Plant Physiology and Biochemistry 54 (2012) 149–155

Contents lists available at SciVerse ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Short communication

Four allantoinase genes are expressed in nitrogen-fixing soybean

Veronica A. Duran, Christopher D. Todd*

Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon, SK, Canada S7N 5E2

ARTICLE INFO

Article history:
Received 30 October 2011
Accepted 2 March 2012
Available online 10 March 2012

Keywords:
Allantoin
Allantoinase
Biological nitrogen fixation
Nitrogen metabolism
Nodules
Soybean
Ureides

ABSTRACT

Soybean (*Glycine max* L. [Merr]) plants export nitrogen from the nodules as ureides during symbiotic biological nitrogen fixation. Ureides also play a role as nitrogen storage compounds in the seeds and are broken down in germinating seedlings. In this work we identified four soybean genes encoding allantoinase (E.C. 3.5.2.5), an enzyme involved in both ureide production in nodules and ureide catabolism in leaves and other sink tissues. We examined ureide content, allantoinase enzyme activity and expression of these genes, which we term *GmALN1* through *GmALN4*, in germinating seedlings and in vegetative tissues from 45 day old soybean plants. *GmALN1* and *GmALN2* transcripts were measured in all tissues, but similar levels of expression of *GmALN3* and *GmALN4* was only observed in nodules. The soybean allantoinase genes seem to have arisen through tandem gene duplication followed by a whole genome duplication. We looked for evidence of the tandem duplication in common bean (*Phaseolus vulgaris* L.) and present evidence that it occured sometime in the bean lineage before these two species diverged, but before soybean became a tetraploid.

1. Introduction

In soybean ureides are products of symbiotic nitrogen fixation, synthesized by the plant in the nodules and exported as a nitrogen source for other tissues. Allantoin and allantoate are the dominant form of nitrogen in the vascular tissue of soybean plants actively fixing nitrogen [1]. These compounds are synthesized via the purine oxidation pathway in the nodule and delivered to leaves and other sink tissues where they are broken down and the nitrogen is reassimilated. Ureide transport is generally associated with legumes of tropical origin, including *Glycine, Phaseolus* and *Vigna* species [2–6] and is distinct from the process utilized by temperate legumes that export amides (glutamine and asparagine) from the nodules to leaves and other sink tissues [2]. Beyond transport of fixed nitrogen ureides are also important in the breakdown and salvage of nitrogen from purines [3] and have recently been implicated in scavenging of reactive oxygen species [7].

Once delivered to sink tissues allantoin is converted to allantoate by the enzyme allantoinase (E.C. 3.5.2.5). Allantoate can be broken down completely to glyoxylate, releasing four molecules of ammonia and two molecules of CO_2. The general pathway for plants, with genetic support identified for each step, appears to be conversion of allantoate to (*S*)-ureidoglycine by allantoate amidohydrolase (E.C. 3.5.3.9), hydrolysis of (*S*)-ureidoglycine to (*S*)-ureidoglycolate by ureidoglycine aminohydrolase (E.C. 3.5.3.-), and conversion of (*S*)-ureidoglycolate to glyoxylate by a ureidoglycolate amidohydrolase (E.C. 3.5.3.19) releasing CO_2 and NH_3 generating (*S*)-hydroxyglycine, which goes through a non-enzymatic deamination producing glyoxylate [8–11]. However, enzymatic activity liberating urea from ureidoglycolate (ureidoglycolate urea-lyase, E.C. 4.3.2.3) has been reported and characterized in chickpea and common bean, but corresponding genes have not been reported [12–14]. Allantoinase is unique in this pathway in that it plays a role in ureide generation for export in the nodules as well as ureide catabolism to generate a nitrogen source in leaves and other nitrogen sinks.

