Regulation of Plant Arginase by Wounding, Jasmonate, and the Phytotoxin Coronatine**

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In mammalian cells, induced expression of arginase in response to wound trauma and pathogen infection plays an important role in regulating the metabolism of L-arginine to either polyamines or nitric oxide (NO). In higher plants, which also utilize arginine for the production of polyamines and NO, the potential role of arginase as a control point for arginine homeostasis has not been investigated. Here, we report the characterization of two genes (LeARG1 and LeARG2) from Lycopersicon esculentum (tomato) that encode arginase. Phylogenetic analysis showed that LeARG1 and -2, like all other plant arginases, are more similar to agmatinase than to arginases from vertebrates, fungi, and bacteria. Nevertheless, recombinant LeARG1 and -2 exhibited specificity for L-arginine over agmatine and related guanidino substrates. The plant enzymes, like mammalian arginases, were inhibited (Kᵢ ~ 14 μM) by the NO precursor N⁶-hydroxy-L-arginine. These results indicate that plant arginases define a distinct group of ureohydrolases that function as authentic L-arginases. LeARG1 and LeARG2 transcripts accumulated to their highest levels in reproductive tissues. In leaves, LeARG2 expression and arginase activity were induced in response to wounding and treatment with jasmonic acid (JA), a potent signal for plant defense responses. Wound- and JA-induced expression of LeARG2 was not observed in the tomato jasmonic acid-insensitive1 mutant, indicating that this response is strictly dependent on an intact JA signal transduction pathway. Infection of wild-type plants with a virulent strain of Pseudomonas syringae pv. tomato also up-regulated LeARG2 expression and arginase activity. This response was mediated by the bacterial phytotoxin coronatine, which exerts its virulence effects by co-opting the host JA signaling pathway. These results highlight striking similarities in the regulation of arginase in plants and animals and suggest that stress-induced arginase may perform similar roles in diverse biological systems.

1-L-Arginine is one of the most functionally diverse amino acids in living cells. In addition to serving as a constituent of proteins, arginine is a precursor for the biosynthesis of polyamines, agmatine, and proline, as well as the cell-signaling molecules glutamate, γ-aminobutyric acid, and nitric oxide (1–3). Two of the most intensively studied pathways of arginine metabolism are those catalyzed by arginase and nitric-oxide synthase (NOS).2 Arginase hydrolyzes arginine to urea and ornithine, the latter of which is a precursor for polyamine biosynthesis. Recent studies in animal systems indicate that increased arginase expression stimulates the production of polyamines that promote tumor cell proliferation (4), wound healing (5), and axonal regeneration following injury (6). Juxtaposed to the growth-promoting effects of polyamines are the cytostatic effects of NO produced by activated macrophages. The switch between the arginase and NOS branches of arginine metabolism is controlled by various inflammatory signals that regulate arginase expression and arginine availability (2, 7–9). Because arginase and NOS compete for a common substrate, increased arginase expression can effectively attenuate the NOS pathway, often with profound physiological consequences. A diversity of human pathogens, for example, induce arginase expression as a means of evading NO-mediated host defenses (10–13). The interaction between the arginase and NOS pathways extends beyond the fact that they both use a common substrate. For example, the intermediate in the NOS-catalyzed production of NO, N⁶-hydroxy-L-arginine (NOHA), functions as a potent inhibitor of arginase (14, 15).

In contrast to our understanding of arginase regulation in animals, very little is known about the potential role of arginase as a metabolic control point for arginine homeostasis in higher plants. The well-established role of NO in plant developmental and defense-related processes (16–18), together with the recent discovery of two arginine-utilizing plant NOSs (19–20), provides a strong rationale for addressing this question. Most studies of plant arginase have focused on its role in mobilizing arginine as a nitrogen source during post-germinative growth (21–28). Arginine can account for 50% of the nitrogen in seed protein, and up to 90% of the free nitrogen in vegetative tissues. In several plant species, including soybean, broad bean, pumpkin, Arabidopsis, and loblolly pine, nitrogen mobilization during seedling development is correlated with large increases in arginase expression (26, 28–29). Seedling arginase catalyzes the breakdown of a significant portion of the arginine pool to ornithine and urea. Ornithine can support the biosynthesis of polyamines, proline, and glutamate, whereas

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‡ The on-line version of this article (available at http://www.jbc.org) contains Figs. S1 and S2.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™ and EBI Data Bank with accession number(s) AY656837 and AY656838.

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1 The abbreviations used are: NOS, nitric-oxide synthase; L-NOHA, N⁶-hydroxy-L-arginine; LeARG, Lycopersicon esculentum arginase; JA, jasmonic acid; MeJA, methyl jasmonate; Pst, Pseudomonas syringae pv. tomato; COR, coronatine; EST, expressed sequence tag; ODC, ornithine decarboxylase; ADC, arginine decarboxylase; COII, coronatine-insensitive 1; CHES, 2-(cyclohexylamino)ethanesulfonic acid; TIGR, The Institute for Genomic Research; dpi, days post-infection.
urea is further catabolized by urease to carbon dioxide and ammonium. The coordinate action of arginase and urease is thought to recycle urea nitrogen to meet the metabolic demands of developing seedlings (26, 30).

