Allantoate amidohydrolase transcript expression is independent of drought tolerance in soybean

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Abstract

Drought is a limiting factor for N2 fixation in soybean [Glycine max (L.) Merr.] thereby resulting in reduced biomass accumulation and yield. Drought-sensitive genotypes accumulate ureides, a product of N2 fixation, during drought stress; however, drought-tolerant genotypes have lower shoot ureide concentrations, which appear to alleviate drought stress on N2 fixation. A key enzyme involved in ureide breakdown in shoots is allantoate amidohydrolase (AAH). It is hypothesized that AAH gene expression in soybean determines shoot ureide concentrations during water-deficit stress and is responsible for the differential sensitivities of the N2-fixation response to drought among soybean genotypes. The objectives were to examine the relationship between AAH transcript levels and shoot ureide concentration and drought tolerance. Drought-tolerant (Jackson) and drought-sensitive (Williams) genotypes were subjected to three water-availability treatments: well-watered control, moderate water-deficit stress, and severe water-deficit stress. Shoot ureide concentrations were examined, in addition to gene expression of AAH and DREB2, a gene expressed during water-deficit stress. As expected, DREB2 expression was detected only during severe water-deficit stress, and shoot ureide concentrations were greatest in the drought-sensitive genotype relative to the drought-tolerant genotype during water-deficit stress. However, expression of AAH transcripts was similar among water treatments and genotypes, indicating that AAH mRNA was not closely associated with drought tolerance. Ureide concentrations in shoots were weakly associated with AAH mRNA levels. These results indicate that AAH expression is probably not associated with the increased ureide catabolism observed in drought-tolerant genotypes, such as Jackson. Further study of AAH at the post-translational and enzymatic levels is warranted in order to dissect the potential role of this gene in drought tolerance.

Key words: Allantoate amidohydrolase, drought, Glycine max, manganese, nitrogen fixation, soybean, ureides, water-deficit stress.

Introduction

Drought is often a yield constraint for crops worldwide. A major benefit of producing soybean [Glycine max (L.) Merr.] is that it grows symbiotically with Bradyrhizobium japonicum and reduces (fixes) atmospheric nitrogen to nitrogen-compounds that the plant can utilize without the need for expensive nitrogen fertilizer. However, N2 fixation is sensitive to water-deficit stress thereby reducing the plant’s ability to fix and utilize nitrogen for biomass accumulation and seed production (see reviews by Serraj et al., 1999; Purcell and Specht, 2004). Although N2 fixation decreases during drought, the products of N2 fixation, ureides (allantoin and allantoate), increase in leaves of drought-sensitive soybean genotypes (King and Purcell, 2001). Experiments have shown that increases in shoot ureide concentration are associated with the inhibition of N2 fixation in the nodules and, subsequently, drought sensitivity (Purcell et al., 2000). Drought-sensitive N2-fixation genotypes subjected to water-deficit stress (i.e. Williams, Biloxi, and KS4895) exhibit a reduction in nitrogenase activity, a decrease in ureide

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degradation, and an increase in shoot ureide concentrations relative to Jackson, a genotype with drought-tolerant N₂-fixation (Purcell et al., 2000; Vadez and Sinclair, 2001). Therefore, lower ureide concentrations in leaves during water-deficit stress appears to prolong N₂ fixation and thereby to confer drought tolerance (Purcell et al., 2000; King and Purcell, 2005). In addition, physiological studies have shown that increases in Mn²⁺ availability to the plant during water-deficit stress increases ureide degradation in leaves and prolongs N₂ fixation (Purcell et al., 2000; Vadez et al., 2000). An enzyme responsible for allantoate breakdown, allantoate amidohydrolase (AAH; EC 3.5.3.9), requires Mn²⁺ as a co-factor for activity (Lukaszewski et al., 1992; Werner et al., 2008) and this enzyme has greater activity in Jackson than in the drought-sensitive genotype KS4895 (Purcell et al., 2000). Therefore, AAH is a likely candidate for an important role conferring drought tolerance in soybean.

