4-23-2013 12:00 AM

Metabolism and transcriptional responses to asparagine in Arabidopsis thaliana

Qianyi Zhang
The University of Western Ontario

Supervisor
Dr. Frédéric Marsolais
The University of Western Ontario

Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

METABOLISM AND TRANSCRIPTIONAL RESPONSE TO ASPARAGINE IN *ARABIDOPSIS THALIANA*

(Thesis format: Integrated-Article)

by

Qianyi Zhang

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

Asparagine aminotransferase transforms asparagine into α-ketosuccinamate, which is further deamidated by an ω-amidase. Serine:glyoxylate aminotransferase, encoded by *AGT1* in Arabidopsis, was identified as asparagine aminotransferase. In the roots of 10-day-old Arabidopsis seedlings treated with 20 mM asparagine, *AGT1* transcript levels increased by 2-fold while *ω-amidase* transcripts were decreased by 30%. Recombinant AGT1 had a substrate preference for asparagine when compared with alanine and serine as amino group donors. An ω-amidase candidate gene, *AT5G12040*, was identified based on amino acid sequence identity with mammalian gene *Nitrilase 2*. RT-PCR of a T-DNA insertion mutant line showed that ω-amidase expression was abolished compared with wild-type. In the roots of 10-day-old seedlings grown without asparagine or with 2 mM asparagine, the amount of ω-amidase substrates, α-ketosuccinamate and α-hydroxysuccinamate, was higher in mutant than in wild-type under both conditions. Kinetic analysis indicated that recombinant ω-amidase has a preference for α-hydroxysuccinamate over α-ketosuccinamate and α-hydroxysuccinamate.

Keywords: AGT1, Arabidopsis, asparagine, asparagine aminotransferase, enzyme kinetics, nitrogen metabolism, Serine:glyoxylate aminotransferase, ω-amidase

Co-Authorship Statement

Zhang, Q., Lee, J., Pandurangan, S., Clarke, M., Pajak, A., Marsolais, F., 2013. Characterization of Arabidopsis serine:glyoxylate aminotransferase, AGT1, as an asparagine aminotransferase. Phytochemistry 85, 30-35.

In chapter 2, Jamie Lee and Sudhakar Pandurangan contributed RT- qPCR data except for AGT1. Matthew Clarke contributed the AGT1 construct and Agnieszka Pajak contributed HPLC measurements of asparagine in roots. Qianyi Zhang contributed enzyme assays of recombinant AGT1 with different substrates, as well as RT- qPCR data for AGT1.

Acknowledgments

I would like to sincerely thank my supervisor, Dr. Frédéric Marsolais, for his support and guidance over the past few years; especially for providing an intellectually inspiring environment to further my academic studies and always being available for research related discussion. I would like to thank my co-supervisor, Dr. Denis Maxwell, for his guidance and helpful suggestions in preparing and editing my thesis. I would like to thank my advisory committee: Dr. Sheila Macfie and Dr. Sangeeta Dhaubhadel for their guidance, input on the direction of research and editing of my thesis.

I am very thankful to the research technicians and staff at Agriculture and Agri-Food Canada (London, Ontario) for their patience, insight and technical support: Aga Pajak, Sudhakar Pandurangan and Alex Molnar. I am grateful for my fellow lab mates, friends and colleagues who were always available to assist with experiments, discuss one another's projects or to just go for coffee or lunch break: Korey Kilpatrick, Mimmie Lu, Gary Tian, Chen Chen, Chenglong Li and Hui Fang.

Lastly, I would like to thank my family and friends, whose support, love and guidance have made me who I am today.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BLAST	Basic local alignment search tool
bp	Base pairs
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
kDa	Kilo-Dalton
K_m	Michaelis-Menten constant
LB	Luria Broth
NAD^+	β-Nicotinamide adenine dinucleotide
NADH	β-Nicotinamide adenine dinucleotide, reduced
OD_{600}	Optical density at 600 nm
PCR	Polymerase chain reaction
ppm	Parts per million
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
RT-qPCR	RT-quantitative PCR
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
T-DNA	Transfer DNA
V_{max}	Maximum reaction rate
V_{max}/K_m	Catalytic efficiency

Chapter 1. Introduction

1.1 *Arabidopsis thaliana*

Arabidopsis thaliana is widely found throughout the world, such as Europe, Asia and North America (Koornneef and Meinke, 2010). Within the last thirty years, Arabidopsis has become the predominant model system for plant biology. The reasons for this include its short generation time, smaller genome size and its ability to self-fertilize. For instance, it takes about eight weeks in a greenhouse to complete the whole life cycle from germination until the seed matures, which is one of the advantages for physiological and biochemical studies. Furthermore, Arabidopsis is easy to manipulate genetically and it has one of the smallest genomes among plants. Stock centres in the UK and USA provide seeds of mutants and wild accessions and DNA material for research. There are abundant genetic resources available, such as The Arabidopsis Information Resource (TAIR) database and extensive genome sequencing information (Somerville and Koornneef, 2002). These factors and the ability to transform Arabidopsis have made it one of the favourite plant model systems for molecular genetic studies. So it has expanded the field of study in plant biology and facilitated the understanding of other plants, allowing the development of potential applications in agriculture.

1.2 Nitrogen metabolism

In plants, nitrogen is one of the most important mineral nutrients required for plant growth and development. Plants have multiple adaptive mechanisms to cope with different kinds of nitrogen availability in soil, such as nitrogen fixation, ammonification and nitrification (Stitt et al., 2002). Plant growth and development, such as leaf

development, root architecture and seed dormancy, can be dramatically affected by the source and amount of nitrogen supplied to plants (Alboresi et al., 2005; Walch-Liu et al., 2000; Zhang and Forde, 1998). As it is important to agricultural productivity, a large amount of research about the biochemical and physiological aspects of nitrogen metabolism has taken place. For example, Wang et al. (2004) determined the role of nitrogen in root signalling using the *nitrate reductase* knock-out mutant in Arabidopsis. They found that nitrate could be used as a signal in starch mobilization and metabolism.

There are various types of nitrogen in the environment that can be used by plants, including nitrate, nitrite and ammonium. Of these, nitrate is the most common source of usable nitrogen that is absorbed, assimilated and redistributed in plants. After uptake, nitrate is known to act as a metabolic signal, which affects nitrogen and carbon metabolism and growth by inducing the transcription of genes encoding enzymes, such as transporters and aminotransferases (Gutiérrez et al., 2008a; Wang et al., 2004). The first step of the nitrogen assimilation process in Arabidopsis root is catalyzed by nitrate reductase, reducing nitrate to nitrite, which is subsequently reduced to ammonium by nitrite reductase. Ammonium then reacts with glutamate (Glu) in a ligase reaction involving glutamine (Gln) synthetase to form Gln. With the formation of Gln, asparagine (Asn) can be synthesized by Gln-dependent Asn synthetase and transported from the root to other sink tissue by a lysine histidine transporter, a high-affinity transporter for amino acids (Chen and Bush, 1997; Hirner et al., 2006) (Figure 1.1). Some studies show that plants have adapted mechanisms to enhance nitrogen acquisition from different ways, including symbiosis with nitrogen-fixing bacteria or alternative pathways using other forms of nitrogen, such as amino acids (Crawford, 1995; Li et al., 2006).

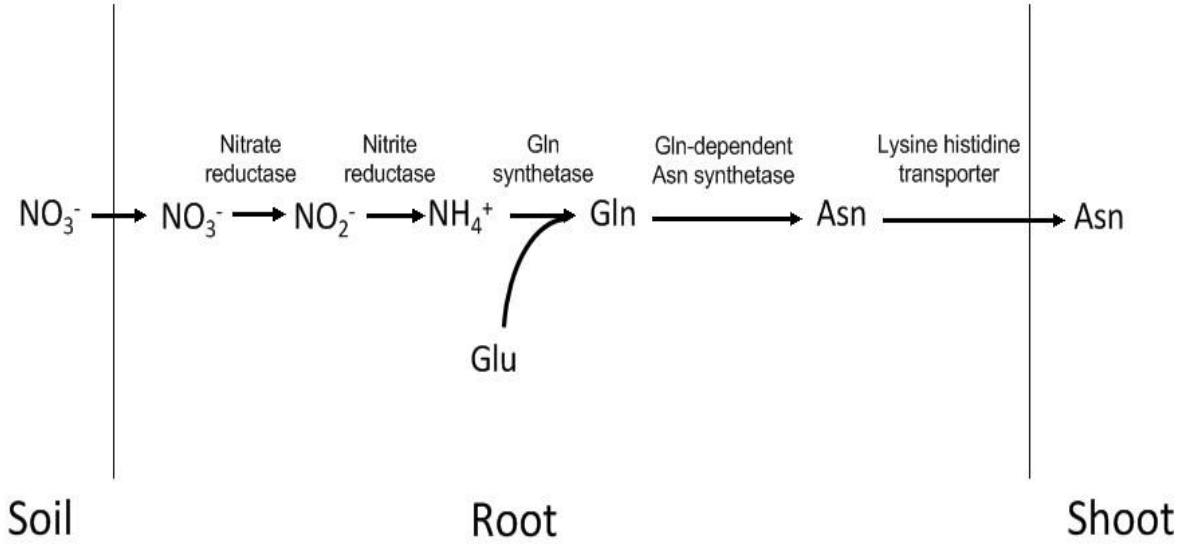

Figure 1.1 Simplified model of the nitrogen assimilation process in root.

Nitrate absorbed from soil into root is reduced to nitrite by nitrite reductase, which is subsequently reduced to ammonium by nitrite reductase. Ammonium can work with Glu to form Gln by Gln synthetase. Asn is synthesized from Gln by Gln-dependent Asn synthetase and transported from root to other tissue by lysine histidine transporter (It is adapted from Chen and Bush, 1997).

1.3 Asparagine

Plants have the ability to take up organic nitrogen from the soil in the form of amino acids (Näsholm et al., 2009). Previous studies have been conducted involving the use of different nitrogen sources, such as amino acids, and have investigated the effects of Asn on growth of Arabidopsis. Asn as the single nitrogen source at a concentration of 5 mM was previously shown to support the growth of the Arabidopsis Columbia ecotype for up to 21 days (Hirner et al., 2006). It was also reported that 3 mM Asn, as the sole nitrogen source, can stimulate growth measured at 20 days in comparison to a control without nitrogen, meaning that plants grown on media without a nitrogen source will eventually consume internal nitrogen reserves whereas Asn can be used as a suitable nitrogen source by Arabidopsis (Forsum et al., 2008). Moreover, Asn is also utilized in other plants. Asn, rather than Gln, is the main molecule that is transported and metabolized in most legumes (Andrews, 1986; Scott et al., 1976), and a study about amino acid metabolism analysis of ears from maize also highlighted the importance of Asn for nitrogen translocation within sink organs for non-legume plants (Canas et al., 2010).

In addition, Asn not only exists in roots but also is found in other sink tissues. It has been shown that Asn can account for more than 80% of the nitrogen transported from root to shoot in *Lotus japonicus* (Waterhouse et al., 1996). There is evidence that the concentration of Asn in Arabidopsis leaves during night is much higher than that in day, which indicates that Asn can be accumulated in the leaves (Schultz et al., 1998). Therefore, Asn can be identified as one of the main storage and transport forms of nitrogen in plants.

1.4 Asparagine degradation

In the nitrogen dissimilation pathways, there are two different degradation mechanisms for Asn. One proceeds via asparaginase, which catalyzes the conversion of Asn to aspartate (Asp) and releases ammonia (Bruneau et al., 2006). The other involves Asn aminotransferase, which yields α-ketosuccinamate (α-KSM), which can be further transformed by ω-amidase to oxaloacetate as well as releasing ammonia (Figure 1.2) (Meister et al., 1952). A previous study about Asn metabolism in pea showed that Asn aminotransferase activity predominates over asparaginase activity in older leaves, and is present in pods, but is very low in young leaves and developing seeds (Ireland and Joy, 1981a).

There are two different types of asparaginase in higher plants, which correspond to biochemical subtypes previously defined on the basis of their dependence on K^+ (Sodek et al., 1980). Each type is represented by a single gene in Arabidopsis. ASPGB1, K^+-dependent asparaginase, has a much higher catalytic efficiency with Asn as substrate than ASPGA1, the K^+-independent asparaginase (Bruneau et al., 2006). Similarly, two asparaginase subfamilies have been reported in *Lotus japonicus*, which are K^+-dependent (LjNSE1) and K^+-independent (LjNSE2) asparaginases (Credali et al., 2011). K^+-dependent and K^+-independent asparaginases are interesting as the exact physiological significance of each of asparaginase is still unclear, although potassium is one of the most abundant inorganic cations in plants. A recent study on *Lotus japonicu*s, showed that K^+-dependent NSE1 asparaginase is more highly expressed than the other asparaginase in sink tissues (Credali et al., 2011). The TILLING (Targeted Induced Local Lesions I.N Genomes) procedure (Perry et al., 2003) was utilized to isolate and characterize mutants

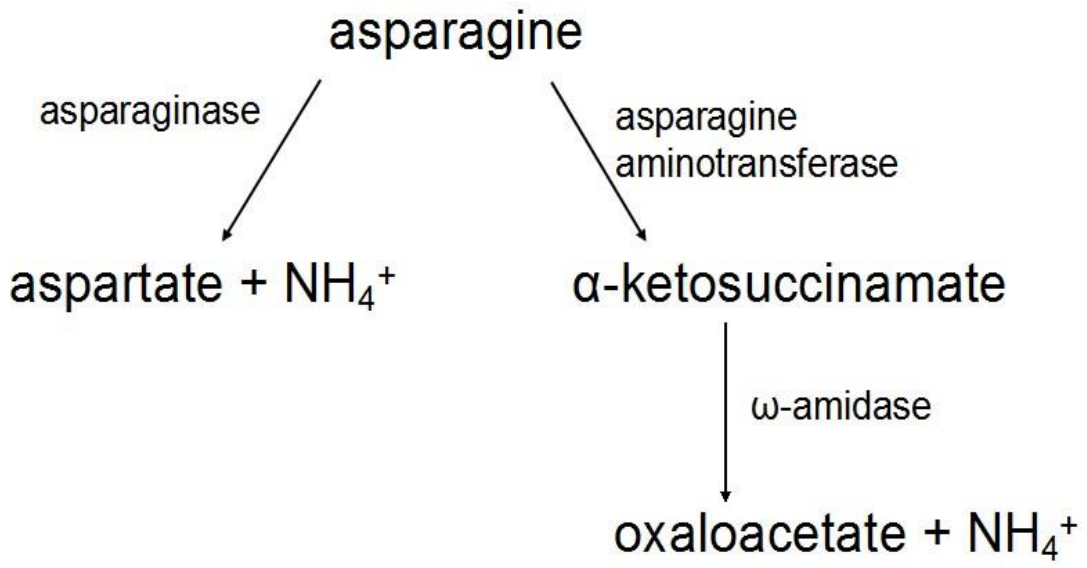

Figure 1.2 Simplified model of the Asn degradation pathway in Arabidopsis.

There are two different pathways of Asn degradation in Arabidopsis. One involves asparaginase and the other proceeds with Asn aminotransferase to make up α-ketosuccinamate, which can be further catabolized by ω-amidase

lacking NSE1 from *Lotus japonicu*s,and the results demonstrated the importance of Asn and K^+-dependent NSE1 asparaginase for nitrogen remobilization and seed production in this species (Credali et al., 2013). Analyses of metabolite and transcript investigation in germinating Arabidopsis seeds have determined that ASPGA1 is involved in the catabolism of free Asn stored during seed desiccation (Fait et al., 2006). However, recent research showed that Arabidopsis mutants lacking asparaginases develop normally compared to wild-type (Ivanov et al., 2012a), indicating that Asn aminotransferase may compensate for the absence of asparaginase in Asn metabolism.

1.5 Serine (Ser):glyoxylate Aminotransferase

Asn aminotransferase has been studied in pea and identified as same protein as Ser:glyoxylate aminotransferase based on its substrate preference and subcellular localization (Ireland and Joy, 1983b, c). Furthermore, studies showed that mutants lacking Ser:glyoxylate aminotransferase in tobacco (Havir and McHale, 1988) and barley (Murray et al., 1987b) did not have Asn aminotransferase activity. This indicated that Ser:glyoxylate aminotransferase can act as Asn aminotransferase in tobacco and barley. In Arabidopsis, Ser:glyoxylate aminotransferase is encoded by *AGT1*, and a mutant that is mutated in this gene also lacks the activity of Ser:glyoxylate aminotransferase (Somerville and Ogren, 1980). Liepman and Olsen (2001) have confirmed that *AGT1* isolated from Arabidopsis encodes a 45 kDa aminotransferase, which is consistent with the subunit size reported for the corresponding enzyme from spinach (Noguchi and Hayashi, 1980), cucumber (Hondred et al., 1985) and pea (Ireland and Joy, 1983b). *AGT1* homologs in many other plants including tobacco, barley, tomato, soybean and rice have

been found, suggesting that *AGT1*is conserved in most plants. So it probably means that AGT1 is also an Asn aminotransferase in Arabidopsis.

1.6 Photorespiration

Ser:glyoxylate aminotransferase has been proved to participate in the photorespiratory pathway (Lee et al., 2007b). Photorespiration is a process in plant metabolism that attempts to ameliorate the consequences of an oxygenation reaction by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). RubisCO acts as a carboxylase instead of an oxygenase when the active site of the enzyme binds to CO_2. The incorporation of a CO_2 molecule into ribulose-1,5-bisphosphate (RuBP), a product from Calvin cycle in photosynthesis, causes a net increase in the carbon of the plant, which produces two molecules of 3-phosphoglycerate (3PGA). In contrast, when RubisCO combines with O_2, as an oxygenase, one molecule of 3PGA and a phosphoglycolate is yielded from RuBP (Figure 1.3). Then phosphoglycolate is dephosphorylated in the chloroplast. Glycolate is transported to the peroxisome where molecular O_2 further oxidizes it to glyoxylate. Glyoxylate is further amidated to the amino acid glycine (Gly). Gly is then transported to the mitochondrial matrix where two Gly are converted into a Ser molecule. The Ser is transported into a peroxisome where it is deaminated to pyruvate, which is further reduced to glycerate. Finally, the glycerate is transported back to the chloroplast, where it is phosphorylated to 3PGA and reused in the Calvin cycle (Figure 2). Compared with the usual carboxylase activity of the Calvin cycle, the oxygenase activity leads to a net loss of carbon in the plant.