The molecular mechanisms by which arginase expression in plants is regulated by developmental or stress-related cues remain to be determined. A prerequisite for addressing this question is the unambiguous identification of genes that encode plant arginase. cDNAs encoding putative arginases has been reported for Arabidopsis (31), soybean (32), and lobolly pine (33). The arginase superfamily is composed of enzymes that hydrolyze various guanidine substrates to a one-carbon nitrogen-containing product (e.g. urea) and a second product that retains the quaternary nitrogen at the site of hydrolysis. The family includes arginase, agmatinase, proclavaminamide amidinohydrolase, formiminoglutamase, as well as several characterized sequences from archaea and eubacteria (34, 35). Because the predicted sequences of plant arginases are more similar to agmatinase and other arginase-like enzymes than to non-plant arginases from vertebrates, fungi, and bacteria, it was suggested that plant genes annotated as arginase may encode agmatinase or another amidinohydrolase activity involved in the production of secondary metabolites (34, 35).

Although an Arabidopsis arginase cDNA can genetically complement an arginase-deficient yeast mutant (31), no direct enzymatic data have been reported for the product of any plant arginase gene.

To begin to assess the role of arginase in arginine homeostasis in higher plants, we identified and characterized two arginase genes (LeARG1 and LeARG2) from tomato. Our results demonstrate that, despite their phylogenetic similarity to agmatinases, the proteins encoded by LeARG1 and LeARG2 have robust amidinohydrolase activity against and high specificity for L-arginine. We report that LeARG2 expression in leaves is strongly induced by wounding and, furthermore, that this induction is mediated by the plant stress signal jasmonic acid (JA). We also document induced expression of arginase in response to Pseudomonas syringae, the causal agent of bacterial speck disease. The bacterial toxin coronatine, which exerts its effects by activating the host JA signaling pathway, was both necessary and sufficient for arginase induction in P. syringae-infected plants. The potential role of stress-induced arginase in higher plants is discussed.

**EXPERIMENTAL PROCEDURES**

**Plant Material and Treatments—**Tomato (Lycopersicon esculentum cv. Castlemart) plants were grown in Jiffy peat pots (Hummert International) in a growth chamber maintained under 17 h of light (200 μE m−2 s−1) at 28 °C and 7 h of dark at 18 °C. Seed for the sterile jai1-1 mutant was obtained from a segregating population as described by Li et al. (36). Flowers and fruits were harvested from plants maintained in a greenhouse. For experiments involving methyl-JA (MeJA) treatment, 3-week-old plants were placed in a closed Lucite box (31 cm×27 cm×14 cm) and treated with 2 μl of pure MeJA (Bedoukian Research) dissolved in 300 μl of ethanol, as previously described (37). A hemostat was used to inflict mechanical wounds near the distal end of leaflet, perpendicular to the midvein. Zhao et al. (38) described the source of coronatine (COR) and application to tomato plants. Briefly, 20 ng of COR (dissolved in 0.1 ml NH4HCO3, 5 ng/μl) was applied to the adaxial surface of leaflets of 3-week-old tomato plants. Control plants were treated with 4 μl of 0.1 ml NH4HCO3. The sources and growth conditions of Pseudomonas syringae pv. tomato strain DC3000 (Pst DC3000) and the mutant strain DC3118 Pst COR were described previously (38). Bacterial suspensions were vacuum-infiltrated into the leaves of 3-week-old plants (38). Three replicate samples were taken for each treatment over a 4-day period. At various times following the treatment, leaf tissue was harvested, frozen in liquid nitrogen, and stored at −80 °C until further use for RNA extraction or arginase assays (see below).

**Identification of Full-length LeARG cDNAs—**A search of the tomato EST (Expressed Sequence Tag) database (version 9.0 released on April 17, 2003) at the Institute for Genomic Research (www.tigr.org/dbd/lgui/) identified two tentative consensus sequences (TC124738 and TC124737) that were annotated as arginase. cDNA clones (EST435583 and EST337938) corresponding to representative members of these two genes were obtained from the Clemson University Genomics Institute. cDNA inserts from each clone were sequenced in their entirety on both strands. The cDNA corresponding to EST435583, which we designated LeARG1, was 1508 bp in length and included 252 bp upstream of the initiator AUG codon and 209 bp in the 3′-untranslated region (excluding 50 poly(A) residues). The presence of an in-frame stop codon (TAA) nine nucleotides upstream of the initiator AUG codon indicated that the cDNA encodes a full-length protein. The cDNA corresponding to EST337938, which we designated LeARG2, was 1360 bp in length and included 19 bp upstream of the initiator AUG codon and 266 bp in the 3′-untranslated region (excluding 58 poly(A) residues). The presence of an in-frame stop codon (TAA) nine nucleotides upstream of the initiator AUG codon indicated that this cDNA also encodes a full-length protein.

Data base searches were performed using the BLAST program available at the U.S. National Center for Biotechnology.

**Arginase Phylogeny—**Members of the arginase superfamily were identified by BLAST searches against non-redundant sequence databases (www.ncbi.nlm.nih.gov/BLAST/) and TIGR plant EST databases (www.tigr.org/dbd/lgui/plant.shtml). Sequences obtained from the TIGR database were composed of gene clusters of homologous genes. A total of 85 sequences were used for construction of the phylogenetic tree (Fig. 1). Sequence accession numbers are listed in Fig. S1 (Supplemental Materials). Amino acid sequences were aligned using PILEUP in the GCG software suite (Wisconsin Package version 10.2, Genetics Computer Group, GCG, Madison, WI). A neighbor-joining phylogeny was constructed from mean character distances using PAUP 4.0, version 4b10 (40). Neighboring-joining bootstrap replicates were run to test the branching order reliability.

