Arginase Is Inoperative in Developing Soybean Embryos

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Abbreviation: DAG, days after germination.

Arginase (EC 3.5.3.1) transcript level and activity were measured in soybean (Glycine max L.) embryos from the reserve deposition stage to postgermination. Using a cDNA probe for a small soybean arginase gene family, no transcript was detected in developing embryos. However, arginase transcripts increased sharply on germination, reaching a maximum at 3 to 5 d after germination. There was low but measurable in vitro arginase specific activity in developing embryos (less than 6% of seedling maximum). During germination arginase specific activity increased in parallel with the sharply increasing arginase transcript level. Seedling arginase activity was largely localized in cotyledons. Arginase activity was assayed in vivo by measuring urea accumulation in a urease-deficient mutant. No urea was detected in developing embryos, whereas accumulated urea paralleled arginase specific activity and transcript level in germinating seedlings. As in planta embryos, cultured cotyledons did not accumulate urea when arginine (Arg) was provided with other amino acids in a “mock” seed-coat exudate. Arg as the sole nitrogen source was converted to urea but did not support cotyledon growth. There appeared to be a lack of recruitment of the low-level arginase activity to hydrolyze free Arg in developing embryos, thus avoiding a futile urea cycle.

Arg, a nitrogen-rich compound, is one of the predominant amino acids in the seed and storage organs of numerous plant species (Van Etten et al., 1967; Polacco and Holland, 1993) and represents a major form of reserve nitrogen. In developing soybean (Glycine max) cotyledons, Arg is both actively synthesized (Micallef and Shelp, 1989b) and supplied from the seed-coat exudate (Rainbird et al., 1984). Arg is incorporated into protein, where it accounts for 18% of the total seed-protein nitrogen, or it remains in the free amino acid pool, constituting more than 60% of the free amino acid nitrogen in developing cotyledons (Micallef and Shelp, 1989a).

During early seedling growth, storage proteins are mobilized to provide amino acids for proteins synthesized in the expanding axes. As part of the overall reconfiguration of free and storage-protein-bound amino acid profiles to serve deposition stage. Our results indicate that the increase of arginase activity upon germination was due to an increase in arginase transcript, and are consistent with the absence of an arginase-catalyzed reaction in developing embryos during the reserve deposition stage.

MATERIALS AND METHODS

Plant Material and Germination/Growth Conditions

Wild-type Eu3/Eu3 soybean (Glycine max L. Merrill cv Williams 82) plants and an otherwise isogenic Eu3/eu3-e1 heterozygote were grown in a controlled-environment chamber at 27°C with a 12-h/12-h light/dark regime at 180 μmol m−2 s−1. The eu3-e1 mutation has been characterized previously (Meyer-Bothling et al., 1987). The homozygous eu3-e1/eu3-e1 mutant lacks the activities of all soybean ureases. Eu3/eu3-e1 heterozygous plants (Stebbins et al., 1991; Stebbins and Polacco, 1995) have urease levels similar to wild-type Eu3/Eu3 plants. All urease-negative eu3-e1/eu3-e1

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2 s

3 mol m

4 EU

5 eu3-e1

6 eu3-e1

7 Eu3

8 eu3

9 eu3

10 Eu3

11 eu3

12 Eu3

13 eu3

14 Eu3

15 eu3

16 Eu3

17 eu3

18 Eu3

19 eu3

20 Eu3

21 eu3

22 Eu3

23 eu3

24 Eu3

25 eu3

26 Eu3

27 eu3

28 Eu3

29 eu3

30 Eu3

31 eu3

32 Eu3

33 eu3
seeds used in this study were derived from selfed *Eu3/eu3-e1* plants. Urease-positive and -negative seeds were distinguished by a seed-chip urease assay (Meyer-Bothling and Polacco, 1987). After removal from pods, immature embryos were separated from the testa, frozen in liquid nitrogen, lyophilized, and stored at −70°C. The relative water content of embryos (milligrams of water per milligram of embryo fresh weight × 100) was taken as the criterion for the physiological state of the embryo.

For seedling studies, seeds were germinated in the dark at 28°C in rolls of germination paper (Anchor Paper, St. Paul, MN) moistened with deionized water. Seeds routinely germinated 1 d after imbibition. Whole etiolated seedlings were frozen immediately in liquid nitrogen and stored at −70°C until use.

