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Functional characterization of the L-type Amino acid Transporters (LATs)
in *Arabidopsis thaliana*

by

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To my mother Shahida A. Akhter and my father Md. Manjur Ali

Abstract

The distribution of amino acids (AAs) in plants is a complex process that involves 67 amino acid transporters (AATs) in Arabidopsis, many of which have yet to be characterized. The L-type Amino acid Transporter (LAT) family, which in Arabidopsis contains five members, remained uncharacterized in plants. This thesis presents the functional characterization of three members (LAT1, LAT4, and LAT5) of the Arabidopsis LAT family. *In planta* studies of knockout and over-expressing lines of these transporters showed increased toxicity to exogenous AAs and variation in AA-mobilization at cellular and whole-plant level compared to WT. Thus, their role in the amino-N distribution in Arabidopsis was investigated in this research. LATs were characterized through *in silico* studies, subcellular localization, organ- and tissue-specific expression, heterologous expression and *in planta* studies. Single knockout mutant or over-expressing lines were used in phenotypic growth studies, organ specific free AA analysis, analysis of nitrogen (N) and carbon (C) content in the seeds, and radio-labeled AA uptake and export studies.

LAT4 is a widely expressed AAT in the whole plant with high levels of expression in the photosynthetic tissues. *In planta* studies of a mutant *lat4-1*, together with the tissue-specific expression, provided evidence that LAT4 mobilizes AAs from leaf mesophyll cells and green carpel cells under low C conditions. LAT5 is also a widely expressed AAT in the whole plant including

expression in the phloem. *In planta* mutant analysis suggested a role for LAT5 in delivering AAs to the leaves under high C conditions. In the *lat5-1* mutant, decreased leaf size and increased accumulation of AAs in the stem was associated with an increased AA-translocation to the siliques and total N content in the seeds. The mutant *lat5-1* showed increased salt and drought stress tolerance and altered free AA content in the seedlings under salt and osmotic stresses indicating that LAT5 plays a role in AA homeostasis under abiotic stresses. In the absence of a knockout mutant, a *LAT1* over-expressing line was analyzed. *In planta* studies suggested a role for LAT1 in mobilizing AAs from leaves.

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List of Abbreviations

°C	Degree Celsius
µE	Micro Einstein
AA	Amino Acid
AAP	Amino Acid Permease
AAT	Amino Acid Transporter
APC	Amino acid Polyamine Choline
ATF1	Amino acid Transporter Family 1
BA	Bacto Agar
C	Carbon
CaMV	Cauliflower Mosaic Virus
CAT	Cationic Amino acid Transporter
cDNA	complementary DNA
DNA	Deoxyribonucleic acid
DPM	Dis-integration Per Minute
DW	Dry Weight
FAA	Formaldehyde: Acetic Acid: Ethanol
FW	Fresh Weight
g; mg; µg	gram; milligram; microgram
GUS	β-Glucuronidase
HPLC	High Performance Liquid Chromatography
hr; hrs	hour; hours

kb	kilo base
kDa	Kilo Dalton
L; ml; µl	Liter; milliliter; microliter
LAT	L-type Amino acid Transporter
M; mM; µM	Molar; millimolar; micromolar
mCi	Milli-Curie
MCS	Multiple Cloning Site
Mins	Minutes
MMG	MES: Mannitol: Magnesium chloride
mRNA	messenger RNA
MS	Murashige and Skoog
N	Nitrogen
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PM	Plasma Membrane
pmol; nmol; µmol	Picomole; Nanomole; Micromole
RT	Room Temperature
RNA	Ribonucleic Acid
rpm	revolutions per minute
RQ	Relative Quantitation
RT-PCR	Reverse Transcriptase-PCR

SD	Standard Deviation
TLC	Thin Layer Chromatography
UTR	Un-Translated Region
WT	Wild-Type

1. General Introduction

1.1. Soil nitrogen

1.1.1. Geo-biological distribution of nitrogen

Total global nitrogen (N) is distributed in three different spheres of the earth: the lithosphere, atmosphere, and biosphere (Stevenson, 1965; Burns and Hardy, 1975). The lithospheric bulk of N (98%) is held in the rocks and minerals in the form of nitrides of iron, titanium, and other metals, or as NH_4^+ ions held in the lattice structure of primary silicate minerals (Stevenson, 1965). This N is completely fixed and contributes very little to the biologically active N cycle except for about 0.2% in the sedimentary rocks that leaches N to the ground water. Atmospheric N (1.9%) that comprises 78% of the atmosphere exists as molecular N_2. The remaining 0.01% of the biospheric N is distributed as biomass N in the ocean and land (Burns and Hardy, 1975). Biologically reactive N is that which is in the soil in both organic and inorganic form, initially derived from the atmosphere, and supports plant growth. Industrially, using the Haber-Bosch process, atmospheric N is converted together with hydrogen gas into ammonia (NH_3), which is used to make fertilizer and is applied to soils to enhance crop production. Another way to fix atmospheric N is by Biological Nitrogen Fixation (BNF). Prokaryotic organisms such as bacteria and actinomycetes, living freely or in association with certain plants, are the main agents of BNF.

1.1.2. Forms of N in the soil

In soil, biologically active N can be broadly grouped into two forms: inorganic and organic. Over 90% of the total N in the surface layer of most soils remains in association with the organic matter (Kelley and Stevenson, 1995; Schulten and Schnitzer, 1998). Inorganic forms of N are either lost from the surface soil or assimilated by the microbes into organic form. Nitrate (NO_3^-), released from inorganic fertilizers or organic N, rapidly disappears from the soil due to its highly mobile nature. Ammonium (NH_4^+) released from inorganic fertilizer or organic N, if in excess of plant uptake, undergoes immobilization

when the carbon/nitrogen (C/N) ratio is high in the soil. In the case of a high C/N ratio, C is in excess to the N-content in the soil, causing rapid utilization of NH_4^+ by the microbes (Mengel, 1996). Fixed NH_4^+, bound between the lattice structures of the clay particles, is used only by the microbes and returned to the soil as microbial biomass (Rosswall, 1982; Balabane and Balesdent, 1996). BNF also adds N to the soil in the organic form. Free-living bacteria or symbiotic bacteria, living in association with plants (eg. Rhizobia and a legume), fix atmospheric N into NH_4^+ which is eventually incorporated into Glu and Gln in the N-fixing cells and is added to the soil in the form of biomass.

A number of studies have been conducted to determine the nature of N compounds present in the soil. Sowden et al. (1977) showed that 33.1- 41.7% of the total soil N occurs in the form of AAs, 4.5%-7.4% N occurs in the form of amino sugars, and 18%-32% occurs in the form of NH_3. The remaining 19%-45% N was classified as nonhydrolysable or un-identified hydrolysable N. While, Sowden et al. (1977) showed 18%-32% of the total soil N in the form of NH_3, they note that some of the NH_3 may have originated from amides, decomposition of AAs and amino sugars, de-amination of purines and pyrimidines, and the release of fixed NH_4^+ from clay particles, all of which will occur during acid hydrolysis. This suggests that the percentage of NH_3-N in soil may be less than what it appears in analytical studies (Schulten and Schnitzer, 1998). A number of subsequent studies conducted in different climatic zones and in various soil types have shown more or less similar compositions of soil N (Stevenson, 1986; Sharpley and Smith, 1995; Sulce et al., 1996; Leinweber and Schulten, 1997).

1.1.3. The potential of soil organic N as a plant nutrient

Soil organic nitrogen (ON) contributes to plant nutrition through releasing N in the form of NH_4^+ and NO_3^- through mineralization. Mineralization of soil ON occurs in three steps: aminization, ammonification and nitrification. The first step of mineralization, aminization, is the formation of amino compounds by the enzymatic hydrolysis of protein. These amino compounds are either utilized by microorganisms or transformed by ammonification. In the ammonification step,

the $-NH_2$ radical of an amino compound is converted to NH_3 through hydrolysis. The NH_3 produced is converted rapidly to an NH_4^+ ion. In waterlogged land, mineralization usually stops at this stage and soil contains N mostly in the form of NH_4^+. Crops such as rice are grown in soil where NH_4^+ is the prevalent form of N. However, in well aerated soils, ammonification is followed by nitrification, which is the final step of mineralization. In this step, NH_4^+ is converted to NO_3^- via NO_2^-. Both aminization and ammonification are carried out by common heterotrophic micro-organisms, whereas nitrification is carried out by nitrifying autotrophs (*Nitrosomonas* and *Nitrobacter*). s

Soil organic N in the form of amino sugars, AAs or peptides, can directly be used as a source of N for plant growth, although it is believed to be predominantly consumed by microbes (Virtanen and Linkola, 1946; Miettinen, 1959; Nasholm et.al., 1998). The range of reported *Km* values for AA uptake is not significantly different between plants and microbes (Lipson and Nasholm, 2001). For some plant species or some particular ecosystems, AAs are the major source of N. For example, AAs contribute 60% of the total N-requirement for *Eriophorum vaginatum* (native community-Tussock tundra) (Chapin et al., 1993) and anywhere from 10-82% for several different arctic tundra species (Kielland, 1994). In experimental conditions, all the plant species tested to date that usually use inorganic N have shown the capacity to take up AAs from soils (reviewed in Nasholm et al., 2009). For example, under controlled experimental conditions, cereal crops have been shown to be able to use AAs as a primary N source. Jones and Darrah (1994) showed that AAs can make up 30-90% of the N-requirement of maize (*Zea mays*), a crop that normally uses NO_3^-. Yamagata and Ae (1996) showed that rice (*Oryza sativa*), which normally uses NH_4^+ as its primary N source, can take up AAs at a rate comparable to or greater than inorganic N. Some studies have shown that plants are able to grow better when a mixture of AAs and inorganic N are used as the N source (Joy, 1969). Soper et al. (2011) demonstrated that in a controlled growth medium, peptides, as sole source of N, can sustain growth and support up to 74% of the maximum biomass compared to that achieved with inorganic N in Arabidopsis.

At the field level, manipulation of a plant's ability to increase organic N uptake has been less explored. The pathway involved in direct peptide and AA uptake from soil and its control at the molecular level is not well studied except for some of the peptide- and AA transporters, which have been identified in the plant roots. Expression analysis in Arabidopsis has identified a number of amino acid transporters (AATs) that are expressed in the roots (Birnbaum et al., 2003; Brady et al., 2007). Some of these AATs have been further characterized to demonstrate their role in organic N acquisition while many remain uncharacterized. The Arabidopsis AAP1, an amino acid permease (AAP) transporter, is localized in the plasma membrane (PM) of root epidermal and root hair cells (Lee et al., 2007). Mutant analysis demonstrated that the AAP1 mediates uptake of neutral AAs and Glu from external sources (Fischer et al., 2002; Lee et al., 2007). Another member of the AAP family, AAP5, which is expressed in roots (Birnbaum et al., 2003), was shown to mediate basic AA uptake from the growth medium (Svennerstam et al., 2008). The Lysine-Histidine Transporter 1 (*LHT1*) that is expressed in roots (Brady et al., 2007; Chen and Bush (1997) and localized in the PM has been demonstrated to mediate AA uptake from external sources both in yeast and in plants (Hirner et al., 2006). In the same study, Hirner et al. (2006) demonstrated that overexpression of the *LHT1* in Arabidopsis increased the root AA uptake several fold and mediated increased seedling growth on a growth medium containing AAs as the sole source of N.

1.2. N acquisition in plants

The predominant forms of N taken up by plants are NO_3^- and NH_4^+, while some species also take up organic N, mostly in the form of AAs and peptides. Plants have separate uptake and transport systems for each of these forms of N.

1.2.1. Nitrate uptake

There are two NO_3^- transport systems exist in plants which take up NO_3^- from the soil (Crawford and Glass, 1998; Tsay et al., 2007): a High Affinity

Transport System (HATS) that takes up NO_3^- at external concentrations below 1 mM and a Low Affinity Transport System (LATS) that mediates uptake at external NO_3^- concentration above 1 mM (Crawford and Glass, 1998). The HATS is again divided into two groups: constitutive-HATS (cHATS), which is active even when plants have not been previously supplied with NO_3^-, and inducible-HATS (iHATS), which is stimulated by the NO_3^- concentration in the external medium. The identified NO_3^- transporters (NRTs) are categorized into two groups: the NRT1 and the NRT2 families. In Arabidopsis, more than 50 members were identified in the NRT1 family, which includes some transporters that are involved in peptide transport. This NRT1 family is also referred to as the PTR (Peptide Transporter) family in some literature (Tsay et al., 2007). Seven members have been identified in the NRT2 family (Miller et al., 2007). In Arabidopsis, NRTl.l has been suggested to be a dual-affinity transporter depending on the phosphorylation state of the transporter (Liu and Tsay, 2003). On the other hand, *NRT1.2* is constitutively expressed in the root epidermis, and a heterologous expression of *NRT1.2* in *Xenopus* oocytes mediated low affinity transport activity (Huang et al., 1999).

In Arabidopsis, NRT2.1 and NRT2.2 are the major constituents of the HATS since HATS activity is impaired when these two transporters are mutated (Filleur et al., 2001; Orsel et al., 2004). The heterologous expression of NRT2 homologs in *Xenopus* oocytes demonstrated that it interacts with another protein, NAR2, for the NO_3^- uptake activity (Zhou et al., 2000; Tong et al., 2005; Orsel et al., 2006). The two-component high affinity uptake system has recently been demonstrated through the analysis of *NAR2.1* mutants, which showed reduced HATS activity similar to the mutants of *NRT2.1* (Okamoto et al., 2006; Orsel et al., 2006). Furthermore, direct protein-protein interactions between NRT2.1 and NAR2.1 were confirmed, as well as the role of NAR2.1 in targeting NRT2 proteins to the PM proposed (Okamoto et al., 2006; Orsel et al., 2006; Miller et al., 2007).

The regulation of NO_3^- uptake activity is induced by NO_3^-. As well, NO_3^- uptake is repressed by the external supply of AAs (Miller et al., 2008) (see details in Section 1.2.3). This regulation which responds to the N supply indicates the presence of a mechanism that coordinates the N uptake process with cellular N status. For instance, the expression of *NRT2.1* is induced by NO_3^-, but it is also regulated through the feedback repression exerted by cellular N metabolites, such as NH_4^+ and AAs (Zhuo et al., 1999; Nazoa et al., 2003; Miller et al., 2007). Moreover, the activity of these transport systems is likely controlled by post-translational events, including phosphorylation and the interaction with 14-3-3 proteins (Miller et al., 2008). In terms of sensing the available NO_3^- levels at the root surface, it has been suggested that NRT2.1 is directly involved in NO_3^- sensing and functions as a negative regulator for the initiation and elongation of lateral roots (Little et al., 2005). The role of NRTl.l in regulating the expression of *NRT2.1* and in the induction of lateral root formation in the NO_3^- rich soils has also been reported (Munos et al., 2004; Krouk et al., 2006; Remans et al., 2006). Nevertheless, a detailed picture of the regulation of NO_3^- uptake and the mechanism of NO_3^- sensing has yet to be fully elucidated.

1.2.2. Ammonium uptake

Studies have shown that the mechanism of NH_4^+ uptake is due to the function of a uniport for NH_4^+ (Ludewig et al., 2002; Loque and Wiren, 2004). Low-affinity and high-affinity transport systems also exist for NH_4^+ uptake in plants (Forde and Clarkson, 1999). The first identified ammonium transporter (AMT), AMT1.1 in Arabidopsis, was isolated by functional complementation of a yeast mutant defective in NH_4^+ uptake (Ninnemann et al., 1994). Another five genes were identified based on the sequence homology to *AMT1.1* gene sequence (Loque and Wiren, 2004). Expression analysis of these genes has shown that members of the AMT1 family are expressed in the roots while *AMT2.1* is expressed in the leaves (Engineer and Kranz, 2007). Among the five *AMT* genes detected, the expression pattern of *AMT1.1* was unique in that the induction was reciprocal between roots and leaves depending on the N availability (Engineer

and Kranz, 2007). Constitutive expression of *AMT1.1* by the CaMV 35S promoter in tobacco and Arabidopsis suggested that transcript level is important in the regulation of *AMT* genes in response to the N-status of plants (Yuan et al., 2007). In addition to N availability, light and sugar levels increase the levels of mRNA for *AMT* genes, such as *AMT1.1, AMT1.2,* and *AMT1.3* (Lejay et al., 2003; Loque and Wiren, 2004; Engineer and Kranz, 2007).

1.2.3. Amino acid uptake

AATs can be categorized into five different super families (Wipf et al., 2002): the Amino acid Transporter1 (ATF1) superfamily, the Amino acid-Polyamine-Choline Transporter (APC) superfamily (also known as Amino acid-Polyamine-Organocation superfamily), the Sodium-dicarboxylate symporter (SDS) superfamily, the Neurotransmitter (NTS) superfamily, and the Major Facilitator (MFS) superfamily. Two of these superfamilies, SDS and NTS, are preferentially Na^+ and Cl^--coupled transporters of AAs and are present in animals but absent in yeast and plants (Wipf et al., 2002). While a phylogenetic analysis showed that plants have AATs in the MFS superfamily, their function in plants remained unknown (Okumoto and Pilot, 2011). In Arabidopsis, the 67 genes that were annotated to encode AATs (Rentsch et al., 2007) belong to the ATF1 and APC superfamily. The ATF1 superfamily in plants includes five families: the Amino Acid Permease (AAP) family, the Lysine Histidine-specific amino acid Transporter (LHT) family, the Auxin-like transporter (AUX) family, the Aromatic and Neutral amino acid Transporter (ANT) family, and the Proline Transporter (ProT) family (Ortiz-Lopez et al., 2000; Wipf et al., 2002). These classifications were based on their substrate specificity. Members of this superfamily mediate H^+-coupled symport of AAs; however, the transport mechanism of a large number of transporters in this superfamily is unknown.

