THE ROLE OF ALLANTOINASE IN SOYBEAN (*Glycine max* L.) PLANTS

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ABSTRACT

Soybean and related legumes export symbiotically-fixed nitrogen from the nodules to the leaves as ureides. The ureide allantoin is hydrolyzed by allantoinase to allantoate then further degraded by other enzymes, releasing ammonia and carbon dioxide. This study aimed to identify allantoinase genes in soybean and their gene expression as well as enzyme activity patterns. The effects of water limitation and allantoin treatment on the expression and activity of allantoinase in N_2-fixing plants were also evaluated. Enzyme activity and ureide content were evaluated using a spectrophotometric assay. Real time RT-PCR was used to quantify the amount of gene products. Four allantoinase genes were identified and were expressed, with *GmALN1* and *2* constantly expressed at higher levels. In seedlings, allantoinase was found to be actively synthesized more in cotyledons than in the embryonic axes, as seen by early enzyme activity and higher *GmALN 1* and *2* transcript levels. Allantoate produced in these tissues appeared to be mobilized to the developing axes. *GmALN1* and *2* were implicated in post-germination nitrogen assimilation during early seedling growth, while *GmALN3* and *4* were consistently expressed at very low levels, with an exception in nodules. Transcript abundance in the nodules of N_2-fixing plants, supported by the high enzyme activity and ureide content observed, suggested an important role in the synthesis and transport of allantoate in these tissues. Allantoinase was also detected in non-fixing tissues but may play a different role in these tissues, most probably functioning in the turnover and salvage of purine nucleotides. The effect of exogenous allantoin during water limitation was investigated. The addition of allantoin prior to water limitation seemed to change the sensitivity of soybean to such stress, prolonging its ureide catabolic activity at least up to 5 days without water. Results of this study will aid in our understanding of how ureide catabolism is regulated during soybean development. This information may help address problems in legume crop improvement specifically in enhancing N_2-fixation and yield capacity and in coping with water limitation stress.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

ALL	Allantoate
ALN	Allantoin
Asn	Asparagine
NH_3	Ammonia
BLAST	Basic Local Alignment Search Tool
BCA	Bicinchoninic acid
H_3BO_3	Boric acid
$CaCl_2{\cdot}2H_2O$	Calcium chloride dihydrate
C	Carbon
CO_2	Carbon dioxide
cm	centimeter
$CoCl_2{\cdot}6H_2O$	Cobalt chloride hexahydrate
$CuSO_4{\cdot}5H_2O$	Copper sulphate pentahydrate
CAM	Crassulacean acid metabolism
cv.	cultivar
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
h	hour
K_2HPO_4	Dipotassium phosphate
Na_2EDTA	Disodium ethylenediaminetetraacetic acid
DO-his	Drop out minus histidine
DO-ura	Drop out minus uracil
EST	Expressed sequence tags
$FeSO_4{\cdot}7H_2O$	Ferrous sulphate heptahydrate
GS-GOGAT	Glutamine synthetase/glutamate-oxoglutarate aminotransferase
GLY	Gloxylate
HCl	Hydrochloric acid
IMP	Inosine monophosphate

kbp	Kilobase pairs
kDa	kilodalton
kV	Kilovolts
KH_2PO_4	Monopotassium phosphate
LB	Lysogeny broth
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
Mn	Manganese
$MnSO_4.H_2O$	Manganese sulphate monohydrate
μF	Microfarad
μg	Microgram
μl	Microliter
μM	Micromolar
mg	Milligram
mL	Milliliter
mM	Millimolar
M	Molar/Molarity
nkat	Nanokatal
nm	Nanometer
nmol	Nanomole
$NiCl_2.6H_2O$	Nickel chloride hexahydrate
NiR	Nitrite reductase
NR	Nitrate reductase
N	Nitrogen
OD	Optical density
PCR	Polymerase chain reaction
PRPP	5-phosphoribosyl-1- pyrophosphate
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
ROS	Reactive oxygen species
g	Relative centrifugal force
RT-PCR	Reverse transcriptase-polymerase chain reaction
rpm	Revolutions per minute

RNA	Ribonucleic acid
s	Second
Ser	Serine
NaOH	Sodium hydroxide
$NaMoO_4 \cdot 2H_2O$	Sodium molybdate dihydrate
TIGR	The Institute of Genomic Research
XMP	Xanthine monophosphate
YPD	Yeast peptone dextrose
$ZnSO_4 \cdot 7H_2O$	Zinc sulphate heptahydrate

CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

Nitrogen plays an important role in plant growth and development as it is an essential constituent of proteins, nucleic acids, porphyrins and alkaloids (Schulten and Schnitzer, 1998). It is required in large amounts by most plants, but is often found limiting in the soil. In the absence of biological nitrogen fixation and fertilizers, the availability of N in the soil is dependent on the amount and type of organic matter available for decomposition (Franco and Munns, 1982). Other factors affecting soil N availability include the presence of microorganisms and the conditions enhancing their activity.

During nitrogen-limiting conditions, legumes and other plants able to form nitrogen-fixing symbioses have an advantage as they are able to access and utilize the large reservoir of N found in the atmosphere as N_2 gas. As a result of its interaction with nitrogen-fixing bacteria, the plant host produces a root nodule, a unique structure which provides a low oxygen environment for sustained reduction of N_2 by the bacterial enzyme nitrogenase (Atkins, 1987). In legume nodules, low oxygen concentration is maintained in the central part of the nodule by the combined high metabolic activity of the N_2-fixing bacteria and the unique morphology of the nodule parenchyma which serves as an oxygen diffusion barrier (Mylona et al., 1995). The development of nodules is highly specific because each host plant is only nodulated by a restricted number of nitrogen-fixing bacteria (Caetano-Anolles and Gresshoff, 1991). Similarly, these nitrogen-fixers also form nodules only on a limited number of host plants.

In symbiosis, ammonium is produced during nitrogen fixation by the bacterial partner, incorporated into organic compounds (nitrogen assimilation) and then exported from the nodule to the rest of the plant (Mylona et al., 1995). The plant in turn provides the bacteria with a carbon supply for use as an energy source. Since nitrogen fixation, catalyzed by the nitrogenase enzyme complex is particularly energy intensive, this exchange of C for N is of critical importance in maintaining this symbiotic relationship.

In general, once the ammonium has been assimilated plants convert it to the amide amino acids asparagine and glutamine. Some species, warm-season legumes in particular, export ureides, particularly allantoin and allantoic acid. Temperate legumes such as pea, lupin, broad bean, alfalfa and clover are classified as amide exporters while tropical legumes of the tribe Phaseoleae such as cowpea, mung bean, common bean and soybean, synthesize and transport ureides from fixed N hence they are known as ureide exporters (Schubert, 1986). These compounds get exported to various plant sink tissues where they get degraded through a series of enzyme-catalyzed steps. In leaves, allantoin is metabolized into allantoate by the enzyme allantoinase. Subsequent enzyme-catalyzed reactions further hydrolyze allantoate, ultimately leading to the release of ammonia for re-assimilation by plant cells.

It has been reported that nitrogen fixation activity of some legumes is sensitive to drought conditions (Sinclair and Serraj, 1995). In the said study, the tropical legumes cowpea and soybean were found to be drought sensitive, their sensitivity hypothesized to be associated with a high ureide concentration in the xylem sap. Drought sensitivity was found to be associated with the type of nitrogenous compounds exported from the nodules, and in the case of the tropical legumes cowpea and soybean, the presence of a high ureide concentration in the xylem sap. There are still many aspects of the ureide pathway which are not well understood, particularly at the molecular level. The recent identification and cloning of two plant allantoinase genes (Yang and Han, 2004) enabled researchers to initiate more studies on the molecular and biochemical basis of ureide metabolism in plants. Molecular analysis and characterization of all the ureide catabolic enzymes involved might elucidate the importance of ureide catabolism to plant growth and development. My research examined how the ureide catabolic enzyme allantoinase is regulated in soybean, a tropical ureide legume. The effect of allantoin treatment and drought on ureide catabolism, particularly its effect on allantoinase gene expression and activity, was also investigated in this study.

1.2. Nitrogen metabolism in non-ureide plants

Nitrate is usually the major form of inorganic N available to higher plants (Andrews, 1986). The assimilation of nitrate requires it to be reduced to ammonium, a two-step reaction catalyzed by the enzymes nitrate reductase (NR) and nitrite reductase (NiR) (Crawford and Arst, 1993). The enzyme NR initially reduces nitrate to nitrite then the process continues inside the

plastid where nitrite is reduced further to ammonia with the help of the NiR enzyme (Crawford, 1995). As ammonia is toxic to plant cells, it has to be rapidly converted to organic compounds.

Miflin and Lea (1976) proposed the glutamine synthetase/glutamate-oxoglutarate aminotransferase (GS-GOGAT) pathway to be the major pathway for ammonium assimilation in higher plants. Glutamate-oxoglutarate aminotransferase (GOGAT), also known as glutamate synthase, works in cycle with glutamine synthetase (GS), facilitating the transfer of the amide group from glutamine to 2-oxoglutarate to form two molecules of glutamate (Figure 1.1, Coruzzi, 2003). Besides glutamate and glutamine, ammonium could also be incorporated into other amino acids such as aspartate and asparagine with the help of the enzymes aspartate aminotransferase (AspAT) and asparagine synthetase (AS), respectively. The α-amino group of glutamate can be transferred to a variety of 2-oxo acid receptors to form these other amino acids but it could also be transferred back to form glutamate when 2-oxoglutarate and other amino acids are available in excess (Forde and Lea, 2007). These reactions are reversibly carried out by transaminases or aminotransferases. A second pathway involving the enzyme glutamate dehydrogenase can also produce glutamate by an amination reaction of 2-oxoglutarate (Figure 1.1, reviewed by Coruzzi, 2003), but this is unlikely that this enzyme plays a major role in nitrogen assimilation in bacteria or higher plants except in circumstances of ammonia excess (Miflin and Lea, 1976).

1.3. Biological nitrogen fixation

Biological nitrogen fixation enables some plants to utilize atmospheric nitrogen during nitrogen-limiting condition by forming symbiotic relationships with specific bacteria. This specific symbiotic mechanism is the sole realm of diazotrophs, bacteria containing nitrogenase, the enzyme which catalyzes the conversion of N from the gaseous to its combined form (Vessey et al., 2004). The most common groups of bacteria involved in these symbiotic interactions include the rhizobia and the actinomycete *Frankia* (Mylona et al., 1995). Among non-legumes, the *Alnus* species (alder trees) have been identified to interact with *Frankia* species (Dalton and Naylor, 1975). Bond (1954) discovered that there are in fact many species of *Frankia* that can colonize the *Alnus* root system and form nodules (Hall et al., 1979).

Legumes like soybean interact with members of the Rhizobiaceae group particularly *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* species (Van Rhijn and Vanderleyden, 1995).
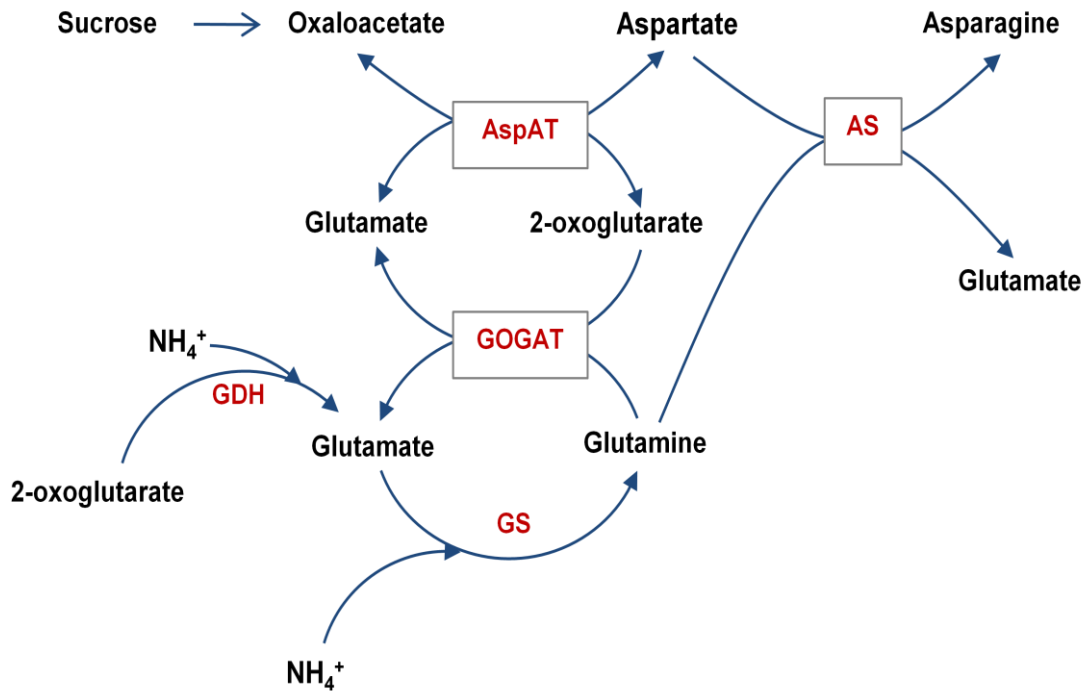
Figure 1.1. Pathways for ammonia assimilation in higher plants. The enzymes involved in the primary pathway are: glutamine synthetase (GS), glutamate synthase (GOGAT), aspartate aminotransferase (AspAT) and asparagine synthetase (AS). The second pathway involves the enzyme glutamate dehydrogenase (GDH). Figure adapted from Coruzzi, 2003.

The interaction of the *Rhizobium* and *Bradyrhizobium* bacteria with the roots of leguminous plants results in the development of a root nodule (Caetano-Anolles and Gresshoff, 1991). Marked curling seems to be the direct response of a root hair cell to the presence of rhizobia, the bacteria being trapped as the tip coils around itself (Pueppke, 1986). The rhizobia then change to their bacteroid form and become surrounded by a symbiosome or peribacteroid membrane which is of plant origin, effectively segregating them from the plant cytoplasm (Day et al., 2001). These steps are coupled with the binding of the constitutively expressed nodD protein to specific plant flavonoids, activating transcription of the bacterial nodulation (*nod*) genes (Heidstra and Bisseling, 1996). The *nod* genes will act together to produce a return signal, a lipo-oligosaccharide known as the Nod factor (Lindström et al., 2002). Nod factors can induce various responses in the root such as root hair deformation, depolarization of the root hair membrane potential, induction of nodulin gene expression, and formation of nodule primordia (Heidstra and Bisseling, 1996).

Nodules provide a conducive environment for the expression of nitrogenase, allowing the bacteria to utilize plant-derived C compounds to fix atmospheric nitrogen to ammonia (Atkins et al., 1992). Carbon is usually available in the form of dicarboxylates like sucrose (Lodwig and Poole, 2003). Sucrose is delivered to the root nodule through the phloem where it is cleaved by sucrose synthase, enters the glycolysis cycle, and ultimately provides malate to the bacteroid (Rogers et al., 2009). In return, ammonia is excreted from the bacteroids to the host plant cell where it is initially assimilated to form glutamine and glutamate. In a study involving lupin nodules, the GS-GOGAT pathway was proposed to be the primary assimilation pathway as implied by the higher activity of the enzymes glutamate synthase and glutamate synthetase compared to that of glutamate dehydrogenase (Robertson et al., 1975). The process of ammonium assimilation for the amide legumes is the same for non-fixing plants, as discussed in Section 1.2. The difference lies in the source of ammonium since non-fixing plants obtain this compound from inorganic N found in soil, while in legumes it is available mostly as products of the nitrogenase reaction.

1.4. Ureide metabolism

1.4.1. Ureide biogenesis

Almost all plants incorporate ammonia into glutamine and asparagine before transporting them into the different sink tissues of the plant. Tropical legumes like soybean (*Glycine max* L.) use glutamine to produce ureides for export during active nitrogen-fixing conditions. Ureides are produced in the nodules from purines mostly via *de novo* synthesis, although it could also be produced through the turnover of RNA and DNA in a process known as the salvage pathway (Schubert, 1986). The salvage pathway regenerates the plant nucleotide pools by interconverting purine bases, nucleosides, and nucleotides derived from plant cell metabolism and catabolism (Zrenner et al., 2006).

In root nodules, the plastids and mitochondria of infected cells synthesize purines using glutamine (Figure 1.2) and other amino acids. Purine biosynthesis starts with the formation of phosphoribosylamine (PRA) from 5-phosphoribosyl-1- pyrophosphate (PRPP) and glutamine (see details of review in Zrenner et al., 2006). *De novo* synthesis of the purine inosine monophosphate (IMP) from PRPP is a ten-step process which involves different enzymes, the amino acids glycine, glutamine and aspartate, 10-formyl tetrahydrofolate, and carbon dioxide.

Almost all the fixed N is subsequently incorporated through the purine pathway to form IMP (Smith and Atkins, 2002). IMP is transformed to xanthosine monophosphate (XMP) by the enzyme IMP dehydrogenase (Figure 1.3). XMP is further metabolized by 5' nucleotidases to xanthosine, which is then transformed to xanthine by inosine/guanine nucleosidase (Zrenner et al., 2006). Xanthine is then oxidized to uric acid in plastids of infected cells by xanthine dehydrogenase. Finally, uric acid is transported to the uninfected cells where it is used to produce allantoin specifically in the peroxisomes but could also be converted to allantoic acid in the cytosol (Smith and Atkins, 2002) through an allantoinase-catalyzed reaction. Figure 1.3 illustrates the pathway for ureide biogenesis in plants.

1.4.2. Ureide transport and catabolism

The ureides allantoin and allantoate are both used as long-distance transport molecules in tropical legumes (Smith and Atkins, 2002). These compounds leave the root nodules via the root xylem and are delivered into different sink tissues of the plant. Usually in leaves, the ureide catabolic pathway starts off with the hydrolysis of allantoin to allantoate. Allantoate is
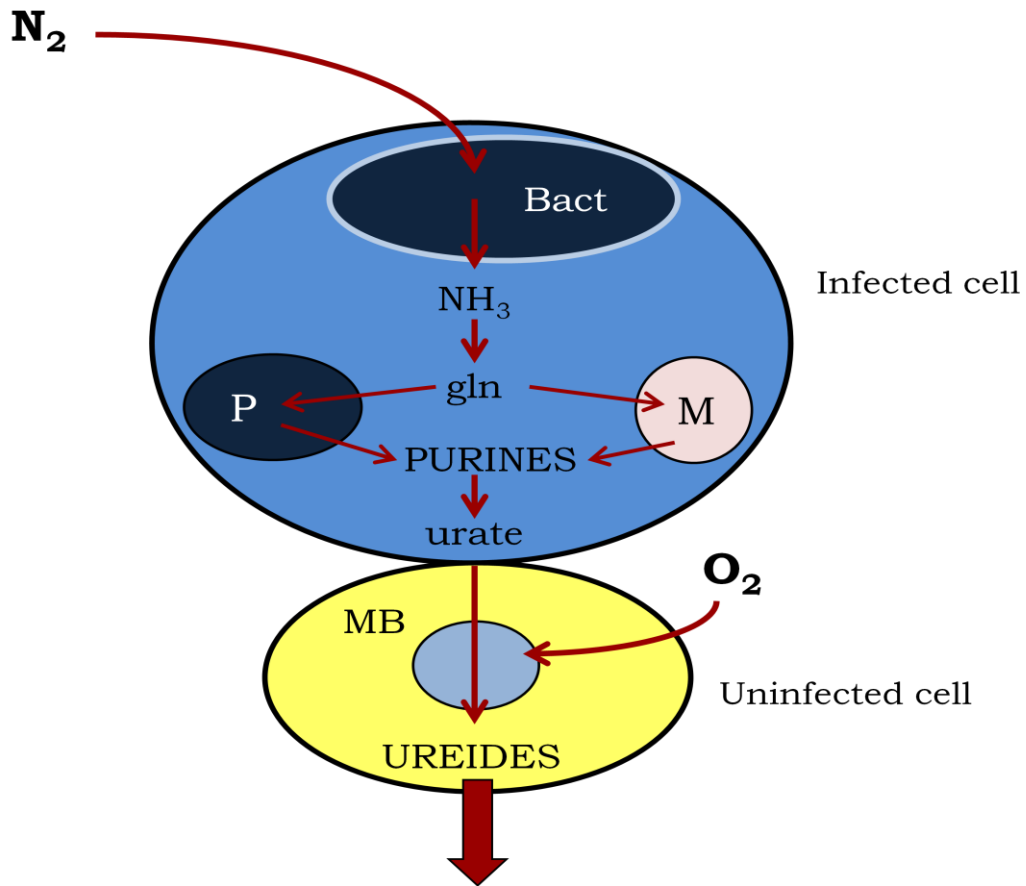
Figure 1.2. Purine synthesis in nodules. Nitrogenase in the bacteroid (Bact) reduces N_2 to NH_3. Ammonia is exported to the plant cytosol where it is assimilated as the amide group of glutamine (Gln). Gln, together with other amino acids, is used by both plastids (P) and mitochondria (M) to form purines. Purines are oxidized within the infected cell to urate. Urate is transported to uninfected cells to be further oxidized in the microbodies (MB) to allantoin and allantoic acid. Figure adapted from Smith and Atkins, 2002.
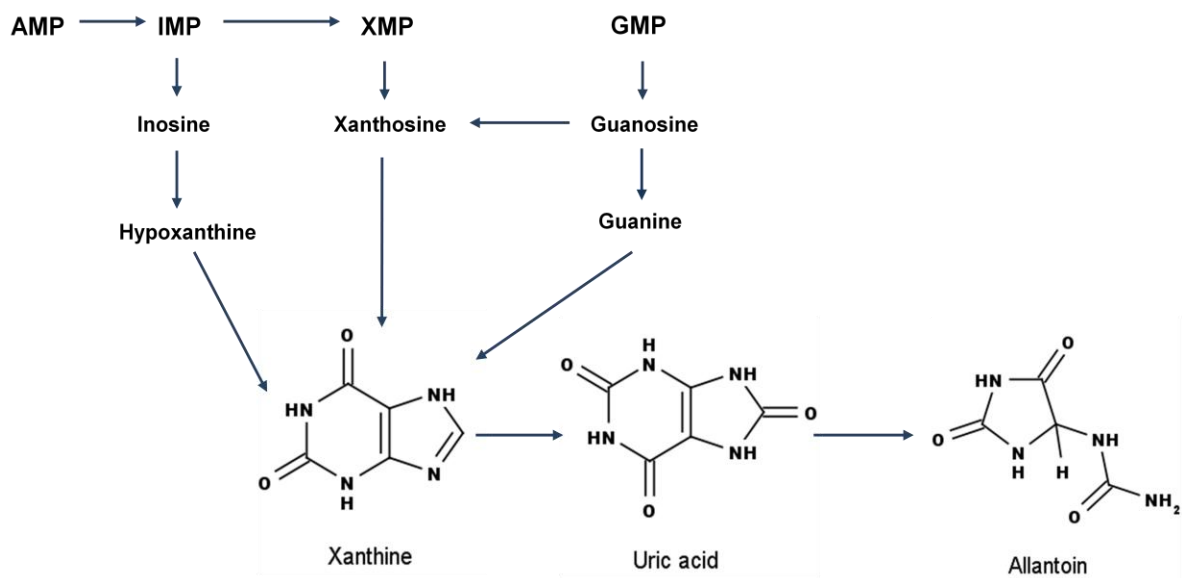
Figure 1.3. Ureide biogenesis in plants. AMP – adenosine monophosphate, IMP – inosine monophosphate, XMP – Xanthosine monophosphate, GMP – Guanosine monophosphate. Figure modified and adapted from Stasolla et al., 2003.

hydrolyzed to either ureidoglycolate or ureidoglycine which is further metabolized to ammonia, carbon dioxide and glyoxylate (Figure 1.4), the final products of purine catabolism (Zrenner et al., 2006).

It is agreed that allantoinase is the single enzyme catalyzing the hydrolysis of allantoin to allantoate (Todd et al., 2006). Allantoin-degrading activities were observed in the ureide legumes soybean (Costigan et al., 1987, Webb and Lindell, 1993, Bell and Webb, 1995) and the common bean, *Phaseolus vulgaris* L. (Raso et al., 2007). However, certain issues have been raised with regards to how allantoate is metabolized. Based on the results from two independent groups studying ureide catabolism, two pathways were proposed as to how this compound is degraded. One pathway suggests that an allantoate amidohydrolase enzyme breaks down allantoate to ureidoglycolate, which in turn is metabolized by ureidoglycolate amidohydrolase to two molecules of ammonium and carbon dioxide without the release of a urea intermediate in soybean (Winkler et al., 1987, Winkler et al., 1988). Aside from soybean, the presence of ureidoglycolate amidohydrolase was also verified in common bean (Wells and Lees, 1991).

Meanwhile, another independent study in soybean (Shelp and Ireland, 1985) proposed a different pathway for the metabolism of allantoate. The enzyme allantoate amidinohydrolase catalyzes the hydrolysis of allantoate to ureidoglycolate and urea (Figure 1.4). Ureidoglycolate in turn is acted upon by ureidoglycolate urea-lyase, releasing one molecule of urea and glyoxylate. The activity of this enzyme has in fact already been confirmed in chickpea, *Cicer arietinum* (Muñoz et al., 2001) and in common bean (Muñoz et al., 2006). Despite the discrepancy between these two initial studies suggesting different pathways, it has been proposed that different soybean cultivars could exclusively use either pathway or potentially both (Vadez et al., 2000).

