Asparagine Metabolic Pathways in Arabidopsis

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Inorganic nitrogen in the form of ammonium is assimilated into asparagine via multiple steps involving glutamine synthetase (GS), glutamate synthase (GOGAT), aspartate aminotransferase (AspAT) and asparagine synthetase (AS) in Arabidopsis. The asparagine amide group is liberated by the reaction catalyzed by asparaginase (ASPG) and also the amino group of asparagine is released by asparagine aminotransferase (AsnAT) for use in the biosynthesis of amino acids. Asparagine plays a primary role in nitrogen recycling, storage and transport in developing and germinating seeds, as well as in vegetative and senescence organs. A small multigene family encodes isoenzymes of each step of asparagine metabolism in Arabidopsis, except for asparagine aminotransferase encoded by a single gene. The aim of this study is to highlight the structure of the genes and encoded enzyme proteins involved in asparagine metabolic pathways; the regulation and role of different isogenes; and kinetic and physiological properties of encoded enzymes in different tissues and developmental stages.

Keywords: Amides and amino acids • Ammonium assimilation • Arabidopsis • Asparagine synthesis and catabolism • Nitrogen metabolism • Transport.

Abbreviations: AS, asparagine synthetase; AsnAT, asparagine aminotransferase; AspAT, aspartate aminotransferase; ASPG, asparaginase; DAA, days after anthesis; Fd, ferredoxin; GDH, glutamate dehydrogenase; GFP, green fluorescent protein; GOGAT, glutamate synthase; GS, glutamine synthetase; GUS, β-glucuronidase; TCA, tricarboxylic acid.

Introduction

Nitrogen is an essential element for plant growth and development, and its efficient utilization has strong effects on biomass and plant productivity. Plants absorb nitrogen in the soil as nitrate and ammonium that are taken up via transporters, and nitrate is reduced to ammonium via nitrate reductase [NAD(P)H-NR, EC 1.7.1.1, EC 1.7.1.2, EC 1.7.1.3] and nitrite reductase [ferredoxin (Fd)-NR, EC 1.7.7.1]. Ammonium is first assimilated into the organic nitrogen glutamine by glutamine synthetase (GS), and then the transfer of the glutamine-amide nitrogen to 2-oxoglutarate by glutamate synthase (GOGAT) yields two molecules of glutamate (GS/GOGAT cycle). Glutamine and glutamate are further metabolized into amides and amino acids such as aspartate by aspartate aminotransferase (AspAT) and asparagine by asparagine synthetase (AS) (Fig. 1). These amides and amino acids glutamine, asparagine, glutamate and aspartate are utilized in photosynthesis to synthesize amino acids, and are also utilized in the biosynthesis of a wide range of higher plants, namely cytosolic GS1 and chloroplastic GS2 (McNally and Hirel 1983). The Arabidopsis genome database (Arabidopsis Genome Initiative 2000) and cloning studies of these amides and amino acids glutamine, asparagine, glutamate and aspartate represent the major intra- and intercellular nitrogen carriers transported in the vascular bundles of plants (Lea et al. 2007, Gaufichon et al. 2010). Of these, asparagine and glutamine act as sources of amide and amino nitrogen for amino acid interconversions and function as efficient nitrogen storage and partitioning compounds. The hydrolysis of the asparagine-amide group by asparaginase (ASPG) yields ammonium and aspartate that are used for amino acid biosynthesis (Ireland and Lea 1999). The transamination of asparagine by serine-glyoxylate aminotransferase (AGT1) releases the amino group and 2-oxosuccinamate (Ireland and Lea, 1999). 2-Oxosuccinamate is hydrolyzed by α-amidase to ammonium and oxaloacetate (Fig. 1). 2-Oxosuccinamate can be rapidly converted to 2-hydroxysuccinimide that is hydrolyzed by α-amidase to ammonium and malate (Ireland and Lea 1999). Finally, the glutamine transamination by glutamine transaminase yields different amino acids and 2-oxoglutarate, which is subsequently hydrolyzed to 2-oxoglutarate and ammonium by α-amidase (Ellens et al. 2015). Ammonium is re-assimilated into glutamine, and the GOGAT cycles glutamine to glutamate. As a result of this glutamine cycle, glutamine and glutamate are continuously synthesized as nitrogen and carbon sources at the expense of ATP, reduced Fd and/or NADH, whilst this cycle appears to be required for diverting carbon skeletons to the tricarboxylic acid (TCA) cycle (Calderón and Mora 1989). Multiple gene family members encode the isoenzymes of each step of nitrogen assimilation into asparagine and nitrogen release from asparagine. Recent analyses of Arabidopsis mutants deficient in particular isogenes and overexpressing lines have provided insight to define the in vivo functions of the encoded isoenzymes which are localized in distinct cells and subcellular compartments. In this study, special attention is focused on asparagine metabolism in Arabidopsis.

Glutamine Synthetase

Glutamine synthetase (GS; EC 6.3.1.2) catalyzes the first step of the ATP-dependent assimilation of ammonium into glutamine (Fig. 1). GS exists in two distinct forms in a wide range of higher plants, namely cytosolic GS1 and chloroplastic GS2 (McNally and Hirel 1983). The Arabidopsis genome database (Arabidopsis Genome Initiative 2000) and cloning studies...
demonstrated a single gene for chloroplastic GS2, GLN2 (At5g35630), and five genes for cytosolic GS1, GLN1;1 (At5g35630), GLN1;2 (At1g66200), GLN1;3 (At1g66200), GLN1;4 (At3g17820), GLN1;5 (At1g48470) [Peterman and Goodman 1991; The Arabidopsis Information Resource (TAIR): https://www.arabidopsis.org/]. Likewise, a single nuclear gene encodes GS2, while GS1 is coded for by a small multinuclear gene family varying from three to five members per haploid in most plant species, with the exception of a second GS2 gene identified on the same or a separate chromosome (Seabra et al. 2010, Castro-Rodriguez et al. 2011, Swarbreck et al. 2011). GLN2 maps to chromosome V (Fig. 2). Two genes, GLN1;1 and GLN1;4, are found on chromosome V; two genes, GLN1;2 and GLN1;5, map to chromosome I; and the fifth gene, GLN1;3, is located on chromosome III (Fig. 2). The GLN genes are composed of 9–12 exons and encode a predicted mature GS2 protein of 42.5 kDa (385 amino acids), GS1 proteins vary from 38.6 kDa (354 amino acids for GLN1;3) to 40.4 kDa (356 kDa for GLN1;2) (Fig. 2; TAIR). A phylogenetic analysis clustered 93 nucleotide sequences from 43 plant species into the GLN1 and GLN2 group. In the GLN1 group, Brassicacea has two GLN1 subgroups, and five GLN1 genes of Arabidopsis are divided into two clusters, one includes GLN1;1, GLN1;2 and GLN1;4, and the other contains GLN1;3 and GLN1;5 (Lothier et al. 2011).