The APC superfamily members in plants were grouped into two families: Cationic Amino acid Transporter (CAT) family with nine members and L-type Amino acid Transporter (LAT) family with five members (Wipf et al., 2002). However, according to the transporter classification (TC) system (Chang et al.,

2004), the APC superfamily members can be reclassified into five families: the Amino acid-Polyamine-organocation (APC) family, the Amino Acid/Auxin Permease (AAAP) family, the Alanine or Glycine: Cation Symporter (AGCS) family, the Hydroxy/Aromatic Amino Acid Permease (HAAAP) family, and the Cation-Chloride Cotransporter family (CCC). The fifth family does not transport AAs (Chang et al., 2004). The APC family, the founding family of the APC superfamily, has members further grouped into three subfamilies: the Cationic Amino acid Transporters (CATs), the Amino acid/Choline Transporters (ACTs), and the Polyamine H^+-Symporters (PHSs) (Okumoto and Pilot, 2011). The PHS family includes the LAT subfamily in Arabidopsis. The transport mechanism, expression pattern, and biological role of the AATs have been discussed in section 1.6.

1.2.4. Peptide uptake

As mentioned previously, the Peptide transporters (PTR) and the NRT1 family of the NO_3^- transporters are related, although the evidence indicates that NO_3^- transporters cannot transport peptides (Huang et al., 1999; Lin et al., 2000; Chiu et al., 2004), while peptide transporters cannot transport NO_3^- (Chiang et al., 2004). All of the PTR (NRT1) transporters in higher plants contain 12 putative transmembrane (TM) spanning regions, with a large hydrophilic loop between TM domains 6 and 7 (Tsay et al., 2007). The position of the long hydrophilic loop between TM 6 and 7 is unique to higher plant PTRs (NRT1) and rat PHTs (Chiang et al., 2004). In most animal PTR transporters (NRT1), the long loop is located between TM 9 and 10, while in fungi, it is between TMs 7 and 8. The function of the long hydrophilic loop in the PTR (NRT1) transporters has yet to be identified (Tsay et al., 2007). The first plant peptide transporter, PTR2 (At2g02040, formerly NRT1), was isolated by complementing a yeast peptide transport-deficient mutant in which PTR2 displayed high-affinity, low-selectivity transport activity for di- and tri-peptides (Rentsch et al., 1995). Heterologous expression in yeast and *Xenopus* oocytes showed that Arabidopsis PTR1 (Dietrich et al., 2004) and PTR2 (Song et al., 1996; Chiang et al., 2004) mediate peptide

transport. A new member of this gene family, *PTR5*, was isolated from Arabidopsis based on its homology with *PTR1* (Komarova et al., 2008). *In planta* mutant and over-expression analysis of PTR1 and PTR5 has demonstrated that these two transporters mediate peptide uptake from external sources (Komarova et al., 2008).

1.3. Regulation of the N-uptake systems in plants

While N-deficiency significantly reduces plant growth and causes chlorosis, excess N delays transition to the reproductive phase and increases susceptibility to diseases, insects, and low temperature (reviewed in Huber and Thompson, 2007). Plants sense their internal N status and regulate the uptake from external sources (Lee and Rudge, 1986; Cooper and Clarkson, 1989; Miller et al., 2007). All high and low affinity transporters for NH_4^+, NO_3^-, AAs and peptides do not remain constantly active in N uptake from the soil. N availability in the environment, as well as, N status within a plant regulate these transporters and thereby net N acquisition. However, the mechanism as to how plants sense and signal their internal N status and regulate the N uptake at a molecular level is still being studied. As NO_3^- taken up by the plants is reduced to NH_4^+ and then both forms of inorganic N are finally assimilated into AA, it has been suggested that the internal pools of downstream N metabolites such as AAs may function as signaling molecules to indicate N status and thereby regulate N uptake and assimilation (Lee and Rudge, 1986; Cooper and Clarkson, 1989; reviewed in Miller et al., 2008). In recent years, several studies demonstrated that free AAs in plants are directly involved in this process (reviewed in Coruzzi and Zhou, 2001; Glass et al., 2002; Miller et al., 2008). These authors postulated that the regulation of N uptake may occur at various steps. This section will briefly discuss the role of AAs in the regulation of N-uptake.

Free AAs may regulate the uptake of both NO_3^- and NH_4^+ through regulating the expression of genes encoding NO_3^- and NH_4^+-transporters. The uptake of both NO_3^- and NH_4^+ has been demonstrated to be negatively influenced

by the exogenous supply of AAs (Lee et al., 1992; Muller et al., 1995; Rawat et al., 1999). Several studies have shown that the expression of high affinity NO_3^- transporters is inhibited by Gln (Quesada et al., 1997; Krapp et al., 1998; Vidmar et al., 2000). Vidmar et al. (2000) showed that exogenous supply of AAs reduces both the transcript abundance of NO_3^- transporters in the root and NO_3^- uptake by the plant. In this study, they specifically identified Gln as the molecule responsible for down regulation of the expression of the NO_3^- transporter (Vidmar et al., 2000). However, another study in *Brassica napus* showed that treating roots with exogenous Gln, Glu, or Asn effectively down regulates the expression of NO_3^- transporter and NO_3^- uptake (Beuve et al., 2004). One of the difficulties with these types of studies is that external AA concentrations are usually very high and the way that they relate to internal AA concentrations is often unclear.

Transporters involved in NH_4^+ uptake have also been shown to be down regulated by AAs. Lee et al. (1992) have demonstrated in maize roots that increased intra-cellular concentrations of Gln suppress the net uptake of NH_4^+, while decreased root Gln and/or Asn concentration are associated with an increased uptake of NH_4^+. In this study, plant roots were treated with exogenous AA or specific inhibitors of Glutamine Synthetase (GS), Glutamate Synthase (GOGAT) and aminotransferases, and the subsequent change in the AA content in the root cells *in vivo* was confirmed by ^{14}N- or ^{15}N-NMR spectroscopy, or by HPLC analysis of tissue extracts. In Arabidopsis, Rawat et al. (1999) showed that Gln, the end product of NH_4^+ assimilation, rather than NH_4^+ itself, is responsible for regulating the expression of *AMT1* in Arabidopsis root. They demonstrated that following the supply of NH_4^+ to N-starved plants, both *AMT1* mRNA level and $^{13}NH_4^+$ influx declined rapidly but remained high when the conversion of NH_4^+ to Gln was blocked with methionine sulfoximine (Rawat et al., 1999).

In addition to down-regulating transporters involved in NO_3^- and NH_4^+ uptake, free AAs are likely to have the potential to regulate N assimilation through the GS/GOGAT pathway which is considered the primary entry port of inorganic N in plants (Lam et al., 1996; Coschigano et al., 1998; Lea and Ireland,

1999; Miflin and Habash, 2002; Suzuki and Knaff, 2005). Enzymes involved in this pathway are known to be feedback-inhibited by free AAs. For example, the first enzyme in the N-assimilation pathway, GS, that produces Gln using Glu as a substrate, has been shown to be feedback-inhibited by various AAs, including Gln, in both plants and microbes (Woolfolk and Stadtman, 1967; Hubbard and Studtman, 1967; Rhodes et al., 1975; Liaw et al., 1993). A free AA pool in the translocation pathway, particularly in the phloem, may regulate N uptake by the plants (Muller and Touraine, 1992; Gessler et al., 1998; Palove-Balang and Mistrik, 2002). However, the mechanism as to how phloem AAs transfer signals to the root and regulate N uptake is not clear. Nitrogen, assimilated into Glu, Gln, Asp, and Asn, is disseminated into the phloem, as these AAs function as N-carriers, to the sink tissues. Studies showed that the concentration of these AAs in the phloem sap is negatively correlated with the root NO_3^- uptake. In soybean, higher concentrations of Asn, Gln, Asp, Glu and some other AAs in the phloem sap were associated with an inhibition in NO_3^- uptake. However, the magnitude of inhibition was lower than that caused by feeding AAs directly into the root (Muller and Touraine, 1992). In beech seedlings, directly feeding Asp and Gln into the phloem reduced the NO_3^- uptake by the roots by more than 50% (Gessler et al., 1998). In maize seedlings, a higher concentration of Gln in phloem, fed through a cut-tip leaf, showed a reduction in the root NO_3^- uptake (Palove-Balang and Mistrik, 2002).

1.4. N metabolism in plants

1.4.1. Assimilation of inorganic N into AAs

In plants, the primary assimilation of inorganic N into the organic form of Gln takes place via the coordinated function of GS and GOGAT using Glu as a substrate (Lea and Miflin 1974; Keys et al., 1978; Miflin and Lea 1980; Stewart et al., 1980) (see detail in section 1.4.4.1.). Andrews (1986) demonstrated that the primary assimilation of N occurs both in roots and shoots. The assimilation of N requires cofactors, reducing equivalents, and C skeletons generated during photosynthesis. Thus, the assimilation of inorganic N into an organic form was

suggested to occur predominantly in the leaf chloroplasts where these components are readily available (Sechley et al., 1992). It was proposed that in plant species that are able to efficiently transport photosynthate to the roots, such as maize and temperate legumes, N assimilation also occurs at a high rate in the root plastids (Oaks, 1992). However, several subsequent studies suggested that primary assimilation occurs mostly in the roots (Andrews et al., 1992; Kruse et al., 2002; Scheurwater et al., 2002).

1.4.2. The role of AAs in plants

AAs play a role in plants in three different ways: as the building blocks of peptides, proteins and enzymes; the substrate of metabolic and hormonal pathways; and as signaling molecules. Neutral AAs are predominant in membrane proteins and confer hydrophobicity and control over the movement of macro and micro molecules in and out of the cells. Acidic or basic AAs in enzymes enhance their catalytic efficiency. Sulfur-containing AAs such as cysteine, in their oxidized state, confer protease resistance to proteins and give them a complex structure and stability. They may also serve as an oxidation-reduction molecule. Seed storage proteins such as α-, β-, and γ-kafirins are examples of some cysteine-rich proteins (summarized from Gruissem and Jones, 2000).

Free AAs within plants will affect the N status by providing signals to regulate N uptake and assimilation by the plant (Lee and Rudge, 1986; Cooper and Clarkson, 1989) (see detail in section 1.3). Amino acid homeostasis plays a role in abiotic stress tolerance (Bonner et al., 1996; Martino et al., 2003; Sharma and Dietz, 2006; Wu et al., 2010), amino-N partitioning (Hernandez-Sebastia et al., 2005), and disease resistance (Song et al., 2004; van Damme et al., 2009; Liu et al., 2010). Proline, histidine, and some N-containing AA-derived metabolites play a role in the defense against heavy metal stress (reviewed in Sharma and Dietz, 2006). Aromatic AAs such as tyrosine may be involved in phosphorylation and play a role in signal transduction.

Uncommon AAs such as α-amino-β-oxoalylaminopropionic acid, mimosine, α- and γ-diaminobutyric acid, canavanine, and β-hydroxy- γ-methylglutamic acid are often accumulated in seeds. No general rules can be made about their function as only a few of them are known to have specific functions in plants. Some plants divert as much as 10% of their resource, biosynthetic capacity, and storage space to the synthesis and accumulation of these uncommon AAs, suggesting that they must provide some selective advantage. Studies reveal that some uncommon AAs such as α-amino-β-oxoalylaminopropionic acid, mimosine, α- and γ-diaminobutyric acid are toxic to human and mammals. Canavanine and β-hydroxy- γ-methylglutamic acid are repellent to some insect larva while 5-hydroxytryptophan and 3, 4-dihydroxyphenyl alanine are toxic to them. Canavanine is also toxic to other higher plants. Therefore, they may play a pivotal role in plant defense against human, insects and other plants. Uncommon AAs can also be useful as taxonomic markers or in establishing phylogeny (reviewed in Bell, 1976).

1.4.3. Classification of plant AAs

All AAs have a carboxyl group and an amino group bonded to the same carbon atom (α-carbon). For all the common AAs except glycine, the α-carbon is bonded to four different groups: a carboxyl group, an amino group, a hydrogen atom and a side chain. In glycine, the side chain is another hydrogen atom. Proline is another exception, being a cyclic AA. AAs have two stereo isomers: L- and D- form, while AA residues in proteins are usually L-stereo isomers. Amino acids reside in a zwitterion form at a neutral pH while being positively or negatively charged at acidic and basic pH respectively. Amino acids differ from each other in their side chains or R-groups which vary in structure, size, and charge, and which influence the solubility of the AAs in water. AAs can be grouped into five classes based on the properties of R-groups, in particular, their polarity, or tendency to interact with water at a biological pH (near pH 7.0) (summarized from Gruissem and Jones, 2000).

1.4.4. Biosynthesis of plant AAs

Amino acid biosynthesis in higher plants can be discussed based on the pathway and C-skeleton used for each group of AAs. For example, inorganic N-assimilation and production of primary AAs Glu, Gln, Asp, and Asn can be discussed as one group. Similarly, the rest of the AAs can be grouped as aromatic AAs, Asp-derived, Glu-derived, pyruvate-derived, and 3-phospho-glycerate-derived AAs. The notable exceptions in this grouping are Ile and Ala. The small neutral AA, Ala, uses the same C-skeleton as branched-chain AA Leu and Val, while the third branched-chain AA Ile uses the same C-skeleton as other Asp-derived AAs . Amino acid biosynthesis in plants is now well documented. The section below discusses the site of the reaction and flow of C and N for the biosynthesis of primary and *de novo* AAs (Gruissem and Jones 2000).

1.4.4.1. Synthesis of N-assimilatory AAs (Gln, Glu, Asp, and Asn)

Plants assimilate inorganic N primarily into Gln via the coordinated function of Glutamine Synthetase (GS) and Glutamate Synthase (GOGAT, also known as glutamine 2-oxoglutarate amidotransferase), using Glu as a substrate. Each of the primary enzymes, GS and GOGAT, are grouped into two classes. The two classes of GOGATs, ferredoxin-dependent GOGAT (Fdx-GOGAT) and NADPH-dependent GOGAT (NADPH-GOGAT) are localized in plastid while the two classes of GS, GS1, and GS2, are localized in the cytosol and chloroplast respectively with non-overlapping functions. The chloroplast-specific GS2 is predominant in leaves, whereas cytosolic GS1 is predominant in the root and cotyledons. The subcellular localization of the two isoenzymes of GS suggested that the primary assimilation of inorganic N into AA in the root may occur either in cytosol or in plastid, while in the leaves their function might be limited in the chloroplast. Furthermore, promoter analysis has shown that cytosolic GS1 is specifically expressed in the phloem, suggesting that it has a role in synthesizing Gln for long distance N transport. Chloroplast-specific GS2 is specifically expressed in the leaf mesophyll cells, suggesting that photo-respiratory NH_4^+ is re-assimilated into Gln in the leaf chloroplast by this enzyme.

The primary assimilation of N into Gln requires continuous regeneration of α-ketoglutarate, the carbon (C) precursor for NH_4^+ assimilation. The synthesis of Asp regenerates the necessary C skeletons for further N assimilation via the GS/GOGAT cycle by transferring the amino group from Glu to oxaloacetate by the enzyme Aspartate Aminotransferase (AspAT). AspAT plays a role in both Asp synthesis and catabolism. Isoforms of AspAT are localized in the cytosol, mitochondria, chloroplasts, and peroxisomes, suggesting that these are the sites where Asp is synthesized and/or catabolized. The amidation of Asp by Gln or NH_4^+ yields Asn, a relatively inactive AA used mostly to store and transport N from the source tissues to the sinks. The enzyme, Asparagine Synthetase (AS), catalyzes the transfer of the amino group, preferably from Gln to Asp-generating Glu and Asn. Under conditions of low C availability or high organic N, plants direct assimilated N into Asn, which has a higher N: C ratio than Gln and therefore can transport and store N more efficiently when C skeletons are limiting. Nitrogen assimilated into Glu, Gln, Asp, and Asn is readily disseminated into plant metabolism because these four AAs usually donate N in the biosynthesis of other AAs, nucleic acids, and N-containing compounds, or else they function as N-carriers to the sink tissues.

1.4.4.2. Synthesis of aromatic AAs (Tyr, Trp and Phe)

In higher plants, aromatic AA-synthesis pathways are considered the major sink of amino-N because about 20% of the carbon fixed by plants flows through these pathways (Gruissem and Jones 2000). In addition to synthesizing aromatic AAs, these pathways are also used to produce numerous secondary metabolites such as the hormone auxin, pigments, defensive phytoalexins, biotic alkaloids, and structural lignins. Therefore, a large share of the N transported by these AAs (i.e., Glu, Gln, Asp, and Asn) is destined towards these pathways.