Meanwhile, certain aspects of the ureide catabolic pathway in higher plants have now been now clarified with studies done in the non-ureide plant *Arabidopsis thaliana*. Urease was earlier identified in seedlings, its role proposed to be attributed to the recycling of urea-nitrogen (Zonia et al., 1995). In 2004, allantoinase activity was identified in seedlings and this allantoin-degrading activity was attributed to a single gene *AtALN* (Yang and Han, 2004). Todd and Polacco (2006) identified and cloned an allantoate amidohydrolase (*AtAAH*) cDNA, its function verified by complementation of the *Saccharomyces cerevisiae dal2* mutants (yeast lacking the allantoate amidinohydrolase gene). The *Arabidopsis* allantoate amidohydrolase (*AtAAH*)

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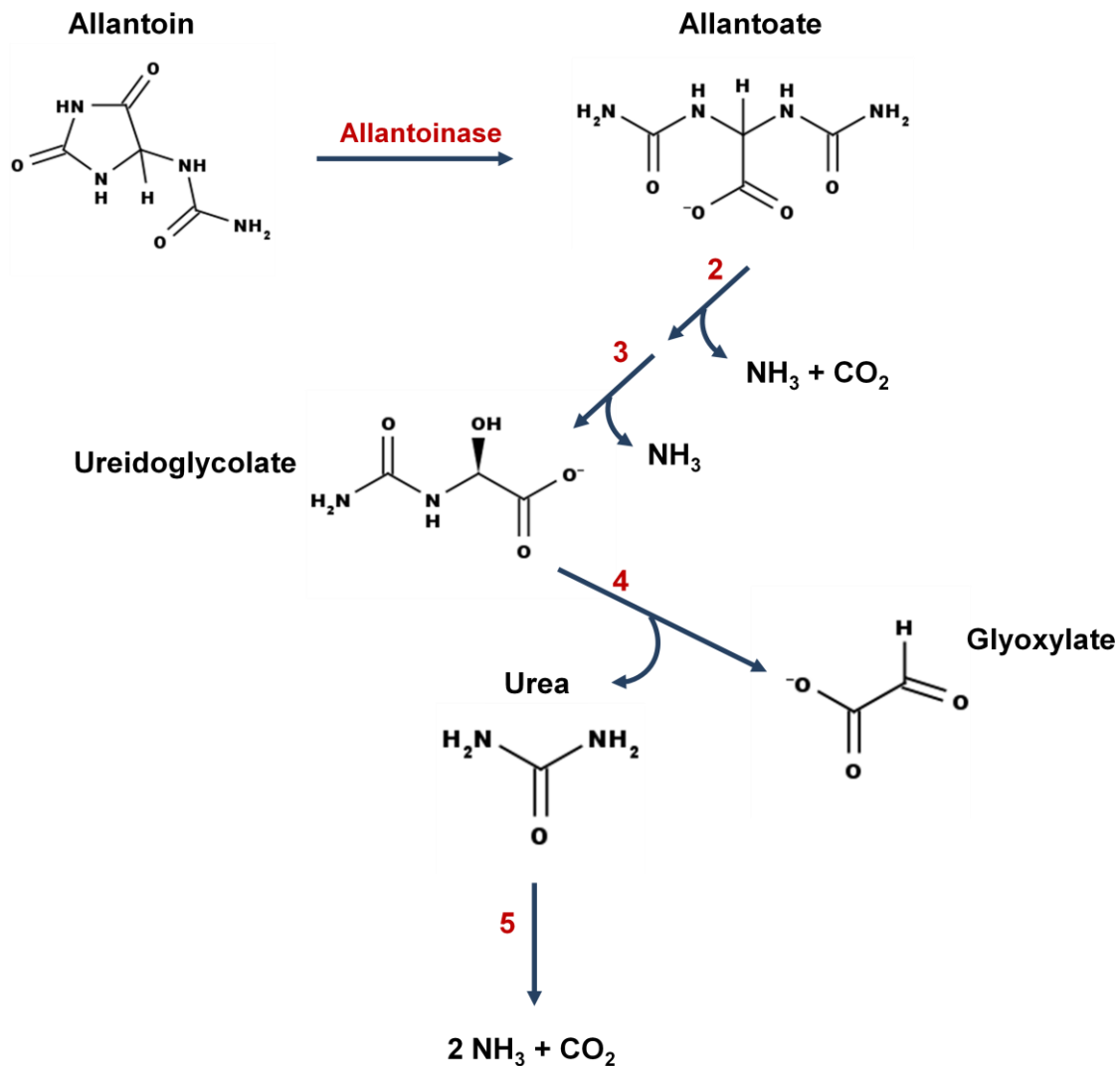
Fig. 1.4. Proposed route for allantoin breakdown in higher plants. Allantoinase catalyzes the breakdown of allantoin to allantoate. Hydrolysis of allantoate involves a series of reactions catalyzed by the enzymes: 2) allantoate amidohydrolase (or allantoate amidinohydrolase), 3) ureidoglycine aminohydrolase, 4) ureidoglycolate urea-lyase (for allantoate amidinohydrolase pathway), OR ureidoglycolate amidohydrolase (for allantoate amidohydrolase pathway) and, 5) urease.

expressed *in planta* as StrepII-tagged variants also rescued the *Ataah* T-DNA mutant (Werner et al., 2008). Werner et al. (2010) recently identified ureidoglycine aminohydrolase and ureidoglycolate amidohydrolase, enzymes which catalyze the final reactions of purine degradation in *Arabidopsis*. The enzyme ureidoglycine aminohydrolase converts ureidoglycine to S-ureidoglycolate, releasing a molecule of NH_3 in the process. Results showed that *Arabidopsis* utilized the enzymes allantoate amidohydrolase, ureidoglycine aminohydrolase and ureidoglycolate amidohydrolase to completely break down allantoate to glyoxylate, releasing four molecules of NH_3 and two molecules of CO_2. Figure 1.4 shows the proposed pathway of ureide catabolism in higher plants, in consideration with the recent findings in *Arabidopsis thaliana*.

It is still not well understood why tropical legumes like soybean utilize the ureide pathway during nitrogen-fixing conditions. The synthesis of purines/ureides is clearly a very complex process for assimilating ammonia, requiring expression of 20 different enzymes as compared with just four for asparagine synthesis (Smith and Atkins, 2002). On the other hand, it has been argued that ureides are more efficient to the N economy of the plant, having a C:N ratio of 1:1 (compared to 2:1 or 2.5:1 in amides). Though the complexity of using the ureide pathway seems to offset any C:N-ratio advantage that it may have, it has not yet been resolved as to which N assimilation pathway is more advantageous for the plants to use and under what conditions it may provide an advantage.

On the issue of nitrogen recycling, plants exporting ureides may have a greater advantage if one would take into account photorespiration. Although photorespiration functions as a scavenging pathway for the loss of carbon due to oxygenase action, a carbon (CO_2) is still lost during the process (Bowsher et al., 2008). Legumes, like any C_3 plant, have higher photorespiration rates compared to C_4 and CAM plants as they do not have systems which concentrate carbon dioxide in photosynthetic cells (Keys, 1986). Photorespiratory CO_2 losses could even be higher for tropical C_3 plants in warmer or dry environments (Bauwe et al., 2010) because entry of CO_2 is hindered (stomata have to be closed to lessen transpiration), decreasing photosynthetic rates. In this respect, plants transporting ureides will be able to prolong photosynthetic activity since fewer carbon skeletons are required for ammonium assimilation.

1.4.3. Effect of water limitation on biological nitrogen fixation

The negative impact of drought stress on nitrogenase activity in legume nodules has long been known (Sprent, 1972) however the mechanism as to how and why it occurs is still poorly understood (Serraj and Sinclair, 1996a, Streeter, 2003). Proposed causal factors include oxygen limitation, carbon shortage and regulation of nitrogen metabolism (Ladrera et al., 2007), however it was pointed out that since the inhibition of biological nitrogen fixation was shown to be more sensitive to water deprivation than photosynthate depletion, signaling for the regulation of biological nitrogen fixation most probably be provided by a nitrogen feedback mechanism involving nitrogen status (Marino et al., 2007). It was proposed that nitrogenous compounds like ureides, asparagine and aspartate are probably involved, either directly by accumulation of products that fail to be exported, or by feedback from the shoot due to an excess in nitrogenous compound supply in the shoots (Vadez et al., 2000).

It has been hypothesized that the observed ureide accumulation in the shoot during water-deficit conditions could be responsible for a feedback inhibition of nodule activity (Serraj and Sinclair, 1996b, Vadez et al., 2000), thereby inhibiting nitrogen fixation. Serraj and Sinclair (1996a) observed an accumulation of ureides in petioles of soybean during water limitation conditions, the drought-sensitive cultivar Biloxi having a higher ureide concentration than the drought-tolerant cultivar Jackson. Following exogenous nitrate application, cv. Jackson still had a lower ureide concentration in its petioles compared to the drought-sensitive soybean cultivar KS4895 (Purcell et al., 2000). Application of exogenous manganese was shown to promote ureide breakdown and prolong N_2 fixation under water deficit, more obviously in the drought-sensitive cultivar, however, King and Purcell (2005) showed that ureide catabolism in a drought-sensitive cultivars (cv. Jackson) was also stimulated by fertilization with Mn. Manganese is a required cofactor of allantoate amidohydrolase activity (Lukaszewski et al., 1992, Purcell et al., 2000). The structural basis for the difference in sensitivity of nitrogen fixation in tropical legumes under water stress is not clearly understood (Todd et al., 2006, Figuieredo et al., 2007). It was proposed that the differences among genotypes in terms of their ureide degradation capability might be associated with ureide accumulation and N_2 fixation sensitivity to soil water deficits, and could be explained by the existence of two pathways for ureide degradation in soybean (Vadez and Sinclair, 2001).

Earlier, Pate et al. (1969) had suggested that a lack of water for export may cause products of fixation to accumulate in the nodule and impair further functioning of the nodule, thus inhibiting nitrogenase activity by a negative feedback mechanism. Serraj et al. (1999b) have shown ureides and an intermediate compound assumed to be asparagine, to accumulate in soybean nodules during water-limiting conditions possibly causing inhibition of nitrogenase activity as a result of decreased phloem flow in the plant. It was suggested that lack of water for export may cause products of nitrogen fixation to accumulate in the nodule. King and Purcell (2005) point out nodule ureides, nodule aspartate, and several amino acids in leaves, as potential candidate molecules for feedback inhibition of N_2 fixation. Ladrera et al. (2007) implied that inhibition of nitrogen fixation in soybean during early drought is caused by a combination of both reduced carbon flux and nitrogen accumulation in nodules, but not in shoots.

A recent study however showed that ureide accumulation during water deficit is independent from *de novo* synthesis of ureides in nodules, and therefore not associated with nitrogen fixation (Alamillo et al., 2010). Ureides were shown to accumulate in roots, shoots, and leaves even at 14 days after water limitation, when nitrogen fixation has already ceased, and the stressed nodules have suffered senescence. Thus, the accumulation of ureides in shoots and the inhibition of nitrogen fixation appear to follow different kinetics and are probably not causally related. The said study provided new insight with regards to the impact of drought on nitrogen fixation, however, follow-up studies have yet to be done to verify these results.

1.5. Allantoinase
1.5.1. Overview
Allantoinase initiates the first step in the degradation of the ureide allantoin. This enzyme catalyzes the conversion of allantoin (5-ureidohydantoin) to allantoic acid via hydrolytic cleavage of the five-member hydantoin ring (Mulrooney and Hausinger, 2003). Allantoinase belongs to a superfamily of amidohydrolases and includes the enzymes D-hydantoinase, dihydropyrimidines, and dihydroorotase (Kim and Kim, 1998). Amidohydrolases are enzymes which catalyze the hydrolysis of a wide range of substrates containing an amide or ester functional group at carbon and phosphorus centers (Seibert and Raushel, 2005). Dihydropyrimidinases, D-hydantoinases, dihydroorotases and allantoinases, share a similar catalytic function of acting on the cyclic amide ring (Kim and Kim, 1998).

Early studies on allantoinase focused on the identification of the enzyme in various organisms through sequence comparisons and verification of function. Allantoinase was identified in yeast, *Saccharomyces cerevisiae* (Cooper and Lawther, 1973) and bacteria, *Pseudomonas aeruginosa* (Janssen et al., 1982). The fungi *Penicillium chrysogenum* was presumed to have allantoinase as one of its constitutive enzymes since it was able to utilize the ureide xanthine as a sole nitrogen source (Allam and Elzainy, 1969). Allantoinase was identified and cloned from cat flea (Gaines et al., 2004), mosquito (Scaraffia et al., 2008), bullfrog (Hayashi et al., 1994) and trout (Resende et al., 2005). Later studies point out the location of allantoinase within the cell which actually varies between species (Gaines et al., 2004). This enzyme was found in the cytosol and peroxisomes of marine fishes but for freshwater fishes, it was found only in the cytosol (Fujiwara et al., 1989). Allantoinase was found to be localized in the inner mitochondrial membrane of bullfrog liver and kidney tissues (Hayashi et al., 1994, Usuda et al., 1994). Interestingly, most mammals do not have allantoinase as allantoin-degrading enzymes were believed to have been lost during mammalian evolution (Fujiwara and Noguchi, 1995). However, a recent study proved that not all mammals lost their allantoin-degrading enzymes. Genomic comparisons done by Keebaugh and Thomas (2009) confirmed that allantoinase is absent in placental mammals but found in the South American opossum (*Monodelphis domestica*), a marsupial and the platypus (*Ornithorynchus anatinus*). The presence of allantoinase in opossum and platypus suggests that the loss of allantoinase is specific to placental mammals.

Recent studies revealed more information on the structure and activity of allantoinase. The metal content of allantoinase was first verified in the recombinant *E. coli* allantoinase, with the identity of the metal ion believed to be zinc ions (Mulrooney and Hausinger, 2003). This was confirmed in another study wherein functional analyses on the *E. coli* allantoinase also showed the presence of zinc in the metal center and that it is essential for the catalysis and enantio-selectivity of the substrate (Kim et al., 2009a). Meanwhile, a metal-independent protein with allantoinase activity (PuuE) was also identified from *Pseudomonas fluorescens* (Ramazzina et al., 2008). Previously identified allantoinases from various organisms were all homologous to the *Saccharomyces cerevisiae* allantoinase *DAL1* while allantoinase analog *PuuE* did not share significant similarities at the protein sequence level with the yeast allantoinase. This leads to the

hypothesis that *DAL1* is a more ancient allantoinase and has been replaced in some organisms by the PuuE protein (Ramazzina et al., 2008).

The presence of a conserved region consisting of one aspartic acid and four histidine residues is a characteristic shared by functionally related amidohydrolases including D-hydantoinase, dihydropyrimidinase, allantoinase and hydroorotase (Kim and Kim, 1998). This particular region was assumed to play a role in metal-binding, catalysis or structure of the enzyme. Substitution of the conserved amino acid residues in *Bacillus stearothermophilus* D-hydantoinase resulted in an almost complete loss of enzyme activity, an indication that these amino acid residues are critical for the catalytic activity of this enzyme (Kim and Kim, 1998). The cat flea allantoinase was also found to contain the conserved histidine and aspartic residues for zinc-binding and catalytic activity (Gaines et al., 2004). Functional analyses on the *E. coli* allantoinase showed that the presence of zinc in the metal center is essential for catalysis and enantio-selectivity of substrate (Kim et al., 2009a). In addition, active site residues (Asn94 and Ser317) were also found to play a crucial role in dictating activity of the *E. coli* allantoinase as mutation of these two residues completely abolished its activity.

1.5.2. Plant allantoinases

Early studies identified the presence of allantoinase in both germinated and dormant seeds of peanuts (Singh et al., 1970). The enzyme appeared to be particle-bound or associated with a membrane as observed in germinating seedlings of castor bean (*Ricinus communis*) (Ory et al., 1969). Allantoinase proteins have been purified and characterized from common bean fruits (Wells and Lees, 1992). In soybean, early studies also focused on the extraction and purification of allantoinase protein from various tissues. In 1993, Webb and Lindell used anion-exchange chromatography and allantoinase-specific antibodies to extract and purify a 30 kDa protein from soybean seeds. Allantoinase protein purified from root nodules and cotyledons of soybean was found to share the same molecular mass with the protein isolated from seeds (Bell and Webb, 1995). Meanwhile, two proteins with allantoinase activity from common bean (*Phaseolus vulgaris*) were recently identified (Raso et al., 2007). It was suggested that these proteins were bound to cellular components or within ruptured organelles. A recent study found the allantoinase proteins to be localized in the endoplasmic reticulum of *Arabidopsis thaliana* (Werner et al., 2008).

Ureide catabolism is a specialized mechanism used by some plants to assimilate nitrogen hence it is previously thought that ureide-catabolic enzymes like allantoinase were likely to be found only among ureide exporters. Recent studies however indicated that allantoinase are also found in non-ureide plants. The green alga, *Chlamydomonas reinhardtii*, has been shown to be able to use either allantoin or allantoate as a sole N source (Piedras et al., 2000) implying that it has allantoinase and other ureide enzymes necessary for degrading these compounds. Recently, Yang and Han (2004) identified and cloned allantoinases from two non-ureide plants, *Arabidopsis thaliana* and *Robinia pseudoacacia* showing that this enzyme is also found in non-ureide plants. As a result of the identification of these enzymes, we now have more knowledge on the ureide degradation pathway in non-ureide plants specifically in *Arabidopsis thaliana*. Not many studies have been done on the ureide catabolic enzymes at the molecular level. The purpose of my research is to generate molecular tools needed to clarify the regulation of ureide catabolism and nitrogen fixation, particularly during water limitation stress. Knowledge of these mechanisms may help point out various strategies to improve the yield of legume crops, specifically by boosting its nitrogen fixation capacity.

1.6. Objectives

My study investigated the regulation of ureide metabolism in soybean, focusing on allantoinase. Experimental work carried out to achieve the following objectives was done to address the hypothesis that allantoinase activity is regulated at the level of gene expression. My specific objectives were:

1. Identification of allantoinase genes in soybean by BLAST searches and sequence comparisons with known allantoinases, and by functional complementation using *Saccharomyces cerevisiae dal1* yeast

2. Determination of enzyme activity and gene expression patterns in developing seedlings and in mature N_2-fixing and non-fixing plant tissues using colorimetri*c* assays and real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), respectively

3. Investigation of the effect of allantoin treatment and water limitation on allantoinase expression and activity in soybean leaf tissues using colorimetric assays and qRT-PCR methods

CHAPTER 2. MATERIALS AND METHODS

2.1. Plant material

2.1.1. Soybean growth conditions

Soybean seeds (*Glycine max* L. cultivar Williams 82) were obtained from Missouri Foundation Seed (Columbia, MO, USA). Plant tissue samples were generated from germinated seedlings and mature plants at its V4-V5 stage. Vegetative stages are designated as Vn where n refers to the number of trifoliate leaves present in the main stem of a plant. Seeds were wrapped in cheesecloth and washed thoroughly in running water for 15-20 min. Ten seeds were rolled in a sheet of moistened 10 x 15 Anchor brand germination paper (St. Paul, MN, USA), covered with Saran Wrap®, placed in a beaker of distilled water and kept in a growth chamber at 22°C in the light (16 h photoperiod). Dark-germinated seedlings were also subjected to the same growth conditions but the germination paper rolls were covered with aluminum foil. Due to the variability in the onset of germination of the seeds, the lengths of the germinated seedlings were initially evaluated at different time points. A range of values for the desired seedling length was set from these data and used as a basis for the selection of seedling samples (See Results, Table 3.2). The seedlings' cotyledons were removed from the axes (hypocotyl and radical) and each was collected separately. Tissue samples were collected, quick-frozen using liquid nitrogen, and stored at -80°C. Except at Day 0 (imbibed seed), seedling axes and cotyledons were collected separately from seeds germinated for 2, 4, 6 and 8 days.

Mature plant tissue samples were obtained from 45 day-old N_2-fixing and non-fixing plants (V4-V5 stage) grown in a growth chamber set to 16 h photoperiod and with a day/night temperature set to 26/24°C. Nodulation was accomplished by inoculating the seeds with approximately 5 grams of peat inoculant containing the bacteria *Bradyrhizobium japonicum* (Turbo-N, Semences Pro-grain Inc., QC, Canada). After two weeks, plants were watered with a nutrient solution prepared using 2 mM $CaCl_2^. 2H_2O$, 0.5 mM $MgSO_4^. 7H_2O$, 0.312 mM K_2SO_4, 0.5 mM K_2HPO_4 and 25 µM $FeSO_4^. 7H_2O/Na_2EDTA$. The nutrient solution also contained a micronutrient solution prepared by combining 2.5 µM H_3BO_3, 1 µM $ZnSO_4^. 7H_2O$, 0.1 µM

NaMoO$_4$·2H$_2$O, 0.11 µM NiCl$_2$·6H$_2$O, 0.15 µM CuSO$_4$·5H$_2$O, 0.01 µM CoCl$_2$·6H$_2$O and 2 µM MnSO$_4$·H$_2$O. Plants were fertilized once every two weeks until the plants reached 45 days. The nitrogen source was removed to promote the development of nitrogen fixation between soybean and *B. japonicum*. Cobalt chloride was included because it is a nutrient requirement for rhizobia. Ammonium nitrate (25 mM) was added to the nutrient solution used to fertilize non-fixing plants. Non-fixing plants were not inoculated with *B. japonicum*. Leaf, root and nodule (fixing plants only) tissues were obtained from both N$_2$-fixing and non-fixing plants, quick-frozen with liquid nitrogen and stored at -80°C for analysis.

2.1.2. Soybean growth for water limitation experiments

Soybean seeds were inoculated with *B. japonicum* as described in Section 2.1.1. and grown for 5 weeks in a growth chamber set to a 16 h photoperiod and with a day/night temperature set to 26/24°C, watering as required and fertilizing every two weeks with nitrogen-free nutrient solution as described in Section 2.1.1. Each replicate consisted of four treatments: 1) Allantoin treated/No water, 2) Allantoin treated/Plus water (control), 3) No allantoin/No water and, 4) No allantoin/Plus water (control). Eight soybean plants comprised one replicate, two individual plants for each treatment. After growing for 5 weeks the allantoin-treated samples were watered with 700 ml of 25 mM allantoin for 10 days (watered once every three days), the control plants were treated with the equivalent volume of water. After this period, the water limitation stress was applied by withholding water from the allantoin treated and non-treated plants for 5 days while the control plants were watered once every two days. Leaf disc samples were obtained from all plants at days 0, 1, 3 and 5 using a #9 cork borer, sampling one leaf disc from the young, fully expanded leaves of each plant (Per treatment: 2 leaf discs, one from each plant). The experiment was replicated three times using the described procedure.

2.2. Identification of soybean allantoinase genes

2.2.1. Primer design, PCR and cloning of *GmALN* genes

Four allantoinase genes were identified in soybean. *GmALN1* and *2* had been previously identified and cloned in the Todd Lab. Identification of these two genes was based on the TIGR (The Institute for Genomic Research) soybean expressed sequence tag (EST) collection and similarity to known allantoinase sequences. For polymerase chain reaction (PCR) based cloning,

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primers were designed using the primer design tool in Vector NTI 10 (Invitrogen, Carlsbad, CA, USA) and the PCR method was used to allow amplification of cDNAs. PCRs were carried out using an Eppendorf thermal cycler (Eppendorf Canada, Mississauga, ON, Canada) set to the following conditions: (1) 30 s denaturation at 95°C, (2) 30 s annealing at 55°C and, (3) 1 min extension, the three steps comprising one cycle. A 2-min initial denaturation step was done prior to the start of the first cycle. At the end of 30 cycles, an additional 10-min extension step was also added. PCR products were visualized using agarose gel electrophoresis. After running the ethidium bromide-stained gel for 45 min at 70 volts, the gel was exposed to ultraviolet light and the image was captured using a UVP BioDoc-It™ UV transilluminator system (UVP LLC, Upland, CA, USA).

Cloning of the *GmALN* coding sequence into *E. coli* was done using the Fermentas GeneJET PCR cloning kit (Fermentas Canada Inc., Burlington, ON, Canada); ligating products into pJET according to manufacturer's instructions. The reaction was allowed to proceed at 22°C for about 1 h. The ligation mixture was used in bacterial transformation. The bacterial transformation method used throughout this study was electroporation. This technique applies a brief high-voltage pulse to bacterial cells and DNA, resulting in a transient permeability of the bacterial membrane and the uptake of DNA by the surviving bacterial cells (Miller, 1994).

In a clean electroporation cuvette, 2 µl of the ligation mixture and 40 µl *E. coli* competent cells were added. The cuvettes were capped and temporarily set on ice. Bacterial cells in electroporation cuvettes were briefly shocked using a Gene Pulser II® electroporation system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) set to 2.5 kV voltage and a capacitance value of 25 µF. Using a Pasteur pipette, about 950 µl of pre-warmed lysogeny broth (LB) media (previously prepared) was added into the cuvette. Using the same pipette, this liquid was recovered and transferred into a sterile 1.7 ml microfuge tube.

The tubes were incubated at 37°C for 40-60 min at 250 revolutions per min (rpm). After incubation, 10 and 100 µl aliquots were spread on solid LB medium containing 100 µg/ml ampicillin using a sterile, bent glass rod. The remaining samples were quickly spun down in a microcentrifuge at 14,000 *g* for 30 s. The supernatant was poured off in each tube then the bacterial pellet was resuspended in the remaining liquid inside the tube. This bacterial suspension was poured off on the LB-ampicillin plate and the liquid was evenly spread using a sterile, bent glass rod. LB-ampicillin plates with transformed bacteria were incubated at 37°C overnight.

Bacterial colonies on these LB-ampicillin plates were the source of plasmid DNA samples used for yeast transformation.

2.2.2. Isolation of recombinant plasmid DNA

Cultures for plasmid isolation were prepared by picking a single, lone colony (successfully transformed bacteria containing pDR196 plus *GmALN1*) from the previously plated LB-ampicillin plate (any one of the three dilutions may be used) using a sterile toothpick. The toothpick with bacterial smear was dropped into a sterile test tube containing 5 ml of liquid LB media plus ampicillin (100 µg/ml). The same procedure was done for the transformed bacteria containing pDR196 plus *GmALN2* and the pDR196 without any gene insert (empty vector). Transformed *E. coli* were allowed to grow overnight at 37°C in a rotary shaker set to a speed of 250 rpm. Extraction of plasmid DNAs was done on the second day using a GeneJET^{TM} plasmid miniprep kit (Fermentas Canada, Inc., Burlington, ON, Canada).

Restriction digestion analysis and agarose gel electrophoresis were done to check if the transformation procedure was successful. The recombinant plasmids pDR196 – no gene insert (empty vector), pDR196 – *GmALN1* and pDR196 – *GmALN2* were added to separate 0.65 ml microfuge tubes containing 4 µl buffer Tango$^{®}$ per reaction. For a single reaction, 1 µl of *EcoR1* and *BamH1*, were then added following manufacturer's instructions. Recombinant pJET plasmid DNA samples were sent for sequencing at National Research Council – Plant Biotechnology Institute (NRC-PBI) Saskatoon for further verification, the remaining amounts stored at minus 20°C.