Expression studies using GS promoter::GUS/GFP (β-glucuronidase/green fluorescent protein) fusions demonstrated that chloroplastic GLN2 is expressed predominantly in leaf mesophyll cells and low levels in roots and germinated seeds (Peterman and Goodman 1991, Thum et al. 2003). Phytochromes control GLN2 expression (Thum et al. 2003), which is in agreement with the functions of GS2 in primary nitrogen assimilation and photorespiratory nitrogen recycling (Miflin and Habash 2002, Swarbreck et al. 2011). Cytosolic GLN1 genes are preferentially expressed in the vascular tissues of leaves during senescence, suggesting that GS1 play a role in nitrogen remobilization (Bernard and Habash 2009, Guan...
et al. 2015). In Arabidopsis, GS2 can be dually targeted to the chloroplasts and mitochondria (Taira et al. 2004). Predicted signal peptides vary from 30 residues (3.2 kDa) to 45 residues (5.0 kDa), giving different targeting predictions (Table 1). Taira et al. (2004) showed that the GLN2::GFP translational fusion which consisted of the 795 nucleotide sequence encoding the first 181 residues of GS2 precursor directs both the GFP fluorescence and chimeric GLN2::GFP proteins into both the

![Fig. 2 Map position (A) and exon–intron organization (B) of genes for enzymes involved in asparagine metabolic pathways in Arabidopsis. Scale in blue = 1 kbp.](image-url)
mitochondria and chloroplasts. Yloc, Target and ProtComp predict the GS2 protein to be either chloroplastic or mitochondrial, whereas ProteinProwler predicts a higher score for the chloroplast (Table 1). Taira et al. (2004) hypothesized that photorespiratory ammonium released in mitochondria by the glycine decarboxylase complex (EC 1.4.4.2/2.1.2.10) can be directly re-assimilated into glutamine by GS in this organelle. In principle, primary ammonium derived from nitrate reduction and photorespiratory ammonium produced in mitochondria are shuttled by passive transport to mesophyll chloroplasts where GS2 and Fd-GOGAT assimilate ammonium in different plant species (Somerville and Ogren 1980, Walls Grove et al. 1987, Pérez-Delgado et al. 2015). Also, it is postulated that mitochondrial GS provides carbamoylphosphate synthetase (CPSase; EC 6.3.5.5) which uses glutamine for carbamoylphosphate synthesis in the mitochondria (Taira et al. 2004). The carbamoylphosphate-dependent pathway converts ornithine to citrulline, coupled to the subsequent conversion to arginine in chloroplasts (Taira et al. 2004). Following the finding of the dual GS2 targeting, Linka and Weber (2005) examined the hypothetical ornithine–citrulline shuttle and glutamine–glutamate shuttle between mitochondria and chloroplasts. Based on the experimental evidence available, photorespiratory nitrogen and carbon dioxide transfer between mitochondria and chloroplasts by these shuttles requires novel chloroplastic/mitochondrial envelope membrane proteins including amino acid transporters (Linka and Weber 2005).

Initially, GLN1;2 is found to be preferentially expressed in roots and to a lesser extent in seeds, and overall expression levels of GLN1;1 are 8- to 10-fold higher than those of GLN1;3 (Peterman and Goodman 1991). The five isoforms of Arabidopsis GS1 have distinct expression patterns and affinity for the substrates (Ishiyama et al. 2004). Cytosolic GS1 is important for primary ammonium assimilation in roots and re- assimilation of ammonium produced by protein hydrolysis (Thomsen et al. 2014). Studies with Arabidopsis roots showed that GLN1;2 is expressed in the root vasculature, GLN1;1 in the root surface layer, GLN1;3 in the vascular tissues and GLN1;4 in the pericycle cells (Ishiyama et al. 2004). Recombinant GLN1;2 and GLN1;3 have a low affinity for ammonium, while GLN1;1 and GLN1;4 have a high affinity (Ishiyama et al. 2004). In addition, genes for cytosolic GS1 have tissue-specific expression patterns: two genes for cytosolic GS1 (GLN1;1 and GLN1;3) are expressed at high levels in germinating seeds, suggesting a role for glutamine synthesis during nitrogen mobilization. Then, by studying a GLN1 promoter::GFP transgene, GLN1;2 expression is observed in the phloem companion cells and parenchyma of leaf veins (Lothier et al. 2011) but also in the epidermal cells of sepal, veins of petals and stamens, and nodes between the pedicel and siliques (Guan et al. 2015). As observed for these GLN1 genes, a distinct cellular localization of specific GS1 isoforms may be central to maintaining the nitrogen remobilization to developing sink organs. Consistently, Arabidopsis GLN1;2 knockout mutants show impaired seed yields such as fewer siliques, less seeds per siliques and lower dry weight per seed (Guan et al. 2015). In contrast, GLN1;2 appears not to be essential for seed production and instead is involved in ammonium detoxification and nitrogen assimilation under a high nitrate supply (Lothier et al. 2011). A mutation in GLN1;1 does not affect seed yields but impairs root development during seed germination (Guan et al. 2015).

Studies on the regulation of GLN genes and GS proteins point to an important physiological role for light, sugars and amino acids in their regulation. Light induction of the GLN2 mRNA level, mediated in part by phytochromes, can be replaced by sucrose (Oliveira and Coruzzi 1999, Thum et al. 2003). In contrast, light has less effect on the expression of the GLN1 genes GLN1;1, GLN1;2 and GLN1;3 (Oliveira and Coruzzi 1999). A modeling approach showed that GLN2 expression shares light and carbon signal transduction pathways. In light- and dark-grown seedlings of Arabidopsis, carbon and light over-ride light and carbon, respectively, as a major regulator of GLN2 (Thum et al. 2003). In addition, carbon interacts with blue, red or far-red light signaling pathways in both etiolated and light-grown seedlings (Thum et al. 2003). The amides and amino acids glutamate, glutamine, asparagine and aspartate have inhibitory effects on sucrose-induced mRNA accumulation of GLN2, GLN1;1, GLN1;2 and GLN1;3 (Thum et al. 2003). These data indicate that ammonium assimilation into glutamine occurs in response to the nitrogen and carbon metabolic

<table>
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<th>Gene</th>
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<th>AGI</th>
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Subcellular localization was predicted using four programs: ProtComp, ProteinProwler, TargetP and Yloc. Numbers for the chloroplast and mitochondrion indicate the integral score (ProtCom), targeting score (ProteinProwler), prediction score at reliability class 3 (GLN2) and 1 (GLU1) (TargetP) and percentage probability (Yloc). ProtComp: http://www.softberry.com/berry.pl?topic=protcomp&group=programs&subgroup=protloc (ProtComp v 9.0). ProteinProwler: http://bioinf.scmb.uq.edu.au:8080/pprowler_webapp_1-2/ (Version 1.2). TargetP: http://www.cbs.dtu.dk/services/TargetP/. Yloc: http://abi.inf.uni-tuebingen.de/Services/YLoc/webloc.cgi.
state. Specific transcription factors binding to the GS promoter or transcripts have been demonstrated and they may play a role in the fine regulation of GLN expression by sensing carbon and nitrogen conditions. Analysis of the transcript and metabolite network has predicted that the MYB transcription factor CCA1 (Circadian Clock-Associated 1) plays a role in regulating GLN1-3 expression in response to carbon supply (Gutiérrez, 2012). Genes involved in nitrogen uptake and assimilation including GS2 are shown to be transcriptionally regulated by the transcription factor NLP7 (Marchive et al. 2013). Also, several lines of evidence indicate that GS activity is regulated by factors other than transcription. A search for homologous genes involved in the regulation of nitrogen metabolism led to the characterization of the gene and encoded chloroplastic PI1-like protein in Arabidopsis and Castor bean (Hsieh et al. 1998). Arabidopsis GS2 possesses two cysteine residues, Cys306 and Cys371, that are absent in cytosolic isoforms but highly conserved in plastidial GS2 of angiosperms. Choi et al. (1999) suggested that these cysteines are involved in activating GS2 activity via thioredoxins that would prevent formation of internal disulfide bonds and maintain reduced cysteine sulfhydryl groups. In line with this mechanism, light activation of GS2 activity from green algae and Canavalia lineata is known to be due to thioredoxin activity (Tischner and Schmidt 1982, Florenicio et al. 1993).