To date, all the enzymes involved in the aromatic AA synthesis are localized in plastids. The C-skeletons used in the synthesis of aromatic AAs come from glycolysis and the pentose-phosphate pathway. Phosphoenolpyruvate (PEP) from glycolysis (occurs in cytosol) and erythrose 4-phosphate from the pentose

phosphate pathway (which occurs in plastid) produce chorismate via shikimate through a seven-step pathway. At the chorismate step, the pathway divides into two: towards Trp or towards Phe and Tyr. In the plastid, the enzyme involved in producing Phe and Tyr from chorismate can be feedback-inhibited by the end product; and Trp, the end product of the other branch of this pathway, can reverse the inhibition. Glu or Asp, synthesized locally in plastid or transported from source tissues, donate the amino-group necessary for the synthesis of Phe, Tyr, and Trp. Serine can also donate the amino-group for Trp biosynthesis.

1.4.4.3. Synthesis of Asp-derived AAs (Lys, Met, Thr and Ile)

The pathway from Asp to Lys, Thr, and Met has two branches: one leads to Lys and the other leads to Thr or Met. Ile is produced downstream from Thr. In each case, both the C-skeleton and the amino-group are provided by Asp while the components of the side chains come from variable sources. For the synthesis of Thr, the amino-group and C-skeleton of Asp remains the same; only the side chain undergoes rearrangement to form Thr via homoserine. The formation of Lys from Asp requires a pyruvate molecule to be assimilated into the side chain of Asp and rearranged to form Lys. The synthesis of Met from Asp via homoserine 4-phosphate requires a Cys molecule to provide the thiol-group in the side chain of Met while the C-skeleton and the NH_4^+ in the Met come from Asp. In this reaction, the C-skeleton of Cys is released as one molecule of pyruvate and one molecule of NH_4^+. The synthesis of Ile from Thr is initiated by anabolic Threonine Deaminase (TD), which is feedback-inhibited by Ile. In this pathway, the amino-group of Thr is removed by TD to produce 2-ketobutyrate, which undergoes a series of reactions and finally obtains the amino-group from another AA to produce Ile. Thus, Ile obtains the C-skeleton from a Thr and amino-group from a non-specific AA. Lys, Thr, and Ile are synthesized in the plastid while Met is synthesized in the cytosol. Asp-derived AA biosynthesis follows three major routes, creating at least five branch-point enzymes, which makes the regulation of this pathway relatively complex. Lys and Thr can each inhibit this pathway at two different points, regulating the synthesis of all four Asp-derived AAs. The accumulation of Lys can inhibit the pathway either at the early step, regulating the

synthesis of all four AAs, or it can inhibit at a step where Lys biosynthesis will stop and the pathway will divert completely towards Thr, Met, and Ile synthesis. Similarly, Thr accumulation can either stop the entire pathway at an early step or divert the pathway towards Lys synthesis and regulate Thr, Met, and Ile synthesis (Jander and Joshi, 2009).

1.4.4.4. Synthesis of Glu-derived AAs (Arg, His and Pro)

Glutamate is the precursor and source of the C-skeleton for Arg, His, and Pro biosynthesis. Arg is synthesized from Glu via ornithine and citrulline through a pathway consisting of nine steps. In this pathway, Glu, Gln, and Asp are catabolized, which are the potential sources of the amino-group. The synthesis of Arg, based on the localization of the enzymes involved in this pathway, occurs in plastid. The catabolism may occur in mitochondria (reviewed in Slocum, 2005). The biosynthesis of Arg is regulated by the end product Arg, which can inhibit one or more of the enzymes involved in this pathway (McKay and Shargool, 1981; Shargool et al., 1988). Histidine is formed through a series of 11 reactions, beginning with the condensation of ATP and phosphoribosylpyrophosphate (PRPP), which provides the C-skeleton and the side chain for His. In this process, one Gln and one Glu are catabolyzed. The amino-group for His comes from the Glu (reviewed in Stepansky and Leustek, 2006). The enzyme catalyzing the first step is feedback-inhibited by His. The overall level of His present in the cell seems to be controlled by the activity of the first enzyme in the pathway because overexpression of this enzyme increases the total free His content measured in the rosette leaves of Arabidopsis, while overexpression of seven downstream genes in the pathway fails to increase the His level (Rees et al., 2009). In plants, the entire pathway is located inside the chloroplast.

For the biosynthesis of Pro, the carboxyl-group in the side chain of Glu undergoes changes and forms a bond with the amino-group through a cyclization reaction to produce Pro. Both the amino-group and the C-skeleton of Pro come from Glu. The Pro synthesis may also initiate from ornithine; in that case, the biosynthesis of both Arg and Pro from the Glu precursor share the same pathway

up to the ornithine step and branch apart towards Arg or Pro synthesis. The biosynthesis of Pro is regulated both at the enzyme level and through changes in gene expression. The first enzymatic reaction, γ-Glutamyl Kinase activity that phosphrylates the side chain of Glu, is feedback-inhibited by Pro. Under stress conditions, one or more enzymes involved in this pathway show altered expression levels which regulate the Pro synthesis (Verbruggen et al., 1993; Strizhov et al., 1997; Abraham et al., 2003; Armengaud et al., 2004).

1.4.4.5. Synthesis of pyruvate-derived AAs (Leu, Val and Ala)

The Leu and Val synthesis pathway starts from pyruvate and is initiated by the enzyme Acetohydroxy Acid Synthase (AHAS, also known as Acetolactate Synthase). The pyruvate molecule undergoes two steps of reactions before the pathway branches towards Val or Leu. While, the C-skeleton for both Leu and Val comes from pyruvate, the amino-group comes from a non-specific AA. The enzyme AHAS that initiates this pathway is synergistically feedback-inhibited by both Leu and Val. The biosynthesis of Leu and Val occurs in plastid. For Ala synthesis, the C-skeleton comes from a pyruvate and the amino-group comes from Glu. Transferring an amino-group from a Glu to pyruvate producing one molecule of Ala and an α-ketogluterate is carried out by Alanine Aminotransferase (AlaAT). Arabidopsis contains four AlaAT homologues. Two of these homologues encode GGAT1 and GGAT2, which have both AlaAT activity and glutamate:glyoxylate aminotransferase (GGAT) activity (Igarashi et al., 2003; Liepman and Olsen, 2003). In Arabidopsis, GGAT activity occurs in peroxisomes (Igarashi et al., 2003; Liepman and Olsen, 2003). The other two enzymes, AlaAT1 and AlaAT2, have a role in both synthesis and break down alanine (Miyashita et al., 2007). *In silico* predictions suggest that the AlaAT2 is localized to the mitochondria while the AlaAT1 has been confirmed to be localized in the cytosol (Igarashi et al., 2003). Depending on the localization of the AlaAT homologs responsible for the transaminase activity, Ala can be synthesized in the cytosol or peroxisome, while peroxisome can also be the site of Ala degradation.

1.4.4.6. Synthesis of 3-phospho glycerate-derived AAs (Ser, Gly, Cys)

Serine biosynthesis in plants proceeds by two pathways: the phosphorylated pathway and the glycolate pathway. In the phosphorylated pathway, Ser is derived from 3-phosphoglycerate, an intermediate of glycolysis in the plastid. This pathway is thought to contribute to Ser biosynthesis in the dark and in non-photosynthetic tissues. In this pathway, the C-skeleton comes from 3-phosphoglycerate and the amino-group comes from a Glu. In the glycolate pathway, Ser is derived from glycolate via Gly. The glycolate pathway is predominant in the photosynthetic tissues. Genes of the phosphorylated pathway are upregulated by high salinity, flood, and cold (Ho and Saito, 2001).

In plants, Gly can be generated from Ser through a one-step activity of Serine hydroxymethyltransferase. This enzyme catalyzes a reversible reaction, the inter-conversion of Ser and Gly. During photorespiration in the leaves when the high concentration of Gly is produced, the reaction is driven towards Ser biosynthesis. In roots and other non-photorespiratory tissues, the enzyme more likely contributes to Gly biosynthesis. There are five isoforms of this enzyme putatively localized in the cytosol and mitochondria, suggesting that Gly is produced in the cytosol (McClung et al., 2000). There are two more pathways present in plants for Gly biosynthesis via Threonine aldolase or from glyoxalate. These pathways have not been well described.

Cysteine biosynthesis, the final step of sulfate assimilatory reduction and the sole entry point of reduced sulfur into an organic form in plants, proceeds from Ser, which provides the AA backbone for Cys synthesis. Cys is produced from Ser via a two-step reaction. The pathway occurs in plastid, cytosol, and mitochondria (Wu et al., 2007). The first enzyme involved in this pathway can be feedback inhibited by the end product Cys or by the limited supply of sulphide.

1.5. The Transporter Classification (TC) and TC numbers of the AATs

Phylogenetic classifications are based on the DNA (genomic DNA or cDNA) sequence alignment. Thus, these classifications may provide variable

results depending on the analysis such as parameter settings, number of species included, number the of characterized and uncharacterized gene sequences used in the analysis. The section below will discuss the classification of various known membrane transporter proteins in various organisms from bacteria to higher eukaryotes. This classification is now recommended by the transport nomenclature panel of the International Union of Biochemistry and Molecular Biology (IUBMB). In this classification, transporters are grouped on the basis of five criteria and each of these criteria corresponds to one of the five entries within the Transporter Classification (TC) number for a particular transporter. The five criteria in the TC number represent the transporter class, the transporter subclass, the permease family or superfamily, and the subfamily in a family (or the family in a superfamily). The final note delineates the substrate or range of substrates transported as well as the polarity of transport (in or out). Therefore, each subclass of transporters has a two-digit TC number; each family (or superfamily) has a three-digit TC number; each subfamily (or family) has a four-digit TC number; and each transporter type has a five-digit TC number. Any two transporter proteins in the same subfamily that transport the same substrate(s) using the same mechanism are given the same TC number (Saier, 2000).

According to the TC system, there are about 600 entries of transporter families with more than 5,000 transporter proteins (http://www.tcdb.org/). Some of these families are actually large superfamilies with more than 1,000 currently sequenced members. Others are very small families with only one or a few currently sequenced members. This classification has accommodated all the listed families under nine broad categories (http://www.tcdb.org/; Thever and Saier, 2009; Saier, 2000). This categorization in the TC system is primarily based on the mode of transport and energy-coupling source. Among these nine categories, the first five are recognized classes of transporters. No assignment has been made for categories 6 and 7. These are reserved for novel types of transporters that do not fall within categories 1 to 5. Nine major categories of transporters in the TC system are listed in the Table 1.1.

The section below will discuss each category of transporters, and will try to identify all known and putative AAT-families (or subfamilies) that may belong to each category.

TC 1. Channel proteins (Channels and Pores)

Channel proteins form a channel through which solutes pass at a high flux rate when the channel is open (Figure 1.3). There are several types of proteins that form channels in membranes. Porins, ion channels, and pores in the nuclear membrane or channels that mediate protein translocation across the endoplasmic reticulum membrane are the examples of some common selective channels. Some channels consist of a single protein whose transmembrane segments form a pore. Others exist as oligomers of identical or different subunits, each of which forms a pore by itself. Channel proteins are selective for a solute; they mediate a rapid rate of solute permeation; and they maintain a gating mechanism that regulates solute permeation. However, some ion channels are selective for cation or anion, based on the charge of the ion. The specificity of the channels is maintained by a structural feature called the selectivity filter that allows the channels to discriminate among different solutes. The electrochemical gradient of the solute dictates the direction of net ionic flux through the channel. For example, in a physiological condition, a mammalian skeletal muscle cell maintains 4:155 concentration gradients of K^+ across the plasma membrane with higher concentration inside the cell. Therefore, in a resting cell, there will be an outward movement of a K^+- ion through the channel (Lewin et al., 2011). Since there is no energy source other than the energy of the electrochemical gradient of the K^+-ion involved in this movement, this type of transport is called passive transport. Channel proteins have been further grouped into eight categories (TC1.A to H) based on their structures (http://www.tcdb.org/browse.php; Saier, 2000):

1. A. α-Type channels: Transmembrane channel proteins of this class are ubiquitously found in the membranes of all types of organisms from bacteria to higher eukaryotes. These transporters usually catalyze the movement of solutes by an energy-independent process by passage through a transmembrane aqueous pore

without evidence for a carrier-mediated mechanism. These channel proteins consist largely of α-helical spanners, although β-strands may be present and may even contribute to the channel. As per the transporter classification (TC) system, there are 45 different families (or superfamilies) of channels in this group. The voltage-gated ion (Na^+, K^+, Ca^{++}) channel (VIC) superfamily (TC1.A.1), ATP-gated cation channel (ACC) family (TC1.A.7), symbiotic ammonium transporter (SAT) family (TC1.A.26) all belong to this transporter group (Saier, 2000).

1. B. β-Barrel porins: These proteins form transmembrane pores that usually allow the energy-independent passage of solutes across a membrane. The transmembrane portions of these proteins consist exclusively of β-strands that usually form β-barrels. Porin-type proteins are found in the outer membranes of gram-negative bacteria, mitochondria, plastids, and possibly acid-fast gram-positive bacteria. There are 26 different families of porins in this group. Some relevant examples of this transporter group are the Sugar Porin (SP) family (TC 1.B.3), the Mitochondrial and Plastid Porin (MPP) family (TC 1.B.8), and the Short-chain Amide and urea Porin (SAP) family (TC 1.B.16) (Saier, 2000). In the outer envelope of chloroplast, Outer Envelope Porin-16 (OEP 16, TC 1.B.30) and Outer Envelope Porin-24 (TC.1.B.28) subfamilies that mediate AA export through the outer membrane of chloroplast (Pohlmeyer et al., 1997; Philippar et al., 2007) belong to this group (http://www.tcdb.org/browse.php).

1. C. Pore-forming toxins: These proteins and peptides are synthesized by one cell and secreted for insertion into the membrane of another cell, where they form transmembrane pores. They may exert their toxic effects by allowing the free flow of electrolytes and other small molecules across the membrane, or they may allow entry into the target cell cytoplasm of a toxin protein that ultimately kills or controls the cell. Both protein (large) and ribosomally synthesized peptide (small) toxins are included in this category. There are 38 different families of transporters in this group (Saier, 2000). Pesticidal crystal proteins commonly known as δ-endotoxin from *Bacillus thuringiensis* belong to this group (Fabrick et al., 2009;

Hua et al., 2008; Pardo-López, et al., 2006; http://www.tcdb.org/search/result.php?tc=1.C.2.1).

1. D. Non-ribosomally synthesized channels: These molecules, often chains of L- and D-AAs as well as other small molecular building blocks such as hydroxy acids (i.e., lactate and β-hydroxybutyrate), form oligomeric transmembrane ion channels. Voltage may induce channel formation by promoting assembly of the oligomeric transmembrane pore-forming structure. These "depsipeptides" are often made by bacteria and fungi as agents of biological warfare. Other substances, completely lacking AAs, may also be capable of channel formation. There are six families of transporters in this group (Saier, 2000). Syringopeptin proteins (TC 1.D.3.1) produced by the plant pathogen, *Pseudomonas syringae,* that targets host plants and induce necrosis are a common example of this transporter group (http://www.tcdb.org/browse.php).

The rest of the transporter categories within TC1 listed in the Table 1.1 are not discussed further in this section.

TC 2. Electrochemical Potential-Driven Porters

2. A. Porters (uniporters, symporters, and antiporters): Transport systems included in this subclass utilize a carrier-mediated process to catalyze a uniport (a single species is transported either by facilitated diffusion or in a membrane potential-dependent process if the solute is charged), antiport (two or more species are transported in opposite directions in a tightly coupled process, not coupled to a direct form of energy other than chemiosmotic energy), and/or symport (two or more species are transported together in the same direction in a tightly coupled process, not coupled to a direct form of energy other than chemiosmotic energy) (Figure 1.3). This is the most important transporter subclass that includes almost all the well characterized AATs. There are three superfamilies and 101 different families listed so far in this category (Saier, 2000; http://www.tcdb.org/browse.php). Among all the listed familiesthe Major Facilitator Superfamily (MFS, TC 2.A.1) and the Amino acid-Polyamine-Organocation (APC) Superfamily (TC 2.A.3) are the two main transporter

families that mediate AA transport. Therefore, these two transporter families will be discussed in detail. Other families that are involved in AA transport are the Neurotransmitter:Sodium Symporter (NSS) Family (TC 2.A.22), the Dicarboxylate/AA:Cation (Na^+ or H^+) Symporter (DAACS, TC 2.A.23) Family, the Hydroxy/Aromatic AA Permease (HAAAP) Family (TC 2.A.42), the L-Lysine Exporter (LysE) Family (TC 2.A.75), the Branched Chain AA Exporter (LIV-E) Family (TC 2.A.78), the Threonine/Serine Exporter (ThrE) Family (TC 2.A. 79), the Aspartate:Alanine Exchanger (AAEx) Family (TC 2.A.81), the 6TMS Neutral AA Transporter (NAAT) Family (TC 2.A.95). Most of these families have characterized members in bacteria and/or mammals. Their plant counterparts have yet to be identified.

