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Developmental Regulation of Arginase in Loblolly Pine (*Pinus taeda* L.) Seeds During Germination and Early Seedling Growth

by

Christopher Douglas Todd

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Plant Biology

Department of Biological Sciences

Edmonton, Alberta

Spring, 2002

University of Alberta

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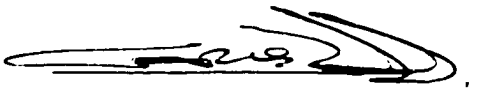

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To My Family

Abstract

A 1366 base-pair arginase cDNA was isolated from a loblolly pine (*Pinus taeda* L.) cDNA expression library constructed from whole seedling tissue collected 9 to 10 days after imbibition at 30°C (DAI_{30}). The cDNA contained an open reading frame predicting a 341 amino acid peptide with a molecular mass of 37.2 kilodaltons (kDa). When expressed in *Escherichia coli*, the loblolly pine arginase protein hydrolyzed arginine to ornithine and urea. The protein produced in *E. coli* was purified and the resultant peptide was used to generate anti-arginase antibodies. The arginase holoprotein was partially purified from 9-12 DAI_{30} shoot poles (cotyledons plus shoot apices) and has a molecular mass of 36.8 kDa. Based on a holoprotein size of 140 kDa, arginase is predicted to form a homotetramer.

Arginase protein and mRNA were found predominantly in the shoot poles (cotyledons plus shoot apices) of the seedling but was also detected in the root pole (hypocotyl and radicle) and the megagametophyte. Following germination, arginase protein and mRNA accumulated in the shoot pole. Arginase appeared to be regulated primarily at the RNA level; however, evidence of post-translational regulation was also observed.

An *in vitro* culture system was utilized to grow loblolly pine zygotic embryos with and without their associated megagametophytes. Removal of the megagametophyte caused a decrease in seedling growth and shoot pole protein accumulation, and caused a decrease in arginase enzyme activity, protein levels and mRNA abundance over the ten day culture period. The seedling was able to initiate arginase gene expression in the absence of the megagametophyte tissue, but the presence of the megagametophyte

maintained and increased arginase transcript abundance during the latter stages of early seedling growth. Removal of the megagametophyte from seedlings grown in its presence caused a rapid decrease in arginase transcript abundance This decrease could be minimized by the replacement of the megagametophyte with exogenous arginine. Application of arginine to seedlings grown in the absence of the megagametophyte was shown to stimulate arginase enzyme activity and induce arginase gene expression.

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BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	bovine serum albumin
CTAB	hexadecyltrimethylammonium bromide
DAI_{30}	days after imbibition at 30°C
DIC_{30}	days in culture at 30°C
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
g	gravity
h	hour
ICL	isocitrate lyase
IMAC	immobilized metal affinity chelation
IPTG	iso-propyl-β-thiogalactopyranoside
kDa	kiloDalton
+M	in the presence of the megagametophyte
-M	in the absence of the megagametophyte
min	minute
MWCO	molecular weight cut off
NBT	nitroblue tetrazolium chloride
nkat	nanokatals
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

pI	isoelectric point
PPD	phenylphosphorodiamidate
PVPP	polyvinylpolypyrrolidone
RACE	rapid amplification of cDNA ends
rpm	revolutions per minute
RT-PCR	reverse transcription – polymerase chain reaction
RuBisCO	Ribulose 1,5-bisphosphate Carboxylase-Oxygenase
S	Svedberg
SDS	sodium dodecyl sulfate
SE	standard error
TAG	triacylglycerol
TBq	TerraBecquerels
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethyldiamine
vol	volume

1. Introduction[1]

1.1 Loblolly Pine

Loblolly pine (*Pinus taeda* L.)[2] is a tree species native to the southeastern United States of America. Its natural range extends from Maryland and Delaware in the north, to northern Florida in the south, from Texas in the west, to the Atlantic coast in the east (Figure 1; Fowells, 1965). Loblolly pine is important to the forest industry, being a major source of fiber for pulp and paper production. It is the principal commercial species in the southeastern United States, comprising 15.7% of the forest land base of the region, with over 9.3 million hectares of managed pine plantation (Carey, 1992). In 1980, nearly one billion loblolly pine seedlings were produced for reforestation, representing 75% of the seedlings produced in the Southeast (Boyer and South, 1984). These numbers are likely to be higher today due to increased usage of loblolly pine as a preferred forestry species in the United States (Boyer and South, 1984). The rapid growth rate of this species combined with the manageability of loblolly pine as a plantation species has resulted in loblolly pine becoming a commercially important plantation species in China (UNDP Report, 1997), South Africa (Hagedorn, 1994) and South America (Becwar and Pullman, 1995).

In the southeastern United States, major forest companies maintain extensive breeding and seed orchards to provide high quality loblolly pine seed for continuation of forestry practices in this region. Clonal propagation of the breeding stock allows for

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[1] A version of this chapter has been published.. Todd C.D., Cooke J.E.K., Mullen R.T., Gifford D.J. (2001a) Plant Mol Biol 45: 555-565; Todd C.D., Cooke J.E.K., Gifford D.J. (2001b) Plant Physiol and Biochem 39: 1037-1045; Todd, C.D. Gifford, D.J. (2002) Planta *In press.*

[2] At the first mention of a species in this thesis both the common name and scientific name will be given. The common name, where feasible, will then be used in subsequent references to the same species.

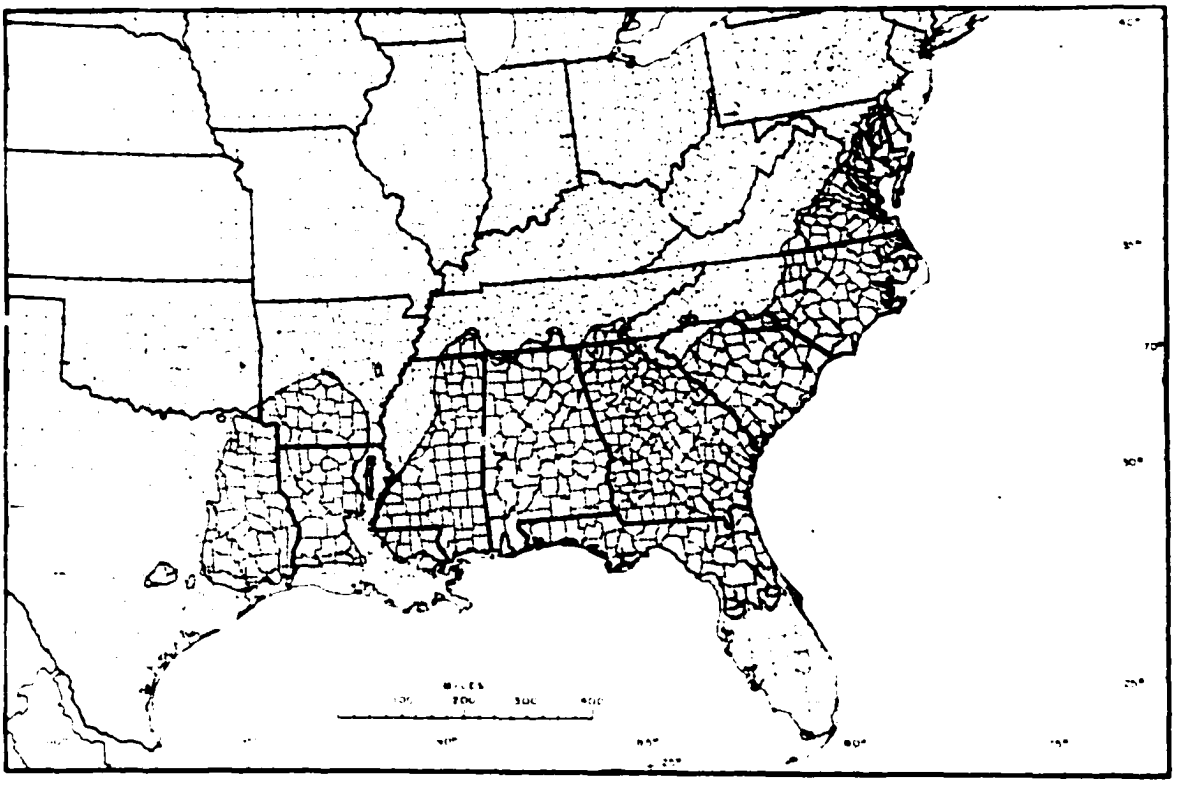


Figure 1: Map of loblolly pine (*Pinus taeda* L.) natural distribution in the southeastern United States of America. Shaded areas indicate loblolly pine's native range. Figure taken from Fowells (1965).

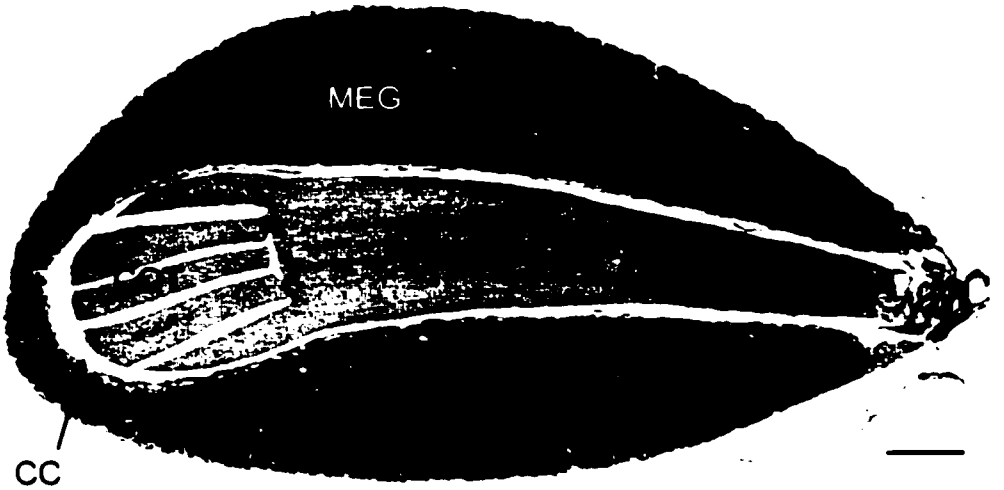
generation of high quality, relatively homogenous, seed stocks for reforestation. These traits also make this seed ideal for understanding physiological, metabolic, biochemical and molecular changes that occur during germination and early seedling growth. Loblolly pine seed forms the basis of the present study.

1.2 Description of the mature seed

The loblolly pine seed is composed of a diploid embryo surrounded by a haploid, maternally derived megagametophyte (Figure 2; Stone and Gifford, 1997). The embryo lies within the corrosion cavity of the megagametophyte, which is formed during seed development. Upon seed imbibition, the embryo and megagametophyte swell and are in direct contact with each other; however, there is no direct anatomical connection between the two; these tissues are not symplastically connected. The micropylar end of the megagametophyte is covered with a thin, tannin-rich, cap-like structure believed to be the remnant of the nucellus and termed the nucellar cap (Singh and Johri, 1972; Tilman-Sutella and Kauppi. 1995a, 1995b). The seed is contained within a hard seed coat, or testa, comprised of the outer two integument layers, and a thin papery layer covering the megagametophyte and nucellar cap believed to be derived from the third, innermost integument layer (Singh and Johri, 1972; Owens and Molder, 1977; Owens et al., 1982).

The embryo itself is composed of the embryonic axis and six to eight cotyledons. The embryonic axis includes the shoot apical meristem, hypocotyl and radicle. The radicle contains the sub-apical root meristem covered by an extensive root cap (Stone, 1999). The root cap itself is believed to be the remnants of the embryonic suspensor (Chamberlain, 1957).

Figure 2: Longitudinal section through a loblolly pine seed after removal of integument layers. Megagametophyte (MEG), cotyledons (COT), embryonic axis (AX) and corrosion cavity (CC) indicated. Scale bar = 400 μm. Figure taken from Stone and Gifford (1997).



The main storage reserves of the conifer seed are triacylglycerols (TAGs) and proteins (Misra, 1994; Stone and Gifford, 1997, 1999) which are partitioned between the embryo and megagametophyte. The majority of the reserves are housed within the megagametophyte; however, the embryo does contain its own storage proteins and TAGs (Simola, 1974; Krasowski and Owens, 1993; Owens et al., 1993; Stone and Gifford, 1997, 1999).

1.3 Seed storage reserves

1.3.1 Triacylglycerol lipid reserves

The major carbon reserves in conifer seeds are lipids, primarily TAGs (Ching, 1966; Kovac and Vardjan, 1981; Hammer and Murphy, 1994, Stone and Gifford, 1999). TAGs are composed of a three-carbon glycerol backbone; each carbon shares an ester bond with a fatty acid. Conifer seed fatty acids are largely unsaturated, the majority being composed of 18 to 20 carbon atoms (Janick et al., 1991, Wolff et al. 1997c). All conifer seeds also contain fatty acids with a Δ5-ethylene bond, which are rare in other plants (Atree et al., 1992; Wolff, 1997). Six different Δ5-olefinic fatty acids exist in conifers (Wolff *et al.*, 1997a), most of which occupy the sn-2 position on the glycerol backbone (Wolff *et al.*, 1997b). In pine seeds, the most abundant fatty acids are linolenic (18:2) acid, oleic acid (18:1) and the Δ5-olefinic fatty acid, pinolenic acid (18:3 Δ5, 9 12) (Janick *et al.*, 1991; Imbs and Pham, 1996; Wolff et al., 1997a).

In loblolly pine, 58% of the storage reserves are TAGs (Stone and Gifford, 1999). The megagametophyte houses 80% of these, constituting 27% of the dry weight of this tissue. However, the 20% of TAGs housed in the embryo make up 40% of the embryo's

dry weight (Stone and Gifford, 1999) and are a very important storage component of the seed. The storage TAGs are located in lipid bodies located in the megagametophyte, embryo hypocotyl and cotyledons (Stone and Gifford, 1999).

1.3.2 Storage protein reserves

Seed storage proteins are classified as such if they are synthesized during seed development, account for a minimum of 5% of the total seed protein and are broken down during germination and early seedling growth (Derbyshire *et al.*, 1976). They provide the nitrogen reserves for the seed. Storage protein classification is based on solubility (Osborne, 1918). Albumins are soluble in aqueous solution. Globulins do not solubilize in water alone, but are soluble in salt solution. Prolamins are soluble in aqueous alcohol solutions. Proteins insoluble in the three previous solutions are classified as glutelins. Glutelins can be dissolved by dilute alkali or acid solutions, or more recently, by chaotropic agents such as sodium dodecyl sulfide or urea (Koie and Nielsen, 1977). Beyond solubility, storage proteins are classified by the ultracentrifugation sedimentation coefficient (S) of their holoprotein and this is incorporated into their nomenclature, such as a 2S albumin (Derbyshire et al., 1976; Shewry et al. 1995).

Storage proteins are also classified or grouped based on their holoprotein structure and most recently by the relatedness of their sequences at the DNA or deduced amino acid level. This does not always agree with classical solubility classification. For example, 11S legumins, a family of storage proteins identified in the Leguminosae (Derbyshire et al., 1976), are salt-soluble (and hence globulins based on Osborne's

solubility characteristics) holoproteins comprised of acidic and basic subunits. Douglas-fir (*Pseudotsuga menziesii*) storage protein cDNAs show between 29 and 39% amino acid identity with legumin cDNAs (Leal and Misra, 1993) and were described as legumin-like by these authors. However, the proteins themselves are not salt-soluble globulins, but require chaotropic agents to solubilize them and are thus glutelins; labeling them as legumin-like storage proteins infers a solubility characteristic, which is not accurate. Furthermore, a legumin is not merely a globulin isolated from a legume, but is so named because of its characteristic holoprotein structure, incorporating disulphide bridged acidic and basic polypeptides arising from a single pre-cursor peptide (Shewry *et al.*, 1995). Strictly speaking, some of this information can be inferred from DNA or amino acid sequences, but the overall holoprotein assembly cannot. Clearly, relatedness at the amino acid level is not sufficient to correctly classify a protein based on the classical definitions.

Mature loblolly pine seeds contain albumins, globulins and glutelins, but not prolamins (Gifford, unpublished). There is an overlap in solubility between the globulin and glutellin fractions, in that the globulins cannot be fully extracted in low salt solutions, but require higher salt concentrations and heat to solubilize them completely. However, high salt solution and heat partially extract the glutelins, which require chaotropic agents to solubilize them completely. This overlap means that a single extraction cannot separate these two fractions and therefore in many studies, including this one, these proteins are extracted and classified based on their solubility in aqueous buffered solutions. Those soluble in buffer are albumins, and those insoluble in buffer are

globulin and glutelins and are extracted with buffers containing chaotropic agents such as SDS.

The majority of the storage proteins in loblolly pine seed are insoluble in phosphate buffer (Groome et al., 1991, Stone and Gifford, 1997), similar to what has been found in most pine species, with the exception of whitebark pine (*Pinus albicaulis*) (Gifford, 1988). Although there are some globulins present in the buffer-insoluble fraction, the main storage proteins found in pines are glutelin-like in their solubility (Gifford, 1988, Groome et al, 1991; Fashu-Kanu, 2001). These proteins are hexameric, comprising subunits that are 51-55 kDa in size, composed of two smaller peptides joined by a disulphide bridge, which can be broken by treatment with a strong reducing agent such as β-mercaptoethanol, resulting in 31-35 kDa and 21-23 kDa subunit peptides (Gifford, 1988; Lammer and Gifford, 1989; Jensen and Lixue, 1991; Allona *et al.*, 1992, Fashu-Kanu, 2001). Other conifers are similar in this regard including several species of *Picea* (Gifford and Tolley, 1989; Misra and Green, 1990; Jensen and Lixue, 1991; Allona *et al.*, 1994c), *Larix* (Jensen and Lixue, 1991), Douglas-fir (Green *et al.*, 1991; Jensen and Lixue, 1991), Himalaya cedar (*Cedrus deodora*) (Jensen and Lixue, 1991) and members of the Cupressaceae (Allona *et al.*, 1994c; Hager and Dank, 1996), but not in any of species of *Abies* (Jensen and Lixue, 1991; Allona *et al.*, 1994c) or in Atlas cedar (*Cedrus atlantica*), though a salt-extractable 55 kDa disulphide-bridged storage protein is present in this latter species (Allona *et al.*, 1994c).

Because of the overlapping solubility characteristics of the insoluble proteins a consensus on nomenclature has not been reached. They have been described as crystalloid proteins based on their similarity of solubility and structural characteristics of

the 11S crystalloid proteins of castor bean (Gifford *et al.*, 1982; Gifford, 1988). The castor bean crystalloid is a 300 kDa 12 S holoprotein made up of 6 identical 50 kDa subunits. Each subunit is composed of a 30 kDa acidic peptide and a 20 kDa basic peptide joined by a disulphide bridge and is so-named for the proteinaceous crystalloid found in protein vacuoles of these seeds (Gifford *et al.*, 1982; Gifford and Bewley, 1983). Under non-reducing conditions, these proteins can also form complexes migrating at approximately 100 kDa, representing a complex of two proteins. Multi-subunit complexes have also been observed in several pines (Gifford, 1988, Fashu-Kanu, 2001); however, in conifers, both subunits are basic when their isoelectric points are determined (Hakman, *et al.*, 1990; Allona et al., 1992). Leal and Misra (1993) suggest that a Douglas-fir large subunit is acidic based on deduced amino acid sequence, but this has not been demonstrated by isoelectric focusing. A further dissimilarity between the crystalloid protein of castor bean and conifer glutelin-like storage protein is the lack of cross-reactivity of the *Pinus* protein with any of three independent sets of antibodies raised to the castor bean crystalloid (Gifford, 1988; Migabo, 1995). Some cross-reactivity was shown between castor bean crystalloid protein and antibodies raised against white spruce conifer storage proteins (Misra and Green, 1994); however, there is a question of non-specificity of these antibodies as outlined by Stone (1999) and it is difficult to classify the spruce proteins as true crystalloids based on these data.

The conifer proteins have also been called legumin-like based on the similarity in DNA sequence and subunit structure described previously. Legumins also contain a large and small subunit joined by a disulphide bridge. Like the crystalloids, the large subunit is acidic and the small subunit basic; however, the legumins do not form

crystalloid deposits (Lott, 1980), are soluble in salt solutions and, therefore, are globulins (Shewry *et al.*, 1995). As discussed above some conifer proteins share amino acid sequence similarity with legumins (Leal and Misra, 1993); however, as also indicated previously, calling them legumin-like may lead to a misinterpretation of their solubility characteristics; describing these proteins as legumin-like-glutelin-like is confusing at best.

Based solely on solubility the major conifer storage proteins are most like glutelins; however, the Douglas-fir cDNA discussed above (Leal and Misra, 1993) shows limited sequence similarity with a family of well characterized cereal glutelin cDNAs (Baker *et al.*, 1996). Rice glutelins are similar to the legumin proteins in structure and sequence, but not solubility (Robert *et al.*, 1985; Takaiwa *et al.*, 1991). It has been suggested that the legumins, the rice glutelins and the conifer storage proteins may have shared a common ancestor protein (Hager *et al.*, 1995). It is clear that the conifer proteins discussed are distinct from these two groups. Stone (1999) suggested that these proteins be given their own classification, coniferalins, to recognize this distinctness.

In loblolly pine seeds, most of the protein is found in the megagametophyte tissue, accounting for approximately 1.9 mg out of an average 2.1 mg total protein per seed (Stone and Gifford, 1997). These proteins are found in protein vacuoles and form crystalloid inclusions in a proteinaceous matrix (Stone and Gifford, 1997). This is similar to many other conifers including jack pine (*Pinus banksiana*) (Durzan *et al.*, 1971), stone pine (*Pinus pinea*) (Gori, 1979), Scots pine (*Pinus sylvestris*) (Simola, 1974), several spruce species (Simola, 1976; De Carli *et al.*, 1987; Misra and Green, 1990; Krakowski and Owens, 1993) and Douglas-fir (Owens *et al.*, 1993).

Approximately 80% of the loblolly pine megagametophyte protein is insoluble in phosphate buffer. In the embryo, only 27% of the protein is phosphate buffer-insoluble and is equally distributed between the shoot pole and the root pole (Stone and Gifford, 1997). The phosphate buffer-insoluble megagametophyte proteins account for the majority of the nitrogen stored within the seed. In addition to the >300 kDa holoprotein which makes up the majority of the protein stores, there are also a 47 kDa storage protein and a 13 to 14 kDa 2S storage protein, both of which are phosphate buffer-insoluble. Together, these four proteins account for 70 to 80% of the total protein in the mature seed (Groome et al., 1991; Stone, 1997) and are the primary source of nitrogen for the developing seedling.

1.3.3 Amino acid composition of conifer storage proteins

Seed storage proteins are typically rich in the amide amino acids glutamine and asparagine (Van Etten *et al.*, 1963) but may also contain high amounts of arginine (Van Etten *et al.*, 1963; Derbyshire *et al.*, 1976; Higgins, 1984). Glutamine and asparagine each contain two nitrogen atoms and arginine, four. These three amino acids all have relatively low carbon-to-nitrogen ratios, making them efficient nitrogen storage compounds (Bray, 1983; Durzan and Steward, 1983). Arginine is particularly abundant in conifer species (Feirer, 1995). Amino acid analysis of storage proteins of maritime pine (*Pinus pinaster*) (Allona *et al.*, 1992, 1994a, 1994b, 1994c) showed that arginine and glutamine content in different storage proteins of this species varies, but is relatively high, especially in the 2S globulins (24.6 molar %) (Allona *et al.*, 1992, 1994b). These amino acids are also high in the storage reserves of jack pine (Durzan and Chalupa,

1968; Ramiah *et al.*, 1971), loblolly pine (King and Gifford, 1997), Eastern white pine (*Pinus strobus*) (Feirer, 1995), and Douglas-fir (Feirer, 1995). A number of conifer storage protein cDNAs have been isolated and deduced amino acid sequences show the same bias towards arginine and glutamine in white spruce (*Picea glauca*) and Engelmann spruce (*P. engelmannii*) (Newton et al., 1992; Rice and Kamalay, 1996), Douglas-fir (Leal and Misra, 1993b; Chatthai and Misra, 1998), incense cedar (*Cedrus decurrens*) (Hager and Dank, 1996), Japanese cedar (*Cryptomeria japonica*) (Wind and Hager, 1996), dawn redwood (*Metasequoia glyptostrobodies*) (Hager and Wind, 1997) and in *Ginkgo biloba* (Hager et al, 1995). Table I indicates the arginine content in some of these seeds, illustrating the arginine content's contribution to the total stored nitrogen in the species described.

The phosphate buffer-insoluble megagametophyte storage proteins of loblolly pine contain over 23.4 mol% arginine and 21.0 mol% combined glutamine/glutamate (King and Gifford, 1997). The acid hydrolysis procedure utilized by King and Gifford (1997) does not allow for differentiation of the amides asparagine and glutamine from aspartate and glutamate. However, assuming that all of the glutamate/glutamine pool is glutamine and all of the aspartate/asparagine pool is asparagine, each containing two nitrogen atoms rather than one, then the contribution of each amino acid to total nitrogen storage can be estimated. Because arginine contains four nitrogen atoms in each molecule it accounts for at least 46% of the total stored nitrogen in these proteins (King and Gifford, 1997). As such, arginine and arginine utilization are likely critical to supplying nitrogen to the seedling during germination and early seedling growth.

Table I: Seed storage protein arginine content

Species	Arginine content (molar %)	Reference(s)
Loblolly pine (*Pinus taeda*)	23.4 %	King and Gifford (1997)
Maritime pine (*Pinus pinaster*)	16.5% - 32.5%	Allona *et al.*, (1992, 1994a, 1994b, 1994c)
Eastern white pine (*Pinus strobus*)	14%	Chattai and Misra, (1998)
White spruce (*Picea glauca*)	8.9% - 15.5%	Dong and Dunstan (1996)
Picea glauca/engelmannii complex	8.0% - 13.4 %	Newton *et al.*, (1992); Newton, (1991a, 1991b); Rice and Kamalay (1996)
Jack pine (*Pinus banksianna*)	32 %	Ramiah *et al.*, (1971)
Incense cedar (*Calocedrus decurrens*)	12.5% - 13.3%	Hager and Dank, (1996)
Japanese cedar (*Cryptomeria japonica*)	13.0% - 13.3%	Wind and Hager (1996)
Douglas-fir (*Psedutsuga menziesii*)	12.7%	Leal and Misra, (1993)
Dawn redwood (*Metasequoia glyptostrobodies*)	13.1 % - 13.4 %	Hager and Wind (1996)
Mean of 200 Angiosperm species	7.2%	Van Etten *et al.*, (1963)

1.4 Seedling growth and development

1.4.1 Dormancy

Seed dormancy is a physiological state which prevents a seed from germinating under germination-promoting conditions (Bewley and Black, 1985). It has also been described as the inherent characteristic of the seed that defines what parameters must be met to induce seed germination (Vleeshouwers *et al.*, 1995). However, since breaking dormancy does not automatically induce germination, it may be more accurate to describe this as a definition of requirements to be met to *permit* germination, rather than *induce* it. Seed dormancy can be separated into primary dormancy and secondary dormancy. Primary dormancy refers to seeds which are dormant at the time of dispersal from the mother plant, while secondary dormancy refers to the acquisition of a dormant state by seeds after dispersal due to an environmental cue (Crocker, 1916). It can also be classified on the basis of whether the dormant state is imposed on the seed by the seed coat or is inherent within the embryo (Bewley, 1997). In coat-imposed dormancy the seed coat constrains the embryo's ability to germinate, possibly through interference with water uptake, gas exchange, or uptake or leaching of specific compounds (Bewley and Black, 1994). Such seeds readily germinate when the seed coat is removed. In contrast, those species that exhibit embryo dormancy are unable to germinate under favorable conditions regardless of whether the seed coat is present or not. Dormancy may benefit the mother plant by reducing sibling competition in space and time by increasing the species' ability to adapt to unpredictable changes in the environment (Nilsson *et al.*, 1994).

The loblolly pine seeds utilized in this study exhibit primary dormancy and require a period of cold, moist incubation called stratification in order to remove the block to germination (Carpita *et al.*, 1983; Schneider and Gifford, 1994). Loblolly pine seed dormancy is coat imposed (Barnett, 1972, 1976; King, 1998, Cooke *et al.*, 2002). It has been shown that during stratification there are changes associated with RNA and protein synthesis (Schneider and Gifford, 1994; Mullen *et al.*, 1996), but a dormancy breaking signal has yet to be identified. The mechanism of dormancy breaking may involve decreasing the physical resistance to radicle protrusion through weakening of the surrounding tissues, similar to what has been observed in white spruce (Downie and Bewley, 1996). For the purposes of this study, any seed germinated in the presence of the seed coat was stratified prior to transfer to germination-promoting conditions whereas mature, unstratified seed was utilized in those experiments in which the testa was removed since these embryos were completely non-dormant under culture conditions.

1.4.2 Germination and early seedling growth

Once any constraints imposed by seed dormancy are lifted the seed may then germinate. For germination to commence the seed must be fully imbibed. This occurs in three phases (Bewley and Black, 1985). The initial phase is characterized by a rapid uptake of water into the seed in response to its physiological condition; the highly desiccated mature seed draws water into its tissue due to the large difference in water potential between the seed and its surrounding environment. As imbibition continues the seed's water potential increases (becomes less negative) and gradually levels off, ending the first phase. The initial phase is a purely physical process which occurs regardless of

metabolic state, in viable and non-viable seeds. Though metabolic activity is not required, initial metabolic processes may begin immediately following imbibition. The seed coat may act as a barrier to water absorption during this phase to slow or inhibit germination (Bewley and Black, 1985); however, in conifers the testa does not significantly inhibit germination by limiting water uptake (Barnett, 1972). Once the physical process of water absorption has ceased the seed enters a lag phase where water content increases very slowly. The seed still absorbs water, but does so in response to the osmotic potential of dissolved solutes in the cells rather than the water potential of the desiccated seed structures. Increased metabolic activity is a characteristic of this phase. In conifers, changes in enzyme activity, protein and nucleic acid profiles have been demonstrated both in the pre-emergent embryo and the megagametophyte tissue (Pitel and Cheliak, 1988; Groome *et al.*, 1991; Mullen *et al.*, 1996). Length of the lag phase can be influenced by external environmental conditions. Dormant seeds may remain in the lag phase for a very long time until dormancy has been broken. The third phase is characterized by an increase in water uptake in preparation for and concurrent with cell elongation in the germinating seed. During this period, seedling growth occurs primarily through cell expansion (Bewley and Black, 1994). In conifers, only the embryo enters this phase. The megagametophyte tissue, while metabolically active, does not undergo cell elongation and thus has no requirement for additional water uptake. Germination in conifer seeds is affected by photoperiod (Kozlowski, 1971) and by light quality (Toole et al., 1962; Johnson and Irgens-Moller, 1964; Tobin and Briggs, 1969). This is likely mediated through a phytochrome interaction, since the effects of red and far red light on germination are reversible (Toole *et al.*, 1962) and phytochrome was detected in mature

seeds of *Pinus palustris* immediately after seed imbibition (Tobin and Briggs, 1969). Radicle protrusion indicates the end of germination and the onset of early seedling growth. All seeds used in this study were imbibed to allow hydration prior to transfer to germination permissive conditions and germinated under constant light. Germination continues until the radicle has exited the seed coat.

1.4.3 Early seedling growth

After germination is completed the embryo, now designated a seedling, enters early seedling growth. For the purposes of this thesis, early seedling growth will be defined as the period between the end of germination, and the exhaustion of the seed storage reserves prior to the seedling's conversion to complete autotrophy. During this period the seed reserves are mobilized (see section 1.5). In pines, the growth of the seedling during this phase is epigeal; the megagametophyte remains attached to the cotyledons which are lifted into the air as the radicle and hypocotyl expand. As the cotyledons fully expand, the megagametophyte is shed, completing this developmental period.