The allantoinase cDNAs were cut from the pJET vector and subcloned into the pDR196 yeast expression vector (Rentsch et al., 1995) for functional complementation in *Saccharomyces cerevisiae*. *EcoR1/BamH11* fragments were subcloned using a Rapid DNA ligation kit (Fermentas Canada Inc., Burlington, ON, Canada) according to the manufacturer's instructions. Again, bacterial transformation using *E. coli* was carried out and the recombinant pDR196 plasmid DNA samples were harvested and isolated as previously described. Restriction digest and agarose gel electrophoresis techniques were employed to check and select for pDR196 plasmid DNA samples with the correct inserts.

2.2.3. Validating *GmALN1*, *2*, *3* and *4* coding sequences

Following the release of the soybean genome in 2008, two additional allantoinase genes *GmALN3* and *4*, were identified using a Basic Local Alignment Search Tool (BLAST, Altschul et al., 1990) search. As this information came during the latter part of this research, verification of enzymatic activity for these genes has not yet been accomplished. However, deduced amino acid sequences for *GmALN3* and *4* were compared with known allantoinase genes (See Results, Figure 3.5) and are highly similar to *GmALN1* and *2* (See Results, Table 3.1).

To verify if *GmALN3* and *4* were expressed in soybean, primers were designed using their nucleotide coding sequences and the primer design tool of Vector NTI Advance™ 10. Gene-specific primers were also designed for *GmALN1* and *2* in order that each primer was specific to only one allantoinase family member. The primer sequences designed for the amplification of an 800 bp internal sequence of the four *GmALN* genes are presented in Table 2.1. Amplification was carried out by PCR using the Qiagen TopTaq PCR kit (Mississauga, ON, Canada). A single reaction was set up by adding in the 5 µl 10X TopTaq PCR buffer, 5 µl thawed deoxyribonucleotide triphosphates (dNTPs), 1.25 µl of 20 µM forward and reverse primers, RNase-free water and 2.5 µl cDNA template in a PCR tube, following manufacturer's instructions. cDNA from various plant samples were made from 5 µg total RNA using Fermentas RevertAid™ First Strand cDNA synthesis kit (Fermentas Canada Inc., Burlington, ON, Canada), following manufacturer's instructions.

PCR tubes were mixed using a mini vortexer (VWR International, Mississauga, ON, Canada) and spun down using a centrifuge. The tubes were then loaded into an Eppendorf thermal cycler pre-set to the following PCR conditions: (1) 30 s initial denaturation at 94°C, (2) 30 s annealing (60°C for *GmALN1* & *2*, 63°C for *GmALN3* and 55°C for *GmALN4*) and, (3) 1 min and 30 s extension time at 72°C. A 2-min initial denaturation step was done prior to the start of the first cycle. An additional 10-min extension step at 72°C was also added after 30 cycles.

The four allantoinase genes have highly similar nucleotide sequences. Restriction digest analysis using *Apa1*, *Nco1* and *HpaII* (Fermentas Canada Inc., Burlington, ON, Canada) was employed to verify if each primer pair specifically amplified the corresponding expected allantoinase gene. *ApaI* was first used to divide the four allantoinase PCR products into two groups: Group 1: *GmALN1* and *2* and Group 2: *GmALN3* and *4*. The gene sequences under Group 1 were treated with *Nco1* to distinguish the *GmALN1* gene sequence from *GmALN2*.

Table 2.1. RT-PCR *GmALN* primers used for the amplification of an 800 bp sequence.

Primer details	Primer sequence (5' to 3')
GmALN1 forward	ATGGATCAGCTGTTGTGGAGGG
GmALN1 reverse	AACAATGTGAACATGAGCTCCTTCT
GmALN2 forward	ATGGATCAGTTTTTGTGGAGGGTG
GmALN2 reverse	GACGATGTGAACATGTGCTCC
GmALN3 forward	ATGGAGCATTTTGTTTGGAGGG
GmALN3 reverse	GACAATGTGAACATGTGCTCCTTCT
GmALN4 forward	ATGGAGCAGTTTGTATGGAGGGT
GmALN4 reverse	AACCACGTGAACATGTGCTCCT

Amplification was done for 30 cycles with a final extension time of 10 min at 72°C. Amplified products were visualized through agarose gel electrophoresis.

Meanwhile, the enzyme *HpaII* was added to Group 2 gene sequences to distinguish *GmALN3* from *GmALN4* gene sequences.

2.2.4. Confirming function of *GmALN* genes using *Saccharomyces cerevisiae dal1* mutant

2.2.4.1. Incorporating *GmALN* genes into the yeast *dal1* mutant

A *Saccharomyces cerevisiae dal1* mutant (Buckholz and Cooper, 1991) was used to confirm the function of the *GmALN* genes. The genotype of the diploid strain used is: MAT a/α his3Δ1/his3Δ1, Leu+, ura3Δ0/ura3Δ0, met15Δ0/+, +/lys2D0. This particular yeast strain (provided by J. Polacco, University of Missouri, St. Louis, MO, USA) has the yeast allantoinase gene replaced with a G^{418} resistance cassette and is not able to grow in a medium with allantoin provided as the sole nitrogen source and was provided by J. Polacco.

The transformation procedure for yeast was adapted from Gietz and Woods (2002). The pDR196 vectors containing *GmALN1* (pDR196-B4) and *2* (pDR196-E34) were first incorporated into the yeast *dal1* mutant. The *dal1* yeast were grown to a concentration of approximately 2.5 x 10^8 cells/ml in yeast peptone dextrose (YPD) media at 30°C. These were used to inoculate a 50 ml pre-warmed 2X YPD liquid media to give a final concentration of 5 x 10^6 cells/ml. The optical density (OD) of the yeast cultures was measured using a Beckman DU-7400 spectrophotometer (Beckman Coulter Canada, Inc., Mississauga, ON, Canada) at 600 nm and an OD_{600} of 1.0 was assumed to correspond to a concentration of 1 x 10^7 cells/ml. The cultures were grown for about 6 h until a concentration of about 2 x 10^7 cells/ml was reached. The cells were then harvested by transferring the yeast cultures in 50-ml sterile centrifuge bottles and spinning them in a J-25 centrifuge (Beckman Coulter Canada, Inc., Mississauga, ON, Canada) at 3,000 *g* for 5 min. The supernatant was discarded and the yeast pellet was washed by resuspending in 25 ml sterile water. The yeast suspension was again spun down in the same centrifuge using the same conditions previously described. One milliliter of sterile water was added and the yeast pellets were again resuspended by gentle shaking. The yeast suspension was distributed in 200 μl aliquots into different microfuge tubes. These were spun in a microcentrifuge at 14,000 *g* for 30 s and the supernatant discarded.

Equal amounts of transformation mix comprising of 240 μl 50% w/v polyethylene glycol, 36 μl 1M lithium acetate and 50 μl 2 mg/ml pre-boiled single-stranded carrier salmon sperm DNA were added to each microfuge tubes containing the pelleted yeast competent cells. Five

microliters of plasmid DNA samples (empty pDR196, pDR196–B4/*GmALN1* and pDR196–E34/*GmALN2*) were diluted with 29 μl sterile water (per one reaction) and added to the different microfuge tubes, mixed and incubated at 42°C in a circulating water bath for 40 min. Transformed *dal1* yeast cells were plated on drop out minus uracil media. The composition of the drop out media include 6.7 g/L yeast nitrogen base without amino acids, 16 g/L Bacto-agar, 20 g/L dextrose plus all the required amino acids except uracil. Successfully transformed mutant yeast should be able to grow in this selective medium. The *dal1* yeast does not have a functional *URA3*, the gene which enables it to produce uracil. However, pDR196 vector contains the *URA3* coding sequence, thus successfully transformed *dal1* yeast should be able to grow in a medium lacking uracil.

2.2.4.2.   Confirmation of successful transformants

Successfully transformed *dal1* yeast cells were first quickly verified using three different media: (1) Yeast peptone dextrose (YPD; positive control), (2) Drop out minus histidine (DO-his; negative control) and (3) Drop out minus uracil (DO-ura; selective media). The amino acid uracil was omitted for DO-ura while for DO-his, histidine was omitted. No growth was expected in DO-his since the *His3* gene of the *dal1* yeast strain had been knocked out, hence requiring an external source of histidine to survive. All yeast strains were expected to grow in YPD as it is the general medium used for growing yeast. As described in Section 2.2.4.1, DO-ura served as the media which selects for the *dal1* yeast with the pDR196 vector. It does not distinguish however, if the incorporated vector contains the gene of interest or not.

2.2.4.3.   Growing transformed yeast *dal1* in a medium with allantoin as the sole nitrogen source

Transformed *dal1* yeast were grown in a minimal media comprising of 1.7 g/L yeast nitrogen base without ammonium sulphate, 20 g/L dextrose, 1.5 ml/L of 100 mM histidine and 2.5 mM allantoin as its sole nitrogen source. Yeast were allowed to grow at 30°C for about 3 to 5 days. This was done to determine if the cloned *GmALN* genes coded for a functional allantoinase enzyme. Transformed yeast cells were grown on both solid media for visual confirmation of functional complementation and later on in liquid cultures to plot growth curves, and obtain

quantitative data. Culture density was monitored by measuring OD_{600} daily for 6 days using a Beckman DU-7400 spectrophotometer.

2.3. Real time quantitative RT-PCR analysis

2.3.1. RNA isolation and quantification

Isolation of total RNA for all plant samples evaluated was done using the E.Z.N.A.[TM] Plant RNA kit from Omega Bio-Tek (VWR International, Mississauga, ON, Canada) based on modification of the manufacturer's instructions. Mortar and pestles were first cooled using liquid nitrogen. A buffer RB plus β–mercaptoethanol solution was then prepared, adding 20 µl β–mercaptoethanol per 1 ml of buffer RB used. Frozen plant samples (approximately 100 mg each) were placed in the mortar and ground into fine pieces. The liquid nitrogen was allowed to evaporate before immediately adding 1.0 ml buffer RB with β–mercaptoethanol and grinding the buffer and plant sample into 'snow'. The plant tissue-buffer mixture was ground as it thawed and then transferred to the Omega[®] homogenizer columns assembled in 2 ml collection tubes. All further steps were done at room temperature unless otherwise indicated. The column-collection tube assemblies were spun at a speed of $\geq$ 14,000 g for 5 min to separate the plant debris from the liquid. The columns were discarded and a 1:1 mixture of the supernatant and 70% ethanol was then prepared in 1.7 ml microfuge tubes, mixed, and loaded into Hi-bind[®] RNA mini columns assembled into clean 2 ml collection tubes. Hi-bind[®] column-collection tube assemblies were spun at $\geq$ 12,000 g for 1 min. Flow-through in the collection tubes was discarded and the remaining plant supernatant-ethanol mixture was loaded into the Hi-bind[®] column-collection tube assemblies. These assemblies were again spun at $\geq$ 12,000 g for 1 min. The flow-through was discarded and 300 µl of RNA wash buffer 1 was added to the columns. The tubes were again spun at $\geq$ 10,000 g for 1 min.

Previously prepared DNA digestion mix containing RNase-free DNase 1 enzyme and E.Z.N.A.[TM] DNase 1 digestion buffer (VWR International, Mississauga, ON, Canada) was then added to the mini column-tube assemblies. An incubation period of 15 min at 25-30°C was done to allow digestion of any genomic DNA left in the samples. The HiBind[®] columns were allowed to stand for 5 min after adding in 500 µl of RNA wash buffer 1. The columns were spun at 14,000 g for 30 s. The flow through was discarded from the collection tubes and 500 µl of RNA wash buffer 2 was added. The tubes were again spun at 14,000 g for 30 s then the flow through

was discarded. This step was repeated to facilitate another washing. The tubes were spun for 1 min to remove the remaining wash buffer in the columns. The collection tubes were discarded and the columns transferred in 1.7 ml microfuge tubes. RNA samples were eluted from the columns by adding diethylpyrocarbonate-treated (DEPC) water and spinning it down at 14,000 g for 1 minute. The columns were discarded and the microfuge tubes containing RNA samples were placed on ice.

Concentration of the undiluted DNA-free RNA samples was quantified using the Nanodrop 2000 (Thermo Fischer Scientific, Waltham, MA, USA) and/or Beckman DU 7400 spectrophotometer at 260 nm. The RNA concentration values obtained for undiluted samples were verified by measuring diluted samples to be used for cDNA synthesis and RNA quality was confirmed by agarose gel electrophoresis.

2.3.2. cDNA synthesis and RT-PCR

Synthesis of cDNA for all plant samples evaluated was done using the Quanta qScript cDNA Supermix kit (VWR International, Mississauga, ON, Canada) which provides a 5X concentrated mix for two-step reverse transcriptase polymerase chain reaction (RT-PCR). This mix contains all the necessary components for first-strand synthesis: buffer, dNTPs, magnesium chloride, random primers, a recombinant RNase inhibitor protein, qScript reverse transcriptase and stabilizers.

For a single reaction, 1 μg total RNA sample was added to a 0.65 ml microfuge tube containing sterile water and 4μl qScript mix in a 0.65 ml. The tubes were mixed using the mini vortexer and the mixture spun down. The tubes were incubated in a Px2 thermal cycler (Thermo Electron Corporation, Marietta, OH, USA) at 25°C for 5 min, 42°C for 30 min then at 85°C for 5 min according to the manufacturer's directions. After the incubation period, the tubes were then taken out of the thermal cycler and chilled on ice. First-strand cDNA samples were amplified using PCR, using the *GmALN* and *CYP2* primers designed for real time quantitative RT-PCR (Table 2.2). Each PCR cycle followed the following steps: (1) 94°C for 30 s – denaturation, (2) 60°C for 30 s – annealing, and (3) 72°C for 1 min and 30 s – extension. A 2-min initial denaturation step was done prior to the start of the first cycle. At the end of 30 cycles, an additional 10-min extension step was also added. PCR products were visualized using agarose gel electrophoresis.

Table 2.2. qRT-PCR primers used for amplification of 200 bp sequence for both *CYP2* reference gene and *GmALN* genes.

Primer details	Primer sequence (5' to 3')
GmALN1 qRT forward	CTGTTGTGGAGGGTGTTGCCTTTGCTA
GmALN1 qRT reverse	GGGGTCACAATGCGTTTGCTG
GmALN2 qRT forward	TGGTCTCTTAAGTGCTGGTGTTCTTGGTG
GmALN2 qRT reverse	GATCAAGGTTATCCTCATTAAGCTCCAAA
GmALN3 qRT forward	GCATCCTCTCGTAAAGTTCCCGCGGAA
GmALN3 qRT reverse	TGCTTAGACTTCCCCTGCTTACTATATCC
GmALN4 qRT forward	ACATCCTCTCGTAAAGTTACCGCGGAG
GmALN4 qRT reverse	TTTCTTGCTTAGACTTCCCCTGCTTAC
CYP2 qRT forward	CCCCTCCACTACAAAGGCTCG
CYP2 qRT reverse	CGGGACCAGTGTGCTTCTTCA

Location of the forward and reverse primers within the nucleotide coding sequence of *GmALN1*, *2*, *3* and *4* are shown in Appendix A.

2.3.3. Analysis of samples using real time quantitative RT-PCR

Real time qRT-PCR was used to evaluate the abundance of *GmALN* transcripts in the different plant tissues examined. The gene *CYP2* was used as a reference gene for this experiment. It is a housekeeping gene coding for cyclophilin and recommended as a reference gene for soybean (Jian et al., 2008). Preliminary testing confirmed *CYP2* to be a good candidate for this study (data not shown). For a given experimental sample, five master mixes were prepared, one each for *GmALN1, 2, 3, 4* and one for the *CYP2* reference gene. Each master mix was prepared by adding to water: forward and reverse qRT-PCR primers and the Perfecta SYBR green supermix (VWR International, Mississauga, ON, Canada) according to manufacturer's directions. The Perfecta SYBR green supermix is a ready-to-use reaction mix already having the necessary components except the template DNA and the specific primers. From the master mix, individual sub-mixes were prepared, each one corresponding to the cDNA samples (e.g. seedling axes sampled at different time points). From this sub-mix, three individual 20 µl-samples were aliquoted into separate wells of a 96-well plate, representing the technical replicates of each cDNA sample (e.g. seedling axes sampled at day 2). The same procedure was done for the other cDNA samples (e.g. seedling axes sampled at other time points). All the samples were loaded on a 96-well flat top PCR microplate and sealed with a clear film (Axygen Scientific, CA, USA). For all qRT-PCR analyses, three biological replicates were performed. Transcript abundance of *CYP2* and *GmALN* genes was also determined in seedling cotyledons, mature N_2-fixing tissues and non-fixing tissues using the same protocol. Leaf tissue samples from the drought experiment were also evaluated using this same protocol. Relative abundance of the PCR products detected was computed using the C_t values generated (Pfaffl, 2001). A C_t value corresponds to that cycle in PCR wherein the abundance level of a particular PCR product (e.g. *GmALN1*) detected exceeded the particular threshold value. The mean C_t value for each sample was calculated from the C_t values of the three biological replicates. Relative abundance was computed using the delta delta C_t ($\Delta\Delta C_t$) analysis, using *CYP2* as the reference gene. For instance, the C_t value of *GmALN1* for Day 2 axes was normalized against the C_t value of *CYP2* gene (using the same cDNA from Day 2 axes). The four soybean allantoinase genes were compared by setting the relative abundance of one sample, typically *GmALN1*, to another (e.g. *GmALN1* expression at day 2 for axes) and normalizing all other data to this value.

2.4. Analysis of allantoinase activity

Plant tissues samples were ground in 50 mM Tricine-HCl buffer (pH 8.0) with 2 mM manganese sulphate using a pre-chilled mortar and pestle. The plant slurry samples were placed in 1.7 ml microfuge tubes and spun at 4°C for 20 min. The supernatant was transferred into clean tubes using a pulled Pasteur pipette. The crude extracts were spun again for 5 min to remove any remaining plant tissue debris. Again, the supernatant was retained and the crude extracts were kept on ice for the duration of the entire assays and used for determination of allantoinase activity, ureide content and soluble protein extracted.

2.4.1. Protein content

Total soluble protein extracted was determined using the Novagen BCA Protein assay kit (Merck, Darmstadt, Germany) according to manufacturer's directions. This procedure involves a reaction between the protein samples and copper sulphate-bicinchoninic reagent. It is based on a biuret reaction, which involves the reduction Cu^{2+} to Cu^{1+} by protein in an alkaline solution. Bicinchoninic acid is a chromogenic reagent that chelates with the reduced copper giving out a purple color that can be detected at 565 nm (Smith et al., 1985).

Diluted protein samples were prepared and 50 µl aliquots were placed in 13 x 100 mm tubes. Five concentrations (0, 125, 250, 500 and 1000 µg/ml) of the bovine serum albumin (BSA) were used as protein standards. Both the protein and standard samples were prepared in duplicates with a fresh protein standard prepared for every assay. One millilitre of the copper sulphate-bicinchoninic acid reagent was added to the tubes then mixed using a mini vortexer. The samples were incubated at 37°C for 30 min and absorbance values for the standard and protein samples were measured at 565 nm using Beckman DU 7400.

2.4.2. Enzyme assays

Allantoinase converts allantoin to allantoate. Further breakdown of allantoate results in the formation of glyoxylate along with 2 CO_2 and 4 NH_3. This assay measures the production of glyoxylate from the hydrolysis of allantoin, followed by chemical conversion of allantoate to glyoxylate (Vogels and van der Drift, 1979). One mole of allantoin will produce one mole of glyoxylate when completely hydrolyzed. Thus, the concentration of glyoxylate detected is equivalent to the amount of allantoate produced in these tissues (Vogels and van der Drift, 1979).

Protein samples were diluted 10-fold in 50 mM Tricine with 2 mM $MnSO_4$ and 35 mM β-mercaptoethanol so that measurement of glyoxylate production was within the linear range of the standard curve. Boiled protein samples (boiled at 100°C for 5 min) were also prepared and treated as previously described. These were used to determine the endogenous glyoxylate equivalents in the samples in the absence of enzyme activity. This value was subtracted from that of the unboiled samples to determine the amount of allantoin hydrolyzed.

Assays were initiated by mixing equal volumes (600 µl) of diluted samples and Tricine-$MnSO_4$-β-mercaptoethanol plus 20 mM allantoin. Samples were incubated at 30°C in a circulating water bath for 30 min. After 30 min, 250 µl of the incubated samples were transferred to 16 x 150 mm Kimax test tubes and 250 µl of 0.15 N hydrochloric acid and 250 µl of 0.33% w/v phenylhydrazine were added, stopping the reaction. A standard curve was generated using glyoxic acid standards (0, 0.03, 0.06, 0.12 and 0.40 µmol/ml of glyoxylate) were treated in the same way. Experimental samples and standards were boiled in a water bath for 2 min then cooled for 10 min before adding 1 ml concentrated hydrochloric acid and 250 µl of 1.67% w/v potassium ferricyanide. Production of a glyoxylate phenylhydrazone causes the samples to develop a red color in the presence of glyoxylate. Color was allowed to develop by incubating the test tubes at room temperature for 10 minutes. The absorbance values measured at 520 nm using a Beckman DU 7400 spectrophotometer, were used to calculate allantoinase activity. All samples were assayed in duplicate and values reported are the mean of at least three independent biological replicates. Data are reported in nanokatals (where 1 katal = 1 mol/s) of allantoin hydrolyzed per second at 30°C.

2.5. Ureide assays

A colorimetric assay was performed to quantify ureides present in different plant tissue samples and to differentiate between the intermediates in ureide catabolism, allantoin, allantoate and ureidoglycolate. The assay is based on differential chemical hydrolysis of ureides and formation of a coloured glyoxylate phenylhydrazone *in vitro* (Vogels and van der Drift, 1970). Diluted crude extracts were subjected to alkaline, heat, acid and alkaline-acid hydrolysis according to Table 2.3.

Table 2.3. Differential analysis of glyoxylate derivatives (Vogels and van der Drift, 1970).

	Allantoin	Allantoate	Ureidoglycolate	Glyoxylate
Assay D: Alkaline-acid hydrolysis	+	+	+	+
Assay C: Acid hydrolysis	-	+	+	+
Assay B: Alkaline hydrolysis	-	-	+	+
Assay A: Standard analysis	-	-	-	+

(+) or (-) indicates the ability to detect each ureide in modifications of the standard analysis

The standard analysis (Assay A) measures glyoxylate. Crude samples were diluted with water (dilution factor varied with tissue types used) and a 250 µl aliquot was added to a 16 x 150 mm Kimax test tube. Five glyoxylate concentrations (0, 0.015, 0.030, 0.060 and 0.120 µM) were used to generate a fresh standard curve each time the assay was performed. One milliliter water, 250 µl of 0.4 M KH_2PO_4 buffer and 250 µl of 0.33% phenylhydrazine were added to the tubes containing the crude extracts and glyoxic acid standards. Samples were vortexed and allowed to stand for 5 min at room temperature and then cooled in an ice water bath. Concentrated hydrochloric acid (1.25 ml) and 1.65% potassium ferricyanide (250 µl) were added and the tubes were vortexed to promote even mixing. Color was allowed to develop for 15 min before absorbance values were measured at 535 nm using a Beckman DU 7400 spectrophotometer. All samples were measured in duplicate.

For alkaline-acid hydrolysis (Assay D), 250 µl of 0.5 N sodium hydroxide and 0.5 ml water were added to a test tube containing 250 µl of diluted crude extract samples. The sample-NaOH mixture was mixed, and then boiled in a water bath for 10 min. The samples were allowed to cool first before 250 µl of 0.65 N HCl was added. The tubes were boiled for 5 min before standard analysis was applied. In the acid hydrolysis method (Assay C), 250 µl of 0.15 N HCl, 0.75 ml water and 250 µl sample were mixed in a Kimax test tube. The tubes were boiled for 5 min then cooled to room temperature before standard analysis was employed. In the alkaline hydrolysis method (Assay B), 250 µl of 0.5 N NaOH and 1 ml water were added to the diluted samples. The tubes were mixed then allowed to stand at room temperature for 2 min. Standard analysis was again applied. Concentrations of allantoin, allantoate, ureidoglycolate and glyoxylate were determined as indicated in Table 2.4.

Table 2.4. Summary of how the concentration for the different glyoxylate derivatives was computed.

Computation Method	Glyoxylate Derivative Measured
[D tubes]: µM ALN + µM ALL + µM UDG + µM GLY − [C tubes]: µM ALL + µM UDG + µM GLY	Allantoin
[C tubes]: µM ALL + µM UDG + µM GLY − [B tubes]: µM UDG + µM GLY	Allantoate
[B tubes]: µM UDG + µM GLY − [A tubes]: µM GLY	Ureidoglycolate
µM GLY in A tubes	Glyoxylate

Standard analysis (A), Alkaline hydrolysis (B), Acid hydrolysis (C) and, Alkaline-acid hydrolysis (D). ALN − allantoin, ALL − allantoate, UDG − ureidoglycolate, GLY − glyoxylate

CHAPTER 3. RESULTS

3.1. Allantoinases in *Glycine max* show high sequence similarities with *Robinia pseudoacacia* allantoinase *(RpALN)*

GmALN1 and *2* were first identified in the Todd lab based on a BLAST search of the TIGR soybean EST collection. The nucleotide coding sequences for each of *GmALN1* and *2* were amplified by RT-PCR, cloned and sequenced (Shahid et al., 2007). Coding and amino acid sequences of these two allantoinase genes were verified later upon the release of the soybean genome data in 2008. *GmALN1* and *2* were among the allantoinase genes identified in soybean, sharing 95.5% identity in nucleotide coding sequences and deduced amino acid sequences.

A search for conserved domains at the National Center for Biotechnology Information's (NCBI) Conserved Domain Database (CDD) was made using the predicted amino acid sequence for *GmALN1* as query (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and identified two conserved domains, cd01315 and PLN02795. Domain cd0315 is a common region to the amidohydrolase enzymes L-hydantoinase and allantoinase and contains one aspartic acid and four histidine residues, amino acids known to be conserved among amidohydrolases (Kim and Kim, 1998). Amino acid sequence alignment showed the amidohydrolase domain to have 42.1% and 42.3% sequence identity with GmALN1 and 2 respectively (Figure 3.1). The second domain (PLN02795) is a conserved motif shared among allantoinases. This domain shares 63.6% amino acid sequence identity with GmALN1 (61.7% for GmALN2) and 63.8% for RpALN (Figure 3.2). The presence of the two conserved protein domains in GmALN1 and 2 implied that the both code for an amidohydrolase enzyme, most likely allantoinase.

