T L A H S F S P P D G R F H L D A A E S W T
V D L S S D S A A T A I D L E S I A T H E I G H L L G L G H T T E K A A V M Y P S I A P
R T R K V D L V L D D V D G V Q Y L Y G A N P N Y N A S A V L A Q N E T S S A T A I
Q L P P G A S L L P I L F F I S S L L P L I L

Fig. 16: Amino acid sequence of the recombinant *Pta-MMP*. N-terminal sequencing of the major band observed after refolding at 37^{o}C overnight has a sequence starting with "SSAMRG" highlighted in pink.

In order to test the proteolytic capacity of this mature protein, myelin basic protein (MBP) degradation assays were used to assay activity. MBP degradation occurred only in the presence of r*Pta-MMP* and was stimulated by the addition of Zn^{2+} (Fig. 17). The use of the zinc chelator EDTA (Fig. 18) or the MMP active site-binding hydroxamate inhibitor GM6001 (Fig. 19) inhibited r*Pta-MMP* in a dose-dependent manner, further confirming its classification as a functional matrix metalloproteinase.

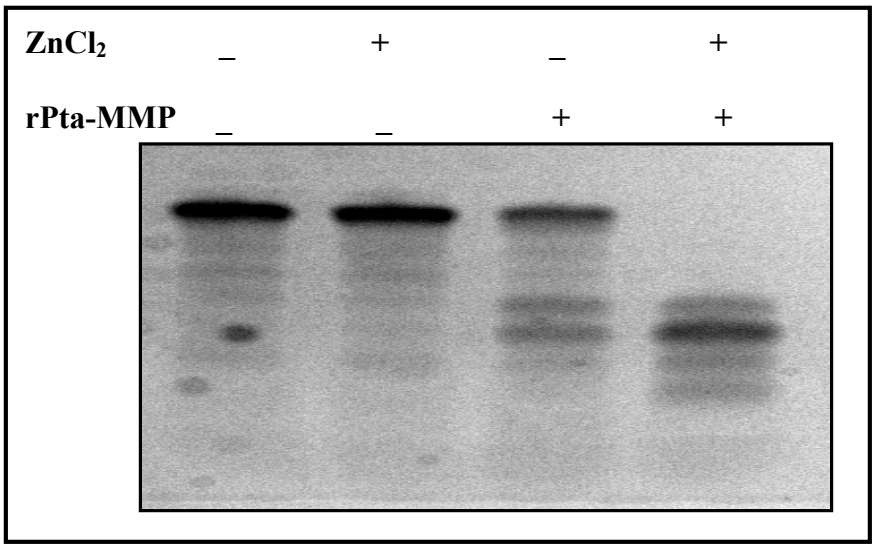

Figure 17: Zinc dependence of metalloprotease activity of r*Pta-MMP*. 1: Control, 2: MBP with 100 µM $ZnCl_2$, 3: MBP and recombinant *Pta-MMP* without $ZnCl_2$, 4: MBP and recombinant *Pta-MMP* with $ZnCl_2$.

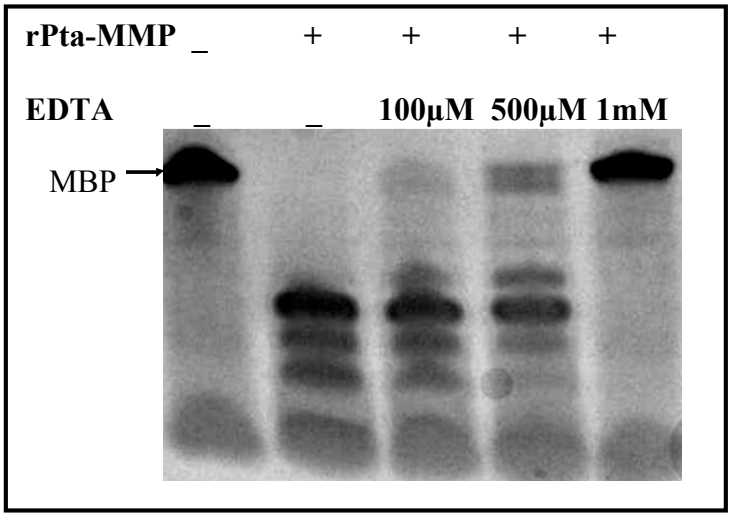

Figure 18: Inhibition of metalloprotease activity of recombinant *Pta-MMP* with 100 µM, 500 µM and 1 mM concentrations of EDTA. Inhibition of activity increases with increase in concentration of EDTA.

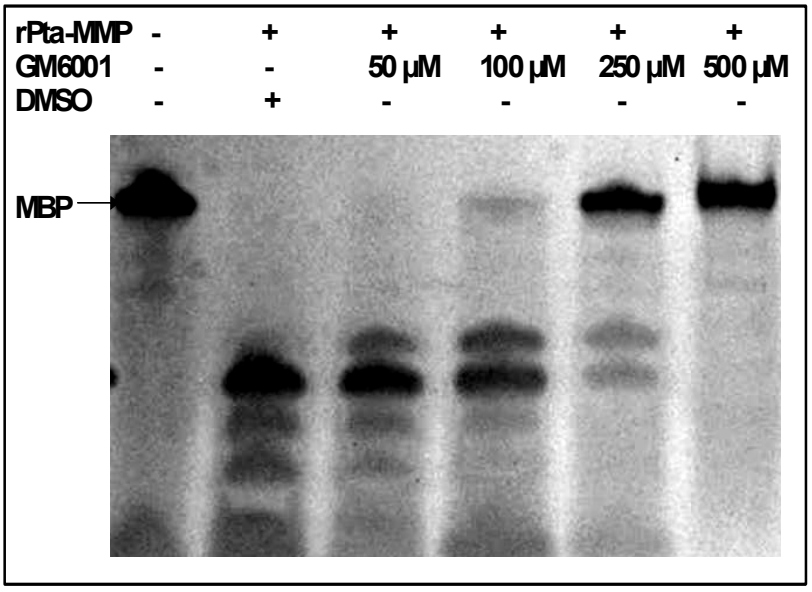

Figure 19: Inhibition of metalloprotease activity of recombinant *Pta-MMP* with 50 µM, 100 µM, 250 µM and 500 µM concentrations of GM6001. Inhibition of activity increases with increase in concentration of GM6001.

3 MMP characterization during Loblolly pine zygotic embryo development

Immature zygotic embryos were separated at different stages of development (Fig. 20) representing proembryos (Stage 1), globular embryos (Stage 2), and early cotyledonary embryos with small cotyledonary nubs (Stage 3), larger cotyledonary embryos in which the cotyledons have overgrown the shoot apex (Stage 4), late cotyledonary embryos during the maturation drying stage (Stage 5), and mature, dry seed embryos (DE).

Real time PCR analysis of pine MMP (*Pta-MMP*) transcript abundance during embryo development was analyzed using primers directed to the 3' end of the EST sequences. Transcript levels were highest during the early stages of embryogenesis. *Pta-MMP* transcripts were present in proembryos at 60% of their maximal embryo level, and increased to peak levels by Stage 3, after which levels declined during Stages 4 and 5, to reach approximately 10% of their maximal level in the dry embryo (Fig. 21). Similar results were found in female megagametophyte samples collected from the same seeds corresponding to the embryo stages. The relative transcript levels in these samples were 20 to 30 times higher at the proembryo and stage 3 relative to the level present in dry female megagametophyte, thereafter decreasing to reach about 5 times at stage 5 (Fig. 22). In order to assess MMP-like protease activity during embryo development, embryo and female megagametophyte protein extracts were assessed using MBP degradation assays (Fig. 23). Due to the small sizes of Stage 1 and Stage 2 embryos, we were unable to obtain sufficient protein to test these stages. Hence, the protease assays started with Stage 3 embryos. Based on the ability to degrade MBP, Stage 3 embryos contained maximal levels of MMP-like activity, which declined in the later stages of embryo development, a pattern that was similar to that of *Pta-MMP* transcript abundance.

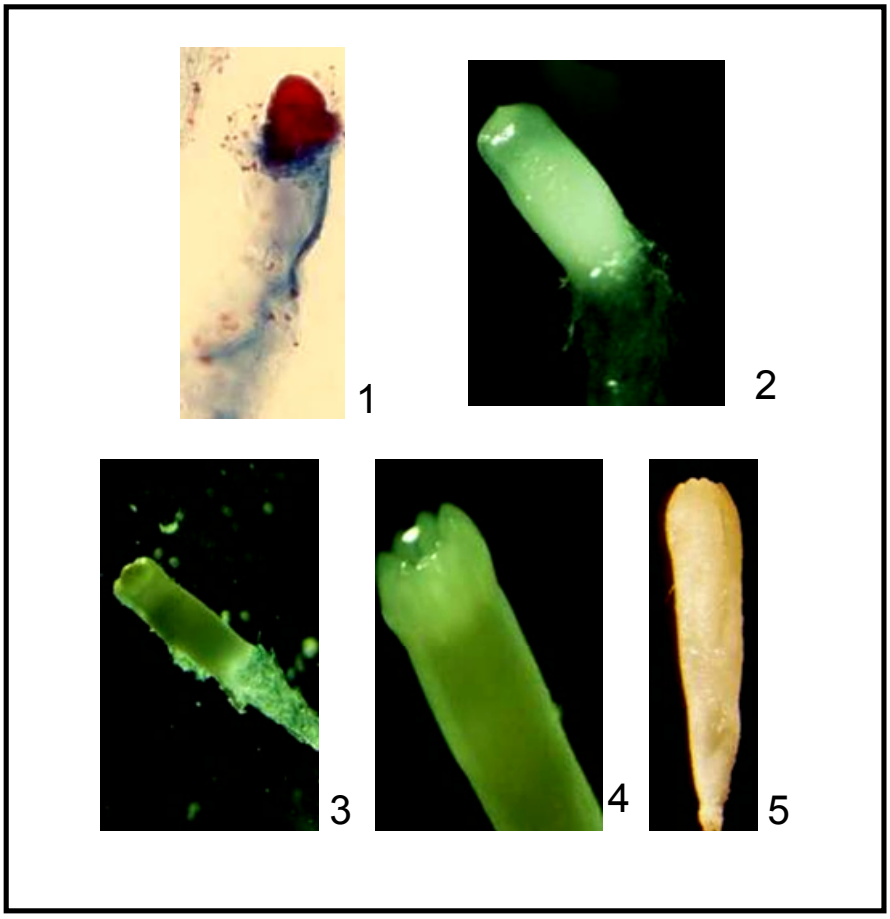

Figure 20: Various stages of zygotic embryo development. 1: Early embryo; 2: Globular embryo; 3: Precotyledonary stage; 4: Cotyledonary stage; 5: Mature embryo.

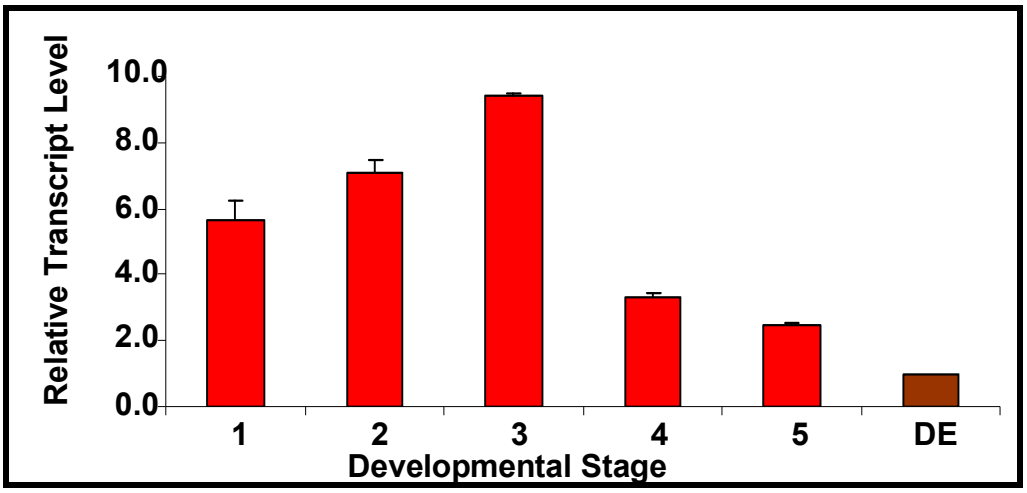

Figure 21: *Pta-MMP* transcript levels at Stages 1, 2, 3, 4, 5 of zygotic embryo development. All the levels are relative to transcript levels in dry embryo (DE).

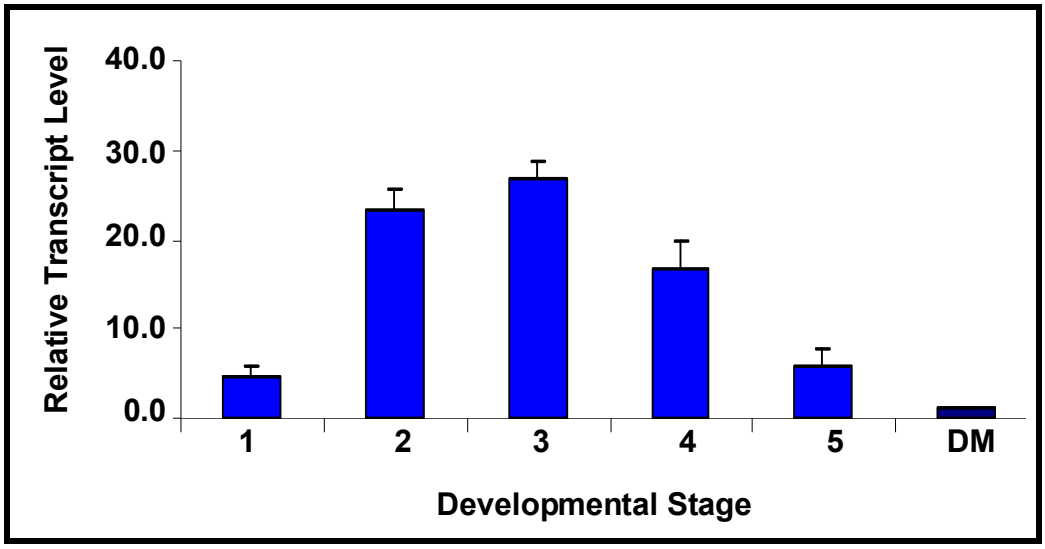

Figure 22: *Pta-MMP* transcript levels at various stages of megagametophyte development stages corrosponding to zygotic embryo developmental stages. All the levels are relative to transcript levels in dry megagametophyte (DM).

