Molecular and Functional Analyses Support a Role of Ornithine-δ-Aminotransferase in the Provision of Glutamate for Glutamine Biosynthesis during Pine Germination[1[W]]

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We report the molecular characterization and functional analysis of a gene (*PsδOAT*) from Scots pine (*Pinus sylvestris*) encoding Orn-δ-aminotransferase (δ-OAT; EC 2.6.1.13), an enzyme of arginine metabolism. The deduced amino acid sequence contains a putative N-terminal signal peptide for mitochondrial targeting. The polypeptide is similar to other δ-OATs from plants, yeast, and mammals and encoded by a single-copy gene in pine. *PsδOAT* encodes a functional δ-OAT as determined by expression of the recombinant protein in *Escherichia coli* and analysis of the active enzyme. The expression of *PsδOAT* was undetectable in the embryo, but highly induced at early stages of germination and seedling development in all different organs. Transcript levels decreased in later developmental stages, although an increase was observed in lignified stems of 90-d-old plants. An increase of δ-OAT activity was observed in germinating embryos and seedlings and appears to mirror the observed alterations in *PsδOAT* transcript levels. Similar expression patterns were also observed for genes encoding arginase and isocitrate dehydrogenase. Transcripts of *PsδOAT* and the arginase gene were found widely distributed in different cell types of pine organs. Consistent with these results a metabolic pathway is proposed for the nitrogen flow from the megagametophyte to the developing seedling, which is also supported by the relative abundance of free amino acids in embryos and seedlings. Taken together, our data support that δ-OAT plays an important role in this process providing glutamate for glutamine biosynthesis during early pine growth.

In seeds of many conifers most of the nitrogen is stored in the storage proteins as Arg, the amino acid with the highest nitrogen content (Allona et al., 1994; King and Gifford, 1997). Nitrogen from Arg is mobilized via the reaction catalyzed by arginase (EC 3.5.3.1), a mitochondrial located enzyme that hydrolyzes Arg to urea and Orn (Goldraij and Polacco, 2000). Studies with pine (*Pinus* spp.) germinating seeds indicate that the urea molecule is broken down by urease (EC 3.5.1.5; King and Gifford, 1997; Todd and Gifford, 2002), releasing ammonium to be assimilated into Gln by the catalytic action of Gln synthetase (GS; EC 6.3.1.2; Suárez et al., 2002). However, the metabolic fate of the Orn is still unknown. Orn-δ-aminotransferase (δ-OAT; EC 2.6.1.13) catalyzes the transamination of the δ-amino group from Orn to 2-oxoglutarate, producing Glu 5-semialdehyde (GSA) and Glu. At physiological pH GSA is in spontaneous equilibrium with Δ^1-pyrroline-5-carboxylate (P5C; Deuschle et al., 2001). GSA/P5C can also be synthesized from Glu by the reaction catalyzed by P5C synthetase (EC 2.7.2.11 and 1.2.1.41) involved in the Pro biosynthesis pathway. In fact, GSA/P5C are intermediates in both Pro synthesis and degradation (Deuschle et al., 2001). Pro is synthesized from P5C in the reaction catalyzed by the enzyme P5C reductase (EC 1.5.1.2), and catabolized by the sequential actions of two mitochondrial enzymes, Pro dehydrogenase (EC 1.5.99.8) that catalyzes the oxidation of Pro to P5C and P5C dehydrogenase (P5CDH; EC 1.5.1.12) that catalyzes the conversion of GSA to Glu (Deuschle et al., 2001).

Glu is a crucial molecule in nitrogen metabolism with two immediate fates: the synthesis of Gln in the reaction catalyzed by GS or the production of Asp through the transamination to oxaloacetate by Asp aminotransferase (EC 2.6.1.1). Gln and Asp are the precursors of Asn in the reaction catalyzed by the Asn synthetase (AS; EC 6.3.5.4). The amides Gln and Asn are crucial molecules for nitrogen distribution and transport in higher plants (Lea and Miflin, 1980; Urquhart and Joy, 1981; Lam et al., 2003), and studies

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with pine seedlings suggest that both amino acids play key and different roles in the mobilization of nitrogen reserves during conifer germination (Suárez et al., 2002; Cañas et al., 2006).

In mammals, the role of Orn aminotransferase in providing Glu for Gln synthesis is well known (Levillain et al., 2004), and it plays an important role providing Glu for detoxification of ammonia under a high protein diet (Boon et al., 1999). On the contrary, there are only a few studies about δ-OAT in plants. Several cDNA clones encoding δ-OAT have been isolated and characterized from *Vigna aconitifolia* (Delauney et al., 1993) and Arabidopsis (*Arabidopsis thaliana*; Roosens et al., 1998). The physiological role of δ-OAT is still not well understood in plants. Although some evidence has associated this enzyme with Pro accumulation observed during salt or hydric stress, contradictory data exist about its contribution to this route. Increased δ-OAT activity in NaCl-treated radish (*Raphanus sativus*) cotyledons (Hervieu et al., 1995) and increased δ-OAT activity and gene expression in young Arabidopsis plantlets exposed to 200 mM NaCl (Roosens et al., 1998) support a contribution of δ-OAT activity to Pro synthesis under salt stress. However, in 4-week-old Arabidopsis plants, although free Pro level increased under salt-stress conditions, the δ-OAT activity was unchanged and δ-OAT mRNA was not detectable (Roosens et al., 1998). Furthermore, Delauney et al. (1993) reported that levels of mRNA corresponding to δ-OAT decreased in response to salt stress in *V. aconitifolia* plants, whereas the transcript levels were elevated in plants supplied with excess of nitrogen.

In this article we report the molecular characterization of δ-OAT from a gymnosperm, Scots pine (*Pinus sylvestris*). Expressional and enzymatic analyses, combined with determination of amino acid contents in pine embryos and seedlings, suggest that the principal role of the enzyme during pine germination and primary seedling development is to supply Glu for Gln biosynthesis.

RESULTS

Molecular Cloning and Characterization of a Pine cDNA Encoding δ-OAT

A set of primers were designed from the sequence information available for ESTs of *Pinus taeda* and *Pinus pinaster*, containing incomplete open reading frames encoding putative δ-OAT. These primers were used to amplify a 579-bp fragment of a cDNA from Scots pine seedlings by reverse transcription (RT)-PCR. The product was sequenced and the deduced amino acid sequence (Supplemental Fig. S1, amino acid residues 272–463) showed similarity to other plant δ-OATs. The sequence was used to design specific oligonucleotides to amplify the complete 5′ region of the mRNA by 5′-RACE. Finally, a new oligonucleotide was designed from the 5′-end available sequence to amplify a cDNA fragment of approximately 2.0 kb by 3′-RACE, as estimated by agarose-gel electrophoresis. The cDNA was cloned and completely sequenced. The cloned insert was 1,967 bp in length and it was shown to contain a full-length open reading frame encoding δ-OAT. The corresponding gene was designated *PsδOAT*. The *PsδOAT* cDNA contained a 5′-untranslated region (UTR) of 323 nucleotides, an open reading frame encoding a polypeptide of 468 amino acid residues, and a 3′-UTR of 237 nucleotides. The molecular mass of the deduced polypeptide was 51.2 kD and the predicted pI was 7.5. Prediction of subcellular localization by using MitoProt (Claros and Vincens, 1996) suggested the existence of a signal peptide of 35 residues for mitochondrial targeting in the N terminus of the pine polypeptide (Supplemental Fig. S1), which agrees with the mitochondrial localization of Arabidopsis δ-OAT (Funck et al., 2008).