3.2. Soybean has four allantoinase genes
3.2.1. *GmALN* genes are located on chromosomes 13 and 15

In 2008, United States Department of Energy's Joint Genome Institute released a draft sequence of the soybean genome which could be accessed at

```
cd0315   --------------------------------------------------DLVIKNGRVVTPDGVR  16
GmALN1   MDQLLWRVLPLLTILVSFGVFFYLQDSYTAQFYALIKLPRDKCSLLPHRHFWISSKRIVTPQGII  65
GmALN2   MDQFLWRVLPLLTILVSFGVFFYLQDSYTAQLFPLIKLPRDKCSLLPHRHFWISSKRIVTPQGII  65

cd0315   EADIAVKGGKIAAIGPDIAN---TEAEEVIDAGGLVVMPGLIDIHVHINEPGRTEWEGFETGTKA  79
GmALN1   SGSVEINDGKIISVVEGHANQGKPKQEEVIDYGDAVIMPGLIDVHVHLDEPGRTEWEGFDTGTRA  130
GmALN2   SGSVEINDGKIISVVEGHAIQGKPKQEEVIDYGDAVIMPGLIDVHVHLNEPGRTEWEGFDTGTRA  130

cd0315   AAAGGITTIIDMPLNSIPPTTTVENLEAKLEAAQGKLHVDVGFWGGLVPG---NLDQLRPLDEAG  141
GmALN1   AAAGGVTTVVDMPLNNYPTTVSKEKLKLKLDAAEDKIYVDVGFWGGLIPENALNTSILEGLLSAG  195
GmALN2   AAAGGVTTVVDMPLNCYPTTVSKEKLKLKLDAAEDKIYVDVGFWGGLIPENALNTSILDGLLSAG  195

cd0315   VVGFKCFLCPSGVDEFPAVDDEQLEEAMKELAKTGSVLAVHAENPE-ITEALQEQAKAKGKRDYR  205
GmALN1   VLGMKSFMCPSGINDFPMTTIDHIKAGLPVLAKYKRPIVVHAEVQQDFENHLELNEDNLDPRAYL  260
GmALN2   VLGVKSFMCPSGIDDFPMATIDHIKAGLSVLAKYRRPIVVHAEIQQDFENHLELNEDNLDPRAYL  260

cd0315   DYLASRPVFTEVEAIQRILLIAKET-------GCRLHIVHLS-SAEAVPLIREARAEGVDVTVET  262
GmALN1   TYLNARPPSWEEAAIKQLVGVTKDTRKGGPLEGAHVHIVHLSDSSASLDLIKEAKSRGDSISVET  325
GmALN2   TYLNTRPPSWEEAAIKQLVSVTKDTRKGGPLEGAHVHIVHLSDSSASLDLIKEAKSHGDSISVET  325

cd0315   CPHYLTFTAEDVPDGGTEFKCAPPIRDAANQEQLWEALENGDIDMVVSDHSPCTPELKLLGKGDF  327
GmALN1   CPHYLAFSSEEIPNGDTRFKCSPPIRDAYNREKLWGAVLEGHIDLLSSDHSPTVPELKLMEEGDF  390
GmALN2   CPHYLAFTSEEIPNGDTRFKCSPPIRDAYNKDKLWEAVLEGHIDLLSTDHSPTVPELKLMEEGDF  390

cd0315   FKAWGGISGLQLGLPVMLTEAVNKRGLSLEDIARLMCENPAKLFGLSHQKGRIAVGYDADFVVWD  392
GmALN1   LKAWGGISSLQFNLPVTWSYGK-KYGLTLEQLSLLWSKKPATLAGLES-KGAIAVGNHADIVVWQ  453
GmALN2   LKAWGGISSLQFNLPVTWSYGK-KYGLTLEQLSLLWSKKPATLAGLES-KGTIAVGNHADIVVWQ  453

cd0315   PEEEFTVDAED--LYYKNKISPYVGRTLKGRVHATILRGTVVYQDGEVVGEPLGQLLL-  448
GmALN1   PELEFDLDDDYPVFIKHSELSAYMGRRLSGKVLETFVRGNLVFKDGKHAPAPCGVQILAK  513
GmALN2   PELEFDLDDDYPVFIKHSELSAYMGRRLSGKVLETFVRGNLVFKDGKHAPAPCGVQILAK  513
```

Figure 3.1. Amino acid sequence comparisons showing GmALN1, GmALN2 and cd01315, a conserved protein domain found among amidohydrolases. GmALN1 and 2 share 42.1% and 42.3% sequence identity with this particular domain. GmALN1, GmALN2 and cd01315 contain the amino acids conserved among amidohydrolases (one aspartic acid/D – boxed in red, four histidine/H residues – boxed in blue). Identical amino acids are highlighted in yellow. Amino acids with similar biochemical properties are highlighted in green.

```
PLN02795   ------------------------------------------RDGCSLLPYPHFVLYSKRVVTPAGVI 26
  RpALN    MDQLVWRVLPMLALLVSFLVFFYLQDSYKAQLSPFIKLPGDECSLLPHRHYWISSKRIVTPQGII 65
  GmALN1   MDQLLWRVLPLLTILVSFGVFFYLQDSYTAQFYALIKLPRDKCSLLPHRHFWISSKRIVTPQGII 65
  GmALN2   MDQFLWRVLPLLTILVSFGVFFYLQDSYTAQLFPLIKLPRDKCSLLPHRHFWISSKRIVTPQGII 65

PLN02795   PGAVEVKGGRIVSVTKREEPRSQPKKPRVLDYGEAVVMPGLIDVHMHLNEPGRTEWEGFPTGTRA 91
  RpALN    SGSVEINEGEIVSIIEGYGKQGNSMQEAVIDYGEAVVMPGLIDVHMHLDEPGRTEWEGFDTGTRA 130
  GmALN1   SGSVEINDGKIISVVEGHANQGKPKQEEVIDYGDAVIMPGLIDVHMHLDEPGRTEWEGFDTGTRA 130
  GmALN2   SGSVEINDGKIISVVEGHAIQGKPKQEEVIDYGDAVIMPGLIDVHMHLNEPGRTEWEGFDTGTRA 130

PLN02795   AAAGGITTLVDMPLNSFPSTTSVETLELKIEAAKGKLYVDVGFWGGLVPENAFNASVLEALLSAG 156
  RpALN    AAAGGVTTVVDMPLNNYPTTVSKETLQLKLEAAEKKLYVDVGFWGGLIPENALNTSILEGLLSAG 195
  GmALN1   AAAGGVTTVVDMPLNNYPTTVSKEKLKLKLDAAEDKIYVDVGFWGGLIPENALNTSILEGLLSAG 195
  GmALN2   AAAGGVTTVVDMPLNCYPTTVSKEKLKLKLDAAEDKIYVDVGFWGGLIPENALNTSILDGLLSAG 195

PLN02795   VLGLKSFMCPSGINDFPMTNATHIKEALPVLAKYKRPLLVHAEVVPDVSPSSLVDGEPGADPRSY 221
  RpALN    VLGVKSFMCPSGIDDFPMTTIDHIKEGLSVLAKYRRPLLVHAEIQQDSKNHLELKG--NGDPRAY 258
  GmALN1   VLGMKSFMCPSGINDFPMTTIDHIKAGLPVLAKYKRPIVVHAEVQQDFENHLELNED-NLDPRAY 259
  GmALN2   VLGVKSFMCPSGIDDFPMATIDHIKAGLSVLAKYRRPIVVHAEIQQDFENHLELNED-NLDPRAY 259

PLN02795   MTYLKSRPPSWEQAAIRQLLEVAKDTRIGGVAEGAHVHIVHLSDAESSLELIKEAKRKGDSVTVE 286
  RpALN    LTYLNTRPPSWEQAAIKELVDVTKDTIIGGPLEGAHVHIVHLSDSSASLDLIKEAKSRGDSISVE 323
  GmALN1   LTYLNARPPSWEEAAIKQLVGVTKDTRKGGPLEGAHVHIVHLSDSSASLDLIKEAKSRGDSISVE 324
  GmALN2   LTYLNTRPPSWEEAAIKQLVSVTKDTRKGGPLEGAHVHIVHLSDSSASLDLIKEAKSHGDSISVE 324

PLN02795   TCPHYLAFSAEEIPDGDTRFKCAPPIRDAANRELLWKALLGGDIDMVSSDHSPSPPDLKLLEEGN 351
  RpALN    TCPHYLAFSSEEIPDRDTRFKCSPPIRDALNKEKLWEAVLEGHIDLLSSDHSPTVPELKLLEEGD 388
  GmALN1   TCPHYLAFSSEEIPNGDTRFKCSPPIRDAYNREKLWGAVLEGHIDLLSSDHSPTVPELKLMEEGD 389
  GmALN2   TCPHYLAFTSEEIPNGDTRFKCSPPIRDAYNKDKLWEAVLEGHIDLLSTDHSPTVPELKLMEEGD 389

PLN02795   FLKAWGGISSLQFVLPATWTHGRARGLTLEQLARWWSERPAKLAGLGSKGAIAPGKDADIVVWDP 416
  RpALN    FLRAWGGISSLQFDLPVTWSYGKKHGLTLEQLSLLWSKKPATFAGLESKGAIAVGNHADIVVWQP 453
  GmALN1   FLKAWGGISSLQFNLPVTWSYGKKYGLTLEQLSLLWSKKPATLAGLESKGAIAVGNHADIVVWQP 455
  GmALN2   FLKAWGGISSLQFNLPVTWSYGKKYGLTLEQLSLLWSKKPATLAGLESKGTIAVGNHADIVVWQP 455

PLN02795   EAEFDLNEDYPIYHKHKSISAYLGTKLSGKVIATFVRGNLVFEEGKHAKKACGSPILAK 475
  RpALN    ELEFDLNDDYPVFIKHPSLSAYMGRRLSGKVLDTFVRGNLVFKDGKHAPAACGVPILAK 512
  GmALN1   ELEFDLDDDYPVFIKHSELSAYMGRRLSGKVLETFVRGNLVFKDGKHAPAPCGVQILAK 513
  GmALN2   ELEFDLDDDYPVFIKHSELSAYMGRRLSGKVLETFVRGNLVFKDGKHAPAPCGVQILAK 513
```

Figure 3.2. Amino acid sequence comparisons showing similarities between RpALN, GmALN1, GmALN2 and PLN02795, a conserved region found in all known allantoinases. Identical amino acids are highlighted in yellow. Amino acids with similar biochemical properties are highlighted in green. Also shown here are the aspartic acid (D, boxed in red) and histidine (H, boxed in blue) residues, amino acids conserved among amidohydrolases.

. Searching this database revealed two more allantoinase genes (*GmALN3* and *4*) in soybean. A BLASTN search in the soybean database using the cloned *GmALN1* nucleotide coding sequence as the query sequence showed that there are four soybean genes with high nucleotide identity to the cloned *GmALN1* in the genome, two each on chromosomes 13 and 15. The two additional allantoinase sequences were designated *GmALN3* and *4*. *GmALN1* and *GmALN3* are located 3.77 kbp apart on chromosome 15 while the other two, *GmALN2* and *4*, are 3.76 kbp apart on chromosome 13 (Figure 3.3). Though situated on different chromosomes, the genes *GmALN1* and *2* shared a higher degree of sequence identity (95.5% sequence identities in nucleotide coding and amino acid sequences, Table 3.1) with each other, and both have a predicted protein sequence of 513 amino acids (Figure 3.4). *GmALN3* and *4* are most similar to each other (Table 3.1), sharing 97.2% sequence identities in nucleotide coding and putative amino acid sequences. Both GmALN3 and 4 lack one asparagine residue (putative protein sequence of 512 amino acids) when compared to GmALN1 and 2.

Like GmALN1 and 2, the predicted amino acid sequences of GmALN3 and 4 contain the cd0315 and PLN02795 conserved domains (sequence alignment not shown). GmALN3 and 4 shared 40.7% and 41.1% amino acid sequence identities with the amidohydrolase domain while for the allantoinase domain, sequence identities of 62.5% and 62.8% were observed for the two allantoinases, respectively. The conserved amino acids (one aspartic acid and four histidine residues) which were believed to play a role in the activity of amidohydrolases like allantoinase were also present in GmALN3 and 4 (Figure 3.4). The active site residues asparagine and serine, previously identified in *E. coli* allantoinase as crucial in influencing enzyme activity (Kim et al. 2009a), were also present in all four allantoinase genes (Figure 3.4).

Soybean allantoinases were also compared to different known allantoinases, the amino acid sequences compared against each other using the alignment tool of Vector NTI 10. The *Robinia* allantoinase RpALN (Yang and Han, 2004) was most closely related to the four soybean allantoinase (Figure 3.5)**,** sharing amino acid sequence similarity of 86.7% with GmALN1, 2 and 4 and 86.3% with GmALN3 (alignment not shown).
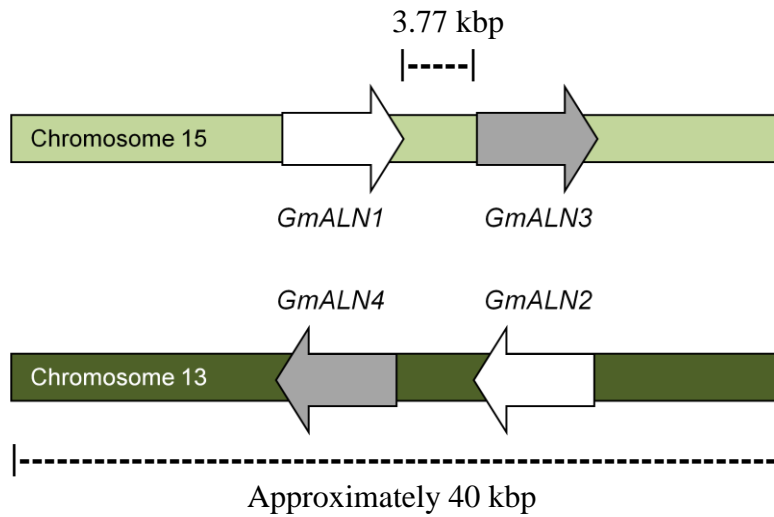
Figure 3.3. Schematic diagram showing the orientation of the four *GmALN* genes on chromosomes 13 and 15. *GmALN1* and *2* are more related to each other, sharing 95.5% (97.2% identity for *GmALN3* and *4*) identity in their predicted protein and nucleotide coding sequences.

Table 3.1. Percent identities of the nucleotide coding and putative amino acid sequences of
GmALN1, *2*, *3* and *4*.

Compared soybean allantoinase genes	Percent identities of sequences	
	Nucleotide coding	Amino acid
GmALN1 and *2*	**95.5**	**95.5**
GmALN1 and *3*	91.4	85.8
GmALN1 and *4*	91.1	85.2
GmALN2 and *3*	90.9	84.8
GmALN2 and *4*	91.1	84.6
GmALN3 and *4*	**97.2**	**97.2**

All sequences were compared using the Vector NTI 11 alignment tool. *GmALN1* and *2* (and also
GmALN3 and *4*) have the highest percent identities in their nucleotide coding and predicted
amino acid sequences.

```
GmALN1   MDQLLWRVLPLLTILVSFGVFFYLQDSYTAQFYALIKLPRDKCSLLPHRHFWISSKRIVTPQGII  65
GmALN2   MDQFLWRVLPLLTILVSFGVFFYLQDSYTAQLFPLIKLPRDKCSLLPHRHFWISSKRIVTPQGII  65
GmALN3   MEHFVWRVLPLLTILISLVVFFYLQDSYRAHLHPLVKFPRNKCSLLPHRHFWITSKRIVTPQGII  65
GmALN4   MEQFVWRVLPLLTILMSLVVFFYLQDSYRAHLHPLVKLPRSKCSLLPHRHFWITSKRIVTPQGII  65

GmALN1   SGSVEINDGKIISVVEGHANQGKPKQEEVIDYGDAVIMPGLIDVHVHLDEPGRTEWEGFDTGTRA  130
GmALN2   SGSVEINDGKIISVVEGHAIQGKPKQEEVIDYGDAVIMPGLIDVHVHLNEPGRTEWEGFDTGTRA  130
GmALN3   SGSVEINEGKIISVIEGYSKQGKSKQEKIIDYGDAVVMPGLIDVHVHLDEPGRTAWEGFDTGTRA  130
GmALN4   SGSVEINEGKIISVIKGYSKQGKSKQEKIIDYGGAVVMPGLIDVHVHLDEPGRTAWEGFDTGTRA  130

GmALN1   AAAGGVTTVVDMPLNNYPTTVSKEKLKLKLDAAEDKIYVDVGFWGGLIPENALNTSILEGLLSAG  195
GmALN2   AAAGGVTTVVDMPLNCYPTTVSKEKLKLKLDAAEDKIYVDVGFWGGLIPENALNTSILDGLLSAG  195
GmALN3   ASAGGVTTVVDMPLNNHPTTVSKETLKLKLEAAENKIYVDVGFWGGLIPENAHNTSILEGLLSAG  195
GmALN4   AAAGGVTTVVDMPLNNHPTTVSRETLKLKLEAAENKIYVDVGFWGGLIPENAHNTSILEGLLSAG  195

GmALN1   VLGMKSFMCPSGINDFPMTTIDHIKAGLPVLAKYKRPIVVHAEVQQDFENHLELNEDNLDPRAYL  260
GmALN2   VLGVKSFMCPSGIDDFPMATIDHIKAGLSVLAKYRRPIVVHAEIQQDFENHLELNEDNLDPRAYL  260
GmALN3   VLGLKSFMCSSGISDFPLTTIHHIKEGLSVLAKYRRPLLVHSEIQQHSKKHLELNDK-GGPRAYL  259
GmALN4   VLGLKSFMCPSGISDFPLTTIHHIKEGLSVLAKYRRPLLVHSEIQQHSKKHLELNDK-GGPRAYL  259

GmALN1   TYLNARPPSWEEAAIKQLVGVTKDTRKGGPLEGAHVHIVHLSDSSASLDLIKEAKSRGDSISVET  325
GmALN2   TYLNTRPPSWEEAAIKQLVSVTKDTRKGGPLEGAHVHIVHLSDSSASLDLIKEAKSHGDSISVET  325
GmALN3   TYLHTRPPSWEEAAIKELVGVTKDTRKGGPLEGAHVHIVHLSDSSASLDLIKEAKSRGDSISVET  324
GmALN4   TYLHTRPPSWEEAAIKELVGVTKDTRKGGPLEGAHVVVHLSDSSASLDLIKEAKRRGDSISVET  324

GmALN1   CPHYLAFSSEEIPNGDTRFKCSPPIRDAYNREKLWGAVLEGHIDLLSSDHSPTVPELKLMEEGDF  390
GmALN2   CPHYLAFTSEEIPNGDTRFKCSPPIRDAYNKDKLWEAVLEGHIDLLSTDHSPTVPELKLMEEGDF  390
GmALN3   CPHYLAFSSEEIPNGDTRFKCSPPIRDAYNREKLWGAVLEGHIDLLSSDHSPTVPQLKLLEEGDF  389
GmALN4   CPHYLAFSSEEIPNGDTRFKCSPPIRDAYNREKLWEAVLEGHIDLLSSDHSPTVPQLKLLEEGDF  389

GmALN1   LKAWGGISSLQFNLPVTWSYGKKYGLTLEQLSLLWSKKPATLAGLESKGAIAVGNHADIVVWQPE  455
GmALN2   LKAWGGISSLQFNLPVTWSYGKKYGLTLEQLSLLWSKKPATLAGLESKGTIAVGNHADIVVWQPE  455
GmALN3   LKAWGGVTSLQFDLPVTWSYGKKHGLTLEQLSLLWSKKPATLAGLESKGAIAVGNHADIVVWKPE  454
GmALN4   LKAWGGVTSLQFDLPVTWSYGKKHGLTLEQLSLLWSKKPATFAGLESKGAIAVGNHADIVVWKPE  454

GmALN1   LEFDLDDDYPVFIKHSELSAYMGRRLSGKVLETFVRGNLVFKDGKHAPAPCGVQILAK  513
GmALN2   LEFDLDDDYPVFIKHSELSAYMGRRLSGKVLETFVRGNLVFKDGKHAPAPCGVQILAK  513
GmALN3   VEFDLNEDYPVFLKHPSLSAYMGRRLSGKVLETFVRGNLVFKKGKHAHAACGVPILAK  512
GmALN4   VEFDLNEDYPVFLKHPSLSAYMGRRLSGKVLETFVRGNLVFKKGKHAPSACGVPILAK  512
```

Figure 3.4. Alignment of the putative amino acid sequence of the four allantoinase in soybean. GmALN1 and 2 share 95.5% identity while GmALN3 and 4 share 97.2% identity. Both GmALN3 and 4 are lacking one asparagine (N, boxed in green). Identical amino acids are highlighted in yellow. Amino acids with similar biochemical properties are highlighted in green. All four soybean allantoinase have the conserved amino acids aspartic acid (D, boxed in red) and histidine (H, boxed in blue) residues. The active site residues asparagine and serine (boxed in black) are also present.

40
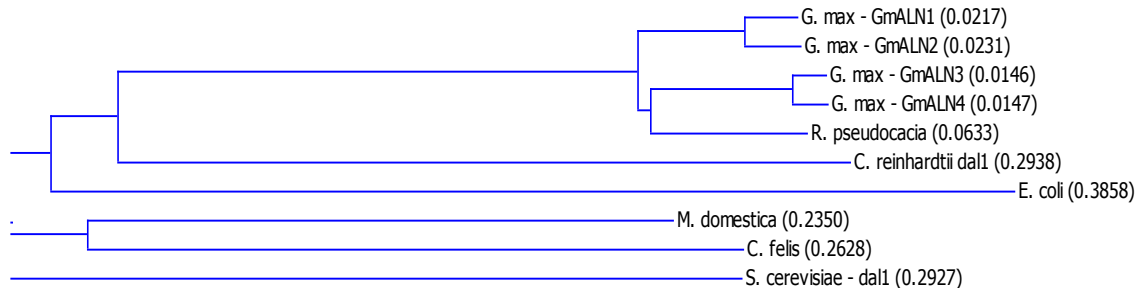
Figure 3.5. Relationship between *GmALN1*, *2*, *3* and *4* with other known allantoinases. Amino acid sequences of all allantoinases shown were compared using the AlignX tool of Vector NTI 11. Values in brackets refer to the calculated genetic distances and are related to the degree of divergence between species, where a lower value implies a closer relationship. Allantoinase from *Robinia pseudoacacia*, a non-ureide legume, is most closely related to the four allantoinase genes of soybean.

3.2.2. Expressing soybean allantoinase in *Saccharomyces cerevisiae dal1* mutants allows allantoin to be used as a sole nitrogen source

Saccharomyces cerevisiae dal1 mutants (Buckholz and Cooper, 1991) are yeast strains lacking a functional allantoinase, hence they are not able to survive if grown in a medium having allantoin as the sole nitrogen source. Allantoinase hydrolyzes allantoin to allantoate, making it available for further degradation, allowing the release of ammonia. To confirm that the putative soybean allantoinase genes do indeed code for allantoinase, they were cloned into the yeast expression vector pDR196 (Rentsch et al., 1995) to conduct functional complementation experiments. The transformed *dal1* mutant yeast carrying a functional soybean allantoinase then should be able to grow on minimal media containing allantoin as its sole nitrogen source. Figure 3.6 depicts the growth of transformed *dal1* yeast expressing *GmALN1* on a solid allantoin medium, compared to the empty vector control, supporting the hypothesis that this gene indeed codes for a functional allantoinase enzyme. The *dal1* yeast was also grown in an allantoin liquid media (Figure 3.7) to demonstrate growth rate differences observed between the *dal1* yeast and strains expressing *GmALN1* or *GmALN2*. Growth observed for *dal1* yeast expressing *GmALN2* in liquid medium was variable for the three independent replicates, but consistently higher than the empty vector control. On synthetic complete media lacking uracil the *dal1* strain expressing *GmALN2* also showed slower growth than the same strain carrying *GmALN1*. The slight growth observed for the *dal1* yeast carrying the empty vector was attributed to the instability of the commercial allantoin used. Strains carrying the soybean allantoinase genes did not grow on media lacking nitrogen completely and did not differ from empty vector controls when either urea or ammonium were provided as N-source (data not shown). These studies were initiated before the identification of *GmALN3* and *4.* Functional complementation of *GmALN3* and *4* is currently underway in the lab.

