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Regulation of loblolly pine (*Pinus taeda* L.) arginase in developing seedling tissue during germination and post-germinative growth

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Abstract

After seed germination, hydrolysis of storage proteins provides a nitrogen source for the developing seedling. In conifers the majority of these reserves are located in the living haploid megagametophyte tissue. In the developing loblolly pine *(Pinus taeda* L.) seedling an influx of free amino acids from the megagametophyte accompanies germination and early seedling growth. The major component of this amino acid pool is arginine, which is transported rapidly and efficiently to the seedling without prior conversion. This arginine accounts for nearly half of the total nitrogen entering the cotyledons and is likely a defining factor in early seedling nitrogen metabolism. In the seedling, the enzyme arginase is responsible for liberating nitrogen, in the form of ornithine and urea, from free arginine supplied by the megagametophyte. In this report we investigate how the seedling uses arginase to cope with the large arginine influx. As part of this work we have cloned an arginase cDNA from a loblolly pine expression library. Analysis of enzyme activity data, accumulation of arginase protein and mRNA abundance indicates that increased arginase activity after seed germination is due to *de novo* synthesis of the enzyme. Our results suggest that arginase is primarily regulated at the RNA level during loblolly pine seed germination and post-germinative growth.

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CTAB, hexadecyltrimethylammonium bromide; DAI_{30}, days after imbibition at 30 °C; DAG, days after germination; FW, fresh weight; PVPP, polyvinylpolypyrrolidone; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE, standard error

Introduction

Storage proteins represent a major source of nitrogen to the seedling during germination and early seedling growth. This is a crucial period in the plant life cycle where a large number of metabolites are required to support rapid growth. A survey of storage proteins reveals that arginine is an important component in many angiosperm species (van Etten *et al.* 1963). Seed storage proteins of conifers are particularly rich in arginine. Analyses of amino acid composition (Ramiah *et al.*, 1971; Allona *et al.*, 1992, 1994a, b; King and Gifford, 1997) or deduced amino acid sequences (Leal and Misra, 1993; Häger and Dank, 1996; Chattai and Misra, 1998) all show high levels of arginine present in these proteins. In loblolly pine (*Pinus taeda* L.) seeds, arginine makes up 23.4% mol/mol of the phosphate buffer-insoluble protein reserves, accounting for nearly half of the stored nitrogen in the megagametophyte storage protein (King and Gifford, 1997). As such, arginine likely represents the major source of nitrogen required for the biosynthesis of nitrogenous compounds including amino acids, nucleotides and polyamines during early seedling development.

We have some knowledge of how loblolly pine mobilizes its protein and lipid seed reserves during germination and early seedling growth. Most of these reserves are located in the megagametophyte, account-

ing for 80% and 75%, respectively, of the protein and lipids stored within the seed (Groome *et al.*, 1991; Stone and Gifford, 1999). The major seed storage proteins of loblolly pine have been identified (Groome *et al.*, 1991; King and Gifford, 1997) and their breakdown patterns characterized (Groome *et al.*, 1991; King and Gifford, 1997; Stone and Gifford, 1997). Breakdown of the phosphate buffer-insoluble storage proteins in the megagametophyte and seedling of loblolly pine coincides with large increases in seedling free amino acid levels, of which arginine is a major component. In the megagametophyte, the primary source of these compounds, a much more modest increase is observed, suggesting that the products of storage protein hydrolysis are rapidly and efficiently transported to the seedling (King and Gifford, 1997).