The Major Facilitator Superfamily (TC2.A.1)

The MFS is a large and diverse superfamily that includes over 10,000 sequenced members (http://www.tcdb.org/browse.php; Saier, 2000). The founding family of this superfamily, the MFS family, contains transporters that mediate AA transport and therefore will be further discussed in this report. Most of these transporters are of 400-600 amino acyl residues in length and possess 12, 14, or 24 putative transmembrane α-helical spanning domains. The mechanistic principles applicable to all MFS carriers have been summarized by Law et al. (2008). MFS permeases exhibit specificity for sugars, polyols, drugs, neurotransmitters, Krebs cycle metabolites, phosphorylated glycolytic intermediates, AAs, peptides etc. They are found ubiquitously in all three kingdoms of living organisms. A genomic analysis of the MFS permeases has been published (Lorca et al., 2007).

The MFS family transporters catalyze uniport, solute:cation (H^+ or Na^+) symport, and/or solute:H^+ or solute:solute antiport. An example of the MFS transport mechanism is the function of the 24 TMs MFS permease, NarK in *Paracoccus pantotrophus***.** The Nark transport complex has two 12 TMs domains, NarK1 and NarK2, both of which are required for normal NO_3^- uptake. The NarK1 catalyzes NO_3^-: H^+ symport, while the NarK2 catalyzes NO_3^-: NO_2^- antiport (Wood et al., 2002). Thus, the protein is a fusion protein of two

homologous but distinct MFS permeases. In Arabidopsis, the NO_3^- uptake transporter, NRT2.1 (TC 2.A.1.8.12) (Wirth et al., 2007) is an example of an MFS superfamily transporter. It also functions in NO_3^- sensing and signaling (Miller et al., 2007; Girin et al., 2010). This transporter system is functional when the NRT2.1 is associated with the NAR2.1 (8.A.20.1.1) in a 2:2 tetrameric complex. The NAR2.1 has an N-terminal and a C-terminal TM and has been annotated as a calcineurin-like phosphoesterase family member (Yong et al., 2010).

The human L-Type Amino acid transporter-3 (LAT3; TC 2.A.1.44.1) and L-Type Amino acid Transporter-4 (LAT4; TC 2.A.1.44.2) belong to the MFS superfamily. The LAT3 mediates transport of large neutral AAs such as Leu, Ile, Val, and Phe by a Na^+-independent, electroneutral, facilitated diffusion process (Babu et al., 2003). The LAT4 with 57% sequence similarity with the LAT3 has similar substrate specificity and mediates Na^+, Cl^-, and pH-independent facilitated diffusion of AAs (Bodoy et al., 2005).

The Amino acid-Polyamine-Organocation (APC) superfamily (TC2.A.3)

The APC superfamily is one of the largest superfamilies of membrane transporter proteins that includes most of the known AATs (Closs et al., 1993; Reizer et al., 1993; Saier, 1998; Saier, 1996; Saier, 2000). The APC superfamily (TC 2.A.3), contains 13 transporter families (TC2.A.3.1 to 13) (http://www.tcdb.org/):

1. TC 2.A.3.1: The AA Transporter (AAT) Family
2. TC 2.A.3.2: The Basic AA/Polyamine Antiporter (APA) Family
3. TC 2.A.3.3: The Cationic AA Transporter (CAT) Family
4. TC 2.A.3.4: The AA/Choline Transporter (ACT) Family
5. TC 2.A.3.5: The Ethanolamine Transporter (EAT) Family
6. TC 2.A.3.6: The Archaeal/Bacterial Transporter (ABT) Family
7. TC 2.A.3.7: The Glutamate: GABA Antiporter (GGA) Family
8. TC 2.A.3.8: The L-type AA Transporter (LAT) Family
9. TC 2.A.3.9: The Spore Germination Protein (SGP) Family
10. TC 2.A.3.10: The Yeast AA Transporter (YAT) Family
11. TC 2.A.3.11: The Aspartate/Glutamate Transporter (AGT) Family

12. TC 2.A.3.12: The Polyamine: H^+ Symporter (PHS) Family

13. TC 2.A.3.13: The AA Efflux (AAE) Family

Half of these 13 families were identified only in bacteria. The CAT (TC2.A.3.3) family is found in both bacteria and eukaryotes and contains 21 members including nine CATs in Arabidopsis. The four families that are found only in eukaryotes are the ACT family (TC2.A.3.4; 13 members), the LAT family (TC2.A.3.8; 26 members), the YAT family (TC 2.A.3.10; 29 members) (Jack et al., 2000), and the PHS family (TC2.A.3.12; 22 members) (Okumoto and Pilot, 2011). The PHS family includes the Arabidopsis LAT family (Okumoto and Pilot, 2011) that was previously classified in the animal LAT cluster (Wipf et al., 2002).

Members of the APC family have diverged in all aspects including topology, structure, number of membrane-spanning domains, transport mechanism, and substrate specificity. Members of this family vary in length, being as small as 350 AA residues and as large as 850 AA residues (Sophianopoulou and Diallinas, 1995). The smaller proteins are generally of the prokaryotic origin while the larger ones are of eukaryotic origin. The majority of homologous integral membrane APC transport proteins appear to exhibit a uniform topology with twelve transmembrane α-helical spanning domains in a single polypeptide (Cosgriff and Pittard, 1997; Ellis et al., 1995; Hu and King, 1998), while the eukaryotic members of the CAT family (TC 2.A.3.3) have 14 TMs (Su et al., 2004; Wipf et al., 2002). This family includes members that function as solute:cation symporters and solute:solute antiporters (Devés and Boyd, 1998; Isnard et al., 1996; Kashiwagi et al., 1997; Verrey et al., 2004). In Arabidopsis, some CAT members have shown H^+-coupled symport activity (eg, CAT1, CAT5, CAT6) (Frommer et al., 1995; Hammes et al., 2006; Rentsch et al., 2007; Su et al., 2004), while CAT8 showed H^+-independent transport of AAs (Yang et al., 2010), suggesting a different type of transport mechanism. Some animal proteins, for example, those in the LAT family (TC 2.A.3.8), associated with a type I transmembrane glycoprotein through a disulfide bond that is essential for the transporter's activity (Devés and Boyd, 1998; Mastroberardino et al., 1998; Torrents et al., 1998). For example, Human LAT1 and LAT2 are

glycoprotein-associated obligatory exchangers (Mastroberardino et al., 1998; Pineda et al., 1999; Rossier et al., 1999); they exchange intra-cellular AAs for extra-cellular AAs (Meier et al., 2002). Members of one family within the APC superfamily (SGP; TC 2.A.3.9) are AA receptors rather than transporters (Cabrera-Martinez et al., 2003) and truncated at their C-termini, relative to the transporters, having 10 TMs (Jack et al., 2000). Some of the animal homologues of the CAT family (TC no. 2.A.3.3) have been found to serve as viral receptors (Kim et al., 1991; Reizer et al., 1993). In the ACT subfamily, only one member, the Bi-directional AA Transporter1 (BAT1), has been characterized so far in plants. In a heterologous system, the BAT1mediated transport of AAs in both directions (Dündar and Bush, 2009); however, the exact mechanism of this transporter is yet to be demonstrated (Okumoto and Pilot, 2011).

The substrate specificities of several APC family permeases have been carefully studied, revealing that while some have exceptionally broad specificity, others are restricted to just one or a few AAs or related compounds (Hu and King, 1998; Isnard et al., 1996; Kashiwagi et al., 1997). One of these transporters, a histidine permease of *Saccharomyces cerevisiae*, surprisingly, has been implicated in manganese transport (Farcasanu et al., 1998). A well-described example of APC family members in prokaryotes is the bacterial AdiC protein that functions as an Arginine/Agmatine antiporter. The recently produced crystal structure of this transporter has revealed the transport mechanism of this transporter class (Casagrande et al., 2008; Tsay et al., 2007; Jack et al., 2000). The overall structure is similar to that of other Na^+-coupled symporters. It forms a homodimer of two AdiC monomers with each subunit consisting of 12 TMs (Fang et al., 2009; Gao et al., 2009). AdiC mediates the influx of Arg with the efflux of agmatine under an acidic environment, resulting in a net export of one proton for each transport cycle (Accardi and Miller, 2004; Iyer et al., 2003).

The Amino acid/Auxin Permease (AAAP) Family (TC 2.A.18)

The Amino acid Permease (AAP) family of the plant ATF1 superfamily belongs to the AAAP (TC 2.A.18) transporter family. The ATF1 superfamily is so far the largest and best-characterized family in plants (Wipf et al., 2002). A

detailed computer analysis indicates that the transporters are built up from 10 membrane-spanning domains, whereas experimental evidence suggests the presence of 11 hydrophobic segments, with the N-terminus exposed to the cytosol (Rentsch et al., 1998; Chang, 1997). Several members of this family were shown to function as proton symporters (Boorer et al., 1996; Boorer and Fischer, 1997). Members of the AAP family recognize a large spectrum of different AAs, including amides and ureides (Fischer et al., 1995; Boorer et al., 1996). Arabidopsis AAP1, AAP2, AAP4, and AAP6 proteins have a preference for neutral and acidic AAs while the AAP3 and AAP5 transport basic AAs in addition to neutral and acidic AAs. Therefore, the AAPs are classified either as neutral and acidic AA transporters or as general AA transporters (Rentsch et al., 1998). No AAT has yet been identified in the rest of the transporter classes 2 to 5 listed in the Table 1.1.

TC 8. Accessory factors involved in transport

8. A. Auxiliary transport proteins: Proteins that in some way facilitate transport across one or more biological membranes but do not themselves participate directly in transport are included in this class. These proteins always function in conjunction with one or more established transport systems. They may provide a function connected with energy coupling to transport, play a structural role in complex formation, serve a stability function, or function in regulation. There are 13 families (TC 8.A.1 to 13) in this class. Among these, members of rBAT transport accessory protein (rBAT) family (TC 8.A.9) interact with L-type AA transporters (TC 2.A.3.8) in animals to mediate AA exchange (Mastroberardino et al., 1998; Torrents et al., 1998; Verrey et al., 1999). Their plant counterparts have yet to be described. The rest of the transporter categories in the transporter class 8 and 9 have been listed in the Table 1.1.

1.6. Distribution of AAs in plants

AAs, either taken up or synthesized in the root, travel via the xylem to the shoot apex, sink leaves and seeds, and by the phloem to the root apex. The distribution of AAs between sources and sinks requires them to cross the PM as

well as the organeller membrane (chloroplast, mitochondria, tonoplast, and peroxisome). Amino acids do not cross membranes through passive diffusion; instead, when crossing a membrane, they require facilitated or active transport by membrane transporter proteins, more specifically, AATs. Plants with various types of source and sink tissues, as well as, with various types of protein and non-protein AAs, require a large number of AATs with export, import, antiport, or facilitator capacity with various substrate affinity and specificity. These transporters, as facilitate the movement of AAs through the membrane, also regulate the distribution of amino-N in the plant. Understanding the distribution of amino-N in plants thus requires understanding the tissue-specific distribution of AATs with their substrate affinity and transport mechanism. This section discusses the amino-N translocation from the root to the seed and describes the characterized and uncharacterized AATs involved in this route.

1.6.1. AA translocation from the root to the leaf

Loading into the xylem

The translocation of AAs from a root cell to a leaf cell occurs via the xylem and involves export from the root cell into the xylem apoplasm, followed by import from the xylem apoplasm into the leaf cell symplasm. AAs produced from inorganic N via the activity of cytosolic GS1 and NADH-GOGAT in roots would be in the root cell symplasm. Thus, in the first step of translocation, AAs, produced in the root cell symplasm or taken up directly from the soil, require crossing the PM in the root cells to be loaded into the xylem (Figure 1.2). This step requires AATs with a net export capacity in the PM of root cells (Figure 1.2). In Arabidopsis, AAP1, AAP5, LHT1, and ProT2 were found to be expressed in the root, and experimental evidences suggested that these AATs are involved in a substrate specific AA-uptake from soil (reviewed in Tegeder and Rentsch, 2010). The AAP1, expressed in the root epidermis and root hairs (Lee et al., 2007), has mediated import of AAs in both *in planta* mutant analysis (Lee et. al., 2007) and heterologous expression studies (Fischer et al., 2002). In a heterologous study, the increased AA-induced current in the AAP1-cRNA-injected oocytes was

inwardly directed, suggesting that the AAP1 mediated the import of AA into the cells (Fischer et al., 2002). In *in planta* studies, mutant Arabidopsis plants deficient in AAP1 showed increased resistance to toxic concentration of exogenous AAs and reduced uptake of ^{14}C-labeled AAs, confirming that AAP1 is an import transporter in the root (Lee et al., 2007). Furthermore, the export of AAs from a root cell into the xylem apoplasm for upward translocation is likely to occur in the pericycle surrounding the vasculature within the endodermis which separates the internal apoplasm of a plant from the root 'free-space.' Thus, AATs expressed in the root epidermis or in the root hair are less likely to mediate the export of AAs for translocation to the shoot. Another member of the AAP family, AAP5, which was detected in roots through microarray analysis (Birnbaum et al., 2003), was also characterized as an import transporter (Svennerstam et al., 2008). A T-DNA insertion line knocking out AAP5 in Arabidopsis showed reduced acquisition of ^{15}N-labeled L-Arg from the growth medium (Svennerstam et al., 2008). Heterologous expression and *in planta* knockout studies confirmed that the LHT1, which is expressed in the root epidermis and cortex (Chen and Bush (1997), is a high affinity AA importer (Hirner et al., 2006). Several studies conducted in heterologous systems showed that ProT2, a proline transporter in the root, mediates the import of AAs and their derivatives (Rentsch et al., 1996; Schwacke et al., 1999; Lehmann et al., 2010). None of the AATs detected in the root have shown AA-export capacity. To date, AATs with export capacity remained to be identified in plants (Lalonde et al., 2003; Okumoto and Pilot, 2011).

Import into the leaf cells

AAs delivered from the root to the shoot may largely be imported into the leaves where they are primarily received by the leaf mesophyll cells. To date, LHT1 is the only well-characterized PM-localized AAT that was demonstrated to import AAs from leaf apoplasm into the mesophyll cells (Hirner et al., 2006). Knocking out LHT1 significantly reduced AA uptake into the mesophyll cell protoplast (Hirner et al., 2006). LHT1 was initially characterized as a Lysine and Histidine- specific AAT (Chen and Bush, 1997), while later studies showed Glu,

Leu, and several other AA transport activity with a preference for Lys and His (Hirner et al., 2006). These studies indicated that LHT1 has a higher affinity for basic (positively charged) AAs. Schultz (1994) demonstrated that Asp, Asn, Glu, and Gln constitute up to 64% of the total AAs in the leaf extracts of Arabidopsis and that Asn and Gln are the two predominant forms of AAs in the xylem and phloem respectively. This suggests that AAs from the root to the leaf travel mostly as Asn and Gln (Schultz, 1994; Lam et al., 1996). Thus, it is likely that, in addition to the LHT1, there are more AATs with higher affinity for Asn and Gln that remain to be identified in the mesophyll cell PM.

1.6.2. AA translocation from the mature root cells to the root apex

Growing root tips receive AAs from mature root cells either symplastically through plasmodesmata or apoplastically. In the case of the apoplastic supply, AAs exported into the xylem apoplasm are likely to undergo a subsequent xylem-to-phloem exchange for translocation to the growing root apex (Figure 1.2). This step requires AAs to cross the PM of phloem parenchyma or phloem companion cells to be imported into the phloem symplasm, and thus requires AATs with import capacity in the PM of these cells. Microarray analysis (Brady et al., 2007) as well as promoter analysis suggested that Arabidopsis AAP2 (Hirner et al., 1998), AAP3 (Okumoto et al., 2004), AAP6 (Hunt et al., 2010; Okumoto et al., 2002), ProT1 (Rentsch et al., 1996), CAT6 (Hammes et al., 2006), and CAT9 (Su et al., 2004) are expressed in the root vasculature and, therefore, may directly or indirectly contribute to delivering AAs to the root apex.

1.6.3. AA translocation from leaves to the seeds

At the post-fertilization stage in Arabidopsis, green carpel cells in the fruit develop distinctive features with well-defined stomata in the epidermal cells for gas exchange, and three layers of mesophyll tissue with photosynthetic capacity (Dinneny and Yanofsky, 2004). Due to the profusion of open stomata in the green fruits, the transpirational pull of xylem sap delivers organic or inorganic N directly from roots to the green carpel cells. Fruit carpel cells, therefore, may play

an important role in seed nutrition. However, as the stream of the xylem sap is towards the leaves due to the higher rate of transpiration in the leaves, N from the root is translocated predominantly to the leaves where it is temporarily stored or metabolized before being transported to the seeds (Tegeder and Rentsch, 2010). Furthermore, loading N-compounds into seeds is a spatially and temporally dynamic process (Patrick and Offler, 2001). In most species, including Arabidopsis, the accumulation of storage compounds occurs concomitantly with the acquisition of dormancy and desiccation tolerance (Goldberg et al., 1993). During this stage, recycled N in leaves derived from photorespiration and leaf senescence become the major source of N for the reproductive sink (Nooden et al., 1988). During seed maturation, up to 80% of seed N may come from leaves, especially from the senescing leaves (Yuan et al., 2005; Aerts and Chapin , 2000).