3.3. Allantoinase plays a role in soybean growth and development
3.3.1. Seedling development
3.3.1.1. Enzyme activity and ureide content

The role of allantoinase in the growth and development of soybean was first investigated in seedlings. Allantoinase activity and gene expression patterns in soybean seeds were investigated prior to and after germination. To control for variable timing of germination, a
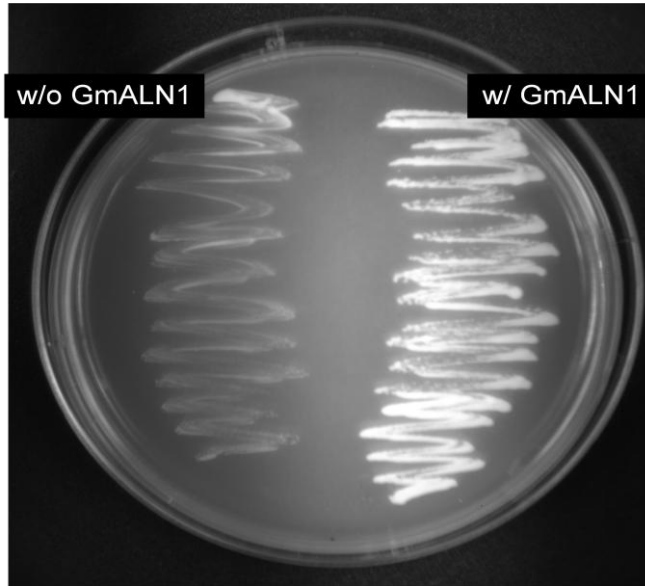
Figure 3.6. Growth of *Saccharomycescerevisiae dal1* mutant on solid minimal media with allantoin as the only nitrogen source. Significant growth was observed with the *dal1* mutant carrying the pDR196 plasmids expressing *GmALN1* DNA when compared to the *dal1* yeast with only the empty pDR196 vector.
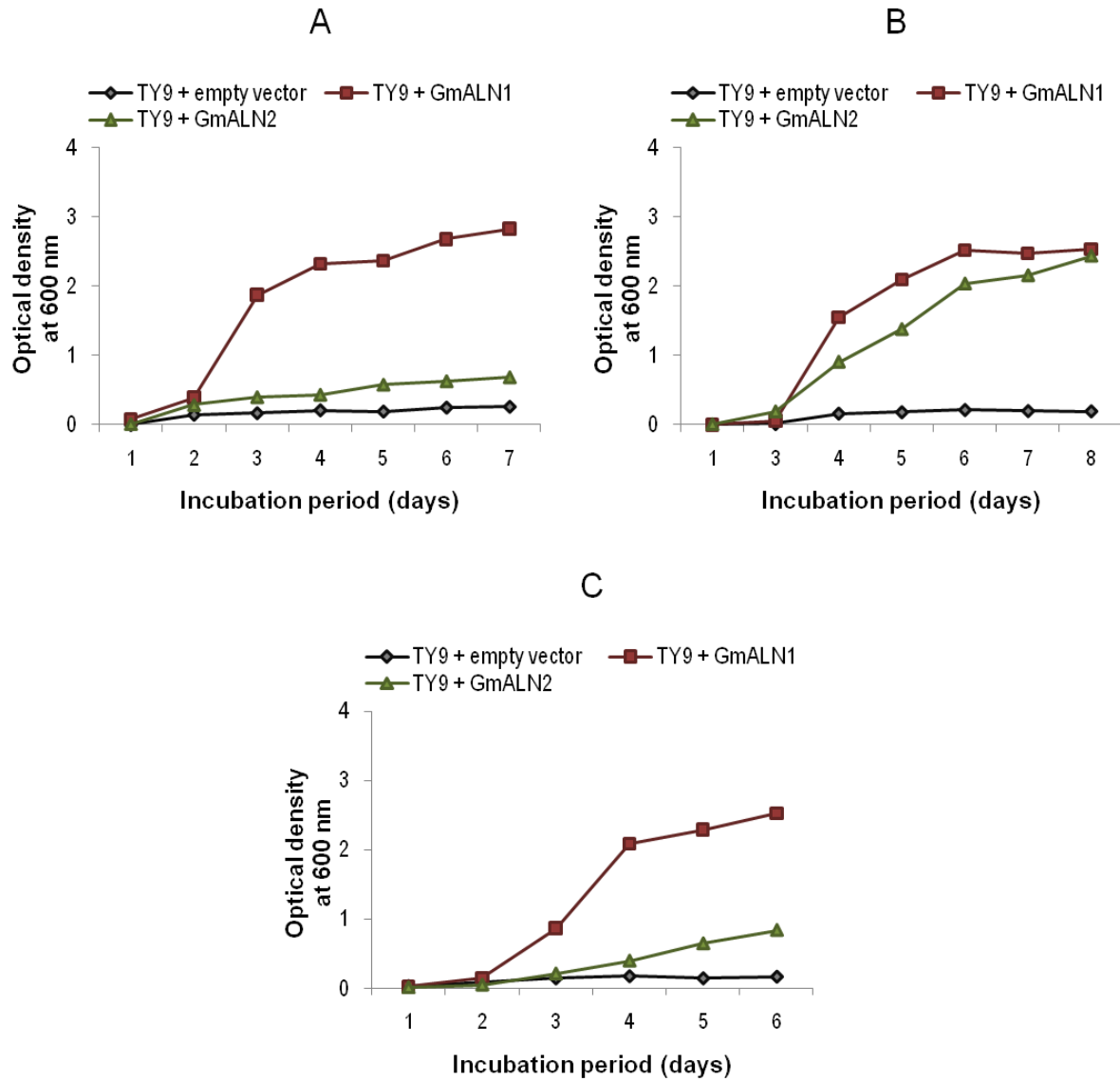
Figure 3.7. Growth of *Saccharomyces cerevisiae dal1* mutant in liquid minimal medium with allantoin as the only nitrogen source grown at 30°C. Graphs A, B and C represent the three independent replicates done for this experiment. Increasing growth was evident for the mutant yeast carrying the recombinant pDR196 plasmid DNA plus *GmALN1* or *2*.

growth experiment was first done to establish standard seedling length (hypocotyl and radicle) values to classify the different time points evaluated in this study. The embryonic axes length of dark and light-germinated seedlings were relatively the same up to four days after seed imbibition (Figure 3.8), after which light-germinated seedlings were slightly longer however the difference observed was not significant either at day 6 (t-test, P-value = 0.36) or day 8 (t test, P-value = 0.83). Based on these data, a range of values was determined, to stage seedlings for all further analyses, similar to the approach taken by Groome et al. (1991). Both the age of the seedlings and these size classes (Table 3.2) were used as a reference in collecting seedling samples for each of the specific time points evaluated for all the data presented for the rest of the thesis.

The role of allantoinase in seedling tissues was assessed initially by measuring its enzyme activity and by determining ureide content. The ureide assay measures allantoin, allantoate and ureidoglycolate and data are presented per milligram of fresh weight assayed. Figure 3.9 depicts the amounts of the ureides allantoin, allantoate and ureidoglycolate found in the embryonic axes and cotyledons of light and dark-germinated seedlings over 8 days. An overall increase in the concentration of total ureides was observed for both seedling types during post-germinative growth. Allantoate was the major ureide found in the axes, ranging from 55 – 88% (22 – 81% in dark axes) of the total ureides detected in light- and dark-germinated seedlings from day 2 to day 8. Allantoate was also the major ureide detected in cotyledons except at day 2 in light cotyledons where allantoin was the most abundant ureide (54%). The presence of ureides and the increasing pattern observed for both axes and cotyledons may indicate that the seedlings actively use these compounds (particularly allantoate) for transporting nitrogen to support its growth and development. Ureides were also detected in imbibed seeds though in smaller amounts compared to the other tissues evaluated, suggesting its possible minor role in nitrogen storage.

Allantoinase catalyzes the breakdown of allantoin to allantoate. Therefore, the presence of high amounts of allantoate in seedlings might be an indication of allantoinase activity. Allantoinase specific activity (nkat/mg protein) and total soluble protein extracted is summarized in Figure 3.10. Variable enzymatic activity was observed for both the axes and cotyledons with the embryonic axes showing relatively higher specific activity compared to the cotyledons of both seedling types (Figures 3.10 C & D). Storage proteins in the cotyledons and embryonic
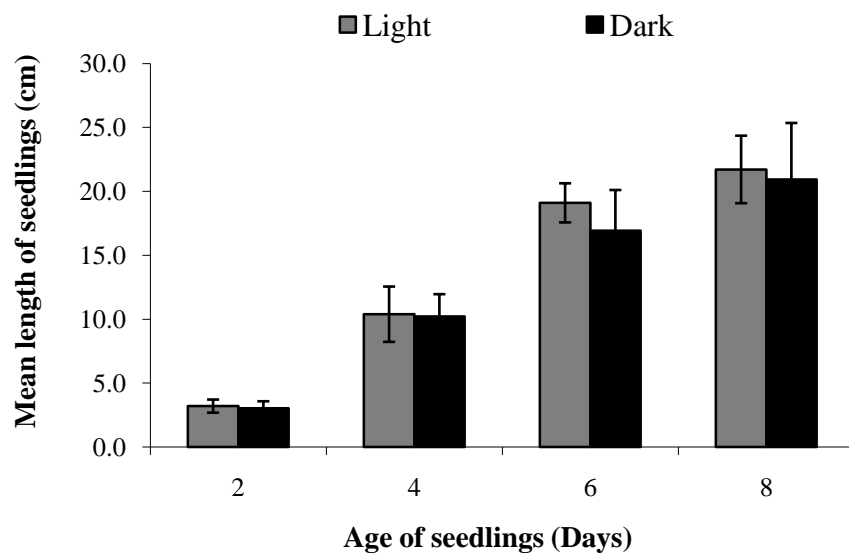
Figure 3.8. Summary of the growth experiment done for *Glycine max* cv. Williams 82 showing the average seedling length of two to eight – day old seedlings. allantoinase in both light and dark-3), bars indicate standard deviation of the mean

Table 3.2. Range of seedling lengths set for soybean seedlings based on growth experiment.

Age of Seedlings, (days)	Range of Accepted Seedling Length (cm)	
	Light-germinated seedlings	*Dark-germinated seedlings*
2	2 – 4	2 – 4
4	8 – 13	9 – 12
6	18 – 21	14 – 20
8	19 – 24	16 – 25

A – Axes, Light

B – Axes, Dark

C – Cotyledons, Light
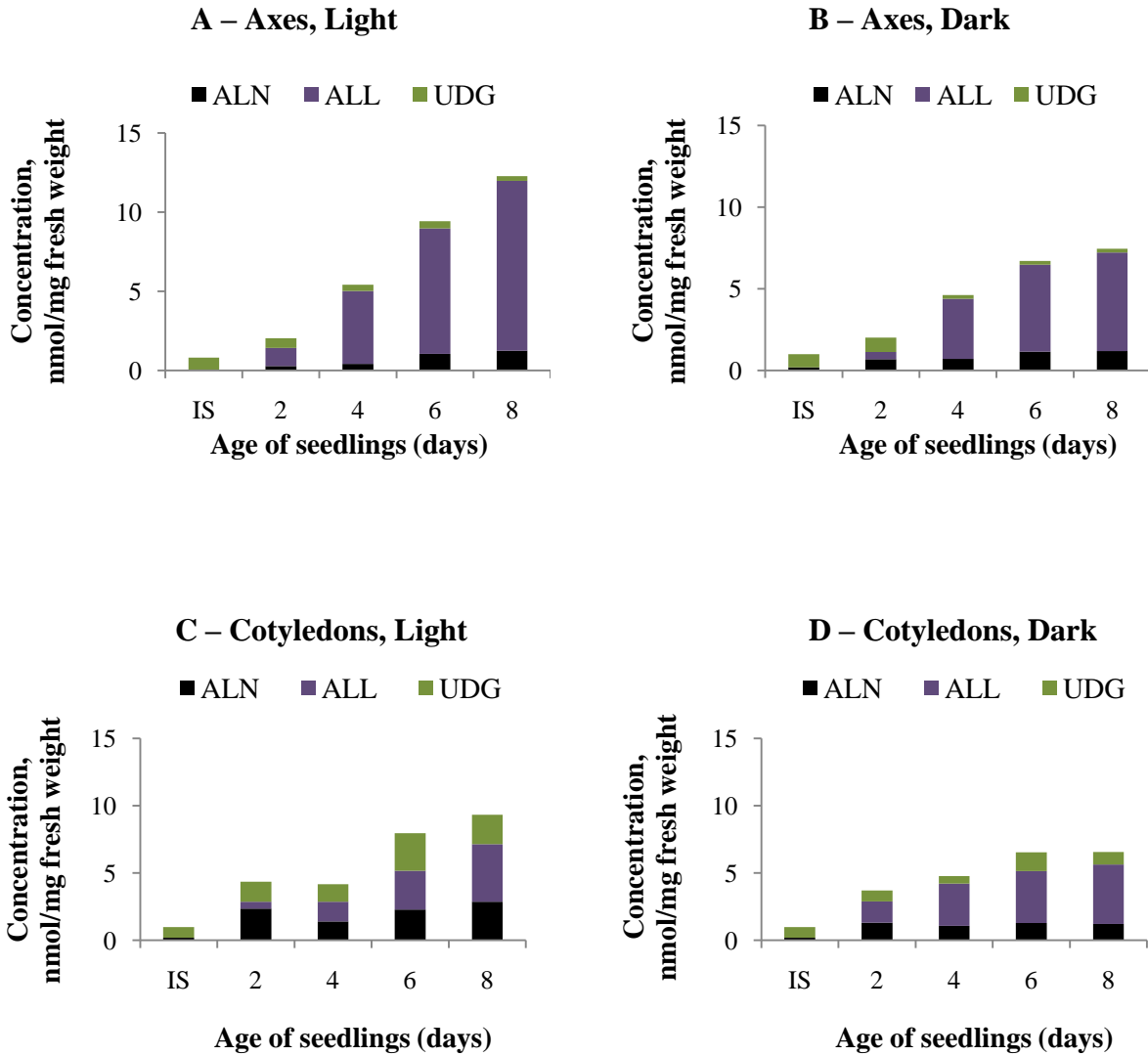
D – Cotyledons, Dark

Figure 3.9. Concentration of the ureides allantoin (ALN), allantoate (ALL) and ureidoglycolate (UDG) detected in the axes (A – light, B - dark) and cotyledons (C – light, D - dark) of light- and dark-germinated soybean seedlings. Axes and cotyledons for imbibed seed (IS) were not evaluated separately. Germination occurs on day 2. Data presented are the mean of three independent experiments (n=3). Refer to Appendix B for the complete actual values presented in this figure.
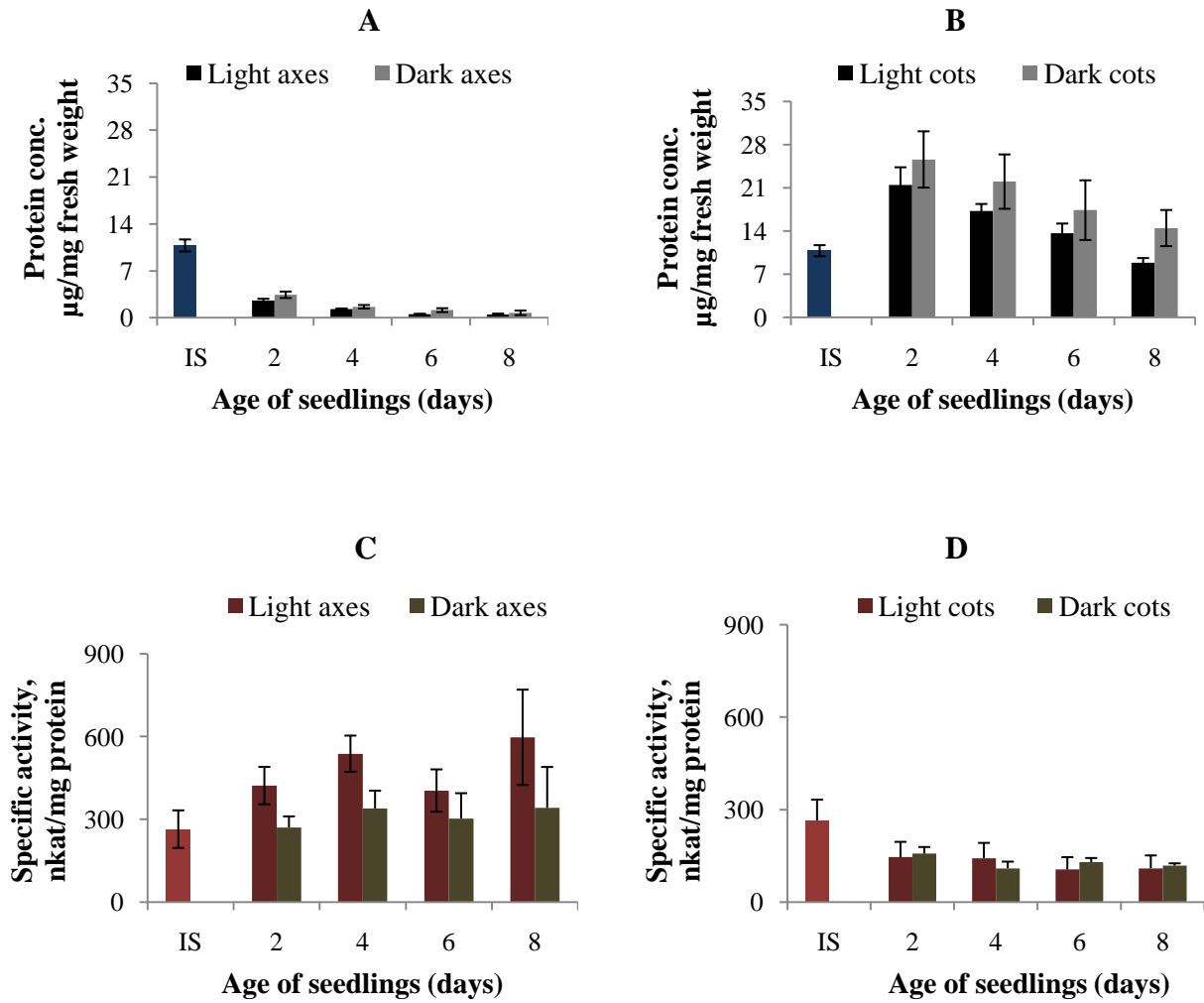
Figure 3.10. Protein content (A – axes, B – cotyledons) and specific activity of allantoinase (C – axes, D – cotyledons) measured in light and dark-germinated soybean seedlings sampled at different time points. Axes and cotyledons for IS (imbibed seed) were not evaluated separately. Data presented are the mean of three independent experiments (n=3). Bars represent standard error of the means

axes are degraded during germination, releasing amino acids which serve as precursors for protein synthesis as well as providing nitrogen for important macromolecules including ureides. Decreasing soluble protein concentrations in both the axes and cotyledons (Figures 3.10 A &B), suggest that it is likely being used for growth and development. It is apparent that the cotyledons have substantially more storage protein than the embryonic axes and this is likely reflected in the lower specific activity values for enzyme activity in the cotyledons (Figures 3.10 C and D). In this case, specific activity values may not accurately describe the enzyme activity of allantoinase in both seedling tissues as it is normalized against their protein content. The ratio of the allantoinase protein to other metabolically active proteins found in the embryonic axes is not likely to be the same as in cotyledons because the majority of proteins in cotyledons are known to be storage proteins. From another perspective, the cotyledons showed higher allantoinase activity than the seedling axes when activity was expressed as a function of tissue fresh weight. Both embryonic axes and cotyledons (Figures 3.11 A & B) showed a decreasing pattern in allantoinase activity as the seedlings grew and developed.

3.3.1.2. Transcript abundance

Apart from slightly higher enzyme activity and ureide content in light-germinated seedlings, no major difference was observed between seedlings germinated in the light and in the dark and the overall ureide content and enzyme activity patterns were similar (Figures 3.9, 3.10, 3.11). Therefore, gene expression patterns for soybean allantoinase genes were evaluated using light-germinated seedling samples only. The presence of at least one allantoinase transcript is expected, as allantoinase activity was noted in all the seedling tissues examined. All of the soybean allantoinase genes were detected using RT-PCR and qRT-PCR and were variably expressed in both the embryonic axes and cotyledons (Figures 3.12 A & B), with higher levels detected in the cotyledons. *GmALN1* and *2* were consistently expressed in abundance while *GmALN3* and *4* were always expressed at low levels relative to *GmALN1* and *2*. The relatively high *GmALN1* and *2* transcript levels in cotyledons likely reflects the active synthesis of allantoinase, illustrated in the increase or early allantoinase activity measured in the same tissue (Figure 3.11 B).

A – Axes
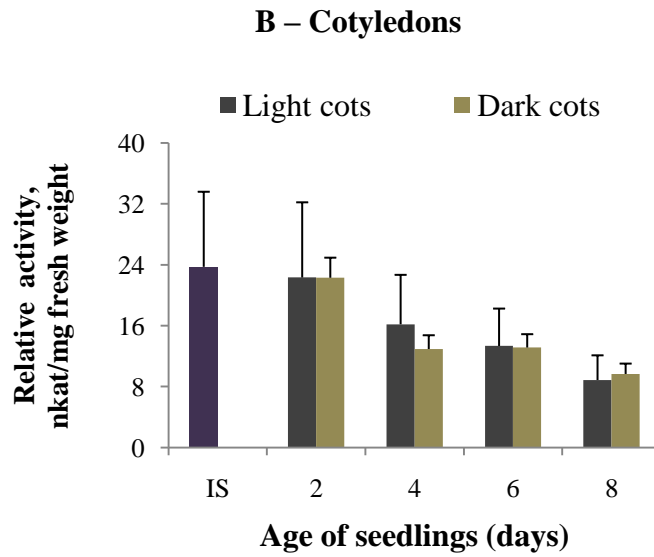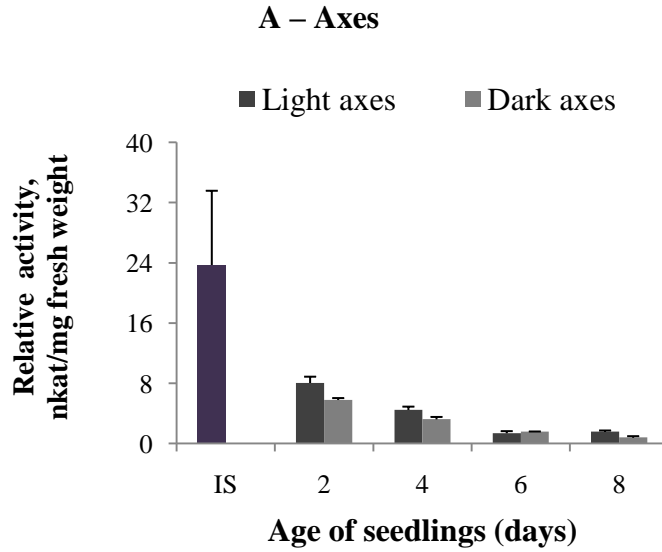
B – Cotyledons

Figure 3.11. Relative activity of allantoinase in axes (A) and cotyledons (B) of light and dark-germinated seedlings sampled at different time points. Axes and cotyledons for IS (imbibed seed) were not evaluated separately. Data presented are the mean of three independent experiments (n=3). Bars represent standard error of the means

A – Axes
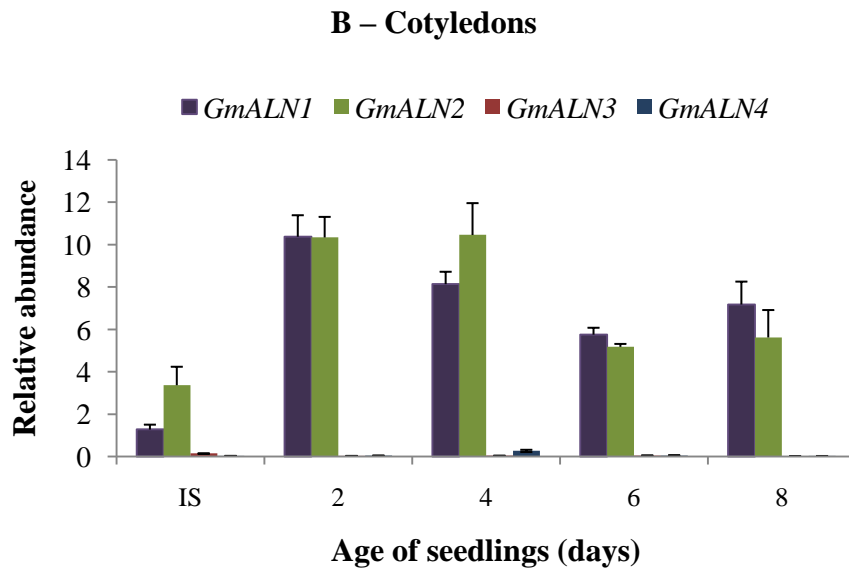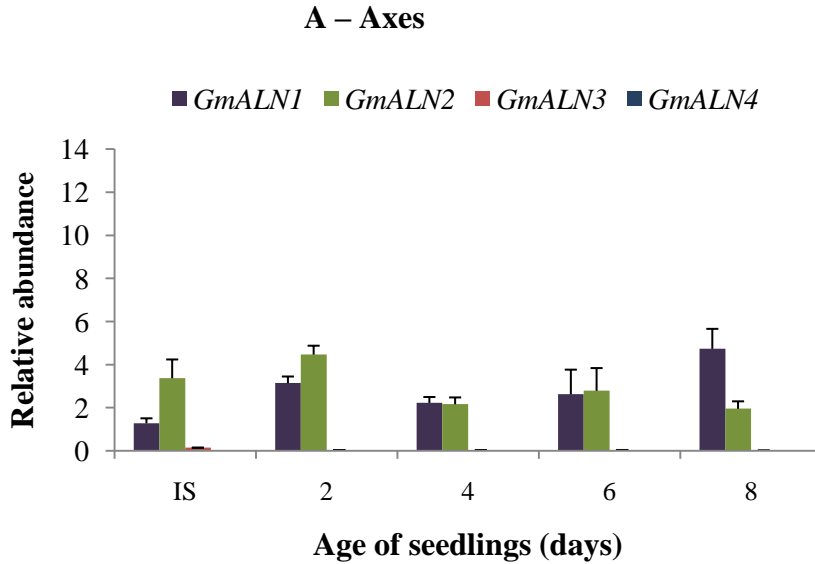
B – Cotyledons

Figure 3.12. Relative abundance of allantoinase transcripts in the axes and cotyledons of light-germinated seedlings. Relative abundance of *GmALN3* and *4* range from 0.01 – 0.27 in cotyledons (0.005 – 0.02 in axes), complete values shown in Appendix C. Values are normalized to the relative abundance of *GmALN1* for IS (Imbibed seed). Axes and cotyledons for IS were not evaluated separately. Data presented are the mean of three independent experiments (n=3). Bars represent standard error of the means.

3.3.2. Mature tissues of N$_2$-fixing and non-fixing soybean

3.3.2.1. Enzyme activity and ureide content

Patterns of allantoinase gene expression and enzyme activity were also evaluated in various mature V4-V5 tissues of N$_2$-fixing and non-fixing soybean. As expected, nodules of N$_2$-fixing plants showed the highest ureide concentration, approximately three times as much as the other tissues, with allantoate (75%) making up the bulk of the total ureides (Figure 3.13 A). Total ureides measured in the roots and leaves of N$_2$-fixing plants were relatively similar, but allantoin was the major ureide found in the leaves (50% of the total ureides in young leaf, 62% in mature leaf; Refer to Appendix D) and in root tissues, allantoate was most abundant (54%). In mature V4-V5 tissues of non-fixing plants (Figure 3.13 B), the concentration of ureides was less than the comparable sample from N$_2$-fixing tissues. One exception to this was the ureide content of the young leaf tissues (mean total ureides, non-fixing = 1.32 nmol/mg FW), which was closer to the amount detected in N$_2$-fixing young leaf (mean total ureides, N$_2$-fixing = 2.41 nmol/mg FW). T test analysis (P-value = 0.30) indeed showed no significant difference between the ureide content of the two young leaf samples.