**Expression and Purification of Recombinant LeARG1 and LeARG2—**Basic molecular techniques were performed as described in Sambrook et al. (41). A PCR-based approach was used to construct the expression vector that added a C-terminal His tag to the LeARG coding sequence. Forward and reverse primers were designed to contain Ndel and XhoI restriction sites, respectively. For preparation of the LeARG1 construct, the sequence of the forward primer was 5′-GGATAATCCATGAGTGGTGAGGAAGATATG-3′ and that of the reverse primer was 5′-CCGCTCGAGGATTGTATCTCTGACGAGGCAGAGACGAGGCGAAGATCTG-3′. PCR amplification of EST435583 (LeARG1) and EST337938 (LeARG2) yielded a 1.0-kilobase product that was subsequently cut with Ndel and XhoI subcloned into the same sites of the expression vector PET-23b (Novagen, Madison, WI). The resulting construct placed an additional eight amino acids (LEHHEHHHHH) in the C terminus of the protein. His-tagged recombinant proteins were expressed in BL21(DE3) harboring the plasmid. An overnight culture (1 ml) was inoculated into 50 ml of Terrific Broth medium supplemented with 200 μg/ml ampicillin. Bacteria were grown at 37 °C in a shaker at 250 rpm for 4 h to a cell density of about 1.2 A600 and then isopropyl-1-thio-β-d-galactopyranoside was added to a final concentration of 0.25 mM. The induced culture was incubated for 4 h at 37 °C. Cells were collected by centrifugation and stored at −20 °C until further use.

Purification of the His-tagged LeARG1 and LeARG2 was performed at 4 °C except where otherwise noted. Bacterial cells expressing the construct were harvested from 50 ml of culture medium, followed by resuspension in 2 ml of Tris buffer (50 mM, pH 8.0) containing 0.1 mM phenylmethylsulfonyl fluoride. Cells were first incubated with 2.5 mg of lysozyme for 60 min at room temperature and then lysed using three 2-min pulses from a probe-type sonicator (Bronson Sonifier Model 450). Cell homogenates were centrifuged at 20,000 × g for 10 min. The resulting supernatant was collected, and the buffer was exchanged with binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) with a 5-ml spin column prepared with Sephadex G-25 (Amersham Biosciences) and equilibrated with binding buffer. Nickel-charged resin columns having a 1-ml bed volume (Qiagen) were conditioned with 10 mM imidazole. Nickel-bound proteins (LeARG1, LeARG2, and full-length protein solution (2 ml in binding buffer), the column was washed with 10 ml of binding buffer and 10 ml of washing buffer (80 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). His-tagged arginase was eluted with elution buffer (400 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) and collected in 2-ml fractions. Arginase eluted in the first two fractions as determined by analysis of fractions on SDS-polyacrylamide gels. Imidazole was removed from the protein samples with a 5-ml spin
column packed with Sephadex G-25 and equilibrated with 100 mM Tris-HCl buffer (pH 7.5). Protein concentrations were determined by the Bradford method (42), using bovine serum albumin as a standard. The purity of recombinant protein was assessed by SDS-polyacrylamide gel electrophoresis and staining of gels with Coomassie Brilliant Blue R-250.

Enzyme Assays—Frozen tomato leaves (~1.5 g) were ground in liquid nitrogen with a mortar and pestle and then homogenized in 10 ml of 100 mM Tris-HCl (pH 7.5) containing 1% (w/v) 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 20,000 × g for 10 min at 4 °C, and the supernatants were used as the enzyme source. Recombinant LeARG enzyme was prepared as described above. Protein concentrations were determined as described above. Arginase activity was measured with a spectrophotometric assay for detection of urea (43), with minor modifications. The enzyme solution was activated with 1 mM MnCl

Sequence alignments between the plant arginases showed a strong conservation in nearly all members of the arginase superfamily (34, 35). To address this issue, we determined the enzymatic activity of recombinant proteins having calculated molecular weights of 37,048 and 36,851, respectively. The deduced amino acid sequences of LeARG1 and LeARG2 were 89% identical to each other, and 70–87% identical to arginase sequences reported from Arabidopsis (31), soya bean (32), and loblolly pine (33). Sequence alignments between the plant arginases showed a high degree of amino acid sequence identity over almost the entire length of the polypeptide. The greatest sequence diversity between plant arginases was at the N-terminal end. This region of the plant arginase exhibits features of a mitochondrial targeting peptide (32), which is consistent with the localization of the enzyme to this organelle (22, 32).

Amino acid sequence alignments between 85 amidohydrolases from diverse organisms indicated that the arginase superfamily is divided into four major groups: (i) L-arginases from vertebrates, fungi, and bacteria (referred to here as non-plant arginases); (ii) plant arginases, including LeARG1 and LeARG2; (iii) agmatinases and agmatinase-like enzymes; and (iv) several hypothetical arginase-like proteins from archea and eubacteria (Fig. 1). All plant arginases (14 sequences from 11 species) formed a monophyletic cluster that was clearly distinguishable from other members of the superfamily. The alignments revealed 20 amino acid residues dispersed along the length of the protein that are conserved in all plant arginases but are not found in the 71 non-plant sequences analyzed (Fig. S1, Supplemental Data). In agreement with previous phylogenetic studies of the arginase family (34, 35), the plant sequences were more closely related to the agmatinase group than to non-plant arginases. For example, the two tomato proteins were 25–26% identical to mammalian agmatinases and 15–20% identical to mammalian arginases.