The amino acid sequence of the polypeptide was compared to the primary structure of other δ-OATs from eukaryotes (Supplemental Fig. S1). All plant and animal polypeptides in the alignment include a putative N-terminal transit peptide for mitochondrial targeting with a barely conserved sequence, whereas the N-terminal extension is absent in the yeast (*Saccharomyces cerevisiae*) enzyme and the enzymes from other members of the *Saccharomycetaceae* family. However, the absence of an N-terminal transit peptide may not be a common character in the fungus kingdom, since a cDNA encoding δ-OAT from the mushroom *Agaricus bisporus* has been recently characterized and an N-terminal transit peptide is also predicted for the protein (Wagemaker et al., 2007). In spite of the evolutionary distance the primary structure of the mature protein is quite well conserved in plants, with an average identity of about 62%, and also when it is compared to mammals (50.2% and 48.8% of identity with human and rat, respectively), whereas the similarity to the *Escherichia coli* enzyme was very low (27%).

Similar to other aminotransferases belonging to the subclass III and other pyridoxal phosphate (PLP)-dependent enzymes, Orn aminotransferases share a common protein scaffold around the cofactor binding site (Yonaha et al., 1992; Delauney et al., 1993; Schneider et al., 2000). A number of anchor residues are highly conserved and many of them are involved in the interaction with PLP. Thus, in the aminotransferase subclass III the K289 residue binds PLP while the E227 and D260 residues interact with the 3′-OH group and N-1 of PLP, respectively. These critical residues are conserved in the primary structure of δ-OATs including plant, animal, fungal, and bacterial (data not shown) enzymes. The presence of these conserved residues in the deduced amino acid sequence of *PsδOAT* suggests that it encodes a functional δ-OAT in pine.

Recombinant PsδOAT Protein Exhibits δ-OAT Activity

To verify whether the protein encoded by *PsδOAT* is a functional δ-OAT enzyme, a truncated version of the

cDNA with a deletion of the codons 1 to 30 and encoding the putative mature mitochondrial polypeptide was cloned under the T7 promoter control in the plasmid pET11a. A His tag was added following the first Met residue of the recombinant polypeptide to facilitate polypeptide detection and purification. Therefore, a recombinant polypeptide was generated with a predicted molecular mass of 48.9 kD. Although several algorithms for signal peptide identification predicted a processing site between residues 35 and 36, we decided to construct a form of the protein truncated between position 30 and 31, since two Ser residues in positions 33 and 34 are conserved in the primary structure of many δ-OAT from plants. Both residues are also present in the yeast polypeptide, even though the yeast protein lacks a signal peptide according to algorithm prediction. Furthermore, the analysis of the crystal structure of human δ-OAT supports the notion that residues preceding the Pro 38 in the human protein do not make contact with the body of the δ-OAT dimers that form the hexameric structure and are far from the active site (Shen et al., 1998). Thus, these authors suggested that changes in length of this region should not have any influence on enzyme activity.

Soluble proteins extracted from *E. coli* cultures transformed with the recombinant pET11a plasmid were analyzed by western blot using antibodies against the His tag and assayed for δ-OAT (Fig. 1A). The expression of a recombinant protein of the expected size (49 kD as estimated in SDS-PAGE gels) was confirmed by detection with anti-His-tag antibodies in bacterial extracts and purified fractions (see Fig. 1A, lanes 2 and 3). Enzyme activity was detected in both extracts and values of specific activity were proportional to the relative amount of recombinant protein present in the enzyme preparations. Thus, when the recombinant protein was enriched by purification on nickel-nitrilotriacetic silica spin columns the specific activity increased by 18.5 times (Fig. 1A, bottom of lane 3). The enrichment in recombinant protein after purification was also confirmed by protein staining after SDS-PAGE (Fig. 1B). Both the 49-kD polypeptide and δ-OAT activity were undetectable in bacterial protein extracts from noninduced cultures (Fig. 1, A [lane 1] and B [lane 3]). Therefore, the detected levels of δ-OAT activity were a consequence of the recombinant protein expression and accumulation. Experiments were carried out to determine the kinetic constants of the recombinant protein for both substrates Orn and 2-oxoglutarate. In both cases no Michaelis-Menten kinetics were obtained, suggesting a positive cooperative behavior of the enzyme ($n = 1.5$ for 2-oxoglutarate and $n = 2.3$ for Orn). The measured kinetic values ($S_{0.5} = 0.8$ mM for 2-oxoglutarate and $S_{0.5} = 6.3$ mM for Orn) are in the range of those previously reported (Drejer and Schousboe, 1984; Sekhar et al., 2007).

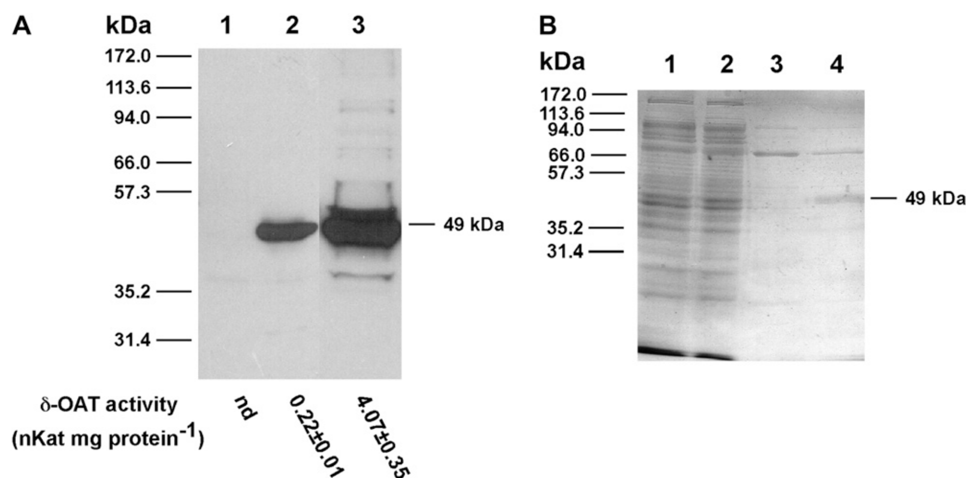

Figure 1. Expression of recombinant pine δ-OAT in *E. coli* and enzyme activity determination. *E. coli* cells were transformed with a construct in the pET11a plasmid encoding an N-terminal His-tagged polypeptide with the coding region of *PsδOAT* cDNAs lacking the first 30 amino acids. Cultures of transformed cells were incubated with isopropyl-β-D-1-thiogalactopyranoside to induce the expression of the recombinant protein. A, Soluble proteins were extracted under nondenaturing conditions and analyzed by western blot with anti-His-tag antibodies: 1, soluble proteins extracted from noninduced *E. coli* cells; lane 2, soluble proteins extracted from induced *E. coli* cells; lane 3, recombinant δ-OAT-enriched fraction after purification. Fifteen micrograms of soluble protein were loaded in each lane. Enzyme activity was tested for different aliquots and results are shown at the bottom of each lane. Enzymatic activity (nkatal mg protein^{-1}) values are the averages of three replicates, ±SE; nd, no activity detected. B, The fraction enriched in recombinant δ-OAT was also visualized with Coomassie Blue stain: 1, 15 μg of soluble proteins extracted from noninduced *E. coli* cells; lane 2, 15 μg of soluble proteins extracted from induced *E. coli* cells; lane 3, 2 μg of proteins eluted from the nickel-nitrilotriacetic silica spin column when soluble protein from a noninduced culture was used; lane 4, 2 μg of proteins eluted from the nickel-nitrilotriacetic silica spin column when soluble protein from an induced culture was used. The molecular masses (kD) of the protein markers are indicated on the left side. The estimated molecular mass of the recombinant protein is indicated on the right.