Nodules were expected to have the highest allantoinase activity as they are known to be the site of ureide synthesis and allantoate is a major export product. Nodules were found to have the highest allantoinase activity and the highest ureide concentration among the various mature V4-V5 tissues examined, with allantoate as the major ureide present. Figure 3.14 A shows the level of allantoinase activity measured for both fixing and non-fixing plant tissues expressed as specific activity. In N$_2$-fixing plants, root tissues showed the highest allantoinase specific activity followed by nodules and young leaves. The amount of protein found in the root tissues was low relative to those found in the leaves and nodules (data not shown), giving it a higher specific activity value. Hence, like in seedlings, specific activity alone might not accurately describe allantoinase activity in these mature tissues. To see this in a different perspective, enzyme activity was then normalized using the fresh weight of plant tissues assayed (relative activity). When normalized against fresh weight, allantoinase activity was greatest in nodules and young leaf tissues of N$_2$-fixing plants (Figure 3.14 B).

A – N$_2$-fixing plants

B – Non-fixing plants
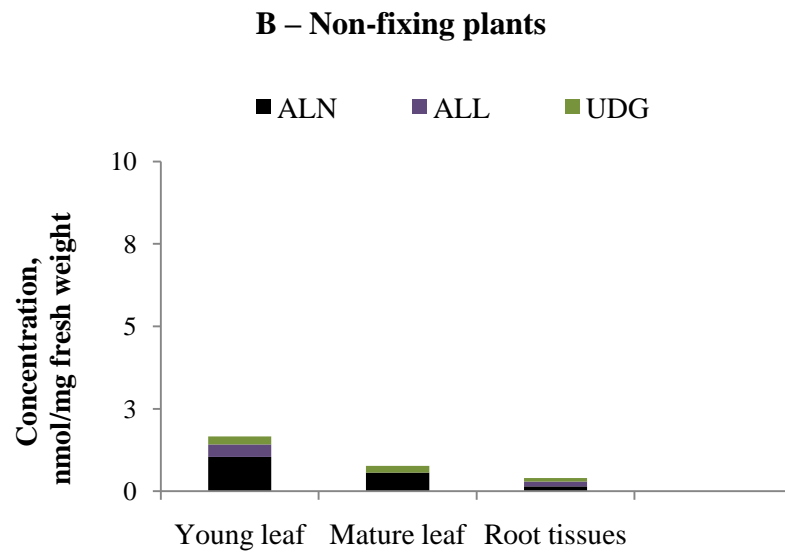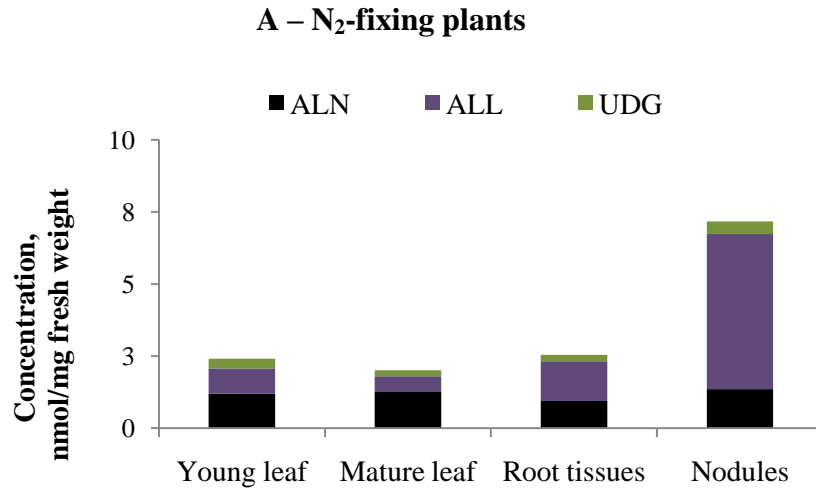
Figure 3.13. Concentration of the ureides allantoin (ALN), allantoate (ALL) and ureidoglycolate (UDG) detected in mature V4-V5 tissues of 45-day old N$_2$-fixing and non-fixing soybean. Data presented are the mean of three independent experiments (n=3).

A

B
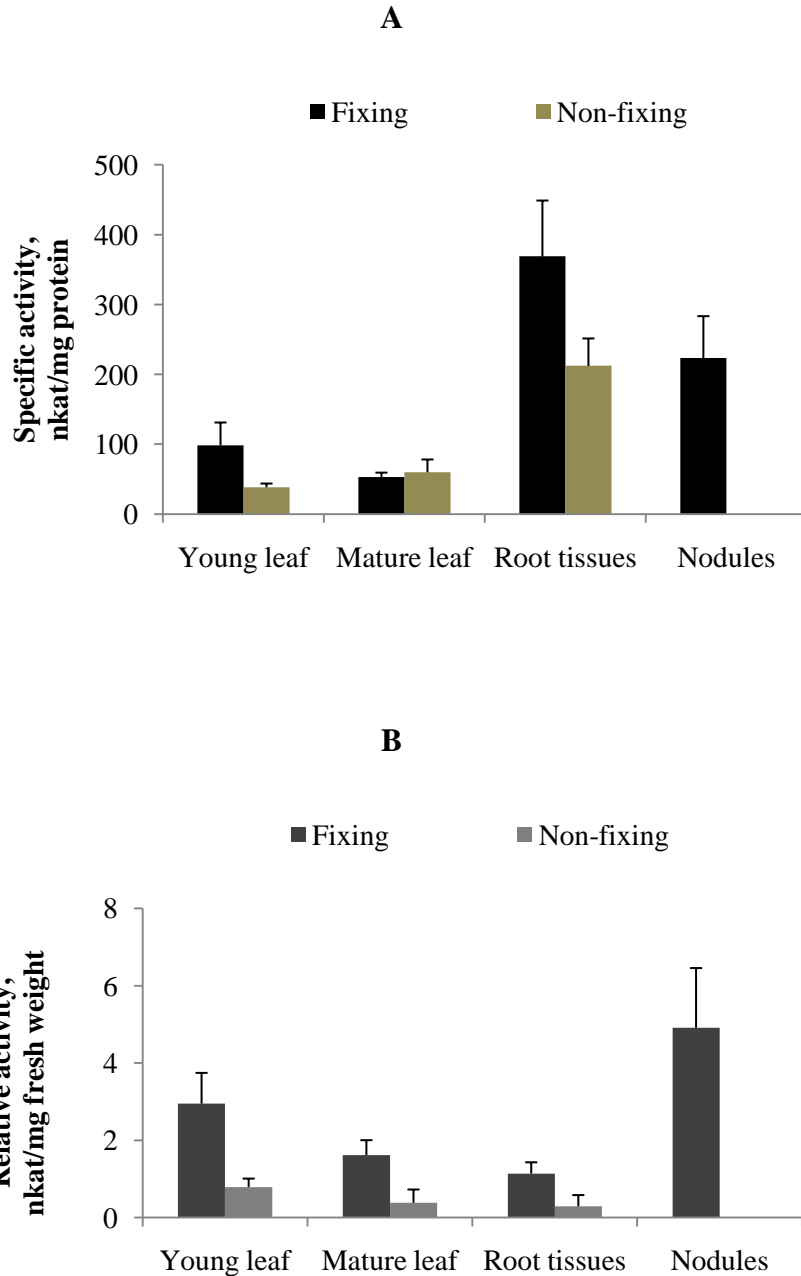
Figure 3.14. Allantoinase activity expressed as specific activity (A) and relative activity (B) measured in various mature V4-V5 tissues of 45-day old N_2-fixing and non-fixing soybean plants. Data presented are the mean of three independent experiments (n=3). Bars represent standard error of the means.

3.3.2.2. Transcript abundance

Allantoinase gene expression was determined using quantitative RT-PCR. All of the allantoinase genes were expressed in leaf and root tissues of both N_2-fixing and non-fixing plants. Like in seedlings, *GmALN1* and *GmALN2* were still consistently expressed at greater levels compared to *GmALN3* and *4* for both non-fixing and N_2-fixing tissues (Figures 3.15 A & B). The most striking observation is the transcript levels of *GmALN3* and *4* in nodules of N_2-fixing tissues. In all other tissues examined *GmALN3* and *4* consistently had low expression levels. Based on these data, I suggest that *GmALN3* and *4* play an important role in the synthesis of ureides and export of allantoate from root nodules. Meanwhile, allantoinase also likely plays an important role in non-fixing plants since small amounts of ureides were found in its various tissues (Figure 3.13 B) and both allantoinase activity (Figures 3.14 A & B) and allantoinase transcripts (Figure 3.15 B) showed gene expression in the leaf and root tissues. In this case, allantoinase may be functioning in the turnover and salvage of purine nucleotides.

3.3.3. Leaf tissues of drought-stressed plants treated with or without allantoin
3.3.3.1. Enzyme activity and ureide content

Nitrogen fixation is highly sensitive to drought (Sinclair and Serraj, 1995, Thavarajah and Ball, 2006), hypothesized to be affected by the build-up of ureides in the leaves of drought-sensitive cultivars which export ureides. As ureides are primarily produced as a result of nitrogen fixation in legumes, drought consequently affects ureide catabolism also. This experiment served as a preliminary study which aimed to understand the effect of drought on ureide catabolism, focusing specifically on its effects on allantoinase. In this study, the effect of ureide application and water limitation on allantoinase gene expression and activity was observed in the young leaves of N_2-fixing plants. Young leaves, which require more nitrogen to support their growth and development, are sink tissues and are known to be a major site of ureide catabolism. This study further validated that catabolism of ureides primarily occurred in the leaves of N_2-fixing soybean, in particular, younger leaves. In this experiment, four N_2-fixing

A – Fixing tissues
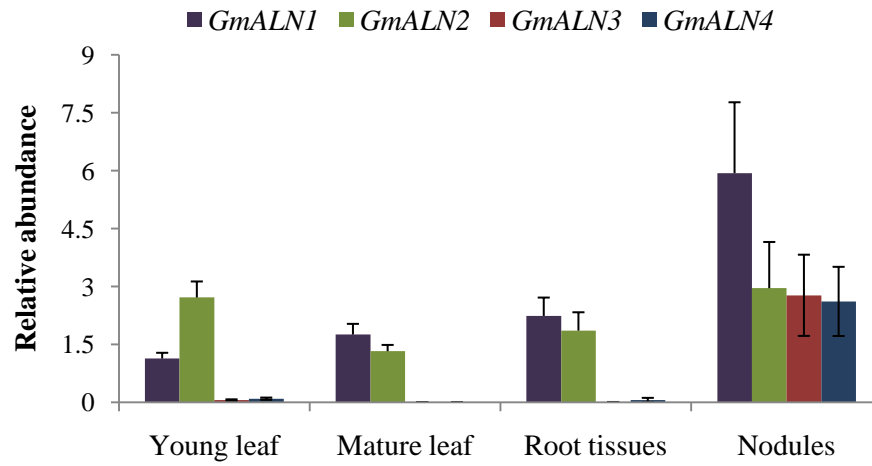
B – Non-fixing tissues
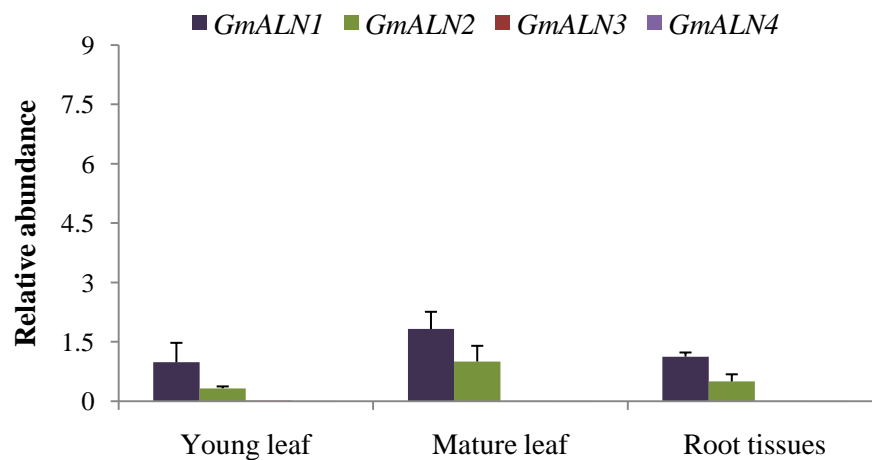
Figure 3.15. Relative abundance of allantoinase transcripts in various V4-V5 tissues of N_2-fixing and non-fixing plants. Relative abundance of *GmALN3* and *4* range from 0.002 – 0.1 in fixing tissues (0.001 – 0.02 in non-fixing tissues, Refer to Appendix D for actual values).Values are normalized to the relative abundance of *GmALN1* in N_2-fixing young leaf. Data presented are the mean of three independent experiments (n=3). Bars represent standard error of the means.

plants (35 days old) were treated with 25 M allantoin for a 10 day period (watering once every three days), the other four plants receiving a water control. After this period, stress was applied by withholding water from both treated and non-treated plants, while the control plants were regularly watered.

The data for the ureide content and enzyme activity are presented here on a per mg protein basis and not by fresh weight because the weight of the leaf samples decreased over time as it dried out, hence this was not a good parameter to normalize the data. Figure 3.16 shows the concentrations of total ureides detected in the young leaves of allantoin treated and non-treated N_2-fixing plants. Variable ureide concentrations were observed in allantoin treated plants and non-treated plants for both control and water-stressed plants at various time points evaluated. When compared to control plants, a decrease in allantoate concentration was observed for non-treated plants as well as the noticeable accumulation of ureidoglycolate (Figures 3.16 C & D). Meanwhile for allantoin treated plants, allantoate concentration seemed not to be affected when water-limiting conditions were applied at least up to Day 3 (Figure 3.16 D), possibly indicating that the allantoinase enzyme was still functional. This assumption was supported by higher enzyme activity data obtained for allantoin treated plants (Figure 3.17 A), conforming with the higher allantoate concentrations measured in these plants. Both the ureide content and enzyme activity data showed that water limiting conditions, at least up to 3 days, did not seem to affect allantoin treated and non-treated plants.

3.3.3.2. Transcript abundance

Expression of allantoinase genes precedes the formation of 4 active enzymes. In this experiment, all allantoinase genes were expressed with *GmALN1* and *2* still being consistently expressed at higher levels than *GmALN3* and *4*, as noted in previous experiments. Since the genes *GmALN3* and *4* have very low transcript levels, only *GmALN1* and *2* will be discussed when comparing gene expression levels for the different treatments used in this experiment. In all the treatments evaluated, transcripts for *GmALN1* were always found to be more abundant than *GmALN2* transcripts. Pre-treatment with allantoin prior to water limitation stress had no effect on the expression of allantoinase genes (Figures 3.18 A & C). Moreover, withholding water for 5 days did not seem to have an effect on transcript abundance levels of these two genes.
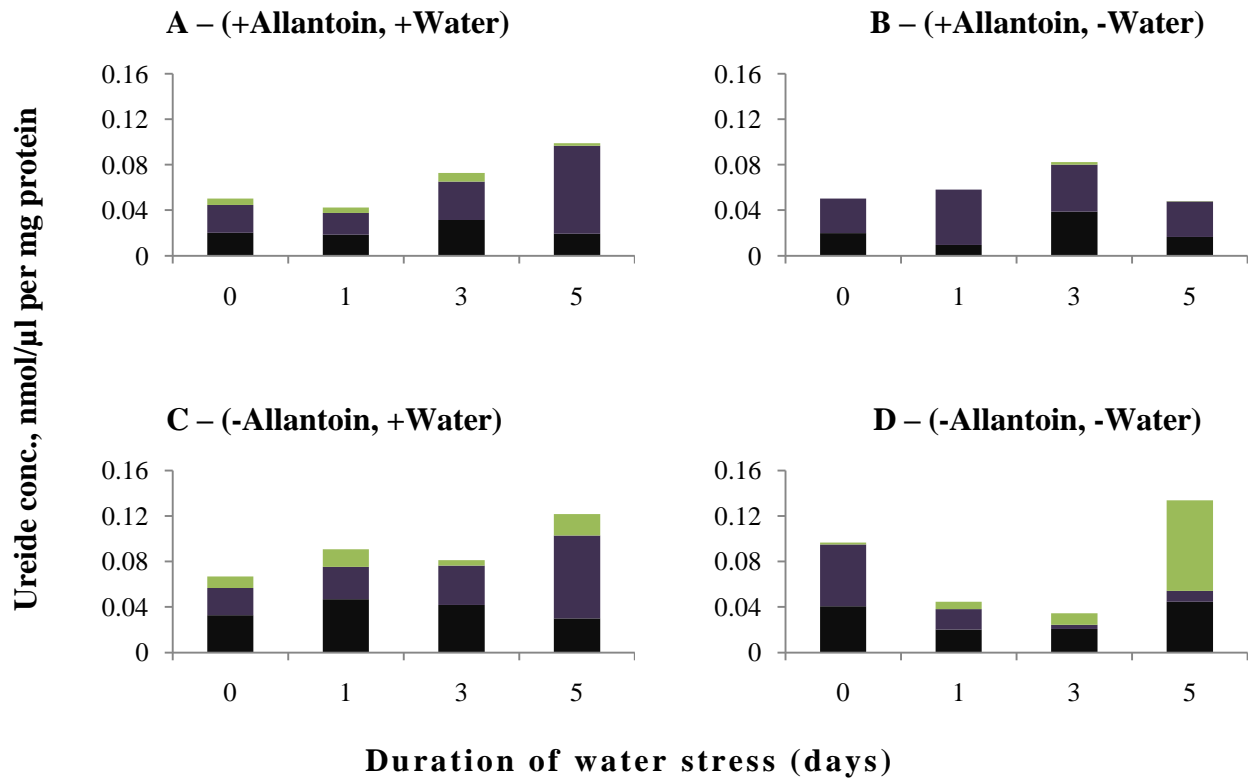
Figure 3.16. Total ureides per mg protein measured in young leaf tissues of 45-day old N_2-fixing soybean plants subjected to water limitation stress. Ureides measured include allantoin (black), allantoate (purple) and ureidoglycolate (green). Comparison was made between allantoin-treated (A & B) and non-treated plants (B & D). Data presented are the mean of three independent experiments (n=3).

A – Allantoin treated plants

B – Non-treated plants
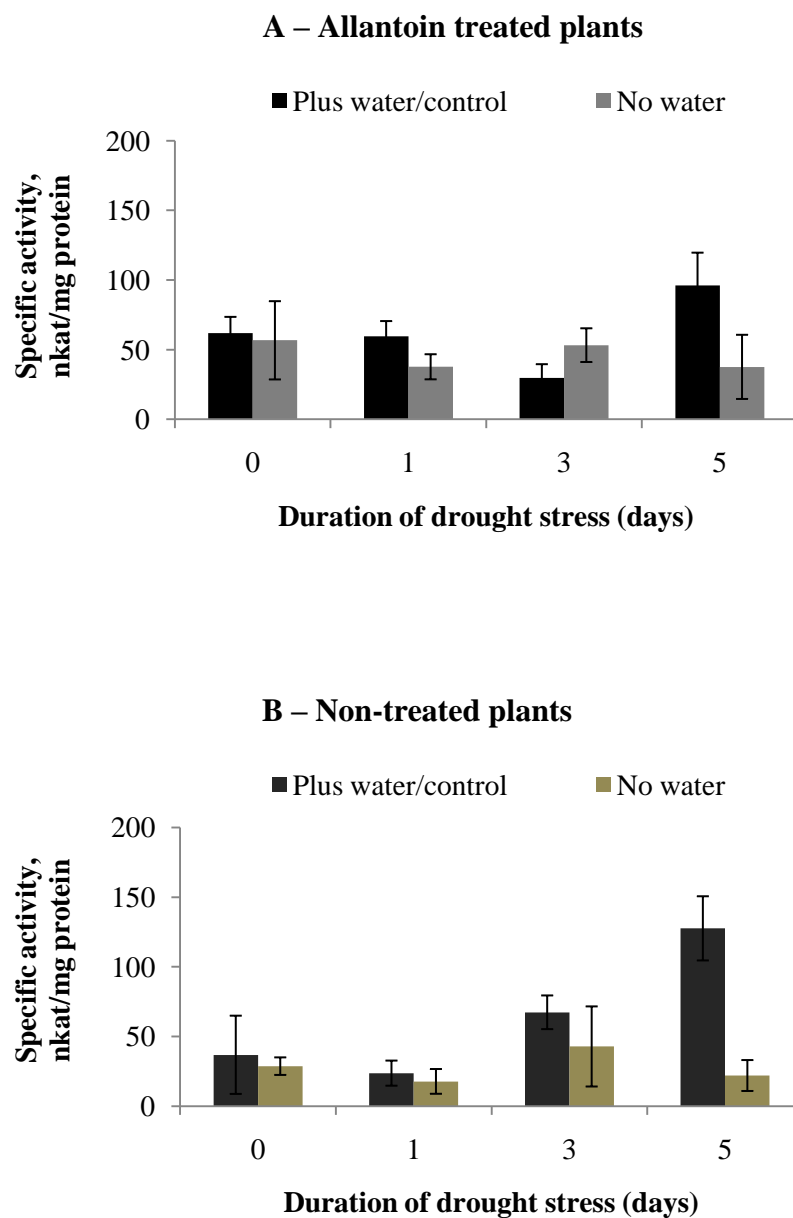
Figure 3.17. Effect of allantoin treatment on allantoinase activity (specific activity) in young leaves of 45-day old N_2-fixing soybean subjected to drought stress. Comparison was made between allantoin-treated (A) and non-treated plants (B). Data presented are the mean of three independent experiments (n=3). Bars represent standard error of the means
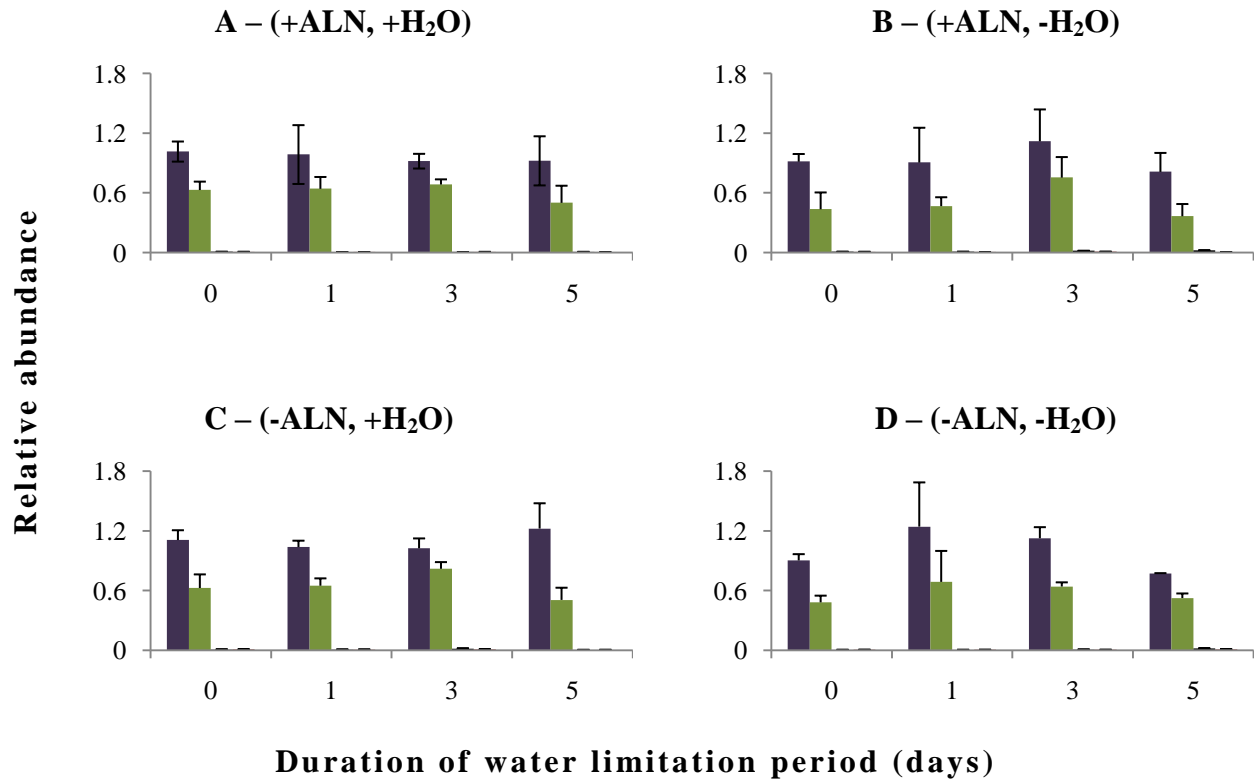
Figure 3.18. The effect of allantoin on the relative abundance of allantoinase transcripts in young leaves of 45-day old N_2-fixing soybean during water limitation period. Data are normalized to *GmALN1* at Day 0 value, for allantoin-treated plants (control). *GmALN1* (purple), *GmALN2* (green), *GmALN3 and 4* (low levels of transcripts were detected, See Appendix). Data presented are the mean of three independent experiments (n=3). Bars represent standard error of the means

The relative abundance of *GmALN1* and *2* showed that water limitation did not affect allantoinase gene expression of both allantoin treated and non-treated plants, at least up to Day 5. Ureide content and specific activity values however pointed out that the stress affected allantoinase activity of non-treated plants over time. In fact, there was a significant difference observed in the decline of allantoinase specific activity at Day 5 (t test, P-value = 0.042). Meanwhile in allantoin treated plants, there was no significant difference (t test, P-value = 0.18) in the observed decline in allantoinase activity. It is predicted that this decline would continue if water was withheld for a longer period.

