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Dual functioning of plant arginases provides a third route for putrescine synthesis

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ABSTRACT

Two biosynthetic routes are known for putrescine, an essential plant metabolite. Ornithine decarboxylase (ODC) converts ornithine directly to putrescine, while a second route for putrescine biosynthesis utilizes arginine decarboxylase (ADC) to convert arginine to agmatine, and two additional enzymes, agmatine iminohydrolase (AIH) and *N*-carbamoyl putrescine aminohydrolase (NLP1) to complete this pathway. Here we show that plants can use ADC and arginase/agmatinase (ARGAH) as a third route for putrescine synthesis. Transformation of *Arabidopsis thaliana ADC2,* and any of the arginases from *A. thaliana* (ARGAH1, or ARGHA2) or the soybean gene Glyma.03g028000 (*GmARGAH*) into a yeast strain deficient in ODC, fully complemented the mutant phenotype. *In vitro* assays using purified recombinant enzymes of AtADC1 and AtARGAH2 were used to show that these enzymes can function in concert to convert arginine to agmatine and putrescine. Transient expression analysis of the soybean genes (Glyma.*06g007500, ADC;* Glyma.03g028000 *GmARGAH*) and the *A. thaliana* ADC2 and ARGAH genes in leaves of *Nicotiana benthamiana,* showed that these proteins are localized to the chloroplast. Experimental support for this pathway also comes from the fact that expression of *AtARGAH*, but not *AtAIH* or *AtNLP1*, is co-regulated with *AtADC2* in response to drought, oxidative stress, wounding, and methyl jasmonate treatments. Based on the high affinity of ARGAH2 for agmatine, its co-localization with ADC2, and typically low arginine levels in many plant tissues, we propose that these two enzymes can be major contributors to putrescine synthesis in many *A. thaliana* stress responses.

1. Introduction

A distinguishing feature of eukaryotic cells is the presence of membrane-bound organelles and vesicles. In plants, these sub-cellular spaces have multiple functions that enable the specialized compartmentation of metabolic processes such as carbon fixation, respiration, energy capture, biosynthesis of primary metabolites, and the sequestering of waste products or defensive metabolites that are normally released only upon cell lysis [1–3]. One particular advantage of this organizational strategy is that the compartmentation of enzymes prevents futile cycling of metabolites by controlling the access of enzyme substrates to enzyme targets. An additional feature of plant metabolism is the redundancy of certain metabolic pathways [4–6], as is also the case for polyamine biosynthesis. In one pathway, ornithine decarboxylase (ODC) converts ornithine directly to putrescine [7] (Fig. 1). A second pathway utilizes arginine decarboxylase (ADC) to convert arginine to agmatine, and two additional enzymes, agmatine iminohydrolase (AIH) and *N*-carbamoyl putrescine aminohydrolase (NLP1) to synthesize putrescine [8,9].

Putrescine serves as a precursor for the other common plant polyamines, spermidine, spermine, and thermospermine. Spermidine is required for the hypusination [10] of the three members of the *elF5A* family of conserved transcription elongation factors [11] that are present in all eukaryotes [12]. Overexpression of spermidine synthase in *Arabidopsis thaliana* increases tolerance to a wide range of abiotic stresses [13]. Thermospermine plays an essential role in normal vascular development of plants [14,15]. Overexpression of spermine synthase results in an increased level of spermine and increased tolerance against *Pseudomonas viridiflava* [16]. *Arabidopsis thaliana* has lost ODC, and thus the second known pathway, but instead has two ADC's [17]. Given the multiple roles of polyamines, it is hardly surprising that homozygous mutants of both arginine decarboxylases in *A. thaliana* are lethal [18]. Homozygous *AIH* mutants are embryo defective, consistent with the hypothesis that putrescine synthesis is essential for plants [19].

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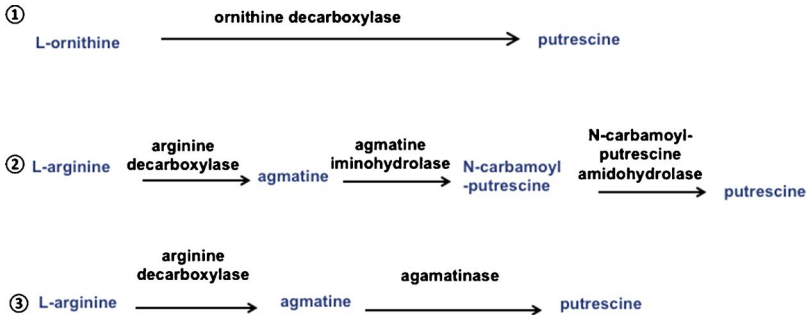

Fig. 1. Biosynthetic strategies for the synthesis of putrescine.

However, homozygous mutants of genes encoding the terminal enzyme of the cytosolic putrescine pathway (NLP1) have no reported phenotype, and are available from the Arabidopsis Biological Resource Center (ABRC). These results suggest an absolute requirement for *N*-carbamoylputrescine in plant metabolism, and that a second mechanism of synthesis for putrescine in *A. thaliana* is necessary.