1.5 Seed reserve utilization

1.5.1 TAG breakdown

Since this thesis focuses primarily on the utilization of storage protein reserves, only a brief overview of lipid reserve catabolism will be presented and will focus on what is known about the events occurring in conifers. Following imbibition, during germination and early seedling growth, the megagametophyte lipid reserves, primarily

TAGs, are broken down by the plant (Ching, 1966; Kovac and Vardjan, 1981; Groome et al, 1991; Hammer and Murphy, 1994). Free fatty acids are released by lipases (Hammer and Murphy, 1994), which in the pine seed are likely metabolized through the β-oxidation spiral to generate succinate (Mullen and Gifford, 1995a, 1995b, 1997; Migabo, 2000). Succinate may then enter the Krebs cycle in the mitochondria to produce malate, which can be moved to the cytoplasm and undergo gluconeogenesis to produce carbohydrates, primarily sucrose (Stone, 1999) for transport to the seedling.

1.5.1.1 Sugar movement

As loblolly pine's megagametophyte lipid reserves are broken down there is only a minor increase in the megagametophyte's soluble carbohydrate pool, but a major increase in the pool size in the seedling (Stone, 1999; Stone and Gifford, 1999). Similar observations have been made in Douglas-fir (Ching, 1966). When the megagametophyte is removed from germinating seedlings this pool does not accumulate in the seedling, suggesting that these carbohydrates are moved to the seedling from the megagametophyte (Stone, 1999). Likewise, radiolabeled carbohydrate compounds applied to the megagametophyte during early seedling growth accumulate in the seedling (Stone, 1999), confirming the movement of these compounds from the megagametophyte to the seedling. In loblolly pine the major transport compound is sucrose. Sucrose accumulates in the megagametophyte during TAG breakdown but the majority of the sugar is found in the seedling (Stone and Gifford, 1999). This is different than in some non-conifer seeds where sucrose is metabolized to fructose and glucose prior to or during transport (Weber *et al.*, 1995, 1997) and where a large pool of soluble carbohydrate builds up in the storage

tissues (*e.g.* endosperm) prior to transport to the embryonic axis (Huang and Beevers, 1974). In some conifer species sucrose also accumulates in the seedling following germination (Ching, 1966; Murphy and Hammer, 1988), and in sugar pine (*Pinus lamberiana*) and Norway spruce (*Picea abies*) the incoming sucrose is converted to starch for storage (De Carli et al, 1987, Murphy and Hammer, 1994). In loblolly pine limited starch accumulation occurs, reaching roughly 10% of the starch accumulating in pinyon pine (Stone, 1999, Hammer and Murphy, 1994), perhaps due to the much greater triacylglycerol content of sugar pine seeds (Stone and Gifford, 1999).

1.5.2 Protein breakdown

For the nitrogen stored within the proteins of embryo and megagametophyte to be used by the plant, they must be converted to individual amino acids. In the megagametophyte, the seed storage proteins are broken down following germination (Guitton, 1964; Durzan and Chalupa, 1968; Ramiah *et al.*, 1971; Lammer and Gifford, 1989; King and Gifford, 1997). This occurs through the combined action of both endo-lytic and exo-lytic peptidases in the megagametophyte (Salmia and Mikola, 1975, 1976a, 1976b; Salmia, 1981; Gifford *et al.*, 1989, Gifford and Tolley, 1989). The free amino acids that arise from protein hydrolysis must move from the megagametophyte to the seedling. In conifers this movement cannot be symplastic as the megagametophyte and embryo are not symplastically connected. In contrast to many angiosperm species where the products of storage protein breakdown are converted to an alternate form, often glutamate or glutamine, before transport (Stewart and Beevers, 1967; Kern and Chrispeels, 1978; Lea and Mifflin, 1980; Bray, 1983), in loblolly pine transport from the

megagametophyte to the seedling seems to occur without much prior conversion of the amino acids (King and Gifford, 1997); conversion appears to occur in the seedling itself. Interestingly, the relative amino acid complement of loblolly pine storage proteins does not entirely match the composition of the free amino acid pool in germinating seedlings (King and Gifford, 1997); thus there is the possibility that some amino acids are converted prior to transport as suggested by Ramiah *et al.* (1971). It is difficult, however, to determine if such conversions occur solely in the megagametophyte or seedling, or both. In the case of arginine, it is metabolized in the cotyledons of the seedling (King and Gifford, 1997). The accumulation of a large asparagine pool in loblolly pine seedlings during the latter stages of early seedling growth (King and Gifford, 1997) is consistent with the model of arginine utilization proposed by Avila *et al.* (2001), whereby nitrogen to be stored or transported is converted to asparagine in seedling tissues rather than in the megagametophyte. Alanine aminotransferase and aspartate aminotransferase activities increase in both the megagametophyte and seedling tissues during early seedling growth of jack pine (*Pinus banksianna*) (Pitel and Cheliak, 1988). Whether these conversions are required for transport or are involved in general metabolism in both tissues is difficult to determine. Moreover, both pyruvate and oxaloacetate, the α-keto acids corresponding to alanine and aspartate, are major intermediates in carbon metabolism. Thus, the conversions may be required for integration of carbon and nitrogen metabolism rather than for amino acid transport.

Similar to the movement of sucrose from the megagametophyte to the seedling (Stone and Gifford, 1999), in loblolly pine (King and Gifford, 1997) and radiata pine (*Pinus radiata*) (Fashu-Kanu, 2001), free amino acids do not accumulate in the

megagametophyte, but are transported to the seedling, against a concentration gradient, resulting in accumulation of a much greater free amino acid pool in the seedling than in the megagametophyte (King and Gifford, 1997; Fashu-Kanu, 2001). It has been suggested that this process may eliminate inhibition of protein breakdown in the megagametophyte by free amino acid pools, possibly arginine (King, 1998). With respect to this hypothesis, it should be noted that the mechanisms of storage protein hydrolysis in the seedling and the megagametophyte must then differ, since the seedling continues to break down its storage protein even as the amino acid pool accumulates. Alternatively, the threshold of inhibition of storage protein breakdown in the seedling must be high enough to allow the seedling to permit breakdown of the seedling reserves. Without such mechanisms, storage protein breakdown in the seedling would cease as amino acids from the megagametophyte accumulate. The timing of reserve breakdown in the seedling might then be important to this process, permitting seedling reserve breakdown prior to achieving a large free amino acid pool. This may be sufficient to prevent inhibition of seedling protein reserve hydrolysis. The megagametophyte continues to degrade its storage proteins long after the seedling reserves have been depleted and it is during this period when seedling amino acid levels are highest.

1.6 Arginine and arginase

1.6.1 Arginine metabolism in plants

Arginine catabolism in plants is linked to the production of polyamines, such as putrescine and agmatine, the protein amino acids, glutamate and proline, and the non-protein amino acid, ornithine. It is also linked to primary nitrogen assimilation through

the production of ammonia. As shown in Figure 3, arginine can undergo a decarboxylation to form CO_2 and the polyamine agmatine in a reaction catalyzed by the enzyme arginine decarboxylase (E.C. 4.1.1.19) or it can be hydrolyzed by the enzyme arginase (L-arginine amidinohydrolase E.C. 3.5.3.1) to form urea and the non-protein amino acid ornithine (Figure 3; Verma and Zhang, 1999 and references therein). Arginine is able to undergo extensive metabolic conversion in plant cells. Products of ^{14}C-labelled arginine in white spruce buds include amino acids, polyamines, monosubstituted guanidines as well as protein, organic acids and neutral compounds (Durzan, 1968a, 1968b, 1969). Still, the accumulation of these compounds can be explained by the production of intermediates originally arising from either the decarboxylation or hydrolysis of arginine.

Decarboxylation of arginine to agmatine is the first step in the direct synthesis of polyamines from arginine. Polyamines are nitrogen rich compounds that have been implicated in important developmental changes in plants, including flowering, fruit formation and root nodulation (Evans and Malmberg, 1989; Verma and Zhang, 1999). In conifers, much of the interest in polyamine metabolism has focused on its role in embryo development (Feirer, 1995) and somatic embryogenesis (Amarasinghe and Carlson, 1994; Amarasinghe *et al.*, 1996). Polyamine levels rise during ovule development in *Pinus strobus* and are associated with increased amino acid metabolism (Feirer, 1995), but the exact role of these compounds, either as an alternate nitrogen store or as a signal directing tissue organization is yet unknown. The arginine decarboxylase reaction is not the sole pathway to polyamine formation from arginine. One product of arginine hydrolysis by arginase, ornithine, can also undergo a decarboxylation reaction catalyzed

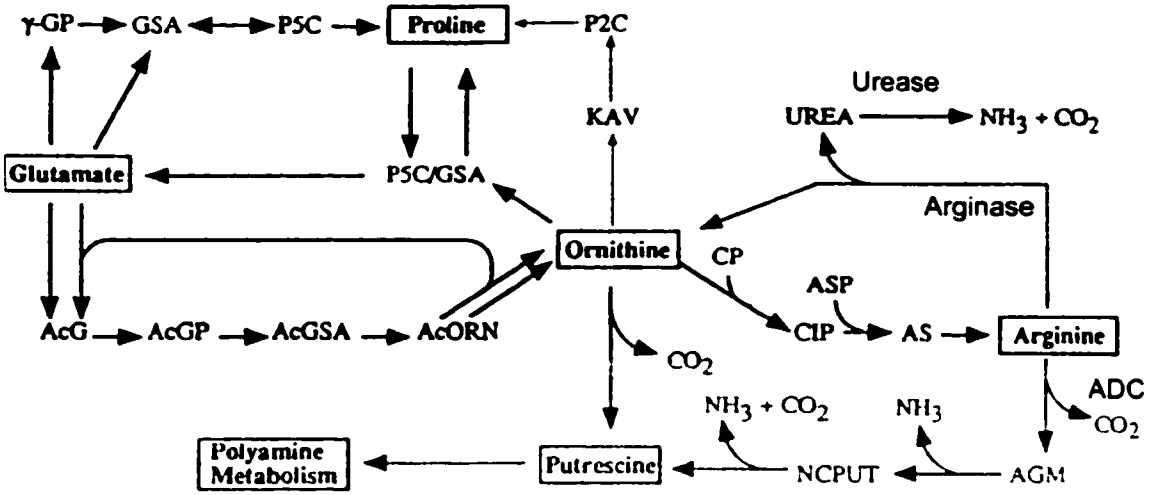


Figure 3: Potential metabolic routes for arginine. The enzymes arginase, arginine decarboxylase (ADC) and urease are indicated. Intermediates: γ-GP, γ-glutamyl phosphate; GSA, γ-glutamyl-5-semialdehyse; P5C, Δ^1-pyrroline-5-carboxylate; KAV, α-keto-δ-aminovalerate; P2C, Δ^1-pyrroline-2-carboxylate; AcG, *N*-acetylglutamate; AcGP, *N*-acetylglutamate-5-phosphate; AcGSA, *N*-acetylglutamate-5-semialdehyde; AcORN, *N*-acetylornithine; CP, carbomyl phosphate; CIT, citrulline; ASP, aspartate; AS, arginosuccinate; AGM, agmatine; NCPUT, *N*-carbamoylputrescine. Figure adapted from Verma and Zhang (1999).

by ornithine decarboxylase (E.C. 4.1.1.17) to form putrescine. Thus the production of polyamines from arginine in and of itself does not indicate the metabolic route taken. Ornithine is a key intermediate in the conversion of the carbon backbone of the arginine molecule to either polyamines or amino acids and is itself a precursor of arginine (Figure 3). Urea produced in the arginase reaction is broken down by the enzyme urease (urea amidohydrolase, E.C. 3.5.1.5) to produce ammonia and carbon dioxide. The ammonia, present as the cation ammonium (NH_4^+), is the primary substrate for essentially all nitrogen assimilation in plants (Mifflin and Lea, 1976, Oaks, 1994; Lea and Ireland 1999).

During development of soybean cotyledons arginine accumulates in the developing tissue due to *in situ* biosynthesis (Micallef and Shelp, 1989a). Application of radiolabeled ornithine showed that the intermediates of arginine biosynthesis appear in the seedling (Micallef and Shelp, 1989b) and that radiolabeled arginine is both incorporated into protein and is degraded by an arginase-mediated pathway (Micallef and Shelp, 1989c). These results predict that a functional urea cycle involving both the synthesis and degradation of arginine occurs in these tissues at this time. Involvement of arginase in the urea cycle is a mechanism for excretion of urea in animal systems (Thompson, 1980; Jenkinson et al., 1996). Micallef and Shelp suggested that multiple pools of arginine exist in the cotyledons during development and storage reserve deposition based on differing concentrations of radioisotopic precursors and products. This may indicate that though all the intermediates of an arginine cycle are present in the cell, synthesis of different compounds may be occurring in separate subcompartments, preventing this cycle from occurring. Thus, the apparently wasteful synthesis and

degradation of arginine to be used for storage reserve deposition might not be occurring as predicted. Shargool *et al.* (1988) present a model for the compartmentalization of arginine and ornithine biosynthesis whereby arginine synthesis from ornithine occurs in the plastids and chloroplast and arginine degradation occurs in the mitochondrion. A similar model based on different routes of arginine metabolism in different tissues during seed development has been proposed (Polacco and Holland, 1993), and Goldraij and Polacco (1999) have shown that arginase may be present, but is inactive in the embryonic axis of developing soybean embryos This would prevent the simultaneous synthesis and degradation of arginine destined for protein synthesis during embryo development. They have also suggested that portioning of arginase to the mitochondria may avoid development of a futile urea cycle once arginase has become active (Goldraij and Polacco, 2000). In these cases the developmental stage examined is one in which arginine synthesis for the deposition of storage proteins and storage of nitrogen is desired. Studies based on developing seeds are useful in demonstrating the metabolic pathways that arginase may take and to illustrate the need for spatial and temporal regulation of the enzymes contained therein to prevent a functional urea cycle. In a germinating seed, the reverse net reaction, the breakdown of storage proteins and utilization of arginine as a nitrogen source, would require the use of the arginase-mediated reaction to free the nitrogen contained within arginine. Inhibition of a functional urea cycle would then be required to prevent the energy requiring re-synthesis of arginine from ornithine. As the focus of this thesis is the regulation of arginase during germination and early seedling growth, the remainder of the introduction will focus on arginase-mediated breakdown and utilization.

The urea released from arginine hydrolysis is an important nitrogen source to developing seedlings. Inhibiting urea metabolism blocks or delays seedling germination and early seedling growth (Zonia *et al.*, 1995; King and Gifford, 1997). It has been well established that the route of nitrogen assimilation in plants is through the action of the enzyme glutamine synthetase (GS, E.C. 6.3.1.2) (Mifflin and Lea, 1976; Lea and Ireland, 1999). The ammonium released from urea hydrolysis is incorporated into the amide glutamine, which can then be used as a nitrogen donor, primarily in transamination reactions, and to recycle glutamate via the enzyme glutamate synthase (glutamine: 2-oxoglutarate amino transferase, GOGAT, E.C. 1.4.1.14, 1.4.7.1), to eventually distribute the nitrogen through any nitrogenous compound in the plant. In angiosperms, two forms of GS exist; GS1 is found in the cytosol and GS2 is chloroplastic (Oaks, 1994; Ireland and Lea, 1999). However, presently only cytosolic GS (GS1) has been identified in conifers (Avila *et al.*, 1998; Canovas *et al.*, 1998). In pines multiple forms of both GS1 and GOGAT have been identified (Canton *et al.*, 1993; Garcia-Gutiérrez *et al.*, 1995, 1998; Canovas *et al.*, 1998) and have been associated with the fate of the assimilated nitrogen. A model of glutamine utilization from the various GS1 isoforms has been proposed in which arginine derived ammonium is assimilated by one of two GS1 isoforms depending on the desired metabolic fate (Avila *et al*, 2001). This is illustrated in Figure 4. Both forms function in the cotyledons where ammonium to be utilized immediately is assimilated through GS1a and GOGAT. Nitrogen to be transported to other parts of the plant is converted to glutamine by GS1b in the vascular tissue. The glutamine produced is then used to synthesize asparagine directly from aspartate and glutamine through the action of asparagine synthetase (AS, E.C. 6.3.5.4) rather than using

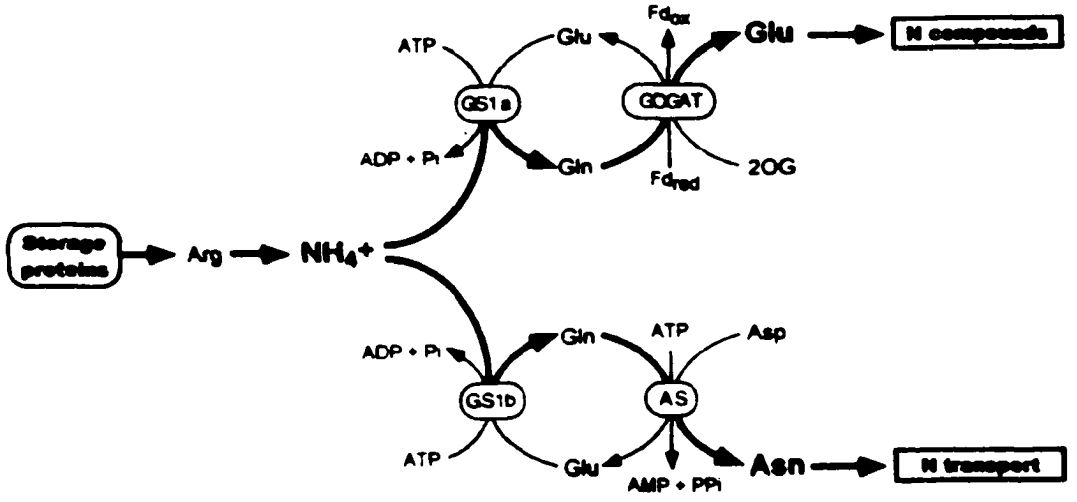


Figure 4: Proposed glutamine cycles in pine seedlings. Enzymes: AS, asparagine synthetase; GOGAT, glutamate synthase/glutamine : 2-oxoglutarare aminotransferase; GS1a and GS1b, glutamine synthetase. Intermediates: Arg, arginine; Asn, asparagine; Asp, aspartate; Gln, glutamine; Glu, glutamate; 2OG, 2-oxoglutarate. Figure taken from Avila *et al.* (2001).

GOGAT, regenerating glutamate to continue ammonium assimilation; asparagine is a common nitrogen transport and storage molecule in plants. Another possible route of nitrogen assimilation would use the more traditional GS-GOGAT cycle, where glutamine is recycled into glutamate to continue with the uptake of ammonium or to act as a nitrogen donor in a suite of different transamination reactions. However, this route is likely less active, since during early seedling growth of loblolly pine there is a very large increase in the asparagine component of the free amino acid pool over time (King and Gifford, 1997). The two proposed pathways probably both function at the same time, but in different tissue types, with the asparagine synthetase catalyzed route being linked to nitrogen movement through the vascular system (Avila *et al.*, 2001).

1.6.2 Arginase in plants

The enzyme arginase splits the amino acid arginine into the non-protein amino acid ornithine and urea in a hydrolytic reaction. In mammals arginase functions in the urea cycle (Thompson, 1980), generating urea for excretion of excess nitrogen (for a comparative review of arginases, please see Jenkinson et al., 1996). Arginases have been identified in all the kingdoms (Jenkinson et al. 1996) and their activity has been demonstrated in many plant species. In plants, arginase plays a very different role, mobilizing the nitrogen stored within arginine during fruit development (Alabadi *et al.*, 1996), in storage organs such as tubers (Wright *et al.*, 1981) or bulbs (Boutin, 1982) and following seed germination (Matsubara and Suzuki, 1984; Kang and Cho, 1991; King and Gifford, 1997). In germinating seeds, it is free arginine resulting from storage protein hydrolysis that the enzyme acts upon (Splittstoesser, 1969; Matsubara and

Suzuki, 1984; King & Gifford, 1997). Arginase activity has been described in Arabidopsis (*Arabidopsis thaliana*) (Zonia et al., 1995), iris (Boutin, 1982), Jerusalem artichoke (Wright et al., 1981), broad bean (Jones and Boulter, 1968; Kollöfel and Van Dijke, 1975; DeRuiter and Kollöfel, 1983), jack bean (Downum et al., 1983), pea (Taylor and Stewart, 1981), pumpkin (Splittstoesser, 1969), soybean (Downum et al, 1983; Matsubara and Suzuki, 1984; Yu and Cho, 1990; Goldraij and Polacco, 1999), tomato (Alabadi et al., 1996), Scots pine (Pietilä et al, 1989), stone pine (Guitton, 1957, 1964) and loblolly pine (King and Gifford, 1997). However the protein has been purified from relatively few sources (Wright *et al.*, 1981; Boutin, 1982; Kang and Cho, 1990; King 1998), and to apparent homogeneity only twice, from soybean axes (Kang and Cho, 1990) and loblolly pine (King, 1998). Prior to this work, two plant arginase cDNAs had been cloned, from Arabidopsis (Krumpelman at al. 1995) and soybean (Goldraij et al. 1998).

In loblolly pine, arginase activity is found primarily within the expanding cotyledons following germination (King and Gifford, 1997). There is evidence that a functional urease is active in developing loblolly pine seedlings to provide ammonium from the resultant urea (Gifford, unpublished). Ornithine, which contains the other two nitrogen atoms and the bulk of the carbon skeleton can undergo several different conversions, primarily to the amino acids glutamate and proline, or it may enter polyamine biosynthesis (Verma and Zhang, 1999). The fate of ornithine in loblolly pine seedlings is currently under investigation. Previous work has shown that not much proline accumulates in seedling tissues post-germination (King and Gifford, 1997), suggesting that proline is not a major end product of ornithine. As mentioned previously,

the vast increase in the asparagine pool in the seedling suggests that the nitrogen stored as arginine may be converted to asparagine for transport and/or storage. Both ornithine and urea may contribute to this process. Ammonium derived from urea could be utilized in asparagine synthesis through GS1b and asparagine synthetase (Figure 4). Ornithine can be converted to glutamate (Figure 3) which then may participate in either the GS1a or GS1b reaction (Figure 4). Arginase represents the first step in the mobilization of both the carbon and nitrogen stored as arginine in seed storage proteins as well as re-mobilizing arginine in senescing tissues. As such, this enzyme is likely of critical importance for nitrogen mobilization in loblolly pine and other plants.

1.6.3 Regulation of arginase

Although arginase activity has been detected in many higher plants there has been limited work on the factors or mechanisms involved in arginase regulation. Matsubara and Suzuki (1984) demonstrated that soybean cotyledons incubated with the seedling axis had greater arginase activity than isolated cotyledons. The source-sink relationship between the cotyledons and seedling axis might possibly suggest that the lack of an effective sink caused feedback inhibition of arginase in the cotyledons, but this was not investigated. The polyamine compounds spermidine and agmatine have been found to increase arginase activity *in vitro* (Kang and Cho, 1990), but there are no data concerning the levels of these compounds *in vivo* during seed germination, nor whether heightened polyamine levels in plant tissue have an effect on arginase regulation. There has been even less work done on the molecular basis for arginase regulation in plants, owing largely to the lack of arginase sequence data; however, it has been demonstrated that

increases in arginase activity following germination of soybean is matched by an increase in arginase mRNA levels (Goldraij and Polacco, 1999). How arginase regulation is achieved has yet to be demonstrated in a plant system.

The lichen, *Evernia prunasti*, has three forms of arginase: a constitutive arginase, present in the thallus at all times; an inducible, but still cellular, arginase; and a secreted form of the enzyme (Legaz and Vicente, 1982; Martin-Falquina and Legaz, 1984; Vicente and Legaz, 1985). Though always present in the lichen, the activity of the constitutive arginase does increase when arginine is presented in the media (Martin-Falquina and Legaz, 1984). The inducible form of the enzyme is not normally present, but its synthesis is induced by arginine and its activity is inhibited by urea (Legaz and Vicente, 1982; Vicente and Legaz, 1985). Thus, the inducible form appears to be tightly regulated, responding to both the presence of its substrate, arginine, but also by accumulation of one of its products, urea.

In fungal systems arginase plays primarily a catabolic role, degrading arginine for use as a nitrogen source, but still balancing arginine hydrolysis with the requirement for protein synthesis (Davis, 1986). In *Neurospora crassa*, controlling the breakdown of arginine is accomplished by compartmentalization of arginine itself (Weiss, 1973). In *Neurospora*, arginase is a cytosolic enzyme, but arginine is sequestered to the vacuole (Weiss, 1973), displacing ornithine as it enters this compartment (Weiss and Davis, 1977). Since the enzymes of the urea cycle are present in the cytosol, this compartmentalization of the metabolites prevents futile cycling of arginine synthesis and degradation (Davis, 1986). Abundance of ornithine in the cytosol may promote arginine synthesis over breakdown as arginine can be produced from ornithine through the latter

half of the urea cycle, providing arginine for protein synthesis and sequestering any excess arginine produced in the vacuole, releasing more ornithine. When nitrogen is limiting, arginine can then be released from the vacuole and replaced by ornithine in this organelle, providing nitrogen, in the form of urea until external nitrogen sources are once more available.

In the yeast *Saccharomyces cerevisiae* all the urea cycle enzymes are cytosolic, but their activities are modulated by the cell's requirement for arginine breakdown or synthesis. Accumulation of arginine in the cytosol results in increased activity of both arginase and ornithine aminotransferase (Whitney and Magasanik, 1973, Magasanik, 1992). Likewise, arginine catabolism in the cytosol inhibits the activity of arginine biosynthetic enzymes (Eisenstein *et al.*, 1986). In this way *S. cerevisiae* avoids a urea cycle, but with a mechanism that is different from *Neurospora*. Though they accomplish this task in different ways both species exhibit inducer exclusion, they limit the uptake of arginine into the cell when preferred nitrogen sources such as glutamine or ammonium are available (Davis, 1986; Cooper *et al.*, 1992).

The regulation of arginase in non-mammalian systems is probably best understood in *Saccharomyces cerevisiae*. Precise control of arginase involves both nitrogen catabolite repression, which regulates synthesis of enzymes, and nitrogen catabolite inactivation and inducer exclusion, which regulates the synthesis of amino acid permeases controlling metabolite levels in the cell (Magasanik, 1992). Transcription factors which control arginase expression have been identified (Messenguy and Dubois, 1988), as have the DNA binding sequences in the corresponding promoter regions (Messenguy *et al.*, 1991). The arginase gene (*CAR1*) is induced in yeast by the presence

of amino acids, not solely arginine, involving both an extra-cellular and intracellular amino acid sensing system (Klasson *et al.*, 1999). In addition to the transcription factors responsible for activation of arginase gene expression, at least three gene products (CARGRI, CARGRII, CARGRIII) repress arginase transcription, as well as transcription of ornithine aminotransferase, when exogenous nitrogen is available to the cell (Messenguy *et al.*, 2000). Moreover, the chromatin state of the arginase promoter may be involved in arginase transcription, as these products assemble as part of a high molecular weight complex with histone de-acetylase activity (Messenguy *et al.*, 2000).

No work has been done regarding the mechanism or regulation of arginase in conifer systems; however, a developmental profile of arginase activity shows that accumulation of arginase in the cotyledons occurs at the same time as the increase in the free amino acid pool in the seedling tissue, a large component of which is arginine (King and Gifford, 1997). This occurs following germination and is temporally coordinated with breakdown of seed storage proteins in the megagametophyte and the shoot pole (Groome *et al.*, 1991; King and Gifford, 1997; Stone and Gifford, 1997). This thesis will deal with understanding the mechanism of arginase regulation and how it is achieved during conifer seed germination and early seedling growth.

1.7 The present study

The focus of this study is to better our understanding of how arginase is regulated during germination and early seedling growth and to understand this regulation within the context of the developmental changes occurring during this time period. At the inception of this study, arginase had been well described in the plant literature at the biochemical

level, yet little or no information about arginase regulation at the molecular level was available and none from a conifer species. Previously, King and Gifford (1997) had demonstrated that arginase is active in the seedling following germination and early seedling growth. To increase our understanding of how this enzyme is regulated at the molecular level an arginase cDNA was isolated and characterized. Other molecular tools generated in the course of this work include polyclonal antibodies raised against recombinant pine arginase expressed in *Escherichia coli* and a loblolly pine actin fragment for use in comparisons of arginase mRNA abundance.

The developmental regulation of arginase was examined during germination and early seedling growth of whole loblolly pine seed. Throughout this period the megagametophyte remains in contact with the seedling, supplying free arginine to the seedling. A major part of this thesis involved examining the role that the megagametophyte plays in the regulation of arginase in the seedling. To accomplish this, an *in vitro* culture system was developed to allow growth of isolated embryos with and without their associated megagametophyte tissues. Seedlings grown under these conditions were characterized and the effect of the megagametophyte on the arginase was determined.

The final part of the thesis involves the manipulation of this experimental culture system to explore the relationship between the megagametophyte and the seedling with respect to arginase regulation. This was performed to test the hypothesis that arginase is responding to arginine derived primarily from the megagametophyte. To summarize this work a model of arginase regulation in the seedling was proposed, taking into account the contribution of both the seedling and the megagametophyte to this process.

2. Materials and Methods

2.1 Water quality and chemicals

Unless otherwise stated, all aqueous solutions used in this study were prepared using distilled, de-ionized Milli-Q water (resistance 18 megohm· cm^{-1}) obtained using a Milli-Q Filtration Water System (Millipore Corporation, Bedford, MA, USA).

Fine chemicals were purchased from BDH (Toronto, ON), Bio-Rad Laboratories (Canada) Ltd. (Mississauga, ON), Fisher Scientific (Nepean, ON), Sigma Chemical Company (St. Louis, MO, USA) and VWR (Mississauga, ON). Radiochemicals were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Bacterial media reagents were purchased from Difco Laboratories (Detroit, MI, USA). Restriction enzymes and PCR reagents were purchased from Amersham Pharmacia Biotech, MBI Fermentas Inc, (Burlington, ON) and Qiagen Inc. (Mississauga, ON).

2.2 Seed Material

2.2.1 Stratification, germination and early seedling growth

Loblolly pine seed was collected from a single open-pollinated clone (11-9) in the fall of 1992 and was a gift from Westvaco (Summerville, SC). Seeds were stored at –20 °C until use. Prior to use, seeds were rinsed under cold tap water for 60 minutes in a loosely tied cheesecloth bag, washed in a dilute Tween 20 solution for 30 minutes, rinsed under tap water for an additional 60 minutes, surface sterilized in 1% sodium hypochlorite for 5 minutes and rinsed with de-ionized water. Following sterilization, the seeds were subjected to a 35-day chilling period (stratification) between two layers of

moist Kimpack (Seedborough Equipment, Chicago, IL) in the dark at 2°C to relieve seed dormancy (Scheneider and Gifford, 1994). After stratification, seeds were surface sterilized again and imbibed at 30°C for up to 12 days under continuous fluorescent light (19 μmol m^{-2} s^{-1}) in Kimpack-lined germination trays in a Controlled Environments Ltd. (Winnipeg, MB) seed germinator. Seed germination was considered complete with the emergence of the radicle from the seed coat, four days after imbibition at 30°C (DAI_{30}) and indicated the onset of early seedling growth.

Seed tissues were staged according to radicle length as described in Mullen et al. (1996). In this thesis the term embryo will be used to described the mature sporophyte at seed maturity and throughout stratification. After transfer to germination-promoting conditions the same tissue is referred to as a seedling.

2.2.2 *In vitro* culture of isolated seed parts

Mature embryos were cultured with (+M) and without (-M) megagametophytes. For those cultured in the presence of the megagametophyte the hard outer seed coat and inner papery layer were removed aseptically. The nucellar cap was removed, leaving only the megagametophyte and embryo. For embryos cultured without the megagametophyte, a single cut was made along the longitudinal axis of the megagametophyte, allowing it to be split in two. The embryos, including the root cap, were then removed and placed on the culture medium.