Glutamate Synthase

Glutamate synthase (GOGAT, glutamine: 2-oxoglutarate aminotransferase) transfers the glutamine-amide group to the 2-position of 2-oxoglutarate, forming two molecules of glutamate (Fig. 1). In Arabidopsis as well as in other higher plants, GOGAT exists in two distinct forms located in the chloroplasts or plastids: one that uses reduced Fd (Fd-GOGAT, EC 1.4.7.1) and one that uses NADH as the electron donor (NADH-GOGAT, EC 1.4.1.14) (Suzuki and Knaff 2005). A search of the Arabidopsis genome database (Arabidopsis Genome Initiative 2000) revealed two genes, GLU1 (At5g04140) and GLU2 (At2g41220), coding for Fd-GOGAT and one gene, GLT (At5g53460), coding for NADH-GOGAT (Fig. 2), while several plant genomes contain two NADH-GOGAT genes (Tabuchi et al. 2007, Blanco et al. 2008). GLU1 maps to the top of chromosome V at the 1,129,852 to 1,138,442 region. GLU2 maps to a separate chromosome II at the 17,177,664 to 17188751 region, and GLT maps to the same chromosome (V) at the 21,700,202 to 21,710,066 region (Fig. 2). The third form of GOGAT that is dependent on NAPDH as the electron donor (NAPDH-GOGAT, EC 1.4.1.13) is found in bacteria (Vanoni et al. 2005, Vanoni 2012). Prokaryotic NAPDH-GOGAT is encoded by two genes: gltB coding for the large α subunit (~150 kDa) and gltD coding for the small β subunit (~50 kDa), giving an active (αβ), heterodimer (Vanoni et al. 2005). GLU1 and GLU2 are each composed of 33 exons coding for Fd-GOGAT proteins of 165 kDa (Fig. 2). These polypeptides contain a class II (purf)-type glutaminase domain that releases the glutamine-amide group at the N-terminal region together with the FMN- and sulfur center-binding regions at the C-terminal region along the polypeptide. The GLU1-encoded glutaminase activity might be sensitive to in vivo chloroplast conditions and releases ammonium from glutamine during a shortage of NADPH (Yoneyama et al. 2015). The amino acid sequences of GLU1- and GLU2-encoded Fd-GOGAT isoforms are 80% identical (TAIR). These Fd-GOGAT polypeptides share up to 40% similarity with the prokaryotic α subunit but contain no region similar to the prokaryotic β subunit (Suzuki and Knaff 2005). GLT is composed of 20 exons encoding a single polypeptide of NADH-GOGAT with a high molecular mass of 240 kDa (Fig. 2). NADH-GOGAT polypeptide also contains a glutaminase domain at the N-terminal region as well as an FMN/FeS synthase domain at the C-terminal region (Vanoni et al. 2005). NADH-GOGAT shows a high sequence similarity to the α subunit and β subunit of NAPDH-GOGAT (Suzuki and Knaff 2005).

Other than these sequence similarities, archaeal genomes contain an open reading frame similar to that of the gltB α subunit which would be active with bacterial-type Fds, while a sequence similar to that of the gltB β subunit is either found or absent (Suzuki and Knaff 2005, Kameya et al. 2007, Pire et al. 2014). Specific complex formation between cyanobacterial Fd-GOGAT and Fd occurs for the multiple electron transfer steps (Suzuki and Knaff 2005, Shimura et al. 2012). Assuming that these sequence similarities are associated with their evolution, bacterial gltB-like genes evolved to cyanobacterial Fd-GOGAT by endosymbiosis, followed by plastid gene transfer to plants. In this scenario, gltB and gltD would have been fused to a single gene coding for NADH-GOGAT in yeasts, fungi and plants (Suzuki and Knaff 2005).

GOGAT plays two nitrogen assimilatory roles: one for primary ammonium assimilation in leaves and roots, and the other for re-assimilation of photorespiratory ammonium. Expression studies demonstrated that GLU1 is the major Fd-GOGAT gene expressed in the mesophyll and vascular bundle of leaves and to lesser extents in non-photosynthetic tissues such as roots (Suzuki and Rothstein 1997, Potel et al. 2009). On the other hand, GLU2 is expressed only at low constitutive levels irrespective of the tissues examined. GLT encoding NADH-GOGAT is expressed at higher levels in roots than in leaves, with an overlapping presence of cytosolic GS1 (Lancien et al. 2002). Exposure of dark-grown Arabidopsis seedlings to white light increases GLU1 mRNA levels, whereas GLU2 mRNA levels remain constant (Suzuki and Rothstein 1997, Coschigano et al. 1998). The induction of the GLU1 mRNA level is mediated in part by phytochromes, and sucrose mimics phytochrome effects although to lesser extents than red light (Ziegler et al. 2003). In contrast, light has no effect on NADH-GOGAT levels (Suzuki and Rothstein 1997). As a result, Fd-GOGAT accounts for >95% of the total GOGAT activity in photosynthetic tissues, and NADH-GOGAT represents only a residual activity.

The role of Fd-GOGAT in photorespiratory ammonium reassimilation has been shown using mutants deficient in Fd-GOGAT (Somerille and Ogren 1980, Wallsgraves et al. 1987, Suzuki and Rothstein 1997, Coschigano et al. 1998). The glu1 mutants lacking GLU1-encoded Fd-GOGAT and containing <5% of the wild-type Fd-GOGAT activity become chlorotic.
under atmospheric conditions but grow in high CO₂ that suppresses photorespiration in the mesophyll (3,000 p.p.m.) (Somerville and Ogren 1980, Suzuki and Rothstein 1997, Coschigano et al. 1998). This conditional lethal phenotype unequivocally shows that the GLU1-encoded Fd-GOGAT isomerase is essential for photorespiratory ammonium re-assimilation coupled with chloroplastic GS2. The altered GLU1 expression has also been shown to result in changed profiles of amino acids and nitrogenous compounds such as Chls and nucleotides (Coschigano et al. 1998, Potel et al. 2009, Kissen et al. 2010).

The GATA transcription factors, encoded by the paralogs GNC and CGA1 and induced in a phytochrome-dependent manner, have been shown to target GLU1 and key Chl synthesis genes, associated with the nitrogen flux to enhanced Chl production (Hudson et al. 2011). Recent studies demonstrated that the GLU1-encoded Fd-GOGAT is dually targeted to the mitochondria and chloroplasts (Jamai et al. 2009). This dual targeting presumably involves a twin pre-sequence mechanism of the N-terminal initial triplet (Met/Lys/Met) (Jamai et al. 2009). ProtComp and Yloc recognize Fd-GOGAT protein to be chloroplastic or mitochondrial, while Target and ProteinProwler predicts it to be chloroplastic (Table 1). The location of Fd-GOGAT in the mitochondria rather than in the chloroplasts is predicted to different extents by four programs (Table 1). In the mitochondria, Fd-GOGAT may be required for photorespiratory mitochondrial reactions. It is hypothesized that a protein–protein interaction of Fd-GOGAT with serine hydroxymethyltransferase (SHMT; EC 2.1.2.1) maintains the SHMT activity. The reversible reaction of SHMT consists of transferring a methyl group of serine to tetrahydrofolate, thus forming glycine and 5,10-methylenetetrahydrofolate (5,10-mTHF) (Voll et al. 2006). The role of Fd-GOGAT bound to SHMT1 may consist of reducing the sensitivity of SHMT activity to a binding inhibitor, 5-formyltetrahydrofolate (5-CHO-THF), or lowering 5-CHO-THF accumulation (Jamai et al. 2009). Protein–protein interactions of Fd-GOGAT have also been shown: Fd-GOGAT in spinach exists as a 250 kDa heteroprotein complex with UDP-sulfoquinovose synthase (SQDI; EC 3.13.1.1) involved in sulfite metabolism (Shimojima et al. 2005). The FMN-binding domain appears to be essential for a specific binding to SQDI and channeling sulfite to the single GDH1 gene (At5g18170) coding for the α subunit, and GDH3 (At3g18170) coding for a root-specific β subunit, and GDH4 (At1g51720) coding for NADPH-GDH (Purnell et al. 2005, Fontaine et al. 2012). NADH-GDH in Arabidopsis can be separated into two homohexamers composed of either α or β subunits and five heterohexamers containing both α and β subunits (Purnell et al. 2005).