In germinating seeds free arginine is broken down by the enzyme arginase (L-arginine amidinohydrolase, E.C. 3.5.3.1) (Splittstoesser, 1969; Matsubara and Suzuki, 1984; King and Gifford, 1997). Arginase activity has been described in *Arabidopsis* (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) and loblolly pine (King and Gifford, 1997). In loblolly pine, arginine hydrolysis occurs primarily within the expanding cotyledons after germination (King and Gifford, 1997). The products of arginine hydrolysis are the non-protein amino acid ornithine and the nitrogenous compound urea. Urea can be further hydrolyzed by the enzyme urease to produce ammonium and carbon dioxide. We have evidence that a functional urease is active in developing loblolly pine seedlings (King, 1998). Incorporation of ammonium into glutamine by glutamine synthetase converts two of the four nitrogen atoms contained within arginine to a form that is easily utilized for biosynthesis of required metabolic compounds. The second product of arginine hydrolysis, ornithine, which accounts for the other two nitrogen atoms, can be metabolized via several different pathways, either to amino acids, such as proline and glutamate, or to polyamines. The fate of ornithine arising from arginine breakdown during germination of loblolly pine seeds has yet to be determined. In loblolly pine seeds arginase appears to play a major role in supplying nitrogen to the seedling after germination. Considering the amount of nitrogen stored as arginine in seed storage proteins of loblolly pine and other conifers, this enzyme and the reactions following arginine hydrolysis are likely of critical importance in post-germinative development.

To understand how arginase is involved in metabolizing the large amount of arginine entering the seedling we have isolated an arginase cDNA clone. With this cDNA and anti-arginase antibodies as tools, we report the developmental regulation of this enzyme from the viewpoints of enzyme activity, protein level and messenger RNA. Our results indicate that primary developmental regulation of this enzyme is accomplished at the RNA level during loblolly pine germination and early seedling growth. However, there appears to be potential for some post-translational regulation to occur after 8 DAI_{30} (days after imbibition at 30 °C).

Materials and methods

Seed material

Loblolly pine seeds from a first-generation select clone (11–9) were a gift from Westvaco (Summerville, SC). Seeds were stored at −20 °C, run under cold tap water for 1 h, washed in a dilute Tween 20 solution for 30 min, rinsed under tap water a second time, surface-sterilized in 1% sodium hypochlorite for 5 min, rinsed with de-ionized water and 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. 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. Seed tissues were staged as described by Mullen *et al.* (1996), used immediately or frozen in liquid nitrogen and stored at −70 °C until needed. Embryos/seedlings were divided into megagametophytes, shoot poles and root poles as per King and Gifford (1997).

Protein extraction and enzyme assays

A known number of seed parts were extracted with a cold mortar and pestle in cold 50 mM sodium phosphate buffer (pH 7.5). The homogenate was centrifuged at 16 000 × g at 4 °C for 20 min and the

supernatant removed as the buffer-soluble protein fraction. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Protein extraction for assay of arginase activity and the resultant assay was performed as described by King and Gifford (1997).

Construction and screening of a loblolly pine seedling cDNA library

A cDNA library was made from poly(A)$^+$ RNA extracted from 9–10 DAI_{30} seedlings in the manner described by Mullen and Gifford (1997) using a UNI-ZAP XR cDNA synthesis kit (Stratagene, La Jolla, CA) and Gigapack II Gold packaging extracts (Stratagene). The library was amplified to 3.2×10^{10} plaque-forming units/ml and stored at -70 °C until use. 2.5×10^5 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. 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). Clone identity from the rescued phagemids was confirmed using the *pico*Blue colony screening protocol utilizing the same antiserum dilution. DNA minipreps, restriction digests and agarose gel electrophoresis were performed as per Sambrook *et al.* (1989) to size positive inserts prior to sequencing.

DNA sequencing

DNA sequencing 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 University of Alberta Biological Sciences DNA synthesis facility. Analysis of DNA and predicted amino acid sequences was performed using the online BLAST server (National Center for Biotechnology Information, Bethesda, MD) and DNAman software (Lynnon BioSoft, Vandreuil, Quebec).

SDS-PAGE and immunoblotting

Protein separation by SDS-PAGE was performed as described by Mullen and Gifford (1997). Immunoblotting was accomplished using a Mini Trans-Blot Electrophoretic Cell (BioRad Laboratories (Canada) Ltd., Mississauga, ON) from 12% SDS-PAGE gels. Protein was transferred to BioRad TransBlot supported nitrocellulose for 14 h at 30 V or for 1 h at 100 V as per the manufacturer's instructions. After transfer, membranes were stained with Ponceau S stain and protein visualized after washing with several changes of water. The location of the protein standards (Low Mol. Wt Stds, BioRad) were marked with a sharp pencil and the membranes were blocked for 1 h in 20 mM Tris-Cl (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 (1:5000 dilution) for 1 h. Blots were then washed 3 times for 20 min each in the antibody diluent followed by incubation with a 1:3000 dilution of secondary antibody for 1 h. 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 (BioRad) was used as the secondary antibody and bands were visualized in a solution containing 100 mM Tris-Cl (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 BioRad goat anti-rabbit conjugated horseradish peroxidase secondary antibody and the LumiGlo chemiluminescence substrate kit (KPL, Gaithersburg, MD) as per the manufacturer's directions. Signals were detected on Kodak X-Omat AR film (Eastman Kodak Company, Rochester, NY).