Genomic Organization

The genomic organization of *PsδOAT* in pine was investigated by Southern-blot analysis using a 579-bp cDNA fragment derived from the coding region of the *PsδOAT* cDNA as a probe, encoding from amino acids 272 to 463. Genomic DNA was digested with restriction enzymes that did not have a recognition site in the probe sequence (*Eco*RV, *Xba*I, *Hin*dIII, and *Eco*RI). When washed at moderate stringency, only a single fragment showed strong hybridization to the probe in each restriction digest (Fig. 2). This result suggests that a single gene for δ-OAT is present in the Scots pine genome.

Expression Analysis of *PsδOAT*

To get insights into the specific function of the enzyme encoded by *PsδOAT* during germination and early development in pine, the relative transcript abundance was determined by northern-blot analyses in pine embryos and seedlings at different stages of development, including three embrionary and four seedling stages (Fig. 3). The blots were also probed with labeled cDNAs for a pine arginase gene (*ARG*; Todd et al., 2001) and a cytosolic $NADP^+$-dependent isocitrate dehydrogenase gene (*ICDH*; B. Pascual, F.M. Cánovas, and F. Gallardo, unpublished data). *PsδOAT* gene expression was undetectable in dry embryos and barely detectable in wet embryos (Fig. 3, *PsδOAT* sections, E0 and E1 lanes) but induced following imbibition to reach high levels in germinated embryos (Fig. 3, *PsδOAT* section, G lane). In the seedlings, relatively high levels of *PsδOAT* transcripts were detected in the cotyledons of young seedlings 8 d after imbibition (Fig. 3, *PsδOAT* sections, C1 lane) that progressively decreased at later stages of development. The abundance of *PsδOAT* transcripts was also high in the hypocotyls during plant development with particularly increased levels in seedlings with lignified stems (Fig. 3, *PsδOAT* sections, H4 lane). Similarly, *PsδOAT* gene expression was also high in radicles but progressively decreased at later stages of development as was observed in the cotyledons. In comparison to *PsδOAT*, the expression pattern of *ARG* was quite similar (Fig. 3, *ARG* sections). A related pattern of gene expression was also observed when the mRNA levels of *ICDH* were analyzed in the different pine organs during development (Fig. 3, *ICDH* sections), with the exception of cotyledons where increased expression levels were observed at later stages (Fig. 3, *ICDH* sections, C1 to C4 lanes).

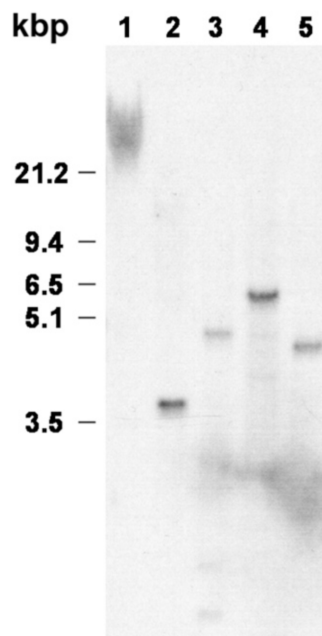

Figure 2. Southern-blot analysis of Scots pine genomic DNA. Aliquots of 20 μg of genomic DNA isolated from pine cotyledons were digested with different restriction enzymes, fractioned in an agarose gel, and transferred to a nylon membrane. Hybridization was performed with a 579-bp probe obtained by labeling the purified cDNA fragment. After hybridization, membranes were washed at moderate stringency. 1, Undigested pine genomic DNA; lanes 2, 3, 4, and 5, digestions with *Eco*RV, *Hin*dIII, *Xba*I, and *Eco*RI, respectively. Numbers on the left indicate size in kilobase pairs of λ-DNA size markers.

We complemented our expression studies in pine seedlings by examining the distribution of *PsδOAT* expression in different cell types of radicles, hypocotyls, and cotyledons by in situ hybridization. The results indicate that *PsδOAT* mRNAs were localized in all cell types of the analyzed sections (Fig. 4, A–C). Although some cells showed stronger signals in phloem and surrounding the resin ducts (labeled with arrowheads), this could be a result of higher expression of the gene or a more condensed cytoplasm due to the absence of vacuoles. When the sections were hybridized with the *ARG* riboprobe a similar pattern of distribution was found (Fig. 4, G–I). No signals of specific expression associated to a particular cell type of cotyledons, hypocotyl, and radicle were observed. The specificity of the signals was confirmed by hybridization with sense probes (Fig. 4, D, E, F, J, K, and L). In contrast, other pine genes involved in nitrogen metabolism exhibited specific expression patterns associated to individual cell types. For example, *GS1a* mRNAs are exclusively localized in photosynthetic cells whereas *GS1b* transcripts are associated to vascular bundles (Ávila et al., 2001; Suárez et al., 2002).

δ-OAT Activity in Pine Embryos and Seedlings

δ-OAT activity was determined in pine embryos and seedlings to investigate whether the enzyme is accumulated during germination, as suggested by the analysis of the mRNA levels. Enzymatic analyses were performed in protein extracts prepared from dry, imbibed, and germinated embryos and cotyledons, hypocotyls and radicles of seedlings germinated for 8 d after imbibitions (developmental stage 1). The δ-OAT activity was determined as P5C formation (Fig. 5). Although the enzyme activity was already detected

in dry and imbibed embryos (Fig. 5, E0 and E1), an increase of activity was observed in embryos following seed germination (Fig. 5G). Statistical significance of differences in activity was evaluated by ANOVA analysis (with $\alpha = 0.05$). All differences were statistically significant, except values for E0 and E1, suggesting that similar levels of δ-OAT activity are found in both stages. Higher levels of δ-OAT activity were also detected in different organs of the seedlings. Nevertheless a slight increment in activity level from cotyledons to radicle was observed.

Amino Acids and Ammonium Levels in Pine Embryos and Seedlings

We are interested in determining the potential function of δ-OAT in the nitrogen flux that takes place from the megagametophyte to the developing seedling during pine germination. To further investigate this process, the levels of individual amino acids were quantified in plant extracts derived from embryos and seedlings. The amino acid and ammonium contents in imbibed and germinated embryos and cotyledons, hypocotyls, and radicles of seedlings at developmental stage 1 are shown in Table I. Total amino acid content was higher in germinated (37.63 μmol g^{-1} fresh weight [FW]) than imbibed (25.66 μmol g^{-1} FW) embryos. In the seedlings, much higher levels were found in cotyledons (93.45 μmol g^{-1} FW) and hypocotyls (67.56 μmol g^{-1} FW) than in roots (27.08 μmol g^{-1} FW), possibly reflecting the influx of nitrogen from the megagametophyte. Pro (40.6%) and Arg (23.0%) are the predominant amino acids in imbibed embryos but their relative abundance markedly decreased early during germination and Gln (55.3%) became the most abundant amino acid in germinated embryos. The amino acid profiles were quite different in the organs of analyzed pine seedlings, however, and interestingly, about 80% of the total content was represented by only a few amino acids. Thus, Gln (16.06%) and particularly Arg (63.2%) were predominant in cotyledons, Asn (45.18%) and Glu (34.5%) in hypocotyls, and Asn (19.61%), Glu (38.88%), and Gln (20.35%) in radicles. It must be remarked that Pro was present at high levels in imbibed embryos but declined in germinated embryos and it was undetectable in cotyledons, hypocotyls, and radicles of seedlings. Orn levels were low at all analyzed stages and organs. Finally, the levels of ammonium remained invariable in the analyzed germination stages.