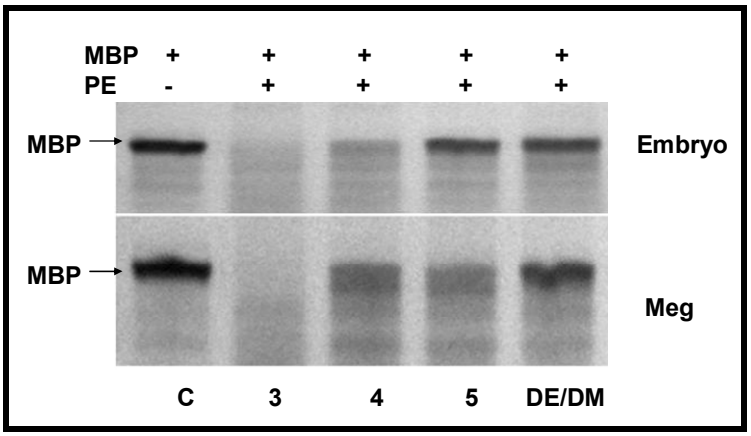

Figure 23: Metalloprotease activity in protein extracts of developing zygotic embryos and megagametophytes at stages 3, 4 and 5 of development. (C: MBP control; numbers 3, 4 and 5 represent the respective stages of development of the embryos/ or megagametophytes used for protein extraction, DE: Dry Embryo, DM: Dry Megagametophyte). No additional $ZnCl_2$ was used for these assays.

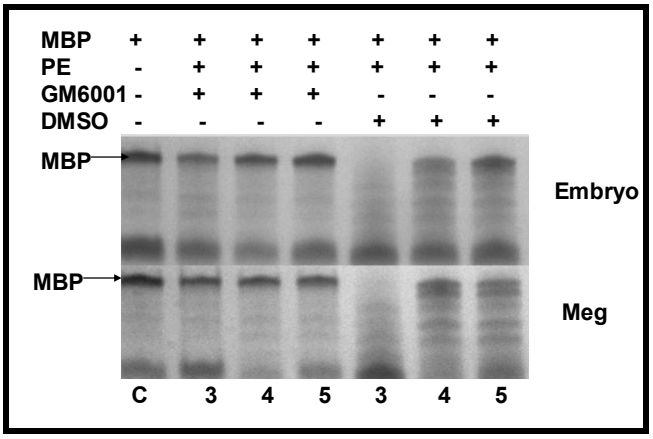

Figure 24: Inhibition of metalloprotease activity in protein extracts of developing zygotic embryos and megagametophytes at stages 3, 4 and 5 of development with 500 µM GM6001. (C: MBP control; numbers 3, 4 and 5 represent the respective stages of development of the embryos/megagametophytes used for protein extraction).

This pattern was also mirrored in the different stages of female megagametophyte, where maximum activity was detected at stage 3; however the activity in stage 4 and stage 5 samples seemed to be higher than the corresponding embryo stages. This detectable activity embryo and female megagametophyte extracts could be inhibited by GM6001 (Fig. 24), However, inhibition by GM6001 was not complete, indicating that in addition to *Pta-MMP* activity, other metalloproteases may be represented in this extract.

4 MMP characterization in zygotic embryos during Loblolly pine seed germination

Preliminary studies were carried out to identify levels of plant growth regulators that would modify Loblolly pine seed germination (Fig. 25 and Table 3). Seeds imbibed in 1 μM GA_3 and 100 μM ABA showed significant difference in germination efficiency (Fig. 26). Other hormone treatments such as NAA 10^{-7} M, NAA 10^{-9} M, BA 10^{-8} M, BA 10^{-9} M, and 2,4-D 10^{-6} M also showed comparable efficiency in modulating germination (Table 3). However for further analysis, 1 μM GA_3, and 100 μM ABA were selected due to their maximum efficiency. Using these preliminary results, Loblolly pine seed germination was assessed in water-imbibed, 1 μM GA_3-imbibed, and 100 μM ABA-imbibed seeds, up to 11 Days after Imbibition (DAI) (Fig. 27). Control, water-imbibed seeds started to exhibit completion of germination (radical protrusion) by 8 DAI, with a major increase in the completion of germination rate between 9-10 DAI, and a maximal level of approximately 50% total seed completed germination by 10-11 DAI. In contrast, GA_3-imbibed seeds completed germinated earlier (7 DAI), with the major increase in completion of germination rate between 7-8 DAI, and final completion of germination rate of 80% by 10-11 DAI. While the two above treatments exhibited significant total percentage of seed completing germination over the assay period up to 11 DAI, the ABA-treated seeds were significantly inhibited from completing germination (Figs. 26, 27 and Table 3).

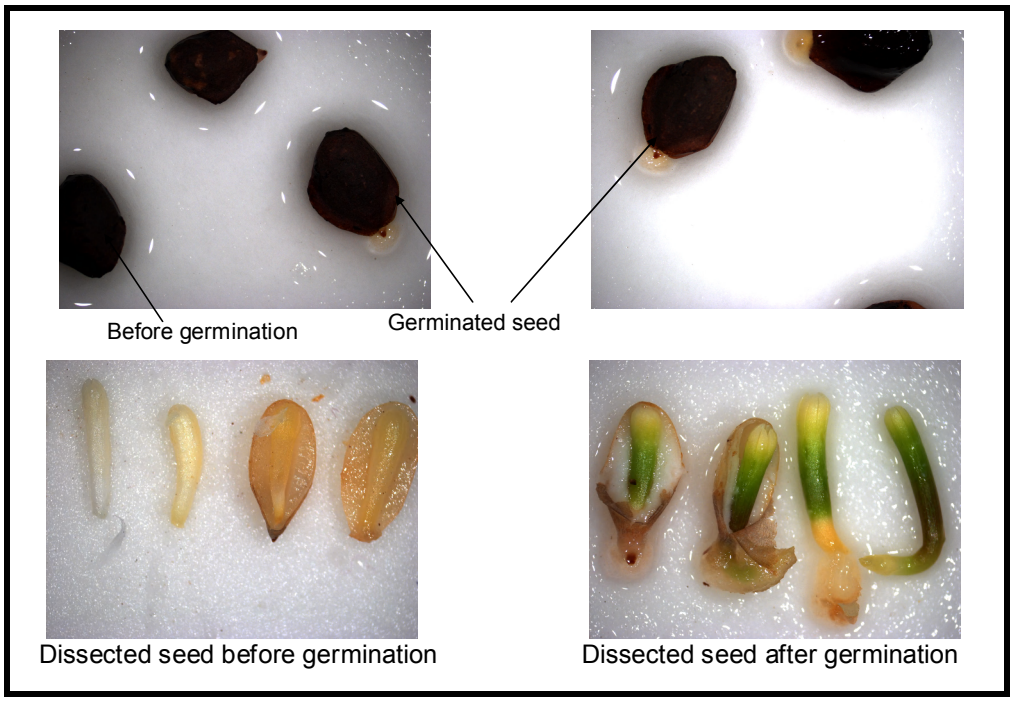

Figure 25: Pine seeds before and after germination

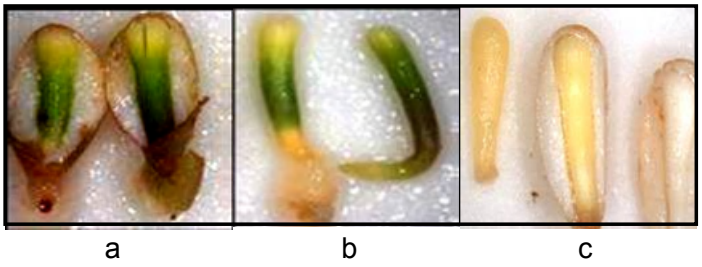

Figure 26: Dissected seeds on day of germination in water imbibed seeds. a: Water-imbibed seeds; b: GA_3-treated seeds; c: ABA-treated seeds.

Table 3: Effect of different concentrations of various hormones on the germination efficiency of pine seeds.

Treatment (M)	Average Seed Germination Day 7	Average Seed Germination Day 8	Average Seed Germination Day 9	Average Seed Germination Day 10	Average Seed Germination Day 11	% Cumulative Seed Germination
Control	0.67	3	7.33	22.33	24.67	49.34
GA3 10^{-5}	1.33	8.67	12.67	23	27.33	54.66
GA3 10^{-6}	6	24.67	30.67	39	42.33	84.66
GA3 10^{-7}	0.33	4	7.67	24.33	28	56
GA3 10^{-8}	0.67	5.33	10.67	20	23.67	47.34
GA3 10^{-9}	0.33	6	13.67	22	25	50
BA 10^{-5}	2.67	5.33	14.33	20	29	58
BA 10^{-6}	0.67	6.67	12.33	22.67	25.67	51.34
BA 10^{-7}	1	6.33	9	20.67	21.33	42.66
BA 10^{-8}	0.67	7.33	12.33	27.67	35	70
BA 10^{-9}	1	7.67	17.33	19.33	32	64
ABA 10^{-4}	0	0	0.67	1.33	7.33	14.66
ABA 10^{-5}	0.67	2	4	7	10.67	21.34
ABA 10^{-6}	2.33	5.33	8.67	12	16.67	33.34
NAA 10^{-5}	0.67	5	11	18.67	24.67	49.34
NAA 10^{-6}	1	5.33	9.67	19.33	28.33	56.66
NAA 10^{-7}	1.33	4	13	25	32.33	64.66
NAA 10^{-8}	1	4	7.67	15.33	22	44
NAA 10^{-9}	0.33	7.67	16.33	29	36.33	72.66
2,4-D 10^{-5}	0.33	3	7	17	20.33	40.66
2,4-D 10^{-6}	1.33	5.67	17.33	25	35	70
2,4-D 10^{-7}	0.33	5.33	15	23.33	29.33	58.66
2,4-D 10^{-8}	2	8	13.67	20.33	28	56
2,4-D 10^{-9}	2	4.67	7.33	19	20.67	41.34

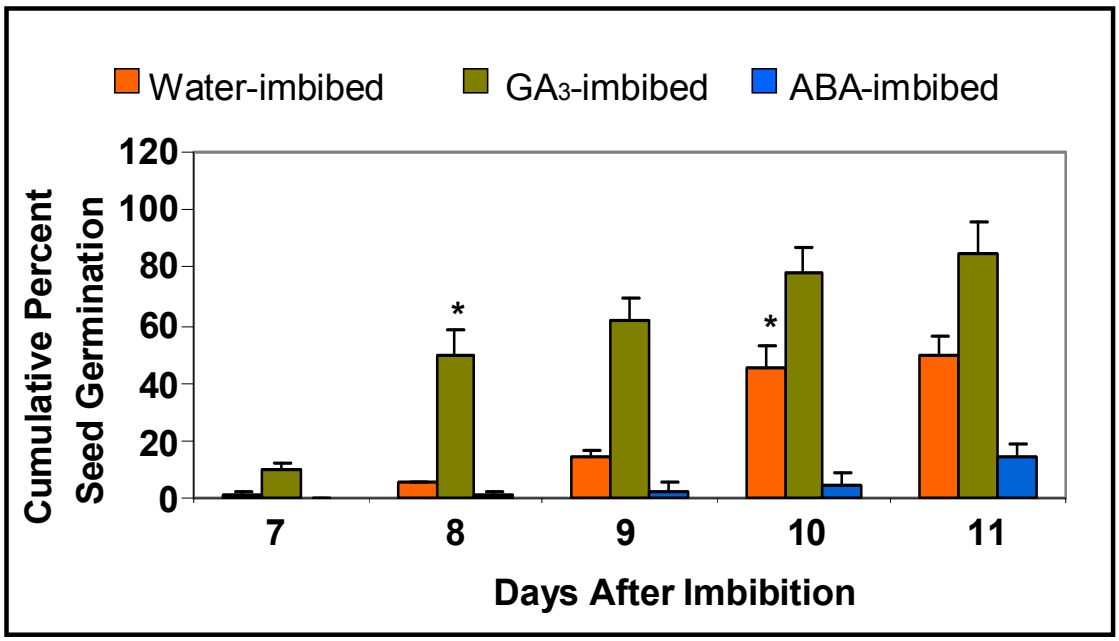

Figure 27: Temporal pattern of seed germination after imbibition in water/ hormone solutions. Asterisks represent the days at which major increases in germination were observed for the water- and GA_3-imbibed seeds.

Minimal germination was observed for the ABA-imbibed seeds by 9-10 DAI, with approximately 12% total seed germination by 11 DAI. A major increase in seed germination occurred later, by 14 DAI. The temporal patterns of germination capacity for the different treatments were then assessed for their relationship to *Pta-MMP* gene expression and MMP-like activity.