Loading AAs from leaf mesophyll cells into the phloem minor vein

The transport of AAs from leaves to seeds occurs via the phloem. Loading AAs from leaf mesophyll cells into the phloem minor vein may occur both symplastically and apoplastically. While symplastic loading via the plasmodesmata can be rate-limiting (Lalonde et al., 2003), it is improbable in some species due to the fact that solute concentration in sieve elements and companion cells in the phloem can be much greater than those in the surrounding source cells. This facilitates the hydrostatic pressure necessary in the phloem to drive long distance transport of solutes (Geiger et al., 1973; van Bel, 1993; Turgeon, 2000; Lalonde et al., 2003). Thus, in many species including Arabidopsis, loading assimilates into the phloem occurs apoplastically (see review Lalonde et al., 2003; Turgeon and Wolf, 2009). In the apoplastic loading, AAs are exported from mesophyll cells into the apoplasm, followed by active uptake into the sieve element-companion cell complex of the phloem (Delrot et al., 2001; Lalonde et al., 2003; Ortiz-Lopez et al., 2000; Williams and Miller, 2001; Winter et al., 1992). Based on expression analysis, Arabidopsis AAP5 (Brady et al., 2007; Fischer et al., 1995), CAT6 (Hammes et al., 2006), CAT9 (Su et al., 2004) and ProT1 (Grallath et al., 2005; Rentsch et al., 1996) have been speculated to be involved in loading AAs from the apoplasm into the phloem minor vein. AATs

with net export capacity in the mesophyll cell PM have not been identified to date (Figure 1.2).

Phloem-to-xylem transfer

Amino acids, loaded into the phloem from source tissues in the leaf, may undergo exchange between the phloem and xylem for upward translocation to the seed (Figure 1.2). The importance of this transfer process for N distribution within plants has been demonstrated in a number of physiological studies (Pate et al., 1975; Schobert and Komor, 1987; Atkins, 2000). In Arabidopsis, expression of the AAP2 (Hirner et al., 1998; Zhang et al., 2010) and AAP6 (Hunt et al., 2010; Okumoto et al., 2002) along the vascular transport strand in the stem indicated their involvement in active exchange of AAs between the xylem and phloem. Exchange between the phloem and xylem is essentially an exchange between the symplasm and apoplasm, and thus requires AATs in the PM of phloem companion cells with net export capacity. Hirner et al. (1998) demonstrated that the AAP2 is expressed in the phloem in the stem, and in funiculi in the silique. They proposed a role for the AAP2 in acquiring AAs assimilated in the green tissues in silique, in the retrieval of AAs passively leaking out of the phloem, and in the xylem-to-phloem transfer along the path. However, a recent study showed that the AAP2 is an import transporter and is involved in the phloem loading and delivering AAs to the seed (Zhang et al., 2010). Okumoto et al. (2002), through a promoter analysis, demonstrated that the AAP6 is expressed in the xylem parenchyma, and proposed a role in retrieving AAs from the xylem apoplasm. In heterologous systems, the AAP6 mediated a high affinity import of AAs (Okumoto et al., 2002). An *in planta* study showed that knocking out the AAP6 significantly reduces total AA concentration in the phloem compared to WT Arabidopsis (Hunt et al., 2010), suggesting that the AAP6 indirectly contributes to loading AAs into the phloem. Thus, an AAT in the phloem with a net export capacity that may mediate the exchange of AAs from the phloem to the xylem has yet to be identified.

Xylem to phloem transfer

Since AAs are delivered to the seed via the phloem, all AAs coming either from roots or leaves via the xylem must be loaded into the phloem prior to being delivered to the seeds. An increase in the AA concentration in the phloem towards the seed suggests a xylem-to-phloem transfer of AAs near the reproductive organ (Jeschke and Hartung, 2000).This process requires AATs in the seed-phloem with import capacity. Based on the expression pattern and mutant analysis, the AAP2 directly (Hirner et al., 1998; Zhang et al., 2010) and the AAP6 indirectly (Okumoto et al., 2002; Hunt et al., 2010) may contribute to this process of phloem-loading near the reproductive sink.

Loading into the seeds

In Arabidopsis, the phloem terminates at the funiculus, and outer integument cells in the seed work as a symplastic extension of the funicular phloem (Baud et al., 2008). However, transferring AAs from the outer integument to the inner integument, from the inner integument to the embryo, and from the embryo to the endosperm, may be apoplastic (Kim and Zambryski, 2005). The seed embryo is separated from the mother plant phloem by three apoplastic borders that require AATs with export and import capacity at each border (Baud et al., 2008) to transfer AAs from the funicular phloem into the embryo (Figure 1.2). While AATs with export capacity in the integument and endosperm remain unknown, the AAP8 plays a role in importing AAs into the endosperm and supplying the developing embryo with AAs during early embryogenesis (Schmidt et al., 2007). Using both knockout mutant and reduced transcript varients (using RNAi), Schmidt et al. (2007) demonstrated that the loss of the AAP8 reduces the average number of seeds and the number of normal seeds by ~50% with a noticeable feature of an arrested embryo at the globular stage. This seed phenotype was found correlated with a significantly reduced Asp and Glu concentration in the young siliques of the mutant (Schmidt et al., 2007). The Arabidopsis AAP1, a H^+/AA symporter with moderate or low affinity for neutral AAs and Glu (Boorer et al., 1996; Fischer et al., 2002), is expressed in the

developing embryo (Hirner et al., 1998; Sanders et al., 2009). Through a mutant analysis, Sanders et al. (2009) demonstrated that the AAP1 plays role in importing AAs into the embryo. In mature and desiccated seeds, the total N and C content was reduced in the mutant while the total free AA level was strongly increased. Analysis of AAs separately in embryos and seed coats/endosperm of mature seeds showed that knocking out the AAP1 caused AAs to accumulate in the seed coat/endosperm (Sanders et al., 2009). A promoter analysis showed that Arabidopsis CAT6 is expressed in the seed, suggesting a role in AA allocation (Hammes et al., 2006); however, the tissue-specific localization of CAT6 in the seed is less clear.

In Arabidopsis, the endosperm degenerates during early seed maturation and the embryo becomes the final sink of storage N (Hill et al., 2003), while in other species, the endosperm serves as the source of nutrients during seed germination. Regardless, the transfer of AAs from the endosperm into the embryo is important in terms of seed protein content and yield achievement. Thus, the identification of AATs with net export capacity in the inner and outer integument cells is important.

1.6.4. Intra-cellular AA transport

Chloroplast

During primary assimilation, NO_3^- taken up by plants is transported to the plastids or chloroplast where it is reduced to NH_4^+ and subsequently assimilated into Gln via Glu. Re-assimilation of recycled NH_4^+, derived from photorespiration or protein hydrolysis, also occurs in the chloroplast. During photorespiration in C_3 plants, the 2-carbon compound produced through the oxygenase activity of Rubisco needs to be converted to a 3-carbon compound so that the photosynthetically fixed carbon can be rescued and fed back into the Calvin cycle (Figure 1.3). This occurs through a complex series of biochemical reactions taking place in the chloroplast, peroxisome, and mitochondria. The 2-carbon compound (2-phosphoglycolate) finally leads to the formation of Gly and a 3-carbon compound (3-phosphoglycerate) (see review Maurino and Peterhansel,

2010; Peterhansel and Maurino, 2011). During this process, both N and C are released in the form of NH_3 and CO_2 in the mitochondria. This photorespiratory NH_3 is recaptured by the GS2/Fdx-GOGAT pathway in the chloroplast. In a mature leaf, the amount of photorespiratory N may exceed the amount of N fixed through primary N-assimilation by up to 10-fold (Keys et al., 1978). As well, storage plastids in non-photosynthetic tissues convert photosynthates (derived from source tissues) into storage compounds for use during development. Thus, chloroplasts/plastids are vital organelles for N assimilation, storage, and remobilization (see review Lopez-Juez and Pyke, 2005; Tetlow et al., 2005; Pudelski et al., 2010).

Nitrogen, stored in the form of storage proteins, peptides, or AAs are mobilized primarily in the form of AA. Most AAs required for protein synthesis are produced in the chloroplast/plastid. Plants follow a basipetal growth pattern, where developing tissues depend on mature tissues for the supply of AAs for protein synthesis. Therefore, AAs, synthesized in the chloroplast/plastid in mature cells, are subject to both intra-cellular and long-distance transport. AAs biosynthesized in the chloroplast/plastid cannot cross the inner and outer membrane without a membrane transporter or a channel protein. Plants thus need both AA export- and import-systems in the inner and outer membranes of chloroplast. In the outer membrane of the chloroplast, a low affinity channel, Outer Envelope Protein 16 (OEP16), facilitates AA transport. In Arabidopsis, several OEP16-family genes have been identified (see review Philippar and Soll, 2007; Duy et al., 2007; Pudelski et al., 2010) that may mediate AA transport through the outer membrane of the chloroplast/plastid. Micro-array analysis and *in silico* sub-cellular localization analysis have identified putative AATs that may be localized in the inner chloroplast membrane (http://rarge.psc.riken.jp/chloroplast/; Abraham and Ohlrogge, 2002). However, experimental evidence for the role of these inner membrane AATs is inadequate, except for the identification of a Glutamate/Malate antiporter (DiT2 coupled with DiT1) in the inner chloroplast membrane that mediates the Glu export from the stroma in exchange for malate (Figure 1.3) (Linka and Weber 2005; Philippar and

Soll 2007; Weber and Fischer 2007). Unidirectional AATs are required in the inner chloroplast membrane to achieve a net export of AAs from chloroplast to the site of protein synthesis or to the developing sink tissues.

Mitochondria

Plant mitochondria are also important in the intra-cellular N-metabolism including the synthesis and catabolism of AAs (reviewed in Mackenzie and McIntosh 1999; Bowsher and Tobin 2001). Mitochondria, together with chloroplasts and peroxisomes, manage both photorespiration and photosynthesis along with many other metabolic pathways (Raghavendra and Padmasree 2003). Mitochondria also play an important role in the remobilization of storage N during seed germination (Picault et al., 2004). Identification of transporter proteins involved in the transport of N compounds in the inner and outer mitochondrial membrane may thus contribute to a better understanding of the role of mitochondria in N metabolism and distribution.

Millar and Heazlewood (2003) reported 45 genes encoding mitochondrial carrier proteins in Arabidopsis. A comprehensive *in silico* screen allowed the identification of 13 additional members in this family of membrane transporter protein in Arabidopsis (Picault et al., 2004). These structurally related proteins are involved in the transport of solutes like nucleotides, phosphate, and di- and tricarboxylates across the mitochondrial membrane (HaferKamp, 2007). How many of these transporters are directly or indirectly associated with the transport of N-compounds has yet to be determined. In Arabidopsis, 17 genes have been speculated to encode transporters involved in AA transport through the inner and outer envelope of mitochondria (Murcha et al., 2007).

The outer membrane of mitochondria is permeable to solutes up to a size of 4-5 kDa through porins (Benz 1994). The mitochondrial porins in the outer membrane, also called VDAC (Voltage Dependent Anion Channels), were characterized as relatively non-specific general diffusion pores that may mediate AA transport through the outer envelope (see review Mannella, 1997). It was primarily assumed that porins normally remain in the open state; however, in recent years evidence has emerged that porins are normally closed and highly

regulated by metabolites, substrates, nucleotides, and modulator proteins located in the inter-membrane space (see overview and references therein, Liu and Colombini, 1992; Lee et al., 1994). A porin, specific for AAs or peptides, in the outer envelope of mitochondria has yet to be identified in plants. In the inner mitochondrial membrane, two basic AA carriers, BAC1 and BAC2, have been experimentally shown to be involved in AA-transport (Catoni et al., 2003; Hoyos et al., 2003). These carrier proteins mediate Arginine/Ornithine (or Citrulline) antiports in the inner mitochondrial membrane (Figure 1.3). In order to exchange Gly and Ser with the peroxysome during photorespiration, there might be an exchanger or a Gly/Ser antiporter in the inner mitochondrial membrane that is yet to be identified. A recent report has shown that the GABP (also known as BAT1) in Arabidopsis is localized in the mitochondrial membrane (Michaeli et al., 2011) and mediates import of certain AAs while exports other AAs (Dundar and Bush, 2009), suggesting that it might be a bi-directional facilitator. Transporters in the inner membrane involved in net AA import and export have yet to be identified.

Tonoplast

Temporary storage of AAs in the vacuole and their subsequent release into the cytoplasm requires AATs in the tonoplast membrane with export and import capacity. So far, the CAT2 in Arabidopsis is the only known AAT in the tonoplast membrane (Su et al., 2004), where it might be involved in loading AAs from the cytoplasm into a vacuole or unloading AAs from a vacuole into the cytoplasm, or both. The direction of transport that the CAT2 may mediate in the tonoplast membrane needs to be clarified. Arabidopsis PTR2, PTR4, and PTR6, members of the PTR/NRT1 family, were shown to be localized in the tonoplast membrane (Weichert et al., 2011). While the PTR2 mediated transport of di- and tri-peptides in experimental conditions (Song et al., 1996), heterologous expression analysis of the PTR4 and PTR6 did not demonstrate any peptide-transport activity in yeast or in *Xenopus* oocytes (Weichert et al., 2011).

1.7. Goals of this thesis

The prevailing AA transport system in plants requires both cellular import and export of AAs, whereas most of the known plant-AATs were thought to be H^+/AA symporters. While a large number of the annotated AATs in plants remain to be characterized, many of the characterized AATs were studied in heterologous unicellular systems such as mutant strains of *Saccharomyces cerevisiae* or oocytes of *Xenopus laevis* (see list in Chapter 5, Table 5.1; Table 5.2). The *in planta* organ and tissue specific expression analysis of an AAT, together with its role in the heterologous system, is important in understanding its biological importance in the plant. However, the content and composition of AAs in and out of a plant cell in a physiological environment may differ from the experimental conditions provided in heterologous uptake studies. As the substrate specificity or affinity and the transport direction of an AAT in a membrane may vary with the change in the relative abundance or concentration gradient of the substrate(s), the role of an AAT at a physiological condition can be different than the role it plays in a heterologous system. Some AATs may require additional components for the tertiary folding and localization and binding partners for mediating the AA-transport activity in the membrane, which may not be available in a heterologous system. While AA exporters, exchangers, or antiporters in the AA translocation route in other species have been well studied, these transporters remain poorly studied in plants. *In planta* characterization of AATs using knockout mutants and over-expressing lines can be more useful in revealing the biological importance of such AATs. This research utilized both heterologous systems and *in planta* mutant analysis to investigate the role of LATs in plants and allowed the development of specific hypothesis about their functional role in Arabidopsis. The specific objectives of this research were to:

- Identify the expression level and pattern and sub-cellular localization of three LAT transporters (LAT1, LAT4, and LAT5) through organ-specific transcript level and promoter: reporter analysis, and through investigating the intra-cellular localization using an epitope tag in the C-terminus.

- Investigate the AA transport activity and direction (exporter/importer/bi-directional) of these LATs by expressing them in a unicellular heterologous system and by *in planta* radio-labeled AA uptake studies.

- Characterize the biological importance of the Arabidopsis LATs through analyzing T-DNA single knockout mutants and over-expressing lines at phenotypic and metabolite level under various growth conditions.

- Investigate the potential role of LATs in translocating AAs from source to sink tissues by analyzing the change in the free AA content and total N content in the reproductive sink in the T-DNA single knockout mutant and over-expressing lines.

Table 1.1. List of transporter classes with the various categories within each TC.

Transporter Class (TC)	**Transporter Type**	**Categories within a TC**
1.	Channel proteins	1. A. α-Type channels 1. B. β-Barrel porins 1. C. Pore-forming toxins 1. D. Non-ribosomally synthesized channels 1. E. Holins 1. F. Vesicle fusion pores 1. G. Viral fusion pores 1. H. Paracellular channels
2.	Electrochemical potential driven porters	2. A. Porters (uniporters, symporters, and antiporters) 2.B. Non-ribosomally synthesized porters 2.C. Ion gradient-driven energizers
3.	Primary active transporters	3. A. Di-phosphate bond hydrolysis-driven transporters 3. B. Decarboxylation-driven transporters 3. C. Methyl transfer-driven transporters 3. D. Oxido-reduction-driven transporters 3. E. Light absorption-driven transporters
4.	Group translocators	
5.	Transport electron carriers	5.A. Transmembrane 2-electron transfer carriers 5.B. Transmembrane 1-electron transfer carriers
6.	No assignment	
7.	No assignment	
8.	Accessory factors involved in transport	8. A. Auxiliary transport proteins 8. B. Ribosomally synthesized protein/peptide toxins that target channels and carriers 8. C. Non-ribosomally synthesized toxins that target channels and carriers
9.	Incompletely characterized transport system	9. A. Transporters of unknown biochemical mechanism 9. B. Putative but uncharacterized transport proteins 9. C. Functionally characterized transport proteins with unidentified sequences

(This system of classification was approved by the transporter nomenclature panel of the International Union of Biochemistry and Molecular Biology in Geneva, 28-30 November 1999 (Saier, 2000)).