Embryos with and without megagametophytes were cultured on sterile solid media in Petri dishes for 5 days, then transferred to Magenta Boxes (Sigma Chemical Company) for the remaining period. The media consisted of a basal medium described

by Becwar et al (1990) modified for somatic embryo germination by Rutter *et al.* (1998a, 1998b). The final composition of the medium was 100 μg/ml KNO_3, 745 μg/ml KCl, 170 μg/ml KH_2PO_4, 370 μg/ml MgSO· $7H_2O$, 440 μg/ml $CaCl_2$, 16.9 μg/ml $MnSO_4$· H_2O, 8.6 μg/ml $ZnSO_4$· $7H_2O$, 0.025 μg/ml $CuSO_4$· $5H_2O$, 0. 83 μg/ml KI, 0.025 μg/ml $CoCl_2$· $6H_2O$, 6.2 μg/ml H_3BO_3, .25 μg/ml Na_2MoO_4· $2H_2O$, 27.84 μg/ml $FeSO_4$· $7H_2O$, 37.24 μg/ml Na_2EDTA, 0.1 μg/ml thiamine, 0.1 μg/ml pyridoxine, 0.5 μg/ml nicotinamide, 30 mg/ml sucrose, 0.8 mg/ml NH_4NO_3, 0.1 mg/ml myo-inositol, 2 mg/ml phytagel and 5 mg/ml activated charcoal. All chemicals used for the medium were plant tissue culture tested from Sigma Chemical Company. Seedlings were grown under continuous fluorescent light (19 μmol m^{-2} s^{-1}) at a constant 30°C for up to 10 days. Seedlings were staged by time. Under culture conditions germination, as indicated by elongation of the radicle of isolated embryos or emergence of the radicle from the megagametophyte was generally complete after 2 days in culture at 30°C (DIC_{30}).

2.2.3 Tissue collection for biochemical and molecular analysis

At different stages of development seed tissues were separated into megagametophytes and whole embryos or seedlings and harvested. Embryos and seedlings were analyzed as whole seedlings, or were split into root poles and shoot poles by making a single transverse cut below the cotyledons. Shoot poles consisted of the cotyledons and epicotyl. Root poles consisted of the hypocotyl and radicle. In each case a known number of seed parts were harvested; the number of seed parts collected varied according to the type of analysis and the developmental stage of the tissue. Tissues were

quick frozen with liquid N_2 immediately after collection and stored at −70°C unless otherwise indicated.

2.2.4 ^{35}S-methionine radiolabeling experiments

Seedlings were labeled with [^{35}S]-methionine (>37 TBq mmol^{-1}, Amersham Pharmacia Biotech) for 3h at 30°C unless otherwise noted. Radioactivity was applied directly to the cotyledons after removal of the megagametophyte. After labeling, tissues were rinsed with distilled water and buffer soluble proteins were extracted in cold 50 mM phosphate buffer (pH 7.5) as described in section 2.3.1. and radioactive incorporation was determined by TCA precipitation as described in Gifford and Bewley (1984).

2.2.5 Removal of megagametophytes from 6 DIC_{30} seedlings

Seedlings were grown with their associated megagametophytes as described in section 2.2.2. After six days in culture (6 DIC_{30}) the seedlings were gently removed from the media to allow the megagametophyte to be pulled from the cotyledons. This was performed by making a small longitudinal incision on the outside of the megagametophyte, allowing it to be removed without damaging the cotyledons. Once the megagametophyte had been removed, the seedlings were gently returned to their Magenta boxes and cultured for up to four more days (10 DIC_{30} total) before being collected for analysis.

2.2.6 Application of exogenous arginine to *in vitro* grown seedlings.

Seedlings were grown with (+M) and without (-M) megagametophytes as described in section 2.2.2 or had their megagametophytes removed as described in section 2.2.5. After 6 DIC_{30}, seedlings were misted with a 10 mM or 100 mM arginine solution (pH 7.5) until the cotyledons were covered with a thin layer of solution. Seedlings were incubated in the presence of the exogenous arginine for up to 24 hours before being collected as described in section 2.2.3..

2.3 Biochemical analyses of storage protein utilization

All biochemical assays were performed using a Beckman DU-65 Spectrophotometer (Beckman Instruments Inc., Fullerton, California).

2.3.1 Protein extraction and quantification

Protein was extracted from different tissue types by a method similar to that described in Gifford *et al.* (1982). A known number of seed parts (section 2.2.3) were homogenized in a cold mortar and pestle with 1 mL (total volume) 50 mM sodium phosphate buffer (pH 7.5) and centrifuged at 14,000 *g* in a Beckman microcentrifuge (Beckman Instruments Inc.) for 20 min at 4°C. The resultant supernatant contained the buffer-soluble protein fraction. Remaining pellets were washed three times with 1 mL phosphate buffer, centrifuging for 5 minutes after each wash. Phosphate buffer-insoluble protein was extracted from the washed pellet by resuspending the pellet in 1 mL Laemmli buffer (62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol (Laemmli, 1970) and boiling for 5 minutes in a heat block. After centrifuging at 20,000 *g* for 20 minutes at

room temperature the supernatant contained the buffer-insoluble fraction. Proteins in each fraction were quantified using the method of Lowry *et al.* (1951), using bovine serum albumin (BSA) as the standard. For quantitative protein extractions the insoluble pellet was washed three times with 1 mL Laemmli buffer, boiled for 5 min each time and centrifuged for 5 minutes at 20,000 *g* at room temperature. The pooled washes for both the soluble and insoluble fraction were assayed for protein concentration using the same method (Lowry *et al.*, 1951).

2.3.2 Arginase (L-arginine amidinohydrolase EC 3.5.3.1)

2.3.2.1 Arginase activity

Arginase activity in cell-free extracts was assayed using a modification of King & Gifford (1997). Assay pH and temperature optima for loblolly pine arginase were determined previously (King, 1998). To prepare the extracts, a known number of seed parts were homogenized in 1 ml (total volume) freshly prepared cold arginase extraction buffer (50 mM Tris HCl (pH 9.0), 0.75 mM maleate, 0.5 mM $MnCl_2$) and spun for 20 min at 4°C at 16,000 *g*. The resultant supernatant was drawn off with a pulled glass Pasteur pipette, avoiding the lipid layer, and used for the assay. The enzyme was pre-activated by incubation for 60 min at 30°C after extraction to ensure that the solution had equilibrated at 30°C and that the enzyme was allowed to bind manganese present in the extraction buffer. Enzyme activity was determined by incubating 100 µL of the activated extract with 0.285 M arginine (pH 9.7), 1mM $MnSO_4$ and 1 mM maleate in a total volume of 1.5 mL for 30 min at 30°C. The assay was terminated by the addition of 0.7 volumes 87% (v/v) acetic acid. A second aliquot from each extract was used to

determine endogenous urea in the tissue samples prior to assay of arginase activity. This was accomplished by either boiling the extracts prior to assay to inactivate the enzyme, or by addition of the acetic acid prior to the assay, also inactivating arginase. Endogenous urea and total urea were measured using the colorimetric method of Geyer and Dabich (1971). Urea produced by arginase during the assay was calculated by subtracting the endogenous urea levels from total urea after the assay. Arginase activity is expressed in nanokatals (nkat). For each assay a fresh urea standard curve (0-20 μg/mL in Milli-Q water) was prepared immediately prior to use. For each standard curve, R^2 values always exceeded 0.99.

2.3.2.2 Arginase immunotitration

Immunotitration of enzyme activity was performed as described by Mullen and Gifford (1995). Shoot poles of 9 DAI_{30} seedlings were extracted in 50 mM Tris-HCl (pH 7.5) with 1 mM $MnSO_4$. Increasing amounts of serum were added to equal aliquots of extract containing equal amounts of enzyme activity. All samples were made up to an equal volume prior to incubation with gentle agitation at 25°C for 1 h, then at 4°C overnight. Any precipitated material was pelleted by centrifugation for 20 min at 15,000 *g*, and an aliquot of the resulting supernatant was assayed for arginase activity as described in section 2.3.2.1.

2.4 Protein electrophoresis and analysis

2.4.1 SDS-PAGE

One dimensional protein separation by SDS-PAGE was performed using a mini-PROTEAN II electrophoresis system (Bio-Rad) as described by Laemmli (1970). Polyacrylamide gels (12%) were poured 0.75 mm thick and run at 200 V. Molecular markers consisted of : phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa (Bio-Rad) unless otherwise stated. Following electrophoresis, gels were stained with Coomassie Blue R (Sigma) using the method of Burk *et al.* (1983) to visualize the protein bands. For fluorography, gels were fixed in 10% (v/v) acetic acid, 40% (v/v) methanol for 30 minutes and soaked for 2 hours in EN^{3}HANCE autoradiography enhancer (NEN, Boston, MA, USA). Gels were rinsed several times with distilled water and dried under vacuum for two hours at 60 C. Dried gels were exposed to Kodak X-OMAT AR film overnight at -80 C before developing. [^{14}C]-methylated molecular weight markers for labeled gels were: myosin, 200 kDa; phosphorylase b, 92.5 kDa; bovine serum albumin, 69.0 kDa; ovalbumin, 46.0 kDa; carbonic anhydrase, 31 kDa; and lysozyme, 14.4 kDa (Amersham Pharmacia).

2.4.2 Immunoblotting

Immunoblotting from 12% SDS-PAGE gels was accomplished using a Mini Trans-Blot Electrophoretic Cell (Bio-Rad). Protein was transferred to Bio-Rad TransBlot supported nitrocellulose for 14 hours at 30V or for 1 hour at 100V as per the manufacturer's instructions (Bio-Rad). After transfer, membranes were stained with

Ponceau S stain (Sambrook *et al.*, 1989) and protein visualized after washing with several changes of water. The location of the protein standards were marked with a sharp pencil and the membranes were blocked for 1 hour in 20 mM Tris-HCl (pH 7.8) 150 mM NaCl, 5% (w/v) Carnation non-fat dried milk. Antibody diluent consisted of the same, with the addition of 0.05% Tween 20. Membranes were incubated with a primary antibody solution for one hour. Blots were then washed 3 times for 20 minutes each in the antibody diluent followed by incubation with a 1:3000 dilution of the secondary antibody for one hour. After removal of the secondary antibody, blots were washed 3 times again, excess wash solution was removed by blotting on Whatman 3MM paper and antibody detection was performed.

For colorimetric detection goat-anti-rabbit conjugated alkaline phosphatase (Bio-Rad) was used as the secondary antibody and bands were visualized in a solution containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$, 0.3 mg/mL nitroblue tetrazolium and 0.15 mg/mL BCIP. Chemiluminescent detection was performed using Bio-Rad goat anti-rabbit conjugated horseradish peroxidase secondary antibody and the LumiGlo chemiluminescence substrate kit (KPL labs, Gaithersburg, MD) as per the manufacturer's directions. For some chemiluminescent blots, Bio-Rad Precision Protein Standards were utilized in conjunction with Streptactin-conjugated horseradish peroxidase (Bio-Rad), allowing molecular markers to be visualized directly on the film; Chemiluminescent detection of these blots was the same as described above. Signals were detected on Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY).

2.5 cDNA isolation

2.5.1 Isolation of an arginase cDNA from a loblolly pine seedling cDNA library

A cDNA library had been constructed previously from poly(A)+ RNA extracted from 9-10 DAI_{30} seedlings (Mullen, 1995) using a UNI-ZAP XR cDNA synthesis kit (Stratagene Cloning Systems, La Jolla, CA) and Gigapack II Gold packaging extracts (Stratagene). The library had been amplified to 3.2 x 10^{10} plaque forming units/ml and stored at -70°C until use. Over 2.5 x 10^5 plaques from the amplified seedling library were transferred to NitroPlus nitrocellulose membranes (MSI, Westboro, MA) and screened with loblolly pine arginase antiserum (King, 1998) at a 1:1000 dilution using a *pico*Blue immunoscreening kit (Stratagene) according to the manufacturer's recommendations. Colorimetric detection was performed with Bio-Rad goat-anti-rabbit conjugated alkaline phosphatase as described in section 2.4.2. All plaque lifts were performed in duplicate and only those showing a strong positive signal on both filters were selected. Plaques were removed from the plates with a sterile pipette tip and suspended in SM buffer (Sambrook *et al.* 1989) before the next round of screening. Positive clones were selected and isolated through two further rounds of plaque screening and excised from the λZAP vector to form pBluescript SK^- insert carrying phagemids using the ExAssist excision kit (Stratagene). Rescued phagemids were transfected into *E. coli* strain SOLR with ExAssist Interference-Resistant Helper Phage using the manufacturer's single-clone excision protocol (Stratagene). This resulted in pBluescript SK^- double stranded phagemids with the cloned cDNA inserts. Helper phage was not able to grow in the nonsuppressing Su- SOLR strain, thus rescued colonies contained only the clone of interest. Clone identity from the rescued phagemids was confirmed

using the *pico*Blue colony screening protocol utilizing a 1:1000 primary antiserum dilution and colorimetric detection as described above. Colonies were screened in duplicate. Plasmid DNA was isolated from individual clones and cDNA inserts were released by EcoRI/KpnI digestion. Inserts were sized by agarose gel electrophoresis prior to sequencing.

After identification of full length cDNA clones encoding loblolly pine arginase cDNAs, glycerol stocks were prepared from single colonies by mixing 10 mL of a late log phase culture with 4.5 mL LB:glycerol (1:1). Aliquots of this mixture were frozen in liquid nitrogen and stored at –80°C until needed. A full length clone, designated pARS20, was used for the remainder of this study. A cesium chloride plasmid prep was performed as described in Sambrook *et al.* (1989) and stored at –20°C until required. All further experiments utilizing the arginase cDNA were performed from this plasmid preparation.

2.5.2 Isolation of a partial loblolly pine actin clone

A partial loblolly pine actin cDNA was isolated as follows. Forward and reverse primers (CTO7 and CTO8, Table II) were designed from the predicted coding sequence of a lodgepole pine actin sequence (Kenny *et al.* 1988). RT-PCR was performed from 10 ng shoot pole total RNA from seedlings cultured in the presence of the megagametophyte tissue for six days (6 DIC_{30}+M) using the Qiagen OneStep RT-PCR Kit according to the manufacturer's recommendations. The reaction conditions consisted of a 30 min reverse transcription step at 50°C followed by 15 min at 95°C to denature the reverse transcriptases and activate the thermostable polymerase. Amplification was achieved

using 30 cycles consisting of a 1 min denaturation step at 94°C, 1 min annealing step at 57°C and 1 min extension step at 72°C. A final extension step at 72°C for 10 min was performed and then the products were held at 4°C until they were separated on a 1% agarose gel and visualized by ethidium bromide staining. A single band of approximately 310 basepairs was produced which was excised from the gel. The isolated DNA fragment was purified using the Qiagen Qiaex II gel isolation kit, cloned into the pCR-Script Amp SK(+) vector using the pCR-Script Amp Cloning Kit (Stratagene) and transformed into XL-10 Kan competent cells (Stratagene) according to the manufacturer's protocol. Plasmid DNA was isolated from individual transformants and sequenced. A single clone was chosen from several positives, designated pLpAct1 and sequenced to entirety. A glycerol stock was made as described in section 2.5.1.

2.6 DNA Sequencing

DNA sequencing of the arginase cDNA was performed by the Arizona State University DNA sequencing facility from the 3' and 5' ends of the cDNA inserts from the T7 and M13 reverse oligonucleotide primers using an ABI Prism 377 sequencer (Perkin Elmer Biosystems, Foster City, CA). Further rounds of sequencing were performed using custom oligonucleotide primers generated at the Department of Biological Sciences Molecular Biology Service Unit.

All other DNA sequencing was performed using the DYEnamic ET terminator cycle (Amersham Pharmacia Biotech) sequencing premix kit utilizing 4µl per 20 µl reaction incorporating 5 pmol sequencing primer and 0.1-0.2 pmol template DNA. The reaction consisted of 30 cycles: 95°C for 30 sec; 50°C for 15 sec; 60°C for 60 sec; and

were held at 4°C until use. The resultant DNA was precipitated according to manufacturer's recommendations and analyzed on an ABI Prism 373 sequencer (Perkin Elmer Biosystems) at the Department of Biological Sciences Molecular Biology Service Unit.

2.7. Genomic analysis of arginase cDNA

2.7.1 DNA Isolation

Genomic DNA was isolated by a protocol modified from Porebski et al. (1997). Briefly, 0.5 mg of fresh tissue (>12 DAI_{30} cotyledons) was homogenized in the presence of 50 mg PVPP with a small amount of washed sea sand in 5 ml CTAB buffer (0.2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 0.3% (v/v) β-mercaptoethanol) pre-warmed to 60°C. The solution was transferred to 15 ml sterile polypropylene tubes and incubated for 60 min in a 60°C shaking water bath with occasional mixing by inversion. The solution was extracted 3 times with 24:1 $CHCl_3$:octanol, spinning 20 min each time at maximum speed in a Sorvall GLC-1 clinical centrifuge (Sorvall, Newtown, CT, USA). After each extraction the supernatant was removed to a fresh tube. Salt concentration was adjusted to 0.2 M with 5 M NaCl and nucleic acids precipitated with 2 vol ice cold 95% ethanol for 20 min at -20°C. The DNA was centrifuged, washed with 70% ethanol, vacuum dried and resuspended in 300 µL TE buffer (10 mM Tris-HCl, 1mM EDTA (pH 7.5)). The nucleic acids were then incubated with 3µL RNAse A (10mg/ml) at room temp for 60 min, followed by 0.5 µL proteinase K (10mg/ml) for 30 min. The solution was extracted with an equal volume of 1:1 phenol:chloroform and spun at 20 000g, 5 min at room temperature. The supernatant (250µL) was removed to a

fresh tube and precipitated with 16.6 µL 3 M sodium acetate (pH 4.8) and 500 µL absolute ethanol at room temperature for 5 min., spun at 10,000 g 1 min., washed with 70% ethanol, dried under vacuum at ambient temperature and resuspended in 100 µL TE overnight at 4°C. DNA was quantified using a Beckman DU-65 spectrophotometer and analyzed for RNA contamination on ethidium bromide stained agarose gels.

2.7.2 Southern blotting

For Southern blotting, 15 µg of genomic DNA was digested with restriction enzymes, run on a 0.7% agarose gel and transferred to Zeta-probe nylon membranes (Bio-Rad). Radioactive probes were generated using a Pharmacia Biotech oligolabeling kit (Amersham Pharmacia Biotech) and ^{32}P α-dCTP (110 TBq mmol^{-1}) (Amersham Pharmacia Biotech) as per the manufacturer's instructions. Removal of unincorporated nucleotides was performed using Bio-Rad Micro-Spin P-30 columns (Bio-Rad) as per the manufacturer's directions. Transfer, hybridization and wash conditions were performed as per Sambrook et al. (1989). Blots were exposed to Kodak X-OMAT AR film at -80°C with intensifying screens.

2.8 RNA analysis

2.8.1 RNA extraction

RNA was isolated from approximately 50 mg tissue using the RNeasy Plant Mini Kit (Qiagen) using a modified protocol (Mackenzie et al. 1997). RNA integrity was determined by formaldehyde-agarose gel electrophoresis through 1.2% gels and visualized with ethidium bromide staining.

2.8.2 Northern blotting

Radioactive probes were generated as described in section 2.7.2.. For northern analysis, RNA was transferred to Zeta-Probe nylon membranes (Bio-Rad) by capillary action (Sambrook *et al.*, 1989). Blots were washed briefly in 2 X SSC, air dried and RNA was fixed to the membrane by baking for 30 min at 80°C under vacuum. Prior to hybridizations the blots were re-hydrated in 2 X SSC. Membranes were pre-hybridized in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, 1 mM EDTA for 1 hour at 65°C prior to addition of probe. Hybridization was performed overnight in fresh solution at 65°C. Filters were washed twice with 50 mM Na_2HPO_4 (pH 7.2), 5% SDS, 1mM EDTA, for 20 minutes at 65°C, and twice with 50 mM Na_2HPO_4 (pH 7.2), 1% SDS, 1mM EDTA at 65°C for 20 minutes each before being exposed to Kodak X-OMAT AR film at -80°C with intensifying screens. Signal strength was quantified using AlphaEase 5.1 software on an AlphaImager documentation system (Canberra Packard Canada, Mississauga, ON).

2.8.3 RT-PCR

RT-PCR was performed using a Qiagen OneStep RT-PCR Kit in a MJ Research Minicycler (MJ Research, Watertown, MA, USA) according to the manufacturer's instructions. PCR and RT-PCR primers are listed in Table II. Unless otherwise stated RT-PCR was performed from 10 ng total RNA.

2.9 *In vitro* transcription and translation

In vitro transcription and translation was performed from 1 μg pARS20 DNA using the T3 TNT Coupled Reticulocyte Lysate System (Promega Corporation, Madison,

WI) and Redivue L-[^{35}S]methionine (>37 TBq mmol^{-1}, Amersham Pharmacia Biotech) as per the manufacturer's instructions. Translation products were diluted 50-fold and analyzed by SDS-PAGE and fluorography. Control reactions containing no DNA were also performed.

2.10 Immunoprecipitation of *in vitro* and *in vivo* radiolabeled protein

Immunoprecipitation of *in vitro* and *in vivo* labeled proteins was performed as per Mullen & Gifford, (1997). Aliquots of radiolabeled protein were diluted to 1 mL with buffer A (50 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 150 mM NaCl, 20 mM methionine, 3 mM EDTA, 1mM iodoacetamide, 1 mM PMSF, 2 μg mL^{-1} aprotinin. 25 μg mL^{-1} leupeptin), microfuged at 14,000 *g* for 15 minutes and the supernatants brought to 1 M NaCl by the addition of 50 mg solid NaCl. The solution was pre-cleared by addition of 50 μL pre-immune serum followed by a 1 h incubation on ice. The pre-immune complexes were then incubated with 50 μL of a 10% suspension of *Staphylococcus aureus* Cowan I cells (Sigma) which had been pre-washed three times in buffer A for 30 minutes on ice. The mixture was microfuged for 15 minutes and the supernatant used for immunoprecipitation.

The supernatant was incubated with 50 μL antiserum for 1 h at room temperature and then overnight at 4°C with gentle agitation. Antibody-antigen complexes were collected by the addition of 20 μL *S. aureus* cells pre-washed three times in buffer A. After 1 h at room temperature, cells were collected by centrifugation, washed five times with buffer B (same as A, but 0.1% (v/v) Triton X-100) and three times with buffer C (same as A, but no Triton X-100). After a final centrifugation the supernatant was

removed and the pellet was boiled for 10 minutes in Laemmli buffer (Laemmli, 1970) to dissociate and denature all the proteins contained therein. The resulting proteins were separated by SDS-PAGE and analyzed by fluorography (section 2.4.1).

2.11 Partial purification of loblolly pine arginase

2.11.1 IgG Purification

Anti-arginase antibodies generated previously (King, 1998) were purified for use in construction of an immuno-affinity matrix. IgG purification was accomplished using an Affi-Gel Protein A MAPS II Kit (Bio-Rad). Five mL of Affi-Gel Protein A was poured into a 1x10 cm glass EconoColumn (Bio-Rad) and equilibrated with 25 mL of the provided binding buffer (Bio-Rad). The pH of the eluent was determined to be greater than 9.0 to ensure the column was equilibrated. Approximately 4.3 mL anti-arginase antiserum was diluted 1:2 with 8.6 mL binding buffer and applied to the pre-equilibrated column. After sample application the column was washed with 15 volumes binding buffer. Absorbance at 280 nm was used to determine that no residual proteins were being washed from the column prior to IgG elution. The column was eluted under acidic conditions with 16 volumes Elution Buffer (Bio-Rad). Fractions (4.5 ml each) were eluted into 0.5 mL 1M Tris-HCL (pH 9.0) to neutralize the strongly acidic elution buffer. Absorbances at 280 nm were taken to determine which fractions contained the eluted antibody. Purified IgGs eluted in the first three fractions which were then combined. Approximately 25.3 mg IgGs was collected in 15 mL (total volume) as estimated by absorbance at 280 nm. The antibody solution was concentrated in an Amicon Bioseparations Centriplus YM-50 (MWCO 50,000) Regenerated Cellulose Centifugal

Filter Device (Millipore Corporation, Bedford, MA, USA) by spinning at 3,000 *g* for 60 min at room temperature. The solution was concentrated to approximately 4 mL with an IgG concentration of roughly 5.5 mg $(mL)^{-1}$. IgGs were desalted on EconoPac 10DG desalting columns (Bio-Rad) according to the manufacturer's recommendations and used immediately for creation of an immunoaffinity matrix.

2.11.2 Immunoaffinity column preparation

Creation of an anti-arginase immunoaffinity column was performed using the Affi-Gel Hz Immunoaffinity Kit (Bio-Rad). The purified IgGs (section 2.11.1) were transferred to Affi-Gel Hz coupling buffer using EconoPac 10DG desalting columns (Bio-Rad) according to the manufacturer's recommendations. After buffer exchange the IgGs were oxidized by combining 6.5 mL of the recovered solution with 1.96 mL of a freshly prepared sodium periodate solution (20.8 mg $(mL)^{-1}$ in Milli-Q water) with end over end mixing for 1 h at room temperature. The oxidized IgGs were then desalted again using the EconoPac 10DG desalting columns equilibrated with coupling buffer (Bio-Rad). Five mL of Affi-Gel Hz was washed twice with 10 mL coupling buffer and allowed to settle. Excess buffer was removed from above the surface of the matrix and discarded. The desalted, oxidized IgG solution (approximately 6.5 mL) was incubated with the washed Affi-Gel Hz for 16 hours at room temperature with constant end over end mixing. Following incubation the mixture was poured into a 1x10 cm glass EconoColumn (Bio-Rad) and the flow through was collected. The column was washed with 8 mL 50 mM Tris-HCL (pH 7.5), 500 mM NaCl to remove any loosely bound antibodies. Absorbance at 280 nm of the flow through and wash fractions was recorded

to determine total unbound IgGs. This value was subtracted from the total amount of IgG utilized in preparing the column to determine that the Affi-Gel Hz matrix retained approximately 5.7 mg IgGs. The column was then washed with 100 mL 50 mM Tris-HCl (pH 7.5) containing 0.02% sodium azide and stored at 4°C until use.

2.11.3 Partial purification of native loblolly pine arginase

Approximately 5 g of frozen 9-12 DAI_{30} shoot poles were ground under liquid nitrogen to powder and then extracted in 24 mL (total) cold STMG buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 mM $MnCl_2$, 10% (v/v) glycerol). The homogenate was then further disrupted with a Polytron homogenizer (Brinkmann, Westbury, NY) at maximum speed for 25 s at 4 °C. The homogenate was centrifuged at 25,000 *g* for 20 min at 4 °C in a Beckman J2-21 M/E centrifuge and the supernatant filtered through Miracloth (Calbiochem, La Jolla, CA). The filtrate was incubated in a 60 °C shaking water bath for 20 min, then immersed in an ice-water bath for 5 minutes prior to centrifuging again at 25,000 *g* for 20 min at 4 °C. The recovered heat-treated supernatant was filtered through Miracloth and loaded onto a 1.5x100 cm EconoColumn containing Sephacryl S-200 HR resin (Sigma Chemical Company) previously equilibrated with STMG buffer. Protein was eluted at a constant flow rate of 2.5 mL $(min)^{-1}$ and 2.5 mL fractions were collected. The absorbance of each fraction at 280 nm was recorded and fractions were assayed for arginase activity as described in section 2.3.2.1. Those fractions containing discernable arginase activity were pooled and loaded onto a 2.5x100 cm EconoColumn containing Sephacryl S-300 HR resin (Sigma Chemical Company) previously equilibrated with STMG buffer. Protein was eluted at a constant flow rate of

4.5 ml $(min)^{-1}$ and 5 mL fractions were collected and used to determine absorbance at 280 nm and arginase activity. Those fractions containing arginase activity were pooled and incubated with 5 mL of anti-arginase coupled Affi-Gel Hz (section 2.11.2) for 3 h at room temperature and then 12 h at 4°C. The slurry was then poured into a 1x10 cm glass EconoColumn (Bio-Rad) and allowed to settle. Flow through was collected and the column was then washed with 25 mL STMG until A_{280} values returned to close to zero. The column was then eluted with 0.2 M Glycine-HCl (pH 2.5). Two mL fractions were collected and neutralized with the addition of 0.2 mL 1 M Tris-HCl (pH 9.0) and assayed for arginase activity. Dilute fractions were concentrated using Millipore Ultrafree-MC microcentrifuge spin concentrators. The concentrated solution was diluted 1:1 with Laemmli buffer, boiled for 5 minutes and these fractions as well as aliquots of protein from the previous purification steps were analyzed by SDS-PAGE (section 2.4.1).

2.11.4 Molecular mass determinations

The subunit molecular mass of arginase was determined using 12 % separating gel SDS-PAGE essentially by the method of Weber and Osborn (1969). The following molecular mass standards (Bio-Rad) were used: phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

2.12 Expression of the arginase cDNA in *Escherichia coli*

2.12.1 Generation of 6xHis-tagged arginase

Standard DNA manipulations were performed as per Sambrook et al. (1989). The coding sequence of the loblolly pine cDNA was amplified by PCR utilizing Taq DNA polymerase (Qiagen) in an MJ Research Minicycler according to the manufacturer's instructions. The forward primer, CTO 5 (Table II) was designed to introduce a BamHI restriction site into the PCR product near the N-terminal region of the coding sequence and the reverse primer, CTO 6 (Table II) introduced a Sal I restriction site downstream of the loblolly pine stop codon. These restriction sites were then used to directionally clone the PCR product into the pQE-31 *Escherichia coli* expression vector (Qiagen). The resulting constructs were transformed into the *E. coli* M15 strain, which already contained the pREP4 repressor plasmid. Transformations were performed according to the manufacturer's protocol. Those transformants harbouring an insert-carrying pQE-31 construct as well as the pREP4 repressor plasmid were then tested for their ability to express the fusion peptide in *E. coli*. All of the protocols utilized during the expression of the recombinant protein were performed using the QIAexpress Expression System (Qiagen). Protein minipreps identified several transformants that expressed a protein of the predicted size (~38 kDa) and a second smaller peptide at high levels. One of these transformants showing high levels of the 38 kDa band after induction with 1 mM IPTG, designated pHAR-2, was used for further purification.

2.12.2 Purification of 6xHis-tagged arginase

Four 1.5L cultures inoculated with the pHAR-2 transformants were grown to an $O.D._{600}$ of 0.6 at 37°C and then adjusted to 1mM IPTG to induce expression of the fusion protein. The cultures were incubated for an additional 4 hours and the cells were collected by centrifugation at 4500*g* at 4°C. The wet weight of the collected cells was recorded. Purification of the his-tagged protein was accomplished from 21.4 g (wet weight) *E. coli*.

The cell pellet was re-suspended in 100 ml native lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (pH 8.0)) and adjusted to 10 mg/ml lysozyme (Sigma-Aldrich, Oakville, ON). The suspension was incubated on ice for 30 minutes and then sonicated with six 10-second bursts (on ice) with a microtip-equipped Sonic Dismembrator Model 300 (Fisher Scientific). The lysate was centrifuged at 18,000 *g* for 30 minutes at 4°C in a JA-20 rotor in a J2-21M/E centrifuge (Beckman). Since the majority of the arginase protein was previously determined to be in the insoluble pellet (data not shown), this pellet was quick frozen in liquid nitrogen and then stored at –20°C until use. After thawing the pellet to room temperature it was suspended in 50 mL denaturing lysis buffer (8M urea, 100mM NaH_2PO_4, 10 mM Tris-HCl (pH 8.0)) and stirred for 30 minutes at room temperature. The solution was then spun at 18,000 *g* for 20 minutes at 25°C and the supernatant was collected and poured through several layers of cheesecloth to remove any loose debris. The collected supernatant was highly viscous and retained some insoluble particles not separated during the previous spin. The solution was diluted to 100 mL with denaturing lysis buffer and spun at 24,000 *g* for 20 minutes at 25°C in a Beckman JA-20 rotor. This pelleted the remaining insoluble matter

and the cleared supernatant was collected. This solution was incubated with 8 mL of a 50% slurry of Ni-NTA agarose (Qiagen Inc.) and gently mixed at room temperature for 60 min. After incubation, the resin was pelleted in a GLC-1 clinical centrifuge (Sorvall) at 1000 rpm and washed with 70 mL (total) denaturing wash buffer (same composition as the lysis buffer but pH 6.2). The slurry was poured into a 1x10 cm glass EconoColumn (Bio-Rad) and allowed to settle. Once the wash buffer had been collected the column was eluted in 20 mL denaturing elution buffer (same composition, but pH 4.3). Two mL fractions were collected and analyzed by SDS-PAGE.