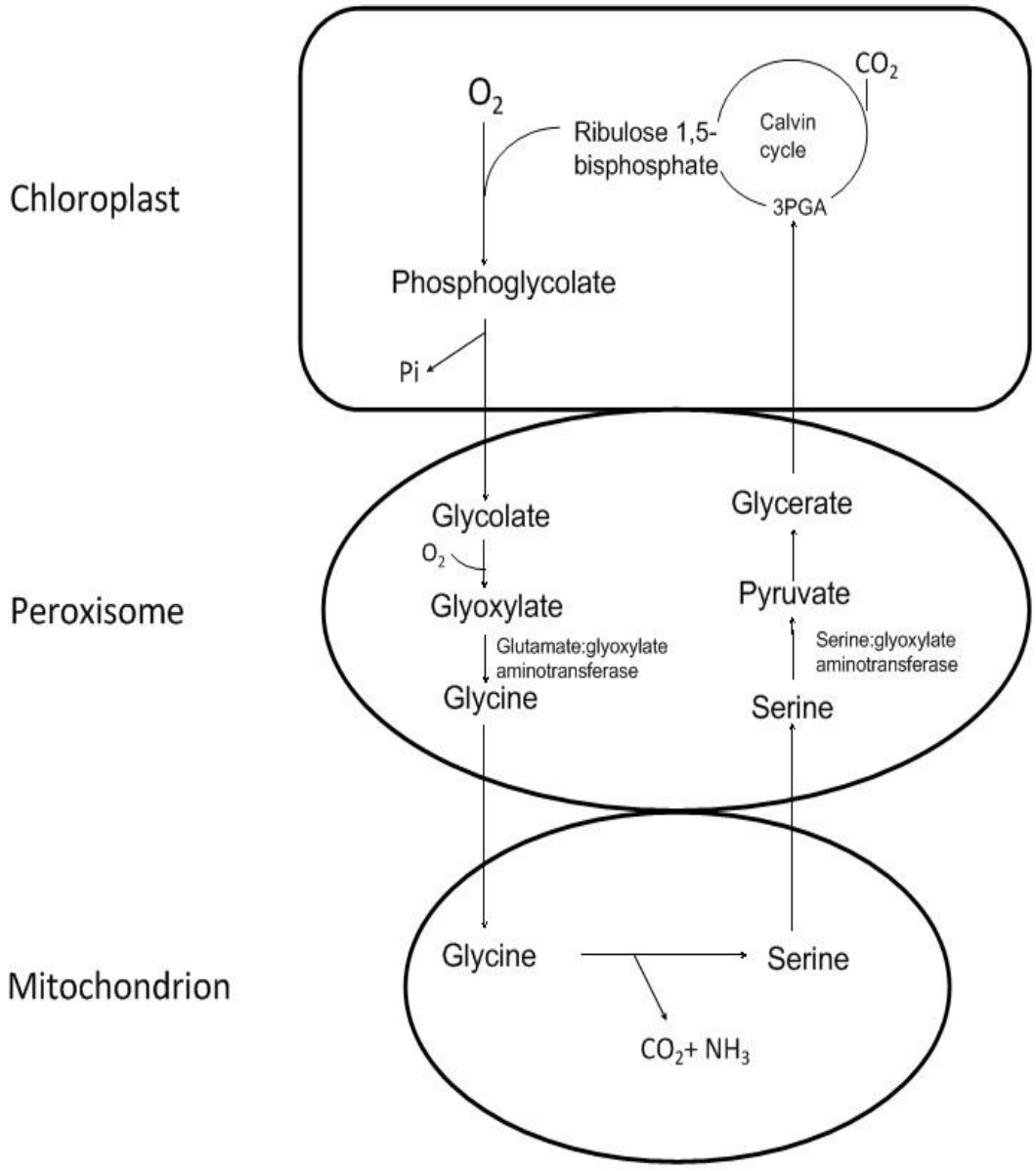

Figure 1.3. Simplified model of photorespiration.

Phosphoglycolate, product of photorespiration, is dephosphorylated in the chloroplast. Glycolate is transported to the peroxisome where molecular O_2 further oxidizes it to glyoxylate. Glyoxylate is further amidated to the amino acid glycine. Glycine is then transported to the mitochondrial matrix where two glycines are converted into a serine molecule. The serine is transported into the peroxisome where it is deaminated to pyruvate, which is further reduced to glycerate. Finally, the glycerate is transported back to the chloroplast, where it is phosphorylated to 3-phosphoglycerate (3PGA) and reused in the Calvin cycle. (It is adapted from Chrispeels and Sadava, 2003).

Although photorespiration is a wasteful process because it reduces the rate of photosynthetic CO_2 assimilation, it significantly deals with low CO_2 or high temperature stress for plants. For example, photorespiration happens on hot dry days when a plant is forced to close its stomata to prevent excess water loss. If the plant continues to attempt to fix CO_2 when its stomata are closed, the CO_2 will get used up and the O_2 ratio in the leaf will increase relative to CO_2 concentrations. When the CO_2 levels in the leaf drop to around 50 ppm atmosphere, RuBisCO starts to combine O_2 with ribulose-1,5-bisphosphate (RuBP) instead of CO_2 (Fernie et al., 2012).

In the photorespiratory pathway, two aminotransferases located in the peroxisome participate in these steps (Figure 2). One is Glu:glyoxylate aminotransferase that uses glyoxylate as the acceptor to produce Gly. The other is Ser:glyoxylate aminotransferase which can use Ser to form pyruvate and release ammonia. Previous studies have shown that Ser:glyoxylate aminotransferase can use various amino acids serving as donors, such as alanine (Ala), Ser, Asn but not Glu, with the 2-oxoacid glyoxylate as an amino acceptor (Husic et al., 1987; Liepman and Olsen, 2001b; Somerville and Ogren, 1980). The key role of Ser:glyoxylate aminotransferase in the photorespiratory pathway has been demonstrated by the air-sensitive Arabidopsis mutant, with a mutated *AGT1* gene (Somerville and Ogren, 1980). The rates of photosynthesis decrease a lot in mutant, which accumulates Ser and Gly when grown in an environment promoting photorespiration. The mutant can be stimulated to photosynthesize again when grown under conditions containing a high concentration of CO_2, where photorespiration is reduced (Somerville and Ogren, 1981). This confirms that AGT1 plays a significant role in photorespiration.

1.7 ω-Amidase

α-Ketosuccinamate (α-KSM) is produced from Asn transamination and transformed further to oxaloacetate and ammonia by ω-amidase (Krasnikov et al., 2009b). Moreover, this enzyme can also hydrolyse α-ketoglutaramate (α-KGM) as well as α-hydroxysuccinamate (α-HSM) to α-ketoglutarate and malate, respectively (Jaisson et al., 2009). Previous study has found that α-KSM can be reduced rapidly to α-HSM in pea leaves (Lloyd and Joy, 1978b). α-KSM is unstable and easily forms a dimer that can further yield a large amount of hetero-aromatic products, some of which may be toxic (Cooper et al., 1987; Stephani and Meister, 1971).

In addition, ω-amidase plays a role in converting the molecules α-KGM to α-ketoglutarate, which can be further utilized to form Gln, a significant substrate in the methionine salvage pathway in mammals (Krasnikov et al., 2009a). In the methionine salvage pathway, the methyl and sulfur of methionine are combined into 5'-methylthioadenosine during polyamine and ethylene biosyntheses, which is further transformed into α-keto-γ-methylthiobutyrate. Then methionine is formed by the transamination with Gln (Figure 1.4a). On the other hand, α-ketoglutarate, produced from the activities of Gln transamination and ω-amidase deamidation, can form Gln again by the action of Glu dehydrogenase or Glu synthase as well as Gln synthetase. Thus, the cycling of five-carbon units including α-ketoglutarate, α-KGM, Glu and Gln can be linked to the cycling of methionine in this process (Figure 1.4b). This Gln cycle is unlike other aminotransferase reactions that are freely reversible. The benefit of transamination reactions using Gln is that the reactions will be going into the direction of alpha-keto acid conversion to the corresponding amino acid, forming a reaction cycle with α-KGM. Then

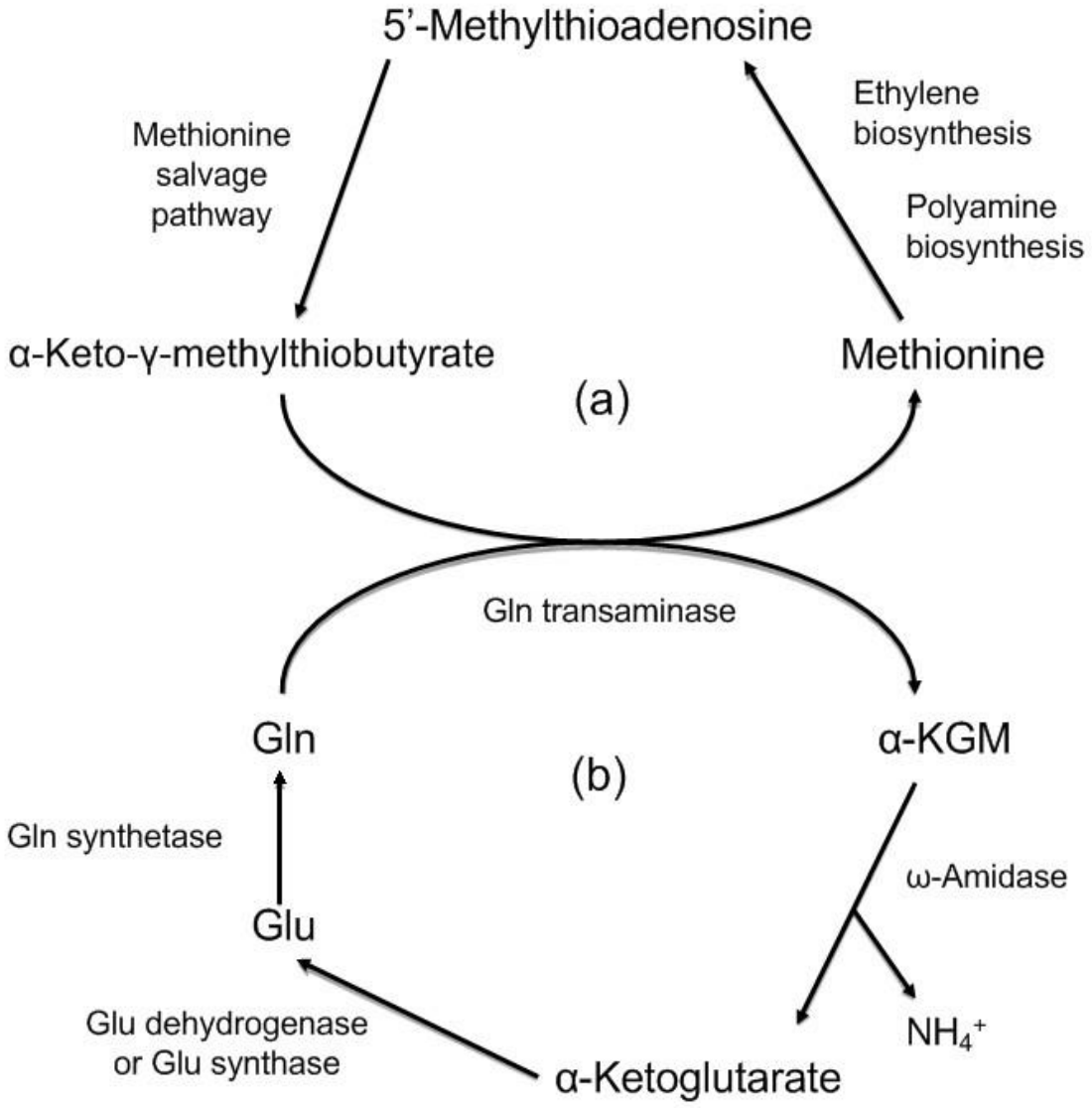

Figure 1.4. Proposed role of ω-amidase in Gln transamination connected to methionine salvage pathway.

(a) 5'-Methylthioadenosine is produced during polyamine and ethylene biosyntheses, which provides α-keto-γ-methylthiobutyrate in the following methionine salvage pathway. In mammals, the pathway is completed by a transamination with Gln. (b) α-KGM, product of Gln transamination, is hydrolyzed to α-ketoglutarate and ammonium by ω-amidase. The α-ketoglutarate is converted to Glu, which is further transformed to Gln via Gln synthetase, thus closing a cycle of Gln transamination (It is adapted form Krasnikov et al., 2009a).

α-KGM can be removed by ω-amidase (Cooper, 2004; Cooper et al., 1987) (Figure 1.4). It has been shown that ω-amidase cooperates with a Gln-dependent aminotransferase, Gln transaminase K (GTK), in mammals during Gln transamination (Cooper, 2004). The Gln transamination product, α-KGM, is present in rat tissues in micromolar concentration (Duffy et al., 1974). Previous results have indicated that excess α-KGM is toxic to rat brain (Vergara et al., 1973). Thus, ω-amidase may metabolize potentially toxic α-KGM and α-KSM to other biological compounds, such as α-ketoglutarate as well as oxaloacetate, which implies that ω-amidase is important in nitrogen metabolism.

Although a candidate gene of ω-amidase has been reported in Arabidopsis (Gerdes et al., 2011b), which was not further characterized, the activities of recombinant ω-amidase with α-KSM and α-KGM have been measured in mammals (Jaisson et al., 2009; Krasnikov et al., 2009a). Despite its versatility and high specific activity, ω-amidase activity has been little studied in Arabidopsis. Understanding the characteristics and function of ω-amidase in plants is necessary as this enzyme plays an important role in Asn transamination, by acting downstream to break down the product of Asn aminotransferase.

1.8 Hypothesis and Objectives

This study investigated the role of aminotransferase-like enzymes in the Asn metabolic pathway in Arabidopsis. As previously mentioned, Ser:glyoxylate aminotransferase (AGT1) and ω-amidase are hypothesized to be involved in Asn metabolism. It is hypothesized that the transcript levels of both genes may be regulated by Asn. It is also hypothesized that an *ω-amidase* mutant will over-accumulate substrates

of ω-amidase in the root, including α-KSM and α-HSM, compared with wild-type Arabidopsis.

The objectives of this study are to (1) quantify AGT1 as well as ω-amidase transcript levels in roots that are treated with Asn; (2) characterize recombinant AGT1 purified from *E. coli* with different amino acids including Asn as substrate; (3) identify an ω-amidase candidate gene in Arabidopsis and characterize the activities of recombinant ω-amidase from *E. coli* with different substrates; (4) investigate the concentrations of ω-amidase substrates in mutant and wild-type roots treated with or without Asn.

1.9 References

Alboresi, A., Gestin, C., Leydecker, M. T., Bedu, M., Meyer, C., Truong, H. N., 2005. Nitrate, a signal relieving seed dormancy in *Arabidopsis*. Plant, Cell Environ. 28, 500-512.

Andrews, M., 1986. Nitrate and reduced-N concentrations in the xylem sap of S*tellaria media, Xanthium strumarium* and 6 legume species. Plant Cell Environ. 9, 605-608.

Bruneau, L., Chapman, R., Marsolais, F., 2006. Co-occurrence of both L-asparaginase subtypes in *Arabidopsis*: *At3g16150* encodes a K^+-dependent L-asparaginase. Planta 224, 668-679.

Canas, R. A., Quillere, I., Lea, P. J., Hirel, B., 2010. Analysis of amino acid metabolism in the ear of maize mutants deficient in two cytosolic glutamine synthetase isoenzymes highlights the importance of asparagine for nitrogen translocation within sink organs. Plant Biotechnol. J. 8, 966-978.

Chen, L. S., Bush, D. R., 1997. LHT1, a lysine- and histidine-specific amino acid transporter in *Arabidopsis*. Plant Physiol. 115, 1127-1134.

Chrispeels, M. J., Sadava, D. E., 2003. Plants, genes, and crop biotechnology. Jones and Bartlett Publisher, Boston.

Cooper, A. J. L., 2004. The role of glutamine transaminase K (GTK) in sulfur and α-keto acid metabolism in the brain, and in the possible bioactivation of neurotoxicants. Neurochem. Int. 44, 557-577.

Cooper, A. J. L., Raps, S. P., Meister, A., 1987. Flurometric determination of α-ketosuccinamic acid in rat tissues. Anal. Biochem. 167, 312-320.

Crawford, N. M., 1995. Nitrate: nutrient and signal for plant growth. Plant Cell 7, 859-868.

Credali, A., Díaz-Quintana, A., García-Calderón, M., Rosa, M., Márquez, A., Vega, J., 2011. Structural analysis of K^+ dependence in L-asparaginases from *Lotus japonicus*. Planta 234, 109-122.

Credali, A., García-Calderón, M., Dam, S., Perry, J., Díaz-Quintana, A., Parniske, M., Wang, T. L., Stougaard, J., Vega, J. M., Márquez, A. J., 2013. The K^+-dependent asparaginase, NSE1, is crucial for plant growth and seed production in *Lotus japonicus*. Plant Cell Physiol. 54, 107-118.

Duffy, T. E., Cooper, A. J. L., Meister, A., 1974. Identification of α-ketoglutaramate in rat liver, kidney, and brain: relationship to glutamine transaminase and ω-amidase activities. J. Biol. Chem. 249, 7603-7606.

Fait, A., Angelovici, R., Less, H., Ohad, I., Urbanczyk-Wochniak, E., Fernie, A. R., Galili, G., 2006. Arabidopsis seed development and germination is associated with temporally distinct metabolic switches. Plant Physiol. 142, 839-854.

Fernie, A. R., Bauwe, H., Eisenhut, M., Florian, A., Hanson, D. T., Hagemann, M., Keech, O., Mielewczik, M., Nikoloski, Z., Peterhänsel, C., Roje, S., Sage, R., Timm, S., von Cammerer, S., Weber, A. P. M., Westhoff, P., 2012. Perspectives on plant photorespiratory metabolism. Plant Biol., 1-6.

Forsum, O., Svennerstam, H., Ganeteg, U., Näsholm, T., 2008. Capacities and constraints of amino acid utilization in *Arabidopsis*. New Phytol. 179, 1058-1069.

Gerdes, S., El Yacoubi, B., Bailly, M., Blaby, I. K., Blaby-Haas, C. E., Jeanguenin, L., Lara-Nunez, A., Pribat, A., Waller, J. C., Wilke, A., Overbeek, R., Hanson, A. D., de Crecy-Lagard, V., 2011. Synergistic use of plant-prokaryote comparative genomics for functional annotations. BMC Genomics 12 Suppl. 1, S2.

Gutiérrez, R. A., Stokes, T. L., Thum, K., Xu, X., Obertello, M., Katari, M. S., Tanurdzic, M., Dean, A., Damion, C. N., McClung, C. R., Coruzzi, G. M., 2008. Systems approach identifies an organic nitrogen-responsive gene network that Is regulated by the master clock control gene CCA1. Proc. Natl. Acad. Sci. U. S. A. 105, 4939-4944.

Havir, E. A., McHale, N. A., 1988. A mutant of *Nicotiana sylvestris* lacking serine:glyoxylate aminotransferase: substrate specificity of the enzyme and gate of [2-^{14}C] glycolate in plants with genetically altered enzyme levels. Plant Physiol. 87, 806-808.

Hirner, A., Ladwig, F., Stransky, H., Okumoto, S., Keinath, M., Harms, A., Frommer, W. B., Koch, W., 2006. *Arabidopsis* LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. Plant Cell 18, 1931-1946.