Allantoate amidohydrolase is important in purine salvage, and is found in many plant species that do not fix nitrogen. This gene was first cloned from plants using the model organism Arabidopsis. Todd and Polacco (2006) demonstrated the functional complementation of the Arabidopsis gene in a yeast strain unable to utilize ureides. The yeast strain transformed with AtAAH was able to utilize allantoin as the sole nitrogen source, whereas the untransformed mutant yeast strain grew poorly in the presence of allantoin. Furthermore, Todd and Polacco (2006) showed that an Arabidopsis mutant lacking the functional AAH was not able to breakdown ureides. Recently, Werner et al. (2008) cloned the soybean homologue of AAH. They demonstrated that the AAH protein is involved in allantoate catabolism in vivo and that Mn²⁺ is required for optimal activity.

It is hypothesized that AAH gene expression in soybean determines the shoot ureide concentrations during water-deficit stress and is responsible for the differential sensitivities of the N₂-fixation response to drought among soybean genotypes. From this hypothesis, it is predicted that AAH mRNA levels in shoots are (i) higher in drought-tolerant versus drought-sensitive soybean genotypes, (ii) differentially expressed in response to water-deficit stress, and (iii) inversely correlated with shoot ureide concentrations. Therefore, the objectives were to examine AAH mRNA levels and ureide concentrations in shoots during well-watered and water-deficit stress for two soybean genotypes differing in drought tolerance.

Materials and methods

Drought-tolerant (Jackson; PI 548657) and drought-sensitive (Williams; PI 548631) soybean genotypes were planted in 1.0 l volume pots containing 2 parts N-free peat-perlite-vermiculite mixture (LB2, Sungro Horticulture Inc., Bellevue, WA) to 1 part Captina silt loam (fine-silty, siliceous, mesic Typic Fragiudult) soil collected from the University of Arkansas Experiment Station near Fayetteville, AR. The soil was saturated with 1.0 l of a N-free nutrient solution (deSilva et al., 1996), drained overnight, and the soil water capacity determined as soil weight. One seed was planted per pot and soil inoculated with Bradyrhizobium japonicum (strain USDA 110).

Growth chamber conditions included day/night temperature of 25/23 °C and a 16 h photoperiod beginning at 06.00 h with metal halide lamps providing approximately 300 μmol m⁻² s⁻¹ photosynthetically active radiation at plant height. Plants were watered as needed for 30 d after emergence to maintain 70% soil water capacity (SWC). The experiment consisted of a 2 × 3 factorial with randomized complete block design with five blocks for Experiment One and 2 blocks for Experiment Two. Two genotypes (Jackson and Williams) were examined over three water treatments on the basis of SWC: well-watered at 70%; moderate water deficit at 50%, and severe water deficit at 30%. To reach each percentage SWC for simultaneous sampling, the soil was dried in several intervals. The first interval was to allow all pots, except for the 70% SWC treatment, to reach 50% SWC. During the second interval, water was added to maintain corresponding pots at 70% and 50% SWC, while the remaining pots were allowed to dry to 30% SWC. The entire drying period was 4 d.

At the V7 stage of development (Fehr and Caviness, 1977), fully expanded trifoliolate leaves at the fifth node were collected from each genotype, water treatment, and replication combination. Three 1 cm diameter leaf punches were collected and combined into one sample per leaf. The tissue was dried at 55 °C, and a 30 mg subsample was ground to pass a 1 mm sieve. Ureide concentration was determined using the colorimetric procedure of Young and Conway (1942) as described by deSilva et al. (1996).

RNA was collected from the remaining leaf tissue, frozen in liquid N₂, and stored at –80 °C before extraction. Total RNA was isolated from leaves using TriReagent (Molecular Research). Total RNA (15 μg) from each sample was separated on 1% agarose formaldehyde gels. The accumulation of transcripts by RNA-blot hybridization was measured using standard techniques (Sambrook et al., 1989) and 32P-labelled probes as described previously (Gomez et al., 2005).