Real time PCR analysis of *Pta-MMP* transcript abundance in embryos during seed germination was studied up to 10 DAI (Fig. 28). Transcript levels declined significantly to approximately 5-10% of the levels of dry embryo in water-, GA_3-, and ABA-imbibed seeds, during the first day of imbibition, after which they began to increase throughout the entire germination time frame. At 2 DAI, *Pta-MMP* transcript levels for all three treatments were similar levels, and by 3 DAI, transcript levels in water- and GA_3-imbibed seed embryos were similar, and higher than those in ABA-treated embryos. At 8 DAI, *Pta-MMP* transcript levels were significantly greater in GA_3- and ABA-imbibed embryos than in water-imbibed embryos. However, by 10 DAI, transcript levels in embryos of all 3 treatments were similar, although they were still lower than the levels that were present in mature, dry seed embryos. In the corresponding female megagametophyte samples, however, no major changes in *Pta-MMP* transcript levels was detected (Fig. 29)

In order to assess MMP-like activity in embryos during germination, embryo protein extracts were characterized using MBP degradation assays (Fig. 30). In this case, the key time points of 8 DAI (increased germination rate for GA_3-imbibed seeds) and 10 DAI (increased germination rate for water-imbibed seeds) were targeted for analysis, with little MMP-like activity observed immediately on imbibition. Embryos from water-imbibed seeds displayed minimal MMP-like activity at 8 DAI, which increased substantially by 10 DAI. In contrast, embryos from GA_3-

imbibed seeds already contained substantial MMP-like activity by 8 DAI. However, little activity was observed in the female megagametophyte protein samples (Fig. 31). In the case of the embryo samples, the activity could be completely inhibited by the active site hydroxamate inhibitor GM6001 (Fig. 32). In contrast to the above two cases, minimal MMP-like activity was observed in embryos from ABA-treated seeds over the 10 DAI period. These results indicated an association of *Pta-MMP* gene expression with imbibition, and MMP-like activity with seed germination capacity.

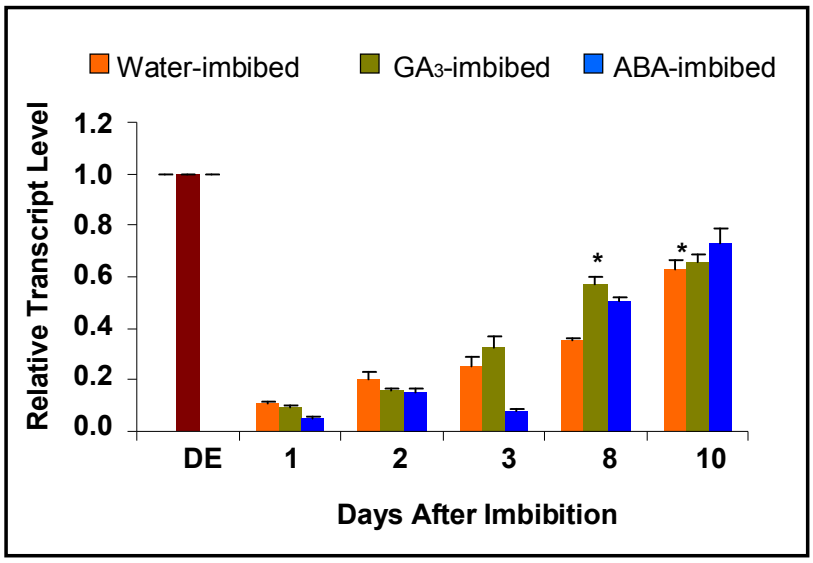

Figure 28: *Pta-MMP* transcript levels in embryos of water/hormone treated seeds at various stages of seed germination measured by real-time PCR using *Pta-MMP* specific primers and tubulin as endogenous normalization control. All the levels are relative to the transcript levels in dry embryo. Asterisks represent the days at which major increases in germination were observed for the water- and GA_3-imbibed seeds.

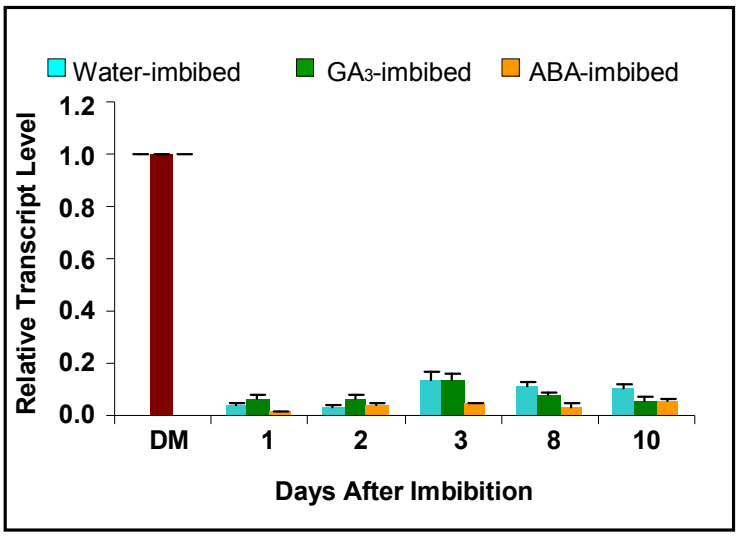

Figure 29: *Pta-MMP* transcript levels in megagametophytes of water/hormone treated seeds at various stages of seed germination measured by real-time PCR using MMP specific primers and tubulin as endogenous control. All the levels are relative to the transcript levels in dry megagametophyte.

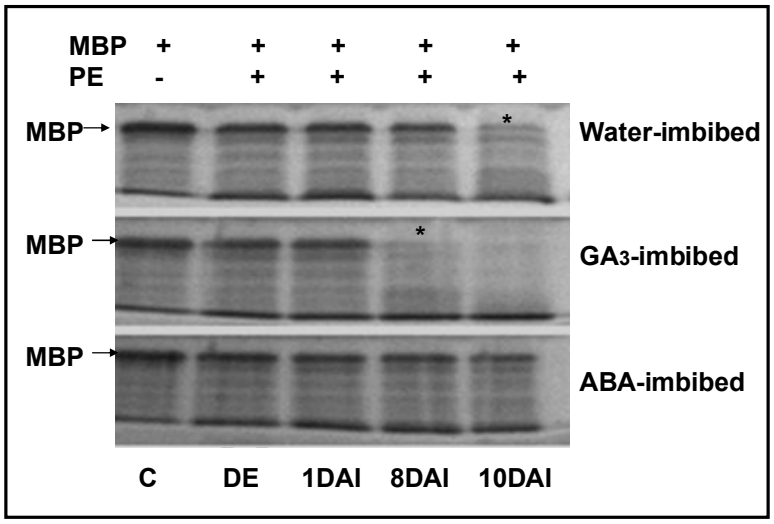

Figure 30: MMP-like activity in protein extracts of embryos at various stages of germination. Asterisks represent the days at which major increases in germination were observed for the water- and GA_3-imbibed seeds. (DAI: Days After Imbibition; DE: Dry Embryo PE: Protein Extract MBP: Myelin Basic Protein).

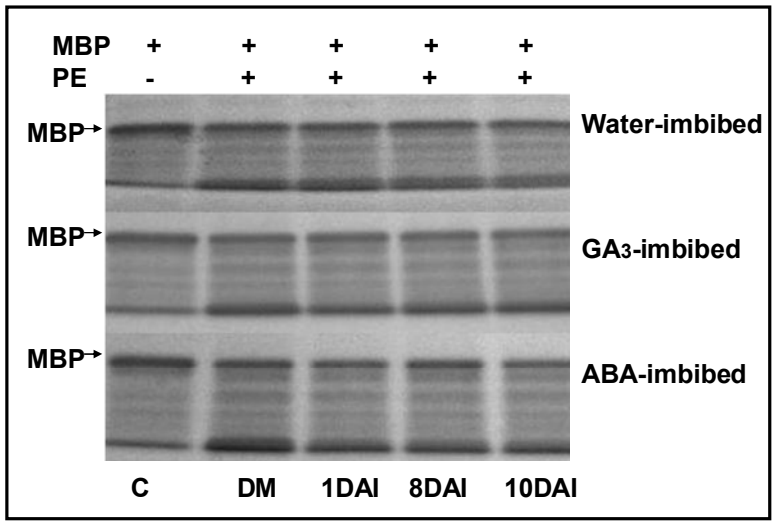

Figure 31: MMP-like activity in protein extracts of megagametophytes at various stages of germination. (DAI: Days After Imbibition; DM: Dry Megametophyte; PE: Protein Extract; MBP: Myelin Basic Protein)

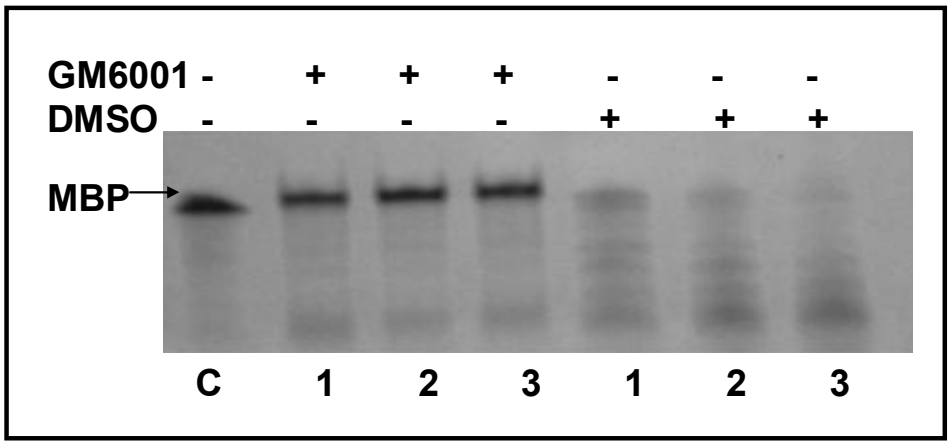

Figure 32: Inhibition of metalloprotease activity in protein extracts of embryos at various stages of germination with GM6001. (C: MBP control, 1: Water imbibed embryo at day 10, 2: GA_3-treated embryo at day 8, 3: GA_3-treated embryo at day 10).

5 Effect of GM6001 treatment on pine seed germination

To further examine the relationship between MMP-like activity and seed germination, the impact of GM6001 on Loblolly pine seeds was assessed. The difference between the treatments was detectable at day 15 (Fig. 33). The MMP inhibitor was tested at 5, 50 and 500 μM during seed imbibition over a period of 19 days, as was the GM6001 solvent, DMSO (Fig. 33). While seeds germinated well during water-imbibition, the DMSO solvent partially inhibited germination by approximately 37%. However, this was minor when compared with GM6001, which significantly reduced germination further in a dose-dependent manner, with 500 μM more inhibitory than 50 μM, which was more inhibitory than 5 μM. Over the 19 day incubation period, increases in cumulative seed germination for both DMSO control and 5 μM GM6001 occurred. However, there was little change in cumulative seed germination for the 50 μM and 500 μM GM6001 treatments (Fig. 33). Some seed germination was observed in all treatments by 9 DAI, but subsequent seedling growth was inhibited by increasing concentrations of GM6001 (Fig. 34). The MBP degradation assays in soluble protein isolates of embryos at day 15 indicated a dose dependent inhibition of metalloprotease activity in the GM6001-treated seeds, while high metalloprotease activity was detected in water-imbibed and DMSO-treated embryos (Fig. 35). The seedlings derived from seeds treated with 5 μM GM6001 were shorter in length (up to 2 cm) than seedlings from control seeds treated with water or DMSO (3-5 cm). Seeds treated with 500 μM GM6001 showed minor radicle protrusion (Fig. 36). These results further suggested a role for MMP activity in seed germination and subsequent seedling elongation and growth.

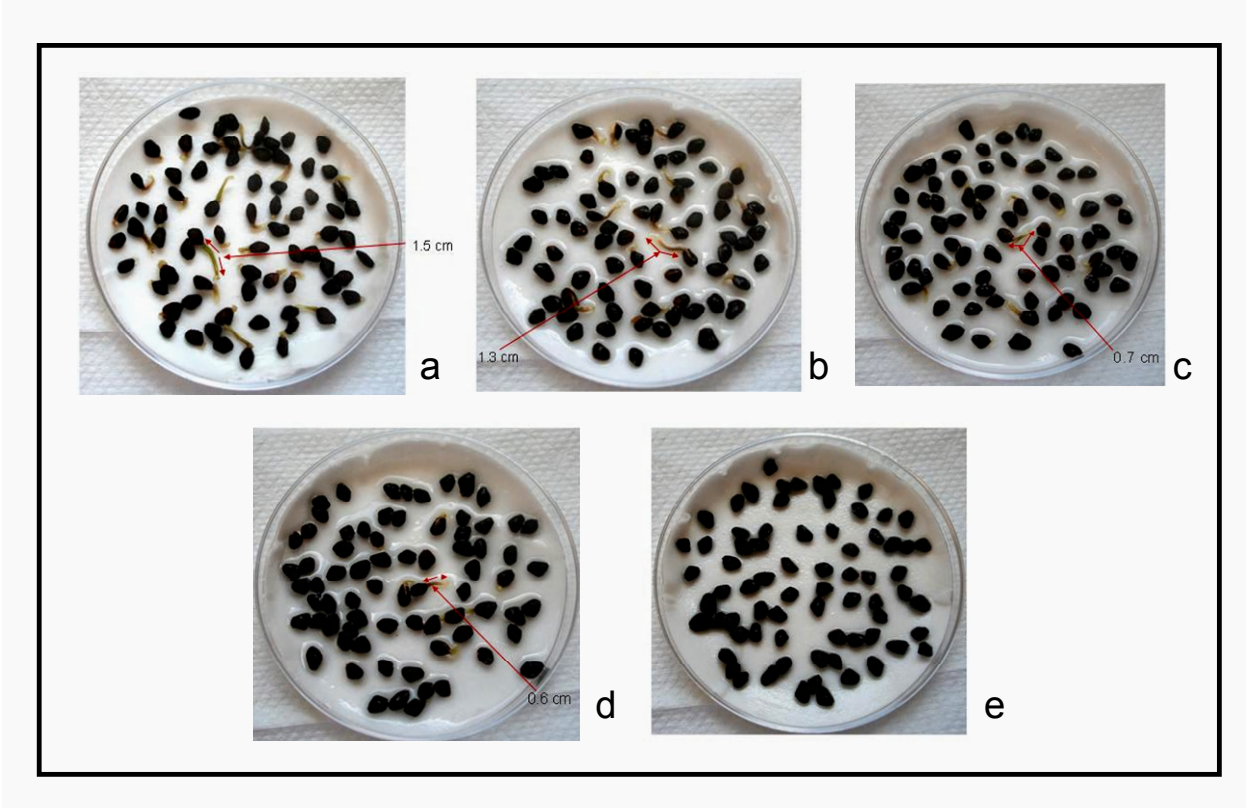

Figure 33: Difference in germination and seedling growth in seeds treated with various concentrations of GM6001 solutions. a: water imbibed, b: DMSO-treated, c: 5 µM GM6001-treated, d: 50 µM GM6001-treated, e: 500 µM GM6001-treated. 5 µM and 50 µM GM6001-treated seeds show reduced size of radicle compared to water-imbibed and DMSO-treated seeds, while 500 µM GM6001-treated seeds fail to show any elongation of radicle.