### Arginase Activity Assay

Embryos or seedlings (1.5–2.0 g fresh weight) were ground and powdered in a mortar with liquid nitrogen. The fine powder was resuspended to a final volume of 4 mL in cold 0.1 M Tris-maleate, pH 7.0, containing 1 mM EDTA and 0.1 mM PMSF. The suspension was maintained on ice and disrupted (three 15-s cycles) in a homogenizer (Ultra Turrax, Tekmar, Cincinnati, OH) and centrifuged at 13,000 rpm for 10 min at room temperature to activate arginase.

Arginase activity was assayed by measuring the Arg-dependent production of urea. One milliliter of standard assay medium contained 160 mM L-Arg (adjusted to pH 9.7 with KOH), 33 μM phenyl phosphorodiamidate (a urease inhibitor, Liao and Raines [1985]), and about 0.1 to 0.3 mg of extract protein. The reaction mixture was incubated for 30 min at 30°C. Aliquots (400 μL) were removed and the reaction was stopped by adding 1 N H2SO4 to 600 μL. Urea released was determined colorimetrically (Schimke, 1970). Arginase activity was expressed as nanomoles of urea released per minute per milligram of protein. Protein was determined by the method of Lowry et al. (1951).

### Nucleic Acid Techniques

Genomic DNA was isolated from soybean leaves according to the method of Dellaporta et al. (1983). For DNA analysis 8 μg of DNA was digested overnight with EcoRI, BamHI, XbaI, or EcoRV at 37°C, and then separated on a 1% agarose gel. DNA was blotted to a nitrocellulose membrane and hybridized at high stringency in 50% (v/v) formamide, 100 μg mL⁻¹ sonicated salmon-sperm DNA, 100 μg mL⁻¹ yeast RNA, 5× Denhardt’s solution (1× Denhardt’s is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 50 mM NaPO₄, pH 6.5, 5× SSC (1× SSC is 0.15 mM NaCl and 0.015 mM sodium citrate), and 0.2% SDS at 42°C for 14 to 16 h. The filter was washed in 2× SSC, 0.2% SDS at room temperature for 15 min, followed by 0.2× SSC, 0.2% SDS at 65°C for 30 min. Low-stringency hybridization conditions were identical to high-stringency conditions except that formamide was reduced to 25%. The filter was washed in 2× SSC, 0.2% SDS at room temperature for 15 min, followed by a second wash in the same solution at 42°C for 15 min. The dried blot was exposed for 3 to 5 d to radiographic film (X-Omat, Kodak) with intensifying screens at −70°C.

Total RNA was isolated from embryos or seedlings according to the method of Murfett et al. (1994). For RNA analysis 8 μg of RNA was separated in a 16.2% formaldehyde and 1% agarose gel and transferred to a nylon membrane. Before hybridization the membrane was stained with 0.3 mM sodium acetate, pH 5.2, containing 0.03% methylene blue to reveal RNA. Hybridization conditions and washes were identical to the high-stringency conditions used for DNA analysis. The RNA blot was exposed to radiographic film as indicated above.

### Cotyledon Culture

Developing urease-positive (*Eu3/Eu3* and *Eu3/eu3-e1*) and -negative (*eu3-e1/eu3-e1*) cotyledons (80–100 mg fresh weight), derived from selfed *Eu3/eu3-e1*, were collected and their phenotype was identified by a seed-chip assay performed on excised embryonic axes (Meyer-Bothling and Polacco, 1987). The tests and axes were aseptically removed and each embryo was halved into separate cotyledons. Cotyledons derived from the same embryo were used to compare different nitrogen sources. Cotyledons were cultivated in 3 mL of culture medium as described by Thompson et al. (1977), except that vitamins were at half-strength, Gly was omitted, and 5.9 mM K₂SO₄ was used as the sulfur source.

A mock seed-coat exudate was made up according to the reported five main amino acids in the in vivo soybean seed coat exudate and used to provide amino acids to the cotyledon (Rainbird et al., 1984). The amino acid mixture was 12.6 mM Gln, 4.5 mM Asn, 1.1 mM Ser, 0.7 mM His, and 0.6 mM Arg (*N* = 40 mM L⁻¹). Arg was provided as the sole nitrogen source at either 10 mM (*N* = 40 mM L⁻¹) or at 0.6 mM. In both cases the results with respect to urea evolution, growth, and total protein after 6 d of culture were identical. For cotyledon protein determination, the 3% HClO₄ pellet (see above) was boiled for 15 min in 1 mL of 1.5 N NaOH. The insoluble material was removed by cen-
trifugation and the solubilized protein was determined in the supernatant by the Lowry method (Lowry et al., 1951).