Three probes were used for RNA-blot analysis. Probes for AAH and DREB2 were obtained through a standard PCR procedure using gene-specific primers (Carlson et al., 2005; Chen et al., 2006). The AAH probe was amplified from Williams EST clone Gm-c1019-6420 (GenBank no. AW704657) using primer sequences 5'-CACCAACCA-CAACCACTATTCTGCCAC-3' (forward) and 5'-ACAGTTACCATATCATTTATGAGTTTTCCAC-3' (reverse) (See Supplementary Fig. S1 at JXB online). The soybean DREB2 gene was used as a positive control to validate that plants were responding differentially to water availability at the transcript level of gene expression; DREB2 was amplified from genomic DNA of Jackson. DREB2 (GenBank No. DQ208968), primers were 5'-ATGGAAAGACCCGGTTAGA-3' (forward) and 5'-ATCTTCAGGTTGGGATACT-3' (reverse) and amplified a 480 bp amplicon probe starting at the start codon.
(ATG) and ending near the stop codon (TAG). Probes were sequenced to confirm identity. Levels of gene expression were calculated as a ratio of AAH or DREB2 to the 18S ribosomal RNA from rice (*Oryza sativa* L.).

**Results**

The response of Jackson (drought-tolerant) and Williams (drought-sensitive) to water availability was as expected regarding ureide concentration between both experiments across water treatments. For well-watered and moderate-water stress, Jackson and Williams had similar ureide concentrations within an experiment: $\sim 10 \, \mu\text{M}$ and $\sim 50 \, \mu\text{M} \, \text{mg}^{-1} \, \text{dw}$ for Experiments One and Two, respectively (Fig. 1). As expected, Williams had a 2–3-fold greater ureide concentration during severe-water stress relative to well-watered conditions, whereas Jackson showed very little increase in ureide concentrations during severe-water stress.

In addition to the observation of expected levels of ureides in water-stressed samples, expression patterns of a known drought-induced gene were also as anticipated. The *DREB2* gene encodes a transcription factor that binds to a drought-responsive element found in the promoters of many plant genes thought to be active in water-stress responses (Sakuma *et al.*, 2002). Transcripts encoding *DREB2* are strongly induced in response to water stress in *Arabidopsis* (Liu *et al.*, 1998) and soybean (Chen *et al.*, 2006). Therefore, *DREB2* was a useful, positive control to validate that changes in gene expression resulting from water-deficit stress could be detected using our experimental approach. The transcript level of soybean *DREB2* was similar between genotypes and $\sim 2$-fold greater at severe water-deficit stress compared with the control plants (Figs 2, 3). The data support that ureide concentration for Jackson and Williams and *DREB2* responded to water availability as expected from previous research (Purcell *et al.*, 2000; Vadez and Sinclair, 2001).

Although ureide concentrations were lower for Jackson relative to Williams (Fig. 1), *AAH* transcript levels were similar between the drought-tolerant and drought-sensitive soybean genotypes (Fig. 2). In addition, and contrary to the observation of shoot ureide concentrations differing among water-deficit treatments, *AAH* transcript levels remained stable regardless of water-deficit stress (Fig. 2). Furthermore, the *GmAAH* probe was gene specific, as indicated by the detection of only one band from Southern blot analysis (data not shown). Therefore, the disparity between transcript expression and ureide concentration may not be accounted for by a cross-hybridization of the probe with the expression of one or more homologous genes during Northern blot analysis.

Since *AAH* breaks down allantoate, a ureide, to a non-ureide (ureidoglycolate), we expected to observe *AAH* transcript levels increasing with a concurrent decrease in shoot ureide concentrations. Linear regression analysis indicated that the response of ureide concentration to *AAH* expression was significant ($P=0.02$), however, the relationship was weak ($R^2=0.15$) (Fig. 4).

![Fig. 1. Shoot ureide concentrations versus water availability and cultivar. The experiment was conducted twice in a growth chamber. Drought-tolerant (Jackson) and drought-sensitive (Williams) soybean genotypes were subjected to three water-availability treatments: well-watered control (70% soil water capacity, SWC), moderate water-deficit stress (50% SWC), and severe (30% SWC) water-deficit stress. Data were averaged within treatment and cultivar for five replications in Experiment One (A) and two replications in Experiment Two (B). Bars indicate the standard error of the mean.](https://academic.oup.com/jxb/article-abstract/60/3/847/447517)

![Fig. 2. Northern blot analysis of allantoate amidohydrolase (AAH), DREB2, and 18S ribosomal RNAs. Gene expression between experiments was similar, therefore, only data from Experiment One are shown. RNA from five replications was combined at equal concentrations of 15 $\mu$g. The same membrane was exposed with all three probes independently. Gene expression was examined for drought-tolerant (Jackson) and drought-sensitive (Williams) soybean genotypes over three water availability treatments: well-watered control (70% soil water capacity, SWC), moderate water-deficit stress (50% SWC), and severe (30% SWC) water-deficit stress.](https://academic.oup.com/jxb/article-abstract/60/3/847/447517)
Discussion