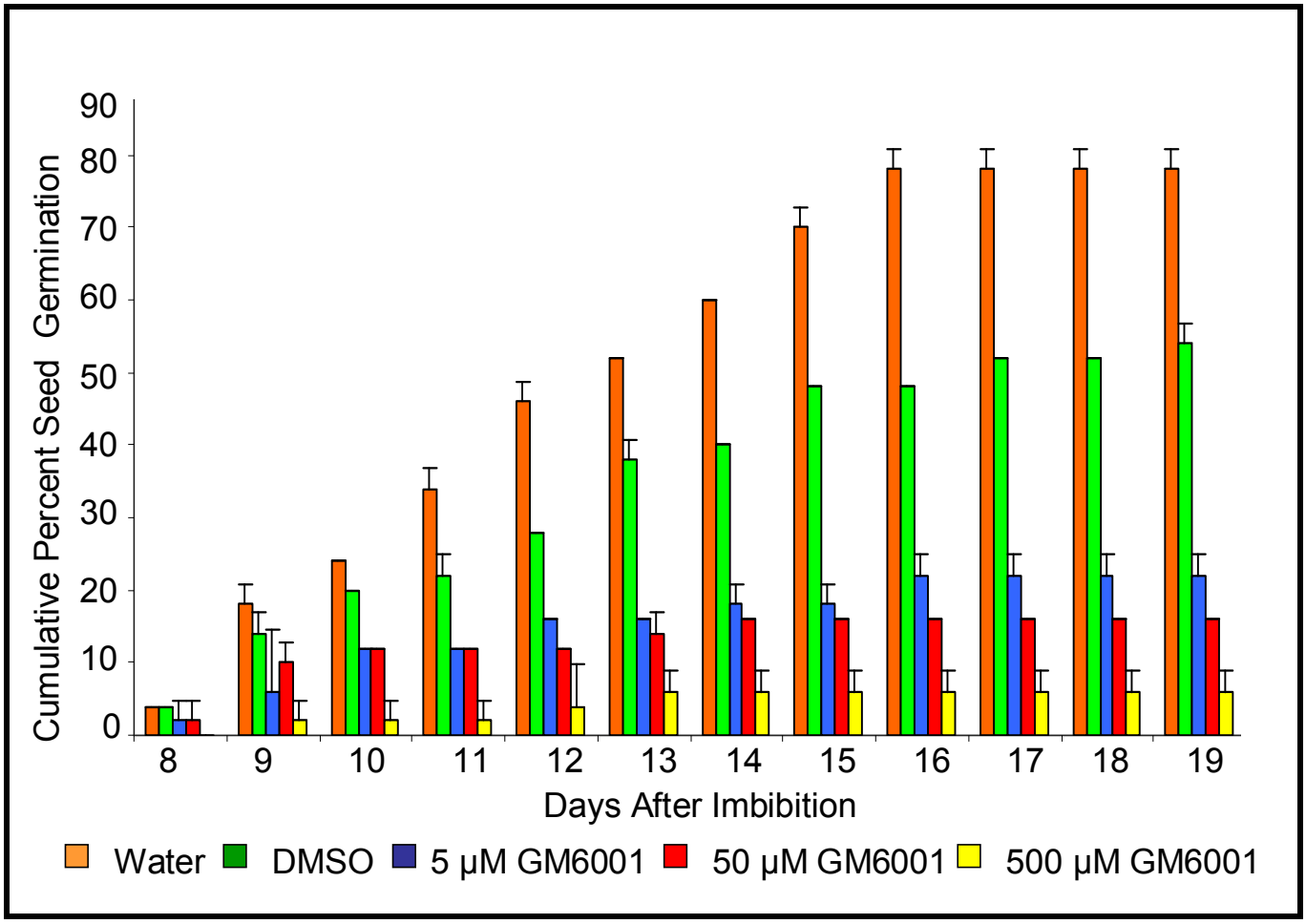

Figure 34: Effect of GM6001 treatment on pine seed germination. Temporal pattern of seed germination after imbibition in water/DMSO/GM6001 solutions.

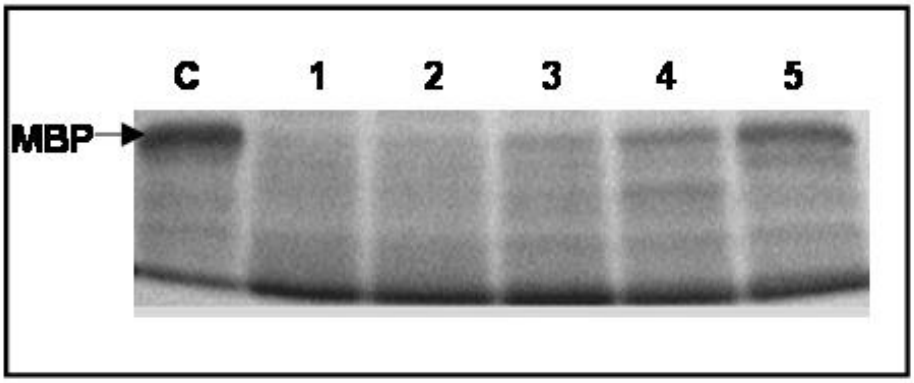

Figure 35: Metalloprotease activity inhibition in protein extracts from embryos of GM6001-treated pine seeds. Inhibition of protease activity increases with increased GM6001 concentration. (C: MBP control; 1: Seeds soaked in water; 2: Seeds soaked in DMSO; 3: Seeds soaked in 5 µM GM6001; 4: Seeds soaked in 50 µM GM6001; 5: Seeds soaked in 500 µM GM6001).

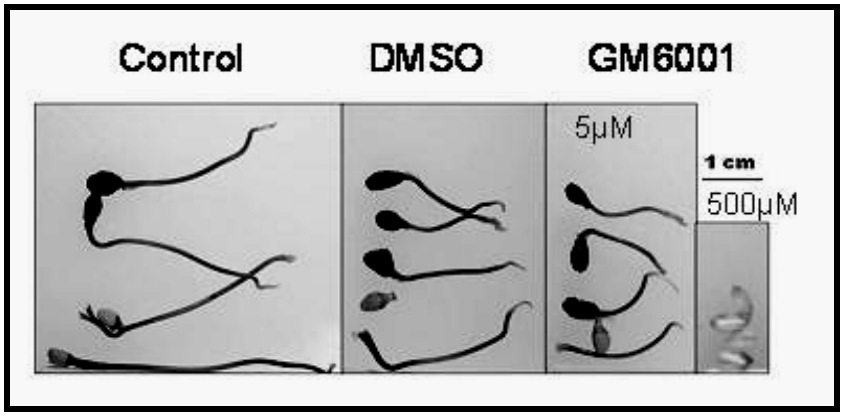

Figure 36: Difference in seedling length from seeds germinated with imbibition in water/ DMSO/ GM6001 solutions on day 19th after imbibition.

6 Cloning of a full length genomic sequence for *Pta1-MMP*

In order to isolate a full length coding sequence, primers designed near the 5' end of the available ESTs were used to isolate a full length MMP sequence from genomic DNA using Genome Walker technology. This allowed the identification of several hundred additional nucleotides, which contained a putative start codon. Primers were then designed to the 5'UTR, and in combination with 3' UTR primers directed against the ESTs, and used to amplify a 1,205 bp fragment (Fig. 37). When translated, this generated an ORF of 364 amino acids with no introns, of which the first 25 amino acids encoded a predicted signal peptide. This Loblolly pine MMP was designated *Pta1-MMP* (**Genbank Accession Number FJ389668**). An alignment of the amino acid sequence of Pta1-MMP over the length of the final, processed, catalytic domain, (detected by N-terminal sequencing as described previously) revealed a difference of 4 amino acid sequences between the two sequences, suggesting that these two may be allelic (Fig. 38). Alignment of the *Pta1-MMP* sequence with 5 Arabidopsis MMPs and human *MMP-7* revealed common characteristics, with putative signal peptides at the N terminus, in agreement with their postulated role as secreted, extracellular proteins (Fig. 39). All contained a characteristic cysteine switch PRCGXXD motif, a structural Zn^{2+}/ Ca^{2+}-binding site (non-catalytic), and a catalytic domain Zn^{2+}-binding motif (HEI/LGHXLGXGH), followed by the conserved methionine residue of the Met turn. In addition, Pta1-MMP and the At-MMPs also contained the conserved DLESV/IA sequence of unknown function, which appears to be unique to plants. Further software analyses of the predicted Pta1-MMP peptide suggested that the preprotein has six potential N-linked glycosylation sites, and is secreted as a zymogen, in an inactive form.

GCAGGGTACAGGGATTCGCAGGGAAAAAAAGGAAAAATTTC**ATG**GCGAGGCGAGAAATGATTTTAATTAT
AGTTGCTGCGTATTGTTTTAGCGTAATCATGTCGGGTGCCTATGGATTTCAGCCGAAAACTATTCCGAAT
ATATACTACCCAGCCCCGGGTTTTATGAATTCTAATTCTGCTGTAGCGGCCGGGGCCTGGGAAGGTTTCA
GGAACCTGACGAACGCCTGCAAGGGCGACCGGATGCAGGGCCTGCCGGACCTGAAGCGCTACTTCCGCCG
CTTCGGTTACCTCAGCGCGCAGAACAACGTGACCGAGGACTTCGACGAGGCCGTGGAGAGCGCGGTGCGG
ACATACCAGAAAAACTTCGGGCTCAACGTTACCGGGGTCCTGGACGAGGCCACGATTTCGCAGCTCATGG
TGCCGCGGTGCGGCCGCGAGGACATCATCAACGGGAGCTCCGCCATGCGGGGACGTGGTCTGTTCCCGTT
CTTCCCGGGGAGCCCCCGCTGGGGACCCGACAAGAGGGTGCTCAGTTACGCCTTCTCGCCGGACCACGAG
GTGCTATCGGAGATCAGCCTGGCAGAGCTCAGCACTGTGGTCGGGCGCGCCTTCAAGCGCTGGGCCGACG
TGATCCCCATAACGTTCACCGAGAGCTCCGATTATTCCTCCGCCGATATCAAGGTCGGGTTCTACAGCGG
CGACCACGGCGACGGGCACCCCTTCGACGGACCCCTCGGGACCCTCGCCCATTCCTTCTCCCCGCCCGAC
GGCCGCTTCCACCTGGACGCCGCCGAGTCGTGGACCGTCGACCTCTCGTCCGACTCAGCTGCCACAGCCA
TCGACCTGGAGTCCATCGCCACCCACGAGATCGGCCACCTGCTCGGCCTCGGCCACACGACCGAGAAAGC
CGCCGTCATGTACCCCAGCATCGCGCCGCGAACCAGAAAAGTCGACCTCGTCCTTGATGACGTGGATGGC
GTTCAGTATGTGTACGGCGCCAACCCCAACTATAACGCCTCTGCCGTCCTCGCCCAGAACGAGACCAGCT
CTGCAACTGCCATACAACTCCCCCCTGGAGCAAGACTGCTCCCAATTCTTTTCTTCATTTCGTCTCTGCT
GCCATTAATTCTG**TGA**CTGCACTTTCCCCTTACTTTACCAATCGAATTCCCGCGACCGCCATGGCGGCTC
GGGAGCATGCGACGT

Figure 37: Amplified *Pta1-MMP* nucleotide sequence (1,205 bp), with start and stop codons indicated, representing an ORF of 364 amino acids. The primer binding regions used for real time PCR are underlined, and nucleotide differences in these regions between the *Pta1-MMP* genomic sequence and the ESTs are shaded.

```
rPta-MMP      78 SSAMRGRGLYTFFPGSPRWGPDKRVLSYAFSPDHEVLSEISL
Pta1-MMP     139 SSAMRGRGLFPFFPGSPRWGPDKRVLSYAFSPDHEVLSEISL
                 *********  ********************************

rPta-MMP     120 AELSTVVGRAFKRWADVIPITFTESSDYSSADIKVGFYSGDHGDGHPFDGPLGTLAHSFS
Pta1-MMP     181 AELSTVVGRAFKRWADVIPITFTESSDYSSADIKVGFYSGDHGDGHPFDGPLGTLAHSFS
                 ************************************************************

rPta-MMP     180 PPDGRFHLDAAESWTVDLSSDSAATAIDLESIATHEIGHLLGLGHTTEKAAVMYPSIAPR
Pta1-MMP     241 PPDGRFHLDAAESWTVDLSSDSAATAIDLESIATHEIGHLLGLGHTTEKAAVMYPSIAPR
                 ************************************************************
rPta-MMP     240 TRKVDLVLDDVDGVQYLYGANPNYNASAVLAQNETSSATAIQLPPGASLLPILFFISSLL
Pta1-MMP     301 TRKVDLVLDDVDGVQYVYGANPNYNASAVLAQNETSSATAIQLPPGARLLPILFFISSLL
                 *************** ****************************** ************

rPta-MMP     300 PLIL
Pta1-MMP     361 PLIL
                 ****
```

98.4% identity in 244 residues overlap

Figure 38: Alignment of the processed, mature r*Pta-MMP* amino acid sequence with the amino acid sequence of Pta1-MMP. The N-terminal sequence of the mature, processed peptide as deduced earlier is highlighted in pink.