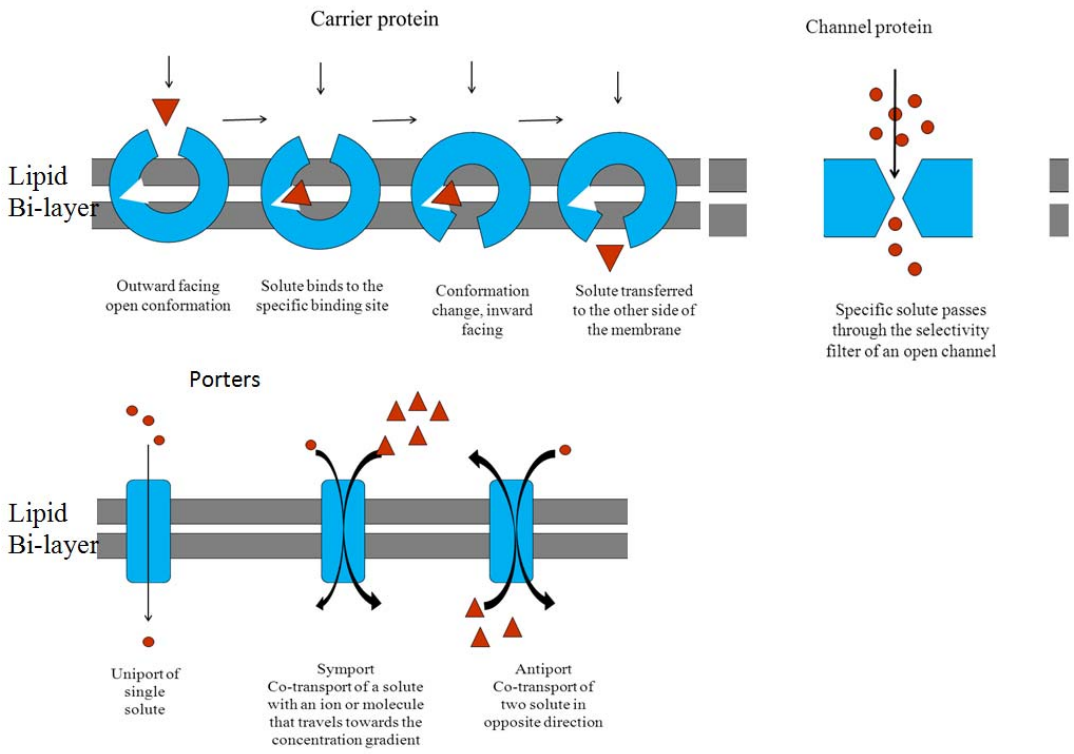

Figure1.1. A cartoon presentation of various types of membrane transporter proteins.

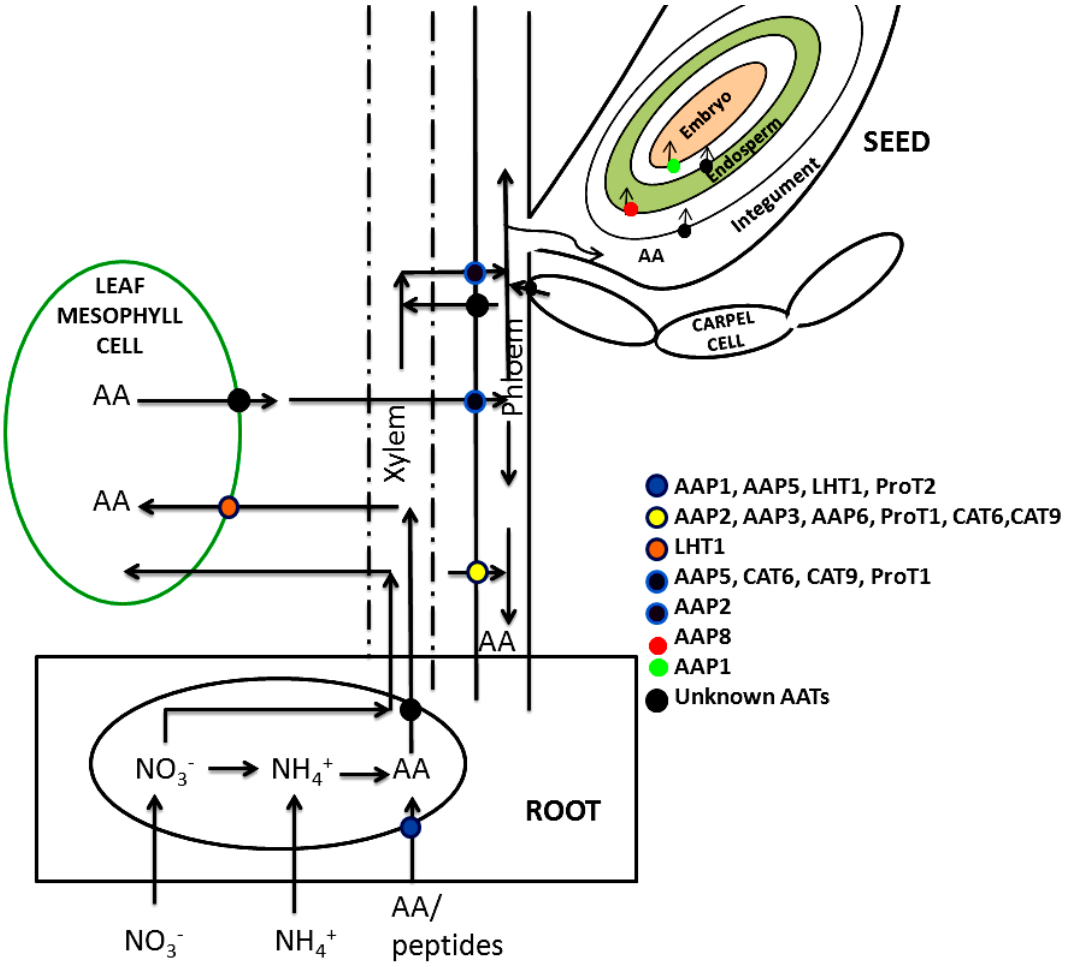

Figure 1.2. A simplified model of AA-transport in Arabidopsis shows positions of organ- and tissue-specific known and unknown AATs involved in the route. Dark filled circles represent positions where AATs are yet to be identified. This figure represents the plasma membrane crossing between symplasm and apoplasm for AA translocation from a root cell to the seed embryo (see Figure 1.3 for organeller membrane crossing). Arrows indicate the direction and movement of AAs. Transporters for the other forms of N have not been specified in this figure.

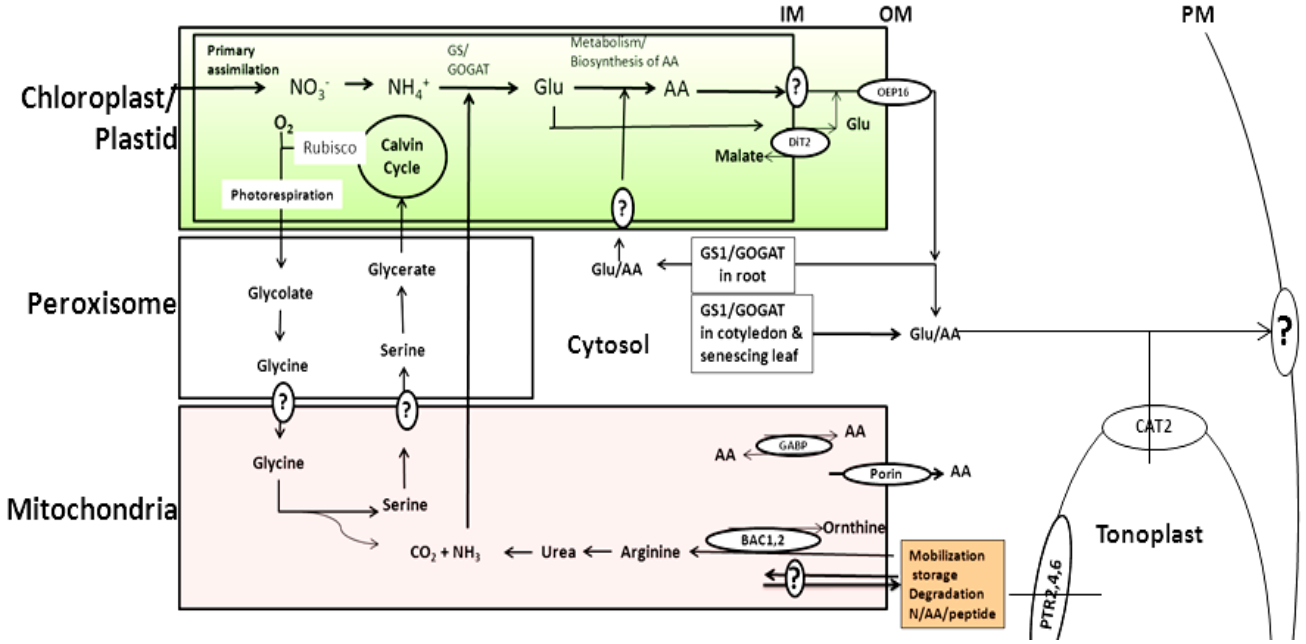

Figure 1.3. A simplified model of the intra-cellular AA transport shows movement of AAs in and out of membrane-bound organelles in a plant cell. Based on the prevailing concept of intra-cellular AA-movement, AATs yet to be identified have been indicated with a "?" mark within a circle. All known AATs in Arabidopsis, to date, have been specified in the membrane of each organelle. To keep it simple, N metabolism in chloroplast and plastid has been shown together in the same organelle. IM, inner membrane; OM, outer membrane; PM, plasma membrane

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2. Functional characterization of the L-type Amino acid Transporter 4 (LAT4) in *Arabidopsis thaliana*

2.1. Introduction

In plants, inorganic nitrogen (N) taken up in the form of NO_3^- and NH_4^+ is primarily assimilated into the amino acid (AA) glutamine (Gln), which then functions as a source of the amine group in the biosynthesis of various other AAs (Coruzzi and Last, 2000). The primary assimilation of inorganic N takes place via the conjugated function of Glutamine Synthetase (GS) and Glutamate Synthase (GOGAT) using glutamate (Glu) as the substrate (Lea and Miflin, 1974; Miflin and Lea, 1980; Stewart et al., 1980). Assimilation of N requires cofactors, reducing equivalents, and carbon (C) skeletons generated during photosynthesis. Thus, although N-acquisition occurs through the roots, primary N-assimilation occurs in both roots and shoots and both of these organs play a role as a primary source of N (Andrews, 1986; Andrews et al., 1992; Kruse et al., 2002; Scheurwater et al., 2002).

Inorganic N, assimilated into AAs in the roots, is translocated to the growing shoots via the xylem. AAs, translocated from the roots towards the shoots, are primarily accepted by the photosynthetic tissues in the leaves due to the high rate of transpiration in the leaves. As well, photosynthetic tissues in the leaves are the sites where photorespiratory N is largely recaptured. In a mature leaf, the amount of photorespiratory N may exceed the amount of N fixed through primary assimilation by up to 10-fold (Keys et al., 1978). Nitrogen, thus acquired in the leaves, is temporarily stored or metabolized in the leaves and exported in the form of AAs to the sink tissues.

Export of AAs from the leaf tissues can occur symplastically or apoplastically. In the symplastic movement, AAs from the leaf mesophyll cells move through the plasmodesmata along the concentration gradient to the adjacent sink tissues or to the distant sinks via the phloem. While symplastic loading of AAs into the phloem via the plasmodesmata can be a rate-limiting process

(Lalonde et al., 2003), in some species the solute concentration in the phloem sieve elements and companion cells are increased compared to the surrounding source cells to achieve the hydrostatic pressure necessary for the long distance transport of solutes (Geiger et al., 1973; van Bel, 1993; Turgeon, 2000; Lalonde et al., 2003). In such cases, symplastic phloem loading against the concentration gradient is unlikely to occur. In many species, including Arabidopsis, the loading of assimilates into the phloem occurs apoplastically (reviewed in Lalonde et al., 2003; Turgeon and Wolf, 2009). During apoplastic loading, AAs are exported from mesophyll cells into the apoplasm, followed by an active uptake into the sieve element-companion cell complex of the phloem (Delrot et al., 2001; Lalonde et al., 2003; Ortiz-Lopez et al., 2000; Williams and Miller, 2001). The export of AAs from the mesophyll cells via the phloem thus requires amino acid transporters (AATs) in the plasma membrane (PM) of mesophyll cells with a net export capacity, and AATs in the PM of companion cells in the phloem minor vein with a net import capacity. Based on expression analysis, it is speculated that the Arabidopsis AAP5 (Brady et al., 2007; Fischer et al., 1995), CAT6 (Hammes et al., 2006), and ProT1 (Grallath et al., 2005; Rentsch et al., 1996) are involved in loading AAs from the apoplasm into the phloem minor vein, while AA-exporters in the leaf mesophyll cells remains unknown. The Arabidopsis GABP (also known as BAT1) that mediates bi-directional AA-transport activity in heterologous systems (Dundar and Bush, 2009) is localized in the mitochondrial membrane (Michaeli et al., 2011). Over expression of a plant-specific type I membrane protein, Glutamine Dumper1 (GDU1), in Arabidopsis, induced secretion of Gln from the leaf hydathodes (modified stomata that mediates passive exudation of surplus water) and increased the AA-content in the leaf apoplasm and xylem sap (Pilot et al., 2004). Over-expression of the GDU1 also mediated increased tolerance to exogenous AAs and reduced net uptake of AAs in seedlings, suggesting that the GDU1 may have AA export capacity (Pratelli et al., 2009). While the subcellular localization of the GDU1 is yet to be resolved, a promoter analysis showed *GDU1* expression in the vascular tissues throughout the plant (Pilot et al., 2004; Pratelli et al., 2009), where it may play a role in

unloading AAs from the phloem symplasm into the xylem apoplasm during phloem to xylem exchange of AAs. However, AATs with export capacity in the source tissues, such as leaf mesophyll cells or in any other plant tissues, have yet to be identified (Lalonde et al., 2003; Okumoto and Pilot, 2011).

Arabidopsis contains 67 putative AAT-encoding genes, many of which are yet to be characterized (Rentsch et al., 2007). The L-type Amino acid Transporter (LAT) family (TC2.A.3.12), containing five members in Arabidopsis, is the second largest family after the Cationic Amino acid Transporter (CAT) family (nine members) in the APC superfamily. In animals, LATs function as exchangers of intra-cellular AAs for extra-cellular AAs in association with a glycoprotein (Mastroberardino et al., 1998; Pineda et al., 1999; Rossier et al., 1999; Meier et al., 2002) while LATs in plants remain to be characterized. In this research, LAT4 (At1g31830) was functionally analyzed. *In silico*, heterologous, and *in planta* studies were performed to determine the biological importance and transport direction of the LAT4. From the studies conducted in this chapter, it was hypothesized that the LAT4 plays a role in mobilizing AAs from source tissues. Based on experimental evidences, the role of LAT4 in Arabidopsis is discussed in this chapter.

2.2. Materials and Methods

2.2.1. Computer-aided analysis

The transmembrane domain prediction for the LAT4 was performed using the TMHMM v.2.0 online prediction tool (http://www.cbs.dtu.dk/services/TMHMM/). The cartoon representation was produced using TMRPres2D (http://bioinformatics.biol.uoa-gRTMRPres2D). The AA sequence alignment of the LAT4, with *Homo sapiens* LATs (*Hs*LAT1 and *Hs*LAT2), was performed using the ClustalW2 multiple alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The protein sequences of *Hs*LATs were taken from the NCBI protein database. A conserved domain search was performed using the NCBI online conserved domain search tool (http://www.ncbi.nlm.nih.gov/). The amino acid sequence of the LAT4 was taken

from the plant membrane protein database (http://aramemnon.botanik.uni-koeln.de/).

For the phylogenetic tree, DNA sequences of the Arabidopsis AATs were taken from the plant membrane protein database. Sequences were aligned using the CLUSTALW online sequence alignment tool. Only the well-characterized AATs were analyzed along with the five functionally unknown LATs, to determine the relationship between the LATs and other known AATs in *Arabidopsis thaliana*. Maximum parsimony analysis was performed using PAUP 4.0b10 with all DNA characters unweighted. Gaps were scored as missing characters. The heuristic tree search was executed using the default program settings of the Tree-bisection-reconnection branch-swapping algorithm and simple addition of the sequence.

2.2.2. Construction of vectors for yeast complementation, promoter analysis and sub-cellular localization study

The expression vector containing the Myc epitope in the C-terminus of the *LAT4* was constructed using the following steps. The *LAT4* ORF (open reading frame) was amplified through a one-step RT-PCR using *SuperScript III* One-Step RT-PCR with a Platinum *Taq* High Fidelity (Invitrogen) kit and *DNAse*-treated total RNA extracted from multiple seedlings of WT as template. Appropriately designed forward and reverse primers (forward primer 5'-AATCTGGATGACGTTTCTAGAATGCA GAAGCG-3' and reverse primer 5'-AATAACTAAGGACATTCGCTAGCACGTATTAGA-3') were used to add an *XbaI* site prior to the start codon, and an *NheI* site to replace the stop codon. The PCR product was run on an agarose gel to check the amplicon size and then was phenol:chloroform purified. The purified and digested PCR product was sub-cloned into *XbaI/NheI*-digested pRTL2/Myc vector (Shokey et al., 2006) to obtain the pRTL2/LAT4-Myc expression vector. The final expression vector with the *LAT4-Myc* reading frame driven by the Cauliflower Mosaic Virus 35S promoter (CaMV-35S) was confirmed through PCR, restriction analysis, and sequencing. For yeast complementation studies, the *LAT4* ORF was amplified through a one-

step RT-PCR as mentioned above. Forward (5'-GAATTCCCGTTTAAAATCTGGTTGACG-3') and reverse (5'-GTCGACGACGAGGGAAATAACTAAG-3') primers with added *EcoRI* and *SalI* sites respectively in the 5' end were used for PCR. The purified and digested PCR product was cloned into the yeast expression vector pDR196 (Rentsch et al., 1995) between *EcoRI* and *SalI* in the multiple cloning site (MCS). The final expression vector was confirmed through PCR, restriction digestion and sequencing.