In experiments with radiolabeled Arg, \(1.1 \times 10^9\) dpm \(L-\text{guanido-}^{14}\text{C}\)Arg (51.5 Ci/mol, DuPont NEN) were added to the culture medium. Treatment with Dowex 50 W-X8 columns separated \({}^{14}\text{C}\)urea in cotyledon extracts and in the culture medium from unreacted \(L-\text{guanido-}^{14}\text{C}\)Arg, as indicated by Daghigh et al. (1994), and then measured by liquid scintillation counting. The eluate was confirmed to be urea by its conversion to \(^{14}\text{CO}_2\) with added urease.

**RESULTS**

**Arginase Sequence and Copy Number**

Using a full-length Arabidopsis arginase cDNA (Krumpelman et al., 1995) as a probe, we have previously reported (Goldraij et al., 1998) the cloning of a 1324-bp cDNA fragment (\(AG1\)) encoding a soybean seedling arginase. Figure 1 shows a comparison of the deduced amino acid sequences of the two enzymes. They are similar in size (36.5 and 38.6 kD for Arabidopsis and soybean, respectively) and are 78% identical. The most notable differences are in the N-terminal region. Considering that all plant arginases reported so far are mitochondrial enzymes (Polacco and Holland, 1993), this region constitutes a putative transit peptide. In addition, both sequences have characteristics common to N-terminal transit peptides, such as abundance of hydroxylated and positively charged residues (Hartl et al., 1989).

To examine the copy number of the soybean arginase gene and the presence of related genes, we used \(AG1\) as a probe to perform DNA analysis. Under high-stringency conditions, genomic DNA digested with \(XbaI\) and \(EcoRI\) showed two and three bands, respectively, whereas \(BamHI\) and \(EcoRV\) digestions showed four or more bands (Fig. 2A). The cloned \(AG1\) had only one restriction site for \(BamHI\) and \(EcoRV\), indicating the presence of more than one arginase gene and/or the presence of intron restriction sites for the enzymes used in the analysis. Additional bands appeared in all DNA digestions when the hybridization was performed at low stringency (Fig. 2B), indicating the existence of more distantly related genes in the soybean genome.

**Arginase Expression in Developing Embryos and in Germinating Seeds**

Arginase expression was studied at the transcript level in developing embryos and in germinating soybean seeds. Total RNA isolated from immature embryos, quiescent seeds, and whole 0- to 6-DAG seedlings was subjected to blot analysis and probed with \(AG1\) (Fig. 3). Hybridization revealed a single band migrating slightly faster than the 1.38-kb standard, which is consistent with the size of the arginase cDNA. The transcript appeared at 1 DAG and stayed at the same level at 2 DAG. It accumulated to higher levels in the 3- to 5-DAG interval and then decreased slightly by 6 DAG. In contrast, no transcript was seen in developing embryos or in quiescent seeds, although the amount of RNA analyzed was nearly four times greater. A nearly identical pattern was obtained for arginase specific activity of the same samples used in the RNA blot experiment (Fig. 4). Arginase activity was barely detectable in developing embryos and in quiescent seed, but a sharp and constant increase was seen from 2 DAG until reaching a maximum at 5 DAG. Therefore, the increase of arginase activity is consistent with an increase in the transcript level, indicating that expression of the arginase gene(s) is a developmentally controlled process coincident with germination and seedling development.

**Figure 1.** Sequence alignment of plant arginas. Deduced amino acid sequence of soybean seedling arginase (Goldraij et al., 1998) was compared with that of Arabidopsis arginase (Krumpelman et al., 1995). Amino acids conserved between the two proteins are highlighted. The N-terminal region, which exhibits the weakest identity, is a putative transit peptide. Dashes are gaps for optimization of alignments.
The organ distribution of arginase expression and activity was investigated in 3- to 4-DAG seedlings. The results showed that the highest arginase specific activity was in the cotyledon, followed by the hypocotyl and radicle, with only about 30% and 15% of the cotyledon specific activity, respectively (Fig. 5A). The arginase activity levels of the different seedling organs roughly corresponded to their arginase transcript levels (Fig. 5B). Because the total activity in the cotyledon was about 50 times higher than in the radicle or hypocotyl, we concluded that arginase was localized mainly in the cotyledons.