RNA isolation and gel electrophoresis

Total RNA was isolated from 50 mg FW tissue from mature and stratified embryos and 2–12 DAI_{30} shoot poles using the RNeasy Plant Mini Kit (Qiagen, Mississauga, ON) as per the manufacturer's instructions. RNA isolated was quantified using a Beckman DU-65 spectrophotometer and RNA integrity was determined by ethidium bromide staining of 1.2% agarose-formaldehyde denaturing gels performed as per Sambrook *et al.* (1989). For northern blot analysis 10 μg total RNA was run for each sample. Prior to transfer for hybridization, gels were washed twice in de-

ionized water for 5 min and once in 10× SSC for 40 min.

RNA blotting and hybridization

RNA was transferred to Zeta-probe nylon membranes (BioRad) via capillary action as per Sambrook *et al.* (1989). Blots were washed briefly in 2× 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× SSC. Membranes were pre-hybridized in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, 1 mM EDTA for 1 h 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, 1 mM EDTA, for 20 min at 65 °C, and twice with 50 mM Na_2HPO_4 (pH 7.2), 1% SDS, 1 mM EDTA at 65 °C for 20 min each before being exposed to Kodak X-Omat AR film at −80 °C with intensifying screens. Radioactive probes were generated using a Pharmacia Biotech oligolabeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) and ^{32}P α-dCTP (3000 mCi/mmol) (Amersham Pharmacia) to a specific activity greater than 1×10^8 dpm/μg as per the manufacturer's instructions. Removal of unincorporated nucleotides and determination of radioactive incorporation prior to hybridization was performed using BioRad Micro-Spin P-30 columns.

Genomic DNA isolation and Southern blotting

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-Cl, 1.4 M NaCl, 20 mM EDTA, 0.3% (v/v) 2-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. 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-Cl, 1 mM EDTA (pH 7.5)). The nucleic acids then incubated with 3 μl RNAse A (10 mg/ml) at room temperature for 60 min and then 0.5 μl proteinase K (10 mg/ml) for 30 min. The solution was extracted with an equal volume of 1:1 phenol/chloroform and spun at 20 000 × *g*, 5 min at room temp. The supernatant (250 μl) 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* for 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. 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 (BioRad). Transfer, hybridization and wash conditions were performed as per Sambrook *et al.* (1989).

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 (Amersham Pharmacia) as per the manufacturer's instructions. Translation products were diluted 50-fold and run on 12% SDS-polyacrylamide gels. Following electrophoresis, gels were fixed in 10% (v/v) acetic acid, 40% (v/v) methanol for 30 min and soaked for 2 h in EN^{3}HANCE autoradiography enhancer (NEN, Boston, MA). Gels were rinsed several times with distilled water and dried under vacuum for 2 h at 60 °C. Dried gels were exposed to Kodak X-Omat AR film overnight at −80 °C before developing. Control reactions containing no DNA were also performed.

Results

cDNA isolation

A loblolly pine cDNA library constructed from 9–10 DAI_{30} seedling tissue was screened with antiserum raised against a loblolly pine arginase subunit. Four potential positive clones were identified. Each was sequenced from both ends of the insert and homology searches were performed using the BLAST on-line search tool (NCBI). All four cDNAs showed highest identity with plant arginases. Comparison of the obtained sequences with each other showed greater