DISCUSSION

In angiosperms, it has been proposed that δ-OAT plays an important role in Pro biosynthesis during vegetative development and in response to different types of stresses (Delauney et al., 1993; Roosens et al., 1998). However, the contribution of the enzyme to Glu biosynthesis in plants has been barely considered. In mammals, it has been shown that Arg-derived Orn can be converted to Glu providing a sufficient amount of this particular amino acid for ammonium detoxification and γ-aminobutyrate biosynthesis (Boon et al., 1999; Levillain et al., 2004), and very recently it has been shown that Arabidopsis plants are able to use Arg and Orn as the only nitrogen source without a concomitant increase in Pro levels (Funck et al., 2008). Due to the above mentioned, we decided to investigate whether δ-OAT could have a similar role in the generation of Glu that is required for ammonium assimilation by GS in germinating pine embryos (Ávila et al., 2001; Cánovas et al., 2007).

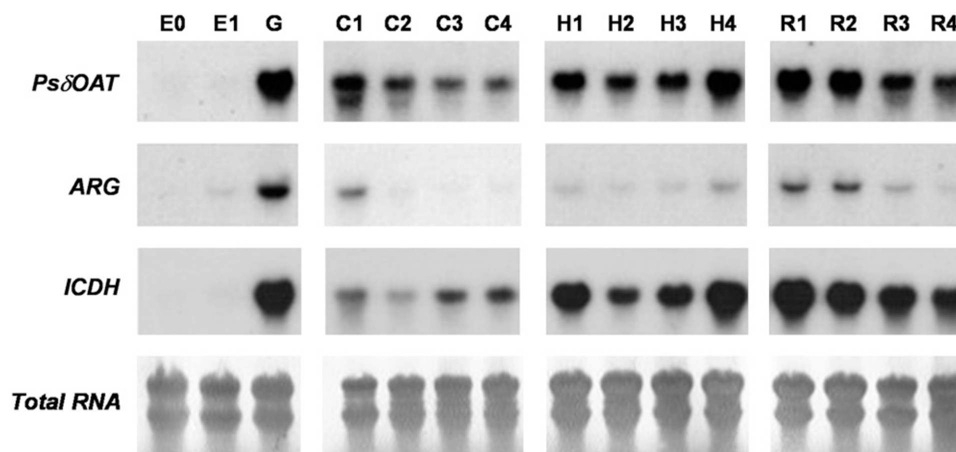

Figure 3. Expression of genes involved in nitrogen mobilization from Arg during pine germination. Samples of total RNA (10 μg) were transferred onto the nylon membrane and hybridized with probes for δ-OAT (*PsδOAT*), arginase (*ARG*), and cytosolic NADP^{+}-dependent isocitrate dehydrogenase (*ICDH*) genes. E0, Dry embryo; E1, embryo after 24 h of imbibition in water; G, germinated seed; C1 to C4, cotyledons from seedlings 8, 12, 30, and 90 d after imbibition; H1 to H4, hypocotyls from seedlings 8, 12, 30, and 90 d after imbibition; R1 to R4, radicles from seedlings 8, 12, 30, and 90 d after imbibitions. Membrane was stained with methylene blue to visualize ribosomal RNA as a loading control (*Total RNA*).

A cDNA clone has been isolated that encodes a functional pine δ-OAT enzyme according to the primary structure of the protein, conservation of critical residues, and the assays with recombinant protein produced in *E. coli*. The pine polypeptide, like other δ-OATs in plants and mammals, has a putative N-terminal transit peptide for mitochondrial targeting. This structural feature is consistent with the localization of δ-OAT and arginase activities in plant isolated mitochondria (Taylor and Stewart, 1981; Goldraij and Polacco, 2000) and the recently published targeting of δ-OAT/GFP chimeric protein to the mitochondria in Arabidopsis (Funck et al., 2008). Arginase and δ-OAT enzymes have also been located in the mitochondria of mammalian cells (Levillain et al., 2004). The common mitochondrial localization of arginase and δ-OAT suggests that these enzymes may have a similar metabolic role in the Arg metabolism of plants and animals.

Southern-blot analysis with a *Psδ**OAT*** probe together with the occurrence of only one contig of EST sequences in the available large collection of pine unigenes (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi) indicates that a single copy of this gene is present per pine haploid genome. Similarly, a single gene appears to be present in the