In *Escherichia coli* and mammals, putrescine can be synthesized in a two-step process from arginine by arginine decarboxylase, which makes agmatine; and agmatinase, which converts agmatine to putrescine with the release of urea [20–22]. Enzymes with agmatinase activity are members of the ureohydrolase superfamily that also include enzymes with arginase, forminoglutamase, and proclavaminate amidoinohydrolase activities [23]. Plant arginases are unique in that they are phylogenetically more similar to bacterial agmatinases, than to bacterial or mammalian arginases [9,24]. However, characterization of the two tomato arginase/agmatinase enzymes indicated that, at high substrate concentrations, these enzymes exhibited the highest catalytic efficiency for the conversion of arginine to ornithine and urea [24]. Since plant arginases are known to be strongly conserved, it has been assumed that an agmatinase-dependent pathway for polyamine biosynthesis does not exist in plants.

Here we show that three arginases (ARGAH), two from *A. thaliana* and one from soybeans, are capable of working in concert with arginine decarboxylase to efficiently convert arginine to putrescine. This second route for putrescine synthesis in *A. thaliana,* and a third route for putrescine synthesis in soybeans, is localized to the plastid. In *A. thaliana,* ADC2 is significantly upregulated in response to drought, oxidative stress, wounding and methyl jasmonate. We also note that it is ARGAH2, and not AIH and NLP1, that increase in response to the expression of ADC2.

2. Materials and methods

2.1. Phyre2 analysis

Three dimensional structure of *A. thaliana* ARGAH2 were predicted using Phyre2by threading the predicted protein models of ARGAH2 using the 3D template of *Deinococcus radiodurans* [25]. The predicted active site region of arginases/agmatinases from *A. thaliana,* soybeans, and poplar were aligned with active site region of *D. radiodurans* agmatinase using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Superposition of 3D structures was performed using Chimera (http://www.cgl.ucsf.edu/chimera/) [26].

2.2. Phylogenetic analysis

Protein sequences of ADC and agmatinase/arginase were obtained from the Phytozome database [27]. The amino acid sequence alignment was created using MUSCLE [28]. Phylogenetic trees were constructed by MEGA 6.06 [29] using the maximum likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model. The reliability of the trees was tested using a bootstrapping test with 1000 duplicates. Alignment files were generated using the MULTALIN interface http://multalin.toulouse.inra.fr/multalin/multalin.html [30].

2.3. DNA sources and constructs

Genes were amplified using gene-specific primer pairs listed in Table S3 following the manufacturer's guidelines for the use of Phusion® high fidelity DNA polymerase. The sequence of Glyma.03g028000 (*GmARGAH*) was codon-optimized for expression in yeast and synthesized by GenScript, Pistcataway, NJ. *AtADC1* was amplified from genomic DNA of *A. thaliana* Col-0. cDNA clones of *A. thaliana ADC2* (At4G34710) *ARGAH1* (At4G08900) *ARGAH2* (At4G08870) were obtained from the ABRC (www.arabidopsis.org).

2.4. Subcellular localization analysis

Full length sequences of *AtADC2, AtARGAH2, AtADC1, GmADC,* and *GmARGAH,* were cloned into plant expression vectors pGWB6, or pGWB5 using the GATEWAY® recombination system [31] Inserts were verified by sequencing and vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 [32]. Samples were imaged using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) using the Leica Application Suite Advanced Fluorescence (LASAF) program at 12–72 h after infiltration. Images were acquired in the XYZ plane in 1 μm steps with a 20X and the 63X oil objective (NA 1.40) using the sequential scan mode to eliminate any spectral overlap in the individual fluorophores. Specifically, GFP was excited at 488 nm and detected at 510 nm mCherry was excited at 561 nm and detected at 610 nm. Chlorophyll autofluorescence was used to localize plastids in the cells using laser excitation at 633 nm, and emission of light at 670 nm. GFP signals were false-colored green, and mCherry signals were false-colored red. Background fluorescence from untransformed leaves of plants at similar laser excitation settings were acquired and subtracted from images to identify fluorescence generated by tagged proteins. Images were merged using ImageJ [33]. Images were acquired from multiple leaf sections at different time points following infiltration (24–72 h) on at least two separate occasions for each construct.

2.5. Yeast complementation assays

The *spe1* yeast knockout strain, which lacks ornithine decarboxylase (YSC6273-201936543), was obtained from GE Darmacon, Lafayette, CO. BY4741 served as a wild-type control. Yeast strains were maintained on enriched medium (YEPD, Amresco) or minimal medium (SC, Sunrise Science) supplemented with 1 mM putrescine (Sigma-Aldrich). The full-length sequence of *AtADC2* was cloned into yeast expression vector pAG303GPD-ccdB (Addgene) using the GATEWAY® recombination system. Full length sequences of *AtARGAH2*, *AtARGAH1* and *GmARGAH* were cloned into the yeast expression vector pYES-DEST52 (Invitrogen) in a similar manner. The pAG303-ADC2 plasmid construct was introduced into competent yeast *spe1* mutant cells by electroporation. The resulting transformants were selected on SC lacking histidine and containing 1 mM putrescine. Positive colonies were used to produce competent cells. The pYES-DEST52-ARGAH2 plasmid