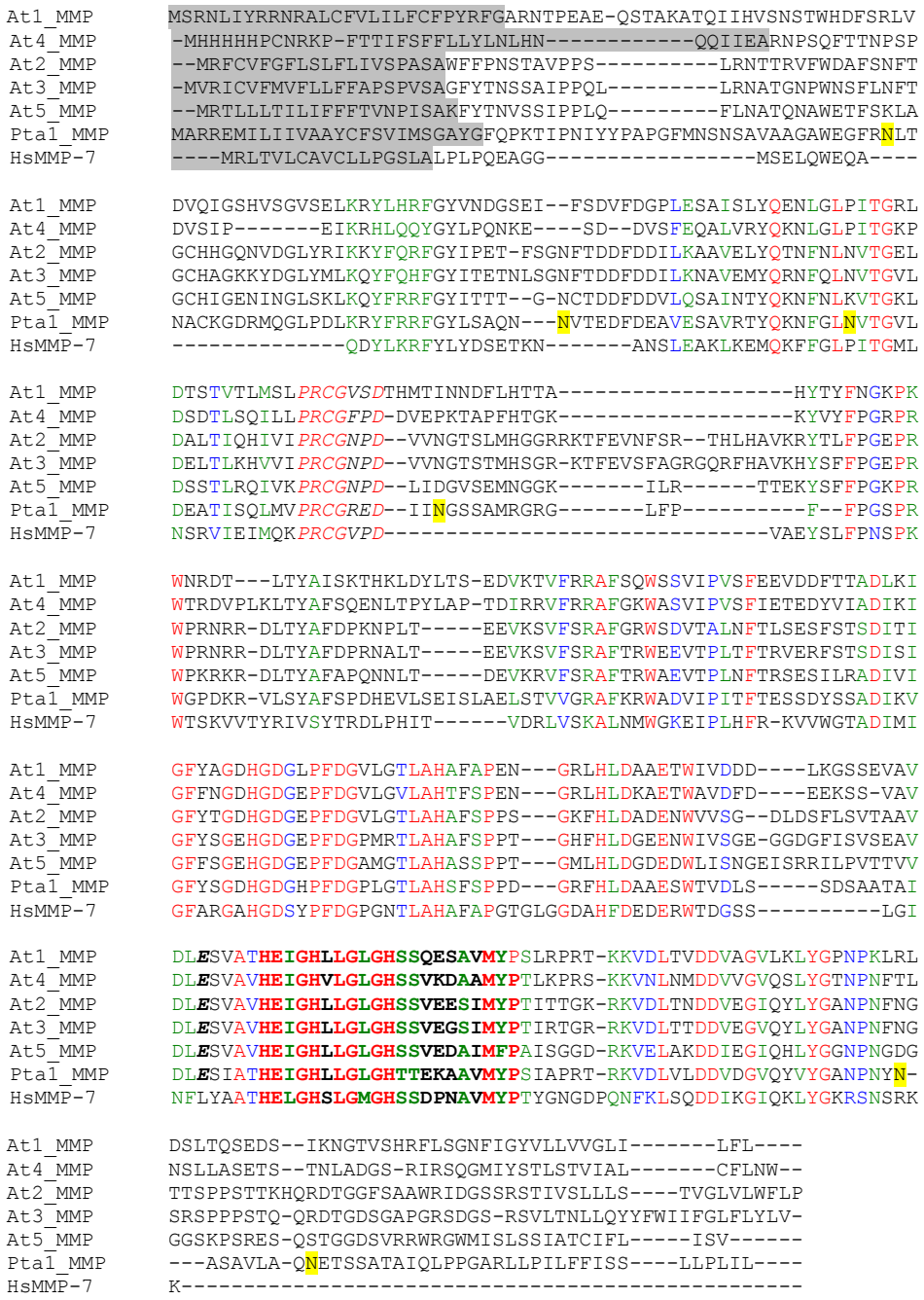

Figure 39: Amino acid sequence alignment of *Pta1*-MMP with Arabidopsis MMPs and human *MMP-7*. The predicted amino acid sequences of At1-MMP to At5-MMP were aligned with the *Pta1*-MMP sequence and human *MMP-7* sequence using clustalW at NPS@: Network Protein Sequence Analysis. The cysteine switch motif PRCGXXD is italicized, Zinc-binding sequence HEXGHXXGXXH is shown in boldface. The conserved Glu residue in plant MMPs is boldface and italicized.

Phylogenetic tree analysis using the region from the cysteine switch to the catalytic region zinc-binding domain of several predicted plant MMPs indicated that Pta1-MMP clusters near At4-MMP and At1-MMP, as well as the predicted rice MMP gene OSJNBa0010C11.5 (Fig. 40).

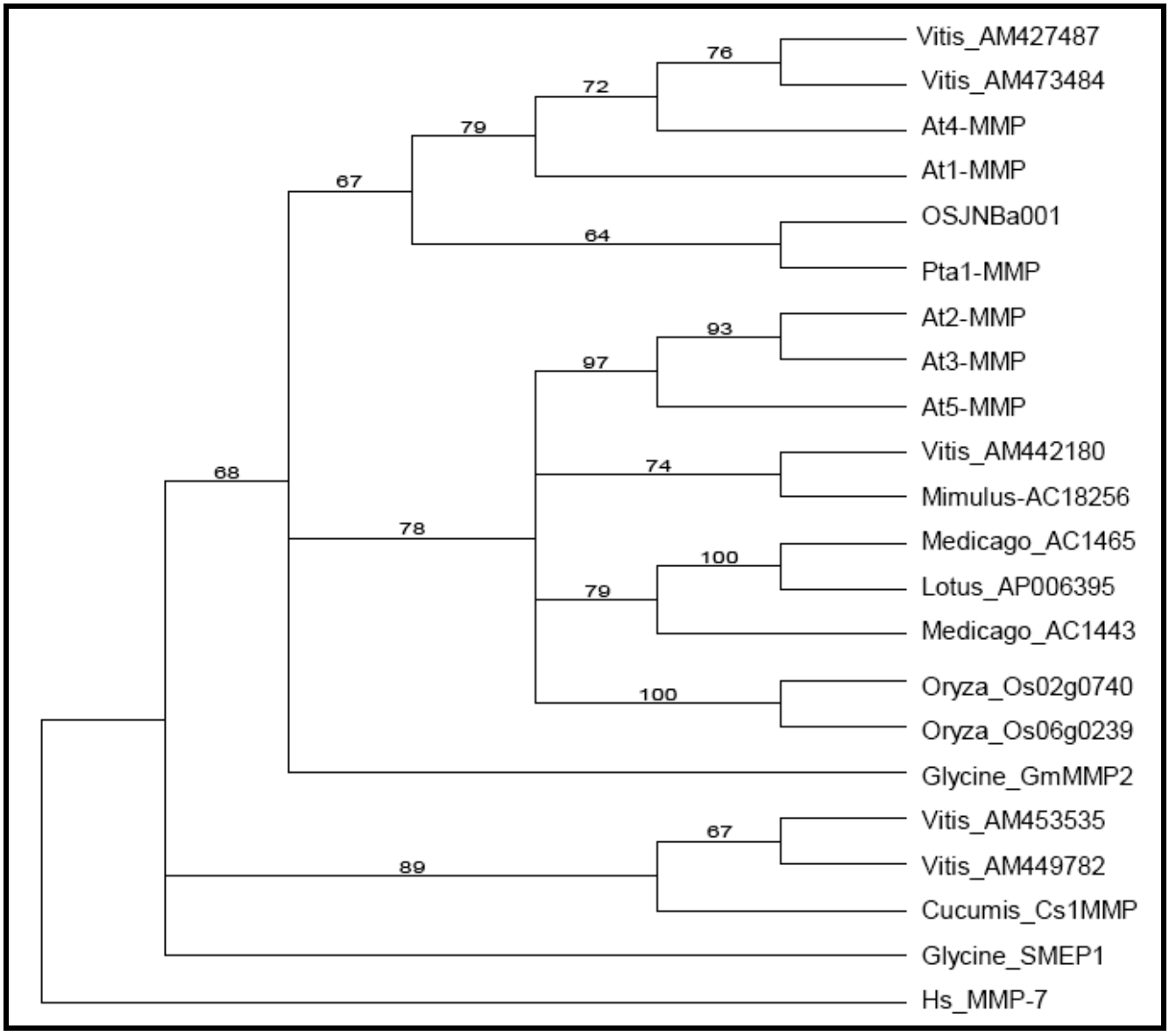

Figure 40: Phylogenetic tree of selected plant MMP fragments and human *MMP-7* generated using PAUP version 4.0. All analyzed fragments are of similar size and span the conserved region from the cysteine switch to Met-turn motif (Using data from Flinn, 2008). Bootstrap values shown as percentages.

V

Discussion

Identification of a seed-expressed Loblolly pine MMP

A key result from this project was the identification and analysis of a Loblolly pine MMP. To our knowledge, this study is the first to describe and characterize a conifer MMP, and also the first study on a seed expressed MMP. While plant MMP ESTs are available in databases such as Genbank (www.ncbi.nih.gov/Genbank), TIGR (http://www.tigr.org/db.shtml), TAIR (www.arabidopsis.org), Phytozome (www.phytozome.net), PlantGDB (www.plantgdb.org), etc., there are only a few clones that have been fully sequenced or functionally characterized, namely *At1-MMP, At2-MMP, At3-MMP, At4-MMP and At5-MMP* from *Arabidopsis*, *GmMMP2* and *SMEP1* from Soybean, *Cs1-MMP* from Cucumber and the variant, *MtMMPL1* from *Medicago* (Maidment *et al.,* 1999; Golldack *et al*., 2002; Liu *et al*., 2001; Pak *et al*., 1997; Delorme *et al*., 2000; Combier *et al*., 2007) . The Loblolly pine MMP studied here was initially identified using several ESTs representing non-seed libraries (www.fungen.org), and subsequently confirmed during the current project to be present in seed tissues. The alignment of the three ESTs identified from the database showed very high identity amongst themselves over the predicted ORF and 3' UTR region, except at the extreme 5' and 3' ends (Fig. 12). Taking into account some potential frame shifts and potential base call misreads or allelic variation, they are believed to represent the same gene. As such, the longest clone (STRS1_39_H03) was used to design primers for the amplification of the ORF for the production of recombinant protein.

Full length genomic clone identification

All of our initial gene expression and recombinant protein analysis was based on the sequence information obtained from identified EST sequences, which were not full length. The existing sequence information was used to obtain the 5' end sequence. Our genomic clone, designated as *Pta1-MMP*, was isolated using a primer designed to the 5' UTR as well as a 3' UTR primer designed from our EST sequences. As such, we assume that the ESTs represent *Pta1-MMP* transcripts. In addition, an alignment of the amino acid translation of *Pta1-MMP* with the amino acid sequence of the mature active r*Pta-MMP* revealed a difference of only only 4 amino acids (Fig. 38). This data, along with a small number of nucleotide substitutions between *Pta1-MMP* and the ESTs suggest that these are allelic. The *Pta1-MMP* protein sequence contains all of the basic characteristic structural features expected of an MMP, that is, a cleavable N-terminal signal peptide, a characteristic PRCGXXD cysteine switch motif in the propeptide domain, a non-catalytic Ca^{2+}/Zn^{2+}-binding domain, a catalytic Zn^{2+}-binding domain with the Histidine triad and the catalytic Glu residue, a Met-turn, as well as the plant-specific DLESV/IA sequence. These characteristics have been detailed for several other full length plant MMP sequences (Table 4 - from Flinn, 2008).

An analysis of some plant MMP sequences suggest that the mature MMP protein may be cell surface-attached through a GPI-anchor or C-terminal membrane spanning domain (Table 4 - Flinn, 2008). To date, there has however been no demonstration of extracellular localization or membrane attachment for any plant MMP. From our analysis, we have been unable to clearly determine if *Pta1-MMP* contains a C-terminal transmembrane domain. To date, nearly all plant

MMPs studied contain a functional HEIGH catalytic domain sequence. However, the *MtMMPL1* gene in *Medicago* contains a Gln instead of a Glu in the catalytic domain. This type of substitution has so far only been found in legume species (Combier *et al*., 2007) and is not present in *Pta1-MMP*. In animal MMPs, Gln-Glu substitutions are associated with a dramatic decrease in protease activity (Rowsell *et al*., 2002; Crabbe *et al*., 1994) however; no protease activity study was performed with *MtMMPL1*.

The complete coding sequence of *Pta1-MMP* was determined from genomic DNA, which indicated that this gene contains no introns, as has been described for all other plant MMPs studied to date (Pak *et al*., 1997, Maidment *et al*., 1999; Delorme *et al*. 2000; Liu *et al*., 2000; Combier *et al*., 2007; Flinn, 2008). Where genomic sequence is known, all the plant MMPs reported so far are intronless unlike the mammalian MMPs (Clark *et al*., 2004). As far as the human MMPs are concerned, the genes show a highly conserved modular structure. The collagenases, stromelysin-1, and stromelysin-2 genes, each contain 10 exons and 9 introns in 8–12 kbp of DNA (Ravanti *et al*., 2000; Fini *et al*., 1987 and Collier *et al*., 1988). The exon-intron junctions are closely conserved, suggesting that the insertion of introns predated the formation of the separate enzymes. The length and number of introns however varies amongst MMPs of different organisms. For example, the nematode MMP, *Hg-MMP*, consists of 19 introns, with a total intron length of 3680 bp and the longest intron comprising 1072 bp (Kovaleva *et al*., 2004). In certain cases, the introns are known to have regulatory sites, for example substitution of the first intron of human *MMP-2* abolished ischemia-induced *MMP-2* transcription *in vivo* indicating the existence of a transcription regulatory site in that intron (Lee *et al*., 2005). This implies

structural as well as functional evolutionary consequences due to absence of introns in MMPs in plants.

Phylogeny

A phylogenetic tree analysis was carried out on selected plant MMP protein sequences containing at least the cysteine switch and the catalytic domain (Fig. 40). To compare fragments of similar lengths and therefore generate a more reliable tree, only sequences spanning the region from the cysteine switch to the M- turn were considered. *Pta1-MMP* clustered with Arabidopsis *At1-MMP* and *At4-MMP*, which are maximally-expressed during seed/silique development (*At1-MMP, At4-MMP*) and subsequent germination (*At4-MMP*) (Flinn, 2008), suggesting that *Pta1-MMP* is an ortholog to one of these.