Expression analysis showed that NADH-GOGAT and cytosolic GS1 are both expressed in the vascular bundle (Ishiyama et al. 1998, Konishi et al. 2014). As such, it has been proposed that the GS1/NADH-GOGAT cycle is involved in ammonium assimilation primarily in non-photosynthetic organs for nitrogen transport in the vascular bundle from roots to leaves and from sources to sinks (Tobin and Yamaya 2001, Lu et al. 2011). Consistently, the glt-T mutants deficient in NADH-GOGAT display impaired fresh weight and Chl contents together with reduced leaf glutamate content as low as 70% of the wild-type level (Lancien et al. 2002). There is an ammonium-dependent induction of GLN mRNA in Arabidopsis roots, and in the nadh-gogat-2 mutant there is a shoot biomass reduction of 25–30% under 5 mM ammonium and these pieces of evidence suggest a key role for the NADH-GOGAT in ammonium assimilation in roots (Konishi et al. 2014). In high CO₂ that suppresses photorespiration, the glu1 mutants grow and flower but they are unable to synthesize the wild-type level of Chl in response to exogenously added inorganic nitrogen (nitrate and ammonium) at either high or low concentrations (Lancien et al. 2002). This implies that a residual activity of GLU2-encoded Fd-GOGAT and NADH-GOGAT remaining in the glu1 mutants cannot substitute for the lack of GLU1-encoded Fd-GOGAT in primary ammonium assimilation. In addition, the Arabidopsis GLU1 promoter directs the GUS expression in the vascular cells and root meristem (Ziegler et al. 2003).

Both the reduced levels of glutamate in the leaf and the inability of the glu1 mutants to assimilate inorganic nitrogen in the presence of the NADH-GOGAT activity (Lancien et al. 2002) suggest that the GLU1-encoded Fd-GOGAT is involved in ammonium assimilation into glutamine and then into glutamate for transport via the vascular tissue to leaves.

An alternative pathway of glutamate formation involves the reductive amination of 2-oxoglutarate by glutamate dehydrogenase (NADH-GDH; EC 1.4.1.2) for ammonium detoxification (Skopelitis et al. 2006). NADH-GDH also catalyzes the oxidative deamination of glutamate to 2-oxoglutarate to generate energy by fueling 2-oxoglutarate into the TCA cycle. Arabidopsis genomes contain four genes: GDH1 (At5g18170) and GDH2 (At5g07440) coding for the β and α subunits of NADH-GDH, respectively, GDH3 (At3g18170) coding for a root-specific β subunit, and GDH4 (At1g51720) coding for NADPH-GDH (Purnell et al. 2005, Fontaine et al. 2012). NADH-GDH in Arabidopsis can be separated into two homohexamers composed of either α or β subunits and five heterohexamers containing both α and β subunits (Purnell et al. 2005). The native NADH-GDH isoenzyme proteins are located in the mitochondria of phloem companion cells of leaves and roots (Fontaine et al. 2012). The role of NADH-GDH in plant cells remains controversial (Lea and Mifflin 2003). However, several lines of evidence indicate that NADH-GDH provides 2-oxoglutarate to the TCA cycle in response to carbohydrate shortage. A double knockout mutant (gdh1-2/gdh2-1) displays a conditional lethal phenotype during darkness mimicking carbon deficiency, while the single gdh mutants behave like the wild-type lines, indicating that both of the homohexameric NADH-GDH isoenzymes (1 and 7) catalyze glutamate catabolism (Miyashita and Good 2008). The differences in the transcript and metabolite profiles occurring in the roots of the gdh1-2 gdh2-3 triple mutant under continuous darkness are indicative of a perturbation of the TCA cycle in the roots resulting either from the lack of 2-oxaloacetate production or from accumulation of pyruvate (Fontaine et al. 2012). Previously, 13N and 15N labeling experiments have shown that the GS/GOGAT cycle is the primary pathway of ammonium assimilation into glutamine and glutamate (Ratcliffe and Shachar-Hill 2001, Masclaux-Daubresse et al. 2006, Purnell and Botella 2007).

Aspartate Aminotransferase

Aspartate aminotransferase (AspAT; EC 2.6.1.1) catalyzes the pyridoxal 5'-phosphate-dependent reversible transamination...
reaction between aspartate and 2-oxoglutarate yielding glutamate and oxaloacetate (Fig. 1). AspAT plays a primary role for amino acid synthesis associated with shuttling of carbon skeletons derived from the TCA cycle. Analyses of nucleotide and protein databases from different cellular organisms show two types of AspAT: prokaryotic AspAT and eukaryotic AspAT. In higher plants, AspAT is a homodimer and is present as a family of isoforms that are targeted to different subcellular compartments (Wilkie et al. 1996). AspAT in Arabidopsis is encoded by a small gene family consisting of five genes of eukaryote type called AspAT1–AspAT5 and a prokaryote type PTA (de la Torre et al. 2006; TAIR). Two genes encode cytosolic AspAT2 and AspAT4: AspAT2 (At5g11520) maps to chromosome V and AspAT4 (At1g62800) maps to chromosome I (Fig. 2). AspAT2 probably encodes the major cytosolic AspAT and aspAT2 mRNA levels are abundant in leaves, roots, flowers and siliques. In contrast, AspAT4 mRNA cannot be detected, suggesting that AspAT4 remains at low levels in Arabidopsis (Schultz and Coruzzi 1995). Chloroplastic AspAT is encoded by two genes: AspAT3 (At5g11520) and AspAT5 (At4g31990). AspAT3 maps to chromosome V (Fig. 2) and the signal peptide sequence analysis has predicted that it encodes either a plastidial or a peroxisomal AspAT (Schultz and Coruzzi 1995). AspAT3 mRNA is found at high levels in different tissues including light- and dark-adapted seedling leaves (Schultz and Coruzzi 1995). AspAT5 is located on a separate chromosome IV. AspAT5 is thought to be localized to amyloplasts (Wilkie and Warren 1998) because of a high DNA sequence similarity of AspAT5 to alfalfa AspAT2 encoding amyloplast-located AspAT2 (Robinson et al. 1994), AspAT1 (At2g30970) encoding mitochondrial AspAT1 maps to chromosome II (Fig. 2) and is expressed ubiquitously throughout the tissues and life cycle of the plant (Schultz and Coruzzi 1995). AspAT1 and AspAT3, coding for mitochondrial and plastidic AspAT, respectively, are both expressed in etiolated seedlings, and light exposure does not change the mRNA levels. Likewise, both AspAT1 and AspAT3 are expressed in the light-grown seedlings, and a dark treatment does not change the mRNA levels, suggesting a relevant inter-organelle function. The other prokaryote-type PT-AspAT (PTA) (At2g30970) is mapped to chromosome II (Fig. 2). PTA encodes a prokaryotic-type PT-AspAT (EC 2.6.1.78) and its nucleotide sequence is unrelated to other eukaryotic AspAT genes from plants and animals but is closely related to cyanobacterial enzymes (de la Torre et al. 2006, Graindorge et al. 2014). An accumulation of PT-AspAT protein in developing chloroplasts has been described (de la Torre et al. 2006).