Hondred, D., Hunter, J. M., Keith, R., Titus, D. E., Becker, W. M., 1985. Isolation of serine:glyoxylate aminotransferase from cucumber cotyledons. Plant Physiol. 79, 95-102.

Husic, D. W., Husic, H. D., Tolbert, N. E., Black, C. C., 1987. The oxidative photosynthetic carbon cycle or C2 cycle. Crit. Rev. Plant Sci. 5, 45-100.

Ireland, R., Joy, K., 1981. Two routes for asparagine metabolism in *Pisum sativum* L. Planta 151, 289-292.

Ireland, R. J., Joy, K. W., 1983a. Purification and properties of an asparagine aminotransferase from *Pisum sativum* leaves. Arch. Biochem. Biophys. 223, 291-296.

Ireland, R. J., Joy, K. W., 1983b. Subcellular localization of asparaginase and asparagine aminotransferase in *Pisum sativum* leaves. Plant Physiol. 72, 1127-1129.

Ivanov, A., Kameka, A., Pajak, A., Bruneau, L., Beyaert, R., Hernández-Sebastià, C., Marsolais, F., 2012. *Arabidopsis* mutants lacking asparaginases develop normally but exhibit enhanced root inhibition by exogenous asparagine. Amino Acids 42, 2307-2318.

Jaisson, S., Veiga-da-Cunha, M., Van Schaftingen, E., 2009. Molecular identification of ω-amidase, the enzyme that is functionally coupled with glutamine transaminases, as the putative tumor suppressor Nit2. Biochimie 91, 1066-1071.

Koornneef, M., Meinke, D., 2010. The development of *Arabidopsis* as a model plant. Plant J. 61, 909-921.

Krasnikov, B. F., Chien, C. H., Nostramo, R., Pinto, J. T., Nieves, E., Callaway, M., Sun, J., Huebner, K., Cooper, A. J. L., 2009a. Identification of the putative tumor suppressor Nit2 as ω-amidase, an enzyme metabolically linked to glutamine and asparagine transamination. Biochimie 91, 1072-1080.

Krasnikov, B. F., Nostramo, R., Pinto, J. T., Cooper, A. J. L., 2009b. Assay and purification of ω-amidase/Nit2, a ubiquitously expressed putative tumor suppressor, that catalyzes the deamidation of the α-keto acid analogues of glutamine and asparagine. Anal. Biochem. 391, 144-150.

Lee, Y.-H., Foster, J., Chen, J., Voll, L. M., Weber, A. P. M., Tegeder, M., 2007. AAP1 transports uncharged amino acids into roots of *Arabidopsis*. Plant J. 50, 305-319.

Li, B., Xin, W., Sun, S., Shen, Q., Xu, G., 2006. Physiological and molecular responses of nitrogen-starved rice plants to re-supply of different nitrogen sources. Plant Soil 287, 145-159.

Liepman, A. H., Olsen, L. J., 2001. Peroxisomal alanine:glyoxylate aminotransferase (AGT1) is a photorespiratory enzyme with multiple substrates in *Arabidopsis thaliana*. Plant J. 25, 487-498.

Lloyd, N. D. H., Joy, K. W., 1978. 2-Hydroxysuccinamic acid: a product of asparagine metabolism in plants. Biochem. Biophys. Res. Commun. 81, 186-192.

Meister, A., Sober, H. A., Tice, S. V., Fraser, P. E., 1952. Transamination and associated deamidation of asparagine and glutamine. J. Biol. Chem. 197, 319-330.

Murray, A. J. S., Blackwell, R. D., Joy, K. W., Lea, P. J., 1987. Photorespiratory N donors, aminotransferase specificity and photosynthesis in a mutant of barley deficient in serine:glyoxylate aminotransferase activity. Planta 172, 106-113.

Näsholm, T., Kielland, K., Ganeteg, U., 2009. Uptake of organic nitrogen by plants. New Phytol. 182, 31-48.

Noguchi, T., Hayashi, S., 1980. Peroxisomal localization and properties of tryptophan aminotransferase in plant leaves. J. Biol. Chem. 255, 2267-2269.

Perry, J. A., Wang, T. L., Welham, T. J., Gardner, S., Pike, J. M., Yoshida, S., Parniske, M., 2003. A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus*. Plant Physiol. 131, 866-871.

Schultz, C. J., Hsu, M., Miesak, B., Coruzzi, G. M., 1998. *Arabidopsis* mutants define an in vivo role for isoenzymes of aspartate aminotransferase in plant nitrogen assimilation. Genetics 149, 491-499.

Scott, D. B., Farnden, K. J. F., Robertson, J. G., 1976. Ammonia assimilation in lupin nodules. Nature 263, 703-705.

Sodek, L., Lea, P. J., Miflin, B. J., 1980. Distribution and properties of a potassium-dependent asparaginase isolated from developing seeds of *Pisum sativum* and other plants. Plant Physiol. 65, 22-26.

Somerville, C., Koornneef, M., 2002. A fortunate choice: the history of *Arabidopsis* as a model plant. Nat. Rev. Genet. 3, 883-889.

Somerville, C. R., Ogren, W. L., 1980. Photorespiration mutants of *Arabidopsis thaliana* deficient in serine-glyoxylate aminotransferase activity. Proc. Natl. Acad. Sci. U. S. A. 77, 2684-2687.

Somerville, C. R., Ogren, W. L., 1981. Photorespiration-deficient mutants of *Arabidopsis thaliana* lacking mitochondrial serine transhydroxymethylase activity Plant Physiol. 67, 666-671.

Stephani, R. A., Meister, A., 1971. Structure of the dimeric α-keto acid analogue of asparagine. J. Biol. Chem. 246, 7115-7118.

Stitt, M., Müller, C., Matt, P., Gibon, Y., Carillo, P., Morcuende, R., Scheible, W. R., Krapp, A., 2002. Steps towards an integrated view of nitrogen metabolism. J. Exp. Bot. 53, 959-970.

Vergara, F., Duffy, T. E., Plum, F., 1973. α-Ketoglutaramate, a neurotoxic agent in hepatic coma. Trans. Assoc. Am. Physicians 86, 255-263.

Walch-Liu, P., Neumann, G., Bangerth, F., Engels, C., 2000. Rapid effects of nitrogen form on leaf morphogenesis in tobacco. J. Exp. Bot. 51, 227-237.

Wang, R., Tischner, R., Gutiérrez, R. A., Hoffman, M., Xing, X., Chen, M., Coruzzi, G., Crawford, N. M., 2004. Genomic analysis of the nitrate response using a nitrate reductase-null mutant of *Arabidopsis*. Plant Physiol. 136, 2512-2522.

Waterhouse, R. N., Smyth, A. J., Massonneau, A., Prosser, I. M., Clarkson, D. T., 1996. Molecular cloning and characterisation of asparagine synthetase from *Lotus japonicus*: dynamics of asparagine synthesis in N-sufficient conditions. Plant Mol. Biol. 30, 883-897.

Zhang, H., Forde, B. G., 1998. An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. Science 279, 407-409.

Chapter 2. Characterization of Arabidopsis serine:glyoxylate aminotransferase, AGT1, as an asparagine aminotransferase.

2.1 Introduction

There are two catabolic pathways of Asn, a major storage form of organic nitrogen that is commonly transported throughout the plant to sink tissue (Lea et al., 2007). One proceeds via asparaginase, which catalyzes the hydrolysis of Asn to Asp and releases ammonia (Michalska and Jaskolski, 2006b). The other goes through Asn aminotransferase and results in the formation of α-ketosuccinamic acid. Results from a recent study showed that Arabidopsis mutants lacking asparaginases develop relatively normally (Ivanov et al., 2012b). This suggests that Asn aminotransferase may be able to compensate for their absence, and may contribute significantly to Asn catabolism.

Early research on Asn metabolism in pea indicated that asparaginase is predominant in young leaves and developing seeds, while Asn aminotransferase predominates in older leaves and pods (Ireland and Joy, 1981b). Asn aminotransferase was characterized from pea leaves and appeared to be the same protein as the peroxisomal photorespiratory enzyme, Ser:glyoxylate aminotransferase [EC 2.6.1.45], based on its substrate preference and subcellular localization (Ireland and Joy, 1983a, d). Indeed, tobacco and barley mutants lacking Ser:glyoxylate aminotransferase were devoid of Asn aminotransferase activity (Havir and McHale, 1988; Murray et al., 1987b). These mutants also lacked Ala:hydroxypyruvate aminotransferase activity but had normal Glu:glyoxylate and Ala:glyoxylate activities. Ser:glyoxylate aminotransferase has a high level of Ala:glyoxylate aminotransferase activity, however, the latter activity is also

catalyzed by the peroxisomal Glu:glyoxylate aminotransferases (Igarashi et al., 2006) and the recently characterized chloroplastic or mitochondrial Ala aminotransferases (Niessen et al., 2012). In Arabidopsis, Ser:glyoxylate aminotransferase is encoded by *AGT1*, and plants mutated in this gene do not have Ser:glyoxylate aminotransferase activity (Somerville and Ogren, 1980). Although it is probable that AGT1 is also an Asn aminotransferase in Arabidopsis, when the catalytic activities of recombinant AGT1 purified from *Escherichia coli* and of native AGT1 purified from leaves were characterized, Asn was not included as substrate (Kendziorek and Paszkowski, 2008a; Liepman and Olsen, 2001a). As part of photorespiratory nitrogen cycling, Asn aminotransferase was hypothesized to have a relatively small contribution to net Asn catabolism (Lea et al., 2007). However, studies with [^{15}N]Asn have revealed a small but significant contribution of Asn to photorespiratory Gly formation (Ta and Joy, 1985, 1986; Ta et al., 1985). The significance of Asn transamination is related to an import of transported Asn into photorespiration. According to this view, photorespiratory nitrogen metabolism is not a closed cycle but receives inputs from external nitrogen. This has also been demonstrated for Ala (Betsche, 1983; Masclaux-Daubresse et al., 2006). Increased flux from Asn or Ala will compensate for the withdrawal of Gly or Ser for the biosynthesis of other amino acids, including Cysteine. In this way, the amino group from Asn can be used directly for the biosynthesis of other amino acids. In addition, it is possible that *AGT1* is expressed in other tissues than the leaf and may contribute to Asn metabolism in a different context from photorespiration. Indeed an examination of *AGT1* transcript expression during development reveals relatively high levels in the root cortex and epidermis, developing siliques, and dry seed (Gifford et al., 2008; Nakabayashi et al.,

2005; Schmid et al., 2005) [visualized with the Arabidopsis eFP Browser at the Botany Array Resource, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Winter et al., 2007)].

The results of recent research have shown that plants can acquire organic nitrogen as amino acids from the soil (Näsholm et al., 2009). LHT1 and AAP1 act as high and low affinity transporters for the uptake of neutral amino acids in Arabidopsis root, respectively (Hirner et al., 2006; Lee et al., 2007a; Svennerstam et al., 2007). While Asn can support the growth of Arabidopsis as a unique nitrogen source until ca. 21 days (Forsum et al., 2008), it can inhibit growth as compared with Glu, Gln, Asp or γ-aminobutyric acid (Hirner et al., 2006). Growth on Asn as a single nitrogen source for ten days inhibits root elongation and delays root hair formation as compared with control conditions without nitrogen (Ivanov et al., 2012b). The *aspga1-1*/*-b1-1* mutant in which both asparaginase genes have been inactivated is more sensitive to this inhibition, and this is correlated with an over-accumulation of Asn in root by approximately 2-fold, as compared with the wild-type. These data support a function of asparaginases in Asn catabolism in root. As an organic form of nitrogen, like Glu or Gln (Gutiérrez et al., 2008b), Asn may elicit transcriptional changes regulating nitrogen metabolism and growth. Some may be specific to Asn, based on the distinct root physiological response.

The objective of the present study was to characterize the catalytic activity of recombinant AGT1 purified from *E. coli* with Asn as substrate. In addition, the effect of Asn on transcript levels of *AGT1*, asparaginases and several other nitrogen metabolic gene markers was investigated in root.

2.2 Experimental method

2.2.1 Plant material and growth conditions

Arabidopsis ecotype Columbia seeds were grown on vertical plates at 22 °C under continuous light (approximately 70 µmol m^{-2} s^{-1}), as previously described by Ivanov et al. (2012b), on a defined medium containing 5 mM potassium nitrate for 10 days (Wang et al., 2003). At the end of the 10-day period, seedlings were transferred to plates containing the same medium without nitrogen as control or with 20 mM Asn as the sole source of nitrogen. Root tissue was harvested after 2 h, immediately frozen in liquid nitrogen and stored at −80°C until free amino acid or RNA extraction was performed. For gene expression analyses, the experimental unit consisted in a plate with approximately 30 seedlings.

2.2.2 AGT1 construct

A full-length *AGT1* cDNA (stock number U10095) was obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/). The plasmid was used as template for amplification by PCR for subcloning into the bacterial expression vector pQE30 (Qiagen, Toronto, ON). The PCR was performed using primers SGAT*Bam*HI-Fw, 5'-CTACGGATCCATGGACTATATGTATGGACCAGG-3', and SGAT*Pst*I-Rvs, 5'-GTAGCTGCAGTTAGATTCTAGAGGGAATGAGAGG-3', and Pfx50 DNA polymerase (Life Technologies, Burlington, ON). Purified full-length products were cloned into the pCR4Blunt-TOPO vector and the cDNA was sequenced with a 3130XL Genetic Analyzer (Life Technologies). The insert was digested with *Bam*HI and *Pst*I and

subcloned at the corresponding sites of the polylinker in pQE30. The construct was transformed into XL10-Gold cells (Agilent Technologies, Mississauga, ON).

2.2.3 Protein purification

Recombinant AGT1 was produced and purified as described by Bruneau et al. (2006) with minor modifications. Cells were lysed with a French press. Purified protein was desalted in 50 mM Tris-HCl pH 8.0, containing 10 mM ethylenediaminetetraacetic acid, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 14 mM β-mercaptoethanol using a PD-10 column (GE Healthcare Biosciences, Baie d'Urfé, QC), and concentrated using an Amicon Ultra-4 centrifugal filter (Millipore, Billerica, MA). The purified protein was flash frozen in liquid nitrogen and stored at −80 °C in 10% glycerol (v/v). Protein quantification and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as previously described (Bruneau et al., 2006).

2.2.4 Aminotransferase assays

Aminotransferase activities were assayed at 30 ºC and measured by monitoring the decrease in NADH absorption using auxiliary enzymes (Figure 2.1) by spectrophotometry at 340 nm with a Power Wave XS 96-well plate reader (Bio-Tek Instruments, Winooski, VT). The path length was measured to correct the calculated enzyme activities using the path length correction feature in KC junior software. Ala:glyoxylate and Ala:hydroxypyruvate aminotransferase activity was determined in a continous assay by monitoring the production of pyruvate using lactate dehydrogenase (Figure 2.1a and -b). Likewise, Ser:glyoxylate, Ser:pyruvate, Gly:pyruvate and Gly:hydroxypyruvate aminotransferase activities were determined in a continous assay by

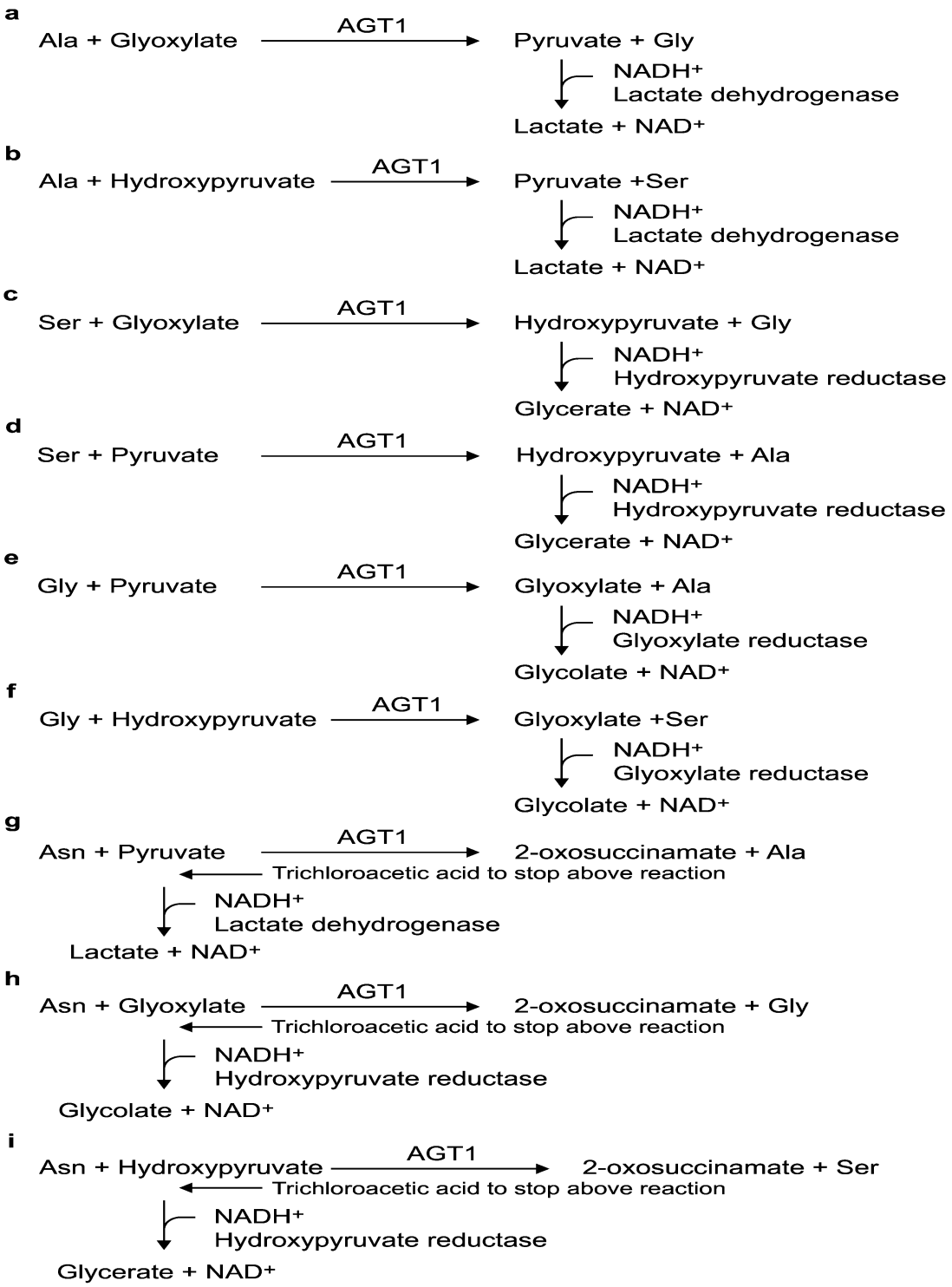

Figure 2.1 Aminotransferase reactions monitored with each pair of substrate.