It is also likely that pine has other MMPs yet to be discovered. The pine genome is large and highly complicated (Dean, 2007) and several projects directed towards targeted sequencing of the pine genome are underway (Dean, 2007), which include, the NSF Genomics of Loblolly Pine Embryogenesis Project which focuses on creating a cDNA library from Loblolly pine zygotic (natural) and somatic (lab cultivated) embryos, representing a combined set of transcripts expressed during all stages of embryogenesis to generate Expressed Sequence Tags (ESTs) which will be clustured and assembled to construct a non redundant pine EST database for identification of unique transcripts (http://www.tigr.org/tdb/e2k1/pine/index.shtml); Accelerating Pine Genomics (APG), the information generated in this project and the molecular resources themselves are providing the foundation for physical mapping and sequencing of the pine

genome (http://www.mgel.msstate.edu/apg.htm); Allele Discovery of Economic Pine Traits (ADEPT), whose goal is to develop an infrastructure for identifying alleles at candidate gene loci that can be used to greatly accelerate Loblolly pine tree improvement (http://dendrome.ucdavis.edu/adept/summary.html); The Pine Genome Initiative (PGI) under Dendrome, which focuses on multiple aspects of Pine genomics including, Genome structure and organization, Functional Genomics, Translational genomics and Genetic stocks development and curation (http://pinegenomeinitiative.org/deliver.html). A BAC library for the Loblolly pine genotype "7-56' is now complete. It is composed of 1,797,505 individually archived clones and affords roughly 8X coverage of the enormous (21.7 Gb) pine genome http://www.mgel.msstate.edu/apg_bac.htm. Methylation analysis suggests >220,000 genes in pine however, methyl-filtration provided poor enrichment. In terms of complexities regarding gene families, 37% of random genomic DNA reads show primary homology to ESTs with average repetitiveness 7X. 10-12% of the genome looks unique with little homology to ESTs (Jeffrey F.D. Dean, December 6, 2007).

Table 4. Representative putative full-length functional MMP ORFs identified in Genbank NR. The cysteine-switch and zinc-binding motifs of *Arabidopsis* MMPs were used for a BLAST search against the Genbank NR database. The ORFs were identified and cysteine switch and catalytic domain zinc-binding motifs determined. They were also analysed with a variety of predictive software programs for signal peptide cleavage site (SignalP 3.0 - http://www.cbs.dtu.dk/services/SignalP/), furin cleavage site prediction (ProP 1.0 - http://www.cbs.dtu.dk/services/ProP/), GPI anchor prediction (BIG-PI Plant Predictor - http://mendel.imp.ac.at/sat/gpi/plant_server.html) and C-terminal transmembrane domain prediction (Localizome Transmembrane Topology Predictor - http://localodom.kobic.re.kr/LocaloDom/index.htm). The GPI-anchor prediction includes both primary (X) and secondary prediction (Y) sites when present. (From Flinn, 2008)

Plant Species	Gene identifier	Genebank accession no.	Length in amino acids	Signal peptide cleavage site	Cys- switch motif	Predicted furin binding site	Zn-binding motif	GPI-anchor prediction	C terminal prediction
Arabidopsis thaliana	At1-MMP (At4g16640)	NM_117765	364	RFG-AR	PRCGVSD	No	HEIGHLLGLGH	No	Yes
	At2-MMP (At1g70170	NM_105685	378	ASA-WF	PRCGNPD	NRR-DL	HEIGHLLGLGH	WRIDG	Yes
	At3-MMP (At1g24140	NM_102260	384	VSA-GF	PRCGNPD	NRR-DL	HEIGHLLGLGH	No	Yes
	At4-MMP (At2g45040	NM_130068	342	IEA-RN	PRCGFPD	WTR-DV	HEIGHVLGLGH	TNLAD	No
	At5-MMP (At1g59970	NM_104689	360	ISA-KF	PRCGNPD	No	HEIGHLLGLGH	QSTGG	Yes
Glycine max	SMEP1	U63725	305	VSA-HG	PRCGVPD	No	HEIGHLLGLGH	No	No
	GmMMP2	AY057902	357	SDG-VS	LRCGVPD	No	HEIGHLLGLDH	NVEDS	Yes
Cucumis Sativus	Cs1-MMP	AJ133371	320	NTS-SP	PRCGVQD	No	HEIGHLLGLQH	No	No
Vitis vinifera	N/A	AM427487	354	IIP-DY	PRCGVSD	No	HEIGHLLGLAH	No	Yes
	N/A	AM473484	353	CQP-GR	PRCGMRD	No	HEIGHLLGLGH	No	No
	N/A	AM442180	373	VSA-RF	PRCGNAD	No	HEIGHLLGLGH	TTNDS	No
	N/A	AM453535	319	ANG-EN	PRCGVAD	No	HEIGHLLGLAH	No	No
	N/A	AM449782	303	ATS-SD	PRCGVPD	No	HEIGHLLGLGH	No	No
Medicago truncatula	MtrDRAFT_AC144345g5	AC144345	368	VSA-RF	PRCGVAD	No	HEIGHLLGLGH	DRDSS	Yes
	MtrDRAFT_AC146588g23	AC146588	373	VSA-RL	PRCGVAD	MKK-VV	HEIGHLLGLGH	NIGNG	No
Oryza sativa	Os02g0740700	NM001054610	372	AMA-FP	PRCGVAD	No	HEIGHLLGLGH	EMDGS	Yes
	OSJNBa0010C11.5	AC069300	355	VHG-HG	PRCGVGD	No	HEIGHVLGLGH	TSSSS	No
	Os06g0239100	NM_001063794	371	AFA-LP	PRCGVAD	No	HEIGHLLGLGH	MDSAG	No
Mimulus Gutattus	N/A	AC182562	374	ASA-NF	PRCGNAD	No	HEIGHLLGLGH	RDTSG	No
Lotus japonicus	N/A	AP006395	382	VVS-AR	PRCGVAD	TKK-VV	HEIGHLLGLGH	PERDA	Yes

Recombinant *Pta-MMP* expression and protein characterization

As efforts to clone the full length MMP gene were taking place, we realized that based on previous plant studies, enough information was available from the ESTs to generate a recombinant protein for functional studies. The STRS1_39_H03 open reading was amplified, containing the propeptide region, as well as the cysteine switch and catalytic domain. Therefore, following bacterial induction and expression, recombinant *Pta-MMP* (r*Pta-MMP*) was generated. The recombinant protein carrying the His-tag was affinity purified, and the newly purified r*Pta-MMP* displayed a MW of ~43 kDa on an SDS-PAGE prior to refolding, somewhat higher than the predicted molecular weight (Fig. 15, lane 1). This could be due to the presence of the His-tag, which may affect mobility under the SDS-PAGE conditions used. During refolding of the denatured protein, the His-tag was cleaved, along with the prodomain, as autocatalytic activation occurred. During refolding, the protein was dialysed through a 20 kDa dialysis membrane and hence it was expected that a majority of the His-tag was removed from the buffer. However, we are unsure how much residual His-tag remained in our recombinant *Pta-MMP* preparation.

All MMPs are expected to be secreted as inactive zymogens, requiring activation through the removal of the prodomain containing the cysteine switch motif. When the cysteine residue of this motif is associated with the catalytic domain zinc atom, the enzyme is inactive. Thus, the dissociation of the cysteine residue from the zinc atom is viewed as the switch that leads to enzyme activation. Activation of the enzyme with aminophenyl mercuric acetate (APMA) causes the cysteine to become dissociated from the zinc (Fig. 8) and the proteinase undergoes

autocatalytic cleavage (Snoek-van Beurden *et al*., 2005). The cysteine switch model has important physiological implications in that it allows for the selective activation of one or a small number of MMPs at certain sites. As MMPs are proteases, they could be deleterious to cell functions if they were constitutively active; therefore the cysteine switch mechanism of their activation is a way of regulating their activity when and as required. It would however be interesting to generate a recombinant, constitutively active MMP by modifying the cysteine residue of the cysteine switch motif, and study its impact *in vivo* on plant growth and development.

Activation of recombinant MMP has been achieved through the use of the MMP activator APMA (Maidment *et al*., 1999, Itoh *et al*., 1996). In our experiments, the activation of r*Pta-MMP* did not require APMA, as autocatalytic activation occurred during refolding. MMP autocatalytic activation has been observed in recombinant At1-MMP while refolding at 37 ^{0}C (Maidment *et al*., 1999), as well as with recombinant Cs1-MMP (Delorme *et al*., 2000) and during refolding of human *MMP-19* (Stracke *et al*., 2000). Autocatalytic activation was found to be inhibited by O-phenanthroline (Maidment *et al*., 1999). In the present study, r*Pta-MMP* was generated as a putative inactive zymogen, containing the cysteine switch, and it underwent suspected autocatalytic activation during refolding, as indicated by the reduced size on refolding (Fig. 15, lane 3), as well as the N-terminal sequence information that we obtained.

MMP-like activity

The protease activity of r*Pta-MMP* was confirmed using MBP degradation assays. Protease activity was analyzed by incubating 0.25 mg/ml of Bovine MBP with r*Pta-MMP* at a concentration of 128 nM for 24 hrs at 37^0C. The activity was tested with and without addition of 100 µM $ZnCl_2$. The activity was significantly enhanced in the presence of additional $ZnCl_2$ (Fig. 17). Similar conditions were applied to *At1-MMP* protease activity assays, however, additional zinc did not have an effect on the activity**.** It can be hypothesized that any residual His-tag, possibly still present in the sample following refolding, bound some of the zinc required for the metalloproteinase activity and hence, additional zinc was required for enhanced activity.

In a study to test the effect of excess zinc on the activity of *MMP-2* and *-9*, it was found that excess amounts, in this case zinc released from 2.0 g of zinc oxide-eugenol inhibited metalloproteinase activity (Santos *et al*., 2004).

The protease activity of r*Pta-MMP* was found to be inhibited by a chelating agent (EDTA) , as well as the MMP active site binding inhibitor GM6001, in a dose dependent manner (Figs. 18 and 19). Plant MMPs have displayed the requirement for zinc ions, as well as inhibition by zinc chelators such as EDTA, and hyroxamate compounds such as BB94 (Graham *et al*., 1991; Maidment *et al*., 1999; Delorme *et al*., 2001; Liu *et al*., 2001). EDTA was found to efficiently block the activity of r*Pta-MMP* at a concentration of 1 mM (Fig. 18). We tested GM6001 (also known as Ilomastat or Galardin) for its efficiency in inhibiting r*Pta-MMP*. This inhibitor has not been tested on any of the plant MMPs and we tested different concentrations (5 µM, 50 µM, 250 µM and 500 µM). Dimethylsufoxide (DMSO), the solvent for GM6001 was used as control at

20% total concentration, adjusted according to the amount present in 500 μM GM6001. GM6001, which is an MMP active site, zinc binding inhibitor, was able to inhibit r*Pta-MMP* activity completely at a concentration of 500 μM (Fig. 19). No inhibition was observed with DMSO alone. An EDTA-free protease inhibitor cocktail containing inhibitors of cysteine, serine and aspartic proteases was added to block non-metalloprotease activity. GM6001 has been used to inhibit MMP activity in several organisms such as *Xenopus* (Webber *et al*., 2002) and *Hydra* (Leontovich *et al*., 2000) and recombinant MMP-2 (Kuramochi, 2008) although at concentrations as low as 1 μM. GM 6001's Ki value *in vitro* for human MMP-1 is 0.4 nM, MMP-2 0.5 nM, MMP-3 27 nM, and the Ki for MMP-9 is 0.2 nM (AMS Scientific, CA). We found 500 μM to be the most efficient concentration for complete inhibition of r*Pta-MMP* activity**.** Our requirement for the relatively higher amounts of GM6001 needed to completely inhibit the activity of r*Pta-MMP* may be related to the presence of the higher amounts of zinc that we used in the assay buffer. This inhibitor has not been tested in any other plant systems; therefore it is not possible to compare the amount required for inhibition of r*Pta-MMP* with the inhibition of any other plant MMP. Taken together, the structural sequence information and the biochemical characterization of the recombinant protein suggest that *Pta-MMP* is a functional plant MMP.

MMP characterization during Loblolly pine seed development

Transcript profiling during seed development

In order to characterize *Pta1-MMP* expression during embryo development and seed germination, relative transcript abundance was measured in zygotic embryos and megagametophytes separately, at various stages of embryo development and seed germination.

Primers were designed proximal to the 3' end of our EST assembly, which was earlier confirmed to be expressed in the seed, in order to avoid the conserved region between cysteine switch motif and the zinc binding motif. The transcript abundance measured in these samples was relative to transcript abundance in dry seed samples, i.e., fully mature and dried zygotic embryo, and the associated megagametophyte. As screening of available plant EST databases, such as Genbank (www.ncbi.nih.gov/Genbank), TIGR (http://www.tigr.org/db.shtml), TAIR (www.arabidopsis.org), Phytozome (www.phytozome.net), PlantGDB (www.plantgdb.org) reveal very few hits for MMP sequences, it suggests that plant MMPs have a generally low transcript abundance.

Transcript abundance studies in plants have shown that MMP expression is associated with growth (Golldack *et al*, 2002), development (Delorme *et al*., 2000) or stress related conditions (Liu *et al*., 2001). The expressions of *SMEP1* (Pak *et al*., 1997) and *GmMMP2* (Liu *et al*., 2001) showed enhanced levels in mature leaves. The expression of *Cs1-MMP* was detected at the boundary of senescence and programmed cell death (PCD) as well as in senescing male flowers (Delorme *et al*., 2000). A study of expression of *At-MMP* genes in various tissues of *Arabidopsis* (Maidment *et al*., 1999) revealed that *At1-MMP* displayed strong levels of expression in flower, root and stem. *At2-MMP* showed strongest expression in roots, while *At3-MMP* was strongest in leaf and root, with expression in leaf vascular bundles. The expression of *At3-MMP* increased in flower and leaf at later stages of development. *At-4 MMP* had strongest expression in stem (Maidment *et al*., 1999), and according to Genevestigator analysis, is also present in developing and germinating seeds (Flinn, 2008). In addition, *At5-MMP* had strong expression levels in leaf, root and stem (Maidment *et al*., 1999).