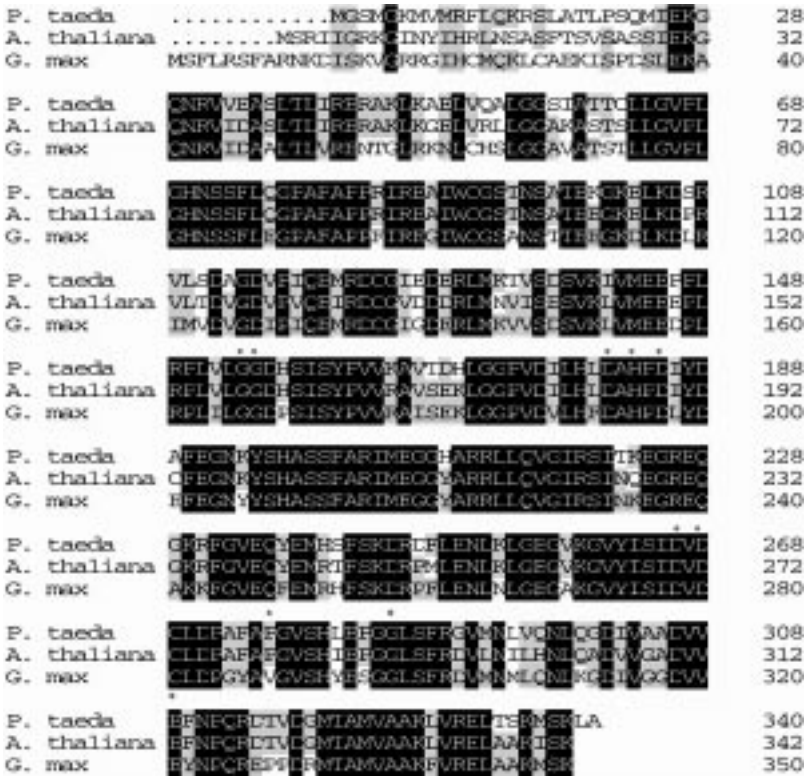

Figure 1. Amino acid alignment of plant arginases. Deduced amino acid sequences of the ARS20 cDNA isolated from loblolly pine, *Pinus taeda*, (GenBank accession number AF130440) aligned with arginase sequences from *Arabidopsis thaliana* (U15019) and soybean, *Glycine max* (AF035671). Identical amino 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).

than 95% identity among all four identified sequences, likely indicating that they encode the same message. Because of this the largest cDNA clone, designated pARS20, was chosen and sequenced to entirety.

pARS20 encodes a 1366 nucleotide full-length cDNA. The insert contains a 341 amino acid open reading frame (Figure 1) 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 bp downstream of the stop codon. The initial methionine codon (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.

The 341 amino acid peptide encoded by pARS20 has a predicted molecular mass of approximately 37 kDa and a pI of roughly 6.4, similar to the deduced size and pI of the *Arabidopsis* and soybean cDNAs. Sequence alignments at the amino acid level 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 1). 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).

Genomic analysis

Southern blot analysis of the loblolly pine genome using the arginase cDNA as a probe (Figure 2) suggests that there is a single copy of this gene. The ARS20 cDNA contains no *Eco*RI or *Kpn*I sites, but does contain two *Hin*dIII recognition sequences, one very near to the 5′ end. Similar results were obtained digesting the DNA with additional enzymes (not shown). No

Figure 2. Southern blot of genomic DNA Genomic DNA digested with *Eco*RI (E), *Hin*dIII (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 $-80\,^{\circ}$C with an intensifying screen.

additional bands were present when the final high-stringency wash was omitted, even when blots were overdeveloped.

Developmental regulation and tissue localization

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). Figure 3A demonstrates 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, but it appears that protein continues to accumulate beyond 8 days,