AspAT2, AspAT3 and AspAT4 have an amino acid sequence similarity of 72% (AspAT3/AspAT4) up to 81% (AspAT2/AspAT3) (TAIR). However, AspAT1 has only 53–55% similarity to ASP2-ASP4. Likewise, AspAT5 shares a low sequence similarity of 49–53% to AspAT2-AspAT4, and PTA has a low amino acid similarity to other AspATs (de la Torre et al. 2006; Fig. 2). A comparison of intron/exon organization shows that the AspAT and PTA genes differ in the nucleotide length, from 2,676 nucleotides (AspAT3) to 3,803 nucleotides (AspAT2) encoding exons varying between 10 (AspAT1) and 12 (AspAT2, AspAT4 and AspAT5) (Fig. 2). The phylogenetic tree distinguishes two classes of AspAT by Neighbor–Joining analysis of the 34 full-length amino acid sequences available in the databases from prokaryotes and eukaryotes (de la Torre et al. 2006, de la Torre et al. 2014a). One group is formed by AspAT from Escherichia coli and eukaryotes including protozoa, animals and plants. This group consists of AspAT1–AspAT5 regardless of cellular localization. The second group is comprised of AspAT from archaea, bacteria, and plant PT-AspAT from Arabidopsis, Pinus pinaster and Oryza sativa. Plant AspATs of subfamily I are closely related to the bacterial AspAT of subfamily I such as the E. coli enzyme, suggesting that eukaryotic AspATs have evolved by duplication of a common aminotransferase ancestor. In contrast, it is hypothesized that PTA is derived from cyanobacterial endosymbiosis integrated by means of Chlamydia pathogens (de la Torre et al. 2014a).

AspAT plays specific roles other than forming aspartate for protein synthesis. AspAT produces aspartate for synthesis of the transport amide asparagine by the amination of oxaloacetate which derives from phosphoenolpyruvate carboxylase (PEPCase) activity, and the resulting 2-oxoglutarate is re-aminated into glutamate by the GS/GOGAT cycle (Vance and Gantt 1992) (Fig. 1). This is consistent in that the higher expression of plastidal PvAAT-2 is associated with increased glutamine and asparagine (Silvente et al. 2003). Also, aspartate is required for the synthesis of the aspartate family amino acids lysine, methionine and threonine in the plastids (Azevedo et al. 2006, Galili 2011). Another role of AspAT consists of transferring carbon and reducing equivalents via the malate/aspartate shuttle across the organelle envelopes, and between the bundle sheath and mesophyll cells of C₄ plants (Givan 1980, Leegood 1997). A constant expression of AspAT1 for mitochondrial AspAT, AspAT2 for cytosolic AspAT and AspAT3 for plastidial AspAT in the various plant tissues (leaves, roots, flowers and siliques) (Schultz et al. 1998) is consistent with this multior-ganelle flux involving the cytoplasm, chloroplasts and mitochondria. In particular, when plants are placed under darkness or stresses that cause limited energy production, metabolic pathways of oxaloacetate or 2-oxoglutarate are of primary importance in energy production, coupled to the anaplerotic reactions that act to replenish TCA cycle intermediates (Galili 2011). Also, photo-heterotrophic organs such as floral organs, siliques and germinating seeds depend on specific amino acid metabolic pathways, and the anaplerotic reactions (oxaloacetate generation) are of primary physiological importance (Allen et al. 2009).

Mutants defective in the cytosolic AspAT2 (aat2) or chloroplastic AspAT3 (aat-3) have no phenotype apart from shorter roots (Schultz et al. 1998). Nevertheless, the reduced levels of aspartate transported in the phloem together with aspartate and asparagine in siliques indicate that cytosolic AspAT2 is involved in the synthesis of aspartate/asparagine for seed storage (Miesak and Coruzzi 2002). Overexpression of either chloroplastic or cytosolic AspAT is shown to enhance total free amino acid contents in seeds (Zhou et al. 2009). In addition, cytosolic AspAT2 may be involved in response to pathogen infection. AspAT2 expression is rapidly induced after 24 h infection with the necrotrophic pathogen Botrytis cinerea, and...
overexpression of AspAT2 results in a higher amount of spreading lesions (Brauc et al. 2011). It is thus assumed that the enhanced glutamate consumption by AspAT activity caused a reduced production of the defense compounds arginine, proline and γ-aminobutyric acid (GABA) derived from glutamate, favoring nutrient acquisition by the pathogen (Brauc et al. 2011). By overexpressing AspAT, changes in the amino acid biosynthetic pathways have been examined. Transgenic Arabidopsis lines expressing the transgene CaMC35S::GmAAT5 encoding the soybean chloroplastic AspAT contain decreased levels of free aspartate and glutamate in seeds (Murooka et al. 2002), while the levels of glutamine, asparagine and threonine are enhanced. Levels of the aspartate family lysine and methionine remain unchanged, and isoleucine is decreased in content (Murooka et al. 2002). It is hypothesized that excess amino acids are deaminated and metabolized as ammonium and carbon sources (Murooka et al. 2002).

In contrast to the plant-type AspAT3 in the chloroplasts, the second prokaryotic AspAT located in the chloroplasts operates as an aminotransferase between glutamate and prephenate or between aspartate and prephenate, yielding 2-oxoglutarate and arogenate or oxaloacetate and arogenate, respectively (EC 2.6.1.77/78) (Maeda et al. 2011, de la Torre et al. 2014b, Graindorge et al. 2014). Arogenate is a precursor for the aromatic amino acids phenylalanine and tyrosine (Tzin et al. 2009). Using glutamate as a nitrogen donor, PTA exhibits similar specific constant values (Kcat/Km) for oxaloacetate (classical AspAT activity) and prephenate (PAT activity) (Graindorge et al. 2014). Thus, PTA could be involved in the biosynthesis of not only aspartate-derived amino acids but also aromatic amino acids. The kinetic parameters highlight its high affinity for aspartate and glutamate compared with other plant AspATs (de la Torre et al. 2014b). Under steady-state photosynthesis conditions, a high glutamate/2-oxoglutarate ratio in the stroma would drive biosynthetic transaminations which favor aspartate biosynthesis. It can be assumed that, under low nitrogen stress conditions where aspartate or glutamate availability is limited, only PTA ensures the biosynthesis of aspartate-derived amino acids and other nitrogen compounds in the plastids (de la Torre et al. 2014a).

**Asparagine Synthetase**

The major pathway of asparagine synthesis takes place by ammonium assimilation into the amide position of glutamine and then into the amide nitrogen of asparagine (Lea et al. 2007, Gaufichon et al. 2010). Asparagine synthetase (AS; EC 6.3.5.4) catalyzes the ATP-dependent transfer of the glutamine-amide group to the amide position of aspartate, forming asparagine and glutamate (Fig. 1). Structural analyses have characterized two distinct asparagine synthetases in different cellular organisms. Asparagine synthetase-A (AS-A; EC 6.3.1.1) encoded by asnA uses exclusively ammonia as an amide donor, producing asparagine in prokaryotes (Reiter and Magasanik 1982). The other type of AS, asparagine synthetase-B (AS-B; EC 6.3.5.4) encoded by asnB uses either ammonium or glutamine as an amide donor in both prokaryotes and eukaryotes, while glutamine is a preferred substrate in mammals, yeasts, algae and plants (Gaufichon et al. 2010). All plants examined contain orthologs to the prokaryote asnB in *Escherichia coli* consisting of a small ASN gene family of two or three genes (Gaufichon et al. 2010).