Sequence alignments showed that all plant arginases contain six invariant His and Asp residues, which, based on x-ray structures of rat and Bacillus caldovelox arginase, bind the Mn²⁺ cofactor (Fig. 2) (45, 46). The plant enzymes showed conservation of some, but not all, amino acid residues in non-plant arginases that bind L-arginine. In the B. caldovelox arginase, Asp-126, His-139, Thr-240, and Glu-271 (positions corresponding to the B. caldovelox enzyme) form hydrogen bonds with the guanidino group of the substrate. With the exception of Thr-240, which appears to be replaced by a Ser in plant arginases (Ser-279 of LeARGs), these residues are conserved in nearly all members of the arginase superfamily (34, 35). This conservation did not appear to extend to residues that interact with the ε-amino and ε-carboxylate groups of L-arginine. Asn-128, Ser-135, and Asn-137 in the B. caldovelox enzyme form hydrogen bonds with the ε-carboxylate oxygen of L-arginine, whereas Glu-181 and Asp-178 bind the ε-amino group. These residues are conserved in all non-plant arginases, but not in plant arginases and agmatinases (Fig. 2). These observations suggest that residues comprising the active site of the enzyme are not strictly conserved between the plant and non-plant arginases.

LeARG1 and -2 Are Authentic L-Arginases—The sequence differences between plant and non-plant arginases, together with the overall similarity of the plant proteins to agmatinases, raised the question of whether LeARGS metabolize L-arginine (34, 35). To address this issue, we determined the enzymatic activity of histidine-tagged derivatives LeARG1 and -2 that were expressed in Escherichia coli and purified by nickel-affinity chromatography. The apparent molecular weight of both recombinant proteins was in agreement with the calculated molecular mass of ~37.0 kDa as determined by SDS-polyacrylamide gel electrophoresis (Fig. S2, Supplemental Data). Enzymatic assays based on the spectrophotometric detection of urea showed that both LeARG1 and -2 have robust hydrolytic activity against L-arginine (Table I). The apparent Km of L-arginine was 32 ± 4 and 29 ± 6 mM for LeARG1 and LeARG2, respectively. The optimum pH of both enzymes was ~9.5, with no activity detected at pH 7.0 or pH 12.0 (data not shown).

We next compared the substrate preference of LeARG1 and -2 to that of bovine liver arginase, which has high specificity for L-arginine (47). The two tomato enzymes were essentially identical among the range of substrates tested; L-arginine was the optimal substrate for both enzymes (Table I). Homoarginine
was metabolized by LeARGs at \(\sim 14\%\) of the rate obtained with L-arginine. Both enzymes were weakly active against D-arginine and showed only trace activity with agmatine and L-canavanine. No activity was detected against 4-guanidinobutyrate, 3-guanidinopropionate, guanidinoacetate, or creatine (data not shown). We conclude that LeARG1 and -2, despite their sequence similarity to agmatinase and phylogenetic distinction from non-plant arginases, are genuine L-arginases that have high specificity for this substrate.

**Inhibition of LeARG by NOHA—** A hallmark of mammalian arginase is its inhibition by NOHA, a stable intermediate produced during the conversion of arginine to NO by NOS (14, 15). Because NOHA also is a substrate for plant NOS (19, 20), it was of interest to determine whether this compound similarly inhibits plant arginase. We found that addition of 200 \(\mu M\) NOHA to *in vitro* reactions inhibited the activity of the recombinant LeARG1 and -2 by greater than 90\% (Table II). NOHA behaved as a competitive inhibitor, with \(K_i\) values of 12 \(\pm\) 4 and 16 \(\pm\) 5 \(\mu M\) for LeARG1 and -2, respectively. These values are significantly lower than those for inhibition of rat liver arginase by NOHA (14, 15). Thus, the plant enzymes appear to be more sensitive than their mammalian counterparts to NOHA-mediated inhibition. Relatively high concentrations of the NO-releasing agent sodium nitroprusside and L-ornithine, the product of arginase, had only modest or no inhibitory effect on LeARG activity. Sulfhydryl-reactive compounds were also less effective than NOHA in inhibiting enzyme activity (Table II). These results suggest that the inhib-
The regulatory effect of NOHA on plant arginase is specific.

**Tissue-specific Expression of Arginase Genes**—Genomic DNA blot analysis showed that full-length LeARG1 and LeARG2 sequences cross-hybridized to one another under high stringency wash conditions (Fig. 3A). Thus, gene-specific probes that recognize the untranslated region of LeARG1 and -2 transcripts were developed. Hybridization of these probes to genomic DNA indicated that LeARG1 and LeARG2 are single copy genes (Fig. 3A). RNA blot analysis showed that the steady-state level of LeARG1 and -2 mRNA was highest in mature unopened flowers, with weaker expression observed in immature flower buds, mature opened flowers, and small (<0.5 cm) green fruit (Fig. 3B). Only LeARG1 transcripts were detected in roots. Neither gene was expressed to significant levels in leaves or stems of healthy plants. These expression patterns are in good agreement with the relative abundance of LeARG1 and LeARG2 ESTs found in various tissue-specific cDNA libraries (www.tigr.org).