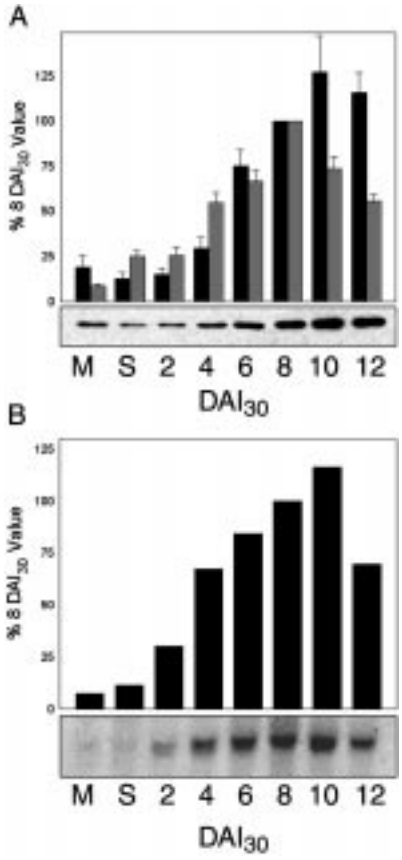

Figure 3. Relative changes in arginase enzyme activity and protein and mRNA levels. A. Developmental profile of arginase protein and enzyme activity is shown for whole embryos isolated from mature (M) and stratified (S) seed and for shoot poles from 2–12 DAI_{30} seedlings. Immunoblot shown is a representative 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 BioRad 670 densitometer and normalized to the 8 DAI_{30} band (black). Values shown were generated from three independent replicates $\pm$ SE. B. Developmental profile of arginase transcript levels from 10 μg total RNA hybridized with probes generated from the loblolly pine arginase cDNA. Blots were exposed for 4 days at $-80\,^{\circ}$C with intensifying screens. Stages and tissues are the same as in A. 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). Relative changes in arginase mRNA levels were quantified as above and normalized to the 8 DAI_{30} band.

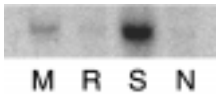

Figure 4. Tissue distribution of arginase mRNA. Northern analysis of total RNA isolated from 10 DAI_{30} megagametophytes, root poles, shoot poles and 31 DAG needles was performed as in Figure 3. Message was detected in all 10 DAI_{30} tissues examined, but not in expanding needle tissue.

by which time specific activity has begun to decline (Figure 3A). Western blots of all tissues and stages examined showed that the antibodies are highly specific for the 37 kDa arginase subunit, even when color reactions were overdeveloped or chemiluminescent blots were overexposed (data not shown).

Northern analyses were performed using total RNA isolated from mature and stratified loblolly pine embryos and shoot poles from 2–12 DAI_{30} seedlings. Arginase transcripts are detectable at low levels in mature and stratified seed and increase following germination 4 DAI_{30}. Arginase mRNA levels reach a peak 10 DAI_{30} and then begin to decline (Figure 3B). The bands detected by northern analysis migrate very close to the 1.38 kb RNA marker, confirming that pARS20 likely encodes a full-length cDNA. Northern blots performed using RNA obtained from 10 DAI_{30} megagametophytes, root poles, shoot poles and 31 DAG needles demonstrate that arginase mRNA is most abundant in the cotyledons, less abundant in the megagametophyte tissue and barely detectable in the root pole at the developmental stages chosen (Figure 4). Over 85% of the arginase message in seedlings 10 days after imbibition resides in the expanding cotyledons and shoot meristem (data not shown). No message was detected in expanding primary needles.

In vitro *transcription and translation*

To confirm that we had obtained the entire arginase coding sequence, *in vitro* transcription and translation of pARS20 was performed. Radiolabelled translation products were separated by SDS-PAGE and detected by fluorography. Transcription and translation of the arginase clone resulted in a major product of approximately 37 kDa, the size of the band detected by immunoblotting, as well as a minor translation product which migrates faster (Figure 5). No protein was detected in control reactions containing RNAse-free water in place of plasmid DNA (Figure 5, lane 3).

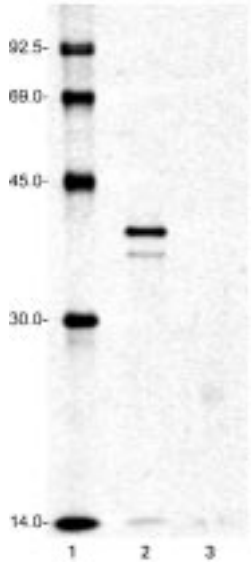

Figure 5. In vitro transcription and translation of pARS20. Transcription and translation of the arginase cDNA was performed from the T3 promoter of the insert carrying pBluescript plasmid (pARS20). Translation products were diluted 50-fold and 7.5 μl was run on 12% SDS-polyacrylamide gels. Gels were subjected to fluorography and exposed overnight. Lanes (from left): 1, ^{14}C-labelled molecular weight standards; 2, pARS20 translation products; 3, control reaction containing no plasmid DNA. Molecular masses of markers (in kDa) are indicated to the left.