The Arabidopsis genome databases (Arabidopsis Genome Initiative 2000) has provided the genome organization and primary structure for AS. Three genes encode AS localized in the cytosol: ASN1 (At3g47340) maps to chromosome III at the 17,448,400–17,452,600 bp region, while ASN2 (At5g65010) and ASN3 (At5g10240) map to the opposite ends of chromosome V at the 25,986–25,990 bp region and the 3,212,200–3,216,900 bp region, respectively (Fig. 2). ASN1 is composed of 10 exons for a 584 amino acid polypeptide of 65.5 kDa (Fig. 2). ASN2 and ASN3 have 13 exons and 14 exons, and encode a predicted polypeptide of 578 amino acids (65.0 kDa) and 578 amino acids (65.2 kDa), respectively (Fig. 2). AS2 and AS3 have a higher overall amino acid sequence similarity to each other (92%) than to AS1 which exhibits a 88% and 87% similarity to AS2 and AS3, respectively (TAIR). AS1, AS2 and AS3 show a sequence similarity of about 65–67% to *E. coli* AS-B, but only 14–16% to *E. coli* AS-A (Gaufichon et al. 2010). Phylogenetic relationships of AS have been analyzed from 57 annotated DNA sequences and expressed sequence tag (EST) contigs of *asnB* from plants (Gaufichon et al. 2010). The *asnB* nucleotide sequences are grouped into two classes, i.e. class I and class II, and each class is divided into dicot and monocot subclasses. Arabidopsis ASN1 is clustered to the dicot subclass of class I while Arabidopsis ASN2 and ASN3 are clustered to the separate monocot subclass of class II (Gaufichon et al. 2010). These ASN genes also differ in their expression patterns in different organs in response to internal and external signals. Contrasting regulations by light and metabolites have been demonstrated for the expression of ASN1 and ASN2. ASN2 is the major expressed gene in developing seedlings and green leaves, and its steady-state mRNA levels are high in the light and in the light-grown seedlings (Thum et al. 2003, Gálvez-Valdivieso et al. 2013, Gaufichon et al. 2013). In contrast, ASN1 mRNA and ASN3 mRNA levels remain low under the same conditions, suggesting that ASN2 expression has a role distinct from those of ASN1 and ASN3. In the *asn2-1* knockout and *asn2-2* knockdown lines, the ASN2 disruption causes a defective growth phenotype with a lower leaf biomass. In the absence of or with reduced levels of asparagine, the *asn2* mutants accumulate ammonium, GABA and alanine. The data indicate that a physiological role for AS2 is related to primary nitrogen assimilation for use in nitrogen storage and export, so that the defect in the synthesis of neutral asparagine may be linked to an impaired nitrogen cycling (Gaufichon et al. 2013). The ASN gene expression is rapidly induced by stress (Wang et al. 2004, Baena-González et al. 2007). Stresses such as dark, salt and cold treatments enhance ASN2 mRNA levels, causing a concomitant increase in internal ammonium (Wong et al. 2004). A correlation between ammonium and ASN2 mRNA accumulation suggests that AS2 may also be involved in a recapturing of lost or excess ammonium. In contrast to the ASN2 expression patterns, dark-grown seedlings
and reproductive organs accumulate ASN1 mRNA, and ASN2 mRNA levels are induced in the dark and in dark-grown seedlings as well as other class II ASN genes (Thum et al. 2003, Gaufrichon et al. 2013). These transcriptional controls are mediated by phytochromes, and sucrose or glucose mimics the light effects in the signal transduction pathways to repress ASN1 mRNA levels (Thum et al. 2003). Asparagine serves as a primary nitrogen storage and transport compound used to remobilize nitrogen from sources to sinks. In a model of seed storage protein synthesis in Arabidopsis, it has been demonstrated that the transgenic lines expressing the 3SS-ASN1 construct promote seed protein storage (Lam et al. 2003). The overexpressing lines contain elevated total and soluble protein contents in the seeds, and young overexpressing lines show higher tolerance for growth under limited nitrogen conditions (Lam et al. 2003).

Asparagine has a lower carbon content (2N:4C) than glutamine (2N:5C), and it is the primary nitrogen carrier exported in the vascular bundle. As such, it has been argued that lower photosynthesis in the dark would favor asparagine synthesis via ASN2 induction in the dark in response to changes in physiological conditions, i.e. light vs. dark, and high carbon vs. low carbon (Thum et al. 2003). Carbon metabolism controls amino acid synthesis by supplying the carbon skeletons through the glycolysis, TCA cycle and anaplerotic pathways. Amino acids in turn enter carbon metabolism for the generation of energy sources in response to the photosynthetic conditions. Nitrate, ammonium and downstream metabolites such as amino acids regulate the expression of genes involved in nitrogen assimilation. Nitrate up-regulates ASN2 and other class II ASN genes (Osuna et al. 2001, Wong et al. 2004, Bläsig et al. 2005, Avila-Ospina et al. 2015) while down-regulating the class II ASN gene (Gálvez-Valdivieso et al. 2013). Also, ammonium exerts contrasting positive effects (Lam et al. 2003) or negative effects (Ohashi et al. 2015) on class II ASN2 expression. Stresses such as dark, salt and cold treatments enhance ASN2 mRNA levels, causing an increase in internal ammonium (Wong et al. 2004), suggesting that ASN2 may be involved in recapitulating lost or excess ammonium. Several lines of evidence indicate that ASN1 is repressed by nitrate and ammonium (Wong et al. 2004), while other class I ASN1 genes are up-regulated by nitrate (Osuna et al. 2001) or ammonium (Ohashi et al. 2015). The repression of ASN1 expression in response to white light and sucrose is at least partially reversed by the addition of the amino acids glutamate, glutamine and asparagine (Wong et al. 2004). A microarray analysis provided evidence that glutamate or a glutamate-derived product is involved in the signal transduction inducing ASN1 expression (Gutiérrez et al. 2008). These findings underline the importance of AS in primary assimilation, ammonium detoxification, recycling and transport of nitrogen in key biological processes including seed germination, vegetative growth, flowering and senescence, and seed filling and maturation.

**Asparagine Catabolism**

Asparagine is a substrate for a few enzymatic or non-enzymatic reactions. Asparagine can be catabolized mainly through two enzymatic pathways in higher plants. The first route involves the hydrolysis of asparagine by asparaginase (ASPG; EC 3.5.1.1) that releases the amide nitrogen as ammonium and aspartate in the cytosol (Ireland and Joy 1983, Gabriel et al. 2012) (Fig. 1). The second route occurs via the transamination of the α-amino group of asparagine to the 2-oxo-acid acceptor, producing 2-oxosuccinamate (Ireland and Joy 1983, Liepman and Olsen 2001) (Fig. 1). 2-Oxosuccinamate can be deaminated to ammonium and oxaloacetate (Fig. 1). Also, 2-oxosuccinamate can be rapidly reduced to 2-hydroxyoxysuccinamate which is deaminated to ammonium and malate. Released ammonium is re-assimilated by the GS/GOGAT pathway into glutamine and glutamate (Ta et al. 1984). Glutamine, glutamate, asparagine and aspartate thus formed are utilized for the biosynthesis of all nitrogenous compounds. As part of nutritional processing, the asparagine carbon skeleton is entirely recovered non-enzymatically in acrylamide, a neurotoxin and a potential carcinogen (Muttucumaru et al. 2014). The depletion of asparagine by ASPG is used as an anticancer therapy (Richards and Kilberg 2006). In this sense, agronomic, genetic and molecular methods on crops would lower the potential for acrylamide formation and reduce the exposure to risks in foods (Halford et al. 2015).