**FIG. 2.** Comparison of cDNA-deduced protein sequences of arginases. Members of the arginase superfamily from Fig. 1 were globally aligned with the PILEUP program in GCG (Wisconsin Package version 10.2, Genetics Computer Group, Madison, WI.). The active site region of a subset of agmatinase (AG), plant L-arginase (PA, bold), and non-plant L-arginase (NA) groups are shown. Alignment of all 85 full-length sequences is shown in Fig. S1 (Supplementary Data). Amino acid residues involved in binding the Mn2+ cofactor are shaded in black; they are conserved in all members of the arginase family. Residues in non-plant arginases that are involved in binding the guanidino moiety of the substrate are denoted with the # symbol and are shaded. Residues in non-plant arginases that form hydrogen bonds with the $\gamma$-carboxylate oxygen and the $\gamma$-amino group of L-arginine are denoted by the * and ^ symbols, respectively, and are shaded in gray. "Plant-specific" residues conserved in all plant arginases, but not found in other family members, are indicated by gray-shaded bold letters.
LeARG2 Expression Is Induced in Response to Wounding and Jasmonic Acid—Although accumulation of LeARG mRNA was not detected in leaves from healthy plants, we found that mechanical wounding effectively induced LeARG2 expression in leaves (Fig. 4A). LeARG2 transcripts were detected within 1 h of wounding, peaked at the 8-h time point, and slowly declined thereafter. No expression of LeARG1 was observed in wounded leaves. Wound-induced expression of LeARG2 was accompanied by an increase of 10-fold in arginase activity 1 day after wounding (Fig. 4B). Although LeARG2 mRNA levels returned to near basal level 1 day after treatment, arginase activity remained elevated for several days. This observation indicates that the enzyme, once synthesized, is relatively stable in plant tissue.

Wound-induced expression of several defense-related genes in tomato leaves requires the biosynthesis and subsequent action of jasmonic acid (JA), a fatty acid-derived signal for stress responses (48). To investigate the role of JA in the regulation of arginase, we measured the effect of jasmonate treatment on LeARG gene expression and total arginase activity. Treatment of plants with methyl-JA (MeJA), a potent volatile form of JA, resulted in strong induction of LeARG2 (Fig. 5A). Expression of LeARG1 was also increased in MeJA-treated leaves, albeit at <10% of LeARG2 mRNA levels. Enhanced expression of LeARG genes was accompanied by a massive increase in arginase activity; following 4 days of exposure to MeJA, arginase activity in leaves exceeded 500 μmol/mg of protein/h (Fig. 5B). Addition of 20 μM NOHA to extracts prepared from MeJA-treated plants inhibited the activity of the native arginase by >80% by (data not shown). Taken together, these results indicate that MeJA-induced increases in the steady-state level of LeARG mRNA account for induction of arginase activity and that LeARG2 is the predominant stress-inducible isoform in tomato leaves.

Induction of JA-responsive genes in tomato depends on a signaling cascade that requires the action of COI1, an F-box protein involved in the ubiquitin-26 S proteasome pathway for regulated proteolysis (49, 50). To determine whether induced arginase expression in leaves is dependent on the JA signaling pathway, we measured arginase activity in tomato mutants (jai1) that lacks COI1 (50). The results showed that jai1 plants are deficient in wound- and MeJA-induced expression of LeARG2, as well as total arginase activity (Fig. 6, A and B). Thus, induced arginase expression in tomato leaves requires an intact JA signal transduction pathway. We also found that arginase activity in untreated jai1 plants (0.16 ± 0.12 μmol/mg of protein/h) was significantly less than that in untreated wild-type plants (7.10 ± 0.88 μmol/mg of protein/h). This observation indicates that, under the growth conditions used, the JA-

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**Table I**

Substrate specificity of tomato arginases

Reaction mixtures (500 μl) containing 10 μl of enzyme source and 250 mM of the indicated substrate in a solution of 50 mM CHES buffer (pH 9.6) and 2 mM MnCl2, were incubated at 37 °C for 30 min. Reactions were terminated by the addition of 500 μl of 15% (v/v) perchloric acid. Enzyme activity was measured by spectrophotometric detection of urea as described under "Experimental Procedures." To correct for effects of non-enzymatic hydrolysis of the substrate, a mock reaction in which the enzyme was omitted was performed in parallel. The resulting spectrophotometric absorbance was subtracted from that obtained with the enzyme-containing reaction. Values shown represent the mean and S.D. of triplet reactions. Values in parenthesis indicate the amount of activity relative to that obtained with L-arginine.

| Substrate            | Activity (μmol/mg protein/h) |
|----------------------|-----------------------------|
|                      | LeARG1                      | LeARG2                      | Bovine arginase             |
|                      | 8848 ± 177 (100%)           | 7018 ± 86 (100%)           | 6608 ± 351 (100%)          |
| L-Arginine           | 1269 ± 2 (14%)              | 916 ± 16 (13%)             | ND*                        |
| Homoarginine         | 195 ± 2 (2.2%)              | 250 ± 5 (3.6%)             | 32 ± 13 (0.5%)             |
| d-Arginine           | 40 ± 3 (0.5%)               | 41 ± 6 (0.6%)              | 12 ± 2 (0.2%)              |
| Arginine             | 3 ± 1 (<0.1%)               | 9 ± 1 (0.1%)               | 14 ± 5 (0.2%)              |

* ND, not detectable.