The Arginase Reaction Is Inoperative in Developing Embryos

The arginase expression patterns (Figs. 3 and 4) suggest that the role of arginase is largely confined to germination and seedling development. We tested this suggestion by an in-vivo approach exploiting mutant eu3-e1/eu3-e1, which lacks all urease activity (Meyer-Bothling et al., 1987). The quantity of urea accumulated by the mutant is a useful indicator of in vivo arginase activity (Stebbins and Polacco, 1995). Virtually identical, low levels of urea were found in developing embryos of the mutant and its urease-positive siblings (all progeny of selfed Eu3/eu3-e1 plants) (Fig. 6).

The lack of urea accumulation in the mutant suggests that the arginase-catalyzed reaction is inoperative in developing embryos.

In contrast, there was a sharp increase in urea upon germination of urease-negative seeds, reaching a peak at 3 DAG. This pattern is roughly in agreement with the pattern for arginase expression and arginase specific activity, albeit with an earlier peak. Based on studies using labeled precursors and an inhibitor (allopurinol) of purine conversion
to ureides (Stebbins and Polacco, 1995), we demonstrated previously that the vast majority of urea generated in soybean seedlings comes from Arg rather than from ureides. As expected, much less urea accumulated in urease-positive seedlings, although the levels were somewhat higher than previously reported.

Arginase Is Conditionally Operative in in Vitro-Cultured Cotyledons

Developing soybean cotyledons were cultured in vitro in defined media to evaluate Arg as a nitrogen source and to test for a functional arginase using the criterion of urea accumulation in the urease-deficient mutant eu3-e1/eu3-e1. We previously determined that Arg uptake was not altered in the urease-deficient mutant compared with the wild type (results not shown). Arg was supplied to immature cotyledons as the sole nitrogen source or included in an amino acid mixture approximating the seed-coat exudate (Rainbird et al., 1984) that nourishes the developing embryo (see “Materials and Methods”).

In both the urease-positive seedlings and in the isogenic urease-deficient sibling, the growth and total protein in cotyledons cultured with Arg as the sole nitrogen source was almost identical to values obtained in cotyledons cultured without nitrogen (Fig. 7A), even though urea derived from Arg accumulated in the urease-deficient mutant (Fig. 7B). Arg is the precursor of urea when it is the sole nitrogen source, as confirmed by the conversion of L-[guanido-14C]Arg to [14C]urea (Table I). Therefore, the products derived from Arg breakdown (urea and Orn) did not support growth and protein deposition beyond developing cotyledons cultured without nitrogen.

The cotyledons used in the experiment shown in Figure 7 received 10 mM Arg. Identical results, including urea accumulation (data not shown), were obtained when Arg was reduced to 0.6 mM. With a mock seed-coat exudate (containing 0.6 mM Arg, see “Materials and Methods”) as the nitrogen source, growth and protein yield were increased about 40% to 50% in both genotypes with respect to cotyledons cultured with Arg (Fig. 7A), whereas no evolution of urea was detected in the urease-deficient mutant (Fig. 7B). As expected, no urea accumulation was seen in urease-positive cotyledons in any of the three treatments assayed. In preliminary experiments, when L-[guanido-14C]Arg was provided in a mock exudate, the label was rapidly incorporated into protein, as previously reported.

Table I. Total urea accumulation in cotyledons cultivated with L-[guanido-14C]Arg

<table>
<thead>
<tr>
<th>Soybean Genotype</th>
<th>Urea</th>
<th>MEa</th>
</tr>
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<tbody>
<tr>
<td>Eu3/Eu3 or Eu3/eu3-e1</td>
<td>29 ± 20</td>
<td>6 ± 0.9</td>
</tr>
<tr>
<td>eu3-e1/eu3-e1</td>
<td>1220 ± 150</td>
<td>8 ± 3</td>
</tr>
</tbody>
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a ME, Mock exudate.
We investigated the expression and functioning of soybean arginase in developing embryos and in germinating seeds. We used the cDNA clone AG1, which encodes a soybean seedling arginase (Goldraij et al., 1998) that is 78% identical to that of Arabidopsis (Fig. 1), the only other plant arginase whose sequence is currently available. High-stringency genomic hybridization indicated that arginase may be a member of a small gene family, whereas low-stringency conditions indicated the presence of more distantly related homologs in soybean (Fig. 2).

Arginase activity increased 20-fold upon germination to a maximum at 5 DAG, whereas RNA blots probed with AG1 revealed a transcript peak at 3 to 5 DAG. In contrast, arginase activity in developing embryos was basal and no transcript signal was detected at any time during the reserve deposition stage. Therefore, the increased arginase activity during germination and seedling development was most likely to be due to de novo enzyme biosynthesis and not to activation of pre-existing enzyme.