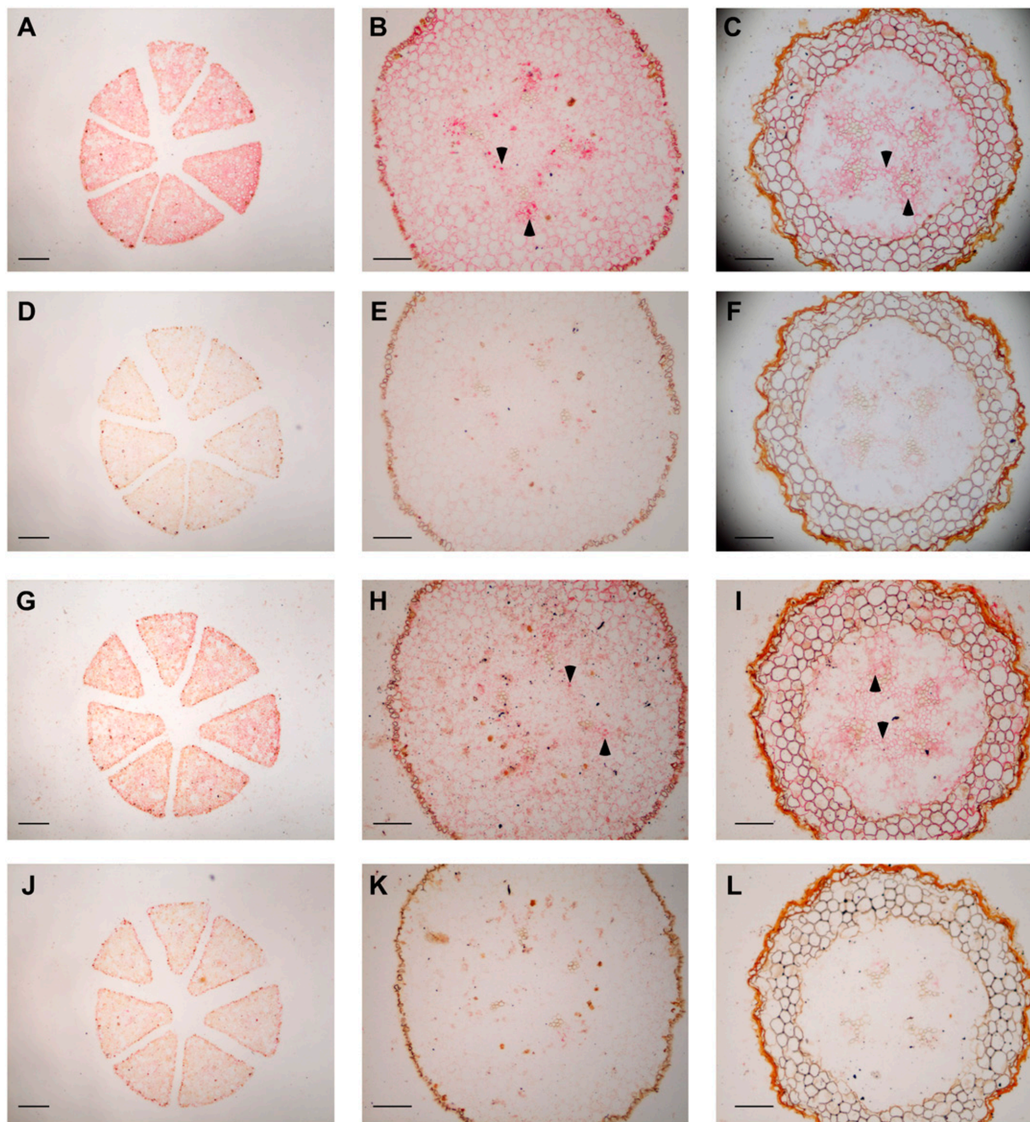

Figure 4. In situ localization of *PsδOAT* and *ARG* transcripts in transversal sections of pine seedlings at developmental stage 1. A to C, Hybridization of cotyledons, hypocotyl, and radicle sections, respectively, with *PsδOAT* antisense cRNA probe. D to F, Hybridization with *PsδOAT* sense cRNA. G to I, Hybridization with *ARG* antisense cRNA probe. J to L, Hybridization with *ARG* sense cRNA. The cRNA probes were visualized in red. Arrowheads label strong signals in phloem cells (arrowheads pointing down) and cells surrounding the resin ducts (arrowheads pointing up). Scale bars represent 0.2 mm in cotyledons sections and 0.1 mm in hypocotyl and radicle sections.

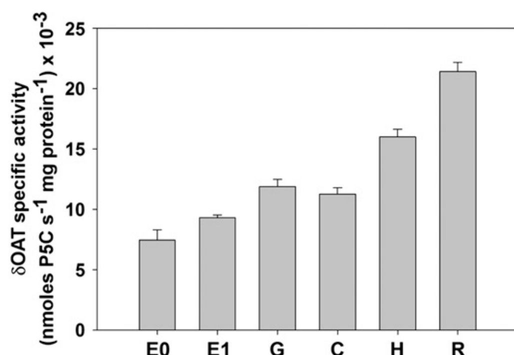

Figure 5. δ-OAT activity in early stages of pine germination. Activity was quantified in protein extracts of dormant (E0), imbibed (E1), and germinated embryos (G), and cotyledons (C), hypocotyl (H), and radicle (R) from seedlings at the developmental stage 1. Activity was determined as P5C formation. Values are means ± SE of three independent replicates. Statistical significance of differences of the average values were evaluated with ANOVA ($\alpha = 0.05$).

completed genome sequences of Arabidopsis (Roosens et al., 1998), rice (*Oryza sativa*), and poplar (*Populus* spp.), suggesting that a single δ-OAT gene is present in higher plant genomes.

Supporting a role of the *PsδOAT* in early pine development, our results demonstrate that gene expression is absent in E0 embryos and barely detectable in E1 embryos, but induced following germination to reach high levels in young seedlings at developmental stage 1. Enzyme activity levels and gene expression were parallels in cotyledons, hypocotyls, and radicle of seedlings, suggesting that changes in activity along the seedling are transcriptionally regulated. Contrarily, a clear discrepancy between δ-OAT activity and gene expression was observed at embryo stages. Despite the very low level of transcript in E1 embryo, a considerable amount of specific activity was detected in the samples. Similar values of activity were also detected in E0 embryos although at this developmental stage the *PsδOAT* mRNA is absent, suggesting the occurrence of δ-OAT protein in the dormant embryo before germination. Similarly, isocitrate dehydrogenase and arginase activities and proteins were detected in pine embryos, contrasting with barely detectable or absent mRNA levels (Palomo et al., 1998; Todd et al., 2001). These observations suggest that during maturation of pine seeds these enzymes accumulate in the embryo, to support Glu biosynthesis from Arg during the very early stages of germination. However, further work must be done to verify this hypothesis and before discarding other possibilities.

The expression patterns of *ARG* and *ICDH* genes are quite similar to the expression pattern of *PsδOAT*, suggesting that the combined action of arginase and isocitrate dehydrogenase would provide Orn and 2-oxoglutarate for δ-OAT activity. The levels of Glu synthases (ferredoxin- and NADH-GOGAT) are very low in the embryo and presumably these enzymes are not involved in Glu biosynthesis in the embryonic tissue (García-Gutiérrez et al., 1995) and probably during very early germination stages. However, arginase and urease activities release ammonium, which is rapidly recycled by the high levels of GS induced in germinating pine embryos (Ávila et al., 2001; Cánovas et al., 2007), requiring Glu as a substrate. The expression pattern of *PsδOAT* strongly suggests that δ-OAT could provide the required amount of Glu to support an active ammonium recycling.

Cytosolic $NADP^+$-dependent isocitrate dehydrogenase is considered a key enzyme in the generation of 2-oxoglutarate for ammonium assimilation and amino acid biosynthesis in higher plants (Hodges, 2002). In germinating pine seedlings, $NADP^+$-dependent isocitrate dehydrogenase activity represents approximately 90% of total isocitrate dehydrogenase activity detected in any tissue and is mostly located in the cytosol (Palomo et al., 1998). These data together with results of gene expression presented in this work suggest that 2-oxoglutarate required for δ-OAT activity is provided mainly by cytosolic isocitrate dehydrogenase.

The decrease in *PsδOAT* transcript levels observed during cotyledon, hypocotyl, and radicle development could indicate the transition from a Glu supply by Orn transamination to a Glu synthesis mainly directed by Glu synthase. Although, the high transcript levels that are present in lignified stems suggest additional roles for δ-OAT in later developmental stages of this organ that would require further research efforts.

At developmental stage 1, *PsδOAT* and the *ARG* mRNAs were widely distributed in photosynthetic and vascular cells of cotyledons, hypocotyls, and radicles. A similar expression pattern has been recently reported for *ARG* transcripts in pine somatic embryos (Pérez-Rodríguez et al., 2006). This broad location of mRNA from both genes suggests that Arg released from the megagametophyte is not metabolized in a specific type of cells. Instead, most of the cells seem to contribute producing Glu and ammonia for nitrogen mobilization.