**Table II**

Effect of various compounds on arginase activity

Arginase activity was measured in the presence of 25 mM L-arginine and various compounds at the indicated concentration. Values represent the mean activity determined from triplet reactions, expressed as a percentage of a control reaction with L-arginine only.

| Compound            | Concentration | Relative activity |
|---------------------|---------------|-------------------|
|                     | mM | % | % |
| Control             | 100 | 100 | 100 |
| L-NOHA              | 0.2 | 7  | 5  |
| Sodium nitroprusside| 2.0 | 102 | 105 |
| L-Ornithine         | 5.0 | 72  | 88  |
| 3-Mercaptopropanate | 5.0 | 24  | 26  |
| 2-Mercaptopropanate | 5.0 | 38  | 38  |
| 2-Mercaptoethanol   | 5.0 | 81  | 80  |
| Mercaptoacetate     | 5.0 | 26  | 26  |

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**Fig. 3.** Tissue-specific expression of LeARG1 and LeARG2. A, genomic DNA blot analysis of LeARG1 and LeARG2. Genomic DNA from tomato was digested with restriction enzymes BamHI (lane 1), EcoRI (lane 2), EcoRV (lane 3), HindIII (lane 4), or XbaI (lane 5), separated by agarose-gel electrophoresis, and transferred to Hybond-N filters. DNA blots were hybridized to 32P-labeled probes corresponding to the full-length LeARG1 cDNA (left panel), or to gene-specific probes that recognize the 5′-untranslated region of LeARG1 (middle panel) or the 3′-untranslated region of LeARG2 (right panel). B, accumulation of LeARG1 and LeARG2 transcripts in various tissues. Total RNA was extracted from roots (R), stems (S), and leaves (L) of 3-week-old plants, and from developing flower buds (B), mature opened flowers (UF), mature opened flowers (OF), and small (<0.5 cm) immature green fruit (GF). RNA blots were hybridized to 32P-labeled gene-specific probes for LeARG1 and LeARG2. As a control for equal loading of RNA, a duplicate gel containing the RNA samples was stained with ethidium bromide (EtBr).
FIG. 4. Induction of tomato arginase in response to wounding. Leaflets on 3-week-old plants were mechanically wounded with a hemostat. At the times indicated, wounded leaves were harvested for extraction of RNA or protein. A control set of unwounded plants (0 point) served as a control. A, 10-μg samples of total RNA were separated on a 1.2% (w/v) denaturing agarose gel. RNA was transferred to a Hybond-N Plus membrane and subsequently hybridized to genespecific probes for LeARG1 and LeARG2. A duplicate RNA gel was stained with ethidium bromide (EtBr) as loading control. B, protein extracts prepared from wounded (closed squares) and unwounded (open squares) plants were assayed for 1-arginase activity. Data points show the mean ± S.D. of three independent assays. Note that the time scale for the experiments shown in A and B are in hours and days, respectively.

FIG. 5. Induction of tomato arginase in response to MeJA treatment. Three-week-old tomato plants were exposed to MeJA vapor in an enclosed Lucite box. At various times thereafter, leaves were harvested for extraction of RNA or protein. A control set of untreated plants (0 point) served as a control. A, total RNA was analyzed by blot hybridization for the presence of LeARG1 and LeARG2 transcripts as described in the legend to Fig. 4. A duplicate RNA blot was hybridized to a probe for eIF4A as a loading control. B, protein extracts prepared from MeJA-treated (closed squares) or mock-treated (open squares) plants were assayed for 1-arginase activity. Data points show the mean ± S.D. of three independent assays. Note that the time scale for the experiments shown in A and B are in hours and days, respectively.

COI1 signaling pathway regulates basal arginase activity in tomato leaves.

Arginase Expression in Response to P. syringae Is Mediated by Coronatine—The importance of arginase in modulating host-pathogen interactions in animals (13) prompted us to test whether arginase expression in tomato is induced by P. syringae pv. tomato, the causal agent of bacterial speck disease. Challenge of plants with the virulent strain Pst DC3000 resulted in strong expression of LeARG2 within 1 day post-infection (dpi) (Fig. 7A). LeARG2 mRNA levels remained elevated for the duration of the time course, and were accompanied by a large increase in arginase activity at 2 dpi (Fig. 7B). Enzyme activity increased further by 4 dpi, at which time infected leaf tissue showed severe disease symptoms (data not shown). LeARG1 expression was not detected in infected leaves (Fig. 7A). Thus, arginase activity in Pst DC3000-challenged plants specifically results from activation of LeARG2.

Full virulence of Pst DC3000 on tomato requires the production of a non-host-specific phytotoxin called coronatine (COR) (51). Recent studies have shown that COR, which is a structural and functional analog of JA, exerts its virulence effects in tomato by activating the expression of JA-regulated anti-herbivore defenses at the expense of salicylic acid-dependent antimicrobial defenses (38). The JA-dependent expression of LeARG2 suggested that induction of arginase activity in response to Pst DC3000 might be mediated by COR. To test this idea, we measured arginase expression in tomato plants infected with a COR-deficient strain of P. syringae (Pst DC3118). The results showed that LeARG2 mRNA levels and arginase activity did not increase significantly in leaves infected with
squares) were assayed for L-arginase activity. Data points show the Pst tively high levels of L-arginase activity (52–54). These studies showing that tomato ovaries and immature fruit contain rela-

especific probes showed that genes in tomato. RNA hybridization experiments with gene-

LeARG1 Information obtained from the EST data base, together with closed circles pared from mock-inoculated plants (0 point) served as a control. A, total RNA was analyzed by blot hybridization for the presence of LeARG1 and LeARG2 transcripts as described in the legend to Fig. 4. A duplicate RNA blot was stained with ethidium bromide (EtBr) as a loading control. B, protein extracts prepared from mock-treated (open squares) or COR-treated (closed squares) leaves were assayed for l-arginase activity. Data points show the mean ± S.D. of three independent measurements.

Pst DC3118 (Fig. 7). To obtain additional evidence for a causal role of COR in arginase induction by Pst DC3000, we measured arginase expression in leaves that were treated with purified COR. Exogenous COR induced LeARG2 mRNA accumulation and arginase activity, but did not have an effect on LeARG1 expression (Fig. 8). These findings demonstrate that induction of arginase activity in Pst DC3000-challenged tomato plants is mediated primarily by COR-induced expression of LeARG2.