(a) Ala and glyoxylate; (b) Ala and hydroxypyruvate; (c) Ser and glyoxylate; (d) Ser and pyruvate; (e) Gly and pyruvate; (f) Gly and hydroxypyruvate; (g) Asn and pyruvate; (h) Asn and glyoxylate; (i) Asn and hydroxypyruvate.

monitoring the production of hydroxypyruvate or glyoxylate using glyoxylate/hydroxypyruvate reductase (Figure 2.1c-f) (Liepman and Olsen, 2001a). Assays contained 0.6 µg AGT1 in 77 mM potassium phosphate pH 8.0, 17 mM NADH, 20 µM pyridoxal 5'-phosphate, 20 mM organic acid, and 0.03 units rabbit muscle L-lactate dehydrogenase (Roche Applied Science, Laval, QC) or 0.05 units of recombinant human glyoxylate reductase/hydroxypyruvate reductase (US Biological, Salem, MA) in a final volume of 250 µl. The reaction was started by adding the amino acid substrate at a concentration ranging between 1 and 20 mM. For reactions with Asn or Gln as the amino group donor, the rate was determined by quantifying the residual organic acid substrates after transamination was stopped (Figure 2.1g-i). The reaction was started by addition of 2-oxoacid, and stopped after 15 min by addition of 100 µl of 10% (w/v) trichloroacetic acid. Asn:pyruvate aminotransferase activity was determined using lactate dehydrogenase (Figure 2.1g). Similarly, Asn:glyoxylate and Asn:hydroxypyruvate aminotransferase activities were measured using hydroxypyruvate reductase (Figure 2.1h and -i). Reverse reactions of Asn aminotransferase activities were monitored by measuring the products pyruvate, glyoxylate and hydroxypyruvate using continuous assays as mentioned previously.

2.2.5 Synthesis of α-ketosuccinamate

α-KSM was prepared by oxidation of Asn as described by Jaisson et al. (2009). Briefly, 5 ml of 0.2 M Asn, pH 7, was incubated overnight at 37 °C under agitation with 10 units of catalase and 10 mg of *Crotalus adamanteus* L-amino acid oxidase (Sigma-Aldrich, Oakville, ON). After incubation, the solution was deproteinized by addition of 250 µl of 7 M perchloric acid and centrifuged at 15,000 × *g* for 15 min at 4 °C. The

product was separated from unreacted Asn by applying the supernatant to a 5 ml Dowex 50WX8-100 cation exchange column (Sigma-Aldrich). The eluate was neutralized with 3 M K_2CO_3 and the $KClO_4$ precipitate was removed by centrifugation at 15,000 × *g* for 15 min at 4 °C. The concentration of the prepared α-KSM was determined by spectrophotometry using an endpoint assay with a recombinant ω-amidase (Zhang and Marsolais, unpublished results) as described in Jaisson et al. (2009).

2.2.6 Amino acid analysis

Free Asn was quantified from root tissue as described in Ivanov et al. (2012b), by HPLC after derivatization with *o*-phthalaldehyde and 3-mercaptopropionic acid.

2.2.7 RNA isolation and gene expression analysis

RNA was extracted from 100 mg of root tissue using the RNeasy Plant Mini Kit (Qiagen). RNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and its quality evaluated from A_{260}/A_{280} ratio and by agarose gel electrophoresis. Ten micrograms of RNA was treated with amplification grade DNase I (Life Technologies). The Thermoscript RT-PCR System (Life Technologies) was used to synthesize cDNA from 4 µg total RNA in a 20 µl reaction volume. For RT-qPCR, *Actin2* was used as reference gene. Primers were:

F: 5'-CACAACAGCAGAGCGGGAAATT-3'
R: 5'-TTCTGGGCATCTGAATCTCTCA-3' for *Actin2*;

F: 5'-TCTCGTTCCTTCTCCTCGCTCATT-3',
R: 5'-GGACGTTACGGTTATGGCTCTCG-3' for *NR1*;

F: 5'-CGAGGAGTATGGTGTGGATTTT-3',
R: 5'-TAGTCCCCAACCAATGTAGAGC-3' for *ASN1*;

F: 5'-CACGAAACCGCAGACATCAAC-3',
R: 5'-TACCCTTTACCTTCCTTCTCTG-3' for *GLN1.3*;

F: 5'-TTGCTTCGGTTCACTTCGTCCTC-3',
R: 5'-CCGGCTGTTGTTTTCGCTTTGT-3' for *LHT1*;

F: 5'-CACAACGAGACCGCGACCG-3',
R: 5'-CACCGTCCACTAGCAGCAAAG-3' for *AGT1*;

F: 5'-GTCAGTATGCCGTAAGAGGTG-3',
R: 5'-CTGGCGAGGAAATGTCAATG-3 for *GGT1*;

F: 5'- TTGACTGAGGCAGCGGCTTA-3',
R: 5'-TCGCTAGCACAAGCCCTGAA-3' for *ASPGA1*;

F: 5'-TGGTGTGTCGTGTACCGGAG-3',
R: 5'- GCAATGAGTCCAGCGAACCC-3' for *ASPGB1*.

The cDNA samples were diluted 8-fold and 2 µl were amplified with 5 µl of SsoFast EvaGreen Supermix in a final volume of 10 µl using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON). In each plate, controls without template were performed in duplicate. Reactions contained primers at a concentration of 0.5 µM. They were carried out in Hard-Shell 96-well clear PCR plates (Bio-Rad Laboratories). The PCR program consisted of an initial step of 3 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 30 s at 58.5 °C. Data was analyzed with the CFX Manager 2.0 software. Data was expressed as the cycle number required for reaching a threshold fluorescence value (C_t). Data was normalized to the mean C_t of the reference gene *Actin2*, for which variation between samples was ≤ 20%. The specificity of primer pairs was confirmed by melt curve analysis in comparison with controls without template. PCR efficiency was calculated from a standard curve of C_t versus the logarithm of

starting template quantity. Each assay was optimized so that efficiency ranged between 100% and 107%, with a coefficient of determination (R^2) > 0.98.

2.2.8 Statistical analysis

Unpaired, two-tailed t-test assuming homogeneity of the variances was performed with the Excel software (Dytham, 2011). Analysis of variance followed by LSD test as post hoc test was performed using SAS version 9.2 (Toronto, ON).

2.2.9 Accession numbers

Sequence data can be found under accession numbers (TAIR ID) At2g13360 for *AGT1*; (TAIR ID) At3G18780 for *Actin2*; (TAIR ID) At1g77760 for *NR1*; (TAIR ID) At3g47340 for *ASN1*; (TAIR ID) At3g17820 for *Gln1.3*; (TAIR ID) At5g40780 for *LHT1*; (TAIR ID) At1g23310 for *GGT1*; (TAIR ID) At3g16150 for *ASPGB1*; (TAIR ID) At5g08100 for *ASPGA1*.

2.3 Results and Discussion

2.3.1 Catalytic activity of recombinant AGT1 with Asn as substrate

To determine whether AGT1 has Asn aminotransferase activity, AGT1 was produced as an N-terminal His-tagged protein in *Escherichia coli* and purified by affinity chromatography on Ni-agarose. The purified protein had an apparent molecular mass of ca. 45 kDa (Figure 2.2), which corresponds to the value predicted for the tagged protein. First, catalytic activities were assayed that had been previously determined with recombinant N-terminal His-tagged AGT1 and native AGT1 purified from leaf (Kendziorek and Paszkowski, 2008a; Liepman and Olsen, 2001a). The apparent V_{max} value determined with Ser and glyoxylate, of 3.32×10^{-8} katal per mg protein, was in the same range as that of the native enzyme purified from leaf, equal to 12.7×10^{-8} katal mg^{-1} (Kendziorek and Paszkowski, 2008a). Apparent kinetic parameters determined for the amino acid donor, at a fixed concentration of 20 mM organic acid acceptor, were comparable for Ser:glyoxylate, Ala:glyoxylate, Ser:pyruvate and Ala:hydroxypyruvate aminotransferase activities (Table 2.1, Figure 2.1). Using glyoxylate as acceptor, Kendziorek and Paszkowski had reported a ca. 2-fold higher value of catalytic efficiency (V_{max} /K_m) for Ala vs. Ser, as compared with ca. 4-fold difference in the present study. K_m values were also similar to those determined in previous studies, with one exception. Liepman and Olsen (2001a) had reported a low affinity of the purified recombinant enzyme for Ala as substrate, with an apparent K_m value of approximately 100 mM, and therefore a strong substrate preference for Ser over Ala. By contrast, Kendziorek and Paszkowski (2008a) had observed similar K_m values for Ala and Ser, as in the present study. They had hypothesized that the N-terminal His-tag in recombinant AGT1 may

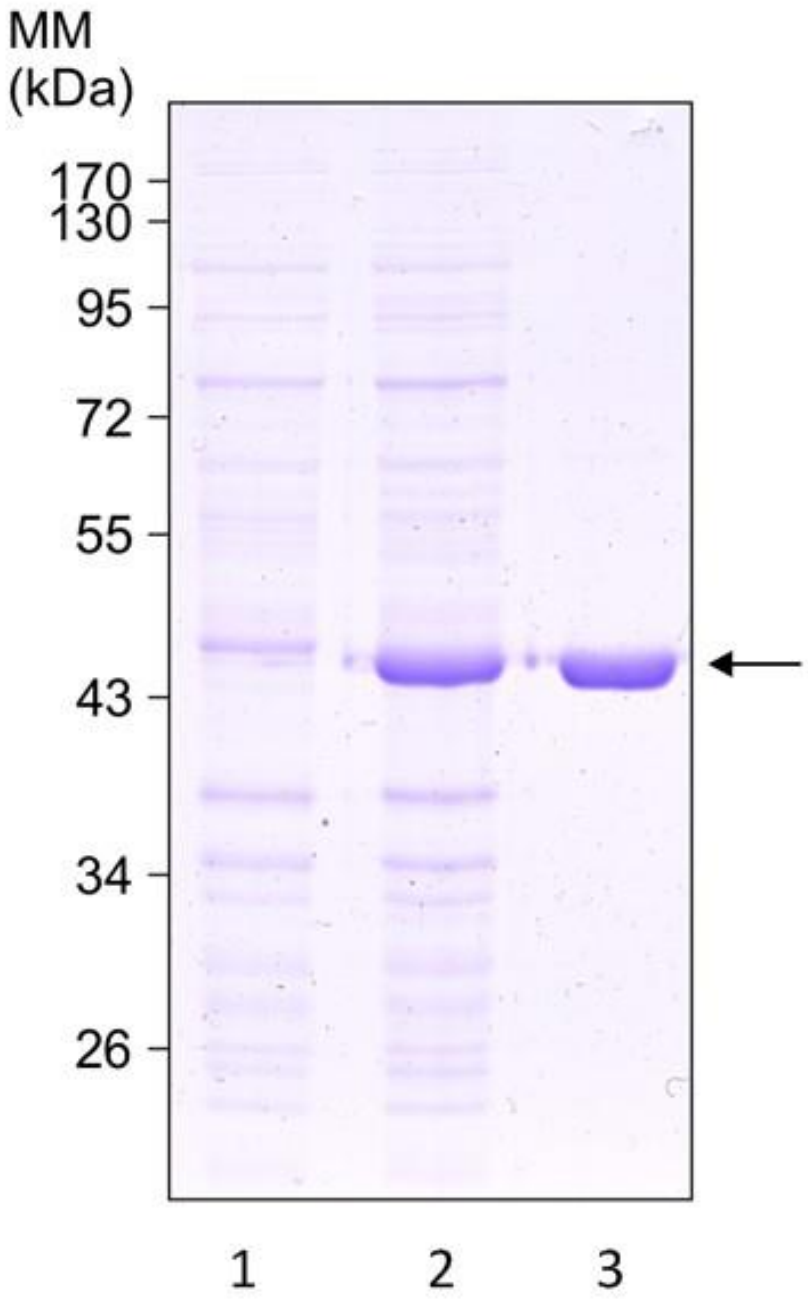

Figure 2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant AGT1.
Lane 1 shows a soluble protein extract from non-induced cells and lane 2 from induced cells. Lane 3 shows the affinity-purified AGT1. Position of the purified protein is marked by an arrow. Molecular mass (MM) markers are indicated on the left.

Table 2.1 Apparent kinetic parameters of recombinant AGT1 with different amino acids as substrates.

Values are the means ± s.d. of three replicates. Activity is expressed per mg protein.

amino acid donors	organic acid acceptors[a]	V_{max} ($\times 10^{-8}$ katal mg^{-1})	K_m (mM)	V_{max} / K_m ($\times 10^{-8}$ katal mg^{-1} mM^{-1})
Ala	glyoxylate	5.02 ± 0.24	0.87 ± 0.09	5.82 ± 0.79
Ala	hydroxypyruvate	7.38 ± 0.61	1.08 ± 0.09	6.86 ± 0.23
Ser	glyoxylate	3.32 ± 0.04	2.47 ± 0.23	1.35 ± 0.12
Ser	pyruvate	3.70 ± 0.19	0.99 ± 0.16	3.80 ± 0.54
Gly	pyruvate	0.84 ± 0.02	0.59 ± 0.02	1.43 ± 0.03
Gly	hydroxypyruvate	0.38 ± 0.04	1.58 ± 0.18	0.24 ± 0.01
Asn	glyoxylate	29.2 ± 1.0	2.82 ± 0.16	10.4 ± 0.20
Asn	pyruvate	70.4 ± 0.5	8.41 ± 0.06	8.38 ± 0.03
Asn	hydroxypyruvate	35.8 ± 0.5	3.79 ± 0.06	9.45 ± 0.02
	LSD[b]	0.81	0.23	0.58

[a] Concentration of organic acceptor was equal to 20 mM except for hydroxypyruvate with Gly at 1 mM. [b] at $p \leq 0.05$.

interfere with binding and catalytic activity with Ala. This conclusion is not supported by the present results, which indicate a slight substrate preference of the N-terminal His-tagged enzyme for Ala over Ser. Activities measured with Gly and pyruvate or hydroxypyruvate as substrates were lower than those of the forward reactions with Ala:glyoxylate and Ser:glyoxylate, respectively. Gly:hydroxypyruvate transamination was detected only at a lower concentration of organic acid (1 mM) as compared with other reactions where organic acid concentration was kept at 20 mM, consistent with the results reported by Kendziorek and Paskowski (2008a). These data indicate that Ser:glyoxylate and Ala:glyoxylate transamination are more likely to occur *in vivo* than are the reverse reactions. Nevertheless, the K_m values for Gly were relatively low, suggesting that Gly may be used as amino group donor under certain conditions.

The catalytic activity of AGT1 was then assayed with Asn as an amino group donor and pyruvate, glyoxylate or hydroxypyruvate as organic acid acceptors (Table 2.1, Figure 2.1). The apparent V_{max} value determined with Asn and glyoxylate, of 29.2×10^{-8} katal mg^{-1}, was in the same range as the specific activity determined with Asn aminotransferase purified from pea leaves, equal to 65.5×10^{-8} katal mg^{-1} (Ireland and Joy, 1983a). While apparent K_m values for Asn were comparable with those determined with Ala or Ser, values of V_{max} were consistently higher, by ca. 6-fold to 9-fold with glyoxylate as the acceptor substrate, 19-fold with pyruvate, and 5-fold with hydroxypyruvate. As a result, values of catalytic efficiency (V_{max} / K_m) were slightly higher with Asn as compared with Ala or Ser. AGT1 had very low catalytic activity with Gln as the amino group donor tested at 20 mM and glyoxylate or pyruvate as acceptors tested at 5 mM, equal to 5 and 4×10^{-10} katal mg^{-1}, respectively. Catalytic activities were

undetectable for the reverse reactions leading to Asn formation. This is consistent with the results of Ireland and Joy (1983a) who measured activities for these reactions that were equal to only a few percent of the value measured with Ser and glyoxylate as substrates. Therefore, AGT1, as an Asn aminotransferase, is expected to function exclusively as a catabolic enzyme in Asn metabolism. Asn aminotransferase activities measured with glyoxylate, pyruvate and hydroxypyruvate as amino group acceptors are consistent with the incorporation of [^{15}N] label from Asn into Gly, Ser and Ala measured in pea leaves, which were reduced by the aminotransferase inhibitor, aminooxyacetate (Ta and Joy, 1985).

The kinetic parameters determined with Asn as substrate (Table 1) were comparable to those of the recombinant K^+-dependent asparaginase, ASPGB1, having a K_m of 6.83 mM and V_{max} of 65.9× 10^{-8} katal mg^{-1} (Gabriel et al., 2012b). By comparison, the recombinant K^+-independent asparaginase, ASPGA1, has a lower V_{max}, of 1.24 × 10^{-8} katal mg^{-1}, and a K_m value of 14.7 mM. Asparaginases have a dual catalytic activity with β-aspartyl dipeptides. However, ASPGB1 has a strong substrate preference for Asn as compared with β-aspartyl-histidine, with a ca. 16-fold higher value of catalytic efficiency.

Asn transamination leads to the formation of α-KSM (Figure 2.1e-g), which can be further deamidated by an ω-amidase. This activity has been characterized from soybean leaves (Streeter, 1977). According to data obtained with radiolabeled precursors in pea leaves, α-KSM can be rapidly reduced to α-HSM, which may also be deamidated (Lloyd and Joy, 1978a). ω-Amidase has been characterized at the molecular level from mouse (Jaisson et al., 2009; Krasnikov et al., 2009a) and a candidate gene has been

reported in Arabidopsis (Gerdes et al., 2011b). Further research is required to identify and characterize an ω-amidase gene and enzyme involved in Asn catabolism in higher plants.

2.3.2 Increased levels of AGT1 transcripts in response to Asn in root

To determine if *AGT1* is transcriptionally regulated by Asn, seedlings were grown for 10 days in a medium containing 5 mM potassium nitrate (Ivanov et al., 2012). At the end of the 10-day period, plants should have essentially depleted the nitrate present in the medium (Wang et al., 2003). Seedlings were transferred to media without nitrogen as control or containing Asn at a concentration of 20 mM for 2 h, and roots were harvested. Under these conditions, the Asn concentration in root was raised from 1.83 nmol per mg fresh weight to 8.71 nmol mg^{-1} (ca. 5-fold) (n = 3; t-test p value ≤ 0.0003). Similar results were obtained with the *aspga1-1*/*-b1-1* mutant: Asn concentration was raised from 1.42 nmol mg^{-1} to 6.02 nmol mg^{-1} (ca. 4-fold) (n = 3; t-test p value ≤ 0.0001). Therefore, the mutant did not over-accumulate Asn under these conditions, unlike after a 10-day growth period (Ivanov et al., 2012b), and experiments were performed only with the wild-type.