A more comprehensive study on cell specificity of expression of *At2-MMP* was carried out by *in situ* hybridizations in leaves and flowers of *Arabidopsis (*Golldack *et al*., 2002). *At2-MMP* was found to be expressed in the phloem, developing xylem, epidermal cells and neighboring mesophyll cell layers of leaves and pistils, ovules and receptacles of flowers. In an *Arabidopsis* mutant (*At2-mmp*-1) carrying a tDNA insertion in *At2-MMP*, neither germination nor development of plants was modified in comparison to the wild type in the juvenile rosette stage. The growth of roots, leaves and shoots was found to be inhibited starting from the onset of shoots, and the plants were characterized by late flowering, in the mutant plants. Besides the flowering, At2-*mmp*-1 plants showed fast degradation of chlorophyll in leaves and early senescence. These results demonstrated the involvement of *At2-MMP* in plant growth, morphogenesis, and development with particular relevance for flowering and senescence (Golldack *et al*., 2002). The expression of *MtMMPL1*, which is a variant MMP with an E to Q substitution in the active site of the zinc binding domain (Combier *et al*., 2007), was only detected in young, developing root nodules was localized in inner nodule tissues within developing infection threads, and was triggered at the onset of infection thread formation.

The level of *Pta1-MMP* transcript present in cDNA derived from 100 ng total RNA was detectable only after 30 PCR cycles in fully mature and dry zygotic embryos, or in the same amount of RNA from the corresponding megagametophyte. The relative transcript abundance of *Pta1-MMP* during Loblolly pine embryo development was highest during the early to mid-cotyledonary stage, i.e stage 3 (Fig. 21) of embryo development. At this stage of development, the conifer embryo goes through an intensive phase of development involving cell division, expansion and differentiation and also suspensor degradation (Singh, 1978). The relative

transcript abundance declined during the later stages of embryo maturation and drying (Fig. 21). The lowest level was found in the fully mature and dry embryo.

The role of MMPs in mammalian embryo development, especially in mouse, has been widely studied and the studies imply developmental regulation of MMP expression in the embryos. An earlier study showed that *MMP-2* participates in the early phase of decidualization and neovascularization required for placentation, during the peri-implantation period, in the mouse uterus (Alexander *et al*., 1996). *MMP-9* is expressed in stromal cells on day 5 and in trophoblast giant cells on day 8, and this is coupled with the expression of *TIMP-3* in the stroma surrounding the embryo, so *MMP-9* and *TIMP-3* may act in concert to regulate trophoblast invasion in the mouse uterus (Das *et al*., 1997). Hence the MMP expression is under tight regulation of TIMPs during mammalian embryo development. In a comprehensive study of MMP expression during *Xenopus* embryo development, it was found that expression of *type IV collagenase, stromelysin-1, stromelysin-3, MT-MMP and XMMP*, was developmentally regulated (Yang *et al*., 1997). *XMMP* mRNA in *Xenopus* egg, was undetected in the blastula stage embryo, induced in gastrula embryo, expressed in neurula embryo, and then down-regulated in pretailbud embryo. In addition, other MMPs also showed a developmentally regulated expression. In blastula embryo, the only MMP gene expressed was found to be a type IV collagenase, which was also expressed in the gastrula, neurula, and pretailbud embryos. Expression of *stromelysin-1, stromelysin-3*, and two different membrane type-MMPs was first detected in the neurula and pretailbud embryos (Yang *et al*., 1997). Besides this, a role for *Hg-MMP* from *Heterodera glycine* has been suggested in hatching of the egg, due to increased levels of expression at that time (Kovaleva *et al*., 2004). These results, demonstrating MMP involvement in patterning and development during

non-plant embryogenesis, suggest that a role for plant MMPs in patterning and development during plant embryogenesis is feasible.

The transcript profiles of *Pta1-MMP* in developing female megagametophytes showed a similar pattern to transcript profiles in the associated developing zygotic embryos (Fig. 22). The relative transcript abundance during Loblolly pine megagametophyte development was highest at stage 3; transcript abundance in zygotic embryos was also highest at stage 3 of seed development, at which time the suspensor cells elongate into the megagametophyte, and a corrosion cavity develops inside the megagametophyte (Bewley and Black, 1994). This process is likely to be accompanied by programmed cell death, and hence, a role for *Pta1-MMP* activity may be suggested here. Programmed cell death has been studied in relation to plant embryo development (Bozhkov *et al*., 2005). There are several events in conifer embryo development which can be expected to involve programmed cell death, e.g. elimination of subordinate embryos from polyembryos, suspensor degradation, and corrosion cavity formation, as discussed and reviewed below.

Polyembryony is a unique feature found in conifer embryos, in which more than one embryo is fertilized inside an ovule, although only one reaches maturity (Singh, 1978). The entire period of post-fertilization ovule development in conifers can be divided into three phases in terms of embryo competition. During the first phase all the embryos in an ovule have similar growth rate and thus have equal opportunity for dominance. The onset of the second, longer phase coincides with one embryo gaining dominance over the other subordinates, which gradually stop growing until the dominant embryo has reached the cotyledonary stage. In the third phase, subordinate

embryos are successively eliminated, while the dominant embryo enters dormancy. In Scots pine (*Pinus sylvestris*), programmed cell death was detected in close correlation with the elimination of subordinate embryos, and the formation of the corrosion cavity (Filinova *et al*., 2002). Furthermore, it was demonstrated that during plant embryogenesis, *metacaspase mcII-Pa* translocates from the cytoplasm to nuclei in terminally differentiated cells that are destined for elimination, where it co-localizes with the nuclear pore complex and chromatin, causing nuclear envelope disassembly and DNA fragmentation (Bozhkov *et al*., 2005). The cell-death function of *mcII-Pa* relies on its cysteine-dependent arginine-specific proteolytic activity.

The role of MMPs in programmed cell death has been proposed in cucumber, owing to its expression at the boundary of senescence at the locations where PCD occurs in cotyledon leaf and male flowers (Delorme *et al*., 2001). MMPs are known to be expressed in association with PCD in mammals. In mice for example, the MMP *stromelysin-3* was not expressed in mammary glands during gestation or lactation. After weaning, however, *stromelysin-3* (Lefebvre *et al*., 1992) and *stromelysin-1* (Lund *et al*., 1996) were specifically expressed in fibroblasts adjacent to degenerating mammary ducts, consistent with a role for these ECM peptidases in basement membrane remodeling common to apoptosis. MMPs have also been implicated in eclosion and molting processes in nematodes (Kovaleva *et al*., 2004).

To date, no published studies have characterized MMP expression and activity in depth during plant embryogenesis. Two of the five Arabidopsis MMP genes (*At1-MMP* and *At4-MMP*) are expressed at near-maximal to maximal levels during seed/silique development**,** suggesting potential involvement in embryogenesis (Flinn, 2008). Due to the absence of large numbers of plant MMPs in EST collections, we assume that the expression of most plant MMPs is relatively

low in normal growing plant tissues and only induced when ECM remodeling is required. This is suggested by their expression during processes requiring ECM remodeling, such as development (Golldack *et al*., 2002, Flinn 2008), stress (Liu *et al*., 2001) and as we have found in our study, during seed development and germination.

MMP gene expression is primarily regulated at the transcriptional level, but there is evidence for modulation of mRNA stability of stromelysin, collagenase and gelatinase A in response to growth factors and cytokines (Overall *et al*., 1991). In general, the MMPs can be grouped as either responsive or constitutive, depending on their response to growth factors, hormones and cytokines. The former group includes stromelysin-1, collagenase-1, collagenase-3 and gelatinase B, while the group that is produced constitutively, or expressed at undetectable levels, include stromleysin-2 and gelatinase A.

MMP-like activity during seed development

MMP-like activity was measured by degradation of MBP in gel assays in zygotic embryos and megagametophytes separately, at various stages of embryo development from stage 3 to stage 5 (Fig. 23). Stages 1 and 2 (Fig. 20. 1 and 20.2) were omitted from this study due to lack of material**.** MMP-like activity paralleled *Pta-MMP* transcript abundance and was high in early cotyledonary embryos and declined during the final maturation stages and transition to dry seed (Fig. 21). A similar pattern was observed for MMP-like activity in megagametophytes at the corresponding developmental stages (Fig. 23). The activity in the megagametophyte protein

sample was higher at all stages compared to that in embryo protein samples at corresponding stages.

The metalloprotease activity assays performed in this study were inhibited by GM6001. The observed inhibition of protease activities may also reflect inhibition of other metalloproteases. GM6001 is a potent MMP inhibitor of the hydroxamate family which binds to the critical active-site zinc atom present in all members of this class of proteinases (Galardy 1993). It is believed that the isobutyl group and tryptophan side chain bind to the sub-sites on the target enzymes which normally bind the extracellular matrix protein substrates (Galardy, 1993). In addition to its inhibition of MMPs, GM6001 inhibits bacterial metalloproteinases, such as thermolysin and *Pseudomonas aeruginosa* elastase (Grobelny *et al*., 1992), as well as the neprilysin, aminopeptidase and dipeptidylpeptidase clans of metalloproteases (Saghatelian *et al*., 2004). Taken together, these results indicate that metalloproteases can display overlapping inhibitor sensitivities, and that GM6001 is not completely specific for matrix metalloproteinases. Hence, while our assays show good correlation with *Pta1-MMP* transcript levels, we can't rule out additional metalloprotease activity. However, the activity and corresponding inhibition of r*Pta-MMP* activity has already been demonstrated in this project. Thus, it is suggested that the activity measurements and inhibition of activity at various stages is at least partly reflect *Pta-MMP* activity.

In this study, we have confirmed *Pta1-MMP* gene expression, as well as MMP-like activity during Loblolly pine seed development. The protein extracts used for MMP-like activity measurements mainly consist of soluble proteins, as the buffer used for extraction does not

contain any strong detergent (Maidment *et al*., 1999)**.** There is a possibility of some membrane-associated proteins being present in the extracts owing to the grinding of tissues in liquid nitrogen, but it is hard to estimate the exact amount of *Pta1-MMP* activity in these assays. Furthermore, in addition to our characterized MMP, the total activity detected in the extracts assayed during this study may well reflect the activity of more than one *Pta-MMP* gene.

Relevance of MMPs during embryo development

It can be expected that modifications to the extracellular matrix will be required as cell division and cell expansion during embryo patterning and growth, and changes in the appearance of the ECM have been observed microscopically during embryo development (Briggs, 1996; Konieczny *et al*., 2005). Extracellular plant proteases, such as the cysteine protease *DEK1* (Lid *et al*. 2002), in aleurone cell development, and the substilisin-like serine protease *ALE1* (Tanaka *et al*., 2001), in the formation of cuticle on embryos and juvenile plants are important for normal embryo and seed development. In addition to these, several other extracellular serine proteases are required for plant development, and are involved in microsporogenesis and pollen development (*LIM9;* Taylor *et al*., 1997), cell wall metabolism or precursor protein processing (*ARA12*; Hamilton *et al*., 2003), in stomatal distribution and density (*SDD1*; Berger and Altmann, 2000), and in proteolytic processing of a rate-limiting protein in brassinosteroid signaling (*BRS1*; Li *et al*., 2001). Furthermore, the extracellular cysteine protease *RCR3* plays a role in disease resistance (Rooney *et al*., 2005), and the extracellular aspartic protease *CDR1* in disease resistance (Xia *et al*., 2004). *Cardosin B*, also an aspartic protease, functions as a proteolytic aid in pistil development, in pollen-pistil interactions, and/or in pathogen protection

(Vieira *et al*., 2001). As these other extracellular proteases are important in various aspects of plant growth and development, our results showing *Pta1-MMP* gene expression and MMP-like activity suggest that additional proteolytic modification of the ECM through MMP activity is involved in various aspects of seed morphogenesis and growth.

Transcript profiling and MMP-like activity during seed germination

In addition to the previously described *Pta1-MMP* transcript abundance and protese activity during seed development, MMP-like activity was also observed during Loblolly pine seed germination. In mature seeds, *Pta1-MMP* transcript levels initially decreased (Fig. 28) as the seeds were imbibed in water/hormone solution, probably owing to degradation of extant mRNA (Bewley *et al.,* 2000). However, the transcript levels and MMP-like activity increased towards completion of germination in embryos of water- and GA_3-imbibed seeds, associated with increased germination rate for both treatments (Fig. 30). GA_3-imbibed seed embryos displayed an increase in *Pta1-MMP* transcript abundance earlier, which correlates with earlier germination than in the water-imbibed seed embryos (Fig. 27). The increase in transcript abundance at the completion of germination is well reflected in the MMP-like activity shown by the protein extracts at these stages of germination in GA_3- treated and water-imbibed samples. Interestingly, ABA-imbibed seed embryos, which were inhibited in germination, also showed an increase in *Pta1-MMP* transcript level during imbibition, but not the corresponding increase in MMP-like activity. These results suggest that MMP-like activity may be under translational or post-translational regulation in the ABA-imbibed seed embryos, inhibiting the synthesis of *Pta1-MMP* transcripts into resulting protein, or blocking the processing/conversion of *Pta1-MMP* protein into functional protease activity. In support of this view, it has been shown that responses

to ABA can be mediated by translational or post-translational protein modifications (Morcillo *et al*., 2001; Chibani *et al*., 2006; Miura *et al*., 2007). The regulation of MMPs in mammalian systems by growth factors such as *TGF-β1, Hepatocyte Growth Factor* (HGF) and inflammatory cytokines is known (Clark *et al*., 2008; Wang and Keiser, 2000). However, based on the current results, we cannot determine if the effects of ABA reflect inhibition of *Pta1-MMP* mRNA translation into protein, inhibition of MMP activation, inhibition by some other mechanism, or a combination of these. It is also possible that another metalloproteinase is responsible for the activity shown by these samples.