Soybean genotypes with drought tolerance have lesser shoot ureide concentrations relative to drought-sensitive genotypes during water-deficit stress (King and Purcell, 2001), and AAH is involved in ureide breakdown in shoots. Therefore, the role of AAH transcript expression relative to the drought-tolerance trait and ureide concentration in shoots was examined. The results of this study indicated that mRNA levels of AAH were weakly associated with shoot ureide concentration and independent of soybean response to water-deficit stress or the drought-tolerance trait in soybean. These data indicate that either AAH does not play a role in drought tolerance or post-translational regulation is involved in AAH activity. There are several lines of physiological and biochemical evidence supporting post-translational regulation of AAH enzymatic activity.

Two enzymes are involved in ureide catabolism: allantoinase (EC 3.5.2.5) and AAH. During ureide catabolism, allantoinase breaks down allantoin to allantoate. Subsequently, allantoate is catabolized by AAH to urideglycolate. Ureide catabolism is proposed to occur in the endoplasmic reticulum (ER). Werner et al. (2008) demonstrated that both allantoinase and AAH proteins are localized to the ER in Arabidopsis, supporting that ureide catabolism occurs in the ER. Allantoin is stored in peroxisomes (Corpas et al., 1987) before transport to the ER for ureide catabolism as proposed by Werner et al. (2008). Increases in ureide shoot concentrations during water-deficit stress may reflect increases in stored allantoin rather than of allantoate (Rice et al., 1990). In addition, AAH mRNA expression is not affected by allantoin concentrations in shoots (Todd and Polacco, 2006; Werner et al., 2008). Furthermore, AAH enzymatic activity is not affected by shoot allantoate levels (Rice et al., 1990). The weak relationship between AAH transcripts and shoot ureide concentrations may have resulted from the sequestration of allantoin in peroxisomes. However, if the process of ureide catabolism is maintained at a constant state in the ER regardless of overall shoot ureide concentrations, then AAH mRNA levels would remain stable. The actual ratio of allantoin to allantoate during water-deficit stress deserves further consideration in the regulation of ureide catabolism.

Several reports show that increases in Mn²⁺ concentration in vitro and in planta result in increases in AAH enzymatic activity (Lukaszewski et al., 1992; Purcell et al., 2000; Vadez et al., 2000; Vadez and Sinclair, 2001), and Mn²⁺ is an important co-factor for optimal AAH enzymatic activity (Lukaszewski et al., 1992; Werner et al., 2008). The drought-tolerant genotype Jackson acquires Mn²⁺ to a greater extent than KS4895, a drought-sensitive genotype (Purcell et al., 2000; King and Purcell, 2005). On the basis of the association of Mn²⁺ with shoot ureide concentrations, Purcell et al. (2000) proposed that differences in shoot ureide concentrations between drought-tolerant and drought-sensitive soybean genotypes may result from regulation of AAH enzymatic activity during water-deficit stress. However in this study, differences in drought tolerance were not associated with differential transcript expression of AAH. This may indicate that differences in AAH enzymatic activity due to Mn²⁺ availability are responsible for the differences in shoot ureide concentration during water-deficit stress. The AAH enzyme is proposed to have a multimeric structure (Agarwall et al., 2007; Werner et al., 2008). The role of Mn²⁺ in enzyme assembly is unknown. It is possible that the regulation of enzyme assembly may be regulated by Mn²⁺ availability, which might account for differences in the response of AAH activity between drought-tolerant and drought-sensitive genotypes. For example, Jackson may be more efficient at supplying Mn²⁺ to AAH, which results in a greater metabolism of allantoate. Greater demand for allantoate may drive the movement of stored allantoin from the peroxisomes to the ER. Therefore, ureide concentrations in the shoots would...
decrease as a result of increased ureide catabolism. Our results, combined with other reports, support focusing attention on the importance of the AAH enzyme in the regulation of shoot ureide concentrations and conferring of drought tolerance in soybean.

Supplementary data

Supplementary data are available at JXB online. Supplementary Fig. S1. Sequence of GenBank Acc. AW704657 (EST name sk54406.y1).

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