The first plant allantoinase genes sequences characterized were isolated from *Robinia pseudoacacia* (*RpALN*) and Arabidopsis (*AtALN*) [15]. The protein is targetted to the endoplasmic reticulum [9] and T-DNA mutants in *AtALN* accumulate allantoin and are unable to grow using allantoin as a sole nitrogen source [8,15]. In common bean allantoinase activity is induced following seed germination, possibly involved in mobilization of nitrogen to developing tissues [16]. Allantoinase gene expression is induced in response to drought and when treated with ABA, and this induction has been proposed as a mechanism leading to drought-induced ureide accumulation [17]. In beginning to examine the potential roles of allantoinase in soybean, a closely related legume, we identified four allantoinase genes. This work examines the importance of the multiple genes in soybean by determining the ureide content, allantoinase activity and allantoinase gene expression in seedlings, in N_2-fixing vegetative tissues and in the same tissues from non-fixing plants. As part of this work we identified a second allantoinase gene in

* Corresponding author. Tel.: +1 306 966 4497; fax: +1 306 966 4462.
E-mail addresses: veronica.duran@usask.ca (V.A. Duran), chris.todd@usask.ca (C.D. Todd).

0981-9428/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2012.03.002

common bean and which has interesting implications for regulation of ureide metabolism in ureide exporting legumes.

2. Results

2.1. Identification of allantoinase genes in soybean

We identified two putative allantoinase sequences in the Gene Index Database [18], amplified the predicted coding sequence from soybean seedling cDNA using RT-PCR, and confirmed their identity by functional complementation of a *Saccharomyces cerevisiae dal1* mutant (data not shown). We designate these sequences *GmALN1* and *GmALN2*. Release of the soybean genome [19] confirmed the nucleotide sequences of *GmALN1* (Glyma15g07910) and *GmALN2* (Glyma13g31430). However, a BLASTN search using the *GmALN1* nucleotide coding sequence as a query revealed two additional allantoinase sequences, we designate *GmALN3* (Glyma15g07920) and *GmALN4* (Glyma13g31420). *GmALN1* and *GmALN3* are located 3.77 kbp apart on chromosome 15 while the other two, *GmALN2* and *GmALN4*, are 3.76 kbp apart on chromosome 13 (Fig. 1). *GmALN3* and *GmALN4* shared the highest identity for both the nucleotide and predicted amino acid coding sequence, followed by *GmALN1* and *GmALN2* (Table 1). RT-PCR with gene-specific primers produced amplicons from both seedling and nodule RNA samples confirming that each of *GmALN1*, *GmALN2*, *GmALN3*, *GmALN4* were expressed (data not shown).

2.2. Allantoinase in seedlings

Allantoinase enzyme activity and ureide content in soybean seedlings was determined in both embryonic axes and cotyledons for up to 8 days following seed imbibition (Fig. 2). Total ureide content increased in both tissues during post-germinative growth. Allantoate was the major ureide found in the axes, ranging from 55 to 88 mol% of the total ureides detected from days 2–8. Allantoate was also the major ureide detected in cotyledons, with the exception of day 2, when allantoin was the most abundant ureide (54 mol%).

Enzyme activity was determined in the same tissues and time points. Allantoinase activity was higher in cotyledons than in embryonic axes on a fresh weight basis (Fig. 2C, D). In both tissues allantoinase activity declined with time. To complement enzyme activity data, transcript levels of the four soybean allantoinase genes were also quantified using qRT-PCR. Data presented were normalized against soybean *CYP2* and data are presented relative to the amount of *GmALN1* transcript in the imbibed seed. *GmALN1* and *GmALN2* were consistently expressed at higher levels than *GmALN3* and *GmALN4* in all samples (Fig. 2E, F), with higher relative levels measured in the cotyledons. *GmALN3* and *GmALN4* were always expressed at relatively low levels, from <1% to 15% of *GmALN1* in imbibed seeds.

2.3. Allantoinase in leaves, roots, stems and nodules

Ureide content, allantoinase enzyme activity and allantoinase transcript levels were also evaluated in mature N_2-fixing and non-fixing tissues, at the V4-V5 stage of development. With the exception of expanding young leaves, ureide concentration was consistently greater in N_2-fixing tissues than in the corresponding tissues from non-fixing plants (Figs. 3 and 4A, B). The greatest concentration was detected in nodules where allantoate was the most abundant compound. Allantoinase activity was detected in all tissues and was consistently greater in N_2-fixing tissues than non-fixing tissues, with the highest activity detected in nodules (Fig. 3C, D), followed by young leaves.