At the corresponding stages in the megagametophyte cDNA samples and protein extracts, there were however no detectable changes in the *Pta1-MMP* transcript levels (Fig. 29) and MMP-like activity (Fig. 31). While a number of cell wall proteins and hydrolases have been identified that are associated with weakening of the endosperm at the time of germination; these are likely to have a mechanistic role in endosperm weakening rather than proteolytic modulation.

The endosperm is considered to be equivalent to the gymnosperm female megagametophyte, despite the developmental differences as described in the introduction section of this thesis. Most research on germination has targeted the angiosperm endosperm tissue rather than the gymnosperm female megagametophyte, and these studies have indicated possible roles for cell wall proteins and hydrolases on the endosperm during germination. The endosperm undergoes dramatic changes during germination *sensu stricto.* At the completion of germination, the storage reserves in the micropylar cells are degraded and the cells become highly vacuolated prior to radicle emergence, while these changes do not occur in adjacent cells of the lateral endosperm until after radicle emergence. These cellular changes are associated with physical weakening of

the endosperm cap tissue. Endosperm weakening is a prerequisite for radicle protrusion in gymnosperm seeds. Indeed, the inner surface of the female megagametophyte cell walls is initially smooth and becomes increasingly degraded following imbibition in seeds of *Picea* (Downie *et al*., 1997). Several cell wall proteins have been implicated in the weakening process. These comprise but are not limited to the expansins, xyloglucans, endotransglycosylase/hydrolases, endo β-mannanases, α-galctosidases and β-mannosidase and cellulases. Hypotheses concerning the functions of the individual cell wall hydrolases in endosperm weakening are mainly based on the association between enzyme activity and gene expression, and endosperm weakening and seed germination. Expansin genes are highly conserved in higher plants and are often present as gene families with multiple members (Cosgrove *et al*., 2002). At least three expansin genes are present in tomato seeds during germination. One of them, *LeEXPA4* (Kende *et al*., 2004), was specifically expressed in the micropylar endosperm cap region within 12 h of imbibition, a time when endosperm weakening has just begun (Chen and Bradford, 2000). Xyloglucan endotransglycosylase/hydrolases (*XTH*) modify xyloglucans, which are major components of primary cell wall in dicots (Carpita and Gibaut, 1993). An XTH gene, *LeXET4* was isolated from germinating tomato seeds (Chen *et al*., 2002). During seed germination, *LeXET4* mRNA was localized exclusively to the endosperm cap region and detectable 12 h after imbibition, with maximal expression at 24 h, (Nonogaki, 2000). Endo-β-mamnnases randomly hydrolyze the internal β-1, 4-D-mannopyranosyl linkage in the backbone of mannan polymers. In the seeds of many plant species, endo-β-mannase activity shows a dramatic increase during germination. Two endo-β-mannases genes, *LeMAN1* (Bewley, 1997) *and LeMAN2* (Nonogaki *et al*., 2000), have been isolated from germinating tomato seeds. While *LeMAN2* mRNA was detectable only in the the endosperm cap 12 to 18h after imbibition,

LeMAN1 was localized to the lateral endosperm and not expressed until germination was completed about 48 h after imbibition. α-Galactosidases remove the galactoside-chains attached to the mannan backbone of gactomannans. α-Galactosidases activity has been detected in the micropylar and lateral endosperm and in the embryo of germinating tomato seeds (Feurtado *et al*., 2001). Transcripts for a gene encoding an α-Galactosidase, *LeGAL*, were present in both the micropylar and the lateral endosperm of tomato, and also in the embryo to the lesser extent, suggesting that *LeGAL* is responsible for the α-Galactosidase activities identified in various tissues of germinating tomato seeds (Feurtado *et al*., 2001). β-Mannosidases hydrolyse the terminal, non-reducing β-D-mannose residues in the oligo-mannans released by the action of endo-β-mannanases and α-galactosidases. β-Mannosidase activity has been detected in the micropylar endosperm of a number of seeds including tomato (Mo and Bewley, 2002), *Datura* (De Miguel *et al*., 2000), and coffee (Da Silva *et al*., 2005). The key enzyme for degrading cellulose is endo-β-1, 4-glucanase, or cellulase. Cellulase activity has been detected in germinating seeds of a number of plant species. In tomato, cellulase activity was detected in germinating seeds, but did not correlate with to the timing of germination (Leviatov *et al*., 1995).

While there is correlative and circumstantial evidence for a role of these cell wall proteins in endosperm weakening, to date β-Glucanase is the only cell wall enzyme for which there is genetic evidence supporting a specific biological function during germination (Nonogaki *et al*., 2007). In tobacco, β-Glucanase expression promotes both testa and endosperm rupture, with latter being sensitive to ABA (Leubner-Metzger, 2002)

Apart from the enzyme activities described above, no MMPs have been reported to be involved in endosperm weakening at the time of germination. Our results also suggest little role for MMP

activity modulation of the megagametophyte during pine seed germination. It is possible that the role of MMP is not part of the program for mechanistic weakening of the megagametophyte and hence, there were no detectable change in MMP transcripts nor metalloproteinase activity at the developmental stages studied in this project.

Additional evidence for MMP involvement in Loblolly pine seed germination was provided by the use of the general MMP inhibitor GM6001 on mature seeds of Loblolly pine. GM6001 blocks MMP activity by binding to the hydroxamate group and thereby preventing the activation of proteinase activity that occurs through binding of hydroxamate to the active-site zinc**.** The rational design of potent inhibitors of MMPs has resulted in the production of numerous agents which inhibit the activity of secreted MMPs. Initially drugs were targeted to the chemical functional group that chelates the active site zinc (II) ion, which is a ubiquitous feature of MMPs. Biochemical studies using collagen-like substrates have shown that six amino acids are primarily responsible for the proteolytic activity of MMPs. These six sub- sites within the enzyme catalytic domain span the locations designated S3-S3'. Peptide and peptide like compounds have been designed that combine backbone features (P1, P1', P2', P3' regions) that would favorably interact with the enzyme sub- sites (S1, S1', S2', S3' pockets) and with functional capabilities of binding zinc in the catalytic site (Borkakoti, 1998). Most drugs designed as MMP inhibitors essentially mimic the collagen substrate of MMPs and thereby work as highly competitive but reversible inhibitors of enzyme activity (Zucker *et al*., 2003). The molecular structure and related details of MMP inhibitors have already been discussed in the introduction section.

In our experiments, we observed a significant decrease in germination efficiency of seeds treated with GM6001 at all different concentrations tested (Figs. 33 and 34). Interestingly, there was

also a reduction in the seedling lengths of the GM6001-treated seeds (Fig. 36). Other hydroxamate inhibitors have been used in several plant MMP protein characterization studies (Maidment *et al*., 1999, Delorme *et al*., 2000), and 5 nM recombinant *At1-MMP* was found to be inhibited by ~90% with 30 nM of BB-94 (Batimastat) (Maidment *et al*., 1999) and *Cs1-MMP* was found to be completely inhibited by 3 μM of BB-94 (Delorme *et al*., 2000). The present study is the first report that we are aware of that has used GM6001 as an MMP inhibitor in plant bioassays.

It is necessary to dissolve GM6001 in DMSO and hence DMSO controls were included in this assay. DMSO has been widely used as a solvent (or carrier) for various molecules, as it acts as a synergist at lower concentrations (less than 0.1%) and has an adverse effect on germination at higher concentration (Erdman and Hsieh, 1969). It is also used as an antioxidant and cryopreservant (Hatton and Wilson, 2007). In the present study, inhibition of germination was observed with 20% DMSO alone (Figs. 33 and 34). Thus, the inhibition of germination could be partially attributed to DMSO. However, the decrease in germination rate in GM6001-treated seeds was much more significant with the addition of GM6001, and dose-dependent, suggesting growth inhibition due to the inhibition of *Pta-MMP* activity by GM6001 *in planta*.

Extracellular proteins implicated in seed germination

Seed germination commences with the uptake of water by imbibition of the dry seed, followed by embryo expansion, culminating in the rupture of the covering layers and emergence of the radicle, which is generally considered as the completion of the germination process (Bewley, 1997). Radicle protrusion at the completion of seed germination depends on embryo growth driven by water uptake. Various extracellular proteins have been shown to be involved in ECM modification and cell wall disassembly during germination, including expansins (Chen and Bradford, 2000), polygalacturonases (Sitrit *et al*., 1999), rhamnogalacturonases (Veronesi *et al*., 2007) and xyloglucan endotransglycosylases (Chen *et al*., 2002). In addition, rapid extracellular proteolytic events can be expected to take place leading up to germination, contributing to cell wall loosening and remodeling, relieving the tissue constraints and allowing radicle protrusion. The Cardosin extracellular aspartic proteases have already been implicated in such a process (Pereira *et al*., 2008), and this current study also implicates MMP involvement in the ECM modification required for germination.

Concluding remarks on MMPs in seed development and seed germination

The purpose of this study was to assess the role of MMPs in embryo development and germination, as these are two important developmental processes in the plant life cycle. Additionally, an in-depth understanding of these processes may lead to development of improved somatic embryogenesis protocols as discussed below. The importance of MMPs in mammalian biological processes, including embryogenesis, tissue remodeling, wound healing, angiogenesis and a variety of diseases (Sternlicht and Werb, 2001; Visse and Nagase, 2003) is

well known. However, little is known about MMPs role in plant development in general, and plant embryo development in particular. The results of this study indicate a role for MMPs during the morphogenesis and expansion phases of seed development, and the expansion phase of germination and early seedling growth.

Possible plant MMP substrates

Proteolytic events are parts of many developmental processes, and require substrates for the proteolytic activity. In absence of knowledge regarding plant MMP substrates, it is difficult to pin-point the function of *Pta1-MMP*, and Pta-MMP activity in embryo development. Several non-plant matrix metalloproteinases have the ability to degrade the plant ECM. A secreted metalloprotease from the plant pathogen *Xanthomonas campestris* degraded the turnip proline-rich glycoprotein gp120 and the hydroxyproline-rich protein gpS-3, as well as extensin from potato and tomato (Dow *et al*., 1998). Furthermore, mammalian collagenase has been shown to degrade the glycine-rich cell wall protein, GRP1.8 in *Phaseolus* (Ringli *et al*., 2001). In addition, plants have several types of proteins that are similar to vertebrate cell surface proteins that are known to be MMP substrates (Cauwe *et al*., 2007). These include plant proteins similar to vitronectin (Sanders *et al*., 1991), fibronectin (Pellenc *et al*., 2004) and integrin (Katembe *et al*., 1997). Interestingly, RGDS tetra peptides, which are known to bind to integrins, can modify somatic embryo development when applied to carrot cultures (Blackman *et al*., 2001), implicating integrin- like molecules in the regulation of embryo development. Whether the above types of proteins represent plant MMP substrates remains to be addressed. It is tempting to hypothesize that *Pta1-MMP* and other Loblolly pine MMPs could be involved in the generation of certain signaling molecules by the cleavage of cell surface peptides, or could be

responsible for the cleavage of ECM proteins required for cell wall stabilization, facilitating cell expansion and growth, or diffusion of extra cellular molecules.

Practical implications: Possible role of MMPs in somatic embryo development and potential for improving SE protocols

Somatic embryogenesis is the only potentially viable technique for mass propagation of gymnosperm trees. Somatic embryogenesis also provides an efficient model system for understanding the underlying events of embryo development. The stages of development of zygotic embryos are similar to those found in somatic embryo. Research conducted utilizing somatic embryogenesis as a model system has in many cases sought to identify genes and proteins expressed during embryo development, with the goal to discover mechanisms regulating embryo development. Of particular interest have been proteins associated with the ECM, as these molecules have direct contact with the outer environment of the cells and thus potentially could be involved in signal transduction mechanisms; extracellular molecules with a regulatory function could be considered for addition to culture medium to affect the growth and development of somatic embryos. Thus, our studies have a potential practical application as they reveal that MMPs are expressed and active during seed development and that active ECM modification and perhaps molecule shedding is taking place. Several such molecules have been identified from conditioned medium from embryogenic cultures and found to have the ability to promote embryogenesis. This implies that indeed, secreted soluble signal molecules play an important role in stimulation of somatic embryogenesis. In Norway spruce, extracellular chitinases (Egertsdotter *et al*., 1993; Dyachok *et al*., 2002) arabinogalactan proteins (AGPs) (Egertsdotter and von Arnold, 1995) and lipo-chitooligosaccharides (LCOs) (Dyachok *et al*.,

2002); phytosulfokines in *Asparagus* (Matsubabyashi and Sakagami, 1996), all have been found to have affected somatic embryogenesis. In carrot, the extracellular chitinase 3 (EP3), has been shown to be capable of rescuing somatic embryos in the mutant carrot cell lines ts11 at the non permissive temperature (De Jong *et al*., 1992). Several extracellular proteases, including serine proteases, cysteine proteases and aspartic proteases have an impact on normal development of embryos as discussed earlier**.** However, MMPs have not previously been studied in relation to somatic embryo development.

Future Directions

- The involvement of MMPs in seed development and germination make them candidates as enhancers of somatic embryogenesis (SE). These could be tested in the SE systems for their effect on somatic embryo development and can also be developed as potential spray molecules for better seed germination.
- A major goal for plant MMP research is to identify MMP substrates in plants. A more systematic research directed towards identification of plant MMP substrates would help elaborate the exact function of these proteases in plants. Some of the ways to identify the substrates include, peptide phage library screening, yeast two hybridization systems, and biochemical strategies such as co-immuno precipitation could also be helpful.
- No TIMP like molecules have so far been identified in plants. Hence it is also important to design studies targeted towards the inhibitors of plant MMPs. Plants contain a lot of flavonoids and several flavonoids have been found to inhibit human MMPs. Plants contain several other molecules which have been found to be inhibitors of mammalian MMPs and are thus used in therapeutic practices. These molecules could potentially be natural inhibitors of plant MMPs; however this needs to be tested experimentally.

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