The his-tagged arginase protein eluted in fractions 2, 3 and 4, as did several other protein bands. These three fractions were pooled and adjusted to a pH of 8.0 with 1.0 M Tris-HCl (pH 9.5). The pooled fractions were then incubated for 60 minutes with 1 mL 50% Ni-NTA agarose at room temperature with gentle agitation. The slurry was poured into a 1x10 cm EconoColumn and allowed to settle. Flow through was collected and the column was washed 3 times with 10 mL wash buffer. The bound protein was then eluted at pH 4.5. Two mL fractions were collected, absorbance at 280 nm was recorded and the fractions analyzed by SDS-PAGE. The majority of the fusion peptide eluted in the first three fractions. A few minor contaminating bands were still present. Since the his-tagged arginase formed insoluble aggregates in non-denaturing buffers it was decided to remove the contaminating proteins by dialysis against a non-denaturing solution. The pooled fractions of interest were adjusted to 10% (v/v) glycerol and then dialyzed against STMG buffer (section 2.11.3) in Spectra-Por dialysis tubing (12,000-14,000 MWCO) (Spectrum Laboratories inc., Rancho Dominguez, CA) previously equilibrated in the same. The solution was dialyzed against two changes of the buffer in a 1L glass

graduated cylinder while stirring at room temperature. During dialysis a flocculent white precipitate (arginase) formed in the tubing. The dialysate was collected and the precipitate pelleted at 24,000 *g* for 20 minutes at 25°C. The supernatant was collected and the pellet washed with 5 mL STMG buffer and collected again. The pellet was then extracted in denaturing lysis buffer at 60°C for 60 minutes and pelleted again. Most of the pellet dissolved, however what remained was re-extracted in the denaturing buffer and collected. These fractions were analyzed by SDS-PAGE. The first urea extraction contained a high amount of the arginase protein as well as a smaller peptide previously determined to be a truncation product (see results) with no discernable contamination. Protein content was determined using the method of Bradford (1976) using the Bio-Rad Protein Assay Reagent (Bio-Rad) and a freshly prepared BSA standard curve. In total, 1540 μg of protein were collected and this preparation was used for antibody production.

2.12.3 Antibody production

Arginase protein samples were diluted 1:1 in Freund's complete (initial injection) or Freund's incomplete (subsequent injections) adjuvant for antibody production. All steps of the immunization procedure were performed by the Biosciences Animal Services, Department of Biological Sciences, University of Alberta. Two female Flemish giant × French lop rabbits were immunized subcutaneously either using 100 or 200 μg protein for the initial injection; two booster injections of 100 or 200 μg protein each were made at 28 day intervals. After the final bleeding the blood was incubated at 37°C for 2 h, then placed at 4°C overnight to allow clotting. The clot was pelleted at 1240 *g* in a GLC-1 clinical centrifuge (Sorvall), and the serum removed. Sodium azide was added to

the serum to a final concentration of 0.02% (w/v), and the serum stored at -80°C until use.

2.13 Standard DNA manipulations

Most standard DNA manipulations were performed according to Sambrook *et al.* (1989). PCR was performed using a Qiagen *Taq* PCR core kit (Qiagen) in a MJ Research Minicycler according to the manufacturer's protocol. All oligonucleotides used as primers are listed in Table II. Plasmid DNA minipreps were performed using a Qiaprep Sin Miniprep Kit (Qiagen) or using the alkaline lysis method of Sambrook et al. (1989).

2.14 Scanning densitometry and DNA gel quantification

Scanning densitometry was performed using a Bio-Rad 670 densitometer (Bio-Rad). Signal strength quantification was also performed using AlphaEase 5.1 software on an AlphaImager gel documentation system (Canberra Packard Canada, Mississauga, ON).

Table II: List of oligonucleotide primers used

Primer Name	Sequence (5' to 3')	Purpose
CTO 1	CTCCACTTCGTCCATTGG	Sequencing
CTO 2	GCCACAATGTCTCCTTGC	Sequencing
CTO 3	CCGCGACTGAGAAAGGGAAAGAATTG	PCR/RT-PCR
CTO 4	CCAGGTGCAAATGCCGGATCAAG	PCR/RT-PCR
CTO 5	GTGCTAGTGGATCCGGGAAAAATGGTGATGAGGTTTCTGC	PCR
CTO 6	TAGTTAGCAGTCGACTGCATCCATGGACTGAGGCTTTTGA	PCR
CTO 7	ATTGGAATGGAAGCACGGGGTATC	PCR/RT-PCR
CTO 8	TTCTGTGGACAATTGAGGGACCTGAC	PCR/RT-PCR
CTO 9	TGCTGAAATGTGCTAAGAGATGCCAAGAT	PCR/RT-PCR
CTO 10	CTTAGTGGTGGTTCTACCATGTTTCCTGG	PCR/RT-PCR

3. Results[3]

3.1 Antibody characterization and arginase tissue distribution

Work completed prior to the inception of this thesis resulted in the purification of the arginase holoprotein from loblolly pine and production of antibodies raised against an electro-eluted subunit (King, 1998). These antibodies were used to screen a seedling cDNA expression library in order to isolate a loblolly pine arginase cDNA clone. Prior to screening, the antibodies were examined to determine their ability to recognize arginase. The antibody serum was tested for its ability to immunotitrate arginase activity from a crude soluble protein extract obtained from 9 DAI_{30} shoot poles (Figure 5). Increasing concentrations of anti-arginase serum decreased arginase activity in a dose-dependant manner, completely abolishing any enzyme activity at a concentration of 60μL serum $(mL)^{-1}$ extract (Figure 5). Pre-immune serum had no effect on the activity of the enzyme, demonstrating that the antibodies generated were able to bind the arginase holoprotein. Antibody specificity for arginase was also demonstrated with immunoblots of soluble protein extracts. Only a single band of approximately 37 kDa was detectable in all tissues taken from 12 DAI_{30} seedlings (Figure 6). The antibodies showed a monospecific affinity for the arginase protein at low serum concentration (1:5000 dilution or greater). The relative distribution of arginase protein in these tissues closely matched the relative levels of arginase enzyme activity in the same tissues (King, 1998), with the majority of arginase protein localized to the cotyledons as expected (Figure 6).

[3] A version off this chapter has been published. Todd C.D., Cooke J.E.K., Mullen R.T., Gifford D.J. (2001) Plant Mol. Biol. 45: 555-565; Todd C.D., Cooke J.E.K., Gifford D.J. (2001) Plant Physiol. and Biochem. 39: 1037-1045; Todd. C.D. Gifford, D.J. (2002) Planta *In press*.

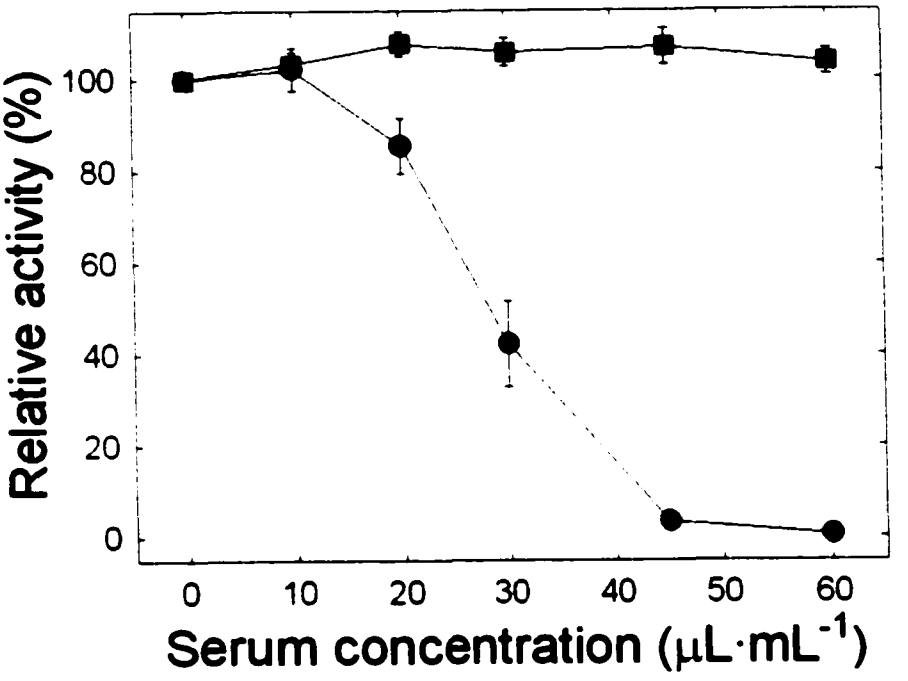


Figure 5: Immunotitration of arginase activity. Shoot poles from 9 DAI_{30} seedlings were extracted in 50 mM Tris-HCl (pH 7.5) with 1 mM $MnSO_4$, and samples containing equivalent enzyme activity were incubated overnight with varying concentrations of pre-immune serum (closed squares) or antiserum (closed circles). Samples were centrifuged to pellet precipitated material, and the supernatants assayed for arginase activity. Values shown as a percentage of activity at a serum concentration of zero. Each value represents the mean ± SE of three independent experiments.

Figure 6: Immunoblot analysis of arginase protein levels in 10 DAI_{30} loblolly pine seedlings. Tissue indicated as follows: M, megagametophyte; H+R, hypocotyl and radicle; C, cotyledons; E, epicotyl. Five micrograms of phosphate buffer soluble protein loaded per lane. The blots were probed with a 1:5000 dilution of the primary antiserum. Blot depicted is representative of additional blots performed with independent protein samples.

M H+R C E

3.2 Arginase cDNA isolation and characterization

3.2.1 Isolation of an arginase cDNA

After testing the arginase antibody preparation, a loblolly pine cDNA library constructed from 9-10 DAI_{30} seedling tissue (Mullen, 1995) was screened with antiserum raised against the loblolly pine arginase subunit. Approximately 2.5 x 10^5 plaques were screened in duplicate in the initial round. In total, three rounds of plaque screening and one round of colony screening resulted in the identification of four potential positive clones. Each was sequenced from both ends of the insert and homology searches were performed using the National Center for Biotechnology Information's online Basic Local Alignment Search Tool (BLAST). All four cDNAs showed highest sequence identity with plant arginases. Comparison of the obtained sequences with each other suggested that they call coded for the same mRNA. Because of this, the largest cDNA clone, designated pARS20, was chosen and sequenced to entirety.

pARS20 encodes a 1366 nucleotide cDNA (Figure 7). The insert contains a 341 amino acid open reading frame (Figure 8) as well as both 3' and 5' untranslated regions and poly-A tail. The loblolly pine arginase sequence includes two potential polyadenylation signals (Joshi, 1987), AATAA and AAATTA, 193 and 226 basepairs downstream of the predicted stop codon. The initial methionine codon (nt. 14-16) is surrounded by a consensus sequence for translation initiation in plants (Kozak, 1984; Lutcke et al. 1987). Interestingly, the third methionine in the amino acid sequence shares a variation on this sequence as well. Nucleotide sequence comparison with known plant arginases yields 56 and 58% nucleotide identity with Arabidopsis and soybean sequences, respectively.