Figure 6 shows a proposed scheme that describes the metabolic reactions involved in the nitrogen flow from the megagametophyte to the developing seedling, as well as the central role of mitochondria in this process. Urea generated inside the organelle is transported to the cytosol, where it could be hydrolyzed by urease (Sirko and Brodzik, 2000). The hydrolysis of urea by the enzyme urease has been reported in loblolly pine seedlings (King and Gifford, 1997; Todd and Gifford, 2002), supporting that Arg catabolism is an important source of ammonium during early seedling development. Furthermore, the role of two cytosolic GS (GS1a and GS1b), GOGAT, and AS in the metabolic fate of this ammonium has also been reported (García-Gutiérrez et al., 1995; Ávila et al., 2001; Suárez et al., 2002; Cañas et al., 2006). However, no previous data have been reported about how the nitrogen contained in the Orn molecule generated from Arg degradation in mitochondria is metabolized.

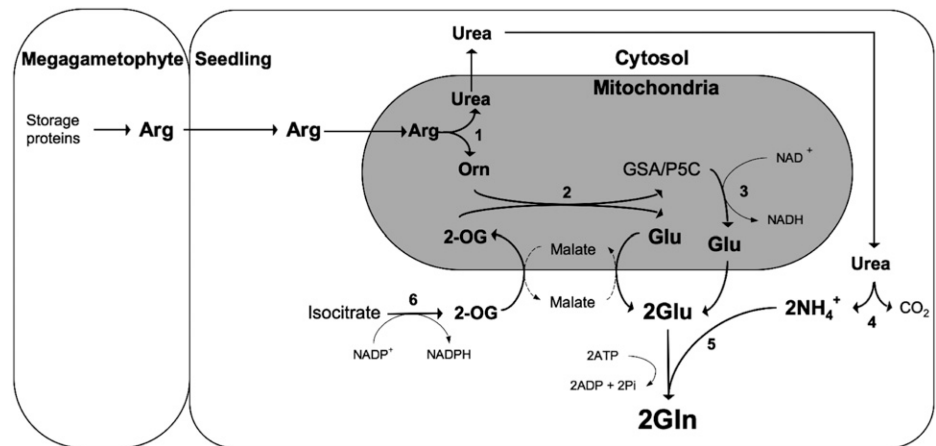

Figure 6. Metabolic scheme of the proposed pathway for Gln biosynthesis from Arg during pine germination. 1, Arginase; 2, δ-OAT; 3, P5CDH; 4, urease; 5, GS; 6, isocitrate dehydrogenase.

Our results strongly support an important role of δ-OAT during early stages of pine seed germination, by synthesizing Glu from Orn and 2-oxoglutarate. Glu can be immediately used to support synthesis of nitrogen-containing molecules, whereas GSA, after spontaneous cycling to P5C, would be oxidized to Glu by the mitochondrial P5CDH activity. Enzymes with P5CDH activity have been purified from cultured plant cells (Forlani et al., 1997a, 1997b) and a nuclear gene encoding a mitochondrial P5CDH has been identified and characterized in Arabidopsis (Deuschle et al., 2001). It has been shown that accumulation of GSA/P5C leads to cell death in human tumor cell lines (Maxwell and Davis, 2000) and plants (Deuschle et al., 2001, 2004), and causes oxidative damage in yeast cells (Nomura and Takagi, 2004). Thus, efficient and coordinated δ-OAT and P5CDH activities into the mitochondria would be essential during pine germination to avoid the accumulation of the highly toxic GSA/P5C. The significant allosteric behavior of the enzyme for Orn ($n = 2.3$) suggests a high sensitivity of the enzyme activity to changes in concentration of this amino acid. This could determine efficient Orn utilization by this pathway above certain levels of free Orn, regulating the contribution of δ-OAT to Orn metabolism.

Table I. *Soluble amino acid composition, pool size, and ammonium content of Scots pine embryo and seedlings at the developmental stage 1*

Amino acid values are expressed as μmol/g FW. All values are the means ± SE of three independent replicates. E1, Imbibed embryos; G, germinated embryos; C1, cotyledons; H1, hypocotyls; R1, radicles.

Amino Acid	E1	G	C1	H1	R1
Asp	0.08 ± 0.02	0.13 ± 0.00	0.21 ± 0.00	0.10 ± 0.02	0.06 ± 0.02
Thr	0.32 ± 0.10	0.47 ± 0.05	0.34 ± 0.12	0.17 ± 0.03	0.24 ± 0.02
Ser	0.47 ± 0.04	1.14 ± 0.07	0.61 ± 0.13	0.88 ± 0.41	0.39 ± 0.06
Asn	0.37 ± 0.11	1.27 ± 0.09	4.13 ± 0.32	30.52 ± 3.85	5.31 ± 0.38
Glu	2.14 ± 0.40	3.36 ± 0.10	3.15 ± 0.37	23.32 ± 2.61	10.53 ± 0.59
Gln	3.28 ± 0.68	20.83 ± 1.00	15.01 ± 0.10	0.00 ± 0.00	5.51 ± 0.38
Pro	10.41 ± 3.03	1.90 ± 0.14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Gly	0.44 ± 0.06	0.42 ± 0.02	0.20 ± 0.02	0.54 ± 0.09	0.19 ± 0.07
Ala	0.49 ± 0.03	0.85 ± 0.07	0.24 ± 0.01	1.13 ± 0.19	0.23 ± 0.02
Val	0.18 ± 0.02	0.91 ± 0.06	1.95 ± 0.08	3.40 ± 0.14	0.00 ± 0.00
Cys	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Met	0.19 ± 0.02	0.22 ± 0.02	0.17 ± 0.02	0.30 ± 0.04	0.34 ± 0.01
Ile	0.10 ± 0.03	0.33 ± 0.04	0.66 ± 0.05	0.98 ± 0.13	1.48 ± 1.17
Leu	0.12 ± 0.04	0.43 ± 0.03	0.91 ± 0.05	0.12 ± 0.02	1.12 ± 0.85
Tyr	0.15 ± 0.01	0.27 ± 0.04	0.18 ± 0.04	0.08 ± 0.06	0.00 ± 0.00
Phe	0.08 ± 0.02	0.37 ± 0.03	0.23 ± 0.03	0.15 ± 0.07	0.00 ± 0.00
Trp	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.04	0.00 ± 0.00	0.00 ± 0.00
Orn	0.20 ± 0.08	0.22 ± 0.11	0.29 ± 0.00	0.14 ± 0.10	0.07 ± 0.00
Lys	0.29 ± 0.08	0.25 ± 0.09	0.53 ± 0.02	0.30 ± 0.12	0.30 ± 0.01
His	0.45 ± 0.08	0.67 ± 0.09	5.48 ± 0.13	1.46 ± 0.11	0.73 ± 0.03
Arg	5.90 ± 0.63	3.59 ± 0.11	59.08 ± 5.81	3.97 ± 0.65	0.58 ± 0.02
Total	25.66 ± 2.21	37.63 ± 1.65	93.45 ± 4.72	67.56 ± 7.98	27.08 ± 3.38
NH_4^+	4.54 ± 0.82	4.62 ± 0.36	6.60 ± 0.07	5.20 ± 0.51	4.52 ± 0.22

Data on amino acid contents in embryo and seedlings at developmental stage 1 fully support the metabolic scheme described above. High levels of Pro accumulated in dormant dry embryo decreased after germination and were undetectable in the seedlings (Table I), suggesting an active catabolism of this amino acid during very early stages of germination, which could also contribute to Glu synthesis by the sequential reaction catalyzed by Pro dehydrogenase and P5CDH. Thus, de novo Pro biosynthesis, another possible metabolic function of δ-OAT, appears to be very limited during early pine growth. In fact, the most abundant amino acids in the embryos, Arg and Pro, seem to be catabolized early during germination to synthesize mainly Gln, which represents more than 50% of total amino acid content in germinating embryos.