For the promoter analysis, the online plant promoter prediction tool (http://www.softberry.com/berry/tssp) was used to predict the promoter region. The promoter region (up to 1316 bp upstream from the start codon) was PCR amplified using *SalI* and *NcoI* sites added to the 5'-end of the forward (5'-GTCGACAGGGACCCATGAATTCAACA-3') and reverse (5'-AACCATGGATGATTCTCCGCTTCTGCAT-3') primers respectively, and cloned into the pCAMBIA1305.1 vector (http://www.cambia.org/) between the *SalI* and *NcoI* site, replacing the CaMV35S promoter preceding the *GusPlus* coding region. The final expression vector with P_{LAT4}:*GusPlus* was confirmed through PCR and restriction analysis.

2.2.3. Quantitative RT-PCR for organ specific expression analysis

For the organ specific qRT-PCR, RNA was extracted from roots, stems, rosette leaves, cauline leaves, senescing leaves, flowers, siliques, and whole plants separately. Total RNA was extracted, using the RNeasy Plant Mini Kit (Qiagen, Darmstadt, Germany), from approximately 100mg (fresh weight) of flash frozen tissues of each organ. To remove contaminating DNA, RNA was treated using Ambion's DNA-*free* kit. The quantity and quality of RNA was checked by using a Nanodrop and Bioanalyzer (Agilent 2100 Bioanalyzer). All samples were adjusted to a final concentration of $50ng.\mu l^{-1}$. For each sample, 2.5 µg of total RNA was used in the cDNA synthesis with oligo-dT and random primers (Invitrogen). SuperScript II Reverse Transcriptase (Invitrogen) was used in the reverse transcription for 50 minutes at $42^{o}C$. Synthesized cDNA was used

to amplify *Elongation Factor 1α (EF1α)* to be used as an endogenous control. A TaqMan Gene Expression Assay (assay ID: At02351062_sH for *LAT4*; assay ID: At02337969_g1 for *EF1α*) was used in the PCR amplification. The PCR program was as follows: 95^{o}C for 10 minutes followed by 40 cycles of 95^{o}C for 15 seconds and 60^{o}C for one minute. The relative quantitation was calculated using a 7500 Fast System SDS software (Applied Biosystems). The average expression level in the whole plant was used as a calibrator during analysis. The final graph was produced using RQ (Relative Quantitation) as an expression level and the mean difference between RQ_{max} and RQ_{min} as an error bar.

2.2.4. GUS Histochemical analysis for organ and tissue specific expression

The GUS staining was performed using a modified protocol described by Geigel and Glazebrook (2002). Samples were prepared by incubating seedlings (three weeks old) or mature plants in a GUS staining buffer (50mM Na_2HPO_4, 2mM Potassium ferricyanide, 2mM Potassium ferrocyanide, 2mM X-Gluc and 0.2% TritonX-100) for 30 minutes to six hrs at 37^{o}C followed by 30 minutes of incubation in 70% ethanol at RT. Samples were fixed in FAA (50% ethanol, 10% Glacial acetic acid, 5% formaldehyde) overnight and stored in 70% ethanol at 4^{o}C until photographs were taken under a dissecting microscope. For the tissue-specific expression analysis, tissues were processed and embedded in wax. Thin cross sections (8µm) were produced using a microtome. Tissue sections were fixed on a glass slide by drying them at 39^{o}C for two days. Samples were de-waxed with toluene and counter-stained with safranin O prior to taking photographs under a light microscope.

2.2.5. Subcellular localization

The transient expression of the C-Myc epitope tagged LAT4 in tobacco bright yellow-2 (BY-2) suspension cells and fluorescence microscopy was done as described in Gidda et al. (2009) in the Mullen lab at the University of Guelph. In the pRTL2-*LAT4*-Myc expression vector, *LAT4* was driven by the constitutive CaMV35S promoter. The transient transformation of BY-2 cells with the pRTL2/*LAT4*-Myc vector was carried out using biolistic bombardment. Six hrs

after bombardment, cells were fixed with formaldehyde, permeabilized with pectolyase and Triton X-100, and then processed for immuno-fluorescence staining by incubating with a mouse anti-Myc antibody and Alexa 488-conjugated goat anti-mouse antibody (excitation 495nm, emission 519nm). Cells were also incubated with Alexa 594-conjugated ConcanavalinA (excitation 590nm, emission 617nm) as a marker stain for the endogenous endoplasmic reticulum (ER).

2.2.6. Isolation of the *lat4-1* knockout mutant

The homozygous T-DNA insertion line (Salk_119707), which has an insertion in the *LAT4,* was obtained from the SALK collections (Alonso et al., 2003) of the Arabidopsis Biological Resource Centre. The T-DNA insertion in the exon of *LAT4* was confirmed through the PCR screening scheme as described in the Salk webpage (http://signal.salk.edu/). One primer specific to the left border sequence of the T-DNA, LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3'), and two primers specific to the *LAT4* sequence (reverse primer 5'-CATCATCACAATGCTCACTGC-3' and forward primer 5'-ATGTTGTGTGGGTTTCCTCTG-3') were used in the PCR screen. The position of the T-DNA between the 1183rd and 1184th bp in the *LAT4* cDNA was confirmed through aligning the gene-specific primers designed by the SIGNAL iSECT tool (http://signal.salk.edu/), and sequences produced using the T-DNA left border specific primer. The sequence alignment was performed using the ClustalW2 online tool (http://www.ebi.ac.uk/). Reverse Transcriptase (RT)-PCR, using 5' and 3'-UTR-specific primers (forward 5'-CCGTTTAAAATCTGGTT GACG-3'and reverse 5'- GACGAGGGAAATAACTAAG-3'), confirmed the absence of the *LAT4* transcript in the Salk line.

2.2.7. Analysis of free AAs through HPLC

For the HPLC analysis of free AAs, samples were prepared as follows. Samples were ground in liquid N. For dry weight, tissue samples were lyophilized for 72 hrs before weighing. One and a half ml extraction buffer (methanol: chloroform: water at 65:25:10 with 20nmol.ml^{-1} internal standard (IS) (Norvaline

and Sarcosine) were added to each sample. In cases when tissue amounts were small, extraction buffer was added during the grinding of freshly weighed samples. Samples were mixed by vortexing and centrifuged to separate the aqueous phase. Five hundred microliter of the supernatant was collected and 100µl chloroform followed by 150µl water were added to the supernatant. Samples were mixed and centrifuged. The upper aqueous phase (400 µl) was passed through a MILLEX GX 0.22µm filter (UFC30GV0S) followed by a MILLIPORE BIOMAX 5KNMWL MEMBRANE filter (UFV5BCC00). The final filtrate was used in the HPLC analysis using a ZORBAX Rapid Resolution HT Eclipse Plus C18 1.8µm 3.0x100mm column. During extraction, all samples were treated equally; volume of reagents added and volume of supernatant harvested were kept constant for all samples to ensure equal extraction efficiency. An Agilent Technologies 1200 series automated liquid chromatography system was used to analyze the samples. Samples were derivatized pre-column with OPA (o-Phthalaldehyde) and FMOC (9-Fluorenylmethyl chloroformate). All chromatographs were processed in the Agilent Chemstation software suite. The area under the signal peaks were calibrated by the standard calibration curve and normalized by the internal standard. The concentration of Val was ignored in the data interpretation due to the possibility that it was sharing the same retention time with another unknown AA and thus might be giving an overestimation of the concentration.

2.2.8. *In planta* ^{14}C- and ^{3}H-Leu uptake study

The ^{14}C- and ^{3}H-Leucine (Leu) uptake study was carried out using homozygous *lat4-1* seedlings with wild type (WT) as control. Seedlings grown in a potting mixture, or on plates (on 0.5xMS medium containing 5mM NO_3^-) were used in the uptake studies. For soil grown, seedlings were removed from the soil and rinsed prior to using in the study. For plate grown, seedlings grown on 100mm X 100mm square plates in a vertical orientation were transferred to liquid MS and acclimatized overnight at RT. Seedlings were then transferred to a starvation medium (0.5xMS without N) and grown in the dark overnight. In all studies (unless otherwise mentioned), ^{14}C-Leu (concentration 0.1mCi/ml stock;

specific activity ~320mCi/mmol; label in the α-carbon) and/or ^{3}H-Leu (concentration 1mCi/ml; specific activity 54.1Ci/mmol; label in the amine-group, procured from Perkin Elmer) was added to the uptake medium (liquid MS without N and C, pH 5.4) with cold Leu to a final concentration of 10µM. Radio-labeled Leu stock was added to the uptake medium at the concentration of 2µl.ml^{-1}. The final concentration was adjusted to 10µM through adding cold Leu. For all studies, only roots were dipped in the uptake, rinsing, and export medium. Thus the radio activity detected in the shoots was a result of the net uptake through the roots. Seedlings were removed from the uptake medium after the respective time point and rinsed three times in sterile water for a total of 10 minutes. Roots and shoots were separated and stored directly in scintillation vials. Shoot tissues were dried at 70^{o}C for two hrs and weighed. Samples were digested with bleach and prepared with HIONIC FLUOR (Perkin Elmer) for counting radioactivity in DPM (Disintegrations Per Minute) in a liquid scintillation counter (Beckman). The data was presented as pmol.mg^{-1}DW.hr^{-1}. The accumulation of radionuclide in the plant tissues detected in DPM was converted to concentration (in pmol) using the following formula: 1pCi / 2.2 DPM= 1pmol of AA/specific activity of the stock (in pCi). For the ^{14}C-Leu uptake study with plate-grown mature plants, *lat4-1* homozygous mutant and WT plants were grown on MS medium (0.5xMS with 5mM NO_3^-, 1% sucrose, 1% agar, pH 5.7) on 245mm x 245mm plates under a regime of 20 hrs of light and four hrs of darkness to induce early flowering. At the flowering stage (34 days old), ^{14}C-Leu, dissolved in 10ml sterile water to a final concentration of 1µM, was added only to the root zone with a micropipette to ensure equal distribution of ^{14}C-Leu for each plant. Plates were transferred to 16/8 hrs of light/dark regime. Plants were grown for eight days after the application of ^{14}C-Leu. This study was designed to test the relative abundance (if any) of the ^{14}C- in various organs. Eight days of growth after the application of the ^{14}C-Leu was considered long enough for the ^{14}C-Leu to be metabolized. This study would allow us to comapare the variation in the long term accumulation of the ^{14}C- between WT and mutant. Plants were removed from the plate and the organs were separated. Samples were separated as leaf, inflorescence (the rest of the plant

including flower and silique except leaves and roots), and root. The root was transferred directly to the scintillation vial without rinsing. All samples were dried and prepared for counting as described in the previous study. The remaining radioactivity in the growth medium was measured and almost nothing was detected.

For the export study, plate grown or soil grown seedlings, starved overnight for light and N, were fed with ^{14}C-Leu for one hr. Feeding seedlings with ^{14}C-Leu was carried out as described above. Both uptake and export were carried out at RT. Seedlings' roots were removed from the uptake medium and rinsed with sterile water for five minutes before being dipped into the export medium. Half-strength liquid MS without N and C was used as an export medium. At the respective time point, 50µl of the export solution was transferred to a glass scintillation vial to measure the radioactivity released by the seedlings.

For the thin layer chromatography (TLC), AAs were extracted from seedlings after four hrs of ^{14}C-Leu uptake. Soil-grown one-month-old seedlings, starved overnight for N and light, were used in the uptake study. AAs (crude) were extracted from leaves immediately after the uptake using methanol:chloroform extraction buffer. Seedlings, not used in ^{14}C-Leu uptake, were used as extraction control (EC) with the ^{14}C-Leu added during grinding to see the nonmetabolic breakdown of Leu (if any) during extraction and handling. Samples, dried in a speed-vac and re-suspended in 70% ethanol, were loaded on a silica gel plate and ran for two hrs in a closed glass jar using Butanol:Acetic acid:water (3:1:1) as a running buffer. The TLC plate was air dried and sprayed with 0.3% ninhydrine in ethanol and baked for 20 minutes at 120^{o}C. ^{14}C-Leu and ^{14}C-Glu were loaded as control. The TLC plate was exposed to a phosphorus storage screen for two weeks at -80^{o}C before scanning the screen using an FLA-5000 image reader.

2.2.9. Protoplast study

To prepare the leaf protoplasts, leaves of a similar size and age from plants that were two months old and grown under an 8/16 hrs of light/dark regime were

used. Plants were kept in the dark for 24 hrs before rosette leaves were collected. Leaf digestion was also carried out in the dark. Protoplast preparation was performed using the protocol described in Yoo et al. (2007). For the size analysis, photos of protoplasts were taken using a ZEISS Scope A1 light microscope attached to an optronics camera. All photos were taken under the 20x objective lens. For each genotype, 100 cells were counted from five photographs from three individual plants. The cell size was measured using a PictureFrame (TM) Software Application 2.3. The software program was calibrated using a stage micrometer under the 20x objective lens prior to measuring the diameter of the cells.

For the ^{14}C-Leu uptake study, cells of each genotype were counted with a haemocytometer and the final concentration was adjusted to $4x10^5$cells.ml^{-1}. The uptake medium was an MMG (4mM Mes, 0.4M Mannitol and 15mM $MgCl_2$, pH 5.6) solution with added ^{14}C-Leu and cold Leu to a final concentration of 10μM. The same stock of the 14C-Leu as described above was used in this study. After one hr of uptake under light, cells were rinsed three times with the MMG solution before radioactivity was counted in $4x10^5$ cells per replication vial. For all studies, the seeds of both genotypes were planted at the same time on the same flat with the exact same treatment of soil, water, fertilizer, and other growth parameters. The conversion of DPM into pmol was carried out using similar calculation described above (section 2.2.8.). Plant growth, transformation, and analysis

2.2.10. Plant growth, transformation and analysis

The Columbia-0 ecotype of *Arabidopsis thaliana* was used in all studies. All plants (unless otherwise mentioned) were grown on well-aerated fertilized soil (peat-based with dolomite limestone and organic fertilizer, sunshine mix) in the growth chamber under a regime of 16/8 hrs of light (~120 μE m^{-2} sec^{-1} light intensity) and dark at 23^{o}C and 60% relative humidity. The 'Plant Prod 20-20-20' all purpose fertilizer containing 20% N was applied to soil during the preparation of pots.The transformation for producing a transgenic Arabidopsis line with P_{LAT4}: *GUS* was carried out as per the modified floral dip transformation protocol

described by Clough and Bent (1998). Transformants were screened on a 0.5x MS medium containing 25µg.ml^{-1} hygromycin and confirmed by PCR using *GUS* specific primers (forward primer 5'-ccgtcccaagcagttacaat-3' and reverse primer 5-gttcagtggttcccagagga-3'). For phenotypic studies, seedlings were grown on a half or full strength MS medium as specified for each experiment (1% agar, pH 5.7) with variable sources and concentrations of C, N, or other abiotic agents as described (eg. Mannitol for osmotic stress). For all phenotypic studies, both WT and mutant were grown on the same plate or flat (soil grown) and provided the same treatment with three to five plate/pot replications. All data analysis and significance tests (two-way T-tests) were performed using PRISM (unless otherwise mentioned).

2.2.11. Heterologous expression of the LAT4 in yeast

The yeast strain, 22Δ8AA (Fischer et al., 2002), was transformed with three independent cDNA clones of the *LAT4* in the expression vector pDR196 (Rentsch et al., 1995). The 22Δ8AA yeast cells were also transformed with a cDNA clone of the *AAP6* in pDR195 (Rentsch et al., 1995) and an empty pDR196 to be used as positive and negative controls respectively. The transformation was carried out using a Yeast Transformation Kit (Sigma) as per manufacturer's instructions. Transformants were selected on a synthetic complete (drop out) medium-lacking uracil and confirmed through a colony PCR using *LAT4*or *AAP6* gene, or pDR196 vector specific primers. Liquid cultures of PCR positive colonies were grown until the log phase. The concentration of cells was counted using a haemocytometer. Cell cultures were diluted to a concentration of 20cells.µl^{-1}. Cells were grown on square plates on minimal BA medium containing $(NH_4)_2SO_4$ (control), Valine (Val), Leucine (Leu), Isoleucine (Ile), Alanine (Ala), Asparagine (Asn), Glutamate (Glu), Arginine (Arg), Proline (Pro), or Putrescine at a 5 mM concentration as the sole source of N. Each genotype had three replications with 100 cells per replication. For each growth condition, all genotypes were grown on the same plate. Plates were incubated at 30^{o}C for three days prior to taking photos. As a second step, minimal BA plates were prepared with 25 mM Ile with or without supplemental 5mM Ala. The same cell cultures

produced above were plated at a volume of 5µl per replication. Each genotype had three replications. Plates were incubated for three to seven days prior to taking photos.