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   1 ggcacgagga gcaatggggt ccatgggaaa aatggtgatg aggtttctgc agaagcgtag
  61 tttggcaact ttaccatcac aaatgataga gaagggccaa aaccgtgttg tggaagcttc
 121 ccttaccctg atcagggaga gagcaaaact caaggcagaa ttggtgcagg cattgggagg
 181 ctcaattgca acgacttgcc ttctaggagt tcctttgggg cacaattcat ctttccttca
 241 aggccctgca ttcgctcctc ctcgcattcg agaagctatt tggtgtggta gtacaaattc
 301 cgcgactgag aaagggaaag aattgaaaga ctcgagagtg ctgtcagatg ctggagatgt
 361 tccaattcaa gaaatgcgag attgtgggat tgaagatgag aggttaatga aaactgtcag
 421 tgactctgta aaaattgtaa tggaggagcc tccacttcgt ccattggttt taggtggcga
 481 tcattcaata tcctacccag ttgttaaggc tgttacagac caccttggag ggccagtgga
 541 tattcttcat ttagatgctc atcctgatat ttatgatgct tttgaaggaa ataagtattc
 601 acatgcttct tcatttgcgc gaattatgga gggtggtcat gcaaggcgac ttttgcaagt
 661 gggcatcagg tctataacaa aggaaggtcg ggagcaaggg aaaagatttg gagtagaaca
 721 atatgaaatg cacagtttca gtaaagatcg tgatttcttg gagaatctga aacttgggga
 781 aggtgtgaaa ggcgtttata tctcaattga tgtggattgc cttgatccgg catttgcacc
 841 tggagtctcg cacctggaac cgggtggtct ctcttttcgt ggtgtcatga accttgtaca
 901 aaatttgcaa ggagacattg tggcggctga tgttgtggaa tttaatccac aacgtgacac
 961 agttgatgga atgacagcaa tggttgctgc aaagcttgta agagagctga cgtcaaagat
1021 gtctaagttg gctcattgaa agcagccatg atctattctg tttcatgata catgagatct
1081 gtaacaggag gaagttctac aattttgtgt gtacttgaga gaataaaggc ctccatgtta
1141 gggttcttct ttgtagaagt gactgaagaa tatcaaaagc ctcagtccat ggatgcatca
1201 attttgaact atcctgtgaa tgcttgacat aaataagtga atgatcaggc tcttcttgga
1261 tagtttcaaa ttatttcgtt tgtctattca tttgttcaaa tttatttaat gagtaaatgc
1321 ttcaatcaat tggtttctgg tgattaaaaa aaaaaaaaaa taaaaa
```

Figure 7: Arginase cDNA nucleotide sequence. Complete nucleotide sequence of ARS20. The predicted initiation and stop codons are highlighted in bold. Potential polyadenylation sites are underlined.

The 341 amino acid peptide encoded by pARS20 has a predicted molecular weight of approximately 37 kDa and a pI of roughly 6.4, similar to the deduced size and pI of the protein sequences deduced from Arabidopsis and soybean cDNAs. Sequence alignments at the amino acid level (Figure 8) show 68% identity with the soybean sequence and over 78% with Arabidopsis. In addition, most of the amino acid divergence between the three deduced amino acid sequences occurs at the N-terminal end (Figure 8). Loblolly pine arginase contains all ten amino acids determined to be invariant within the arginase family as well as a number of other amino acids identified as important for arginase activity (Perozich *et al.*, 1998).

3.2.2 Southern blotting

Southern blot analysis of the loblolly pine genome using the arginase cDNA as a probe (Figure 9) suggests that there is a single copy of this gene. The ARS20 cDNA contains no EcoRI or Kpn I sites, but does contain two Hind III recognition sequences, one very near to the 5' end. Similar results were obtained digesting the DNA with additional enzymes not shown. No additional bands were present when the final high stringency wash was omitted, even when blots were overdeveloped.

3.2.3 Arginase mRNA tissue distribution

To examine the relative distribution of the arginase transcript in different tissues, total RNA was isolated from megagametophytes, root poles and shoot poles of 10 DAI_{30} seedlings. In addition, RNA was isolated from secondary needles 31 days after germination. The RNA was separated by formaldehyde-agarose electrophoresis and

Figure 8: Amino acid alignment of plant arginases. Deduced amino acid sequences of the ARS20 cDNA isolated from loblolly pine, *Pinus taeda*, (GenBank Accession #AF130440) aligned with arginase sequences from *Arabidopsis thaliana* (Acc. #U15019) and soybean, *Glycine max* (Acc. #AF035671). Identical amino acids boxed in black, similar amino acids in gray. Invariant positions within the arginase family (Perozich et al., 1998) denoted with asterisks. Note that the soybean sequence lacks the conserved proline (Pro-276 in *P. taeda* sequence).

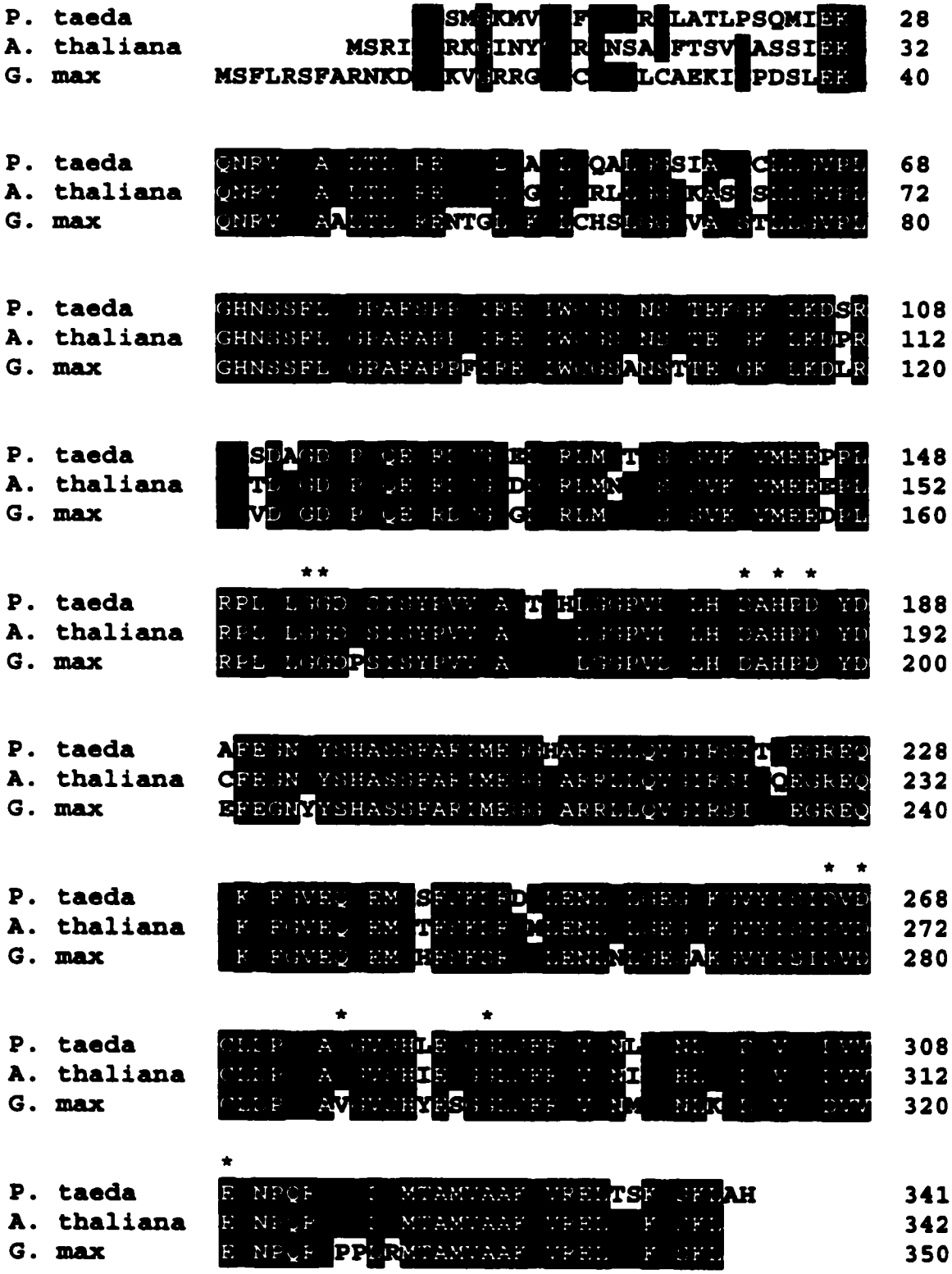


Figure 9: Southern blot of genomic DNA. Genomic DNA digested with EcoRI (E), Hind III (H) or Kpn I (K) hybridized with the pARS20 probe at high stringency wash conditions. 15 μg genomic DNA loaded per lane. Filter was exposed for 4 days at -80°C with an intensifying screen.

probed with radioactive probes generated using the ARS20 cDNA as a template. Relative distribution of the arginase mRNA is shown in Figure 10. The transcript is most abundant in the shoot pole of the 10 DAI_{30} seedlings, is much less abundant in the megagametophyte, and is nearly undetectable in the root pole and in the 31-day-old needles (Figure 10). Over 85% of the arginase message in seedlings 10 days after imbibition resides in the expanding cotyledons and shoot meristem. This relative distribution is similar to the distribution of both arginase protein levels (Figure 6) and enzyme activity (King, 1998).

3.2.4 *In vitro* transcription and translation of the arginase cDNA

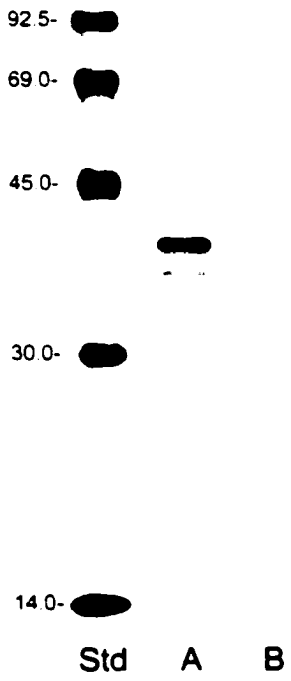
To confirm that the entire arginase coding sequence had been obtained, *in vitro* transcription and translation of pARS20 was performed. Radiolabeled translation products were separated by SDS-PAGE and detected by fluorography. Transcription and translation of the arginase clone resulted in a major product estimated at 37-39 kDa, similar to the size of the band detected by immunoblotting, as well as a minor translation product which migrates faster (Figure 11, lane A). No protein was detected in control reactions containing RNAse free water in place of plasmid DNA (Figure 11, lane B).

3.2.5 Arginase protein post-translational processing

To determine if the arginase protein is processed following translation, the pARS20 transcription and translation product was compared with arginase protein immunoprecipitated from soluble protein extracts obtained from ^{35}S-methionine labeled, 9 DAI_{30} shoot poles. After separation by SDS-PAGE and visualization by fluorography

Figure 10: Tissue Distribution of Arginase mRNA. Northern analysis of total RNA isolated from 10 DAI_{30} megagametophytes (M), root poles (R), shoot poles (S) and 31 DAG needles (N) Ten micrograms total RNA loaded per lane. RNA was visualized by ethidium bromide staining prior to transfer to ensure RNA integrity and equal loading. Blot depicted is representative of additional blots performed using independent RNA extracts.

Figure 11: *In vitro* transcription and translation of pARS20. Transcription and translation of the arginase cDNA was performed from the T3 promoter of the insert carrying pBluescript plasmid (pARS20). Translation products were diluted 50-fold and 7.5 μL was run on 12% SDS-polyacrylamide gels. Gels were subjected to fluorography and exposed overnight. Std, ^{14}C-labelled molecular weight standards. Lane A, pARS20 translation products. Lane B, control reaction containing no plasmid DNA. Molecular weights of markers (in kDa) are indicated to the left.



(Figure 12) it was observed that the immunoprecipitated protein (Figure 12, lane B) migrated slightly faster than the *in vitro* transcription and translation product (Figure 12, lane A, arrowhead), suggesting a slightly lower subunit molecular mass.

To confirm that the size of the translation product generated from the pARS20 plasmid template was the same as that generated *in vivo*, total RNA was isolated from 9 DAI_{30} shoot poles and translated *in vitro* in the presence of ^{35}S-methionine. The translation products were immunoprecipitated. *In vitro* translated total RNA produced a protein band identical in size to that produced by the cloned cDNA (Figure 13, lane A) which was also slightly larger than arginase protein immunoprecipitated from 9 DAI_{30} shoot poles (Figure 13, lane B). The same data were obtained from *in vitro* translated mRNA (data not shown). Closer analysis of the labeled protein immunoselected from the 9 DAI_{30} shoot poles shows that the peptide band produced from *in vitro* translation of 9 DAI_{30} total RNA (Figure 14, lane A) is also present in the immunoprecipitated 9 DAI_{30} soluble protein sample (Figure 14, lane B, arrowhead). This band may be the precursor form of the mature arginase protein subunit.

3.3 Partial purification of loblolly pine arginase

3.3.1 Purification

The initial purification of arginase sized the holoprotein at 140 kDa with a subunit size of 43 kDa, predicting a homotrimer (King, 1998). Subsequent characterization of the antibodies (section 3.1) and cloning of the arginase cDNA (section 3.2.1) suggested that the peptide making up the arginase subunit was somewhat smaller. To address this

Figure 12: Immunoprecipitation of radiolabeled arginase. Lane A –*In vitro* transcription and translation of the arginase cDNA (pARS20) as in Figure 11. Lane B – ^{35}S-methionine labeled arginase protein immunoprecipitated from 9 DAI_{30} shoot poles. Proteins were run on 12% SDS-polyacrylamide gels. Gels were subjected to fluorography and exposed overnight. Molecular weights of markers (in kDa) are indicated to the left.

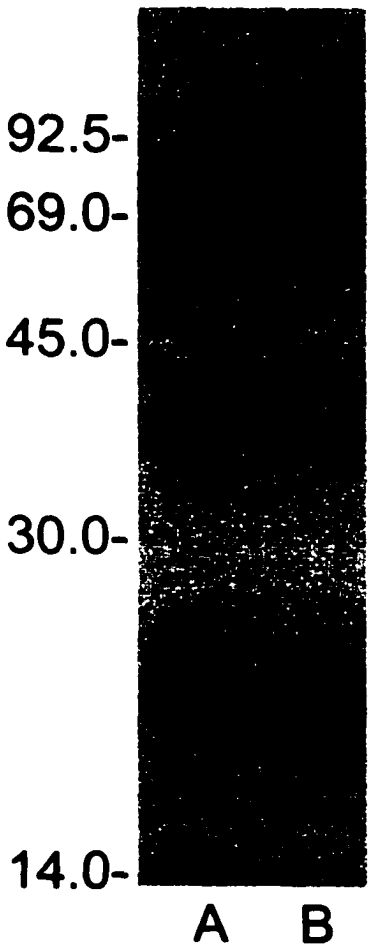


Figure 13: *In vitro* translated and immunoprecipitated arginase. Lane A – Immunoprecipitated ^{35}S-methionine labeled *in vitro* translation products from 9 DAI_{30} shoot pole total RNA. Lane B - ^{35}S-methionine labeled arginase protein immunoprecipitated from 9 DAI_{30} shoot poles. Both samples were immunoprecipitated from equal counts *in vitro* translated/extracted soluble protein. Proteins were run on 12% SDS-polyacrylamide gels. Gels were subjected to fluorography and exposed overnight. Molecular weights of markers (in kDa) are indicated to the left. Arrow to the left indicates the predicted arginase precursor protein.

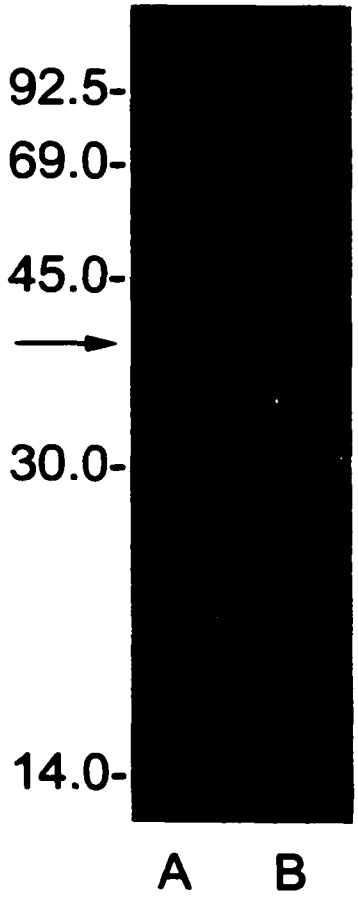
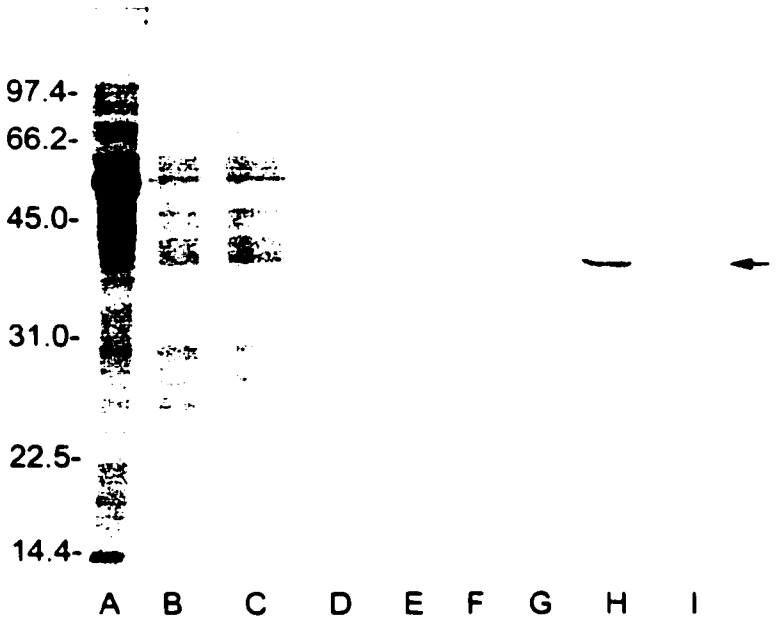


Figure 14: Immunoprecipitated arginase precursor protein. Lane A – Immunoprecipitated ^{35}S-methionine labeled *in vitro* translation products from 9 DAI_{30} shoot pole total RNA. Lane B - ^{35}S-methionine labeled arginase protein immunoprecipitated from 9 DAI_{30} shoot poles. Arrow indicates a faint band from the shoot pole soluble protein immunoprecipitation of the same molecular mass as the product pulled down from *in vitro* translated 9 DAI_{30} shoot pole total RNA.

discrepancy a partial purification of native loblolly pine arginase was performed to identify and size the holoprotein's subunit.

The purification protocol consisted of a heat treatment, separation on two molecular sieving columns and then a final purification step on an immunoaffinity matrix generated from the anti-arginase antibodies used to clone the cDNA (see section 2.11 in Materials and Methods for immunoaffinity matrix construction). Shoot poles from 9-12 DAI_{30} seedlings were homogenized in a buffer containing glycerol and manganese (STMG buffer), to aid in protein stability throughout the process. After a 20 minute heat treatment which removed much of the protein, including several intense bands, (Figure 15, lane B) the extract was passed over a Sephacryl S-200 HR molecular sieving column and separated in STMG buffer. Arginase eluted early in the elution profile in a single peak of activity, separating it from much of the protein contained therein (Figure 16). Those fractions containing arginase activity were pooled and applied to a Sephacryl S-300 HR molecular sizing column and again eluted in STMG buffer. Arginase again eluted in a single peak (Figure 17). Those fractions containing active arginase were then pooled and applied to an anti-arginase immunoaffinity matrix and washed with STMG buffer. Much of the active arginase applied to the column eluted in the flow through and wash fractions with the majority of the protein applied to the column (Figure 18). After A_{280} values of the eluate approached zero, the column was eluted at low pH with 0.2 M Glycine-HCl (pH 2.5) to disrupt the antibody-antigen complex. A small, but discernable peak of arginase activity was detected in several fractions (Figure 18) and these fractions were concentrated and analyzed by SDS-PAGE. One protein band in the predicted size

Figure 15: Partial purification of native arginase. SDS-PAGE profiles of pooled fractions from each stage of the purification process. Samples were electrophoresed on 12 % separating gels under reducing conditions. Lanes A, crude homogenate; B, supernatant from heat-treated homogenate; C, pooled active fractions from the Sephacryl S-200 molecular sieving column; D, pooled active fractions from the Sephacryl S-300 molecular sieving column; E through I, fractions 31 through 35 from the immunoaffinity matrix. The arrow to the right of the gel designates arginase. Molecular mass markers (in kDa) are indicated to the left of the gel.



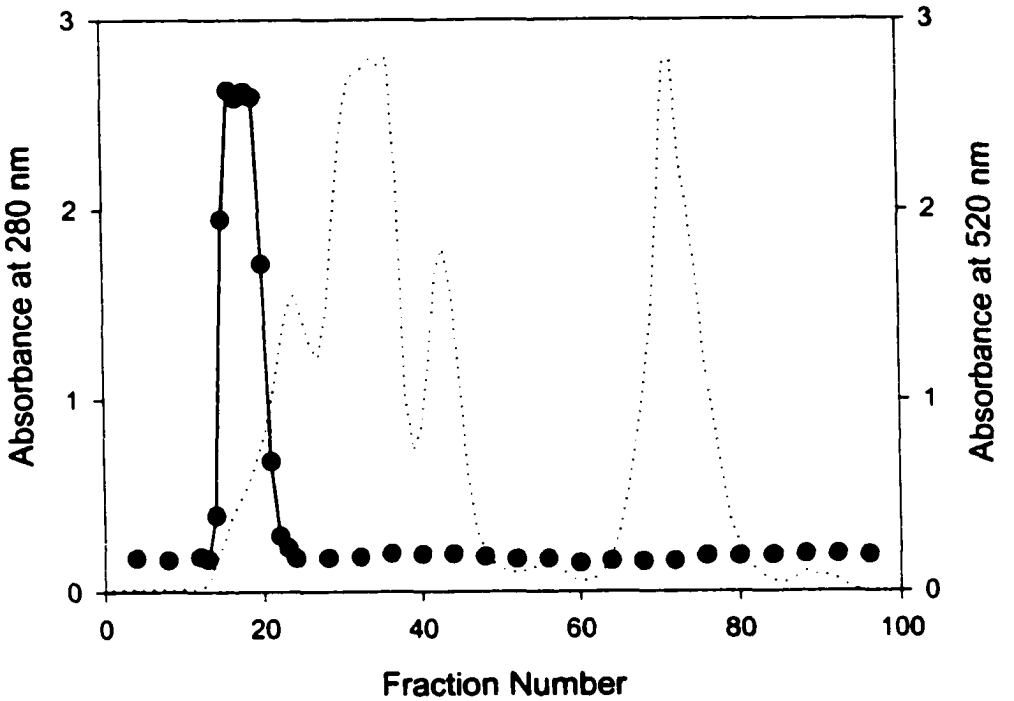


Figure 16: Sephacryl S-200 HR column elution profile. Elution profiles of arginase activity (closed circles) are shown superimposed upon total protein profiles (dotted lines). The latter were estimated by measuring absorbance values at 280 nm. Arginase activity is expressed as an increase in optical absorbance at 520 nm.

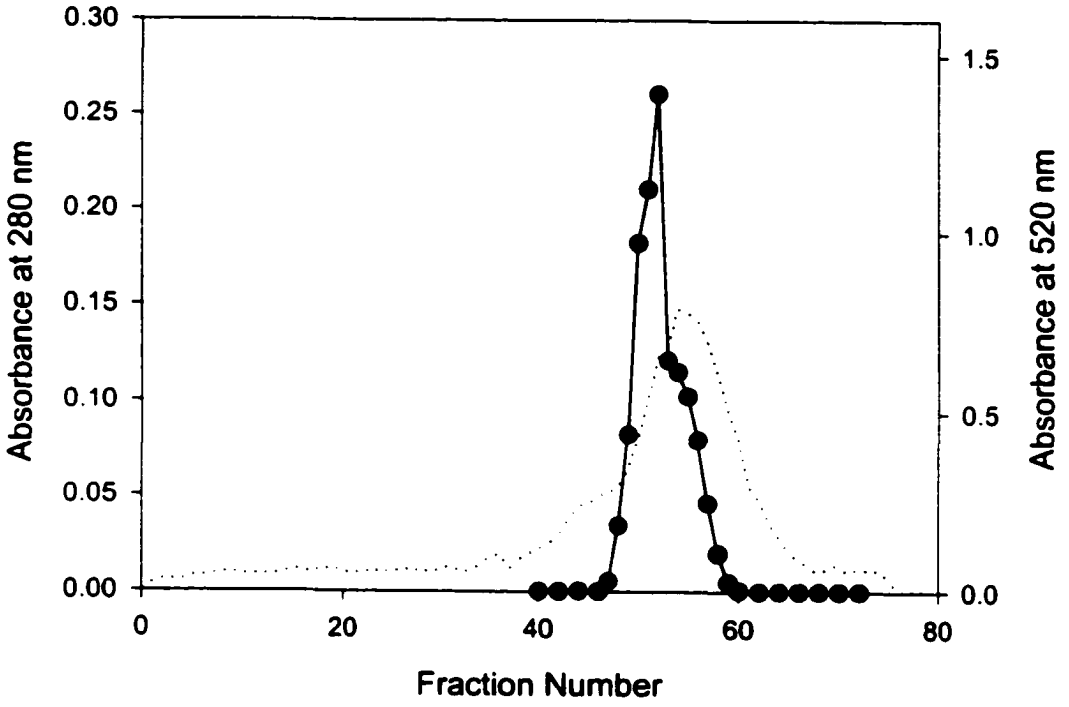


Figure 17: Sephacryl S-300 HR column elution profile. Elution profiles of arginase activity (closed circles) are shown superimposed upon total protein profiles (dotted lines). The latter were estimated by measuring absorbance values at 280 nm. Arginase activity is expressed as an increase in optical absorbance at 520 nm.

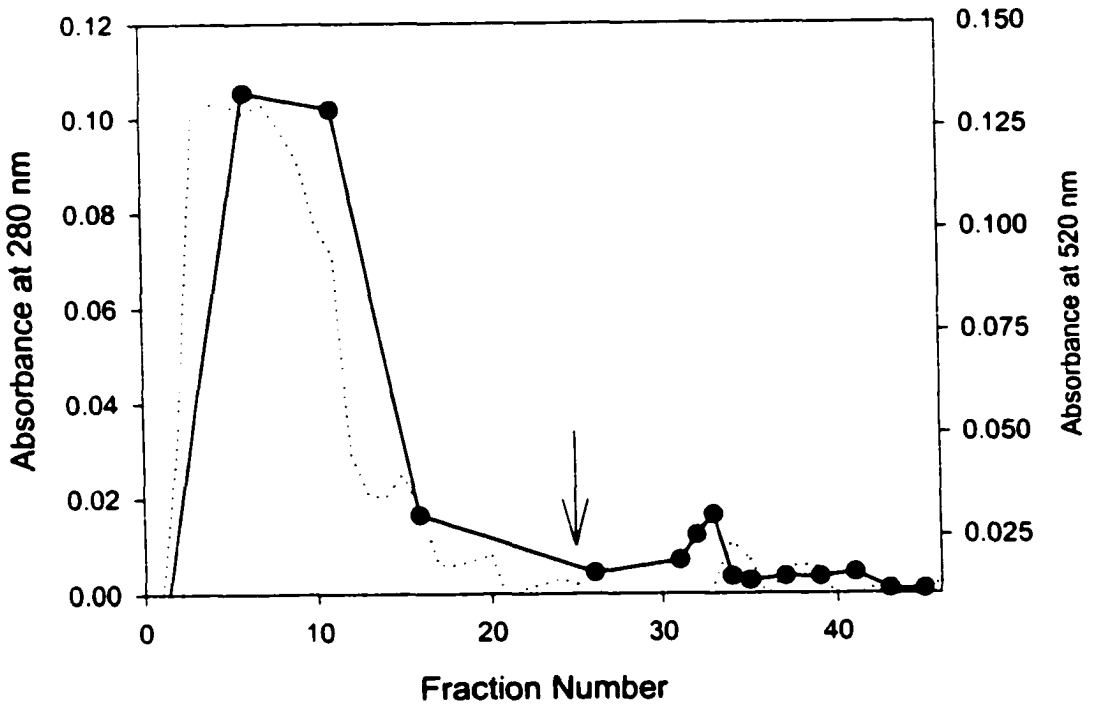


Figure 18: Anti-arginase immunoaffinity matrix elution profile. Elution profiles of arginase activity (closed circles) are shown superimposed upon total protein profiles (dotted lines). The latter were estimated by measuring absorbance values at 280 nm. Arginase activity is expressed as an increase in optical absorbance at 520 nm. Fractions 1-30, flow through and wash fractions. The column was eluted at low pH starting with fraction #31, indicated by arrow.

range was prominent in the fractions containing arginase activity (Figure 15, lanes H and I) and was used to size the arginase holoprotein.

3.3.2 Molecular sizing

The protein band identified as arginase in Figure 15 was used to determine the arginase subunit's molecular mass based on it's relative migration through polyacryiamide gels (Weber and Osborn, 1969) (Figure 19). From these data, the predicted subunit size was determined to be 36.8 kDa. For the remainder of this thesis, this will be referred to as the 37 kDa subunit. Based on a holoprotein size of 140 kDa (King, 1998), arginase is predicted to be a homotetramer rather than a homotrimer predicted previously (King, 1998).

3.4 Developmental regulation of arginase during germination and early seedling growth

3.4.1 Arginase enzyme activity and protein levels

Increased arginase activity during loblolly pine germination and early seedling growth has been demonstrated previously (King and Gifford, 1997). The majority of enzyme activity is confined to the shoot pole, defined as the expanding cotyledons and apical meristem, where it accounts for 94% of arginase activity by 12 DAI_{30} (King and Gifford, 1997). Figures 20A and B depicts the changes in arginase protein levels in mature and stratified embryos and in the shoot poles of loblolly pine seedlings throughout germination and early seedling growth. Arginase protein levels begin to increase after germination has been completed at 4 DAI_{30}. After this, protein levels increase

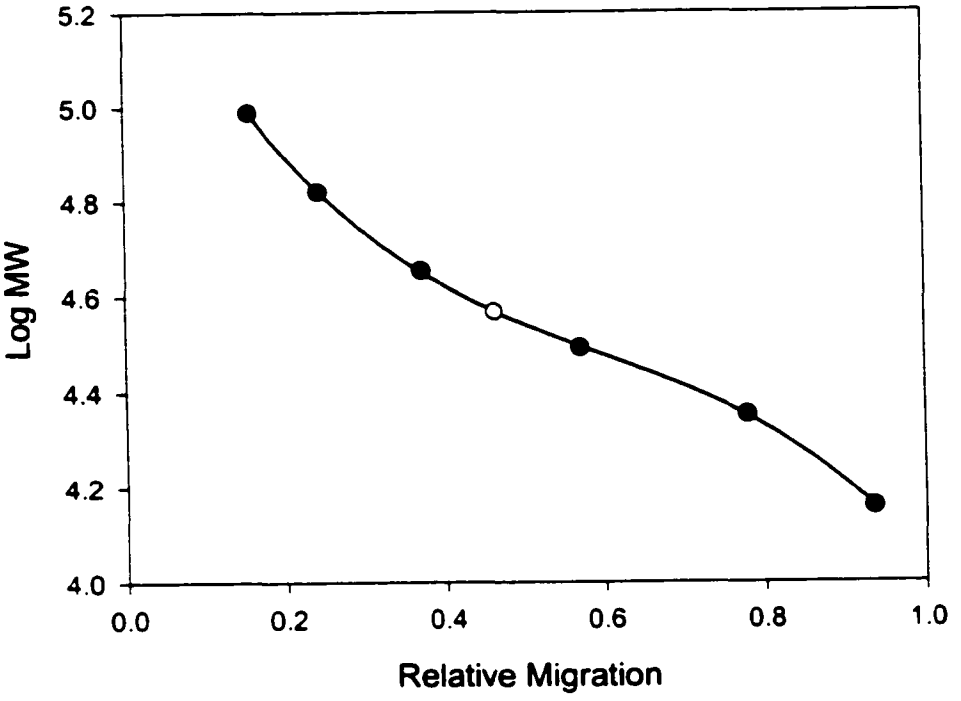
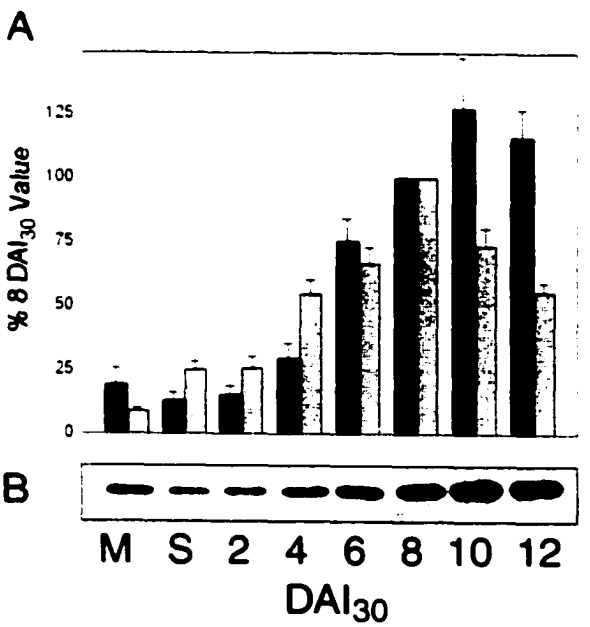


Figure 19: Arginase subunit molecular mass determination. Subunit molecular mass was determined by electrophoresis through 12% SDS-PAGE gels. Relative migration of molecular weight markers (closed circles) and partially purified arginase (open circle) are shown. The cubic regression of the molecular weight markers is indicated by the solid line and the equation of this line was used to determine the molecular mass of arginase based on its relative migration.

Figure 20: Relative changes in arginase enzyme activity and protein levels. (A) Developmental profile of arginase protein and enzyme activity is shown for whole embryos isolated from mature (M) and stratified (S) seed and for shoot poles from 2-12 DAI_{30} seedlings. (B) Immunoblot shown is a representative of similar blots achieved using independent protein extracts. For immunodetection 10 μg protein was loaded in each lane and anti-arginase antiserum was used at a 1:5000 dilution. Chemiluminescent detection was employed and signals were captured on Kodak X-OMAT AR film. Arginase specific activity (in gray) was normalized to the 8 DAI_{30} value for comparison with protein levels quantified from immunoblots using a Bio-Rad 670 densitometer and normalized to the 8 DAI_{30} band (black). Values shown were generated from three independent replicates + SE.

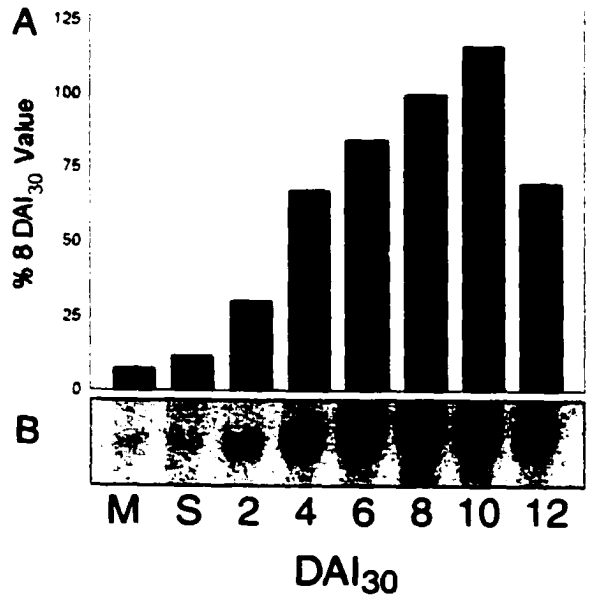


throughout early seedling growth, declining slightly by 12 DAI_{30}. Figure 20A shows changes in arginase enzyme activity and protein levels during germination and early seedling growth normalized against the 8 DAI_{30} values. In loblolly pine shoot poles arginase specific activity remains low until germination has been completed at 4 DAI_{30}, increases to a maximum value around 8 DAI_{30} and falls off by 12 DAI_{30}. The accumulation of arginase protein appears to follow this trend for the first eight days after imbibition, however it appears that protein continues to accumulate to day 10 before declining, by which time specific activity has begun to decline (Fig. 20A). Western blots of all tissues and stages examined showed that the antibodies were specific for the 37 kDa arginase subunit, even when color reactions were overdeveloped or chemiluminescent blots were overexposed.

3.4.2 Arginase transcript abundance

Northern analyses were performed using total RNA isolated from mature and stratified loblolly pine embryos and shoot poles from 2-12 DAI_{30} seedlings. Figure 21 B shows that arginase transcripts are barely detectable at low levels in mature and stratified seed and increase following transfer to germination promoting conditions. Arginase mRNA levels reach a peak 10 DAI_{30} and then begin to decline. For comparison with the protein and enzyme data, relative transcript levels from the blot depicted are shown in Figure 21A, again normalized to the 8 DAI_{30} value. Similar to what is observed for protein accumulation, arginase mRNA appears to begin to accumulate following germination, reaching a peak at 10 DAI_{30} and then declining.

Figure 21: Developmental profile of arginase transcript levels.
Developmental profile of arginase mRNA abundance is shown for whole embryos isolated from mature (M) and stratified (S) seed and for shoot poles from 2-12 DAI_{30} seedlings. Ten μg total RNA per lane was hybridized with probes generated from the loblolly pine arginase cDNA. (A) Relative changes in arginase mRNA levels were quantified as in Figure 20 and normalized to the 8 DAI_{30} band. (B) Blots were exposed for 4 days at -80°C with intensifying screens. Blot shown is a representative of additional blots performed using different RNA samples. Formaldehyde agarose gels were stained with ethidium bromide prior to hybridization to ensure equal loading (not shown).



3.5 Expression of loblolly pine arginase in *Escherichia coli*

3.5.1 Production of a recombinant arginase and demonstration of enzyme activity

To further confirm that the pARS20 encoded a functional arginase subunit and to purify sufficient quantities of the protein to generate antibodies, the arginase coding sequence was cloned into an *E. coli* expression vector which added an N-terminal histidine tag to the peptide. Primers for PCR were designed to incorporate a new, unique BamHI restriction enzyme recognition site within the coding sequence of the cDNA and a SalI site in the 3' untranslated region. These allowed for the amplified fragment to be directionally cloned, in the correct reading frame into pQE-31, an *E. coli* expression vector containing its own initiation codon and a 6xHis epitope, six consecutive histidine codons, used for immobilization of the recombinant protein through metal chelation affinity chromatography. The cloning strategy employed resulted in the removal of the first four amino acids from the cDNA's deduced amino acid sequence and the addition of 13 amino acids, including the engineered start codon and histidine tag in their place (Figure 22). The *LacZ* inducible promoter was used to control expression of the recombinant cDNA. Transformants were tested for the presence of the correct insert by restriction digests of plasmid minipreps. Positive clones were then tested for the ability to produce a protein of the predicted size using protein minipreps (Section 2.12.1). One of these clones, designated pHAR-2, was used for protein purification.

Prior to large scale purification, the solubility of the protein produced by pHAR-2 transformants in *E. coli* was tested. After induction with IPTG, cell pellets were extracted in native lysis buffer (section 2.12.2) and then in a denaturing lysis buffer (section 2.12.2) to determine if the target protein was present in the soluble extract or in

Figure 22: N-terminus of His-tagged arginase. The first 20 amino acids of the recombinant His-tagged arginase construct shown aligned with the original N-terminal region of the protein. Amino acids highlighted in gray were derived from the cloning vector and PCR primer. The four amino acids in white, including the predicted initiating methionine were removed from the N-terminal region of the cDNA coding sequence.

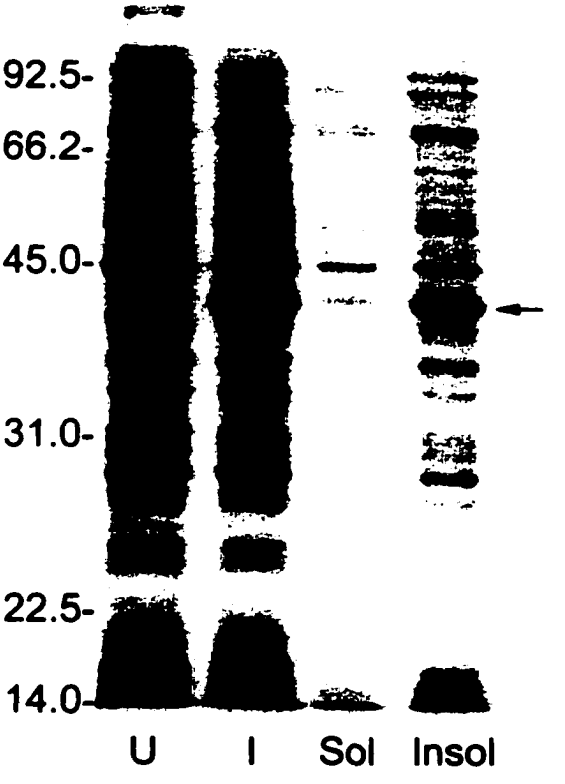
the resultant pellet. As shown in Figure 23, the majority of the 38 kDa protein whose production was induced by IPTG was found in the insoluble pellet. It is estimated that greater than 95% of the recombinant protein was not solubilized in aqueous buffer and therefore purification was carried out under denaturing conditions after removal of the soluble protein fraction.

Even though the vast majority of the recombinant protein was found in the insoluble fraction, some protein appeared to be soluble in aqueous buffer (Figure 23). To confirm that the 38 kDa protein produced was indeed a recombinant arginase, aliquots of the culture used for protein purification were collected before and after induction of the culture with IPTG. The soluble protein fraction from these aliquots was then tested for arginase activity. After induction, the culture showed a > 21-fold increase in arginase specific activity (Figure 24). The small amount of activity in the absence of IPTG was attributed to leaky expression from the plasmid prior to the addition of the inducer.

3.5.2 Purification of a His-tagged arginase from *E. coli*

Six liters of bacterial culture harboring the pHAR-2 plasmid was induced to produce the recombinant arginase protein by induction with 1 mM IPTG. After removal of those proteins soluble in native lysis buffer, the remaining pellet was solubilized in denaturing lysis buffer and the recombinant protein was selected by immobilized metal affinity chelation (IMAC) on a nickel-charged affinity matrix. After elution from the matrix, the majority of the protein selected was the full sized (~38 kDa) recombinant arginase (Figure 25, lane A). There was however, substantial contamination by additional bacterial proteins and by a two peptides approximately 28 kDa in size. The 28 kDa

Figure 23: Solubility of His-tagged arginase. Total *E. coli* protein from pHAR2 cultures; un-induced total protein (U) and total protein after induction with IPTG (I). Total induced protein split into soluble (Sol) and insoluble (Insol) fractions. Arrow to the right indicates the induced fusion protein. Molecular size markers (in kDa) indicated to the left.



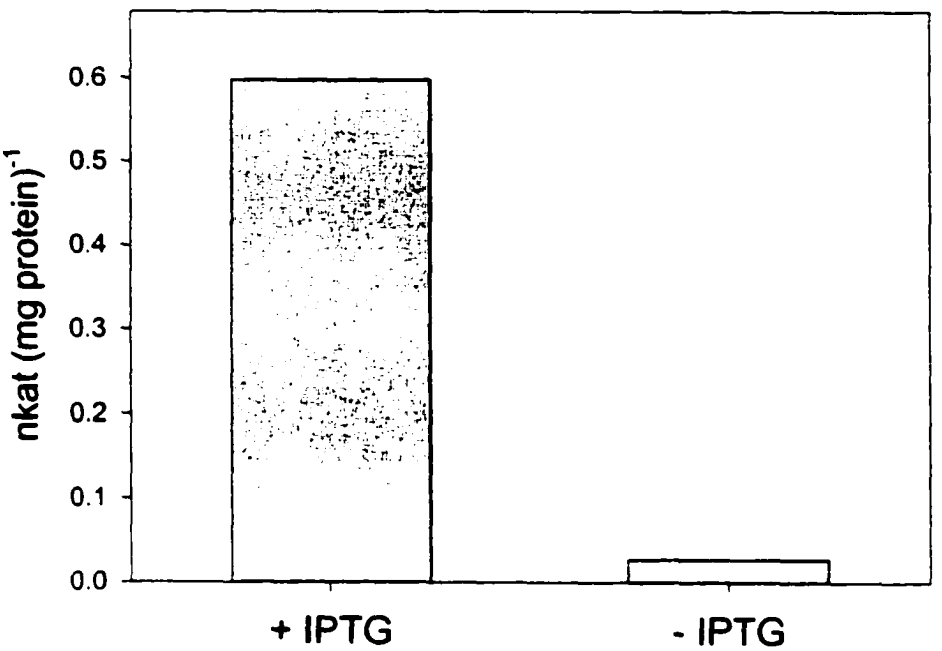
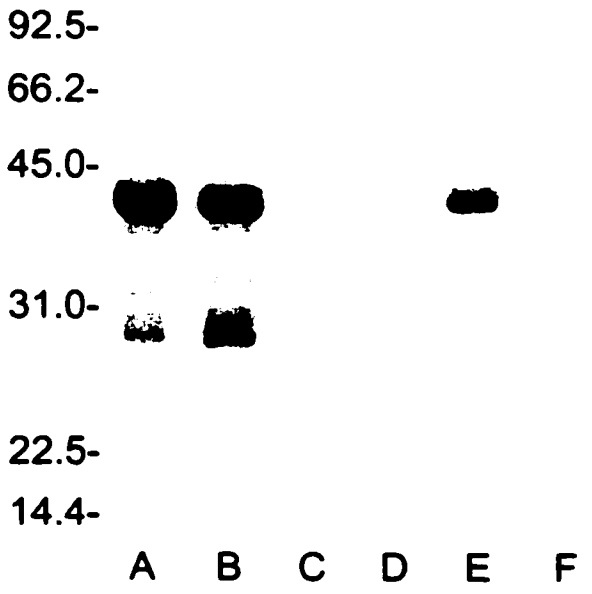


Figure 24: Induction of arginase activity with IPTG. Arginase specific activity from the induced and un-induced pHAR2 culture used for protein purification. Enzyme activity is shown as nanokatals per mg protein assayed.

Figure 25: Purification of His-tagged arginase. SDS-PAGE profiles of pooled fractions from each stage of the purification process. Samples were electrophoresed on 12 % separating gels under reducing conditions. Lanes A, pooled fractions after first pass over immobilized metal affinity column; B, pooled fractions after second pass over immobilized metal affinity column; C, first Tris-buffer extraction of precipitated protein pellet following dialysis to remove urea; D, second Tris-buffer extraction of precipitated protein pellet following dialysis to remove urea; E, first urea-buffer extraction of precipitated protein pellet following dialysis; F, second urea-buffer extraction of precipitated protein pellet following dialysis. Molecular mass markers (in kDa) are indicated to the left of the gel.



peptides were determined in preliminary experiments to be a truncated translation product of the protein of interest, however similar sized proteins have been identified as bacterial contaminating proteins using this technique (Yu and Owttrim, 2000). The proteins eluted from the nickel charged column were subjected to an additional round of IMAC, which resulted in removal of some of the bacterial proteins, but retained the band of interest and the smaller peptide (Figure 25, lane B). Taking advantage of the insolubility of the recombinant protein in non-denaturing buffers, the urea was dialyzed away, causing aggregation and precipitation of the recombinant protein. The precipitate was collected, extracted twice with native lysis buffer and twice with denaturing lysis buffer. Most of the pHAR-2 translation product was solubilized in the first urea extraction (Figure 25, lane E) and showed that contaminating bacterial proteins had been removed, but the truncated peptide remained. A small amount of these two proteins was present in the second urea wash. Since the goal of this purification was to generate sufficient protein to raise antibodies, the proteins in the first urea wash, representing the full length translation product and a truncated version of the same peptide, were used to raise antibodies in rabbits. In total, 1540 μg of recombinant arginase protein was recovered and used for immunization. The resultant antibody serum was mono-specific for arginase as determined by western blotting; dilutions of 1:20,000 or greater of the serum resulted in strong signals from western blots. These antibodies were used for the remainder of the thesis.

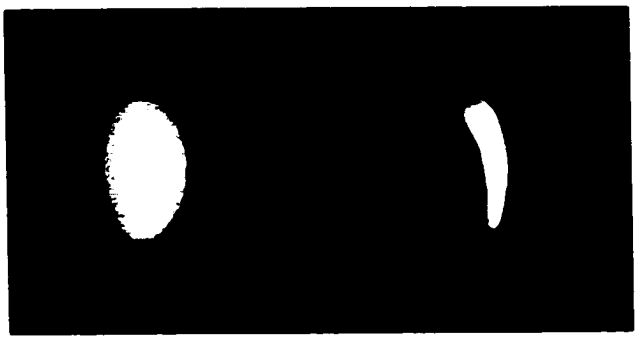
3.6 *In vitro* culture of loblolly pine embryos

To test the hypothesis that the megagametophyte is involved in the developmental regulation of arginase in the embryo or seedling an *in vitro* culture system was developed that permitted the growth of loblolly pine embryos with and without their associated megagametophyte tissue (depicted in Figure 26). The solid media the embryos were cultured on was derived from Becwar *et al.* (1990) as modified for germination of loblolly pine somatic embryos by Rutter *et al.* (1998a, 1998b).

3.6.1 Growth of pine embryos with and without the megagametophyte

Intact embryos were cultured *in vitro* with and without their associated megagametophytes for up to 10 days at 30°C. Germination was completed by 2 days in culture at 30°C, DIC_{30}, regardless of the presence of the megagametophyte. For the first four days in culture both embryo types elongated at approximately the same rate regardless of the presence of the megagametophyte (Figure 27). After 4 DIC_{30} however, the presence of the megagametophyte tissue resulted in increased seedling growth (Figure 27). The lack of the megagametophyte strongly affected cotyledon development. In the absence of the maternal tissue, cotyledon expansion was dramatically reduced (Figure 28). Seedling growth as demonstrated by fresh weight increases was initially similar, however after approximately six days in culture those seedlings cultured in the presence of the megagametophyte showed a marked increase in fresh weight gain over those cultured as isolated embryos. By 10 DIC_{30} seedlings cultured in the presence of the megagametophyte had a fresh weight of more than double that of those cultured in its absence, 77.1 ± 1.7 mg per seedling as compared to 32.3 ± 2.5 mg per seedling (Figure 29).