Following germination, after the G developmental stage, when most of the nitrogen storage material in the embryo is exhausted, the megagametophyte seed storage proteins are broken down and amino acids are moved to the cotyledons of the developing seedling (King and Gifford, 1997), where they are used as a source of nitrogen. At developmental stage 1 the megagametophyte only contacts with the cotyledons, whereas the hypocotyl and radicle have emerged from the seed. Therefore the flux of nitrogen from the megagametophyte to the seedlings occurs via the cotyledons to the hypocotyl and radicle. This transfer of Arg from the megagametophyte to the seedling through the cotyledons could cause the observed gradient from the cotyledons to the radicle.

The high levels of Gln observed in cotyledons and radicles of seedlings and Asn in hypocotyls and radicles are also consistent with our previously reported data on the role of *GS* and *AS* genes in nitrogen mobilization (Cánovas et al., 1998, 2007; Suárez et al., 2002; Cantón et al., 2005). The efficient recycling of the ammonia released during storage protein mobilization by GS activity would maintain the stable levels of ammonium observed in embryos and seedling. Concomitant to this ammonium reassimilation, Glu has to be efficiently supplied to support high levels of Gln synthesis. If, as we propose, δ-OAT activity is a major supplier of Glu during early stages of germination this could explain why Orn levels do not increase either during germination, as observed for ammonium. The lack of Gln in the hypocotyl and the higher levels of Glu may reflect its utilization to support a high synthesis of Asn in this organ through the activity of a previously described Gln-dependent AS (Cañas et al., 2006), recycling Glu to the free amino acid pool.

CONCLUSION

The results shown in this article strongly suggest that a large fraction of Arg-derived Orn during pine germination is converted to Glu. The provision of two molecules of Glu from Orn catabolism meets the demands strictly required to fulfill the role of cytosolic GS in the assimilation of ammonium ions released in the urease reaction. The results reported here indicate that δ-OAT plays an important role in this metabolic pathway. Since Arg appears to be necessary to increase the levels of arginase protein and transcript during pine germination (Todd and Gifford, 2002, 2003), further research work is needed to investigate whether Arg and/or other metabolites are regulating the expression of genes encoding δ-OAT and other enzymes downstream the pathway.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Scots pine (*Pinus sylvestris*) seeds were obtained from Servicio de Material Genético, Ministerio de Medio Ambiente. Seeds were immersed in deionized water for 24 h, under continuous aeration and germinated in soil, in a controlled culture chamber (Ibercex H-900-B, ASL, SA) at 24°C with cycles of 16 h light/8 h dark. Illumination was provided by fluorescent lamps (Sylvania F-48T12/CW/WHO) at a flux density of 150 μmol m^{-2} s^{-1}. Plants were watered regularly but no nitrogen fertilization was added. Samples were always harvested at the same time in the light/dark cycle. Seven developmental stages were defined as previously described (Cañas et al., 2007): E0 or dry embryos were sampled from dormant seeds, E1 or imbibed embryos were sampled from seeds after 24 h of being immersed in deionized water, G or germinated embryo were harvested 4 d after imbibition, when the radicle was emerging from the seed. Developmental stages 1 and 2 are seedlings 8 and 12 d after imbibition, respectively, and correspond to early stages where the first needles have not yet developed and the vascular system in the hypocotyl consists of a few poorly lignified vascular bundles. Developmental stage 3 corresponds to seedlings 30 days after imbibition, showing new needles and a developed vascular system in the hypocotyl with an increased number of xylem and phloem elements and extensive lignifications, but still not forming a closed ring. Developmental stage 4 corresponds to seedlings 90 days after imbibition characterized for a highly lignified stem with a developed vascular ring.

Isolation of a Pine cDNA Clone Encoding δ-OAT

To clone a full-length cDNA for δ-OAT, two primers were designed from EST sequences available for *Pinus pinaster* (AL750399) and *Pinus taeda* (BG039825) in databases: OATf, 5′-GTTATTAGCCTGTGAGTGGG-3′ and OATr, 5′-CAACGATCACATGGTTCAGG-3′. A 579-bp cDNA fragment was amplified from Scots pine seedlings and cloned in the pBluescript vector (Stratagene) that encoded the carboxyl end of a polypeptide with similarity to δ-OAT from other plant species (from amino acid residues 272 to 463 in the pine polypeptide). A reverse primer derived from the 579-bp fragment was used to amplify the 5′ end of the mRNA by 5′-RACE RT-PCR (Marathon cDNA amplification kit; Clontech): OAT5r, 5′-CACTACATCAGGACGGA-CACTCTCC-3′. This partial cDNA clone was 1,178 bp in size and it included 323 bp from the 5′-UTR and 855 bp encoding the N terminus of a δ-OAT polypeptide. Finally, a δ-OAT near full-length cDNA was isolated by 5′-RACE RT-PCR with a specific forward primer designed from the 5′-UTR region of the partial clone and including eight nucleotides from the PCR adaptor (Marathon cDNA adaptor) at the 5′ end: OAT3f, 5′-GGGCAGGTGAAA-CAAGTAACAACCAC-3′. PCR were performed with proof-reading *Pfu* polymerase (Stratagene) and cloned in the pBluescript SK(−) plasmid (Stratagene). DNA was sequenced using an automatic sequencer ABI 373 XL Stretch and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Three independently amplified full-length cDNA clones were sequenced for sequence accuracy. The nucleotide sequence of the full-length cDNA was deposited in the EMBL Sequence Database under the accession number AM228955. Amino acid alignment was carried out with Clustal IV in the DNAstar software package.

Recombinant Protein Production in *Escherichia coli* Cells and Partial Purification

To produce an N-terminal poly-His-tagged δ-OAT polypeptide, a forward primer was designed containing nucleotides from positions 414 to 432

preceded by an *Ase*I site, an ATG codon, and the sequence encoding six His residues (5′-AGAATTAATGCACCATCATCATCATCATAATGCAGTCTCTT-CTCAAG-3′). This primer was used in a PCR reaction together with a reverse primer containing a sequence derived from positions 1,724 to 1,742 from the cDNA and incorporating a unique restriction site for *Bam*HI immediately downstream from the translation stop codon (5′-GCCTCTGGATCCTTAT-TTA-3′). Therefore, a cDNA encoding an N-terminal His-tagged δ-OAT protein with a deletion of residues 1 to 30 was synthesized. PCR was carried out with *Pfu* DNA polymerase (Stratagene). The products were digested with *Ase*I and *Bam*HI and cloned in the pET11a vector previously linearized by digestion with *Nde*I and *Bam*HI. Recombinant protein expression in BL21-Codon Plus (DE3) Ril *E. coli* cells (Stratagene), extraction of soluble protein, and partial purification with nickel-nitrilotriacetic silica spin columns (Qiagen) was performed as described elsewhere (Cañas et al., 2007).