It is well established that nitrate, nitrite and ammonium elicit transcriptional responses in Arabidopsis (Patterson et al., 2010; Wang et al., 2004; Wang et al., 2007). The use of a nitrate reductase-null mutant, which over-accumulates nitrate (Unkles et al., 2004), has facilitated the analysis of nitrate-specfic responses. Recently, the nitrate transporter NRT1.1 has been identified as a transceptor mediating transcriptional responses to nitrate (Ho et al., 2009; Wang et al., 2009). Organic nitrogen in the form of amino acids also elicits transcriptional responses. Glu, Gln and Asn positively regulate the levels of *ASN1* transcripts encoding asparagine synthetase (Lam et al., 1994). The

global transcriptional response to organic nitrogen as Glu or downstream metabolites has been investigated in whole plants, and compared to that of inorganic nitrogen (Gutiérrez et al., 2008b). The study relied on a combination of treatments with exogenous nitrate, ammonium, Glu and methionine sulfoximine, an inhibitor of Gln synthetase. Treatment with methionine sulfoximine should block the accumulation of downstream metabolites from inorganic nitrogen and allow distinguishing between regulations by organic or inorganic nitrogen. *ASN1* was induced by Glu or a downstream metabolite, while *ASPGB1* was regulated in an opposite manner. *NR1* encoding nitrate reductase and *Gln1.3* encoding Gln synthetase were repressed by Glu.

RNA was extracted from roots and levels of transcripts tested by RT-qPCR relative to the *Actin2* reference gene (Czechowski et al., 2005). As with Glu, the levels of *NR1* transcripts were reduced by the Asn treatment by ca. 0.5-fold, while *ASN1* transcripts were increased by ca. 2-fold (Table 2.2). *GLN1*.3 transcripts were unchanged by the treatment. Transcripts of *LHT1* implicated in amino acid uptake by the root were slightly elevated, by 1.3-fold. Similarly with Glu, *ASPGB1* transcripts were significantly reduced by Asn, although the fold-change was marginal (1.1-fold). Transcript levels of *ASPGA1*, encoding the K^+-independent asparaginase, were not altered. *AGT1* was induced by the treatment by ca. 2-fold. By contrast, transcripts of *GGT1*, coding for peroxisomal Glu:glyoxylate aminotransferase were unchanged.

The repression of *NR1*, the induction of *ASN1* and possibly the repression of *ASPGB1* appear common to Asn and Glu (Gutiérrez et al., 2008b), and could form part of a universal response to organic nitrogen. The net effect should be to down-regulate nitrate

Table 2.2 Relative transcript expression in root of Asn-treated and control samples as determined by RT-qPCR.

Data was normalized to the mean Cq of the reference gene, *Actin2*. Values are the means ± s.d. of four biological replicates, with each biological replicate the average of three technical replicates.

Gene	0 mM Asn	20 mM Asn	*t*-test *p* value
NR1	1.12 ± 0.09	0.51 ± 0.05	0.0001
LHT1	0.75 ± 0.03	1.01 ± 0.05	0.0001
ASN1	0.51 ± 0.06	1.07 ± 0.11	0.0001
GLN1.3	1.17 ± 0.03	1.07 ± 0.12	n.s.[a]
AGT1	0.45 ± 0.02	0.97 ± 0.05	0.0001
GGT1	0.88 ± 0.34	0.78 ± 0.14	n.s.[a]
ASPGA1	0.91 ± 0.04	1.07 ± 0.12	n.s.[a]
ASPGB1	1.20 ± 0.08	1.08 ± 0.11	0.04

[a] n.s.: not significant.

assimilation and to increase the accumulation of Asn as a storage form of nitrogen. The slight increase in *LHT1* transcripts reveals an absence of feedback on high affinity amino acid uptake by the root. The fact that *GLN1.3* was not responsive to the treatment may be a specific feature of the root. Alternatively, it is possible that Glu, and not Asn represses its expression. The induction of *AGT1* by Asn supports of a possible function of the aminotransferase in Asn metabolism. AGT1 may channel nitrogen from Asn into other amino acids, particularly in a non-photosynthetic tissue where photorespiration should be absent. However, asparaginases clearly contribute to Asn catabolism in root, as indicated by the over-accumulation of Asn in the *aspga1-1/-b1-1* mutant grown on media containing this amino acid as a single nitrogen source for a 10-day period (Ivanov et al., 2012b).

In summary, the data presented here support the conclusion that AGT1 may act as an Asn aminotransferase in Arabidopsis. The recombinant enzyme exhibited a higher level of catalytic rate with Asn than with its prototypical substrates, Ser and Ala. This level of activity was comparable to that of the K^+-dependent asparaginase, ASPGB1. Further research will be necessary to identify and characterize the ω-amidase acting downstream from AGT1 in the breakdown of the product α-KSM. The fact that Asn induces *AGT1* expression in root also supports a possible role of the aminotransferase in Asn metabolism. The system established to measure transcript levels in root will be useful to further investigate transcriptional responses to Asn.

2.4 References

Betsche, T., 1983. Aminotransfer from alanine and glutamate to glycine and serine during photorespiration in oat leaves. Plant Physiol. 71, 961-965.

Bruneau, L., Chapman, R., Marsolais, F., 2006. Co-occurrence of both L-asparaginase subtypes in *Arabidopsis*: *At3g16150* encodes a K^+-dependent L-asparaginase. Planta 224, 668-679.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., Scheible, W.-R., 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol. 139, 5-17.

Dytham, C., 2011. Choosing and Using Statistics: A Biologist's Guide. Wiley-Blackwell, Oxford.

Forsum, O., Svennerstam, H., Ganeteg, U., Näsholm, T., 2008. Capacities and constraints of amino acid utilization in *Arabidopsis*. New Phytol. 179, 1058-1069.

Gabriel, M., Telmer, P. G., Marsolais, F., 2012. Role of asparaginase variable loop at the carboxyl terminal of the alpha subunit in the determination of substrate preference in plants. Planta 235, 1013-1022.

Gerdes, S., El Yacoubi, B., Bailly, M., Blaby, I. K., Blaby-Haas, C. E., Jeanguenin, L., Lara-Nunez, A., Pribat, A., Waller, J. C., Wilke, A., Overbeek, R., Hanson, A. D., de Crecy-Lagard, V., 2011. Synergistic use of plant-prokaryote comparative genomics for functional annotations. BMC Genomics 12 Suppl. 1, S2.

Gifford, M. L., Dean, A., Gutiérrez, R. A., Coruzzi, G. M., Birnbaum, K. D., 2008. Cell-specific nitrogen responses mediate developmental plasticity. Proc. Natl. Acad. Sci. USA 105, 803-808.

Gutiérrez, R. A., Stokes, T. L., Thum, K., Xu, X., Obertello, M., Katari, M. S., Tanurdzic, M., Dean, A., Nero, D. C., McClung, C. R., Coruzzi, G. M., 2008. Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene *CCA1*. Proc. Natl. Acad. Sci. USA 105, 4939-4944.

Havir, E. A., McHale, N. A., 1988. A mutant of *Nicotiana sylvestris* lacking serine:glyoxylate aminotransferase: substrate specificity of the enzyme and gate of [2-^{14}C] glycolate in plants with genetically altered enzyme levels. Plant Physiol. 87, 806-808.

Hirner, A., Ladwig, F., Stransky, H., Okumoto, S., Keinath, M., Harms, A., Frommer, W. B., Koch, W., 2006. Arabidopsis LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. Plant Cell 18, 1931-1946.

Ho, C. H., Lin, S. H., Hu, H. C., Tsay, Y. F., 2009. CHL1 functions as a nitrate sensor in plants. Cell 138, 1184-1194.

Igarashi, D., Tsuchida, H., Miyao, M., Ohsumi, C., 2006. Glutamate:glyoxylate aminotransferase modulates amino acid content during photorespiration. Plant Physiol. 142, 901-910.

Ireland, R. J., Joy, K. W., 1981. Two routes for asparagine metabolism in *Pisum sativum* L. Planta 151, 289-292.

Ireland, R. J., Joy, K. W., 1983a. Purification and properties of an asparagine aminotransferase from *Pisum sativum* leaves. Arch. Biochem. Biophys. 223, 291-296.

Ireland, R. J., Joy, K. W., 1983b. Subcellular localization of asparaginase and asparagine aminotransferase in Pisum sativum leaves Peas. Plant Physiol. 72, 1127-1129.

Ivanov, A., Kameka, A., Pajak, A., Bruneau, L., Beyaert, R., Hernández-Sebastià, C., Marsolais, F., 2012. *Arabidopsis* mutants lacking asparaginases develop normally but exhibit enhanced root inhibition by exogenous asparagine. Amino Acids 42, 2307-2318.

Jaisson, S., Veiga-da-Cunha, M., Van Schaftingen, E., 2009. Molecular identification of ω-amidase, the enzyme that is functionally coupled with glutamine transaminases, as the putative tumor suppressor Nit2. Biochimie 91, 1066-1071.

Kendziorek, M., Paszkowski, A., 2008. Properties of serine:glyoxylate aminotransferase purified from *Arabidopsis thaliana* leaves. Acta Biochim. Biophys. Sin. 40, 102-110.

Krasnikov, B. F., Chien, C. H., Nostramo, R., Pinto, J. T., Nieves, E., Callaway, M., Sun, J., Huebner, K., Cooper, A. J. L., 2009. Identification of the putative tumor suppressor Nit2 as ω-amidase, an enzyme metabolically linked to glutamine and asparagine transamination. Biochimie 91, 1072-1080.

Lam, H.-M., Peng, S. S. Y., Coruzzi, G. M., 1994. Metabolic regulation of the gene encoding glutamine-dependent asparagine synthetase in *Arabidopsis thaliana*. Plant Physiol. 106, 1347-1357.

Lea, P. J., Sodek, L., Parry, M. A. J., Shewry, P. R., Halford, N. G., 2007. Asparagine in plants. Ann. Appl. Biol. 150, 1-26.

Lee, Y.-H., Foster, J., Chen, J., Voll, L. M., Weber, A. P., Tegeder, M., 2007. AAP1 transports uncharged amino acids into roots of Arabidopsis. Plant J. 50, 305-319.

Liepman, A. H., Olsen, L. J., 2001. Peroxisomal alanine : glyoxylate aminotransferase (AGT1) is a photorespiratory enzyme with multiple substrates in *Arabidopsis thaliana*. Plant J. 25, 487-498.

Lloyd, N. D. H., Joy, K. W., 1978. 2-Hydroxysuccinamic acid: a product of asparagine metabolism in plants. Biochem. Biophys. Res. Commun. 81, 186-192.

Masclaux-Daubresse, C., Reisdorf-Cren, M., Pageau, K., Lelandais, M., Grandjean, O., Kronenberger, J., Valadier, M. H., Feraud, M., Jouglet, T., Suzuki, A., 2006. Glutamine synthetase-glutamate synthase pathway and glutamate dehydrogenase play distinct roles in the sink-source nitrogen cycle in tobacco. Plant Physiol. 140, 444-456.

Michalska, K., Jaskolski, M., 2006. Structural aspects of L-asparaginases, their friends and relations. Acta Biochim. Pol. 53, 627-640.

Murray, A. J. S., Blackwell, R. D., Joy, K. W., Lea, P. J., 1987. Photorespiratory N donors, aminotransferase specificity and photosynthesis in a mutant of barley deficient in serine:glyoxylate aminotransferase activity. Planta 172, 106-113.

Nakabayashi, K., Okamoto, M., Koshiba, T., Kamiya, Y., Nambara, E., 2005. Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. Plant J. 41, 697-709.

Näsholm, T., Kielland, K., Ganeteg, U., 2009. Uptake of organic nitrogen by plants. New Phytol. 182, 31-48.

Niessen, M., Krause, K., Horst, I., Staebler, N., Klaus, S., Gaertner, S., Kebeish, R., Araujo, W. L., Fernie, A. R., Peterhansel, C., 2012. Two alanine aminotranferases link mitochondrial glycolate oxidation to the major photorespiratory pathway in *Arabidopsis* and rice. J. Exp. Bot. 63, 2705-2716.

Patterson, K., Cakmak, T., Cooper, A., Lager, I., Rasmusson, A. G., Escobar, M. A., 2010. Distinct signalling pathways and transcriptome response signatures differentiate ammonium- and nitrate-supplied plants. Plant Cell Environ. 33, 1486-1501.

Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., Scholkopf, B., Weigel, D., Lohmann, J. U., 2005. A gene expression map of *Arabidopsis thaliana* development. Nat. Genet. 37, 501-506.

Somerville, C. R., Ogren, W. L., 1980. Photorespiration mutants of *Arabidopsis thaliana* deficient in serine-glyoxylate aminotransferase activity. Proc. Natl. Acad. Sci. U. S. A. 77, 2684-2687.

Streeter, J. G., 1977. Asparaginase and asparagine transaminase in soybean leaves and root nodules. Plant Physiol. 60, 235-239.

Svennerstam, H., Ganeteg, U., Bellini, C., Nashölm, T., 2007. Comprehensive screening of Arabidopsis mutants suggests the lysine histidine transporter 1 to be involved in plant uptake of amino acids. Plant Physiol. 143, 1853-1860.

Ta, T. C., Joy, K. W., 1985. Transamination, deamidation, and the utilisation of asparagine amino nitrogen in pea leaves. Can. J. Bot. 63, 881-884.

Ta, T. C., Joy, K. W., 1986. Metabolism of some amino acids in relation to the photorespiratory nitrogen cycle of pea leaves. Planta 169, 117-122.

Ta, T. C., Joy, K. W., Ireland, R. J., 1985. Role of asparagine in the photorespiratory nitrogen metabolism of pea leaves. Plant Physiol. 78, 334-337.

Unkles, S. E., Wang, R. C., Wang, Y., Glass, A. D. M., Crawford, N. M., Kinghorn, J. R., 2004. Nitrate reductase activity is required for nitrate uptake into fungal but not plant cells. J. Biol. Chem. 279, 28182-28186.

Wang, R., Okamoto, M., Xing, X., Crawford, N. M., 2003. Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol. 132, 556-567.

Wang, R., Tischner, R., Gutiérrez, R. A., Hoffman, M., Xing, X., Chen, M., Coruzzi, G., Crawford, N. M., 2004. Genomic analysis of the nitrate response using a nitrate reductase-null mutant of *Arabidopsis*. Plant Physiol. 136, 2512-2522.

Wang, R., Xing, X., Crawford, N., 2007. Nitrite acts as a transcriptome signal at micromolar concentrations in *Arabidopsis* roots. Plant Physiol. 145, 1735-1745.

Wang, R., Xing, X., Wang, Y., Tran, A., Crawford, N. M., 2009. A genetic screen for nitrate regulatory mutants captures the nitrate transporter gene NRT1.1. Plant Physiol. 151, 472-478.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V., Provart, N. J., 2007. An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS ONE 2, e718.

Chapter 3 Identification and characterization of ω-amidase as an enzyme metabolically linked to asparagine transamination in *Arabidopsis thaliana*.

3.1 Introduction

Transamination of Asn and Gln produces α-KSM and α-KGM, respectively, both of which are substrates of ω-amidase (E.C.3.5.1.3), yielding oxaloacetate or α-ketoglutarate and ammonia (Jaisson et al., 2009; Meister et al., 1952). It has been proven that ω-amidase is involved in nitrogen catabolic pathways including Asn transamination and Gln transamination (Krasnikov et al., 2009b).

There are two different catabolic pathways of Asn, one of which proceeds through asparaginase that catalyzes the hydrolysis of Asn to Asp and releases ammonia (Michalska and Jaskolski, 2006a). The other goes via Asn aminotransferase and results in the formation of α-KSM, which can be hydrolyzed by ω-amidase (Krasnikov et al., 2009b). This suggests that ω-amidase can act downstream from Asn aminotransferase. According to previous studies in pea leaves, α-HSM, reduced further from α-KSM, may also be a substrate of ω-amidase (Lloyd and Joy, 1978b). Studies indicated that α-KSM is not stable and easily forms a homo-dimer, which may further form some toxic aromatic compounds (Cooper et al., 1987; Stephani and Meister, 1971). The Asn transamination and ω-amidase pathway has also been studied in rat liver mitochondria, which showed its importance for Asn metabolism (Moragaa et al., 1989).

The Gln transamination and ω-amidase activities are involved in part of a Gln cycle (Mora, 1990). The involvement of Gln in transamination reactions plays a role in

the methionine salvage pathway (Chapter 1, Figure 1.4). During polyamine and ethylene biosynthesis, methionine is converted to 5'-methylthioadenosine (Albers, 2009). In the methionine salvage pathway, which exists in mammals and plants, 5'-methylthioadenosine is further converted to α-keto-γ-methylthiobutyrate (Cooper and Meister, 1981). The last step of the methionine salvage pathway is a transamination of α-keto-γ-methylthiobutyrate to methionine (Albers, 2009). In the methionine salvage pathway, enzymatic and ^{15}N tracer *in vivo* results have suggested that a distinctive Gln-dependent aminotransferase, Gln transaminase K (GTK) is implicated in the α-keto-γ-methylthiobutyrate transamination (Cooper, 2004; Hoskin et al., 2001). Then α-KGM, the Gln transamination product, can be transformed to an α-ketoglutarate molecule by ω-amidase (Cooper, 2004), indicating that ω-amidase could play an important role in the Gln transamination. In addition, it has been proven that α-KGM can be detected in micromole as concentrations and it might be neurotoxic in rat tissues (Duffy et al., 1974).

Thus, besides the biological significance of Gln and Asn transamination reactions, ω-amidase is important in transforming two potentially toxic molecules to other molecules, α-ketoglutarate or oxaloacetate. ω-Amidase has been characterized experimentally at the molecular level in rat (Krasnikov et al., 2009a; Krasnikov et al., 2009b). A candidate gene has been reported in Arabidopsis but not characterized (Gerdes et al., 2011a). Therefore, the characteristics and function of ω-amidase in Asn catabolism in plants remain unclear.

The objective of the present study was to identify ω-amidase gene candidates in Arabidopsis. According to a previous study, the expression of eight genes, involved in nitrogen metabolism in Arabidopsis, were regulated by exogenous Asn (Chapter 2). As ω-

amidase is related to Asn transamination, an investigation about the effect of Asn on transcript levels of ω-amidase in Arabidopsis was also performed in this study. The catalytic activity of recombinant ω-amidase enzyme purified from *E. coli* was characterized with different amide substrates *in vitro*. Lastly, the amount of substrates of ω-amidase was measured in roots of wild-type Arabidopsis and *ω-amidase* mutant root.

3.2 Experimental methods

3.2.1 Plant material and growth conditions

Arabidopsis ecotype Columbia (wild-type) seeds were grown on vertical plates at 22 °C under continuous light (approximately 70 µmol m^{-2} s^{-1}), on a medium containing 5 mM potassium nitrate for 10 days (Wang et al., 2003). At the end of the 10-day period, seedlings were transferred to plates containing the same medium without nitrogen as control or with 20 mM Asn as the sole source of nitrogen for 2 h. Then roots were harvested. RNA was extracted from roots and levels of transcripts tested by RT-qPCR, relative to the *Actin2* reference gene (Czechowski et al., 2005).