Germination as the signal for the synthesis of arginase is in agreement with a catabolic role. Its substrate, Arg, is a major nitrogenous storage compound in plants. For 379 species analyzed, Arg averaged 7.7 mol % of seed amino acids and 21.1% of total amino acid nitrogen, the highest contribution of any amino acid (Van Etten et al., 1967; Polacco and Holland, 1993). In soybean, Arg contains 18% of seed protein-bound nitrogen (Micallef and Shelp, 1989a). Arg degradation by arginase during germination is consistent with the sharp increase in urea in urease-deficient mutant seedlings (Fig. 6; Stebbins and Polacco, 1995). Urease has been proposed to function coordinately with arginase in the utilization of seed protein reserves during germination (Thompson, 1980). In Arabidopsis, urease functions to recycle urea nitrogen derived from Arg breakdown during seedling development (Zonia et al., 1995).

The preponderance of arginase activity in seedling cotyledons (Fig. 5) indicates that Arg degradation might occur mainly in the cotyledon, prior to the delivery of its nitrogen to the growing points. This is in agreement with observations in pumpkin cotyledons (Chou and Splitsstoesser, 1972), in which Arg constitutes 30% of amino acid nitrogen but is only a minor component of the amino acid nitrogen (1.7%) transported out of the cotyledon.

In contrast to the abundant arginase activity and urea reaction product in developing seedlings, the enzyme does not appear to be active in developing embryos. This conclusion is based mainly on the absence of urea accumulation in urease-deficient developing embryos (Fig. 6). At this stage arginase activity was extremely low but detectable. The absence of any transcript detectable by the seedling axis clone AG1 is consistent with either a lack of arginase transcript in the developing embryo or with a poorly cross-hybridizing minor arginase mRNA.

In vitro cotyledon culture was used to evaluate the capacity of Arg to support growth and protein synthesis in immature soybean cotyledons. A mixture of amino acids containing Arg and resembling the seed-coat exudate (mock exudate) stimulated an increase in fresh weight and protein deposition similar to that obtained with Gln alone (2.5 times the initial fresh weight and 95 mg of protein per cotyledon after 6 d culture), which was previously shown to be the best nitrogen source (Thompson et al., 1977). Increases in fresh weight and protein supported by the mock exudate were not, however, accompanied by Arg breakdown caused by arginase, because no urea accumulated in urease-deficient cotyledons (Fig. 7).

The situation with Arg as the sole nitrogen source (at 0.6 or 10 mM) was exactly reversed. Arg did not support growth or protein deposition in urease-positive or -negative cotyledons, but was actively broken down by arginase, as evidenced by urea accumulation in the mutant. We observed no induction of arginase by Arg. The in vitro arginase specific activity of cultured cotyledons (<11 nmol urea min⁻¹ mg⁻¹ protein) was lower than the specific activity observed in embryos in planta. Because the other amino acids in the mock exudate appear to prevent arginase action, they could prevent uptake of Arg into the cotyledon or into the mitochondrion, the site of all or most plant arginases (Polacco and Holland, 1993). Inhibition of uptake into the cotyledon appears unlikely, because we observed no differences in Arg uptake whether it was provided alone or included in the mock exudate. In the latter case, Arg was abundantly incorporated (35% of total Arg uptake) into perchloric-acid-precipitable material (A. Goldraij and J. Polacco, unpublished results).

Other studies have examined the role of arginase in developing cotyledons of pea (De Ruiter and Kollöfle, 1983) and soybean (Micallef and Shelp, 1989b, 1989c). In both species Arg was reported to be actively incorporated into protein and was also the major constituent of the free amino acid pool. After injection of L-[guanido-¹⁴C]Arg into excised cotyledons, little urea accumulation or ¹⁴CO₂ release was detected in pea despite the in vitro arginase activity detected in developing seeds. Contrary to our results, Micallef and Shelp (1989c) found that approximately 20% of injected Arg was routed to urea and Orn in soybean. Perhaps this activity was the result of arginase being released from mitochondria by mechanical disruption caused by Arg injection.

We propose that arginase is not operative in vivo in developing soybean embryos. Although very low, in vitro arginase activity could be detected and was sufficient to generate large amounts of urea when Arg was the sole nitrogen source in cultured cotyledons. The presence of arginase and a large free Arg pool without a reaction taking place indicates that a regulatory mechanism impedes the reaction. Considering that all plant arginases reported so far are mitochondrial or particulate enzymes (for review, see Polacco and Holland, 1993), this regulation may be achieved by different intracellular locations of sub-
Arginase in Developing Embryos and in Germinating Seeds