**Asparaginase**

The Arabidopsis genome contains two genes for ASPG localized in the cytosol: ASPGA1 (At5g08100) coding for asparaginase A1 and ASPGB1 (At3g16150) coding for asparaginase B1. ASPGA1 is located on chromosome V and ASPGB1 on chromosome III (Fig. 2). ASPGA1 and ASPGB1 consist of 1,602 and 1,542 nucleotides, respectively. Both of the ASPG genes have four exons, and ASPGA1 and ASPGB1 encode a predicted polypeptide of 315 and 325 amino acids, respectively (Fig. 2). ASPGA1 and ASPGB1 share a similar subunit structure and conserved autoproteolytic pentapeptide cleavage site. Autoproteolytic processing exposes a catalytic nucleophile threonine within the consensus sequence GlyThrVal/IleGly at the N-terminal residue of the β subunit (Brunseau et al. 2006). The ASPGA1 precursor undergoes an autoproteolytic cleavage by the hydrolysis between Gly182 and Thr183, leading to the formation of the α subunit (182 amino acids of 19.5 kDa) and the β subunit (133 amino acids of 13.5 kDa). ASPGB1 is cleaved between Glu194 and Thr195 into the α subunit (194 amino acids of 20.7 kDa) and the β subunit (131 amino acids of 13.6 kDa). Thus, the autoproteolytic processing exposes the catalytic nucleophile threonine at the N-terminal residue of the β subunit. ASPGA1 and ASPGB1 share an amino acid sequence similarity of 54–57% and 60–61% for the α subunit and β subunit, respectively. ASPGs in plants are clustered together with bacterial ASPGs into one of four branches by Neighbor-Joining analysis of 76 amino acid sequences available from different organisms (Borek et al. 2004). These two types of ASPGs are evolutionarily distinct and differ in their substrate affinity and specificity. Plant ASPGs belong to the N-terminal nucleophile (Ntn) amidohydrolases and show a hydrolysis activity of isoaspartyl residues.
that arise from the β-aspartyl formation by asparagine deamination (Michalska et al. 2006).

An analysis of steady-state mRNA levels of ASPGA1 and ASPGB1 demonstrated a high expression of both of the genes in the sink tissues such as flower buds, flowers and silicules, but low levels in seedlings (Bruneau et al. 2006). The data point to the importance of asparagine as a nitrogen carrier to the sink tissues especially the developing seeds and fruits. Among the sink tissues, the highest ASPGB1 mRNA levels are associated with the flowers. Afterwards, ASPGB1 mRNA levels decline in the developing silicules at 5 days after anthesis (DAA) (cotyledon expansion stage), 12 DAA (desiccation stage) and 15 DAA (maturity stage). ASPGA1 shares a similar developmental pattern with ASPGB1, although ASPGA1 is expressed to lower levels (Bruneau et al. 2006). By a promoter-driven expression approach, the APGB1::GUS activity is found exclusively in the developing pollen grains in parallel with the ASPGA1::GUS activity (Ivanov et al. 2012). This developmental expression pattern overlaps the microarray data showing that ASPGA1 is expressed to lower levels (Bruneau et al. 2006). Guan et al. (2015) reported high levels of GFP expression in the anthers of stamen from Arabidopsis transformed with a GLN1:2 promoter::GFP fusion, suggesting that ammonium released from asparagine by ASPG activity is assimilated into glutamine for the synthesis of amino acids. An analysis of a translational fusion containing 1,128 bp of 5′-flanking sequence and 82 bp of ASPG in-frame to the GUS gene located the GUS expression in the endosperm cells at the funicular end of the seed, the entry site of asparagine transported through the vascular strand, as well as in the seed pod and testa (Grant and Bevan 1994). The K⁺-dependent ASPG (PvAspG2) overexpression study under the embryo-specific promoter showed that the reduction in relative asparagine content at mid-maturation is associated with reduced seed nitrogen content rather than with increasing nitrogen flux to seed proteins (Pandurangan et al. 2015). Experiments with developing Lupinus arboreus seeds have shown that the maximal activities of ASPG, GS, Fd-GOGAT and NADH-GOGAT take place in the testa and cotyledons at about 40 DAA, except for a delayed peak of Fd-GOGAT (at 47 DAA) (Lough et al. 1992). This is in accordance with a hypothesis that amides and amino acids transported in the vasculature are metabolized in the seed coat (Rochat and Boutin 1991). Nevertheless, the ASPGA1/ASPGB1 double mutant grows normally under high nitrogen conditions, except for a root growth inhibition (Ivanov et al. 2012). This mild defective phenotype may be linked to the AspAT reaction which hydrolyzes asparagine (Ivanov et al. 2012).

In plants, two types of ASPGs are characterized in Arabidopsis by their dependence on K⁺ for catalytic activity: K⁺-independent ASPGA1 and K⁺-independent ASPGB1 (Bruneau et al. 2006). These two types have also been distinguished in other higher plants (Sodek et al. 1980, Ireland and Joy 1983, Lough et al. 1992, Credali et al. 2011). ASPGA1 and ASPGB1 display a variable linker between the α and β subunits, and its functions for autoproteolysis and catalysis have been investigated. ASPGB1 displays 13 additional residues at the C-terminal end of the α subunit (27 residues from Pro168 to Glu194) while ASPGA1 comprises a 14 residue peptide at the C-terminal end of the α subunit (14 residues from Ser169 to Gly182) (Bruneau et al. 2006). The autoproteolytic cleavage of the ASPGB1 precursor is inhibited by the deletion of a short peptide (4–10 residues) in the variable loop (Gabriel et al. 2012). Moreover, the side chain of the aromatic ring of Phe162 in ASPGB1 is in proximity to (3.9 Å) the oxygen atom of the carbonyl side chain of aspartate. Phe162 in ASPGB1 is close to Gly246 (3.5 Å) and Thr245 (3.8 Å) which form the oxyanion hole in stabilizing a negative charge on the oxygen atom of the amide group of the substrate during reaction. In comparison, the Phe162 side chain is located at 4.2 Å to the hydroxyl group of the catalytic Thr195 of ASPGB1. Phe162 is strictly conserved in K⁺-dependent ASPGB1, whereas the corresponding Leu163 in K⁺-independent ASPGA1 can be substituted by valine or isoleucine (Gabriel et al. 2012).

To investigate the mechanism of asparagine hydrolysis, the quaternary structure of ASPGA1 and ASPGB1 has been characterized by referring to the well-characterized plant ASPGs: Lotus japonicus asparaginases LjNSE1 and LjNSE2 (Credali et al. 2011, Credali et al. 2013) and Lupinus luteus asparaginase Lia (Michalska et al. 2006). K⁺-independent ASPGA1 shows a higher amino acid similarity to K⁺-independent LjNSE2 (76%), and K⁺-dependent ASPGB1 has a higher amino acid sequence similarity to K⁺-dependent LjNSE1 (83%) (Credali et al. 2011). The LjNSE1 and LjNS2 precursors undergo an autoproteolysis, giving rise to an N-terminal threonine on the β subunit (Thr196 and Thr186 for LjNSE1 and LjNSE2, respectively). The mature LjNSE1 and LjNSE2 comprise two heterodimers of α and β subunits [[αβ]2], and the molecular mass of α and β subunits of LjNSE1 and LjNSE2 is predicted to be 20.9 and 20.2 kDa and 17.1 and 17.3 kDa, respectively (Credali et al. 2011). K⁺ activation of LjNSE1 induces a structural change involving Glu248, Asp285 and Glu286 that form a negatively charged cavity at the entrance of the active site of the enzyme (Credali et al. 2011). Lupinus luteus K⁺-independent Lia consists of a heterodimer of the α subunit (residues 1–192) and β subunit (residues 193–325) [[αβ]2], created by the autoproteolysis of the precursor peptide (Michalska et al. 2006). K⁺-independent ASPGs are evolutionarily distinct from the bacterial-type ASPGs but show 60% sequence similarity to aspartylglucosaminidases (EC 3.5.1.26) which are classified as the N-terminal nucleophile (Ntn) hydrolases. In this group, the N-terminal nucleophilic residue (threonine, serine or cysteine) is created during the autocatalytic cleavage of the precursor (Michalska et al. 2006). Lia is also one of the Ntn-type amidohydrolases which involve the N-terminal nucleophilic residue: threonine, serine or cysteine (Borek et al. 2004). A comparison of the active site of Lia and E. coli EcAIII protein revealed highly conserved amino acids in the catalytic cavity. These include Asp214 and Gly23 involved in the binding to the α-amino group of the asparagine substrate, Arg211, forming a salt bridge between its guanidinium group and the α-carboxyl group of the aspartate product, and Thr234 and Gly235 as the components of the oxyanion hole that stabilizes the negative charge of the tetrahedral transition state during the catalysis (Michalska et al. 2008). K⁺ appears not to be involved in a direct interaction with the
substrate but is involved in a conformational change of the C-terminal end of the α subunit (Creda et al. 2011, Gabriel et al. 2012). The catalytic efficiency of enzymes can be expressed as $V_{\text{max}}/K_m$, and the kinetic analysis showed that ASPGA1 has higher efficiency with β-aspartyl dipeptide (β-Asp-His) than with asparagine ($V_{\text{max}}/K_m$ of 0.085 and 0.26 for asparagine and β-Asp-His, respectively), while ASPGB1 has a longer variable loop and exhibits higher catalytic efficiency with asparagine than with β-Asp-His ($V_{\text{max}}/K_m$ of 9.27 and 0.62 for asparagine and β-Asp-His, respectively) (Hejazi et al. 2002, Gabriel et al. 2012). It can be hypothesized that Arabidopsis ASPGB1 can be fully activated in vivo on the basis of a high K$^+$ concentration up to 100 mM in the Arabidopsis phloem sap compared with other ions such as sulfur and phosphorus (Gajdanowicz et al. 2011).