δ-OAT Enzyme Assays

Plant tissue was ground in a cooled mortar at 4°C with two volumes of extraction buffer (100 mM potassium phosphate buffer pH 8, 1 mM PLP, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% [v/v] glycerol, and 0.2% [v/v] Triton X-100). To facilitate the extraction, sea sand was added to plant tissues in a ratio 2:1 (w:w, tissue:sand). The extract was centrifuged at 22,000*g* for 20 min at 4°C. The supernatant was dialyzed overnight at 4°C in extraction buffer without Triton X-100 to eliminate the metabolites in the sample. The dialyzed samples were used for determination of δ-OAT activity. The reaction was carried out in a buffer with 100 mM potassium phosphate buffer pH 8.0, 1 mM PLP, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% (v/v) glycerol, 25 mM Orn, and 5 mM 2-oxoglutarate. The addition of 2-oxoglutarate marked the beginning of the enzyme reaction. The incubation was carried out at 37°C for 90 min when protein extracted from embryo and seedling was assayed or 30 min when the assay was carried out with recombinant protein. The reactions were stopped by boiling the samples for 5 min in a water bath. Boiled samples were centrifuged at 16,000*g* for 2 min and the supernatant saved to a fresh tube. P5C was measured in the saved supernatant with the ninhydrin method described by Kim et al. (1994). For 1 mL of sample 0.3 mL of 3 N perchloric acid and 0.2 mL of ninhydrin 2% (w/v) was added. The mixture was boiled for 5 min in a water bath. Under these conditions P5C reacts with ninhydrin to form a reddish pigment soluble in ethanol. This product was recovered by centrifugation at 16,000*g* for 5 min and resuspended in 1.5 mL of ethanol. Absorbance of the colored P5C solution was measured at 510 nm and the concentration of P5C was calculated using the molar extinction coefficient at 510 nm of the P5C-ninhydrin complex ($16.5 \times 10^3\ M^{-1}\ cm^{-1}$) according to Lim et al. (1998). The reaction was lineal at least up to 180 min after starting.

Western-Blot Analysis

SDS-PAGE and electroblotting were carried out as described elsewhere (Cañas et al., 2007). Membranes were incubated 30 min at room temperature with a 1:5,000 dilution of 6x-His monoclonal antibody from mouse (CLONTECH) in TPBS (140 mM NaCl, 3 mM KCl, 5 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, 0.05% [v/v] Tween 20), with 0.05% (w/v) of bovine serum albumin. The membranes were washed three times with TPBS. Antigen-antibody complexes were detected with a secondary peroxidase-conjugated sheep anti-mouse-IgG antiserum (Amersham). The membranes were incubated at room temperature for 30 min with peroxidase-conjugated secondary antibodies diluted in TPBS with 0.05% (w/v) bovine serum albumin. Finally, the membranes were washed twice with TPBS and then twice with PBS (140 mM NaCl, 3 mM KCl, 5 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). Immunodetection was visualized with the Super Signal West Pico Chemiluminescent substrate for horseradish peroxidase (Pierce). The membranes were incubated with a 1:1 (v:v) mixture of luminol/enhancer and peroxide buffer solutions for 5 min. Finally, the membranes were exposed to an x-ray film (X-OMAT AR; Kodak) and developed.

DNA and RNA Extraction and Gel Analyses

Pine genomic DNA was prepared according to Dellaporta et al. (1983). For Southern-blot analysis, 20 μg of genomic DNA were digested separately with *Eco*RV, *Hin*dIII, *Xba*I, and *Eco*RI restriction enzymes. DNA samples were run on 0.8% agarose gel, blotted to nitrocellulose filters, and hybridized to ^{32}P-labeled probe obtained by labeling the 579-bp cDNA fragment described above with the random primer method (Megaprime; Roche). Prehybridizations, hybridizations, and washes at moderate stringency were performed as described elsewhere (Cañas et al., 2007).

Total RNA was isolated following the procedure described by Chang et al. (1993). Northern-blot analysis was performed as described previously (Cañas et al., 2006). The hybridization probes were derived from the Scots pine cDNA clone of *Ps*δ*OAT* (accession no. AM228955), a *P. taeda* cDNA clone of an arginase gene (accession no. AF130440), and a *P. pinaster* cDNA clone of an isocitrate dehydrogenase gene (accession no. AY344584). The partial cDNA fragment of 579 described above was used as a probe for δ-OAT mRNA detection. Digestion of the *P. taeda* full-length cDNA encoding arginase (Todd et al., 2001) with *Hin*dIII released an 847-bp fragment from positions 115 to 992. Southern-blot results in *P. taeda* indicate the presence of only one gene encoding arginase in the pine genome (Todd et al., 2001). This evidence is strongly supported by the presence of only one contig for arginase mRNA in the large collection of EST-derived unigenes in the Gene Index Project database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi). To obtain an *ICDH* probe, a fragment of 372 bp was derived from the 5′ end of the full-length cDNA by digestion with *Eco*RI, including 113 bp from the 5′-UTR and 260 from the coding region. Experiments carried out in our lab have shown the same result in the expression pattern during pine germination and seedling development whether we hybridize with the isocitrate dehydrogenase full-length or a 3′-UTR-derived probe. ^{32}P labeling was carried out with the High Prime system (Roche).

Preparation of Plant Tissue for Microscopy and in Situ mRNA Localization

Seedling tissues were fixed in freshly prepared FAA buffer (5% formaldehyde, 5% glacial acetic acid, 90% ethanol). The samples were then dehydrated in an ethanol series (50%; 70%; 80%; 96%; 100%), treated with increasing concentrations of xylene (1:1 ethanol/xylene, xylene, 1:1 paraffin/xylene), and paraffin embedded in an automatic tissue processor TP1020 (Leica). Thick sections (10 μm) were then obtained from embedded tissues and directly mounted onto poly-L-Lys-coated glass slides (Menzel-Gläser).

As templates for probe synthesis, the following cDNA fragments were used: for δ-OAT a fragment of 831 bp (positions 1,136–1,966 from the Scots pine full-length cDNA) and for arginase a fragment of 734 bp (positions 612–1,345 from the *P. taeda* full-length cDNA). Antisense and sense cRNA probes were synthesized by in vitro transcription and labeled with digoxigenin-11-UTP (Roche) following the procedure described by Langdale (1993). Rehydratation, treatment of the sections before hybridization, hybridization, and visualization of signals were carried out exactly as previously described (Cañas et al., 2007).

Free Amino Acids and Ammonium Analysis

Frozen plant material was ground in a mortar and extracted in 30 mM Tris-HCl, pH 8, 1 mM EDTA, and 10 mM 2-mercaptoethanol. The extract was centrifuged at 22,000*g* at 4°C and the supernatant was recovered and centrifuged again as above. An aliquot of 200 μL was transferred to a fresh tube, 500 μL of methanol were added and mixed for 10 min at 4°C. After centrifugation as above, the supernatant was saved in a fresh tube and the pellet was resuspended in 200 μL of (4:1, v/v) methanol:water, stirred for 10 min at 4°C, centrifuged, and the supernatant reserved. The last step was repeated twice and all the reserved supernatants combined. The volume was reduced to 200 μL by evaporation at 90°C in an oven. Finally the extract was filtered through a 0.2-μm pore filter.

To determine the free amino acid pool content of the samples together with the ammonium content, amino acids and free ammonium were separated with no derivatization with a System Gold HPLC BioEssential high-performance liquid chromatograph (Beckman-Coulter) using a lithium citrate buffer system and followed by a postcolumn ninhydrin reaction detection system. For the identification and quantification of amino acids and ammonium the corresponding standards were used.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AM228955.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Amino acid sequence alignment of eukaryotic δ-OAT.

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