Figure 26: Seed tissue used for *in vitro* culture. Isolated loblolly pine embryo (right) and embryo still encased in the megagametophyte following removal of integument layers and nucellar cap. Scale bar = 2.5 mm.



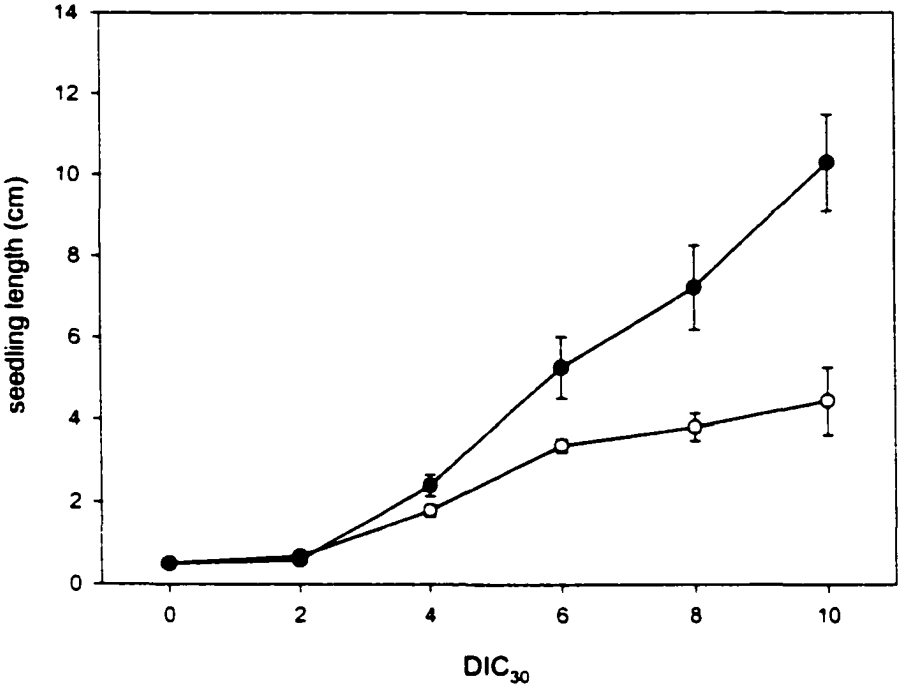


Figure 27: Effect of the megagametophyte tissue on seedling length. Increases in seedling length are shown in the presence (closed circles) and absence (open circles) of the megagametophyte tissue under culture conditions at 30°C. Seedling length is depicted in cm ± SE.

Figure 28: Seedling growth ± megagametophyte tissue. Left to right, 4-10 DIC_{30} seedlings cultured with (Top, +M) and without the megagametophyte (Bottom, -M). Scale bar = 1.0 cm.

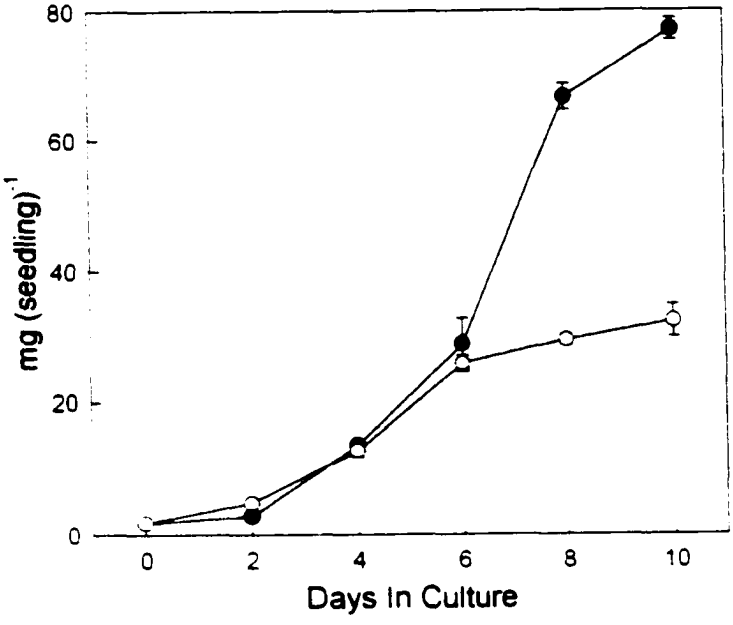


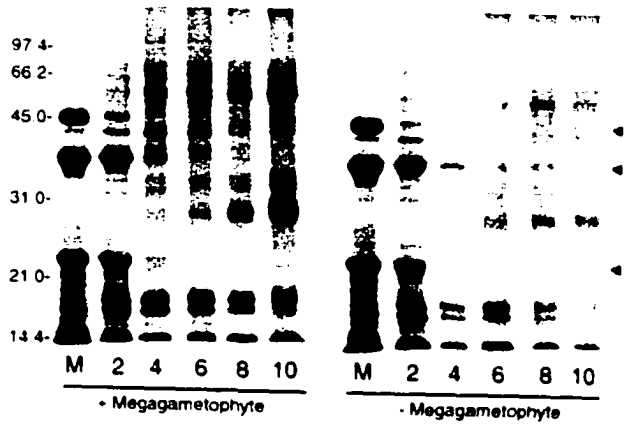
Figure 29: Fresh weight increases in seedlings. Data shown as mg FW (seedling)$^{-1}$ is the mean of 3 independent replicates of 10 seed parts each ± SE. Closed circles – seedlings cultured in the presence of the megagametophyte tissue; open circles – seedlings cultured without megagametophytes. Seedlings were cultured for up to 10 days at 30°C.

3.6.2 The effect of the megagametophyte on shoot pole soluble and insoluble protein

The effect of the megagametophyte tissue on storage protein breakdown and soluble and insoluble protein accumulation in the shoot pole was investigated in the cultured seedlings. SDS-PAGE of shoot pole buffer-insoluble protein shows that seedlings growing in the presence or absence of the megagametophyte broke down their shoot pole storage proteins at relatively the same rate (Figure 30). In both cases the breakdown of the 47 kDa, 37.5 kDa and 22.5 kDa proteins appear to be the same and all three of these proteins have been completely degraded by 6 DIC_{30} in the presence and in the absence of the megagametophyte tissue (Figure 30).

In contrast, the accumulation of phosphate buffer-soluble and -insoluble proteins differed dramatically when the megagametophyte tissue had been removed. The presence of the megagametophyte resulted in a greater accumulation of both pools of protein. Buffer-insoluble protein levels remained the same in both classes of seedlings until 6 DIC_{30}, at which time shoot poles growing in the presence of the megagametophyte accumulated more insoluble protein, reaching a maximum of 428 ± 29 μg protein per seed part by 10 DIC_{30} compared to 97 ± 8 μg protein per seed part by the same stage in the absence of the megagametophyte (Figure 31). Similarly, shoot pole buffer-soluble protein levels increased to much higher levels in the presence of the megagametophyte than they did in the absence of the tissue; this accumulation began somewhat earlier following germination and is evident by 4 DIC_{30} (Figure 32). A maximum value of 1052 ± 74 μg per seed part shoot pole soluble protein was present by 10 DIC_{30} in the presence of the megagametophyte in comparison to a maximum value of 159 ± 2 μg protein per seed part found in the shoot poles of 6 DIC_{30} seedlings grown in its absence (Figure 32).

Figure 30: Breakdown of shoot pole seed storage proteins. Coomassie blue stained developmental profile of seedling shoot pole phosphate buffer-insoluble proteins for up to 10 days in culture with and without megagametophyte tissue. 3μg protein loaded per lane. Molecular weights (in kDa) indicated to the left of the figure. Solid arrowheads to the right of the gel indicate the position of the 47.0, 37.5 and 22.5 kDa seed storage proteins.



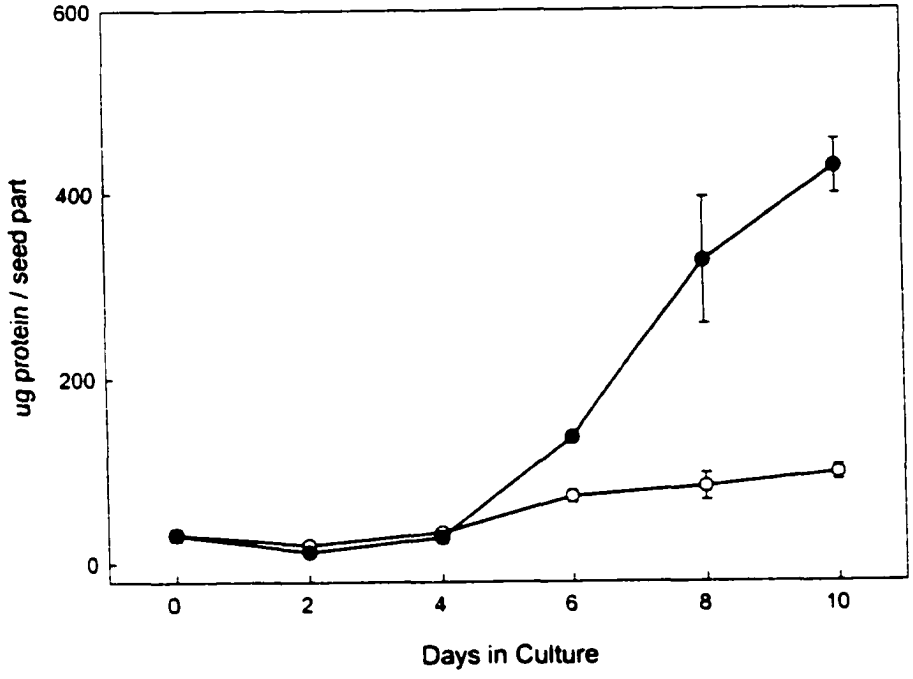


Figure 31: Quantitative changes in shoot pole buffer insoluble protein ± megagametophytes. Changes in phosphate buffer insoluble protein levels in seedling shoot poles cultured with (closed circles) and without (open circles) megagametophyte tissue for up to 10 days at 30°C. Data shown as μg protein per seed part (individual shoot pole). Values are the mean of at least two independent replicates each assayed in duplicate ± SE.

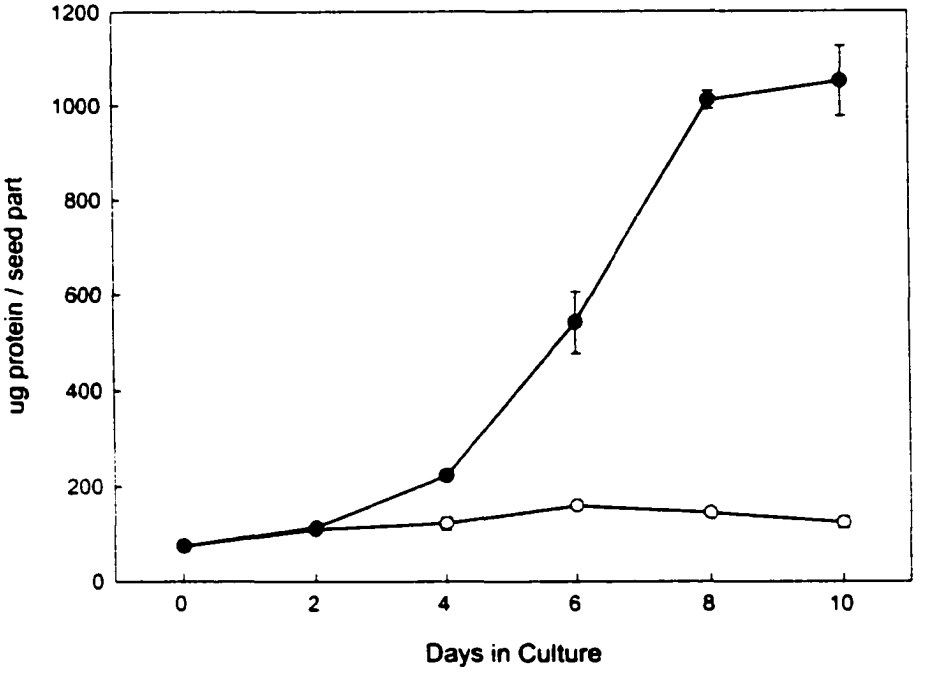


Figure 32: Quantitative changes in shoot pole buffer soluble protein ± megagametophytes. Changes in phosphate buffer soluble protein levels in seedling shoot poles cultured with (closed circles) and without (open circles) megagametophyte tissue for up to 10 days at 30°C. Data shown as μg protein per seed part (individual shoot pole). Values are the mean of at least two independent replicates each assayed in duplicate ± SE.

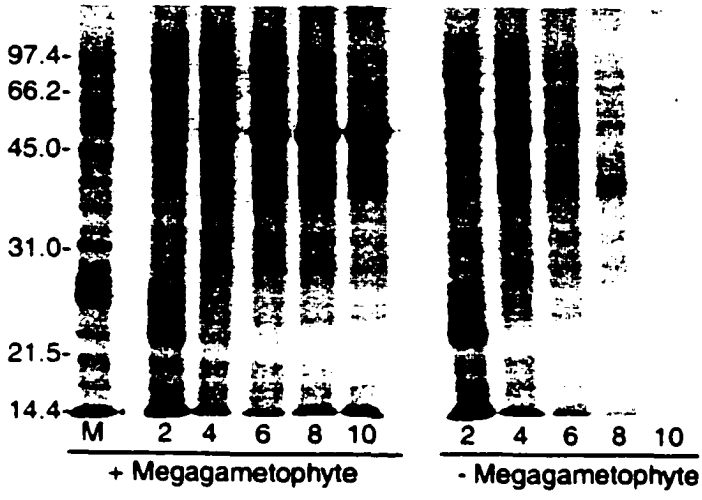
SDS-PAGE soluble protein profiles of shoot poles from seedlings grown in the presence of the megagametophyte clearly show the accumulation of protein bands such as the large subunit of RuBisCo (approx 50 kDa, Figure 33, + Megagametophyte) These were not present to such high levels in shoot poles grown in the absence of the megagametophyte (Figure 33, - Megagametophyte) and decreased from day 4 through day 10.

Phosphate buffer-insoluble protein levels in the megagametophyte were determined in order to understand the changes in the seedling relative to the timing of storage protein breakdown in the megagametophyte. As the megagametophyte buffer-insoluble protein is almost exclusively made up of storage protein, this gives a good indication of the rate of storage protein breakdown in this tissue. Breakdown of megagametophyte storage reserves began to occur as germination was complete at 2 DIC_{30} (Figure 34). The majority of reserve hydrolysis occurred between 4 and 6 DIC_{30}. By 10 DIC_{30}, the megagametophyte protein storage reserves were largely exhausted; the initial 871 ± 13 μg insoluble protein per megagametophyte had diminished to 53 ± 9 μg insoluble protein per seed part (Figure 34).

3.6.3 The effect of the megagametophyte on arginase in *in vitro* cultured seedlings

The presence or absence of the megagametophyte tissue had a dramatic effect on arginase enzyme activity in the *in vitro* cultured seedlings. In the presence of the megagametophyte, arginase enzyme activity per seed part increased from 0.43 ± 0.021 nkat per seed part at 2 DIC_{30} to a maximum value of 6.89 ± 0.42 nkat per seed part by 8 DIC_{30} before beginning to decline (Figure 35). In the absence of the megagametophyte, arginase activity at 2 DIC_{30} was very similar to the activity found in the presence of the

Figure 33: Changes in shoot pole buffer soluble proteins. Coomassie blue stained developmental profile of mature embryo and seedling shoot pole phosphate buffer-soluble proteins for up to 10 days in culture with and without megagametophyte tissue. 8µg protein loaded per lane. Molecular weights (in kDa) indicated to the left of the figure.



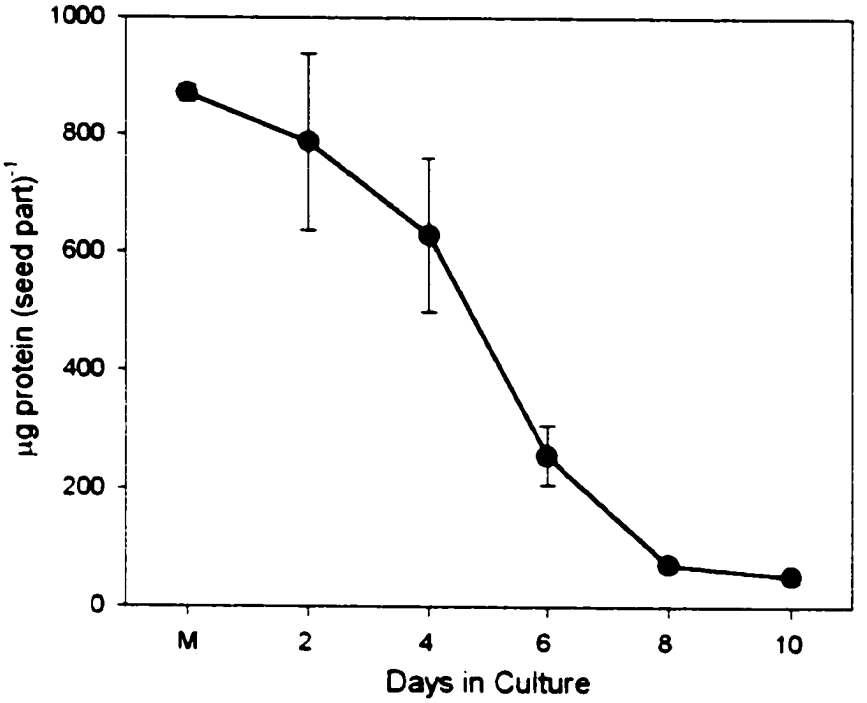


Figure 34: Quantitative changes in megagametophyte buffer insoluble protein. Changes in phosphate buffer insoluble protein levels in megagametophytes cultured with seedlings for up to 10 days at 30°C. Data shown as µg protein per seed part (individual megagametophyte). Values are the mean of at least two independent replicates each assayed in duplicate ± SE.

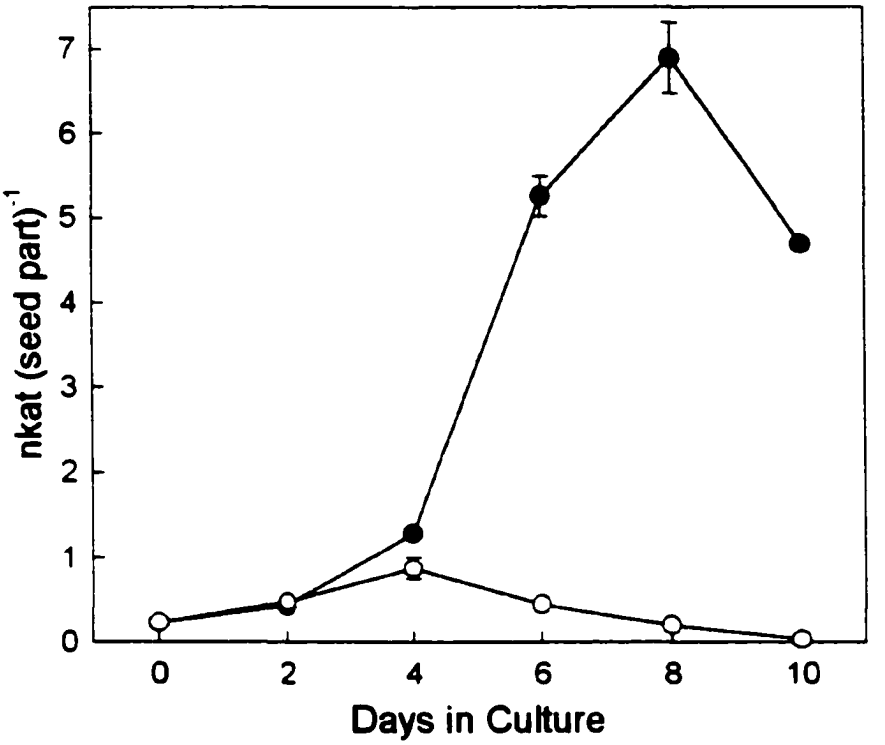
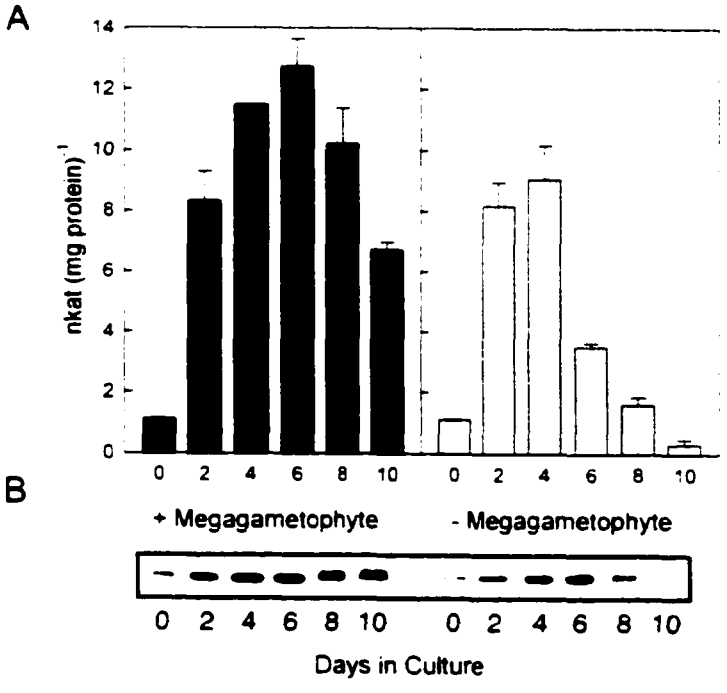


Figure 35: Shoot pole arginase activity per seed part. Cell free extract arginase activity expressed on a per seed part basis. Values shown are the mean of at least two independent replicates assayed in duplicate ± SE. Closed circles – arginase activity in shoot poles cultured with megagametophytes; open circles – arginase activity in shoot poles cultured without megagametophytes. Data is presented in nanokatals.

megagametophyte (0.48 ± 0.01 nkat (seed part)$^{-1}$); however, in the absence of the megagametophyte, enzyme activity only reached a maximum value of 0.87 ± 0.13 nkat per seed part, occurring at 4 DIC_{30}, before declining to almost zero by 10 DIC_{30} (Figure 35).

As shown in section 3.6.2, the presence of the megagametophyte resulted in a large difference in the shoot pole soluble protein pool (Figure 32). The size of this pool likely influences the total amount of extracted arginase in these seedlings. Enzyme specific activity, the amount of enzyme activity per unit protein extracted, is a measure of the relative abundance and activity in these tissues. When specific activity was calculated, both sets of seedlings showed similar initial increases in arginase activity. At 2 DIC_{30}, there was no apparent effect of the megagametophyte tissue (Figure 36A). When cultured with an intact megagametophyte, arginase specific activity reached a maximum value of 12.74 ± 0.75 nkat (mg protein)$^{-1}$ at 6 DIC_{30}, before declining to 6.72 ± 0.26 nkat by 10 DIC_{30} (Figure 36A). In the absence of the megagametophyte, specific activity increased only slightly after 2 DIC_{30} and peaked at 4 DIC_{30}, reaching a maximum of 9.05 ± 1.11 nkat (mg protein)$^{-1}$ (Figure 36A). After 4 DIC_{30}, arginase activity rapidly declined to a value of 0.32 ± 0.16 nkat by 10 DIC_{30}. It is noteworthy that at 2 and 4 DIC_{30}, the relative amounts of arginase activity in both seedling types were similar. Maximum values of arginase specific activity of seedlings grown in the presence of the megagametophyte (12.74 ± 0.75 nkat (mg protein)$^{-1}$) were similar to the values obtained at the peak of enzyme activity from the shoot poles of germinated whole seeds (13.34 ± 0.15 nkat (mg protein)$^{-1}$).

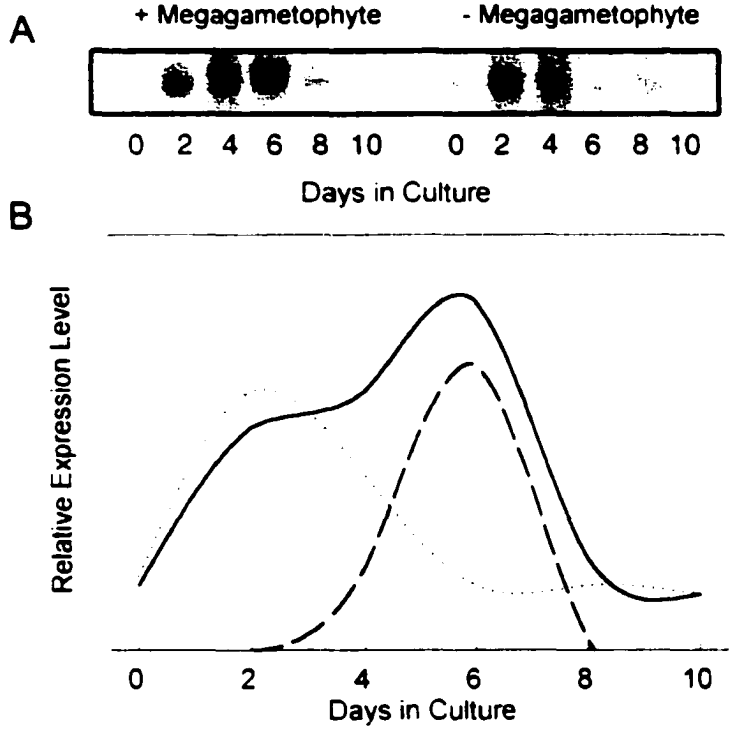
Figure 36: Developmental profile of arginase activity, and protein levels. (A) Arginase shoot pole specific activity expressed as nkat (mg soluble protein)$^{-1}$. Values shown are the mean of at least two independent replicates assayed in duplicate + SE. Black Bars – seedlings grown for up to 10 days in culture in the presence of the megagametophyte; gray bars – seedlings cultured in the absence of the megagametophyte. (B) Accumulation of arginase protein determined by immunoblotting. 8 μg shoot pole soluble protein loaded per lane. Primary antibody incubation performed with a 1:6000 dilution of serum. Blot shown is a representative of additional immunoblots performed with independent protein samples. Tissues and stages are the same as in (A).



In the presence of the megagametophyte, shoot pole arginase protein levels increase following germination at 2 DIC_{30}, and remain high throughout the culture period (Figure 36B). Interestingly, the drop in enzyme specific activity exhibited by 10 DIC_{30} seedlings (Figure 36A) was not mirrored by protein levels (Figure 36B); arginase protein was still relatively abundant in the shoot poles. In the absence of the megagametophyte, arginase protein also began to accumulate following germination, and reached a maximum level in the shoot pole after six days in culture (Figure 36B) and then declined rapidly. By 10 DIC_{30} arginase protein was not detectable in the tissue. The drop in arginase protein observed between 8 and 10 DIC_{30} in the absence of the megagametophyte occurred after the peak of enzyme specific activity at 4 DIC_{30}; however, by 10 DIC_{30} arginase activity in the shoot pole was negligible in the absence of the megagametophyte (Figure 36A).

A developmental profile of arginase mRNA abundance in the shoot pole was generated (Figure 37A). When the seedlings were associated with the megagametophyte, arginase transcript levels increased following germination at 2 DIC_{30} to a maximum at 6 DIC_{30} before declining rapidly to become undetectable by 10 DIC_{30} (Figure 37A). Seedlings cultured in the absence of the megagametophyte did accumulate arginase transcript early on during early seedling growth. The relative level of the message appeared to be very similar at 2 DIC_{30}; however, in contrast to seedlings grown in the presence of the megagametophyte, arginase RNA levels decreased in the shoot poles of seedlings cultured in its absence (Figure 37A) and were very low in shoot poles after 6 DIC_{30} (Figure 37A). It should be noted that the transcript abundance in the absence of

Figure 37: Developmental profile of arginase transcript abundance. (A) Accumulation of arginase mRNA determined by northern blotting. A 1.1 kb fragment from the loblolly pine arginase cDNA was utilized as template for the probe. 10 μg total RNA loaded per lane. RNA integrity and equal loading was determined by ethidium bromide staining after electrophoresis (not shown). Data shown is a representative blot of additional blots performed with independent RNA samples. Tissues and stages are the same as in Figure 36. Blot shown was exposed for 2 days at –50 °C. (B) Graphical representation of arginase transcript abundance determined from the blot shown in (A). The effect of the megagametophyte (dashed line) was determined by subtracting arginase mRNA levels from seedlings cultured without the megagametophyte (dotted line) from total arginase transcript levels obtained from seedlings cultured with the megagametophyte (solid line).



the megagametophyte showed its greatest decrease at the same time those seedlings cultured in the presence of the megagametophyte showed their greatest increase.

The seedling is able to initiate arginase gene expression in the absence of the megagametophyte (Figure 37A). To determine the megagametophyte's contribution to arginase transcript levels, the relative intensities of the autoradiogram signals were quantified and the intensity of the signal in the absence of the megagametophyte was subtracted from the total signal in the presence of the megagametophyte and shown graphically in Figure 37B. This demonstrated that the seedling itself is responsible for the initial increase in arginase mRNA and then, following germination, the megagametophyte's contribution increased to a peak at 6 DIC_{30} and then declined (Figure 37B, dashed line).

3.7 Megagametophyte removal experiments

To test the hypothesis that the megagametophyte was responsible for the increases in arginase enzyme activity, protein levels and transcript abundance demonstrated in section 3.6 an additional set of experiments was performed in which seedlings were cultured in the presence of the megagametophyte as before until 6 DIC_{30}, after which the megagametophyte was removed from the seedling. The effect of this removal was investigated at both the biochemical and molecular levels. Megagametophytes were removed at 6 DIC_{30}, because of the abundance of arginase in the tissue and because after six days in culture the cotyledons were no longer in contact with the surface of the culture media. In a second set of experiments, the megagametophyte was removed and replaced

with exogenous arginine to test the hypothesis that arginine itself was responsible for the observed results.

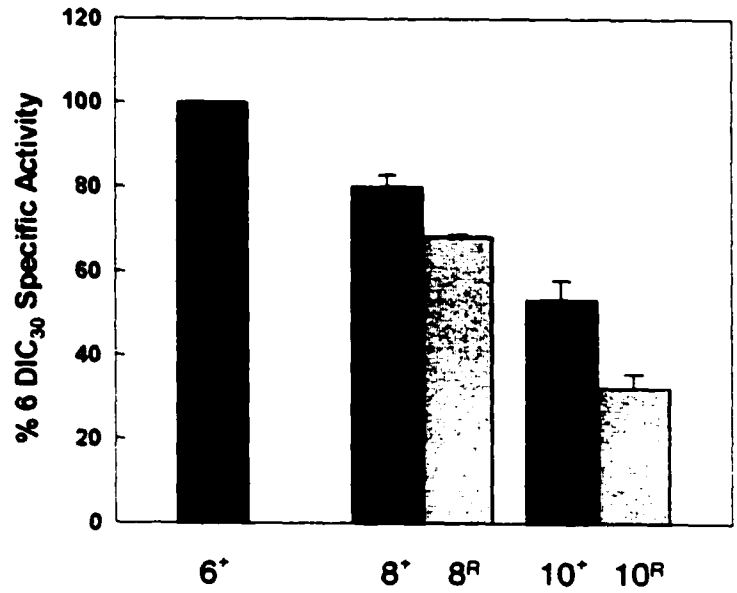
3.7.1 The effect of megagametophyte removal on arginase *in vitro*

Loblolly pine embryos were cultured *in vitro* for six days in the presence of the megagametophyte, at which time some seedlings had the megagametophyte gently removed. The seedlings were then cultured for up to four more days. These plants were compared to control seedlings which had been cultured intact with megagametophytes for the entire period. Shoot pole arginase specific activity was determined at 8 DIC_{30} and 10 DIC_{30}. When the megagametophyte was removed at 6 DIC_{30} there was a slight reduction of arginase enzyme activity over the next four days (Figure 38A) compared to control seedlings. A general decline in specific activity occurred in control seedlings, but this decrease was not as great as when the megagametophyte was absent. After 8 DIC_{30}, seedlings which had their megagametophytes removed exhibited 68% of the specific activity of 6 DIC_{30} seedlings, whereas the activity of control seedlings was 80% (Figure 38A). By 10 DIC_{30}, activity in control seedlings was still greater than 50% of the 6 DIC_{30} value, whereas those seedlings from which the megagametophyte was removed exhibited only 32% of the specific activity determined at 6 DIC_{30} (Figure 32A).

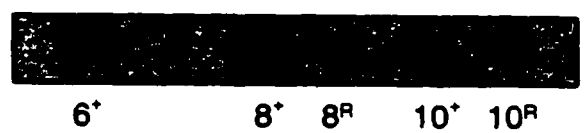
In contrast, there was no appreciable drop in arginase protein levels over the four day period examined (Figure 38B). The decrease in enzyme activity seemed not to be due to loss of arginase protein, since there was little, if any, difference between arginase protein levels over the four day period, regardless of time or whether the megagametophyte had been removed or retained.

Figure 38: Effects of megagametophyte removal on 6 DIC_{30} seedlings. Megagametophyte tissue was removed from seedlings cultured for six days in its presence. Enzyme activity (A) and arginase protein levels (B) shown for seedlings cultured for an additional four days either with or without the megagametophyte tissue. (A) Cell free extract arginase activity of shoot poles which have had their megagametophytes removed (8^R, 10^R) in gray compared to those seedlings in which the megagametophyte tissue was left intact (8^+, 10^+). Data shown as a percentage of the arginase specific activity present at 6 DIC_{30} (6^+), the time point at which the megagametophyte was removed. Values shown are the mean of two independent replicates each assayed in duplicate + SE. (B) Arginase protein levels determined by immunoblotting. Tissues and stages are identical to (A). 6 μg protein loaded per lane. Primary antibody dilution used was 1:15 000. Blot shown is a representative of additional blots performed with independent protein samples.

A



B



Arginase transcript levels showed a different pattern from the enzyme or protein data. When the megagametophyte was removed at the peak of arginase mRNA abundance (6 DIC_{30}), a gradual decline in arginase message occurred in control seedlings (Figure 39, +megagametophyte). The message was clearly detectable 24 and 48 hours later, but was almost undetectable after 96 hours (Figure 39). However, when the megagametophyte was removed at 6 DIC_{30}, arginase mRNA became undetectable in the same tissue within 24 hours and remained undetectable by northern blotting for the rest of the culture period (Figure 39, -megagametophyte).

3.7.2 Replacement of the megagametophyte with arginine

To test the hypothesis that the disappearance of arginase mRNA following megagametophyte removal was due to lack of arginine influx from the megagametophyte, seedlings were cultured to 6 DIC_{30} and had their megagametophytes removed as described above. However, after the megagametophyte was removed, the seedlings were sprayed with 100 mM arginine (pH 7.5) and arginase transcript abundance was determined 12 and 24 h after megagametophyte removal. Replacement of the megagametophyte with the arginine solution caused a more gradual decrease in arginase mRNA as determined by northern blotting (Figure 40) and RT-PCR (data not shown). After 24 hours in the absence of the megagametophyte, no arginase message was detected by northern blotting (Figure 40, lane B); however, after 12 hours in contact with the arginine solution there was still a substantial amount of arginase mRNA present in the tissue (Figure 40, lane C), which in contrast to control seedlings, was not depleted 24 hours following removal of the megagametophyte (Figure 40, lane D).

Figure 39. Arginase transcript levels following megagametophyte removal at 6 DIC_{30}. Shoot pole arginase mRNA levels in seedlings at the peak of arginase transcript levels at 6 DIC_{30} prior to megagametophyte removal (time = 0) and 24, 48 or 96 hours in the shoot poles of seedlings cultured either with or without the megagametophyte. RNA was ethidium bromide stained following electrophoresis to ensure RNA integrity and equal loading (not shown). 10 μg total RNA loaded per lane. Blot depicted is a representative of additional blots performed with independent RNA samples.

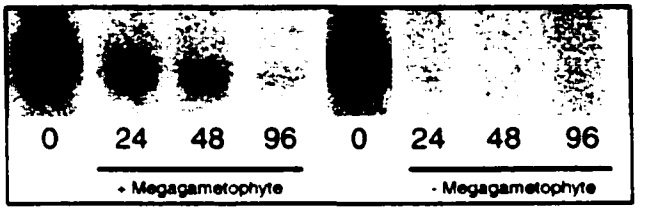
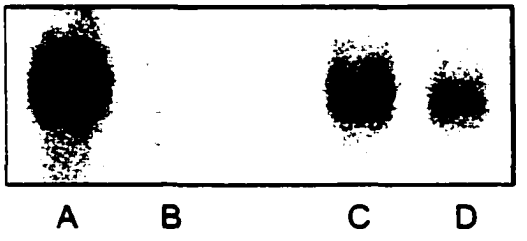


Figure 40: Effect of arginine on transcript abundance after megagametophyte removal. Megagametophytes were removed at 6 DIC_{30} as in Figure 33 and then sprayed with 100 mM arginine. (A) Transcript abundance at 6 DIC_{30}, (B) Transcript abundance 24 hours after megagametophyte removal, (C) Transcript abundance 12 hours after megagametophyte removal and arginine spraying, (D) Transcript abundance 24 hours after megagametophyte removal and arginine spraying. RNA was ethidium bromide stained following electrophoresis to ensure RNA integrity and equal loading (not shown). 10 μg total RNA loaded per lane. Blot depicted is a representative of additional blots performed with independent RNA samples.



3.8 Isolation of a loblolly pine actin fragment for multiplex RT-PCR

At certain stages of development arginase enzyme activity and protein levels are detectable at low levels, whereas arginase RNA is difficult to detect by northern blotting. The final set of experiments was performed using one of these stages. To more readily detect low levels of arginase transcript in these tissues a RT-PCR approach was taken. To provide an internal control for these amplifications a short segment of a loblolly pine β-actin gene was isolated, cloned and used as a template to design gene specific primers for multiplex RT-PCR.

3.8.1 Fragment Isolation

A 260 bp loblolly pine actin fragment was isolated using RT-PCR. PCR primers, designated CTO7 and CTO8 (Table II) were designed within the last two exons of the lodgepole pine β-actin gene (Kenny *et al.*, 1988) and used in a single step RT-PCR reaction using 10 ng of total RNA isolated from 6 DIC_{30} loblolly pine shoot poles cultured in the presence of the megagametophyte. A single PCR product of 310 bp was amplified, isolated, cloned, sequenced and designated pLpAct1. Removal of the CTO7 and CTO8 sequences resulted in 260 bp of loblolly pine actin sequence (Figure 41). In this region the loblolly and lodgepole pine sequences share greater than 99% sequence identity and the isolated fragment shares 87% sequence identity with a *Picea rubens* sequence (Zhou *et al.*, 1999) identified using the NCBI Basic Local Alignment Search Tool. New primers based entirely on the loblolly pine sequence were synthesized and designated CTO9 and CTO10 (Table II).

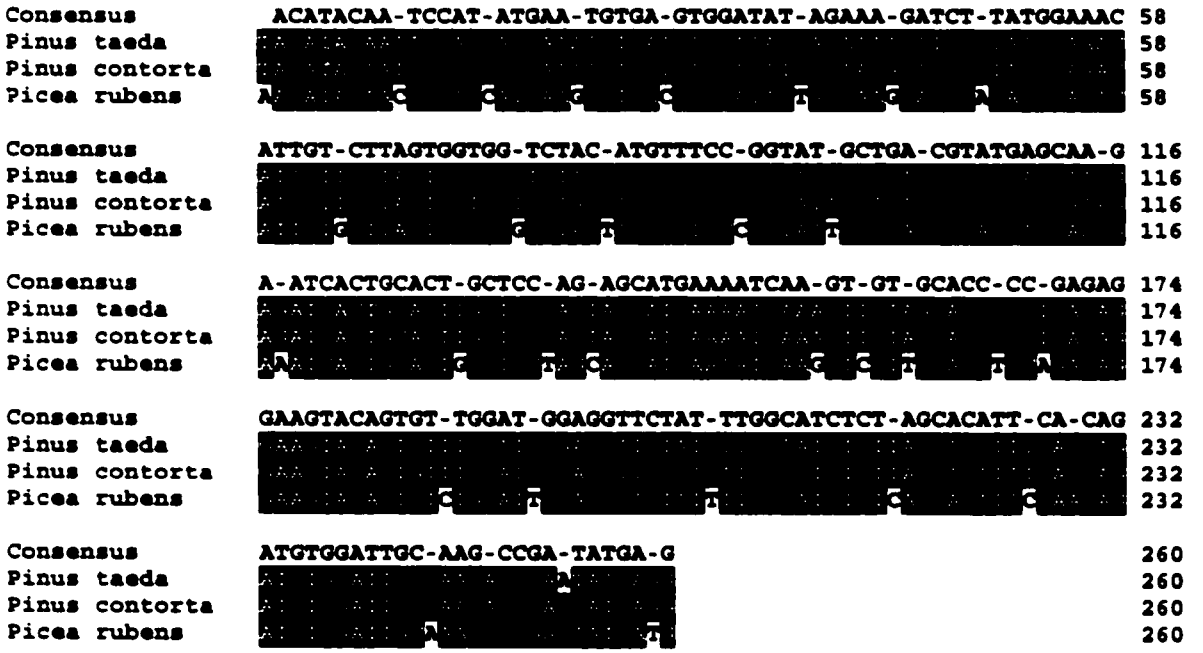


Figure 41: Nucleotide sequence and alignment of cloned actin fragment. DNA sequence of the cloned loblolly pine LpAct1 260 bp fragment aligned with the corresponding regions of *Pinus contorta* (GenBank accession #M36171) and *Picea rubens* (GenBank accession #AF172094). Identical nucleotides are shown highlighted in black and nucleotide consensus sequence included on top.

3.8.2 Arginase and actin multiplex RT-PCR

The PCR/RT-PCR primers CTO3 and CTO4 (Table II) were used in a single step RT-PCR reaction with the CTO9 and CTO10 primers to co-amplify a 510 bp arginase fragment and the 260 bp β-actin fragment from the same 6 DIC_{30} total RNA used to isolate the actin fragment. These primer pairs were also used individually to demonstrate their specificity for the 510 and 260 bp fragments respectively (Figure 42). The addition of both primer pairs to the amplification reaction had no negative effect on the amplification of either PCR product; therefore, these primers were used in all the multiplex RT-PCR experiments that follow.

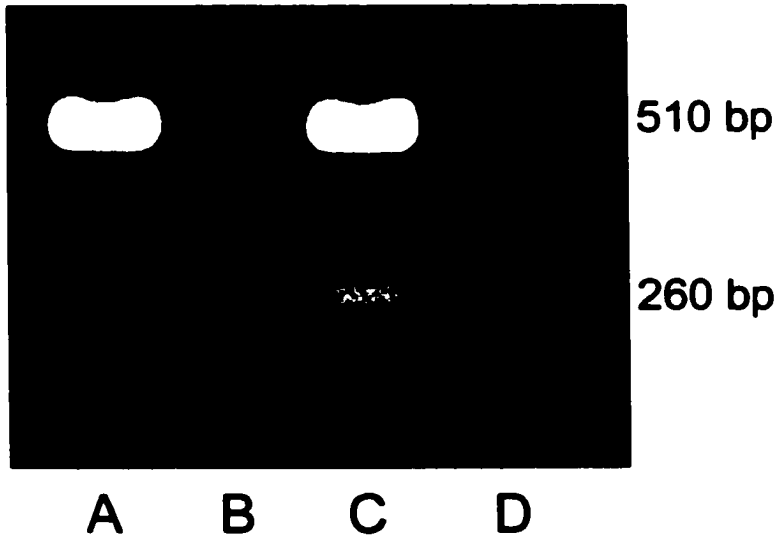
3.9 Induction of arginase by arginine

The results in section 3.7.2 demonstrated that arginine is able to partially substitute for the megagametophyte in maintaining arginase transcript abundance in the shoot pole. To test whether arginine is also able to induce arginase gene expression *in vitro*, isolated embryos were cultured for six days without the megagametophyte and the effects of exogenous application of arginine was investigated.

3.9.1 The effect of exogenous arginine on arginase enzyme activity

Seedlings were grown for six days in culture in the absence of the megagametophyte (6 DIC_{30}-M) before application of 10 mM and 100 mM arginine. Milli-Q water was also applied as a control. After 24 h in contact with the control or arginine solutions shoot pole arginase activity was determined. Application of 10 mM and 100 mM arginine caused a 53 and 78% respective increase in arginase shoot pole

Figure 42: Multiplex RT-PCR. A 510 bp arginase fragment was amplified from 10 ng 6 DIC_{30}+M total RNA in reactions containing arginase specific primers (A and C). A 260 bp actin fragment was amplified from 10 ng 6 DIC_{30}+M total RNA in reactions containing arginase specific primers (B and C). Presence of both primer pairs did not negatively affect the amplification of either band (C). Water control containing both sets of primers, but RNAse free water in place of template RNA shown in lane D.



enzyme activity per seed part over the water-sprayed control (Figure 43A). Enzyme specific activity was also increased 31% over control values with the application of 10 mM arginine and 65% after incubation with 100 mM arginine (Figure 43B). These values are not inflated due to overproduction of urea in the tissues, as endogenous urea levels were determined during the assay procedure to allow for quantification of only the urea produced during the assay.

3.9.2 The effect of exogenous arginine on arginase protein levels

In contrast to its effects on enzyme activity, there was little or no increase in arginase protein levels in the shoot pole due to the application of exogenous arginine (Figure 44). It is important to note, however, that arginase protein levels in the shoot poles of 6 DIC_{30}-M seedlings are relatively high in comparison to enzyme activity (Figure 36). The negligible increase in protein levels may be due to the overabundance of available arginase protein already present in the tissue.

3.9.3 The effect of exogenous arginine on arginase mRNA levels

After six days in culture in the absence of the megagametophyte, little or no arginase mRNA was detected by northern blotting (Figure 37A). To observe the impact of arginine on arginase mRNA levels, RT-PCR was used to amplify a region of the arginase transcript following treatment with either 10 mM or 100 mM arginine. Addition of 10 mM arginine appeared to have little or no effect on transcript abundance when compared to water sprayed seedlings; however, the intensity of the 510 bp band amplified from the 100 mM arginine sprayed seedlings noticeably increased (Figure 45). It is of

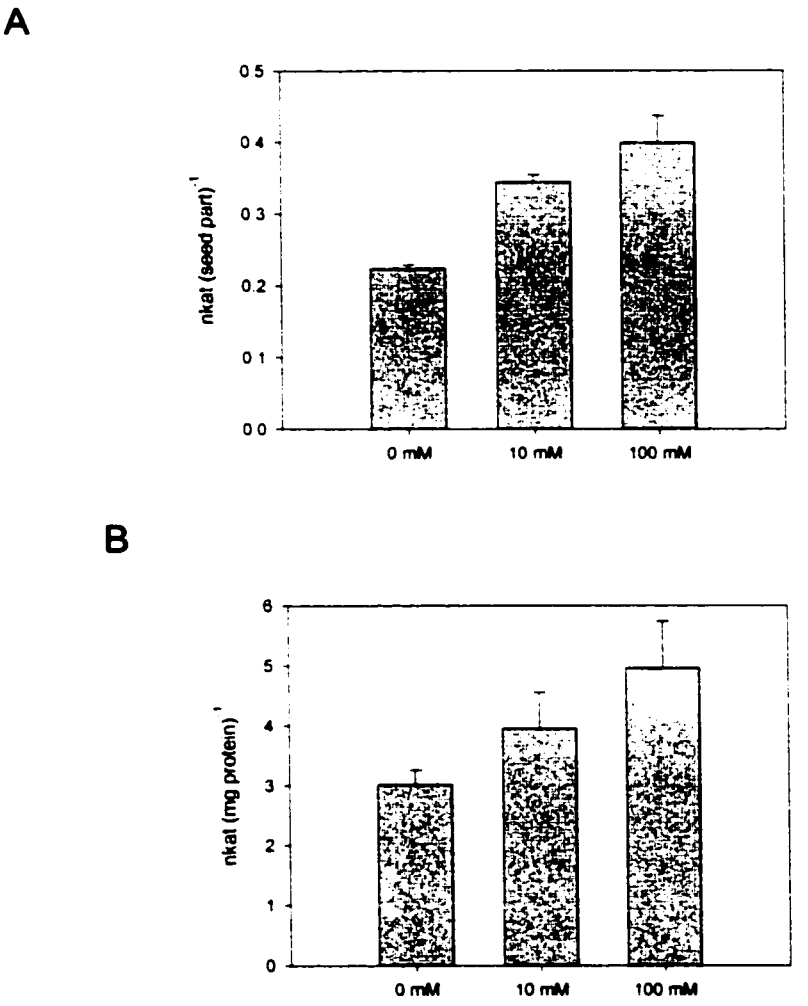


Figure 43: Effect of arginine on arginase activity. Seedlings were grown for six days in culture in the absence of the megagametophyte and then sprayed with either water (0 mM), 10 mM arginine or 100 mM arginine and sampled after 24 h. (A) Arginase enzyme activity per seed part in nkat. (B) Arginase specific activity in nkat (mg protein)$^{-1}$. Values shown determined from two individual replicates each assayed in duplicate + SE.

Figure 44: Effect of arginine on arginase protein levels. Seedlings were grown for six days in culture in the absence of the megagametophyte and then sprayed with either water (0 mM), 10 mM arginine or 100 mM arginine and sampled after 24 h. 6 μg protein loaded per lane. Primary antibody dilution used was 1:15 000. Blot shown is a representative of additional blots performed with independent protein samples.

0 mM 10 mM 100 mM

Figure 45: Effect of arginine on arginase transcript levels. Seedlings were grown for six days in culture in the absence of the megagametophyte and then sprayed with either water (A), 10 mM arginine (B) or 100 mM arginine (C) and sampled after 24 h. Multiplex RT-PCR was performed from 10 ng total RNA. Water control containing both sets of primers, but RNAse free water in place of template RNA shown in lane D.

A B C D

note that arginase mRNA is present in the shoot poles even when not easily detectable by northern blotting. To further illustrate this, Figure 46 shows a comparison of a northern blot and RT-PCR amplification of water-sprayed and 100 mM-sprayed 6 DIC_{30}-M seedlings.

Figure 46: RT-PCR versus northern analysis. (A) Northern blot from water sprayed (left) and 100 mM arginine sprayed (right) 6 DIC_{30} shoot pole total RNA. 10 μg loaded per lane. RNA was ethidium bromide stained following electrophoresis to ensure RNA integrity and equal loading (not shown). (B) Multiplex RT-PCR of the same samples from 10 ng total RNA showing 510 bp arginase fragment and 260 bp actin fragment.

4. Discussion

4.1 Loblolly pine arginase cDNA isolation