**Asparagine Aminotransferase**

Asparagine aminotransferase (AsnAT; EC 2.6.1.45) catalyzes the transfer of the amino group of asparagine to 2-oxo-acid glyoxylate as an amino acceptor producing glycine and 2-oxosuccinamic acid (Fig. 1). Previous biochemical and loss-of-function studies demonstrated that AsnAT in Arabidopsis acts as a serineglyoxylate aminotransferase (Zhang et al. 2013). The reaction consists of the amino transfer between serine and glyoxylate and is designated as AGT1 since this aminotransf

![Figure 2](https://example.com/figure2.png)

**Figure 2** AGT1 protein, and it is located on chromosome II at the region 5,539,240 to 5,541,358 (TAIR). AGT1 displays a high nucleotide sequence similarity to the cDNA sequences from other plants: tomato, rice, soybean, crystalline iceplant and moss (Lipman and Olsen 2001). Peroxosomal proteins such as AGT1 are generally synthesized as the final mature protein and are not processed during or after their import into the peroxisome (Olsen 1998). Arabidopsis AGT1 is targeted to the peroxisomes by a C-terminal tripeptide—peroxisomal targeting signal (Ser399–Arg400–Ileu401) (PTS1)—which shares the Ser/Lys/Leu motif or related sequences such as Ser/Arg/Leu, so that the initial precursor is found after its import into the matrix (Lipman and Olsen 2001). AGT1, produced as an N-terminal His-tagged protein in E. coli, has an apparent molecular mass of 45 kDa (Zhang et al. 2013) which correlates with the molecular mass of the native AGT1 (Lipman and Olsen 2001, Kendziorek and Paszkowski 2008). By comparison with the molecular mass of the purified native enzyme, it is suggested that AGT1 may be active as a homodimer (Kendziorek and Paszkowski 2008).

AGT1 expression is highest in the green tissues including cotyledons, seedlings and leaves where the steady-state mRNA level is 2-fold higher than it is in the flowers and siliques (Lipman and Olsen 2001). During photorespiration, AGT1 in Arabidopsis might play a role in detoxifying glyoxylate to glycine using asparagine as an amino donor, so that the carbon of 2-phosphoglycerate becomes available for plant metabolism during photorespiration. The operation of the photosynthetic glycolate cycle is in accordance with the $^{15}$N kinetic data showing that a substantial part of $^{15}$N(Streeter 1977). To evaluate the in vivo activity, the catalytic efficiencies of the transamination reactions, expressed as $V_{\text{max}}/K_m$ are determined using different amino acids as substrates. When glyoxylate is used as an amino acceptor from asparagine, with alanine or serine as the amino donor with the production of glycine, AGT1 exhibits significantly higher catalytic efficiency for asparagine (10.4 x 10$^{-8}$ katal mg$^{-1}$mM$^{-1}$) than for alanine (5.8 x 10$^{-8}$ katal mg$^{-1}$mM$^{-1}$) and serine (1.4 x 10$^{-8}$ katal mg$^{-1}$mM$^{-1}$) (Zhang et al. 2013). When asparagine is used as an amino donor at the expense of glyoxylate, pyruvate or hydroxypyruvate as an amino acceptor leading to the production of glycine, alanine and serine, respectively, the AGT1 has a slightly higher efficiency for glyoxylate (10.4 x 10$^{-8}$ katal mg$^{-1}$mM$^{-1}$) than for pyruvate (8.4 x 10$^{-8}$ katal mg$^{-1}$mM$^{-1}$) and hydroxypyruvate (9.5 x 10$^{-8}$ katal mg$^{-1}$mM$^{-1}$) (Zhang et al. 2013). These in vitro kinetic data support the transamination between asparagine and glyoxylate and are in agreement with the previous observations showing that the asparagine transamination to 2-oxosuccinamate is most active with pyruvate and glyoxylate followed by oxaloacetate and 2-oxoglutarate (Streeter 1977). To evaluate the role of asparagine for photorespiration, a loss-of-function analysis is carried out using the Arabidopsis asn2-1 knock-out mutant (sn2-1). The lack of asparagine in the vegetative leaves does not cause an apparent difference in the net CO$_2$ assimilation in either 2% or 2% O$_2$, suggesting a similar rate of photorespiration between the sn2-1 and control lines (Gaufichon et al. 2013). Likewise, as part of the photosynthetic nitrogen cycling, it has been hypothesized that AspAT has a small contribution to the net asparagine catabolism (Lee et al. 2007). The asparagine transamination results in the formation of 2-oxosuccinamate, and β-amidase hydrolyzes 2-oxosuccinamate to ammonium and oxaloacetate (Zhang and Marsolais 2014) (Fig. 2). 2-Oxosuccinamate is reduced to 2-hydroxysuccinamate, then deaminated by β-amidase to ammonia and malate. The candidate gene At5g12040 encodes a polypeptide of 40.5 kDa.
(369 amino acids) that is predicted to be cytosolic or targeted to the plastid (Zhang and Marsolais 2014). The recombinant ω-amidase has a catalytic efficiency \( V_{\text{max}}/K_m \) similar to that of Arabidopsis AGT1 for both 2-oxosuccinamate \((12.8 \times 10^{-8} \text{ katal mg}^{-1} \text{ mM}^{-1})\) and 2-hydroxysuccinamate \((15.5 \times 10^{-8} \text{ katal mg}^{-1} \text{ mM}^{-1})\) (Zhang and Marsolais 2014). These data suggest that ω-amidase may act as a critical enzyme associated with asparagine transamination in Arabidopsis.

**Conclusions**

Inorganic ammonium assimilation into asparagine and utilization of asparagine for nitrogen recycling, storage and transport in response to environmental and internal signals are primary processes for Arabidopsis development including seed germination, vegetative growth, flowering and senescence, and seed filling and maturation. To investigate the impact of each step of asparagine synthesis and utilization, multidisciplinary studies have been carried out using Arabidopsis as a model plant. Analyses of structural and regulatory data of genes and encoded enzyme proteins have been combined with studies on their functional properties using screened Arabidopsis mutants generated by T-DNA insertional mutagenesis or overexpression lines. These studies underline the important regulatory controls of genes involved in asparagine metabolism pathways in response to multiple biological signals such as light, dark, sugars and nitrogen metabolites. Also, global profiling of transcripts and metabolites provides insight into the interaction between asparagine and carbon metabolism. Kinetic and catalytic properties linked to protein structure have provided evidence to investigate the physiological functions of the enzymes involved. Expression studies and in silico data indicate that the genes of asparagine metabolism are crucial control points in many aspects of nitrogen metabolism throughout plant development.

**Disclosures**

The authors have no conflicts of interest to declare.

**References**