Similarly, for metabolites, *ω-amidase* mutant (Germplasm: SALK_608_A02) seeds were obtained from Arabidopsis Biological Resource Center (ABRC). Wild-type and mutant seeds were grown under the same environmental conditions as described above, on a medium without Asn as control or containing 2 mM Asn as the single nitrogen source for 14 days. Then root tissue was harvested, immediately frozen in liquid nitrogen and stored at −80°C until RNA or metabolite extraction was performed. For gene expression analyses, the experimental unit consisted of a plate with approximately 30

seedlings. For metabolite analysis, the experimental unit consisted of 3 plates and each plate with approximately 30 seedlings.

For genotyping, wild-type and *ω-amidase* mutant seeds were grown under long day conditions (16 h light, 8 h dark), as described by Ivanov et al. (2012b). After two months' growth, leaves from both genotypes were harvested, and immediately frozen in liquid nitrogen and stored at −80°C until further experiment were performed.

3.2.2 ω-Amidase mutant genotype analysis

Homozygous individuals of *ω-amidase* mutant were identified by PCR genotyping, using gene-specific and T-DNA specific primers. Genomic DNA was extracted from leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Mississauga, ON). The general PCR reaction used *Pfx50* DNA polymerase (Invitrogen) and consisted of denaturing at 95°C for 2 min, 35 cycles at 95°C for 2 min, 60°C for 30 s and 68°C for 60 s, and a final extension at 68°C for 10 min. Position of insertion was determined by sequencing of PCR products amplified with Platinum PCR Super Mix High Fidelity (Life Technologies). Sequencing was performed with a 3130XL GeneticAnalyzer (Applied Biosystems, Streetsville, ON). The following primers were used for genotyping:

G-At5g121040-F, 5'-AAATCGAATTACATTCGGCGTTAA-3',
G-At5g12040-R, 5'-CCTATATGATCATCGGACTAAAGTGA-3'
LB2, 5'-GCTTCCTATTATATCTTCCCAAATTACCAATACA-3'.

3.2.3 Gene expression analysis

The absence of transcript in homozygous *ω-amidase* mutants was confirmed by RT-PCR in reference to the wild-type. Total RNA was extracted from 100 mg of leaf

tissue from wild-type and *ω-amidase* mutant by using the RNeasy Plant Mini Kit (Qiagen). RNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Mississauga, ON). Five micrograms of RNA were treated with amplification grade DNase I (Life Technologies) to minimize any DNA contamination that may have occurred during the RNA extraction process. RNA quality was determined prior to cDNA synthesis by using gel electrophoresis on a 1% (w/v) agarose gel. A Thermoscript RT-PCR System (Invitrogen) was used to synthesize cDNA from 2.5 µg total RNA in a 20 µl reaction volume. A regular PCR was carried out in a final volume of 25 µl, using 2 µl of cDNA as template. The PCR program consisted in an initial step of denaturing at 95°C for 2 min, 35 cycles at 95°C for 30 s, 61°C for 30 s and 68°C for 60 s, and a final extension at 72°C for 5 min. The PCR product was analyzed on 1.5% (w/v) agarose gel. Primers used in PCR were:

Actin2-F: 5'-CACAACAGCAGAGCGGGAAATT-3',
Actin2-R: 5'-TTCTGGGCATCTGAATCTCTCA-3';

ω-amidase-F: 5'-GGTTGAAACGCAAACGCAGAGGAT-3',
ω-amidase-R: 5'-ACGAAGATGGGAGCTTCAAGGTGT-3'.

To determine whether *ω-amidase* is transcriptionally regulated by Asn, RNA was extracted from roots that were treated with or without 20 mM Asn, and used to synthesize cDNA from 3 µg total RNA in a 20 µl reaction volume. For RT-qPCR, *Actin2* was used as reference gene. Primers were: for *Actin2*, same as described above; for *ω-amidase*,

Q-At5g121040-F, 5'-ACGAAGGACTAGCCTTCCATTG-3'
Q-At5g121040-R, 5'-GTAACCAAATCGTGCTCAACTC-3'.

The cDNA samples were diluted 8-fold and 2 µl amplified with 5 µl of SsoFast EvaGreen Supermix in a final volume of 10 µl using a CFX96 Real-Time PCR Detection System

(Bio-Rad Laboratories). In each plate, controls without template were performed in duplicate. Reactions contained primers at a concentration of 0.5 µM. They were carried out in Hard-Shell 96-well clear PCR plates (Bio-Rad Laboratories). The PCR program consisted in an initial step of 2 min at 95 °C followed by 40 cycles of 30 s at 94 °C and 30 s at 58.5 °C. Data were analyzed with the CFX Manager 2.0 software. The PCR product was examined by 1.5% (w/v) agarose gel electrophoresis.

3.2.4 Extraction of metabolites from root

Metabolites were extracted from root tissue as described by Cooper et al. (1987). Briefly, 100 mg of root tissue was homogenized in 3 volumes of ice-cold 3 M perchloric acid. The precipitate was removed by centrifugation at 15,000 × *g* for 15 min at 4 °C. For α-KSM, to 500 µl of the supernatant solution was added 50 µl of 1 M Hepes and the solution was adjusted to pH 7 with KOH. Similarly, for α-HSM, 500 µl of the supernatant solution was treated with 100 µl of 500 mM Hepes-NaCl and then adjusted to pH 6.5 with KOH. The precipitated potassium perchlorate was removed by centrifugation at 15,000 × *g* for 15 min at 4°C, and the deproteinized product was stored at −80°C immediately until further experiment.

3.2.5 Determination of α-ketosuccinamate and α-hydroxysuccinamate

Substrates from above were detected by fluorometry (Cooper et al., 1987). For α-KSM, 200 µl of the tissue extract was treated with 1 µl of purified recombinant ω-amidase (40 µg) and 250 µl of 0.9 M Hepes-KOH, pH 7.3, in a fluorometer tube. After standing for 1 h at 23°C, 550 µl of freshly prepared 0.9 M Hepes-KOH pH 7.3 containing 2.5 µM of NADH was added. After standing for an additional 10 min at 23°C, 2 µl of

malate dehydrogenase (12 units) solution was added and the changes in fluorescence of substrates were measured in a RF-Mini 150 Recording Fluorometer (Hitachi) (excitation, 360 nm; fluorescence emission 460 nm) for exactly 5 min. In addition, α-KSM, prepared as a standard (see section 3.2.9), was detected under the same conditions in order to determine a standard curve. The change in fluorescence in the standard curve was linear from 5.0 to 25 nmol of α-KSM. Conditions for determination of α-HSM were identical to the α-KSM assay except that 0.2 M Hepes-KOH pH 7.2 was substituted for 0.9 M Hepes-KOH pH 7.3, and 3.0 µM of NAD was substituted for 2.5 µM of NADH. Similarly, α-HSM, prepared as a standard (see section 3.2.9), was measured in the same conditions to make a standard curve. The change in fluorescence was linear from 5.0 to 30 nmol of α-HSM.

3.2.6 Cloning of ω-amidase cDNA

A full-length *ω-amidase* cDNA (stock number U21629) was obtained from the Arabidopsis Biological Resource Centre. Primers were designed based on the longest deduced open reading frame (ORF) and sequence alignment to query genes, which were

*Nco*I-Fw, 5'-AATTCCATGGGAATGAAGTCAGCAATTTCATCG-3',
*Pst*I-Rvs, 5'-AATTCTGCAGTCATTTAGAGTCTAAGCGCT-3'.

The general PCR reaction used *Pfx50* DNA polymerase (Invitrogen) and consisted of denaturing at 94°C for 2 min, 35 cycles at 94°C for 30 s, 60.5°C for 30 s and 68°C for 60 s, and a final extension at 68°C for 5 min. PCR products were cloned into pCR4Blunt-TOPO vector (Invitrogen) and the cDNA was sequenced with a 3130XL Genetic Analyzer (Life Technologies) to confirm the proper sequence. Then the insert was subcloned into pProEX-HTb expression vector by restriction enzyme-mediated cloning

using *NcoI* and *PstI* restriction endonucleases (New England Biolabs) and T4 DNA ligase (Invitrogen) following manufacturers' guidelines. *Escherichia coli* (*E. coli*) strain XL10-Gold (Agilent Technologies) was used for cloning and expression of gene in the pProEX-HTb vector.

3.2.7 Protein Expression

Fresh overnight culture containing recombinant plasmid was grown in LB media and used to inoculate 500 mL of LB media. Cultures were incubated at 37°C until an OD_{600} between 0.8 and 1.0 was reached. Expression of the candidate genes was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cells were cultured for 10 h at 37°C. Following expression, cells were pelleted by centrifugation at 5,500 × *g* for 10 min at 4°C and frozen at −20°C to enhance lysis.

3.2.8 Protein purification

Pelleted cells were resuspended in 5 – 10 mL of Native Protein Lysis Buffer (50 mM sodium phosphate monobasic, 300 mM sodium chloride and 10 mM imidazole at pH 8.0), 1 mg/mL lysozyme was added and cells were incubated on ice for 30 min. A French Press (American Instrument Co. Inc.) was used to exert 1200 pound per square inch (psi) pressure on the cells to lyse and enhance protein recovery. Sonication was also performed to break down genomic DNA and reduce viscosity. The lysate was then centrifuged at 15,500 × *g* for 45 min at 4°C to pellet cell debris. To the supernatant, 500 µL Ni-NTA agarose beads (Qiagen) were added to bind the hexahistidine tagged recombinant proteins. The supernatant and nickel beads were incubated at 4°C for 2 h on a slow rotor. The Ni-NTA protein solutions were centrifuged at 3,500 × *g* for 3 min at 4°C and the

supernatant discarded. The pellets were then washed 10 times with Native Protein Wash Buffer. Recombinant proteins were eluted from the beads by the addition of 1.25 mL Native Protein Elution Buffer (50 mM sodium phosphate monobasic, 300 mM sodium chloride and 250 mM imidazole at pH 8.0). The solution was incubated for 10 min at 4°C on a slow rotor and elution repeated with an additional 1.25 mL of Native Protein Elution Buffer. Purified protein was desalted in 25 mM Hepes pH 7.2, containing 30 mM NaCl, 1 mM DL-dithiothreitol using a PD-10 column (GE Healthcare Life Sciences), and concentrated using an Amicon Ultra-4 centrifugal filter (Millipore, Billerica, MA). The purified protein was flash frozen in liquid nitrogen and stored at −80°C with 10% glycerol (v/v).

Protein concentration was determined using the Bio-Rad protein assay solution and bovine serum albumin as a standard. SDS-PAGE was performed using Coomassie Brilliant Blue R250 to stain proteins to confirm the proper protein had been purified by comparison to the predicted molecular mass. Molecular mass and isoelectric point of recombinant protein were predicted using ExPASy Compute pI/MW tool (http://web.expasy.org/compute_pi/).

3.2.9 Preparation and purification of ω-amidase substrates

α-KGM and α-KSM were prepared by oxidation of Gln and Asn as described by Jaisson et al. (2009). Briefly, 5 mL of 0.2 M Gln or Asn, pH 7, was incubated overnight at 37°C under agitation with 10 units of catalase and 10 mg of *Crotalus adamanteus* L-amino acid oxidase (Sigma-Aldrich, Oakville, ON). After incubation, the solution was deproteinized by the addition of 250 µL of 7 M perchloric acid and centrifuged at 15,000

× *g* for 15 min at 4 °C. The product was separated from unreacted Gln or Asn by applying the supernatant to a 5 mL Dowex 50WX8-100 cation exchange column in hydrogen form (Sigma-Aldrich). The eluate was neutralized with 3 M potassium carbonate and the potassium perchlorate precipitate was removed by centrifugation at 15,000 × *g* for 15 min at 4 °C.

α-HSM was prepared from α-KSM as described by Lloyd and Joy (1978). A concentration of 0.2 M sodium borohydride was added to α-KSM that was purified from the last step as described above. Then the mixture was incubated for 30 min at room temperature. The solution was adjusted to pH 2 with hydrochloric acid to decompose residual borohydride. After neutralisation, α-HSM was absorbed on AG 1-X8 Resin (formate form, Bio-Rad Laboratories) and eluted in 2 M formic acid.

3.2.10 ω-Amidase assays

ω-Amidase activities were assayed at 30 ºC and measured by monitoring the changes in NADH or NAD absorption using auxiliary enzymes (Figure 3.1) by spectrophotometry at 340 nm with a Power Wave XS 96-well plate reader (Bio-Tek Instruments, Winooski, VT). The path length was measured to correct the calculated enzyme activities using the path length correction feature in KC junior software. α-KSM aminotransferase activity was determined by monitoring the production of oxaloacetate using L-malate dehydrogenase (Figure 3.1a) (Jaisson et al., 2009). Likewise, α-KGM aminotransferase activity was determined by monitoring the production of α-ketoglutarate using Glu dehydrogenase (Figure 3.1b) (Jaisson et al., 2009). α-HSM aminotransferase

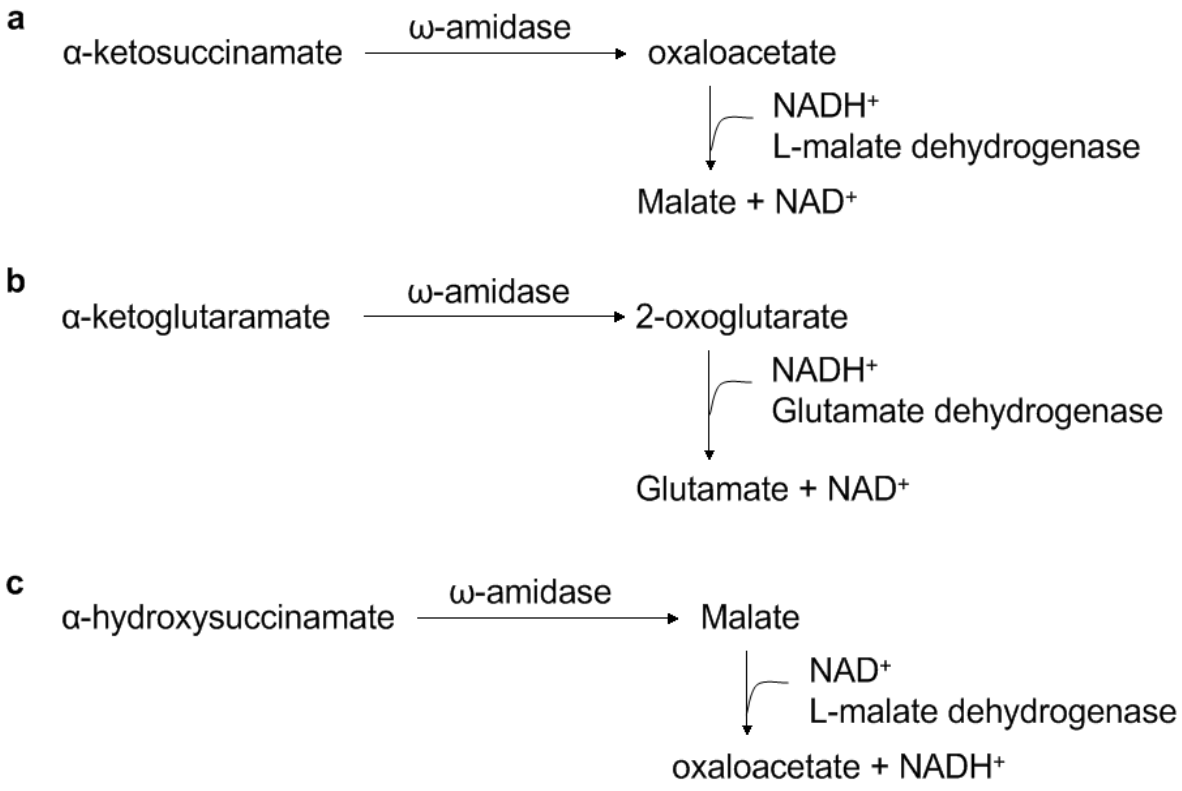

Figure 3.1 Catalytic activities of ω-amidase monitored with three different substrates:

(a) α-ketosuccinamate; (b) α-ketoglutaramate; (c) α-hydroxysuccinamate

activity was measured by the production of oxaloacetate using L-malate dehydrogenase (Figure 3.1c) (Lloyd and Joy, 1978).

α-KSM hydrolysis was followed by measuring the formation of oxaloacetate in a mixture containing 50 mM Tris pH 8.5 as well as 150 µM NADH and 6 units of L-malatedehydrogenase (Roche Applied Science, Laval, QC) in a final volume of 200 µL. For α-HSM hydrolysis, reaction conditions were identical to the α-KSM assay except that 150 µM NAD was substituted for NADH. The hydrolysis of α-KGM was determined by following the formation of α-ketoglutarate in an assay mixture containing 50 mM Tris pH 8.5, 10 mM NH_4Cl, 150 µM NADH, 1 mM ADP, 2 mM $MgCl_2$, 0.5 mg/mL bovine serum albumin (Sigma-Aldrich), 2 units of Glu dehydrogenase (Roche Applied Science, Laval, QC) in a final volume of 200 µL. The reactions were started by adding 2.0 µg of recombinant ω-amidase. The substrates were assayed at a concentration ranging between 1 and 10 mM. The amount of product was linear with time between 0 min to 30 min at 2 mM of substrate and 1.0 µg of recombinant ω-amidase. Also, the activity was linear between 1.0 to 5.0 µg of enzyme at the same substrate concentrations.

3.2.11 Statistical analysis

An Unpaired, two-tailed t-test assuming homogeneity of the variances was performed with the Excel software (Dytham, 2011). Analysis of variance followed by LSD test as post hoc test was performed using SAS version 9.2 (Toronto, ON).

3.2.12 Accession numbers

Sequence data can be found under accession numbers (TAIR ID) At3G18780 for *Actin2*; (TAIR ID) At5g12040 for ω-amidase.

3.3 Results and Discussion

3.3.1 Identification and characterization of ω-amidase candidate genes

Previous study has identified that mammalian Nitrilase 2 is the same as ω-amidase, based on the catalytic activity (Jaisson et al., 2009). The amino acid sequence of Nitrilase 2 was used to BLAST search Arabidopsis proteins in the TAIR database (http://www.arabidopsis.org/) to find ω-amidase candidate genes. Screening of the TAIR database generated several candidate genes based on sequence homology but only the top six genes with highest sequence identity were chosen (Table 3.1). The top candidate gene *AT5G12040* was identified as a candidate gene of ω-amidase based on amino acid sequence identity. The ω-amidase protein was predicted to be expressed in the chloroplast by a subcellular localization prediction tool, Cell eFP Browser (http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi). In addition, it was predicted that there was a chloroplast transit peptide of 63 resudues at the N-terminus of its amino acid sequence, using ChloroP server (http://www.cbs.dtu.dk/services/ChloroP/). Localization prediction with the Target P program also indicated that ω-amidase was a chloroplast protein (http://www.cbs.dtu.dk/services/TargetP/).