At the developmental stage used for cDNA library construction, 9-10 DAI_{30}, arginase mRNA is relatively abundant (Figure 21). This allowed for isolation of an arginase cDNA containing the entire coding sequence. The 1366 bp sequence identified is predicted to be a full length cDNA since the size of the band detected by northern blotting agrees with the size of the cloned insert. This is very similar in size to the full length cDNA clones isolated in Arabidopsis (Krumpelman *et al.*, 1995) and soybean (Goldraij *et al.*, 1998). The initial methionine codon in the loblolly pine sequence identified appears only 14 nucleotides from the 5' end of the ARS20 clone. Both this methionine codon and a second methionine codon (nucleotides 23-26), are surrounded by a variation on the consensus sequence for translation initiation in plants (Kozak, 1984; Lutcke et al. 1987). The deduced amino acid sequence shown in Figure 8 is therefore based on translation from this initial methionine codon. In total there are five methionine codons in the first 24 amino acids of the deduced arginase sequence (Figure 8). Though initially identified as a full length cDNA, there is the possibility that the 5' end of clone is truncated, since there is little 5' untranslated region. This could be resolved by cloning and sequencing of 5' RACE products; however, for the purposes of this thesis, extension of the 5' end was not necessary and therefore not performed.

As indicated, there are five methionine codons in the N-terminal portion of the arginase peptide, two of which share the same level of identity with the consensus sequence for translation initiation in plants (Lutcke et al., 1987). In contrast, neither the Arabidopsis nor soybean sequences, both angiosperms and the only other plant arginase

sequences yet identified, share this feature. It is possible that these alternate sites may produce different arginase peptides or subunits. A smaller form of the peptide was produced by *in vitro* transcription and translation (Figure 11), potentially from an alternate start site, though premature termination of translation may also have occurred. Multiple forms of arginase have been reported for the lichen *Evernia prunasti* (Legaz and Vicente, 1982) and Neurospora (*Neurospora crassa*) (Borkovich and Weiss, 1987) as well as many higher organisms, such as mammals (for review see Jenkinson et al., 1996). In both Neurospora (Marathe *et al.*, 1998) and *Aspergillus nidulans* (Borsuk *et al.*, 1999) multiple forms of arginase are differentially expressed at the transcriptional level from a single arginase gene. It is possible that the multiple methionine codons in the loblolly pine sequence and the proximity of the first methionine to the 5' end of the ARS20 clone may be involved in differential transcription and translation of the arginase gene from different transcription or translation initiation sites. This does not to appear to be the case in the tissues and developmental stages examined in this thesis. In addition, during purification of the arginase holoprotein, no additional arginase isozymes or arginase subunits were identified (King, 1998; Figures 15 to 18), nor were any additional bands detected by immunoblots of the seedling tissue (Figure 6). Moreover, only a single band was detected by northern blotting in all tissues and stages tested. However, multiple putative transcription and translation initiation sites may yet play some role in the regulation or subcellular localization of this protein, possibly in other tissues or in later developmental stages.

Southern blotting suggests that arginase is present as a single copy in the loblolly pine genome. No additional low intensity bands were visible that might suggest

additional, highly related sequences corresponding to other arginase genes; however, this possibility cannot be ruled out since low stringency hybridizations were not performed. Similar to these results, Arabidopsis arginase has been reported as a single copy gene (Zonia *et al.*, 1995); however, a second putative arginase gene may have since been identified in Arabidopsis (J.C. Polacco, personal communication). In contrast, the soybean arginase cDNA hybridized to several bands under high stringency conditions (Goldraij and Polacco, 1999), suggesting that arginase may be a member of a small gene family in that species. Following germination and early seedling growth plants likely still require arginase to perform housekeeping functions. If the loblolly pine sequence identified is indeed present only once in the pine genome, perhaps a complex mechanism of regulation may be required to fulfill multiple roles.

At the nucleotide level, sequence comparisons between loblolly pine arginase and either soybean or Arabidopsis cDNAs show substantial identity. However, alignments between the loblolly pine arginase cDNA and either of the other two plant sequences show that the sequences do not share long regions of nucleotide identity. In fact there are only four regions of identity greater than 12 nucleotides in common between loblolly pine and soybean sequences and no consecutive strings greater than 26 bases. Similar results are obtained when the Arabidopsis arginase sequence is compared to the loblolly pine sequence identified in this thesis. Moreover, when other loblolly pine cDNA sequences are compared with equivalent sequences from other plant species similar results are obtained. For example, when the loblolly pine isocitrate lyase clones ICL 8 and ICL 12 (Mullen and Gifford, 1997), are compared with cDNAs from angiosperm species such as cotton (Turley *et al.*, 1990) or soybean (Guex *et al.* 1992), there is limited

nucleotide sequence identity observed. This observation may explain some of the difficulty encountered with using heterologous probes obtained from angiosperm species in hybridizations with conifer nucleic acids.

When identified, the Arabidopsis arginase cDNA sequence showed very little similarity to the mammalian hepatic and extrahepatic arginases and more nucleotide similarity to other non-arginase related sequences. Consequently, it was suggested that the Arabidopsis sequence may not encode a functional arginase, rather it may encode an agmatinase, a related enzyme which hydrolyzes the polyamine agmatine (Perozich *et al.*, 1998). However, the cloning of a soybean cDNA, AG1, using the Arabidopsis sequence (Goldraij *et al.*, 1998), whose message abundance is in agreement with observed changes in enzyme activity (Goldraij and Polacco, 1999) and shares significant identity with the Arabidopsis sequence, tended to refute that suggestion. However, the most convincing evidence that the plant sequences identified encode functional arginase enzymes comes from the loblolly pine arginase clone isolated during this thesis research. The sequence isolation reported here was performed using antibodies able to immunotitrate arginase activity (Figure 5) and was based on an immunoscreening protocol, rather than DNA sequence homology. Moreover, when expressed in *E. coli*, even with the addition of several amino acids at the N-terminal end of the protein, the pine arginase sequence was capable of producing a protein with functional arginase activity (Figure 24). In addition , both the loblolly pine and Arabidopsis deduced amino acid sequences contain all 10 amino acids determined to be invariant in the arginase gene family (Perozich *et al.*, 1998), providing further evidence that the Arabidopsis sequence is an arginase and not an agmatinase. Interestingly, the soybean cDNA sequence lacks one of these invariant

amino acids, a conserved proline residue (Pro-276 in *P. taeda* sequence), predicted to form an important point in the enzyme's structure (Perozich *et al.*, 1998). Arginase has been identified in all the kingdoms (Jenkinson *et al.*, 1996). Identification of additional plant arginase sequences, potentially from plant genomics initiatives, may allow for a more accurate comparison with non-plant sequences and perhaps help uncover the evolutionary divergence of this enzyme.

4.1.1 Arginase subunit size

The size of loblolly pine arginase predicted from the deduced amino acid sequence is approximately 37 kDa with a predicted pI of 6.4. Initial purification of the loblolly pine holoprotein suggested a subunit size of 43 kDa (King, 1988). The loblolly pine amino acid sequence contains a putative glycosylation site (NSS, amino acids 70-73), a variant of the Asn-X-Ser/Thr sequence demonstrated to be a site for N-linked glycosylation in both natural and synthetic peptides (Hart *et al.*, 1979). It was hypothesized that the discrepancy between the deduced holoprotein size and the molecular mass identified by King (1988) may have been due to glycosylation of the arginase subunit. The secreted arginase of the lichen *Evernia prunasti* has been demonstrated to be a glycoprotein (Planelles and Legaz, 1987), but none of the higher plant arginases described to date have a post-translational modification of this type. The *in vitro* translation product produced from the arginase cDNA is slightly larger than the mature protein immunoprecipitated from 9 DAI_{30} shoot poles (Figure 12). That the *in vitro* translated protein is larger, rather than smaller, than the mature form of the peptide and that the *in vitro* transcribed and translated peptide was translated without addition of

a microsomal membrane fraction, argues against arginase being glycosylated. The discrepancy of the predicted protein size and the subunit size predicted by King (1998) was resolved by partial purification and re-sizing of the arginase protein and is discussed in section 4.2.

Nuclear encoded proteins which function in the mitochondria are translated in the cytosol on free poly-ribosomes and then imported into one of the mitochondrial sub-compartments. Mitochondrial matrix proteins are typically targeted to the mitochondria through an N-terminal mitochondrial targeting sequence which is removed by a mitochondrial processing peptidase following import. While loblolly pine arginase shares considerable deduced amino acid identity with the cDNAs from Arabidopsis and soybean (Figure 8), most of the divergence in the amino acid sequences is found in the N-terminal region of the protein. Plant arginases have been reported to be associated with mitochondria (Kollöfel and Van Dijke, 1975; Taylor and Stewart, 1981, Downum *et al.*, 1983; Matsubara and Suzuki, 1984; Polacco and Holland, 1993) and arginine degradation has been demonstrated in isolated soybean mitochondria (Goldraij and Polacco, 2000). It has been suggested that the N-terminus of plant arginases may be involved in targeting to the mitochondria (Krumpelmann *et al.*, 1995; Goldraij and Polacco, 2000). The N-terminal portion of loblolly pine arginase is rich in basic and hydroxylated residues as well as the amino acids alanine and leucine, typical of plant mitochondrial proteins (Whelan and Glaser, 1997). Furthermore, the N-terminal region of this protein is sufficient to reroute a passenger protein (chloramphenicol acetyl transferase) from the cytosol to mitochondria in tobacco cells (R.T. Mullen, personal communication).

In vitro transcription and translation of the ARS20 clone (Figure 11) or immunoprecipitation of arginase from *in vitro* translated total RNA resulted in a peptide that was larger than the mature form of the protein identified by either western blotting or by immunoprecipitation of radiolabeled arginase from 9 DAI_{30} shoot poles (Figure 12). Removal of an N-terminal mitochondrial targeting sequence *in vivo* would account for the smaller molecular mass demonstrated by the mature form of the protein. When arginase was immunoprecipitated from 9 DAI_{30} seedlings, a faint band of the same size as that produced from immunoprecipitation of total RNA *in vitro* translation products was observed (Figure 14). This band is likely the arginase translation product before import into the mitochondria and removal of the N-terminal targeting sequence. Western blots of all tissues and stages fail to identify this band, even when overdeveloped, suggesting that post-translational targeting and import into the mitochondria functions efficiently and rapidly, not allowing a substantial pool of the arginase pre-protein to accumulate in the cytosol.

4.2 Partial purification of the arginase holoprotein, subunit size and holoprotein structure

To further clarify the discrepancy between the predicted size of the arginase subunit as determined from the cDNA and the size reported by King (1998), the arginase holoprotein was partially purified from 9-12 DAI_{30} shoot poles. Since the purpose of this experiment was not to generate microgram quantities of protein for immunization, it was achieved using substantially less starting material than that of King (1998). Care was taken during the purification not to deplete arginase of manganese, an essential co-factor

of the enzyme in plants (Splittstoesser, 1969; Wright *et al.*, 1981; Boutin, 1982; Carvajal *et al.*, 1996) and which has been demonstrated to be essential for enzyme activity in loblolly pine (King, 1998). The purification strategy was based on the construction of an immunoaffinity matrix using the same anti-arginase serum used for screening the cDNA library; this serum effectively immunotitrated out arginase activity (Figure 5). The tissue homogenization and heat-treatment steps followed that of King (1998), but then the protocol diverged. An ammonium sulfate fractionation was not carried out due to the 61% loss in protein yield observed by King (1998). Further separation involved two molecular sieving steps using Sephacryl S-200 HR and Sephacryl S-300 HR columns. The protein was then incubated with the arginase immunoaffinity matrix in a column. Beyond selectively binding the protein of interest, this step was designed to concentrate arginase by removing it from solution and eluting it in a smaller volume. Though much of the arginase protein did not to bind to the immunoaffinity matrix (Figure 8), sufficient protein was eluted by disruption of the antibody-antigen complexes at low pH to identify arginase by SDS-PAGE after concentrating the eluted fractions. The subunit had a molecular mass of 36.8 kDa (referred to as the 37 kDa subunit) (Figure 19), which was in close agreement with the size of the protein deduced from the cDNA.

The difference in the size of the protein determined by SDS-PAGE in this study and by King (1998) can be attributed to two factors: the polyacrylamide concentration of the separation gel, and the equation of the line determined from the molecular markers. In both experiments the protein size markers used were the same; however, in the 10% polyacrylamide gel used by King (1998), linear separation of the lower molecular weight proteins was not achieved, as the 14.4 kDa marker migrates with the gel front under these

conditions. When the linear equation used included all the markers, not only those in the linear range of separation, the size of the protein subunit was overestimated at 43 kDa, even though on the gel it does not appear to be very close to the 45 kDa marker (King, 1998). In this study a 12% polyacrylamide concentration was used, maintaining all the molecular weight markers on the gel. Due to the small size of the gel (<6 cm separation range) it was decided to include all the markers in the size determination and a cubic, rather than linear equation was used to describe the migration of the markers. When this equation was used to determine the subunit size, the value of 36.8 kDa obtained appeared to fit the migration of the protein through the gel (Figure 15).

The size of the subunit also affects the predicted holoprotein structure. The loblolly pine arginase holoprotein has a molecular mass of approximately 140 determined by FPLC on a Superose 6 column (King, 1998). This makes it one of the smaller plant arginases, similar in size to the 140 kDa arginase purified from Jerusalem artichoke (Wright *et al.*, 1981), but smaller than the 200 kDa iris arginase (Boutin, 1982), the 204 kDa pea arginase (Vardanian and Davtian, 1984) and the 220-240 kDa soybean arginase (Kang and Cho, 1990). In contrast to these plant arginases, the lichen *Evernia prunasti* has more than one form of the arginase protein, a 180 kDa inducible arginase, a 330 kDa constitutive form of the protein and a 245 kDa glycosylated secreted form (Legaz and Vicente, 1982; Martin-Falquina and Legaz, 1984; Planelles and Legaz, 1987), the constitutive arginase being the largest arginase protein described in any plant. Based on a 43 kDa subunit size, loblolly pine arginase was initially predicted to be a homotrimer, even though the size of the subunits does not sum up to this value (King, 1998). Based on the 37 kDa size determined in this work, the pine holoprotein is now predicted to be a

homotetramer. The iris polypeptide is approximately the same size as the loblolly pine protein at 36.5 kDa, yet was reported as a hexameric holoprotein (Boutin, 1982). The loblolly pine arginase subunit is much smaller than the 60 kDa subunit from soybean (Kang and Cho, 1990); however, like loblolly pine arginase, the soybean protein also is reported to be tetrameric. Interestingly, a soybean arginase cDNA has been cloned (Goldraij *et al.*, 1998) with a predicted molecular mass of 38.6 kDa, much more similar in size to both the loblolly pine and iris peptides. There appears to be a small arginase gene family in soybean (Goldraij and Polacco, 1999), and the 60 kDa subunit may represent the product of a different arginase coding sequence than the cloned cDNA. In contrast, the loblolly pine cDNA predicts a subunit of almost exactly the same size as that purified from the cotyledons, and appears to be a single copy gene. The cloned loblolly pine cDNA predicts a 37.2 kDa subunit. The Arabidopsis cDNA, identified initially as a single copy gene, predicts a peptide of 36.5 kDa. (Krumpelman *et al.*, 1995).

The soybean subunit of 60 kDa (Kang and Cho, 1990) is one of the largest purified, the other being isolated from *Mycoplasma arginini* (Claesson *et al.*, 1990). Jenkinson *et al.* (1996) suggested that the *M. arginini* arginase may have arisen from a gene fusion event. It is possible that the soybean peptide may have arisen in a similar manner through the fusion of two arginase genes. An interesting observation about the previously described plant arginases in which the quaternary organization has been deduced is that they all appear to consist of an equal number of subunits (i.e. 4 or 6). Loblolly pine arginase is the same in this regard. When examining the enzyme activity of partially dissociated arginase from iris, Boutin (1982) found that arginase dimers and tetramers as well as the hexameric holoprotein show demonstrable catalytic activity.

Moreover, protein crosslinking showed that arginase probably dissociates into active dimers which then subsequently form the hexameric structure (Boutin, 1982). Active arginase dimers may also explain why the *M. arginini* and soybean peptides remain active if they do indeed represent gene fusion products. The fusion of two subunits may provide a peptide similar to an active dimer of two smaller subunits. Trimeric, tetrameric, hexameric and octameric arginases have been reported in various organisms, but the mammalian enzymes, which have seen the most study, appear to be predominantly trimeric (Jenkinson *et al.*, 1996). Thus, the possible dimeric substructure of plant arginases, while not necessarily setting them apart from all other species, does distinguish them from their mammalian counterparts. Moreover, the tetrameric structure predicted for the loblolly pine arginase is much more like its plant counterparts than the trimeric structure reported earlier by King (1998). To date, too few plant arginases have been either purified or cloned to comment on the evolutionary significance of protein subunit organization.

4.3 Developmental regulation of arginase

4.3.1 Arginase in the megagametophyte

Arginase mRNA is present at very low levels in mature and stratified pine embryos and does not increase substantially until after germination has occurred 4 DAI_{30} (Figure 21). The majority of arginase protein and mRNA is located in the shoot pole following germination, though a minor amount is present in the megagametophyte (Figure 10). It is not likely that arginase in the megagametophyte is required for arginine hydrolysis prior to transport to the cotyledons, since arginine accumulates to very high

levels in the seedling (King and Gifford, 1997), and is found primarily in the shoot pole rather than the root pole (Starchuk and Gifford, 2000), where arginase is most abundant. Arginine does not accumulate to high levels in the megagametophyte (King and Gifford, 1997). During germination and early seedling growth the megagametophyte actively synthesizes enzymes involved in storage reserve breakdown (Mullen and Gifford, 1997; Migabo, 2000). While arginine is relatively abundant in megagametophyte storage proteins, other amino acids such as phenylalanine, methionine and threonine each account for less than 1 % of the nitrogen stored within these proteins. In light of this, perhaps arginine hydrolysis in the megagametophyte is required to provide nitrogen, in the form of ornithine or urea, to be incorporated into some of the less abundant amino acids that may be required for synthesis of enzymes involved in storage reserve mobilization. One or both of these compounds could be utilized as a nitrogen source; however, to be used as a common amino-donor both ornithine and urea are likely converted to glutamine or glutamate, using the enzymes ornithine aminotransferase or urease and glutamine synthetase, respectively. At present we have no information on the activity of these enzymes during germination in the megagametophyte. Moreover, glutamate and glutamine comprise over a fifth of the amino acids in the megagametophyte storage proteins (King and Gifford, 1998) and may or may not require additional nitrogen from arginine to generate sufficient levels for synthesis of the less abundant protein amino acids to continue protein synthesis in the megagametophyte. Still, given the abundance of arginine in the megagametophyte storage proteins, arginase may be involved in converting arginine to alternate amino acids to support protein

synthesis in the megagametophyte as it breaks down the storage reserves and then later as it senesces.

A second potential role for arginase activity in the megagametophyte also exists. Rather than synthesizing amino acids, arginine hydrolysis may be required for polyamine synthesis. Polyamine synthesis during *Pinus strobus* ovule development is associated with amino acid metabolism (Feirer, 1995). Perhaps polyamines are involved in megagametophyte storage reserve hydrolysis and tissue senescence as well. At present, there is no information regarding changes in polyamine levels in the megagametophyte following germination. Both explanations may be plausible, but are merely conjecture.

4.3.2 Developmental regulation of arginase in the shoot pole during germination and early seedling growth

Increases in mRNA levels following germination match accumulation of arginase protein, as demonstrated by immunoblotting (Figure 20). However, there appear to be low levels of protein present in mature and stratified embryos prior to germination before arginase mRNA begins to increase. This basal level of protein likely accounts for low levels of arginase activity detected previously in loblolly pine seeds at these stages (King and Gifford, 1997). It has been suggested that arginase is present at this stage, though in an inactive form (Goldraij and Polacco, 1999). Still, no information regarding arginase protein levels prior to germination was provided. Whether the low level enzyme activity is due to long lived protein present in the mature seed, an alternate arginase, or to a low level, poorly hybridizing mRNA species can not be determined from these data. However, RT-PCR analysis of loblolly pine RNA samples isolated from mature and

stratified seed using primers designed from the cDNA sequence confirms the presence of arginase mRNA in the mature and stratified embryo prior to transfer to germination promoting conditions (data not shown).

The increase in arginase activity in loblolly pine seedlings appears to be due to de novo synthesis of new enzyme as demonstrated by the enzyme, protein and RNA data (Figures 20 and 21). In soybean, a similar relationship has been observed between arginase transcript and measured enzyme activity (Goldraij and Polacco, 1999), yet changes in arginase protein levels were not determined. In loblolly pine seedlings, mRNA and protein accumulation appears to continue beyond the peak of enzyme specific activity (Figures 20 and 21). Post-transcriptional regulation has been previously demonstrated in germinated loblolly pine seeds, where only newly synthesized isocitrate lyase protein appear to be active in the megagametophyte (Mullen and Gifford, 1997). This would not appear to be the case for arginase, since accumulation of mRNA and protein continues beyond the 8 DAI_{30} peak of enzyme specific activity (Figures 20 and 21). Rather, some other factor may be inhibiting the activity of the enzyme. As this protein accumulates in the shoot pole, some sort of check to arginase activity may be required to prevent over-degradation of arginine, which would still be required for protein synthesis during this developmental period. Likewise, the seedling tissue may require a mechanism to prevent over-accumulation of urea and/or ornithine. It is important to note that loblolly pine arginase activity per seed part remains relatively constant from 8 to 12 days after imbibition (King and Gifford, 1997) and that decreases in specific activity are, in part, due to large increases in the shoot pole phosphate buffer-soluble protein pool (Stone and Gifford, 1997). However, one would expect that if the

decrease in arginase specific activity was due solely to this dilution effect, then the relative abundance of arginase protein detected by immunoblotting would follow the same pattern. Clearly, this is not the case in pine cotyledons. Rather, the primary factor in the regulation of arginase following germination appears to be *de novo* synthesis of the enzyme resulting from increased levels of its mRNA for the first eight days following transfer to germination promoting conditions. After eight days enzyme activity decreases, but protein and mRNA levels remain high for at least two days. During the latter stages of early seedling growth, a combination of mRNA abundance, protein synthesis and post-translational regulation may be involved in the regulation of arginine breakdown by arginase.

Expression of the arginase gene in loblolly pine is temporally coordinated with the pattern of storage protein hydrolysis (Groome *et al.*, 1991; Stone & Gifford, 1997) and the rapid accumulation of soluble amino acids, notably arginine, in the seedling (King and Gifford, 1997). Arginase activity, protein and mRNA are predominantly localized in the expanding cotyledons, which remain in contact with the megagametophyte throughout early seedling growth. The accumulation of both the enzyme and its substrate in the cotyledons following germination supports the theory that arginine and arginase are of major importance in post germinative nitrogen metabolism of loblolly pine seeds, particularly during early seedling growth. Moreover, the concomitant import of arginine from the megagametophyte, and accumulation of arginase in the cotyledons, points to the importance of the close relationship between the seedling and the megagametophyte for both nitrogen metabolism and the regulation of arginase.

4.4 Seedling growth and protein reserve breakdown *in vitro*

4.4.1 Growth and development

The utilization of an *in vitro* system to culture zygotic loblolly pine embryos is useful procedure to identify the role played by the megagametophyte in the regulation of important developmental events following seed germination since it allows separation of the megagametophyte (source) from the seedling (sink). To take advantage of this *in vitro* approach a whole plant tissue culture system was generated to allow germination of isolated embryos with and without the megagametophyte. This then allowed characterization of the effect of the megagametophyte on arginase regulation. However, as an experimental tool, the system described is entirely applicable for researching source-sink relationships or other potential communication or signaling events between the two tissues. The conifer seed is a particularly attractive model germination system as the megagametophyte (source) and seedling (sink) are anatomically distinct living tissues and can be cultured independently of one another.