CHAPTER 4. DISCUSSION

4.1. Role of allantoinase in ureide transport in legumes

Allantoinase is the only enzyme catalyzing the hydrolysis of allantoin to allantoate, the first step (Todd et al., 2006). Initial research on the ureide catabolic pathway in ureide legumes focused on the role of allantoinase, mostly in soybean (Hanks et al., 1981, Christensen and Jochimsen, 1983, Polayes and Schubert, 1984, Costigan et al., 1987). The presence of allantoinase and its role in the ureide transport in soybean was evaluated in this study by examining transcript abundance, enzyme activity and also the ureide composition in both young and mature V4-V5 tissues. This is the first study to look at multiple allantoinase genes and the first to look at gene expression in soybean.

The presence of allantoate in different N_2-fixing and non-fixing V4-V5 tissues is an indication of the roles that allantoinase plays in the transport of ureides in mature soybean. A previous study showed allantoate to be the predominant ureide in both nodulated (N_2-fixing) and non-nodulated (non-fixing) soybean plants (Rice et al., 1990). In this study, allantoate was also found to be the dominant ureide in the nodules of N_2-fixing plants (Figure 3.13 A) which is consistent with its role in N export. It was expected that allantoate (and ureides in general) would be most abundant in nodules as ureide biogenesis occurs mostly from purines synthesized *de novo* (Schubert, 1986), occurs in these specialized root structures. The purine salvage pathway may have also contributed to this ureide pool detected in nodules as well as in the other plant tissues examined in this study. All organisms utilize salvage pathways in retrieving purine rings after nucleic acid or coenzyme breakdown, and recycle nucleotides to meet the day-to-day needs of a particular cell (Smith and Atkins, 2002).

Soybean can export more than 80% of N compounds out of the nodules in the form of ureides (Serraj et al., 2001). Most of these ureides are distributed to various sink tissues, first passing through the root xylem. Indeed, ureides (mostly allantoate) were detected in root tissues (Figure 3.13 A) of N_2-fixing plants. Based on the abundance of allantoate in the nodules and root tissues, it could be inferred that allantoinase activity plays an important role in the generation of

allantoate for transport from the nodules to the leaves and other sink tissues. Meanwhile, small amounts of ureides detected in non-fixing tissues could represent products of purine recycling or could be ureides synthesized for transport.

Although degradation of ureides is known to occur rapidly in the leaves, it has been reported that significant levels of allantoin and allantoate are often present in these tissues (Rice et al., 1990). These findings were confirmed in this study where the ureides allantoin and allantoate were found to be present in both young and mature leaves of N_2-fixing tissues (Figure 3.13 A). Total ureides in young leaves were found to be relatively higher than in mature leaves but the difference observed was not significant (T test, P-value = 0.74). More allantoin was found in mature leaves (1.26 nmol/mg FW) than in young leaves (1.20 nmol/mg FW, 50%) however, this difference was also not significant (T test, P-value = 0.91).Younger leaves have higher allantoate (0.87 nmol/mg FW) than in mature leaves (0.54 nmol/mg FW) but the difference in the observed values is also not significant (T test, P-value = 0.75).

Though no significant difference was observed between the total ureides (and allantoin or allantoate alone) measured in young and mature leaves, the observed variation in allantoin and allantoate concentrations in the two leaf types might be due to differences in the rate of ureide catabolism. Higher allantoate levels in young leaves (and higher allantoin in mature leaves) imply higher allantoinase activity compared in mature leaves. Ureide catabolic activity is expected to be higher in the younger leaves as they have higher nitrogen requirements to support growth and development, through cell division and expansion. Moreover, young leaves require significant nitrogen input for the synthesis of Rubisco and other chloroplast proteins as well as for the synthesis of chlorophyll required for these tissues to become photosynthetically competent (Kaschuk et al., 2010). Alternately, the rate of ureide delivery to the leaves might account for differences in allantoate levels in the older and younger leaf tissues. Variations in the type of ureide (allantoin or allantoate) dominating in the leaves could also be due to differences in leaf age as well as plant development and nutritional condition (Costigan et al., 1987).

Previous work in soybean showed that allantoin content in roots and stems of nodulating plants increased significantly during growth and reached a maximum level at the fruiting stage before decreasing (Matsumoto et al., 1977). Meanwhile, leaves were found to have more allantoin as they matured. This was also confirmed in my study where more allantoin was found in mature leaves (62% of the total ureides) than in younger leaves (50% of the total ureides). As

ureide content analysis was only limited to V6-V5 stage, it was not verified whether allantoin content increased in the roots and stems (not evaluated) as the plant advanced to later stages of its development. Allantoate was found to be the dominant ureide in roots (1.37 nmol/ mg fresh weight; 54% of total ureides) and nodules (5.38 nmol/ mg fresh weight; 75% of total ureides) of N_2-fixing soybean plants. Meanwhile in the ureide legume Jack bean (*Canavalia ensiformis*), allantoin dominated in leaves, stems and roots tissues during the flowering stage, having a concentration of 1.59, 1.54 and 1.09 nmol/ mg fresh weight, respectively (Camargos et al., 2009). However, allantoic acid was the dominant ureide during fruiting except in leaves, with concentrations of 1.56 and 1.61 nmol/ mg fresh weight for the stems and roots, respectively.

Ureides are also present in legume seedlings as observed by Quiles et al. (2008) in common bean, with higher amounts detected in the embryonic axes. Allantoate was found to be the major ureide present in both embryonic axes and cotyledons. Allantoate was also found to be the dominant ureide in soybean seedlings, more obviously in the embryonic axes at days 2 to 8 (Figures 3.9 A & B). Though more allantoate was measured in the axes, allantoinase may play a more important role in cotyledons as implied by the higher relative activity in these tissues (Figures 3.11 A & B). The production of ureides in cotyledons is thought to play a role in the export of nitrogenous resources to support seedling growth (Bell and Webb, 1995, Webb and Lindell, 1993) and clearly, allantoinase is involved in this process. Thus, most of the allantoate detected in the embryonic axes likely came from the cotyledons and was most likely produced via *de novo* purine synthesis and nucleotide breakdown as observed in an earlier study in soybean seedlings (Polayes and Schubert, 1984).

All of these results confirmed that soybean uses ureides, commonly allantoate, in facilitating nitrogen transport. In mature tissues for instance, most of the ureide measured in nodules and roots of N_2-fixing plants was allantoate (Figure 3.13 A), implying that this was the type of ureide used in nitrogen transport. Allantoate was also the major ureide used by cotyledons to remobilize nitrogen reserves to the developing embryonic axes (Figure 3.9). Moreover, it was also found that allantoate was commonly used to distribute nitrogen within the developing axes, to support its growth and development. Based on these data, it could be summarized that allantoinase is important to the growth and development of both young and mature soybean plants because it not only played a major role in the transport of ureides across

different plant tissues, but also in the remobilization of nitrogen reserves from source to sink tissues.

4.2. *GmALN1* and *2* are ubiquitously expressed in soybean

Only a few studies have looked at transcript and protein expression patterns of allantoinase as a tool for validating enzyme activity and ureide content data in plants and none in soybean despite its economic importance and ample background research. In 2004, Yang and Han reported allantoinase gene expression patterns together with enzyme activity in *Arabidopsis thaliana* and *Robinia pseudoacacia*, both non-ureide plants. Raso *et al.* (2007) isolated two proteins with allantoinase activity in the ureide legume *Phaseolus vulgaris* and investigated the abundance of these proteins in different plant tissues alongside allantoinase activity and ureide content levels. A major goal of my study was to integrate gene expression patterns of the four putative allantoinase genes in soybean with ureide content and enzyme activity data to provide insight into the regulation of this process.

GmALN1 and *2* were first identified and their predicted protein sequences showed sequence identity with several known allantoinases and highest sequence similarity to the legume allantoinase *RpALN*, the *Robinia* allantoinase (Table 3.1). The presence of the two conserved protein domains (Figures 3.1, 3.2) as well as the active site residues crucial for enzyme activity (Figure 3.4) and its successful functional complementation of the *Saccharomyces cerevisiae dal1* mutant (Figures 3.6, 3.7) provided further evidence that these genes code for this ureide catabolic enzyme. Quantitative RT-PCR experiments revealed consistently high levels of *GmALN1* and *2* transcripts in both seedlings (Figures 3.12 A & B) and mature V4-V5 tissues (Figures 3.15 A & B) of soybean. The highest transcript levels seen were in V4-V5 tissues of N_2-fixing plants particularly in the nodules (Figures 3.15 A). Though protein abundance patterns were not evaluated in this study, results of enzyme activity (Figures 3.14 A & B) and ureide content (Figures 3.13 A & B) analyses implied that *GmALN1* and *2* were the genes that contribute the most to the production of allantoinases except in nodules (Figures 3.12 B, 3.15 B).

In seedlings, a variable pattern for *GmALN1* and *2* transcript levels (Figures 3.12 A & B) was observed as seedlings developed. These two genes were most active in cotyledons, particularly in two- and four-day old seedlings. Early synthesis of allantoinase at these times will lead to the production of allantoate in the presence of allantoin. However, allantoate

accumulation was observed in embryonic axes and not in the cotyledons (Figures 3.9 A & B). I conclude that most of the allantoate produced in the cotyledons from allantoinase activity was remobilized to the axes resulting in the gradual increase in allantoate levels in the axes. The observed increase in transcripts from seed to 4 day-old seedlings (for both axes and cotyledons) suggests allantoinase proteins were actively synthesized during this period to provide N from allantoin for seedling growth but needs to be confirmed to be by Western blot. Declining allantoinase activity (Figures 3.11 A & B) means a decrease in the production of allantoin in seedlings as the seedlings matured, and suggests that allantoinase and ureides are primarily important in the early stages of post-germination growth. Based on the gene expression data, it appears that *GmALN1* and *2* are involved in post-germination nitrogen assimilation during early seedling growth. Since allantoinase may also play a role in the recovery of nitrogen from purines in senescing tissues, it is possible that as the cotyledons senesce after development of true leaves, allantoinase activity and ureide catabolism might resume.

Even though *GmALN1* and *2* were found to be expressed in all soybean tissues of soybean, the role that these proteins play in a given tissue likely depends on the fate of its degradation product, allantoate. For instance, its primary role in source tissues like cotyledons and nodules seems to be in redistributing nitrogen in other plant tissues in the form allantoate (allantoic acid). Meanwhile, in sink tissues like leaves, allantoinase plays a crucial role in providing nitrogen by catabolizing allantoin delivered to these tissues

4.3. *GmALN3* and *4* play a specific role in nodules

Few studies focused on the identification of allantoinase genes and the determination of their gene expression patterns have been done in plants. Only one gene was identified to encode an allantoinase enzyme in the non-ureide plants *Robinia* and *Arabidopsis* (Yang and Han, 2004). Meanwhile, two protein isoforms with allantoinase activity have been identified in *Phaseolus vulgaris* (Raso et al., 2007). The authors however hypothesized that these two protein isoforms resulted from alternate splicing of the transcript from a single gene. Meanwhile in soybean, previous studies also identified two protein isoforms with allantoinase activity, one found in abundance in seeds and the other in nodules (Bell and Webb, 1995). As the existence of four allantoinase genes has been verified in this study, it is very likely that the two allantoinase proteins previously identified in soybean could be encoded by different allantoinase genes.

Likewise, the two allantoinase protein isoforms in common bean described by Raso et al. (2007) could represent more than one gene.

The release of the soybean genome (Schmutz et al., 2008) paved way for the identification of two additional allantoinase genes. The divergence of the four allantoinase genes into two groups was believed to be a product of processes like tandem duplication and whole genome duplication. Results of my gene expression studies suggested an exclusive function for *GmALN3* and *4*. These genes were expressed in all of the soybean plant tissues evaluated but consistently at very low transcript levels. Interestingly, among all the samples evaluated, the expression of these two genes was up-regulated only in the nodules of N_2-fixing plants and in fact, the transcript levels noted were comparable with that of *GmALN2* (Figure 3.15 A).

Though this study was limited to the V4-V5 stage of development in soybean, it is possible that the role of these two allantoinase genes is solely for the synthesis of ureides in nodules for export. Hanks et al. (1981) determined that uricase and allantoinase, enzymes involved in ureide biogenesis, were predominant in the uninfected cell fraction of the nodule. Schubert (1986) suggested that uninfected cells played an important role in ureide biogenesis. Hence, *GmALN3* and *4* were most likely expressed in uninfected cells of nodules as ureides are synthesized in these particular cell types. I hypothesize that the up-regulation of *GmALN3* and *4* in the nodules brings about the synthesis of another functional protein which facilitates allantoin hydrolysis, producing more allantoate for transport. Though very similar, it is tempting to speculate that the amino acid sequence differences in the two soybean allantoinase pairs (GmALN1/2 vs. GmALN3/4) may reflect modifications to be more suited to the unique environment the nodules possess.

Sequence similarity, chromosome orientation and gene expression patterns of *GmALN3* and *4* lead us to hypothesize that tandem duplication occurred in the soybean ancestral allantoinase gene, resulting in two copies of allantoinase on what was to become soybean chromosomes 13 and 15. Over time these two genes accumulated mutations. Mutations in a duplicated gene may result in the formation of a novel gene (neo-functionalization), a pseudogene (non-functionalization) (Monson, 2003) or functional divergence (Ha et al., 2009). The divergence of *GmALN1* and *2* and of *GmALN3* and *4* therefore would represent sequence divergence since the duplication event. In soybean, it appears that gene mutations resulted in a divergence of function, with one of these genes apparently becoming restricted to the nodules.

Subsequently, at least two rounds of polyploidy in soybean (Kim et al., 2009b) resulted in a tetraploid genome, resulting in two abundantly expressed copies (*GmALN1* and *2*) and two nodule-specialized (*GmALN3* and *4*) copies. Hence, duplication, gene mutation and polyploidy in soybean are all contributing events which shaped the role that allantoinase genes play today.

4.4. Regulation of allantoinase

Allantoinase (encoded by *GmALN1* and/or *2*) is present in almost all tissues in soybean. Its presence, however, does not signify the release of ammonia. The hydrolysis of allantoin to allantoate marks the start of ureide catabolism and is followed by a series of enzyme-catalyzed steps that ultimately results in the release of NH_3 for re-assimilation, usually in the leaves. Based on the current findings, it could be presumed that the role of allantoinase in plants is regulated by nitrogen fixation, type of plant tissue (source or sink) and age of the plant tissue.

Ureides are produced mostly in nodules either via the salvage pathway or through *de novo* purine synthesis (Bell and Webb, 1995, Schubert, 1986) hence often thought of as exclusive products of nitrogen fixation. It was hypothesized that the amount of ureides in plants could serve to quantify the dependence of a particular plant on symbiosis (Quiles et al., 2009). On such a basis, it was expected that allantoinase would have a greater role in N_2-fixing tissues. This was in fact already observed in an earlier study where ureides were found to be the major agents of xylary nitrogen transport in N_2-fixing soybean (McClure and Israel, 1979a, McClure and Israel, 1979b). The total ureide concentration measured in the xylem of N_2-fixing soybean was five times higher compared to non-fixing plants (Rice et al., 1990). The current study did not evaluate the ureide content in the xylem sap of N_2-fixing soybean however results confirmed the presence of more ureides in mature V4-V5 tissues, particularly in nodules. Ureide concentration in nodules (7.17 nmol/mg fresh weight) of N_2-fixing plants was found to be four times higher than that in young leaves (1.66 nmol/mg fresh weight; highest concentration) of non-fixing plants (Figures 3.13 B).

The presence of ureides in non-nodulated plants contradicts the idea that ureides are mostly associated with nitrogen fixation (Camargos et al., 2009). The ureides detected in non-fixing tissues most probably were products of the purine salvage pathway, a process allowing utilization of preformed purine bases and nucleosides arising from intercellular breakdown of unstable RNA and nucleotides (Stasolla et al., 2003). Nevertheless, allantoinase may not have a

broad role in these plants, as illustrated by the very low ureide levels measured in leaves and root tissues (Figure 3.13 B).

Depending on the type of tissue (source or sink) where it is actively expressed, allantoinase could either initiate a series of events leading to the release of ammonia or be responsible for producing the N-transport molecule allantoic acid. If present in source tissues like the nodules of N_2-fixing plants, allantoinase is expressed for producing allantoate for transport. This was evident in the high allantoinase activity (Figure 3.14 B) and allantoate levels (Figure 3.13 A) observed in nodules of N_2-fixing plants and the allantoate concentration in roots. Meanwhile, sink tissues like the leaves require nitrogen for growth and development of cells and are sites of high ureide catabolic activity. In these tissues, the role of allantoinase is mostly for addressing that demand and that is to provide nitrogen in the form of NH_3. An earlier study by Polayes and Schubert (1984) suggested that ureides are not produced in the leaves of young soybean seedlings due to the lack of uricase activity in these tissues. Hence, ureides measured in the leaves were assumed to all come from the nodules. A variation in the type of dominant ureide in the two leaf types was observed in this study. Lesser allantoin in young leaves (but not significantly different from mature leaves) of N_2-fixing plants implied higher allantoinase activity or lower rates of ureide delivery. Given that young leaves are initially carbon and nitrogen sinks, the lower allantoin levels observed is more consistent with allantoin breakdown. This was expected because young leaves have higher nitrogen requirement compared to mature leaves and other tissues. Hence when present in the leaves, allantoinase activity will be higher in younger leaves.

In soybean seedlings, allantoinase seemed to play a major role in the remobilization of nitrogen sources from cotyledons to the embryonic axes, as seen in the accumulation of allantoate in the axes (Figures 3.9 A & B). However, the axes were shown to be capable of producing ureides as observed by Quiles *et al.* (2009), wherein the addition of allopurinol, a xanthine dehydrogenase inhibitor, decreased the amount of ureides measured in the axes of common bean seedlings. Moreover, allantoinase activity also increased even after the removal of cotyledons from the axes. Their results also showed an increase in allantoinase activity in the axes. Allantoinase activity was also observed in the embryonic axes of soybean though the relative activity values were very low compared to those in cotyledons (Figures 3.11 A & B). Meanwhile, allantoinase activity in both axes and cotyledons decreased as the seedlings grew,

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implying lesser dependence on ureides. As *de novo* ureide synthesis is a very complicated and expensive process involving 20 different enzymes (Smith and Atkins, 2002), it would likely be beneficial for the growing embryo to channel its resources to maintaining its other metabolic processes.

4.5. Role of ureides in soybean during water limiting conditions

Nitrogen fixation activity of soybean nodules has been shown to be especially sensitive to soil dehydration (Sall and Sinclair, 1991). As nitrogen fixation provides the precursor molecule for the synthesis of ureides, drought consequently affects ureide transport and catabolism. Accumulated ureides in leaves or petioles are hypothesized to act as a systemic signal causing the inhibition of nitrogen fixation in the nodules. In this study, I examined the effect of water limitation on the ureide catabolic pathway by looking into changes in ureide content, allantoinase activity and transcript levels of N_2-fixing young leaves. The effect of ureide application on ureide degradation capacity during conditions when water was limiting, in particular its effect on allantoinase activity, was also evaluated. This was performed to address previous suggestions that feedback from leaf N influenced N_2 fixation. This evaluation was however limited only to young leaf tissues of vegetative N_2-fixing plants, which are known to be the major sites of active ureide degradation.

An increase in ureide levels in the leaf blades and nodules of soybean was observed during drought stress (Serraj et al., 1999a). Meanwhile, Ladrera et al. (2007) observed no ureide accumulation in both drought-sensitive (cv. Biloxi) and drought-tolerant (cv. Jackson) soybean though nitrogen fixation was already inhibited as a consequence of drought. Based on their findings, it was inferred that ureides were not responsible for inhibiting nitrogen fixation, though they might have a role during advanced drought stages (Ladrera et al., 2007). A recent study showed ureide accumulation in the roots, shoots and leaves of N_2-fixing *Phaseolus vulgaris* plants exposed to drought stress (Alamillo et al., 2010). However, this accumulation was only observed 8 days after the stress was initiated. In my study, ureides (ureidoglycolate) were observed to accumulate in water-stressed non-treated plants by Day 5 (Figures 3.16 C & D). The build-up of ureidoglycolate hinted that ureide catabolic regulation was not at the level of allantoinase, but possibly with ureidoglycolate amidohydrolase. Meanwhile, ureides did not accumulate in allantoin-treated plants during the 5-day water-limiting period. As the ureide

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levels obtained were variable among biological replicates (see Appendices F.1, F.2 and F.3 for values of individual ureides), a follow-up study is recommended to validate these findings. Possible reason for variable ureide concentrations could be attributed to the fluctuating or unequal environmental conditions in the growth chamber, which could have led to various state of water stress to soybean plants used in the study.

Decreased allantoinase activity could mean decreased synthesis of functional proteins as a consequence of decreased nitrogen fixation activity, meaning a decrease in the synthesis of ureides. No obvious decrease (or increase) was noted in allantoinase transcript levels (Figures 3.18 B & D) and specific activity values of non-treated water-stressed plants (Figure 3.17 B), except a slight decline in enzyme activity at Day 5. The same trend was observed in allantoin treated plants. The observed decrease in specific activity of allantoinase is significant in non-treated plants (T test, P-value = 0.042) but not in allantoin treated plants (T test, P-value = 0.18). The apparent build-up of ureidoglycolate probably implied that ureide catabolism was affected by water limitation in non-treated plants by Day 5 (Figures 3.16 B & D). Transcript abundance, enzyme activity and ureide content data (Figures 3.18, 3.17 & 3.16) all implied that allantoinase activity was post-transcriptionally regulated.

In studies where the ureide levels of drought-tolerant and drought-sensitive cultivars were compared, it was shown that the presence of more ureides correlated with greater sensitivity to drought stress. Under well-watered conditions, the drought-tolerant soybean cultivar 'Jackson' was shown to have lower ureide concentrations in its petioles compared to the drought-sensitive cultivar Biloxi (Serraj and Sinclair, 1996a). The accumulation of ureides during drought was also lesser for cv. Jackson. These results were also observed in another study (Ladrera et al., 2007) where ureide content measured in the petioles of the drought-sensitive cultivar Biloxi was higher compared to the drought-tolerant cultivar Jackson. The lower ureide content in Jackson was also observed in conditions without drought. These studies implied that the amount of ureides in petioles and leaves are correlated with the sensitivity of a plant to the effects of drought. In my study, I hypothesize that the addition of allantoin to N_2-fixing plants would cause a change in their sensitivity to water limitation stress. It could be said that prior to the application of stress, non-treated plants have more ureides in their leaves than allantoin treated plants (Figures 3.16 A & C), hence based on the findings of the previous studies, have increased sensitivity to water limitation stress.

Allantoin has also been touted as a protectant from reactive oxygen species (ROS). The addition of allantoin and allantoate to the leaves of the *xdh1 Arabidopsis* mutant (which lacks a functional xanthine dehydrogenase gene) significantly alleviated chlorophyll degradation brought about by dark-induced senescence, possibly by scavenging excess ROS (Brychkova et al., 2008). Production of ROS was indeed observed to be rapidly up-regulated in the leaves and roots of water-stressed *Arabidopsis thaliana* plants (Yesbergenova et al., 2005). Applied ureides will be directly targeted to the leaves and are not likely to accumulate in the nodules (Ladrera et al., 2007). Based on the current results, application of allantoin resulted in higher allantoate levels, which was more or less maintained at least up to Day 3. This was supported by the high specific activity values obtained for allantoinase compared to those of non-treated plants (Figure 3.17 A & B). Based on its proposed role as a ROS scavenger, these leaf ureides might have slowed down the decrease in ureide catabolic capacity in the leaves, hence prolonging the survival of the plant.

Current findings supported previous results as to how water limitation affected ureide catabolism in the leaves of soybean. It is however apparent that ureide and allantoinase levels in the nodules also have to be examined to be able to uncover the mechanism of ureide catabolism and nitrogen fixation inhibition in response to water limitation. In the meantime, it could be inferred from the results that leaf ureides may not be primarily responsible for the inhibition of nitrogen fixation in the nodules during early water-limiting conditions. Moreover, application of ureides prior to water limitation stress resulted to prolonged allantoinase activity and continued ureide catabolic activity in the leaves. Additionally, a longer period of water limitation, and/or a less severe water stress might shed new light onto this process.

4.6. Implications for non-ureide transporting plants

The presence of ureides in plants requires the presence of ureide catabolic enzymes such as allantoinase. Ureides were thought to be produced mostly through nitrogen fixation among ureide exporters. In ureide legumes, ureides are mostly derived from *de novo* purine synthesis in the nodules and also from purine salvage pathways. Previous studies however showed that ureides were present in non-fixing soybean (McClure and Israel, 1979a, McClure and Israel, 1979b) and common bean plants (Raso et al., 2007). The concentration of ureides however was very low relative to those found in N_2-fixing plants. Meanwhile, ureides were also detected in

seedlings of soybean (Polayes and Schubert, 1984) and common bean (Quiles et al., 2009), young plants which were not yet fixing N_2. Results obtained in this study identified ureides and allantoinase in soybean seedlings and evaluated non-fixing tissues.

Previous studies also reported the presence of ureides in non-ureide plants, also known as amide exporters. Ureides are also produced in non-legumes such as *Arabidopsis* and the tree legume, *Robinia pseudoacacia* (Yang and Han, 2004). In fact, ureide catabolic genes like allantoinase (*AtALN, RpALN*) were already identified in these plants. The ureide transporter *AtUPS1* was also identified in *Arabidopsis thaliana* (Desimone et al., 2002), implying that this plant has a complete and functional system for the uptake and degradation of allantoin. Amide plants like all other organisms are capable of producing purines *de novo* or through purine recycling (salvage pathway). In some amide plants, uninfected cells differentiate in the nodules and express uricase (Smith & Atkins, 2002), the enzyme which converts uric acid to allantoin (Zrenner et al., 2006). As ureide biogenesis also occurs in uninfected cells in nodules of ureide exporters, it is possible that both ureide and amide plants are capable of ureide synthesis and catabolism.