For the functional analysis of the ω-amidase gene from Arabidopsis, a T-DNA insertion mutant for *ω-amidase* was obtained from ABRC. To confirm its T-DNA insertion position, genomic DNA was extracted from mutant leaf and genotyped. After DNA sequencing, the T-DNA insertion was found in the first exon, 121 bp downstream away from the first ATG or initiation codon (Figure 3.2), which should have an effect on transcript levels. To confirm whether the *ω-amidase* gene was inactivated by the T-DNA

Table 3.1 *ω-Amidase* gene candidates in *Arabidopsis* by BlastP using mouse nitrilase 2 (*Nit 2*) as query sequence from Genbank.

Locus	Annotation	Score	Identities (%)
AT5G12040.1	Nitrilase/cyanide hydratase	320	56
AT5G12040.2	Nitrilase/cyanide hydratase	249	59
AT4G08790.1	Nitrilase/cyanide hydratase	156	33
AT2G27450.1	nitrilase-like protein 1	98	28
AT2G27450.2	nitrilase-like protein 1	94	27
AT3G44300.1	nitrilase 2	87	27

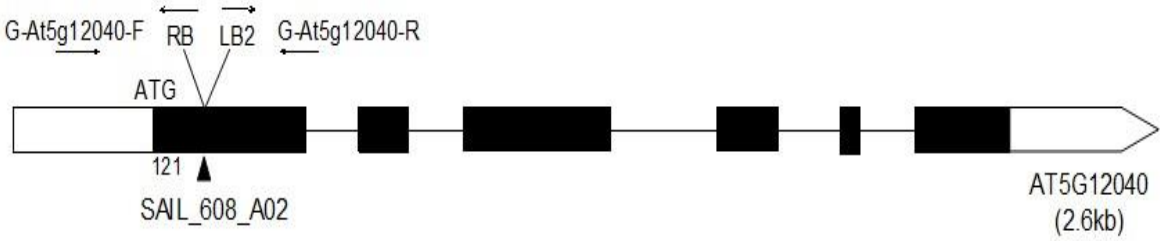

Figure 3.2 Schematic representation of ω-amidase, showing the position of the T-DNA insertion.
Black bars represent exons, white regions represent untranslated regions, triangle indicates the site of T-DNA insertion. Position of insertion is located downstream 121 bp away from the first ATG. G-At5g12040-F and G-At5g12040-R are gene-specific primers. LB2 and RB indicate T-DNA specific primers.

insertion, total RNA from wild-type Arabidopsis and homozygous *ω-amidase* mutant plants was analyzed by RT-PCR, which revealed the absence of detectable transcripts in mutant lines when compared with the wild-type Columbia ecotype (Figure 3.3). This suggests that *ω-amidase* expression is blocked by the T-DNA insertion in mutant, which may further lead to over-accumulation of substrates of ω-amidase in mutant, compared with wild-type.

Further, to determine if *ω-amidase* is also transcriptionally regulated by Asn, RT-qPCR result showed that the levels of *ω-amidase* transcripts were reduced by the Asn treatment by 30% (Table 3.2). Under the same condition, *AGT1* transcripts were induced by ca. 2-fold (chapter 2, Table 2.2). This meant that *AGT1* transcripts were up-regulated while the expression of *ω-amidase* was decreased by external Asn. The net result could be to increase the accumulation of α-KSM to store nitrogen following Asn treatment in root.

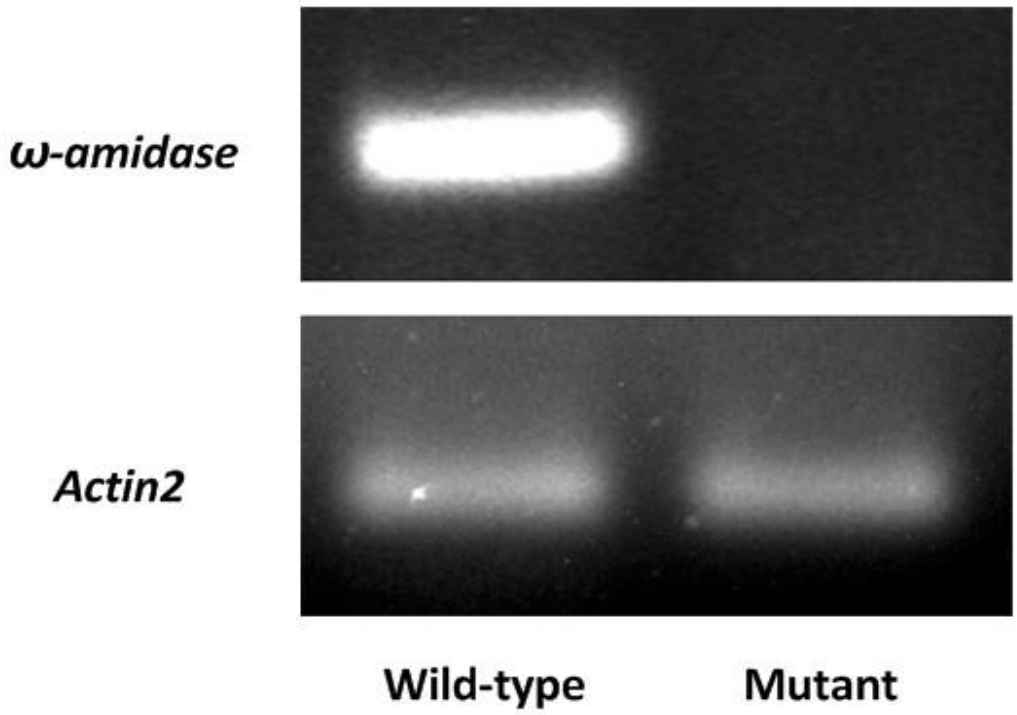

Figure 3.3 Assessment of expression levels of *ω-amidase* from wild-type and mutant by RT-PCR.
The changes in transcript level of *ω-amidase* between wild-type and mutant leaves were assessed visually in comparison to *Actin2*.

Table 3.2 Quantification of *ω-amidase* transcripts in root of Asn-treated and control samples as determined by RT-qPCR.

Data was normalized to the mean Cq of the reference gene, *Actin2*. Values are the means ± s.d. of four biological replicates, with each biological replicate the average of three technical replicates.

Treatment	*ω-amidase*
0 mM Asn	1.40 ± 0.02
20 mM Asn	0.99 ± 0.05
t-Test *P* value	0.0002

3.3.2 Quantification of ω-amidase substrates in root

In general, about ω-amidase substrates in root, the levels of α-KSM were slightly higher than α-HSM (Table 3.3). The concentration of α-KSM in mutant was higher than that in wild-type by 2-fold in control and close to 3-fold in the Asn treatment. Similarly, the amount of α-HSM in the mutant was increased by close to 3-fold in control and over 3-fold in the Asn treatment, compared with wild-type. In addition, the increase of α-KSM concentration following the Asn treatment was larger in the mutant than in the wild-type. This relationship was also observed for α-HSM. The significant interactions for both α-KSM and α-KSM confirmed that the genotypes responded differently to the treatment (Figure 3.4 and 3.5). This suggested that the over-accumulation of ω-amidase substrates may be caused by a reduced expression in the mutant caused by T-DNA insertion. The data support a role for ω-amidase in the degradation of α-KSM and α-HSM *in vivo*.

Previous study showed that after 2 h feeding ^{14}C α-KSM to young pea leaf, about 80% of the ^{14}C α-KSM was consumed and 58% of the metabolized carbon was recovered in α-HSM, while ^{14}C α-HSM fed to pea leaf, most labelled carbon was still detected in α-HSM (Lloyd and Joy, 1978b). More ^{15}N was also discovered in the molecule of α-HSM than in α-KSM when ^{15}N Asn was fed to pea leaf (Ta et al., 1984). These findings suggested that α-KSM was not accumulated but could be reduced to α-HSM. Although the results showed that some α-KSM was detected in root, the concentration of α-HSM was higher than α-KSM in root, which does not rule out the possibility that α-KSM could be transported to sink tissues and decomposed in the latter. In addition, α-KSM can be deamidated to useful oxaloacetate by ω-amidase, which can react with acetyl-CoA to form citrate in the Krebs cycle (Russell, 2012; Streeter, 1977). Furthermore, malate, a

Table 3.3 Determination of ω-amidase substrates extracted from wild-type and mutant roots.

The data were analyzed by two-way ANOVA. Values are the means ± SD of four biological replicates; each biological replicate is the average of three technical replicates. WT presents wild-type Arabidopsis of which ecotype is Columbia. α-KSM and α-HSM mean α-ketosuccinamate and α-hydroxysuccinamate d.f., degrees of freedom.

Genotype	Asn Treatment	Substrates (nmol/mg)	
		α-KSM	α-HSM
WT	0 mM	8.0 ± 0.5	6.2 ± 0.3
	2 mM	10.4 ± 0.6	7.8 ± 0.5
Mutant	0 mM	16.3 ± 0.6	17.8 ± 0.3
	2 mM	27.3 ± 0.8	25.3 ± 0.3
Source of Variation	d.f.	ANOVA *P* values	
Genotype (G)	1	0.0001	0.0001
Treatment (T)	1	0.0001	0.0001
G × T	1	0.0001	0.0001
Error	12		

Figure 3.4 Effect of genotype and treatment on accumulation of α-KSM in wild-type and mutant roots.

KSM means the amount of α-KSM (nmol/mg fresh weight); WT indicates wild-type.

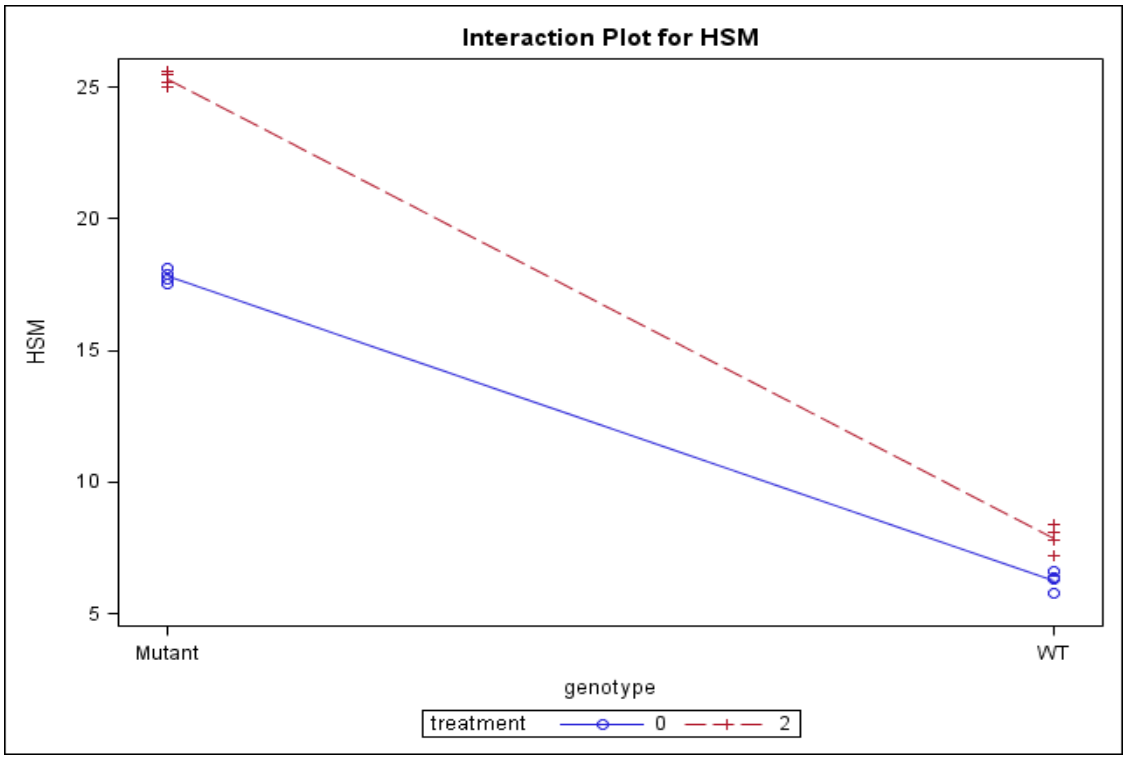

Figure 3.5 Effect of genotype and treatment on accumulation of α-HSM in wild-type and mutant roots.

HSM means the amount of α-HSM (nmol/mg fresh weight); WT indicates wild-type.

critical component in the Krebs cycle, can be produced from oxaloacetate by L-malate dehydrogenase (Russell, 2012). Therefore, being downstream following Asn transamination, ω-amidase plays an important role for transforming α-KSM to oxaloacetate and α-HSM to malate, which could be further utilized to produce energy through the Krebs cycle (Russell, 2012).

3.3.3 Catalytic activity of recombinant ω-amidase

To determine whether ω-amidase has aminotransferase activity, recombinant ω-amidase was produced as an N-terminal His-tagged protein in *E.coli* and purified by affinity chromatography on Ni-agarose. SDS-PAGE was performed to confirm the size and integrity of the purified protein (Figure 3.6). The purified protein had an apparent molecular mass a little below ca. 43 kDa, which corresponded to the predicted size including the additional N-terminal sequence (42.2 kDa).

Kinetic parameters were determined with the three substrates α-KGM, α-KSM and α-HSM (Figure 3.1). For α-HSM, the value of V_{max} was 4.04×10^{-8} katal mg^{-1}, 1.4-fold higher than α-KGM and α-KSM, while its K_m value was the lowest among three substrates. As a result, catalytic efficiency (V_{max} /K_m) value of α-HSM was higher than that of α-KGM by 2-fold, and α-KSM by 1.7-fold (Table 3.4). This suggests that α-HSM could be a slightly better substrate for recombinant ω-amidase, although the values from each substrate were similar. However, V_{max} of α-KGM was 71% lower than that determined with a human ω-amidase protein, and 97% lower than that from rat liver cytosolic ω-amidase (Krasnikov et al., 2009a). Jaisson et al. (2009) also reported that V_{max} with α-KGM, detected with ω-amidase from rat liver, was 19-fold higher while V_{max} value

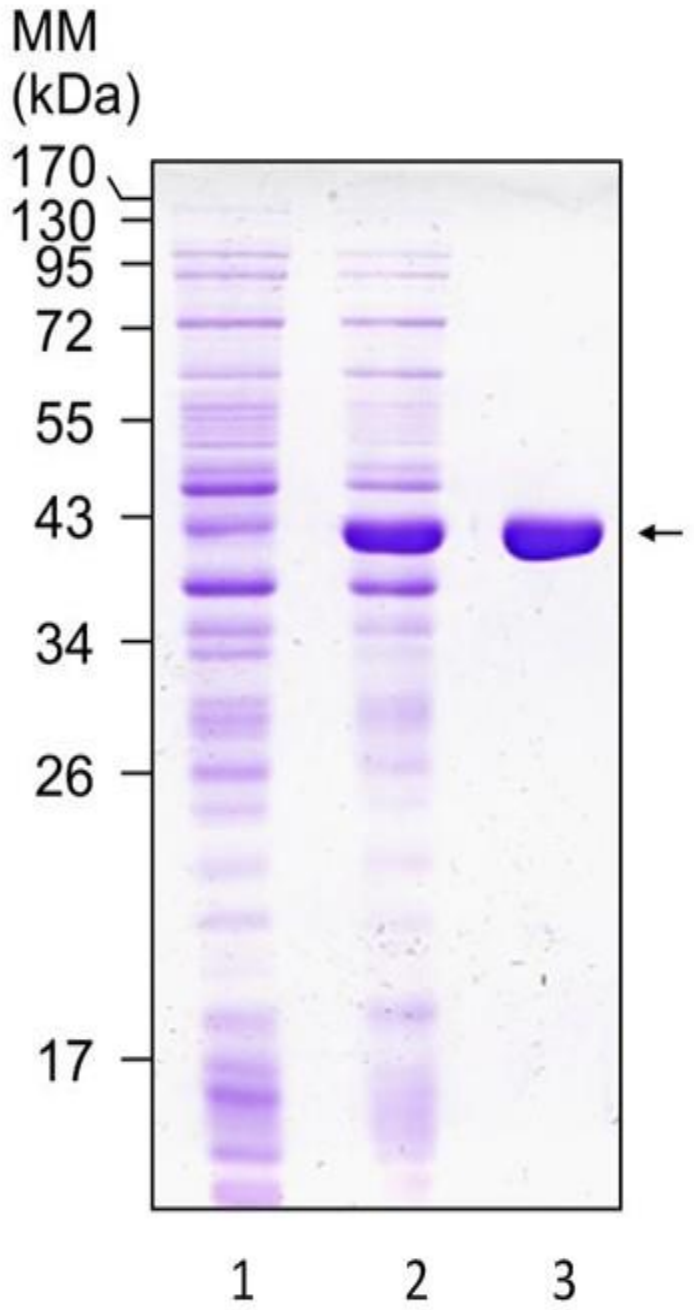

Figure 3.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant ω-amidase.
Lane 1 shows a soluble protein extract from non-induced cells and lane 2 from induced cells. Lane 3 shows the affinity-purified ω-amidase. Position of the purified protein is marked by an arrow. Molecular mass (MM) markers are indicated on the left.

Table 3.4 Apparent kinetic parameters of recombinant ω-amidase.

Values are the means ± s.d. of three replicates. Activity is expressed per mg protein. α-KGM, α-KSM and α-HSM mean α-ketoglutaramate, α-ketosuccinamate and α-ketosuccinamate.

Substrates	V_{max} ($\times 10^{-8}$ katal mg^{-1})	K_m (mM)	V_{max}/K_m ($\times 10^{-8}$ katal mg^{-1} mM^{-1})
α-KGM	2.78 ± 0.02	3.19 ± 0.15	0.87 ± 0.04
α-KSM	2.86 ± 0.03	2.69 ± 0.06	1.06 ± 0.02
α-HSM	4.04 ± 0.05	2.24 ± 0.06	1.80 ± 0.03
LSD ($p \leq 0.05$)	0.07	0.20	0.06

with α-KSM was comparable with the value from this study. One reason could be that the recombinant ω-amidase was expressed with a chloroplast transit peptide at the N-terminus, and it is possible that this peptide affected the specific activity. Present results showed that α-HSM had a higher V_{max}, lower K_m and higher V_{max}/K_m when compared with α-KSM and α-KGM. Therefore, recombinant ω-amidase has a slight substrate preference for α-HSM over the other two substrates.

In summary, the data presented here support the conclusion that ω-amidase may act as a critical enzyme linked to Asn transamination metabolically in Arabidopsis. The recombinant enzyme exhibited a slightly higher level of catalytic rate with α-HSM than with α-KSM or α-KGM, although the value with α-KGM was lower than in those reported in mammalian studies. Further research will be necessary to produce a new recombinant ω-amidase without the chloroplast transit peptide at the N-terminus and investigate its catalytic activity. In the *ω-amidase* mutant, the gene expression was blocked by the T-DNA insertion, which resulted in accumulation of ω-amidase substrates on Asn metabolite in root, supporting the idea that ω-amidase plays an important role in α-KSM and α-HSM metabolism, acting downstream from Asn transamination. The system established to measure metabolites concentrations in root will be useful to further investigate metabolic responses to Asn.