DISCUSSION

Here we report the identification and characterization of two arginase-encoding genes (LeARG1 and LeARG2) from tomato. Information obtained from the EST data base, together with results from genomic DNA blot analysis, indicates that LeARG1 and LeARG2 are likely the only arginase-encoding genes in tomato. RNA hybridization experiments with gene-
specific probes showed that LeARG1 and LeARG2 are expressed to their highest levels in reproductive tissues of healthy plants. This observation agrees with previous studies showing that tomato ovaries and immature fruit contain relatively high levels of L-arginase activity (52–54). These studies have suggested that arginase expression in reproductive tis-

sues of tomato plays a role in the production of polyamines that promote early fruit development.

Our analysis of the enzymatic properties of recombinant LeARG1 and -2 showed that the substrate specificity, pH opt-
tima, and kinetic parameters of the two enzymes were virtually indistinguishable. These properties also are comparable to those reported for the native enzyme purified from tomato ovary (43) and other diverse plant sources (24, 28, 55). The most notable biochemical feature of LeARGs was their high specificity for l-arginine. The clustering of plant arginase sequences into a distinct phylogenetic group (see Fig. 1) suggests that this specificity is a general feature of plant ureohy-
drolases, and therefore that the major role of plant arginase is catabolism of l-arginine to urea and ornithine. Characterization of additional recombinant plant arginases is needed to verify this conclusion.

Phylogenetic analysis showed that LeARG1 and -2 sequences are more similar to agmatinases than to non-plant arginases from vertebrates, fungi, and bacteria. Paradoxically, however, the plant enzymes are highly active against l-arginine but not agmatine or other guanidino substrates. These observations suggest that plant arginases define a distinct group of ureohy-
drolases whose evolutionary history is different from that of non-plant arginases. Sequence alignments showed that some amino acid residues involved in substrate binding are con-
served between the plant and non-plant arginases, whereas others are not. For example, amino acids that interact with the Mn²⁺ cofactor and the guanidino moiety of the substrate are conserved in the plant proteins. However, residues in non-plant arginases that bind the ε-amino and ε-carboxyl groups of l-
arginine, and impart specificity for the L-isomer, are not con-
served in plant arginases. Presumably, the plant enzymes pos-
sess other structural features that provide specificity for l-arginine. In this context, it is noteworthy that the NO bio-
synthetic intermediate, NOHA, functions as a competitive in-
hibitor of both plant and non-plant arginases. This observation provides indirect evidence that the structure of the active site of these two distinct groups of l-arginases is conserved. Eluci-
dation of the three-dimensional structure of plant arginase is needed to determine more precisely the structural relationship between plant and non-plant arginases.

Our results support the hypothesis that L-arginase evolved from a broad specificity agmatinase or agmatinase-like enzyme (35). The sequence differences between plant and non-plant arginases lead us to suggest, however, that different mecha-
nisms acted to progressively specify the plant and non-plant arginases for l-arginine. Such distinctions are likely to reflect
differences in the physiological function of these enzymes in the plant and animal kingdoms. For example, a major role of mammalian arginase is the elimination of waste nitrogen via the urea cycle. In contrast to this detoxification function, the coordinate activity of arginase and urease in plants provides a mechanism to recycle urea-nitrogen in rapidly growing tissue (29, 30). A second significant difference between plant and non-plant arginases is their role in the synthesis of putrescine and higher polyamines. Polyamine biosynthesis in animals and fungi occurs primarily by the ornithine decarboxylase (ODC) pathway in which ornithine produced by arginase is converted directly to putrescine by ODC. Plants, by contrast, use both the ODC pathway and the arginine decarboxylase (ADC) pathway for polyamine synthesis. In the latter route, ADC converts arginine to agmatine, which is then metabolized to putrescine in a two-step process involving agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase. Considerations of the origin and fate of arginine in early evolution led to the proposal that the ODC pathway evolved later than the ADC pathway (35). If this is indeed the case, the evolution of plant arginase from a broad specificity ancestral enzyme may have been influenced by selective pressure for increased polyamine synthesis, or a metabolic function unrelated to polyamine production. It is interesting to note that some plants (e.g. Arabidopsis thaliana) have lost the ODC gene and therefore rely exclusively on the ADC pathway for polyamine biosynthesis (56). The relative contribution of the ODC pathway to polyamine production in plants such as tomato that retain both pathways is not known. If the ODC route is dispensable for polyamine synthesis, alternative functions for plant arginase need to be considered (see below).

Although LeARG1 and -2 both function as L-arginases, the corresponding genes differ in their regulation. Of particular interest was the observation that LeARG2 expression and total arginase activity were strongly induced by wounding. Several lines of evidence indicate that this effect was dependent on the JA signal transduction pathway that mediates numerous stress-related plant responses. First, exogenous MeJA strongly elicited LeARG2 expression and a corresponding increase in arginase activity. Second, wound- and MeJA-induced expression of LeARG2 was abrogated in the jai1 mutant that lacks a functional JA signaling pathway. Third, the pathogen-derived toxin COR was necessary and sufficient for induced expression of LeARG2 in response to P. syringae infection. The ability of COR to function as a potent activator of JA-responsive genes in tomato (38) is consistent with the interpretation that induction of arginase in Pst DC3000-infected plants is mediated by the JA signaling pathway. A low level of LeARG1 expression also was observed in MeJA-treated leaves. However, because the concentration of MeJA used in these experiments was likely well above the physiological level of JA in tomato leaves, increased expression of LeARG1 in these experiments may not be physiologically relevant. This interpretation is supported by the fact that LeARG1 was not induced by wounding, P. syringae infection, or treatment with moderate levels of COR. We thus conclude that LeARG2 is primarily responsible for stress-induced expression of arginase activity in tomato leaves. LeARG1 may have a more general role in arginine homeostasis, consistent with its expression in diverse tissue types. Preliminary experiments conducted with A. thaliana showed that one of the two arginine-encoding genes in this species (i.e. AtARG2) also is regulated by the JA signaling pathway. This finding suggests that stress-inducible arginase may be a general feature of higher plants.