Discussion

cDNA isolation

At the developmental stage chosen for cDNA library construction, 9–10 DAI_{30}, arginase mRNA is relatively abundant (Figure 3B). This allowed for isolation of a full-length arginase cDNA. Southern blotting suggests that arginase is present only 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 we cannot rule this possibility out since low-stringency hybridizations were not performed. Similar to our results, *Arabidopsis thaliana* arginase has been reported as a single-copy gene (Krumpelman *et al.*, 1995). 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.

Loblolly pine arginase shares considerable amino acid similarity with the cDNAs from *Arabidopsis* and soybean (Figure 1). Most of the divergence in the

amino acid sequences is at the N-terminus. 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 from the cytosol to mitochondria in tobacco cells (R.T. Mullen, unpublished data).

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 with the *Arabidopsis* arginase sequence as well when other loblolly pine cDNAs we have identified, such as the 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). This observation may explain some of the difficulty encountered with using heterologous probes obtained from angiosperm species in hybridizations with conifer nucleic acids.

The *Arabidopsis* arginase cDNA sequence shows very little similarity to the mammalian hepatic and extrahepatic arginases and more nucleotide similarity to other non-arginase related sequences. Consequently, it has been suggested that the *Arabidopsis* sequence may not encode a functional arginase (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, tends to refute that suggestion. Furthermore, the sequence isolation reported here, using antibodies able to immunotitrate arginase activity (Todd and Gifford, 1998), supports the theory that the plant sequences identified indeed encode functional arginases. 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). Interestingly, the soybean cDNA sequence lacks one of these, 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). Identification of additional plant arginase sequences may allow for a more accurate comparison with non-plant sequences and perhaps help uncover the evolutionary divergence of this enzyme.

The size of loblolly pine arginase predicted from the deduced amino acid sequence is approximately 37 kDa with a predicted pI of 6.4. 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). 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 been demonstrated to have post-translational modifications. The *in vitro* translation product produced from the arginase cDNA shows similar migration to the purified subunit using SDS-PAGE (Figure 5). That this protein was translated without addition of a microsomal membrane fraction argues against arginase being glycosylated.

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 of the angiosperm sequences 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 5), potentially from an alternate start site. Multiple forms of arginase have been reported for *Evernia prunasti* (Legaz and Vicente, 1982) and *Neurospora crassa* (Borkovich and Weiss, 1987) as well as many higher organisms (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. During purification of

the arginase subunit no additional arginase isozymes or arginase subunits were identified (King, 1998), nor were any additional bands detected by immunoblots of the seedling tissue (Figure 3A). However, the two putative translation initiation sites identified may play some role in the regulation or subcellular localization of this protein, possibly in other tissues or in later developmental stages.

Arginase developmental regulation

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 3B). Increases in mRNA levels following germination match accumulation of arginase protein, as demonstrated by immunoblotting (Figure 3A). 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). 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. 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 measured. 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 cannot 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 these tissues prior to transfer to germination-promoting conditions (C.D. Todd, unpublished data).

In the system we have described mRNA and protein accumulation appears to continue beyond the peak of enzyme activity (Figure 3A, B). Post-transcriptional regulation has been previously demonstrated in germinating loblolly pine seeds, where only newly synthesized isocitrate lyase protein appears to be active in the megagametophyte (Mullen and Gifford, 1997). This would appear not to be the case for arginase since accumulation of the RNA and synthesis of protein continues beyond the 8 DAI_{30} peak of enzyme specific activity (Figure 3). Rather, some other factor may be inhibiting activity of the enzyme. As this protein accumulates in the shoot pole some sort of check to arginase activity may be required to prevent degradation of arginine to be utilized for protein synthesis. 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 activity per unit protein (specific activity) were 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 8 days following transfer to germination-promoting conditions, but a combination of mRNA accumulation, protein synthesis and post-translational regulation may be involved in the latter part of early seedling growth.

Expression of arginase in loblolly pine is temporally coordinated with the pattern of storage protein hydrolysis (Groome *et al.*, 1991; Stone and 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.

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