The advantage of having a system for both pathways has been hypothesized to benefit ureide plants. Ureide legumes were shown to transport allantoin and allantoic acid during N_2-fixing conditions, however, when nitrogen is abundant in the soil, these plants use asparagine and glutamine as nitrogen transport molecules. Soybean was shown to transport fixed nitrogen as ureides during active nitrogen-fixing conditions but use amino acids (Peoples et al., 1991), particularly asparagine (do Amarante et al., 2006) when these plants were fed with nitrate. The use of ureides in assimilating nitrogen is viewed to be an advantage for plants as these compounds better conserve carbon skeletons compared to amides. Meanwhile, no studies have shown amide transporters switching to the ureide pathway under any given condition which may put them at a disadvantage over the ureide exporters. As it has not been verified until now which pathway is most cost effective for the plants to use, I think the ability to switch pathways depending on the type of available nitrogen, would be a definite advantage for the survival of the plant particularly warm-season legumes. As previously discussed in Section 1.4.2, the ureide pathway might enable these plants to photosynthesize longer during higher temperature conditions when the entry of CO_2 is limited due to closed stomata.

4.7. Future directions

Allantoinase triggers the ureide catabolic pathway by initiating the first step, which is the hydrolysis of allantoin to allantoate. Allantoinase genes, specifically *GmALN1* and *2*, were found to be expressed in almost all tissues of soybean, most probably playing a role in purine biosynthesis. However, its presence does not always lead to the release of ammonia as seen in the results presented in this study. As observed by Quiles and his colleagues (2009), allantoin-degrading activity was not the limiting step in ureide catabolism.

The function of allantoinase in a particular plant tissue, as observed here, depends on the fate of its degradation product which is allantoate. Allantoinase facilitates the hydrolysis of allantoin in source tissues like cotyledons and nodules but most of the allantoate produced will not be degraded to release ammonia. Hence in these types of tissues, allantoinase takes part in producing ureides (allantoate) for transport and distribution of nitrogen to sink tissues such as the leaves. In developing tissues (e.g. embryonic axes, leaves), allantoinase takes part in providing nitrogen to support plant growth and development. Meanwhile, it is not known why allantoate is the preferred form of ureide for transport. I suggest that in soybean, ureide catabolism is not regulated at the level of allantoinase. The presence of allantoic acid as the major ureide in various tissues in common bean implied that the regulation of the ureide catabolic pathway is not at the level of allantoinase but in allantoate amidohydrolases (Quiles et al., 2009) and other enzymes catalyzing the downstream steps. These findings were also observed in the current study.

During water-limiting conditions, ureides (ureidoglycolate) accumulated in the leaves of non-treated plants. Ureide levels in allantoin treated plants were variable but did not increase as the duration of the stress advanced. Meanwhile, allantoinase activity was not affected for both treated and non-treated plants except in Day 5 where a slight decline in activity was noted for both treatments (significant only in non-treated plants). Higher specific activity values were however observed for allantoin treated plants confirming the higher allantoate concentrations measured for these plants. Both allantoin application and water-limiting conditions, however, did not have a noticeable effect on the transcript levels of soybean allantoinase genes, implying that regulation was not at the transcription level. Based on the ureide content, enzyme activity and transcript abundance of soybean allantoinases, it could then be inferred that early water-limiting conditions (up to Day 3) had no apparent effect on allantoinase. Addition of allantoin

prior to the water limitation stress however led to an increase in enzyme activity in the leaves, consequently ureide catabolic activity. Finally, allantoin treatment prior to water limitation stress seemed to delay the decay of other ureide catabolic enzymes, probably protecting the enzymes from early inactivation. Alternatively, treatment helped prolong the metabolic activity of the plants.

Research in the Todd lab aims to understand nitrogen metabolism in legumes by focusing on the ureide pathway using soybean, and *Arabidopsis* as models. We are studying ureide catabolism because the regulation of this process is still not clear. An important aspect of this will be to address protein levels in addition to transcript abundance and ureide activity. To accomplish this, a peptide antibody was generated for this study but found not to be specific for allantoinase (data not shown). To address this, expression of allantoinase in a heterologous host and purification of the recombinant protein for antibody production, to complement the transcript and enzyme data, would be beneficial.

In addition to allantoinase, the enzymes allantoate amidohydrolase and ureidoglycolate-urea lyase are also currently being studied in the lab. Our collective results will then provide the molecular tools needed to deal with the other objectives of our research, such as defining the basis for induced inhibition of nitrogen fixation and ureide catabolism due to water limitation. My preliminary experiment to address this particular research question will require follow up experiments to better understand the mechanism of drought-induced inhibition of ureide catabolism.

I anticipate that our efforts will benefit the agriculture industry in the long run. As legumes now have a rising demand as a crop, particularly because of their low demand for nitrogen input and high seed protein content, new knowledge which leads to improvement of legume crops will certainly be a big help to the industry. Understanding the regulatory mechanisms of nitrogen fixation along with ureide catabolism may lead to the development of various strategies which would boost nitrogen fixation capacity of legumes especially in dealing with water-limiting conditions.

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APPENDIX A

NUCLEOTIDE CODING SEQUENCE (SHOWING 1-975 BASE PAIRS ONLY) OF *GmALN1*, *2*, *3* AND *4* SHOWING THE LOCATION OF qRT-PCR PRIMERS USED

Nucleotides marked by black boxes refer to forward primers (reverse primers in red boxes) used for real time qRT-PCR (as previously shown in Table 2.2). Nucleotide coding sequences of *GmALN1*, *2*, *3* and *4* shown below were compared using the Vector NTI 11 alignment tool. Nucleotides common to all four soybean allantoinase sequences are highlighted in yellow while those highlighted in turquoise are nucleotides common to only two or three allantoinase sequences. Similar nucleotides are highlighted in green while non-similar nucleotides are not highlighted.

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GmALN1  ATGGATCAGCTGTTGTGGAGGGTGTTGCCTTTGCTAACAATCCTTGTCTCCTTTGGAGTGTTCTTCTACTTGCAG
GmALN2  ATGGATCAGTTTTTGTGGAGGGTGTTGCCTTTGCTTACAATCCTTGTTTCCTTTGGAGTGTTCTTCTACTTGCAG
GmALN3  ATGGAGCATTTTGTTTGGAGGGTGTTGCCTTTGCTCACAATCCTCATTTCCTTAGTAGTATTCTTCTACTTGCAG
GmALN4  ATGGAGCAGTTTGTATGGAGGGTGTTGCCTTTACTCACAATCCTCATGTCTTTAGTAGTATTCTTCTACTTGCAG


GmALN1  GATTCTTACACAGCACAGTTTTATGCTCTCATAAAGTTACCACGGGATAAGTGCAGCCTTCTTCCCCATCGCCAC
GmALN2  GATTCTTACACAGCACAGTTATTTCCTCTCATAAAGTTACCGCGGGATAAGTGCAGCCTTCTTCCCCATCGCCAC
GmALN3  GATTCTTACAGGGCCCATTTGCATCCTCTCGTAAAGTTCCCGCGGAATAAGTGCAGCCTTCTTCCCCATCGTCAT
GmALN4  GATTCTTACAGGGCCCATTTACATCCTCTCGTAAAGTTACCGCGGAGTAAGTGCAGTCTTCTTCCCCATCGTCAT


GmALN1  TTCTGGATATCCAGCAAACGCATTGTGACCCCACAAGGGATCATTTCTGGTTCAGTGGAGATAAATGATGGGAAA
GmALN2  TTCTGGATATCCAGCAAACGTATTGTGACCCCACAAGGGATCATTTCTGGTTCAGTGGAGATAAATGATGGGAAA
GmALN3  TTCTGGATAACCAGCAAACGCATTGTGACCCCACAAGGGATCATTTCTGGTTCAGTGGAGATAAATGAAGGGAAA
GmALN4  TTCTGGATAACCAGCAAACGCATTGTGACCCCACAAGGGATCATTTCTGGTTCAGTGGAGATAAATGAAGGGAAA


GmALN1  ATAATATCTGTTGTCGAGGGACATGCTAACCAGGGGAAGCCTAAGCAAGAAGAAGTAATTGATTATGGAGATGCT
GmALN2  ATAATATCTGTCGTTGAGGGACATGCTATTCAGGGGAAGCCTAAGCAAGAAGAAGTAATTGATTATGGAGATGCT
GmALN3  ATAATATCTGTCATCGAGGGATATAGTAAGCAGGGGAAGTCTAAGCAAGAAAAAATAATTGATTATGGAGATGCT
GmALN4  ATAATATCTGTCATCAAGGGATATAGTAAGCAGGGGAAGTCTAAGCAAGAAAAAATAATTGATTATGGAGGTGCT


GmALN1  GTTATCATGCCTGGCTTGATTGATGTGCACGTACATCTTGATGAGCCAGGAAGAACTGAGTGGGAAGGATTTGAT
GmALN2  GTTATCATGCCTGGCTTGATTGATGTGCACGTACATCTTAATGAGCCTGGAAGAACTGAGTGGGAAGGATTTGAT
GmALN3  GTTGTCATGCCTGGCTTGATTGATGTGCACGTACATCTTGATGAGCCAGGAAGAACTGCATGGGAAGGATTTGAT
GmALN4  GTTGTCATGCCTGGCTTGATTGATGTGCACGTACATCTTGATGAGCCAGGAAGAACTGCGTGGGAAGGATTTGAT
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GmALN1  ACTGGTACCAGAGCTGCTGCTGCTGGTGGCGTAACCACAGTGGTTGACATGCCTCTGAACAATTATCCTACAACC
GmALN2  ACTGGTACCAGAGCTGCTGCTGCTGGTGGTGTAACCACAGTGGTTGACATGCCTCTAAACTGTTACCCTACAACT
GmALN3  ACTGGTACCAGAGCTGCTTCTGCTGGTGGTGTAACAACAGTGGTTGACATGCCTCTAAACAATCACCCTACGACT
GmALN4  ACTGGTACCAGAGCTGCTGCTGCTGGTGGTGTAACAACAGTGGTTGACATGCCTCTGAACAATCACCCTACAACT


GmALN1  GTGTCAAAGGAAAAACTAAAACTTAAGCTTGATGCTGCAGAGGATAAAATCTATGTTGATGTTGGATTTTGGGGA
GmALN2  GTGTCTAAGGAAAAACTGAAACTTAAGCTTGATGCTGCAGAGGATAAAATCTATGTTGATGTTGGATTTTGGGGA
GmALN3  GTGTCTAAGGAAACACTGAAACTTAAGCTTGAAGCTGCAGAGAATAAAATCTATGTTGATGTTGGATTTTGGGGA
GmALN4  GTGTCTAGGGAAACACTGAAACTTAAGCTTGAAGCTGCAGAGAATAAAATCTATGTCGATGTTGGATTTTGGGGA


GmALN1  GGCCTAATCCCTGAAAATGCACTTAATACAAGTATTCTTGAGGGGCTCTTAAGTGCTGGTGTTCTTGGTATGAAG
GmALN2  GGCCTAATCCCTGAAAATGCACTTAATACAAGTATTCTTGATGGTCTCTTAAGTGCTGGTGTTCTTGGTGTGAAG
GmALN3  GGCCTAATCCCTGAAAATGCACATAATACAAGTATTCTTGAAGGTCTCCTAAGTGCCGGTGTTCTCGGTTTGAAG
GmALN4  GGCCTAATCCCTGAAAATGCACATAATACAAGTATTCTTGAAGGTCTCCTAAGTGCTGGTGTTCTTGGTTTGAAG


GmALN1  TCTTTTATGTGTCCTTCAGGAATAAAATGACTTCCCTATGACTACTATTGACCATATCAAGGCGGGATTGCCTGTG
GmALN2  TCTTTTATGTGTCCTTCAGGAATAGATGACTTCCCCATGGCTACTATTGACCATATCAAGGCAGGATTGTCTGTG
GmALN3  TCTTTTATGTGTTCTTCGGGAATTAGTGACTTTCCTTTGACTACTATTCACCATATCAAGGAGGGATTGTCTGTG
GmALN4  TCTTTTATGTGTCCTTCAGGAATTAGTGACTTTCCTTTGACTACTATTCACCATATCAAGGAGGGATTGTCTGTG


GmALN1  TTAGCAAAATATAAAAGGCCGATAGTTGTGCATGCTGAGGTTCAACAAGATTTTGAAAACCATTTGGAGCTTAAT
GmALN2  TTGGCAAAATATAGAAGGCCTATAGTTGTGCATGCTGAGATTCAACAAGATTTTGAAAATCATTTGGAGCTTAAT
GmALN3  TTAGCAAAATATAGAAGGCCTTTACTTGTGCATTCTGAGATTCAACAACATTCTAAAAAACATTTGGAGCTTAAT
GmALN4  TTAGCAAAATATAGAAGGCCTTTACTTGTGCATTCTGAGATTCAACAACATTCTAAAAAACATTTGGAGCTTAAT


GmALN1  GAGGATAACCTTGATCCACGTGCTTACTTGACCTATCTCAATGCCAGGCCACCTTCATGGGAGGAGGCAGCCATT
GmALN2  GAGGATAACCTTGATCCACGTGCTTACTTGACCTATCTCAATACCAGGCCACCTTCATGGGAGGAGGCAGCCATT
GmALN3  GA---TAAAGGTGGTCCGCGTGCTTACTTGACCTATCTCCACACTAGGCCACCTTCATGGGAGGAAGCAGCCATT
GmALN4  GA---TAAAGGTGGTCCACGTGCTTACTTGACCTATCTCCACACTAGGCCACCTTCATGGGAGGAAGCAGCCATT


GmALN1  AAACAACTTGTAGGTGTTACGAAGGACACAAGAAAAGGTGGTCCTTTAGAAGGAGCTCATGTTCACATTGTTTCAC
GmALN2  AAACAACTTGTAAGTGTTACGAAGGACACAAGAAAAGGTGGCCCCTTTAGAAGGAGCACATGTTCACATCGTCCAC
GmALN3  AAAGAACTTGTAGGTGTTACGAAGGACACAAGAAAAGGTGGTCCTTTAGAAGGAGCACATGTTCACATTGTCCAC
GmALN4  AAAGAACTTGTAGGTGTTACGAAGGACACAAGAAAAGGTGGTCCTTTAGAAGGAGCACATGTTCACGTGGTTCAC


GmALN1  TTGTCTGATTCAAGTGCTTCCTTGGGATCTGATTAAGGAAGCAAAAAGTCGTGGTGACAGCATAAGTGTTGAGACT
GmALN2  TTGTCTGATTCAAGTGCTTCCTTAGATCTGATTAAGGAAGCAAAAAGTCATGGTGACAGCATAAGTGTTGAGACT
GmALN3  TTGTCTGATTCAAGTGCTTCCTTGGATCTGATTAAGGAAGCAAAAAGTCGTGGTGACAGCATAAGTGTTGAGACT
GmALN4  TTGTCTGATTCAAGTGCTTCCTTAGATCTGATTAAGGAAGCAAAACGTCGTGGTGACAGCATAAGTGTTGAGACT
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MEAN UREIDE CONCENTRATIONS OF AXES AND COTYLEDONS OF LIGHT AND
DARK-GERMINATED SOYBEAN SEEDLINGS MEASURED AT
DAYS 0 (IMBIBED SEED), 2, 4, 6 AND 8

The ureides allantoin (ALN), allantoate (ALL) and ureidoglycolate (UDG) were measured using
the glyoxylate assay method developed by Vogels and van der Drift (1970). Data
presented are the mean of three independent experiments (n=3).

Table B.1. Mean ureide concentrations for allantoin (ALN), allantoate (ALL), ureidoglycolate
(UDG) measured in axes and cotyledons of light-germinated seedlings.

AGE OF SEEDLINGS (DAYS)	MEAN UREIDE CONCENTRATION,					
	ALN		ALL		UDG	
	Mean	Standard error	Mean	Standard error	Mean	Standard error
Seedling axes						
Imbibed seed/Day 0	0.1877	0.1213	0	0	0.80050	0.3298
Day 2	0.2846	0.1569	1.1316	0.4847	0.6266	0.1348
Day 4	0.4186	0.4033	4.5972	0.2147	0.3949	0.1482
Day 6	1.0538	0.3597	7.9307	0.3515	0.4556	0.1941
Day 8	1.2352	0.4444	10.7572	1.5108	0.2832	0.1066
Cotyledons						
Imbibed seed/Day 0	0.1877	0.1213	0	0.0000	0.8050	0.3298
Day 2	2.3604	1.4561	0.4963	0.4963	1.5011	1.3306
Day 4	1.3978	0.9356	1.4674	1.0905	1.3004	1.0151
Day 6	2.2876	1.8324	2.8877	1.6706	2.7867	2.3338
Day 8	2.8698	2.5707	4.2836	2.5134	2.1846	1.7315

Table B.2. Mean ureide concentrations for allantoin, allantoate, ureidoglycolate measured in axes and cotyledons of dark-germinated seedlings.

AGE OF SEEDLINGS (DAYS)	MEAN UREIDE CONCENTRATION,					
	Allantoin		allantoate		Ureidoglycolate	
	Mean	Standard error	Mean	Standard error	Mean	Standard error
Seedling axes						
Imbibed seed/Day 0	0.1877	0.1213	0	0	0.8050	0.3298
Day 2	0.6855	0.3283	0.4460	0.3364	0.8679	0.5232
Day 4	0.7058	0.3596	3.6745	0.9903	0.2304	0.1394
Day 6	1.1433	0.5688	5.3300	0.3879	0.2160	0.1121
Day 8	1.1798	0.5295	6.0423	0.5896	0.2151	0.0613
Cotyledons						
Imbibed seed/Day 0	0.1877	0.1213	0	0	0.8050	0.3298
Day 2	1.3182	0.9856	1.5820	0.3777	0.8066	0.5829
Day 4	1.0825	1.0825	3.1308	0.9624	0.5555	0.4000
Day 6	1.2766	1.1799	3.8709	1.0595	1.3949	1.3949
Day 8	1.2148	0.8737	4.4297	0.9079	0.9109	0.8347

APPENDIX C

RELATIVE ABUNDANCE OF *GmALN3* AND *4* IN THE AXES AND COTYLEDONS OF LIGHT-GERMINATED SOYBEAN SEEDLINGS

Values are normalized to the relative abundance of *GmALN1* for IS (Imbibed seed). Axes and cotyledons for IS were not evaluated separately. Data presented are the mean of three independent experiments (n=3).

Age of seedlings (days)	Relative abundance			
	GmALN3		*GmALN4*	
	Mean	Standard error	Mean	Standard error
Seedling axes				
Imbibed seed (Day 0)	0.1458	0.0103	0.0322	0.0077
Day 2	0.0328	0.0042	0.0220	0.0046
Day 4	0.0223	0.0058	0.0113	0.0039
Day 6	0.0312	0.0123	0.0176	0.0096
Day 8	0.0121	0.0028	0.0084	0.0028
Cotyledons				
Imbibed seed (Day 0)	0.1458	0.0103	0.0322	0.0077
Day 2	0.0332	0.0057	0.0462	0.0026
Day 4	0.0422	0.0058	0.2725	0.0535
Day 6	0.0491	0.0129	0.0539	0.0148
Day 8	0.0113	0.0017	0.0294	0.0046

MEAN UREIDE CONCENTRATIONS OF MATURE V4-V5 TISSUES OF N_2-FIXING AND NON-FIXING SOYBEAN PLANTS

The ureides allantoin, allantoate and ureidoglycolate were measured using the glyoxylate assay method developed by Vogels and van der Drift (1970). Data presented are the mean of three independent experiments (n=3).

Table D.1. Mean concentrations of allantoin, allantoate and ureidoglycolate measured in mature V4-V5 tissues of soybean.

	MEAN UREIDE CONCENTRATION, nmol/mg fresh weight					
	Allantoin		Allantoate		Ureidoglycolate	
	Mean	Standard error	Mean	Standard error	Mean	Standard error
Fixing tissues						
Young leaf	1.1986	0.2884	0.8730	0.8452	0.3383	0.2039
Mature leaf	1.2588	0.4224	0.5432	0.4450	0.2147	0.1458
Root tissues	0.9493	0.4244	1.3697	0.5357	0.2242	0.1609
Nodules	1.3584	0.3235	5.3823	2.0714	0.4322	0.1111
Non-fixing tissues						
Young leaf	1.0429	1.0062	0.3755	0.3755	0.2450	0.0473
Mature leaf	0.5676	0.4357	0	0	0.2008	0.0589
Root tissues	0.1501	0.1045	0.1456	0.1456	0.1037	0.0633

Table D.2. Percentage of individual ureides measured out of total ureides detected in mature V4-V5 tissues of soybean.

	PERCENTAGE OUT OF TOTAL UREIDES			
	Total ureides	Allantoin	Allantoate	Ureidoglycolate
Fixing tissues				
Young leaf	2.4099	49.74	36.23	14.04
Mature leaf	2.0166	62.42	26.94	10.64
Root tissues	2.5433	37.33	53.86	8.81
Nodules	7.1730	18.94	75.03	6.03
Non-fixing tissues				
Young leaf	1.6634	62.70	22.58	14.73
Mature leaf	0.7683	73.87	0	26.13
Root tissues	0.3994	37.57	36.45	25.97

APPENDIX E

RELATIVE ABUNDANCE OF *GmALN3* AND *4* IN VARIOUS TISSUES OF

MATURE V4-V5 SOYBEAN PLANTS

Values are normalized to the relative abundance of *GmALN1* in N_2-fixing young leaf. Data

presented are the mean of three independent experiments (n=3).

	Relative abundance			
	GmALN3		*GmALN4*	
	Mean	Standard error	Mean	Standard error
Fixing tissues				
Young leaf	0.0604	0.0200	0.0967	0.0292
Mature leaf	0.0034	0.0028	0.0023	0.0002
Root tissues	0.0063	0.0051	0.0622	0.0560
Nodules	2.7745	1.0515	2.6157	0.8957
Non-fixing tissues				
Young leaf	0.0172	0.0134	0.0038	0.0038
Mature leaf	0.0006	0.0004	0.0019	0.0011
Root tissues	0.0015	0.0003	0.0029	0.0012

APPENDIX F

CONCENTRATION OF UREIDES DETECTED IN YOUNG LEAVES OF 45-DAY OLD N$_2$-FIXING SOYBEAN SUBJECTED TO WATER LIMITATION

The ureides allantoin, allantoate and ureidoglycolate were measured using the glyoxylate assay method developed by Vogels and van der Drift (1970).

Table F.1. Allantoin concentration measured in young leaves of N$_2$-fixing soybean subjected to water limitation.

Treatments	Ureide concentration, nmol per mg protein				
	Allantoin				
	Replicate 1	Replicate 2	Replicate 3	Mean	Standard error
With allantoin, plus water					
Day 0	0.0107	0.022665	0.0067	0.0134	0.0048
Day 1	0.0023	0.034348	0.0138	0.0168	0.0094
Day 3	0.0258	0.039514	0	0.0218	0.0116
Day 5	0.02109	0.036647	0	0.0192	0.0106
With allantoin, no water					
Day 0	0.0156	0.044291	0.0194	0.0264	0.0090
Day 1	0.0172	0	0.0208	0.0127	0.0064
Day 3	0.0860	0.042494	0.0121	0.0469	0.0215
Day 5	0.0504	0	0.0154	0.0219	0.0149
No allantoin, plus water					
Day 0	0.0861	0	0.0120	0.0327	0.0269
Day 1	0.1245	0.001925	0.0142	0.0469	0.0390
Day 3	0.0717	0.038481	0.0150	0.0417	0.0164
Day 5	0.0822	0	0.0078	0.0300	0.0262
No allantoin, no water					
Day 0	0.0489	0.02607	0.0469	0.0406	0.0073
Day 1	0	0.013655	0.0464	0.0200	0.0138
Day 3	0.0159	0.008193	0.0382	0.0208	0.0090
Day 5	0.0712	0.02156	0.0409	0.0446	0.0145

Table F.2. Allantoate concentration measured in young leaves of N_2-fixing soybean subjected to water limitation.

Treatments	Ureide concentration, nmol per mg protein				
	Allantoate				
	Replicate 1	Replicate 2	Replicate 3	Mean	Standard error
With allantoin, plus water					
Day 0	0.0453	0.0468	0.0067	0.0329	0.0007
Day 1	0.0317	0.0255	0	0.0191	0.0031
Day 3	0.0276	0.0168	0.0415	0.0286	0.0054
Day 5	0.0369	0.0286	0.0989	0.0548	0.0042
With allantoin, no water					
Day 0	0.0260	0.0220	0.0310	0.0264	0.0026
Day 1	0.0478	0.0858	0.0205	0.0514	0.0189
Day 3	0.0556	0.0295	0.0244	0.0365	0.0097
Day 5	0.0213	0.0383	0.0543	0.0380	0.0095
No allantoin, plus water					
Day 0	0	0.0312	0.0171	0.0241	0.0070
Day 1	0	0.0371	0.0196	0.0284	0.0088
Day 3	0	0.0628	0.0068	0.0348	0.0280
Day 5	0	0.1322	0.0135	0.0729	0.0594
No allantoin, no water					
Day 0	0.1337	0.0172	0.0120	0.0543	0.0397
Day 1	0.0457	0.0003	0.0087	0.0182	0.0139
Day 3	0	0.0031	0.0080	0.0037	0.0023
Day 5	0.0056	0	0.0234	0.0097	0.0071

Table F.3. Ureidoglycolate concentration measured in young leaves of N_2-fixing soybean subjected to water limitation.

Treatments	Ureide concentration, nmol per mg protein				
	Ureidoglycolate				
	Replicate 1	Replicate 2	Replicate 3	Mean	Standard error
With allantoin, with water					
Day 0	0.0080	0.0082	0	0.0054	0.0027
Day 1	0.0091	0.0056	0	0.0049	0.0027
Day 3	0.0021	0	0.0206	0.0076	0.0066
Day 5	0.0020	0.0047	0	0.0022	0.0014
With allantoin, no water					
Day 0	0	0	0	0	0
Day 1	0	0	0	0	0
Day 3	0	0	0.008836	0.0029	0.0029
Day 5	0	0	0.000112	0.000037	0.000037
No allantoin, with water					
Day 0	0.0084	0.0144	0.0075	0.0101	0.0022
Day 1	0.0149	0.0319	0	0.0156	0.0092
Day 3	0.0097	0	0.0045	0.0048	0.0028
Day 5	0.0135	0.0432	0	0.0189	0.0128
No allantoin, no water					
Day 0	0	0	0.0056	0.0019	0.0019
Day 1	0.0164	0	0.0031	0.0065	0.0050
Day 3	0.0214	0.0066	0.0019	0.0099	0.0059
Day 5	0.2377	0	0.0014	0.0797	0.0790