All of the allantoinase genes were expressed in leaf and root tissues of both N_2-fixing and non-fixing plants. As in seedlings, *GmALN1* and *GmALN2* were still consistently expressed at greater levels compared to *GmALN3* and *GmALN4* in both non-fixing and N_2-fixing tissues (Fig. 3E, F), with *GmALN2* appearing to be relatively more abundant in N_2-fixing tissues than in non-fixing tissues. The most striking observation is the transcript levels of *GmALN3* and *GmALN4* in nodules of N_2-fixing plants. In all other tissues examined *GmALN3* and *GmALN4* consistently had low expression levels (from <1% to <10% of N_2-fixing young leaves).

2.4. Phaseolus vulgaris has more than one allantoinase gene

Two proteins (with unequal masses) showing allantoinase activity have been purified from developing fruits of common bean [20], but only a single gene has been identified previously [17]. It was hypothesized that alternate splicing of a single allantoinase gene might explain the origin of these two protein isoforms [20]. The organization of the soybean allantoinase genes and the presence of a nodule EST sharing sequence identity, but distinct from the previously reported common bean allantoinase, led us to hypothesize that there are two allantoinase genes in *Phaseolus*. Using an RT-PCR based approach with gene-specific primers we generated two separate amplicons from common bean hypocotyl RNA, approximately 550 bp in size. We identify these as *PvALN1* and *PvALN2* where *PvALN2* is the novel sequence. The amplified fragments of *PvALN1* and *PvALN2* share 86.7% sequence identity. When compared to soybean allantoinase genes, *PvALN1* shared greatest sequence similarity with *GmALN1* and *GmALN2* and *PvALN2* was more similar to *GmALN3* and *GmALN4*. Using a common internal coding sequence fragment from all four soybean allantoinase genes, *PvALN1*, *PvALN2* and predicted homologs from *Medicago truncatula*, *Populus trichocarpa* and *Arabidopsis thaliana* we generated a small phylogenetic tree, demonstrating that *PvALN1,* the isoform more abundant in common bean axes and with substantially more EST support, is most similar to *GmALN1* and *2*, whereas *PvALN2* shares higher nucleotide identity with *GmALN3* and *4*. Based on our data it appears that an ancestral allantoinase gene underwent tandem duplication sometime after the two bean species split from *Medicago* (an amide exporting legume), but prior to a whole genome duplication in soybean.

3. Discussion

Soybean utilizes ureide in transporting fixed nitrogen during active nitrogen fixation. Allantoinase initiates the multi-step hydrolysis of allantoin that leads to the release of NH_3 for re-assimilation. In this study, we evaluated allantoinase transcript abundance, enzyme

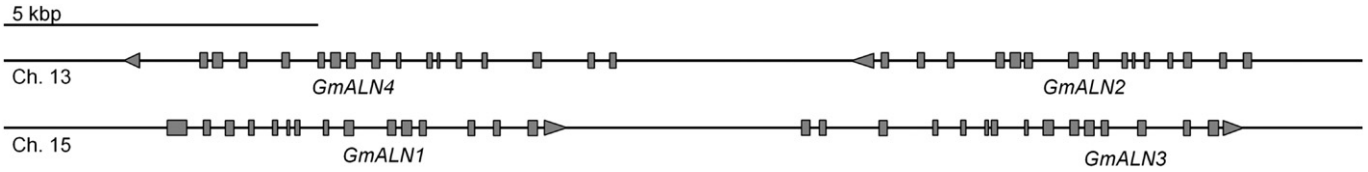

Fig. 1. Chromosome orientation and intron/exon structure of soybean allantoinase genes. *GmALN1* and *GmALN3* are located on chromosome 15, *GmALN3* and *GmALN4* on chromosome 13. Scale bar 5 kbp. Predicted exons are depicted as gray boxes (A).