The megagametophyte storage reserves play a nutritive role, but these reserves are clearly not absolutely required to initiate germination and early seedling growth. Germination occurs independently of storage reserve breakdown, with protein and lipid reserve utilization beginning following radicle emergence. In loblolly pine both the megagametophytic and embryonic storage reserves do not begin to significantly decline until germination is complete (Groome *et al.* 1991; Stone and Gifford 1997, 1999). As shown in Figure 29, accumulation of fresh weight by the seedling for the first six days in culture is identical regardless of whether the megagametophyte is present, even though germination has been completed by 2 DIC_{30}. After 6 DIC_{30} the effect of the

megagametophyte becomes quite pronounced as those seedlings cultured in its presence continue their rapid growth whereas those cultured in its absence show a decreased fresh weight accumulation. Initial growth is likely similar regardless of the presence of the megagametophyte because during this period the embryonic storage reserves are sufficient to support development. As these reserves are exhausted the seedling does not have sufficient building blocks to continue at the same pace. Interestingly, the most striking visible effect on the seedlings is in the growth of the cotyledons. In the absence of the megagametophyte the cotyledons do not fully expand, remaining short and somewhat thicker (Figure 27). As the cotyledons are the tissue that remain in contact with the megagametophyte for the longest period of time it is not surprising that their development is adversely affected by the lack of available storage reserve products, both free amino acids and sugars. In conifers, the cotyledons acquire the ability to photosynthesize soon after emergence (Kozlowski and Pallardy, 1997). Interestingly, seedlings cultured in the absence of the megagametophyte can be transferred to soil and will become completely autotrophic, becoming identical to seedlings cultured in the presence of the megagametophyte, but showing a developmental delay (C.D. Todd, data not shown). This suggests that the seedlings have a mechanism to cope with nutritional deficiency, perhaps delaying or arresting cotyledon development until the seedling is able to assimilate carbon and nitrogen from its environment.

4.4.2 Protein levels

When loblolly pine seedlings are cultured on sucrose without the megagametophyte, the seedling free amino acid pool does not increase over a ten day

culture period (King and Gifford, 1997). This lack of available amino acids should hinder protein synthesis in the seedling. As shown in Figures 31 and 32, seedlings cultured without the megagametophyte tissue show marked reductions in the size of the shoot pole phosphate buffer-soluble and -insoluble protein pools by 10 DIC_{30}. This can also be seen in the SDS-PAGE profile of the buffer-soluble proteins. By 10 DIC_{30}, lack of amino acid input from the megagametophyte results in an indistinct and hazy banding pattern, even when the protein gels contain equal amounts of protein in each lane (Figure 33). The same result is found when radiata pine seeds are cultured without the megagametophyte tissue (Fashu-Kanu, 2001). It appears that by 10 DIC_{30} the lack of free amino acid input from the megagametophyte is causing the cotyledons to senesce, breaking down their proteins to supply other metabolic processes.

In contrast, the lack of the megagametophyte does not impact the breakdown of the shoot pole storage protein reserves. Regardless of the presence or absence of the megagametophyte, the seed storage proteins in the embryo broke down at identical rates. By 2 DIC_{30} the 47.0 kDa band was almost entirely depleted and the 37.5 and 22.5 kDa peptides had begun to disappear (Figure 30). By 4 DIC_{30} only remnants of these proteins remained and by 6 DIC_{30} they were nearly undetectable in the shoot pole. Breakdown of these proteins supplies free amino acids, notably arginine, to the seedling. The pattern of protein hydrolysis in culture is exactly that which is found in germinated whole seeds, with breakdown of the 47.0 kDa band preceding the 37.5 and 22.5 kDa protein bands (Stone and Gifford 1997). In loblolly pine (Stone and Gifford 1997) as well as *Picea excelsa* (De Carli *et al.* 1987) storage protein breakdown is more rapid in the root pole and has been attributed to an increased need for metabolites in this tissue during radicle

expansion (De Carli *et al.* 1987). This may also suggest that the slower rate of storage protein breakdown in the shoot pole may be due not only to reduced need, but also may be impacted by the influx of amino acids from the megagametophyte concomitant with the breakdown of the embryonic reserves. Clearly though, as shown here, this is not the case since regardless of whether the megagametophyte is present these proteins are broken down at the same rate.

4.5 The effect of the megagametophyte on arginase

As shown with both seedling growth and protein accumulation, the megagametophyte appears to play a similar role as far as arginase regulation is concerned. By 2 DIC_{30} there is a slight accumulation of arginase activity in the cotyledons in seedlings cultured both with and without the megagametophyte (Figure 35). In the absence of the megagametophyte, total arginase activity peaks at 4 DIC_{30}; however, when the megagametophyte tissue is present arginase activity continues to climb and peaks at 8 DIC_{30} (Figure 35). The initial increase in arginase activity in the embryos cultured alone demonstrates that the megagametophyte is not required for the seedling to be able to metabolize arginine via arginase. The seedling apparently increases arginase activity in response to a signal accompanying the completion of germination and the onset of early seedling growth. Since the isolated seedlings are able to break down their own storage proteins independently, this signal could perhaps be the accumulation of arginine which results from storage reserve hydrolysis.

The relative importance or abundance of arginase in these seedlings can not be determined solely from measurements of total activity. When expressed on a specific

activity basis (Figure 36A) we can compare the abundance of the enzyme relative to the rest of the protein pool in the cells. Here it is very interesting to note the initial increases in enzyme specific activity. In the absence of the megagametophyte shoot pole arginase specific activity is essentially identical at 2 DIC_{30} in both sets of seedlings. More importantly though, the peak of specific activity, which occurs in the presence of the megagametophyte at 6 DIC_{30}, is only 1.6x greater than that of the 4 DIC_{30} seedlings cultured without the megagametophyte (Figure 36A). This demonstrates that following germination, even in the absence of the megagametophyte, the role that arginase plays in the tissue is of similar relative importance to the role it plays later on during early seedling growth when the megagametophyte is contributing to the seedling free amino acid pool. Again, this relative importance in the absence of the megagametophyte is likely related to the utilization of free arginine resulting from the breakdown of the embryonic storage proteins.

In normal germinating seedlings, increases in arginase activity are due to accumulation of the arginase protein because of increases in the arginase mRNA pool (section 4.3). Thus, *de novo* synthesis of arginase seems to be responsible for the observed increases in enzyme activity. In soybean, arginase appears to be regulated at the RNA level as well (Goldraij and Polacco, 1999). In the culture system described here increases in arginase activity are accompanied by increases in arginase protein levels (Figure 36A and B) and mRNA abundance (Figure 37A). It is interesting to note that during the decline of enzyme specific activity, in either the presence or absence of the megagametophyte, arginase protein levels do not show an immediate decline. This is similar to what was shown using intact seedlings (Figures 20 and 21). Loblolly pine

arginase appears to be a relatively robust and long lived enzyme and may remain in the tissue in an inactive form once synthesis ceases. However it is also possible that arginase is post-translationally down regulated during the latter stages of early seedling growth, as discussed previously.

Arginase steady state mRNA levels in the shoot poles as determined by northern blotting (Figure 37A) showed that mRNA accumulation was independent of the megagametophyte immediately following germination at 2 DIC_{30}. The increase in mRNA after transfer of the embryos to culture conditions indicates that accumulation of arginase transcript in the seedling does not require the megagametophyte. Rather, the increases observed in arginase mRNA levels at 6 DIC_{30} in those seedlings cultured with megagametophytes argues that the seedling initiates this response and the megagametophyte further up-regulates it (Figure 37A and B). Up-regulation is most likely due to influx of amino acids, potentially arginine itself, from the megagametophyte. Thus, increases in the free amino acid (or arginine) pool may be the factor influencing arginase regulation. When whole loblolly pine seeds are germinated, arginase regulation is temporally coordinated with storage protein hydrolysis and accumulation of free amino acids, particularly arginine, in the seedling (King & Gifford 1997). The data gathered from the *in vitro* grown seedlings suggests that free amino acids generated from embryonic storage protein breakdown initiate arginase gene expression. It would then follow that influx of amino acids from the megagametophyte would cause an increase in arginase transcript levels as the requirement for arginine metabolism increases. Support for this hypothesis is demonstrated by the timing of the breakdown of the megagametophyte storage reserves *in vitro* (Figure 34). The greatest

decrease in the megagametophytic protein levels occurs between 4 DIC_{30} and 6 DIC_{30}. This would result in a very large influx of free amino acids to the seedling shoot pole at this time. This is also observed when arginase transcript levels increase to their highest in those seedlings cultured with the megagametophyte (Figure 37A). Likewise as the storage reserves in the megagametophyte are exhausted between 8 and 10 DIC_{30} arginase transcript levels also decline. The effects on seedling growth can also be seen in Figures 27 and 29. Absence of the megagametophyte only has a pronounced effect on those seedlings cultured longer than six days, by which time the embryonic protein reserves have been exhausted.

4.6 The effect of megagametophyte removal on arginase

If the megagametophyte is responsible for maintaining high levels of arginase activity and gene expression then removal of the megagametophyte from seedlings cultured in its presence should have a negative effect on arginase activity and transcript abundance. To test this hypothesis seedlings were cultured in the presence of the megagametophyte for six days, allowing them to reach maximal levels of arginase activity, protein and mRNA. Some seedlings had their megagametophytes removed at 6 DIC_{30} and were cultured for up to four more days. Those seedlings which had their megagametophytes removed did show a faster decline in arginase enzyme activity (Figure 38A). That there is a greater decline after the megagametophyte has been removed does indeed suggest that some factor from the megagametophyte maintains arginase in the shoot poles, even as the megagametophyte reserves are being depleted. Protein levels remained high in both sets of seedlings (Figure 38B), similar to what was

observed previously (compare with Figure 36B, +Megagametophyte samples). The gradual decline in arginase activity is what one would expect from a long lived stable enzyme. It is possible too, that the greater decrease in enzyme activity between those seedlings which had their megagametophytes removed may be active down-regulation of enzyme activity in order to prevent hydrolysis of arginine destined for protein synthesis or to avoid a futile urea cycle. It has been suggested that partitioning of arginase to the mitochondria may also be designed to avoid such a cycle (Goldraij and Polacco 2000). Loblolly pine arginase does appear to be mitochondrial; however, that hypothesis would infer the presence of some block to arginine movement in and out of the mitochondria to avoid a futile cycle. As of yet, no evidence of this block has been shown. Perhaps post-translational inhibition of arginase enzyme activity, observed here, may also contribute to the avoidance of wasteful arginine synthesis and degradation.

When the megagametophyte was removed the effects on arginase mRNA were much more pronounced (Figure 39). Megagametophyte removal after 6 DIC_{30} caused arginase transcripts to disappear immediately within the first 24 hours. In contrast, as the megagametophyte senesces, there is a much more gradual decline in arginase mRNA levels. That megagametophyte removal caused such a rapid and drastic decline in arginase mRNA argues strongly that some factor from the megagametophyte, potentially arginine, is responsible for up-regulating arginase during the latter stages of early seedling growth.

4.7 The effect of arginine on arginase

To determine if the factor influencing arginase gene expression from the seedling was indeed arginine, two sets of experiments were performed. To test the hypothesis that removal of the megagametophyte causes a decrease in arginase message levels due to a lack of arginine transport to the seedling, the megagametophyte was removed after 6 DIC_{30} and replaced with either arginine or a control solution (Milli-Q water). To determine if arginine is able to induce arginase gene expression, seedlings were cultured without the megagametophyte tissue for six days, until there was little or no arginase mRNA present (Figure 37A) and then sprayed with either water or arginine. In the first experiment, the decline in arginase mRNA levels was clearly slower when the megagametophyte was removed and replaced with arginine than in its absence. This suggests that the maintenance of high levels of arginine in the seedling due to import from the megagametophyte plays a role in maintaining high levels of arginase transcript in the tissue. The disappearance of the arginase message in the absence of the megagametophyte can be attributed to the depletion of the arginine pool, which occurs as the embryonic protein reserves are depleted. It appears that the presence of the enzyme's substrate, arginine, is sufficient to maintain arginase gene expression. This is similar to what has been shown in the lichen *Evernia prunasti*, where the presence of arginine further increases the activity of a constitutive arginase already present in the tissue (Martin-Falquina and Legaz, 1984), or in fungal systems where arginase gene expression is induced in response to increased cellular arginine levels (Davis, 1986).

In addition, elevated arginine levels may play a role in the megagametophyte. It has been suggested that high levels of arginine in the megagametophyte cause feedback inhibition of storage protein breakdown in this tissue (King, 1998). However, no

experimental evidence has shown a direct impact of arginine on specific proteases involved in reserve hydrolysis. In spite of this, it is still interesting to consider whether arginine levels in either the seedling or megagametophyte may impact other aspects of germination associated with reserve breakdown and utilization. It has been shown that in de-coated loblolly pine seeds, blocking urea hydrolysis with the urease inhibitor PPD results in decreased seedling growth, soluble protein accumulation and arginase activity (King and Gifford, 1997; King, 1998). This result is believed to be due to feedback inhibition of arginase by urea, though it may be due to potential toxic effects of elevated urea concentrations. Urea has been shown to reduce the activity of the inducible arginase of *Evernia prunasti* both *in vitro* and *in vivo* (Legaz and Vicente, 1980; Vicente and Legaz, 1985), but the arginase of Jerusalem artichoke is unaffected by urea levels *in vitro* (Wright *et al.*, 1981). Since arginase responds to the presence of it's substrate, arginine, it is reasonable to suggest that the results shown by King and Gifford (1997) may also indicate regulation by one of the enzyme's products as well.

When arginine was supplied to seedlings cultured for six days in the absence of the megagametophyte arginase enzyme activity increased on both a per seed part and specific activity basis (Figure 43A and B). Minimal increases in arginase protein levels were observed (Figure 44), but a strong increase of arginase mRNA levels was shown with 100 mM arginine (Figure 45). As discussed previously, arginase protein levels remain high in the tissue after the arginase message declines or disappears, suggesting a slow protein turnover rate. Enzyme activity drops off, suggesting that the protein is somehow inactivated, either reversibly or irreversibly. If this inactivation is a reversible reaction caused by a decrease in arginine levels below a certain threshold, then perhaps

addition of arginine results in the re-activation of the enzyme and could account for the increases in enzyme activity observed with the 10 mM arginine treatment in Figure 43, even in the absence of arginase message. This would also account for the lack of additional arginase protein accumulating in these seedlings (Figure 44). Circumstantial evidence indicates that this may occur through a protein-protein interaction. During the purification of the arginase subunit, total arginase activity and enzyme yield actually increased after separation on the Sephacryl S-200 HR column (data not shown). If, during this step, the arginase protein was separated from a second protein responsible for down-regulating arginase activity, then total enzyme activity might be expected to increase. Alternatively, a change in the enzyme's environment during the purification or dilution factor may account for this observation. In developing soybean seeds, it has been suggested that arginase is present, but inoperative, potentially to avoid a futile cycle of arginine synthesis and degradation (Goldraij and Polacco, 1999). At present, the theory that arginase is negatively regulated by a second protein is entirely speculation; however, this may provide a potential mechanism for the post-translational down-regulation of the enzyme in the presence of high arginase transcript levels observed in 10 and 12 DAI_{30} seedlings or may provide a potential mechanism for the seedlings to avoid a futile urea cycle.

Evidence for regulation of arginase at the RNA level was shown with the addition of 100 mM arginine. At this concentration an increase in arginase mRNA occurred (Figures 45 and 46). This increase in transcript abundance was accompanied by a further increase in enzyme activity (Figure 43), but only a small, increase in arginase protein levels (Figure 44). This might be explained by a combination of further re-activation of

arginase protein coupled with additional protein synthesis, the presence of a large inactive pool of arginase might mask additional protein synthesis. Clearly the additional arginine causes an increase in arginase gene expression, but at present we have no information regarding the efficiency or rates of arginase protein synthesis. There are conflicting reports in the literature regarding arginase induction by arginine. Yu and Cho (1997) reported that excised embryonic axes from germinating soybean show an increase in arginase activity when incubated with increasing levels of arginine over a 24 h period. Interestingly, only a negligible increase in activity was observed when soybean axes were placed on 10 mM arginine-soaked filter paper, but a substantial increase was shown with 40 mM and 100 mM arginine (Yu and Cho, 1997); arginase activity of loblolly pine seedlings was induced by both 10 mM and 100 mM arginine. In contrast, Goldraij and Polacco (1999) reported that arginase is not induced by arginine in excised soybean cotyledons, though a low level arginase activity was present as demonstrated by urea accumulation in urease-negative *eu3-e1/eu3-e1* mutant seeds. However, the maximum arginine concentration utilized in their study was 10 mM, which did not strongly induce arginase activity in excised embryonic axes (Yu and Cho, 1997). Moreover, these two studies used different tissues (embryonic axes vs. cotyledons) and in the former study the seeds were allowed to germinate prior to addition of arginine. In addition, arginase in the isolated cotyledons used by Goldraij and Polacco (1999) may suffer from catabolite repression (Cooper *et al.*, 1992) as the sink tissue, the embryonic axis, was removed, likely causing accumulation of metabolites in the cotyledons, the source tissue. Under these conditions it is conceivable that a buildup of ornithine, urea, or some alternate metabolite might result in repression of arginase. In both studies the conclusions drawn

were based entirely on measurement of enzyme activity. The data generated using cultured pine embryos clearly demonstrate that exogenous arginine causes induction of arginase at both the biochemical and molecular levels.

4.8 A model of arginase regulation

The work presented in this thesis allows for the generation of a model of arginine utilization and arginase regulation during germination and early seedling growth of loblolly pine. This model is summarized in Figure 47. Following germination, breakdown of both embryonic and megagametophytic storage proteins results in free arginine, though the majority of this pool is derived from the megagametophyte and accumulates in the seedling during early seedling growth. The free arginine is hydrolyzed by arginase to produce ornithine and urea. Urea is further hydrolyzed by urease to produce ammonium and carbon dioxide. Arginine acts as a positive regulator of the arginase reaction. This regulation is achieved through a combination of arginase transcript abundance and post-translational regulation. Urea acts as a negative regulator of arginase, though the mechanism is not known.

The seedling and the megagametophyte both contribute to arginase regulation. No signal is required from the megagametophyte to initiate arginase gene expression, as was suggested previously (King and Gifford, 1997); the embryo itself is able to accumulate both arginase transcript and protein in the absence of the megagametophyte tissue. However, the megagametophyte is responsible for the up-regulation of arginase during the latter stages of early seedling growth. Increases in arginase activity are correlated with megagametophyte storage protein hydrolysis and movement of free

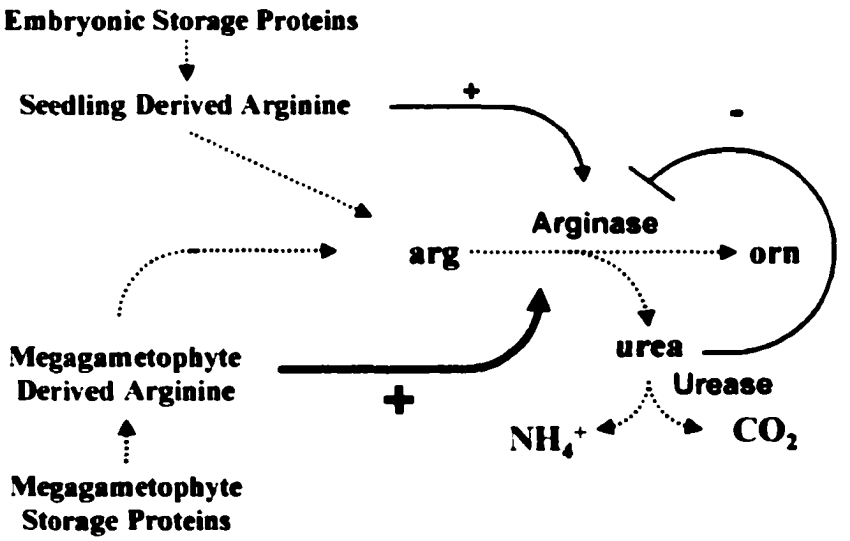


Figure 47. Schematic representation of arginine utilization and arginase regulation. Dotted arrows indicate metabolic flow. Solid arrows indicate positive or negative regulation denoted by (+) and (-) respectively.

amino acids, notably arginine, from the megagametophyte to the seedling. Arginine itself is derived from the breakdown of both embryonic and megagametophytic storage proteins and may be the sole signal responsible for inducing arginase following germination. It is presumed that the nitrogen released by urea hydrolysis is assimilated by the enzyme glutamine synthetase and that ornithine undergoes further metabolic conversions to be incorporated into biosynthesis of polyamines or other amino acids. That arginase is located primarily in the cotyledons suggests that these conversions are required prior to movement of the arginine-derived nitrogen throughout the plant.

4.9 Future work

Quite clearly, the work presented in this thesis has introduced new questions about utilization of nitrogen reserves during conifer germination and early seedling growth. The *in vitro* culture system affords the opportunity to further investigate how arginase is regulated. Since arginase responds to its substrate, arginine, a logical extension of the current work would be to investigate the role of it's products, ornithine and urea, in regulating this enzyme. This could be accomplished through the same type of feeding experiments presented here, or could potentially employ inhibitors, such as PPD, a urease inhibitor, to artificially raise urea levels in the tissue and determine the effects on arginase activity, protein levels and transcript abundance. Use of an arginase inhibitor, such as some of the hydroxy-arginine derivatives, would also likely provide interesting insight into the regulation of arginase, as it would allow arginine to accumulate in the seedling without permitting arginine hydrolysis. This type of experiment may help separate the post-translational component of arginase regulation

from arginase gene expression. In addition, the mechanism of arginase post-translational regulation is an interesting problem.

Arginine is not the sole amino acid moved from the megagametophyte to the seedling. Other amino acids may be involved in arginase regulation. An approach similar to that taken with supplying arginine to the seedlings should be repeated with the other abundant amino acids released from the megagametophyte. This may help shed light on the potential route of arginine-derived nitrogen assimilation. Moreover, the fate of ornithine has not yet been determined. To fully understand the contribution of arginine and arginase to the seedling's nitrogen balance we need to determine how the seedling utilizes ornithine. This might be accomplished by following the fate of radiolabeled arginine, ornithine and other important intermediates. An approach utilizing both carbon and nitrogen isotopic compounds would permit following the route taken by the urea-derived nitrogen, the ornithine derived nitrogen and the carbon backbone of arginine.

The observation that arginine transcripts accumulate in response to arginine is exciting in that it suggests that transcriptional activation of the gene may be occurring. This provides an excellent opportunity to look at the function of a potentially-inducible conifer promoter. Cloning and characterization of the arginase promoter is an important first step to understanding how this regulation is achieved. Identification of important nucleotide sequences or regulatory factors may be the jumping off point for a better understanding not only of arginase regulation, but many other developmentally important genes involved in seed reserve utilization and nitrogen metabolism. Characterization of a conifer promoter active during germination and early seedling growth might also be

useful in the forest industry, allowing for manipulation of somatic embryos during this critical developmental stage.

Finally, it is important to understand arginase localization, both within the seedling and within the cell. Confirmation of arginase's mitochondrial location and identification of which mitochondrial compartment it is active in would aid in developing models of arginase regulation. The identification of potential mitochondrial arginase transporters and their regulation would allow for further refinement of these models and help in understanding how the seedling avoids futile cycles of arginine biosynthesis and degradation. Within the seedling, identification of where arginase is located, its proximity to the megagametophyte or to the vascular system would aid in understanding the bigger picture. The highly specific antibodies generated in this thesis should aid in immunolocalization studies; the cDNA clone provides an excellent tool for looking at patterns of expression, via *in situ* hybridization or RT-PCR. A comprehensive understanding of how arginine is exported to the seedling from the megagametophyte, metabolized and then moved throughout the plant to satisfy the nitrogen requirements of the seedling might then be generated.

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