The physiological function of wound- and JA-induced arginase in plants remains to be determined. In considering this question, we point out that stress-induced arginase in plants has striking parallels to the expression of mammalian arginases that are highly up-regulated in response to wound trauma and pathogen infection. Various inflammatory signals involved in regulating this response have been identified, including cytokines, interleukins, and prostaglandins (2, 3, 8, 57). It is tempting to speculate that the function of stress-induced arginase may be conserved in diverse multicellular organisms. For example, polyamines produced by the arginase-ODC pathway may promote wound healing of plant tissues, in a manner analogous to the role of polyamines in tissue repair in animals (6, 58). This idea is consistent with a large body of evidence indicating that wounding and JA induce the biosynthesis of polyamines and polyamine conjugates in diverse plant species (59–70) and the general role ascribed to polyamines in plant protection against biotic and abiotic stress (71–72).

Wound-induced plant arginase may play a role in protection against insects or other types of herbivores. Putrescine, for example, is a biosynthetic precursor of the potent anti-herbivore toxin, nicotine (63, 67). Ornithine generated via the arginase reaction may be used for the synthesis of proline that is needed to produce hydroxyproline-rich proteins (i.e. extensins). The expression of these defense-related glycoproteins, which reinforce the cell wall at sites of tissue damage, is known to be induced by wounding and JA (73, 74). In consideration of the metabolic demands faced by plants under attack by herbivores, another potential stress-related role for arginase is the production of urea. Herbivore-damaged tomato plants, for example, synthesize massive quantities of anti-nutritive proteinase inhibitors that inhibit the feeding of lepidopteran caterpillars (48, 75). The synthesis and accumulation of proteinase inhibitors requires the availability of large pools of nitrogen-rich amino acids. By analogy to the proposed role of arginase in nitrogen metabolism during post-germinative growth (26, 30), wound-induced catabolism of arginine to ammonium via the coordinate action of arginase and urease may provide a mechanism to divert urea nitrogen into the production of amino acids that are used to support the synthesis of defensive proteinase inhibitors. It is also worth considering the possibility that plant arginase, like wound-inducible proteinase inhibitors, functions in the insect midgut in an anti-nutritive capacity. The pH optimum (−9.5), Km (−30 mM), and high stability of plant arginase suggest that the enzyme would be active within the alkaline and amino acid-rich environment of the insect midgut. By depleting the pool of arginine available for uptake into the intestine, wound-induced arginase may play a significant role in reducing the nutritional quality of damaged leaf tissue. Support for this hypothesis comes from our observation that jai1 tomato plants, which are defective in wound-induced arginase expression (Fig. 6), are severely compromised in defense against herbivore attack (50).

Increasing evidence from mammalian systems indicates that arginase, by virtue of its ability to compete with NOS for a common substrate, plays an important role in attenuating NO production during pathogenesis (13). For example, trypanosomes can evade host defenses by stimulating the expression of macrophage arginase, which effectively inhibits NO production and NO-mediated trypanosome killing (10). Similarly, an arginase expressed by Helicobacter pylori allows this human gastric pathogen to evade the host immune response by suppressing NO synthesis in activated macrophages (11). With these examples in mind, our results suggest that induction of LeARG2 in response to Pst DC3000 infection may represent a virulence strategy of the pathogen to attenuate NO-mediated

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2 H. Chen, B. C. McCaig, M. Melotto, S. Y. He, and G. A. Howe, unpublished results.
host defenses, which are well documented in plants (16–18). This hypothesis is supported by the recent discovery of a pathogen-inducible plant NO synthase (Nos) that uses arginine and NOHA as a substrate, and the demonstrated role of this protein in resistance of tomato to Pst DC3000 (19, 76). We found that induction of Nos expression in Pst DC3000-infected plants was strictly dependent on COR. Previous studies showed that this toxin enhances the virulence of Pst DC3000 on tomato by coordinately activating the host JA signaling pathway for anti-herbivore defense and suppressing the salicylic acid-dependent pathway that is important for defense against Pst DC3000 (38). The results reported here therefore suggest that Pst DC3000 may use COR to suppress both the salicylic acid and NO pathways for plant defense. In considering potential interactions between the arginase and Nos pathways in plants, it is also interesting to note that the Km for inhibition of tomato arginase by NOHA was more than 1000-fold lower than the Km for L-arginine, the enzyme’s natural substrate. The ability of plant Nos to utilize NOHA as a substrate (19, 20) suggests that this hydroxylated form of arginine may accumulate in plant tissues that are actively synthesizing NO. If this is the case, metabolic flux through the arginase pathway would likely be attenuated under conditions that promote NO synthesis. Genetic manipulation of arginase expression transgenic plants will be useful to address this hypothesis, and to gain insight into the physiological function of arginase in healthy and diseased plants.

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