3.4 Reference

Albers, E., 2009. Metabolic characteristics and importance of the universal methionine salvage pathway recycling methionine from 5′-methylthioadenosine. IUBMB Life 61, 1132-1142.

Cooper, A. J. L., 2004. The role of glutamine transaminase K (GTK) in sulfur and α-keto acid metabolism in the brain, and in the possible bioactivation of neurotoxicants. Neurochem. Int. 44, 557-577.

Cooper, A. J. L., Meister, A., 1981. Comparative studies of glutamine transaminases from rat tissues. Comp. Biochem. Physiol., B: Comp. Biochem. 69, 137-145.

Cooper, A. J. L., Raps, S. P., Meister, A., 1987. Fluorometric determination of α-ketosuccinamic acid in rat tissues. Anal. Biochem. 167, 312-320.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., Scheible, W.-R., 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol. 139, 5-17.

Duffy, T. E., Cooper, A. J. L., Meister, A., 1974. Identification of α-ketoglutaramate in rat liver, kidney, and brain: relationship to glutamine transaminase and ω-amidase activities. J. Biol. Chem. 249, 7603-7606.

Dytham, C., 2011. Choosing and Using Statistics: A Biologist's Guide. Wiley-Blackwell, Oxford.

Gerdes, S., El Yacoubi, B., Bailly, M., Blaby, I., Blaby-Haas, C., Jeanguenin, L., Lara-Nunez, A., Pribat, A., Waller, J., Wilke, A., Overbeek, R., Hanson, A., de Crecy-Lagard, V., 2011. Synergistic use of plant-prokaryote comparative genomics for functional annotations. BMC Genomics 12, S2.

Hoskin, S. O., Gavet, S., Milne, E., Lobley, G. E., 2001. Does glutamine act as a substrate for transamination reactions in the liver of fed and fasted sheep? Br. J. Nutr. 85, 591-597.

Ivanov, A., Kameka, A., Pajak, A., Bruneau, L., Beyaert, R., Hernández-Sebastià, C., Marsolais, F., 2012. *Arabidopsis* mutants lacking asparaginases develop normally but exhibit enhanced root inhibition by exogenous asparagine. Amino Acids 42, 2307-2318.

Jaisson, S., Veiga-da-Cunha, M., Van Schaftingen, E., 2009. Molecular identification of ω-amidase, the enzyme that is functionally coupled with glutamine transaminases, as the putative tumor suppressor Nit2. Biochimie 91, 1066-1071.

Krasnikov, B. F., Chien, C. H., Nostramo, R., Pinto, J. T., Nieves, E., Callaway, M., Sun, J., Huebner, K., Cooper, A. J. L., 2009a. Identification of the putative tumor suppressor

Nit2 as ω-amidase, an enzyme metabolically linked to glutamine and asparagine transamination. Biochimie 91, 1072-1080.

Krasnikov, B. F., Nostramo, R., Pinto, J. T., Cooper, A. J. L., 2009b. Assay and purification of ω-amidase/Nit2, a ubiquitously expressed putative tumor suppressor, that catalyzes the deamidation of the α-keto acid analogues of glutamine and asparagine. Anal. Biochem. 391, 144-150.

Lloyd, N. D. H., Joy, K. W., 1978. 2-Hydroxysuccinamic acid: a product of asparagine metabolism in plants. Biochem. Biophys. Res. Commun. 81, 186-192.

Meister, A., Sober, H. A., Tice, S. V., Fraser, P. E., 1952. Transamination and associated deamidation of asparagine and glutamine. J. Biol. Chem. 197, 319-330.

Michalska, K., Jaskolski, M., 2006. Structural aspects of L-asparaginases, their friends and relations. Acta Biochim. Pol. 53, 627-640.

Mora, J., 1990. Glutamine metabolism and cycling in *Neurospora crassa*. Microbiol. Rev. 54, 293-304.

Moragaa, D. A., Macpheequiggley, K. M., Keefer, J. F., Schuster, S. M., 1989. Asparagine catabolism in rat-liver mitochondria. Arch. Biochem. Biophys. 268, 314-326.

Russell, P. J., 2012. Biology : Exploring the Diversity of Life. Nelson Education, Toronto.

Stephani, R. A., Meister, A., 1971. Structure of the dimeric α-keto acid analogue of asparagine. J. Biol. Chem. 246, 7115-7118.

Streeter, J. G., 1977. Asparaginase and asparagine transaminase in soybean leaves and root nodules. Plant Physiol. 60, 235-239.

Ta, T. C., Joy, K. W., Ireland, R. J., 1984. Amino acid metabolism in pea leaves: utilization of nitrogen from amide and amino groups of [^{15}N]asparagine. Plant Physiol. 74, 822-826.

Wang, R., Okamoto, M., Xing, X., Crawford, N. M., 2003. Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol. 132, 556-567.

Chapter 4 Conclusion

Asn is produced by amidation of Asp using either Gln or ammonium as an amino donor. Asn can be considered soluble in acidic and alkaline conditions, and it has a high N:C ratio 2:4, in contrast to other amino acids, such as Gln which has 2:5 (Lea et al., 2007). Asn is a stable substrate for only a few enzymatic reactions related to Asn metabolism, and has little net charge under physiological conditions (Giannino et al., 2008; Yang et al., 2011). In *Lupinus albus,* Asn is one important form of nitrogen that is commonly transported throughout the plant to sink tissues (Atkins et al., 1975). Asn can be used as a single nitrogen source to sustain Arabidopsis growth for up to 3 weeks (Hirner et al., 2006). Therefore, this suggests that Asn is an ideal and efficient substance for the transport and storage form of nitrogen in plants, and is involved in various physiological processes, such as germination, nitrate assimilation and nitrogen fixation (Lea et al., 2007; Sulieman and Tran, 2012).

In Asn catabolism, there are two main degradation pathways, of which one is making use of asparaginase to transform Asn to Asp and release ammonia. Two types of asparaginase, K^+-dependent asparaginases and K^+-independent asparaginases, have been characterized in Arabidopsis (Bruneau et al., 2006). Besides asparaginase, Asn aminotransferase catalyzes the transfer of the amino group of Asn to the corresponding α-keto acid, forming α-KSM. Previous studies have shown that asparaginase activity is high in young pea leaves but decreases when the leaf expands; however, aminotransferase activity remains high throughout leaf development (Ireland and Joy, 1981a). Feeding studies in pea leaf have shown that the two activities are approximately equal when the leaf is half expanded (Ireland and Joy, 1981a). The existence of two metabolic pathways

for Asn in the same tissue suggests that regulatory mechanisms need to be operated. Further research on the subcellular localization of the two Asn catabolism enzymes, asparaginase and aminotransferase in pea demonstrated that asparaginase was purified from cytosolic supernatant while Asn aminotransferase was localized in the peroxisome (Ireland and Joy, 1983c). As Asn can be metabolized in two different subcellular compartments in pea leaf, the regulation of Asn metabolism in plant leaf could mean that when both enzymes are active in young leaf, asparaginase will transform Asn to Asp and release ammonia in cytosol, whereas aminotransferase will generate α-KSM and the corresponding amino acid in peroxisome. With the growth of the leaf, aminotransferase will play an increasing important role in Asn degradation when asparaginase activity decreases. It is possible that Asn metabolism is restricted to the peroxisome in mature leaf.

According to previous studies, Asn aminotransferase is the same enzyme as Ser:glyoxylate aminotransferase, which is involved in photorespiration (Ireland and Joy, 1983b) (Chapter 1, Figure 1.2). Ser:glyoxylate aminotransferase has been characterized as Asn aminotransferase in tobacco and barley (Havir and McHale, 1988; Murray et al., 1987a). An *Arabidopsis* mutant lacking *AGT1*, a gene encoding Ser:glyoxylate aminotransferase, is devoid of Ser:glyoxylate aminotransferase activity (Somerville and Ogren, 1980). Although Ser:glyoxylate aminotransferase has been purified from Arabidopsis and characterized with different amino acids as substrates, such as Ser and Ala (Kendziorek and Paszkowski, 2008b), its catalytic activities with Asn as a substrate had not been characterized. Therefore, one contribution from this study was to determine the activities of recombinant Ser:glyoxylate aminotransferase purified from *E. coli* with

Asn as well as other amino acid substrates. The results showed that recombinant Ser:glyoxylate aminotransferase had a substrate preference for Asn as an amino group donor, when compared with Ala and Ser using the same acceptor substrates. The kinetic parameters of the recombinant Ser:glyoxylate aminotransferase for Asn (Table 2.1) were comparable to those values of recombinant K^+-independent asparaginase, ASPGA1, which had a lower V_{max}, of 1.24×10^{-8} katal mg^{-1}, and a higher K_m value of 14.7 mM (Gabriel et al., 2012a). This means that recombinant Ser:glyoxylate aminotransferase may have a higher affinity for Asn than this asparaginase.

Asn can be transformed by Asn aminotransferase to yield α-KSM, and similarly transamination of Gln produces α-KGM, both of which are substrates of ω-amidase (Meister et al., 1952). ω-Amidase deamidates the α-keto group of α-KSM and α-KGM to methylene group products, which are oxaloacetate and α-ketoglutarate (Meister et al., 1955). Previous studies have demonstrated that α-KSM can be reduced to α-HSM in pea and soybean leaf, and this is also a substrate of ω-amidase (Lloyd and Joy, 1978b; Streeter, 1977). The metabolism of α-KSM by reduction to α-HSM is unclear. The specific enzyme, which could be called α-KSM dehydrogenase, has not been isolated or characterized from any organism. Further research about the characteristics and function of the dehydrogenase is necessary as this enzyme may play a role in Asn metabolism, by reducing the product of Asn transamination.

Mammalian ω-amidase has been studied from rat experimentally, and corresponds to a protein known as Nitrilase 2 (NIT 2) in databases (Krasnikov et al., 2009a; Krasnikov et al., 2009b). The activity of recombinant ω-amidase from rat with α-KSM and α-KGM has been reported (Jaisson et al., 2009). However, despite one ω-amidase

candidate gene being present in Arabidopsis (Gerdes et al., 2011a), the role and function of ω-amidase related to Asn metabolism was still unclear in this species. In this study, AT5G12040 was identified as *ω-amidase* in Arabidopsis based on amino acid sequence identity. Concerning the *ω-amidase* mutant, the location of the T-DNA insertion and its effect on gene expression were measured by DNA sequencing and RT-PCR analysis. The results indicated that ω-amidase transcripts were absent in the mutant when compared with the wild-type. The reason could be that the T-DNA insertion prevents the production of a functional transcript. The results of quantification of ω-amidase substrates in wild-type and mutant roots, treated with 2 mM Asn or without Asn as control, revealed that α-KSM and α-HSM were over accumulated in mutant roots in control and Asn treatment. Enzymatic assays demonstrated that recombinant ω-amidase has a substrate preference for α-HSM. These results support the idea that ω-amidase plays a role in α-KSM and α-HSM metabolism, and can deamidate the products of Asn transamination to release ammonia.

In conclusion, transamination of Asn normally takes place with glyoxylate or pyruvate in Asn metabolism, which transfers the amino group to Gly or Ala (Ireland and Joy, 1983b). Thus, taking into consideration the action of Asn transaminase, the amino group of Asn can be transferred to Ala or Gly, which can be further metabolized to a range of amino and other compounds. Subsequently, the amide group from Asn could be removed to yield ammonia via deamidation of α-KSM or α-HSM by ω-amidase. These pathways may provide a series of reactions for the utilization of both nitrogenous groups of Asn, which can enrich the usefulness of Asn in nitrogen metabolism in plants. Future

studies designed to understand other pathways downstream of Asn transamination should be undertaken.

4.1 Reference

Atkins, C. A., Pate, J. S., Sharkey, P. J., 1975. Asparagine metabolism - key to nitrogen nutrition of developing legume seeds. Plant Physiol. 56, 807-812.

Bruneau, L., Chapman, R., Marsolais, F., 2006. Co-occurrence of both L-asparaginase subtypes in *Arabidopsis*: *At3g16150* encodes a K^+-dependent L-asparaginase. Planta 224, 668-679.

Gabriel, M., Telmer, P., Marsolais, F., 2012. Role of asparaginase variable loop at the carboxyl terminal of the alpha subunit in the determination of substrate preference in plants. Planta 235, 1013-1022.

Gerdes, S., El Yacoubi, B., Bailly, M., Blaby, I., Blaby-Haas, C., Jeanguenin, L., Lara-Nunez, A., Pribat, A., Waller, J., Wilke, A., Overbeek, R., Hanson, A., de Crecy-Lagard, V., 2011. Synergistic use of plant-prokaryote comparative genomics for functional annotations. BMC Genomics 12, S2.

Giannino, D., Nicolodi, C., Testone, G., Frugis, G., Pace, E., Santamaria, P., Guardasole, M., Mariotti, D., 2008. The overexpression of asparagine synthetase A from *E. coli* affects the nitrogen status in leaves of lettuce (*Lactuca sativa L.*) and enhances vegetative growth. Euphytica 162, 11-22.

Havir, E. A., McHale, N. A., 1988. A mutant of *Nicotiana sylvestris* lacking serine:glyoxylate aminotransferase: substrate specificity of the enzyme and gate of [2-^{14}C] glycolate in plants with genetically altered enzyme levels. Plant Physiol. 87, 806-808.

Hirner, A., Ladwig, F., Stransky, H., Okumoto, S., Keinath, M., Harms, A., Frommer, W. B., Koch, W., 2006. *Arabidopsis* LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. Plant Cell 18, 1931-1946.

Ireland, R., Joy, K., 1981. Two routes for asparagine metabolism in *Pisum sativum* L. Planta 151, 289-292.

Ireland, R. J., Joy, K. W., 1983a. Purification and properties of an asparagine aminotransferase from *Pisum sativum* leaves. Arch. Biochem. Biophys. 223, 291-296.

Ireland, R. J., Joy, K. W., 1983b. Subcellular localization of asparaginase and asparagine aminotransferase in *Pisum sativum* leaves. Plant Physiol. 72, 1127-1129.

Jaisson, S., Veiga-da-Cunha, M., Van Schaftingen, E., 2009. Molecular identification of ω-amidase, the enzyme that is functionally coupled with glutamine transaminases, as the putative tumor suppressor Nit2. Biochimie 91, 1066-1071.

Kendziorek, M., Paszkowski, A., 2008. Properties of serine:glyoxylate aminotransferase purified from *Arabidopsis thaliana* leaves. Acta Biochim. Biophys. Sinica 40, 102-110.

Krasnikov, B. F., Chien, C. H., Nostramo, R., Pinto, J. T., Nieves, E., Callaway, M., Sun, J., Huebner, K., Cooper, A. J. L., 2009a. Identification of the putative tumor suppressor Nit2 as ω-amidase, an enzyme metabolically linked to glutamine and asparagine transamination. Biochimie 91, 1072-1080.

Krasnikov, B. F., Nostramo, R., Pinto, J. T., Cooper, A. J. L., 2009b. Assay and purification of ω-amidase/Nit2, a ubiquitously expressed putative tumor suppressor, that catalyzes the deamidation of the α-keto acid analogues of glutamine and asparagine. Anal. Biochem. 391, 144-150.

Lea, P. J., Sodek, L., Parry, M. A. J., Shewry, P. R., Halford, N. G., 2007. Asparagine in plants. Ann. Appl. Biol. 150, 1-26.

Lloyd, N. D. H., Joy, K. W., 1978. 2-Hydroxysuccinamic acid: a product of asparagine metabolism in plants. Biochem. Biophys. Res. Commun. 81, 186-192.

Meister, A., Levintow, L., Greenfield, R. E., Abendschein, P. A., 1955. Hydrolysis and transfer reactions catalyzed by ω-amidase preparations. J. Biol. Chem. 215, 441-460.

Meister, A., Sober, H. A., Tice, S. V., Fraser, P. E., 1952. Transamination and associated deamidation of asparagine and glutamine. J. Biol. Chem. 197, 319-330.

Murray, A. J. S., Blackwell, R. D., Joy, K. W., Lea, P. J., 1987. Photorespiratory N donors, aminotransferase specificity and photosynthesis in a mutant of barley deficient in serine: glyoxylate aminotransferase activity. Planta 172, 106-113.

Somerville, C. R., Ogren, W. L., 1980. Photorespiration mutants of *Arabidopsis thaliana* deficient in serine-glyoxylate aminotransferase activity. Proc. Natl. Acad. Sci. U. S. A. 77, 2684-2687.

Streeter, J. G., 1977. Asparaginase and asparagine transaminase in soybean leaves and root nodules. Plant Physiol. 60, 235-239.

Sulieman, S., Tran, L.-S. P., 2012. Asparagine: an amide of particular distinction in the regulation of symbiotic nitrogen fixation of legumes. Crit. Rev. Biotechnol. 0, 1-19.

Yang, X., Wang, X., Wei, M., Hikosaka, S., Goto, E., 2011. Changes of glutamine and asparagine content in cucumber seedlings in response to nitrate stress. Int. J. of Plant Pro. 5, 1-7.

Curriculum Vitae

Name: Qianyi Zhang

Post-secondary Education and Degrees:

2011 – 2013 M.Sc. Department of Biology – Physiology and Biochemistry
Western University
London, Ontario, Canada

2010 – 2011 York Pre-Graduate Preparation Program
York University English Language Institute
Toronto, Ontario, Canada

2006 – 2010 B.Sc. College of Life Sciences - Biochemistry and Molecular Biology
South China Agriculturale University
Guangzhou, Guangdong, China

Honours and Awards:

2010 – 2012 Western Graduate Research Scholarship – Western University

2008 – 2009 Fourth Prize in Men's Badminton Doubles
Fifth Prize in Men's Badminton Single – College of Life Sciences

2007 – 2008 Comprehensive Scholarship - South China Agricultural University

Related Work Experience:

2011 – 2013 Teaching Assistant – First Year Biology Laboratories
The University of Western Ontario
London, Ontario, Canada

2011 – 2013 Research Participants (RP Student)
Agriculture and Agri-Food Canada – Southern Crop Protection and Food Research Centre
London, Ontario, Canada

Jan – Feb, 2010 Intern – Bacteria Fermentation Preparation
Guangzhou Institute of Microbiology
Guangzhou, Guangdong, China

Publications:

Qianyi Zhang, Frédéric Marsolais. (2013). Identification and characterization ω-amidase as an enzyme metabolically linked to asparagine transamination in *Arabidopsis thaliana* (not published yet).

Qianyi Zhang, Jamie Lee, Sudhakar Pandurangan, Matthew Clarke, Agnieszka Pajak, Frédéric Marsolais. (2013). Characterization of *Arabidopsis* serine:glyoxylate aminotransferase, AGT1, as an asparagine aminotransferase. Phytochemistry *85*: 30 – 35.

Guohui Zhu, Weiying XU, Dan Wu and **Qianyi Zhang**. (2010). Effect of abscisic acid biosynthesis gene NCED6 in glucose induced delay of *Arabidopsis thaliana* seed germination. Plant Physiology Communications. *45*: 21 – 26.