2.3. Results

2.3.1. *In silico* analysis: LAT4 is an integral membrane protein with ten transmembrane domains.

A phylogenetic tree representation of LATs compared to the other characterized AATs in the ATF1 and APC superfamilies in Arabidopsis demonstrates that the LAT family is a separate cluster from the CATs within the APC superfamily (Figure 2.1A).

In Arabidopsis, LAT4 is a 495-AA protein encoded in chromosome1 with a single small intron in the 5' UTR. A secondary structure analysis predicted 10 transmembrane domains of the LAT4 with both the N- and C-termini in the cytoplasmic side (Figure 2.1B). A conserved domain search using the NCBI domain search tool identified putative domains including an AA transporter domain, Glu/GABA antiporter domain, Arg/Agmatine antiporter domain, L-type AA transporter domain, basic AA/polyamine antiporter domain, AA permease domain, Lys/Cadaverine antiporter domain, and Arg/Ornithine antiporter domain in the LAT4 protein sequence.

A sequence alignment of the LAT4 with other members within the same family identified the LAT3 and LAT1 as the closest similar transporters with decreasing homology to the LAT5 and LAT2, respectively. Alignement of the Arabidopsis LATs with animal LATs did not show any significant homology. Aligning the AA-sequence of the Arabidopsis LAT4 with human LATs showed less than 15% sequence similarity. The Arabidopsis LAT4 also lacks the cysteine (Cys) residue, conserved in the second extra-cellular loop of human LATs, which interacts with a heavy chain glycoprotein through disulphide bonding to function as an exchanger (Pfeiffer et al., 1998; Verrey et al., 2000). A PSI-BLASTp search

in the NCBI protein data bank identified bacterial AdiC (PDB Id 3LRB_A; GI: 289526962) as the most similar transporter of the LAT4 (E-value: $3e^{-16}$).

2.3.2. *LAT4* is expressed in all organs with a higher abundance in rosette leaves; and in leaf mesophyll cells, stem cortical cells, and carpel cells in the siliques valve

Analysing the *LAT4* expression using the public database Arabidopsis eFP Browser (At-TAX) showed high levels of *LAT4* mRNA in leaf, stem, flower, silique, whole seedling, and shoot apex. At a tissue-specific level, the Arabidopsis eFP Browser showed an *LAT4* expression in the leaf mesophyll and guard cells, stem epidermis, and fruit carpels (http://bbc.botany.utoronto.ca/efp/). An organ-specific qRT-PCR was performed to see the relative abundance of the *LAT4* transcript in various organs such as root, stem, rosette leaf, senescing leaf, cauline leaf, flower, and silique. The *LAT4* transcript was detected in all organs with a higher abundance in the rosette leaves (Figure 2.2A). A promoter analysis using P_{LAT4}:*GUS* (*ß-glucuronidase*) was performed to identify the organ and tissue-specific expression pattern of the *LAT4*. The GUS histochemical analysis of transgenic mature plants expressing P_{LAT4}: *GUS* showed staining in all organs including root, mature rosette leaves, stem, flower, and siliques (Figure 2.2C, F, J, M, N), which was consistent with the qRT-PCR analysis. At the seedling stage, GUS activity was prominent in the cotyledon leaves as well as in the root (Figure 2.2B). At the mature stage, strong GUS activity was observed in the root (Figure 2.2C), rosette leaf (Figure 2.2F), flower (Figure 2.2M), and silique (Figure 2.2N). In the stem, GUS staining was detected in the surface of the young stem (Figure 2.2J). In the flower, GUS activity was present in the sepals, stigma, and anthers.

Transgenic Arabidopsis plants expressing P_{LAT4}: *GUS* were further studied to elucidate the tissue-specific expression pattern of the *LAT4* promoter. Microtome sections of GUS-stained root, leaf, stem, and silique were examined using a light microscope. In the mature root cross section, GUS activity was noticeable in the lateral root epidermis (Figure 2.2D), while at the seedling stage GUS activity was observed throughout the root cross section with a darker stain

near the epidermal cells (Figure 2.2E). In the leaf, GUS activity was detected in the mesophyll cells (Figure 2.2G, H). In the young stem, GUS activity was detected in the cortex (Figure 2.2K, L). The blue stain of GUS activity was more evident in the cells surrounding the stomata (Figure 2.2L). In developing siliques, the GUS stain was detected only in the green carpel cells in the valve (Figure 2.2O, P). Based on the tissue-specific expression analysis of the *LAT4* promoter, GUS activity was detected mostly in the green tissues such as mesophyll tissues in the leaf, green corticalcells in the stem, and green carpel cells in the silique valve, suggesting that the *LAT4* is expressed in these tissues.

2.3.3. Subcellular localization: with an epitope in the C-terminus, LAT4 localizes to the ER

Multiple prediction programs listed in the plant membrane protein database (http://aramemnon.botanik.uni-koeln.de/) predicted that the LAT4 will be localized in the chloroplast membrane or in the secretary pathway. The Subcellular Proteomic Database (SUBA) predicted LAT4 localization to the mitochondria or PM or extra-cellular (http://suba.plantenergy.uwa.edu.au/). With a Myc-epitope in the C-terminus, the *LAT4* was transiently expressed in the tobacco BY-2 cells to see the intra-cellular localization of this transporter. In these cells, the LAT4 was localized to the endoplasmic reticulum (ER) (Figure 2.3A-F). ER-located proteins typically possess two conserved Lysine residues at the -3 and -5 positions relative to the C-terminus (Benghezal et al., 2000; McCartney et al., 2004). A motif search in the C-terminus of the LAT4 did not identify any "di-lysine" motif. Localizing to the ER suggests that an epitope in the C-terminus of LAT4 may have interfered with its ability to fold and localize properly.

2.3.4. Loss of function of the LAT4 shows reduced shoot growth under normal growth conditions

The Arabidopsis Salk line with a T-DNA insertion in the exon of the *LAT4* locus (At1g31830) (Figure 2.4A) was requested from the Arabidopsis Biological Resource Centre (ABRC). A homozygous line for the T-DNA insertion was

selected using three-primer PCR (Figure 2.4B). The absence of *LAT4* transcript in the Salk line was confirmed through a reverse transcriptase PCR (RT-PCR) (Figure 2.4C). The position of the T-DNA in the *LAT4* exon was confirmed through aligning the DNA sequence produced using a T-DNA left-border-specific primer. This homozygous Salk line with a T-DNA insertion in the *LAT4* locus was designated as *lat4-1*.

Under normal growth conditions, *lat4-1* showed reduced shoot growth compared to wild type (WT) on agar plates with a complete MS medium (Figure 2.4E) as well as in fertilized soil (Figure 2.4D; see Figure 2.5 and 2.6 for quantitative data).

2.3.5. Mutant seedlings are more sensitive to exogenous AA toxicity

Mutant *lat4-1* seedlings were grown on Leu, Ile, or Val with a NO_3^- supplement. In the presence of NO_3^- in addition to Leu, Ile, or Val, the mutant showed reduced growth compared to WT on AA concentrations of 1mM and higher (Figure 2.5A for Leu). In the presence of 1mM Leu, Ile, or Val in the growth medium, WT seedlings obtained 71%, 26%, and 11% biomass, while *lat4-1* seedlings obtained 34%, 17%, and 4% biomass, respectively, compared to the WT grown on 1mM NO_3^- (Figure 2.5B). The variation in the growth reduction between WT and *lat4-1* seedlings in the presence of 1mM Leu, Ile, or Val was 37%, 9%, and 7%, respectively, which was a significant reduction compared to the corresponding WT (Figure 2.5B). However, in the presence of 1mM NO_3^- alone, the biomass reduction in *lat4-1* compared to WT was also a significant reduction of approximately 50% (45%). In the presence of 2mM Leu, Ile, or Val, *lat4-1* seeds barely germinated and were reduced in biomass compared to WT. On 2mM Leu, Ile, or Val, WT seedlings obtained 33%, 17%, and 4% growth, while *lat4-1* seedlings obtained 0.7%, 5%, and 0.6% growth, respectively, compared to WT grown on 1mM NO_3^-. In the presence of 2mM Leu, Ile, or Val, the magnitude of growth reduction in *lat4-1* compared to that in WT was 47, 3.4, and 7 times greater, respectively. This growth variation was found consistent regardless of the NO_3^- concentration added to the growth medium. The mutant *lat4-1* showed the

same sensitivity to 2mM Leu, Ile, or Val when the concentration of the NO_3^- supplement was 1 mM, 2mM, or 3mM (data not shown).

2.3.6. In the absence of a C supplement in the growth medium, mutant shows a drastic reduction in root and shoot growth

As published in the public database (http://bbc.botany.utoronto.ca/efp/), Covington and Harmer (2007) demonstrated that under constant optimum light intensity (120μE), the Arabidopsis *LAT4* shows minimum expression, while Edwards et al. (2006) demonstrated that if the light intensity is reduced to half (60 μE) with other parameters remaining constant, *LAT4* induction increases by more than 250%. In this database, another study by Michael et al. (2008) demonstrated that the *LAT4* is induced up to 400% when seedlings are kept constantly in the dark compared to being kept under optimum light (demonstrated by Covington and Harmer, 2007). To test whether the induction of the *LAT4* in the dark is due to the low C content during darkness or due to other factors regulated by the light/dark regime, seedlings were grown on plates under prolonged light (20hrs light and 4hrs dark) without a C-supplement in the growth medium.

In the presence of 1% sucrose in the media, the *lat4-1* mutant showed significant reduction in total biomass (Figure 2.6A, B) and root length (Figure 2.6A, C) compared to WT. The mutant obtained 68% of the dry weight of biomass and 88% of the root length compared to WT, representing a significant decrease in biomass and root length respectively (Figure 2.6B, C). However, in the absence of the C supplement in the growth medium, both the root length and biomass of *lat4-1* showed further reduction compared to WT (Figure 2.6A-C). In the absence of sucrose, *lat4-1* showed a significant 51% reduction in the biomass and 52% reduction in the root length, compared to WT. In the absence of sucrose in the growth medium, WT showed a nonsignificant change in the biomass (-24%) and root length (+4%) compared to WT grown in the presence of sucrose, while *lat4-1* showed a significant 54% (P-value <0.01) and 56% (P-value <0.001) reduction in biomass and root length, respectively, compared to *lat4-1* grown in the presence of sucrose.

2.3.7. In the absence of a C supplement in the growth medium, free AA concentration increases in the shoots and decreases in the roots in *lat4-1*

Free AAs were analyzed using High Performance Liquid Chromatography (HPLC) in the roots and shoots of WT and *lat4-1* seedlings, grown in the presence or absence of sucrose in the growth medium (Figure 2.6A). With sucrose in the growth medium, *lat4-1* showed a 2% and 23% decrease in the total free AA content in the shoots and roots, respectively, compared to WT (Table 2.1). While in the *lat4-1* shoots, the concentration of some AAs (Asp, Arg, Tyr, Cys, Val, Met, Trp, Ile, Leu, Lys) increased, the concentration of others (Glu, Asn, Ser, Gln, His, Gly, Thr, Ala, Phe) decreased, compared to WT.

On the other hand, in the absence of sucrose in the growth medium, the total free AA concentration in *lat4-1* seedlings was increased by 176% in the shoots and decreased by 80% in the roots compared to WT (Table 2.1). The mutant *lat4-1* showed a significant increased content of Ser (448%), Thr (330%), Arg (220%), Ala (252%), Tyr (420%), Cys (818%), Met (277%), Trp (367%), Phe (488%), Ile (437%), and Leu (459%) in the shoots, with a significant decrease in His (90%), Tyr (47%), Met (56%), Trp (58%), and Ile (66%) in the roots. In general, the concentration of almost all AAs detected showed an increase in shoots and a decrease in roots of *lat4-1* compared to WT (Table 2.1).

In the absence of sucrose in the growth medium, the total free AA concentration in the shoots decreased by 40% in WT compared to that in the presence of sucrose, whereas in *lat4-1*, it was increased by 41% compared to that in the presence of sucrose. In the roots of WT and *lat4-1*, the free AA concentration decreased by 57% and 89%, respectively, in the absence of sucrose compared to that in the same genotype in the presence of sucrose (Table 2.1). Thus, the absence of sucrose in the growth medium caused a decrease in the free AA concentration in both roots and shoots of WT, while in *lat4-1* it was increased in the shoots with a corresponding decrease in the roots.

2.3.8. ^{14}C- and ^{3}H-Leu uptake study: *lat4-1* accumulates more Leu under low carbon conditions

Leu was used as a substrate in the *in planta* uptake studies due to its ability to cause toxicity in *lat4-1* seedlings (Figure 2.5A). A ^{14}C-Leu uptake study was conducted using three-week-old plate-grown seedlings starved overnight for both N and C. The study showed a significantly higher accumulation of ^{14}C- in *lat4-1* compared to WT (Supplementary Figure 2.1A). Plate-grown seedlings largely depend on the sucrose supplement in the growth medium as a source of C and may show a low rate of photosynthesis and transpiration. With a view to avoid the concern that the flow of C and, to some extent N, in a plate-grown seedling fluctuates from that in a soil-grown seedling, the study was repeated using soil-grown seedlings, which would allow an easy interpretation of the result from the normal AA translocation standpoint. As the preliminary study (Supplementary Figure 2.1A) indicated that the LAT4 may have a role in exporting Leu, a mixture of ^{14}C-Leu and ^{3}H-Leu was used in the subsequent studies to trace both the -NH_2-group and the C-skeleton of Leu. Measuring the accumulation of both the ^{14}C- (in the α-C) and ^{3}H- (in the -NH_2 group) was useful to investigate whether *lat4-1* showed an increased accumulation of a deaminated C-skeleton or it is true for both the –NH_2 group and the C-skeleton of Leu.

When soil-grown normal seedlings were used in the uptake study, no variation was observed in ^{14}C- or ^{3}H- accumulation between the *lat4-1* and WT seedlings (Figure 2.7A, D). However, when seedlings were kept in the dark for 24 hrs prior to the study, *lat4-1* accumulated significantly more ^{14}C- and ^{3}H- (60% and 80%, respectively) compared to WT (Figure 2.7B, E). With a view to investigate if the magnitude of ^{14}C- and ^{3}H- accumulation in *lat4-1* changes when plants are allowed to export for an extended period, dark-treated seedlings as in Figure 2.7B and E were subjected to two hrs of uptake followed by two hrs of export. The mutant *lat4-1* accumulated significantly increased ^{14}C- (80%) and ^{3}H- (67%) compared to WT (Figure 2.7C, F), showing an increase in the ^{14}C- accumulation and a decrease in the ^{3}H-accumulation compared to those not subjected to export. A TLC analysis of AAs extracted from seedlings after four

hrs of ^{14}C-Leu uptake showed that a noticeable part of ^{14}C- in WT ran faster (Supplementary Figure 2.1E, indicated by a black arrow) than ^{14}C-Leu, while it didn't appear the same in the *lat4-1* sample, suggesting a rapid metabolism of the ^{14}C- Leu in WT compared to *lat4-1*.

To test further, a separate export study was conducted using both plate-grown and soil-grown seedlings. No noticeable variation was detected between WT and *lat4-1* seedlings in ^{14}C-Leu export for a period of one hour. Supplementary Figure 2.1B shows the data obtained from the export study performed using plate grown seedlings. Keeping in mind that if the LAT4 mediated export of ^{14}C-Leu in the WT plants, this could have occurred during the rinsing of roots prior to counting the radioactivity, the remaining radioactivity in the plates was measured. If export occurred during the rinsing period, both WT and *lat4-1* should have similar levels of remaining radio-activity in the uptake medium. The remaining radioactivity in the WT plate was significantly higher than that in the *lat4-1* plate (data not shown)..

Another study was conducted with mature plants to determine if any specific organ showed higher accumulation compared to the others. Mature plants grown on a plate were supplemented with ^{14}C-Leu at the flowering stage in the root zone. After eight days of ^{14}C-Leu application, radioactivity was measured in the leaves, root, and inflorescence. In the whole plant, *lat4-1* showed a higher accumulation of ^{14}C- compared to WT. Organ-specifically, *lat4-1* leaves, and inflorescence showed significantly higher accumulation compared to WT (Supplementary Figure 2.1C).

In a separate study using soil-grown dark-treated seedlings, a mixture of ^{14}C-Glu and ^{3}H-Leu was used to test if *lat4-1* shows a higher accumulation of ^{14}C-Glu. The mutant *lat4-1* accumulated 29% and 40% more ^{14}C- and ^{3}H- respectively compared to WT (Supplementary Figure 2.1D).

2.3.9. Leaf protoplasts in *lat4-1* are smaller in size but accumulate more ^{14}C-(Leu) compared to WT.

Leaf protoplasts, produced from mature rosette leaves of two-month-old plants grown under a 8/16 hrs of light/dark regime, were significantly smaller in size for the *lat4-1* line compared to WT (Figure 2.8A). While the average diameter of protoplasts in WT was 0.51µm, it was 0.38 µm in *lat4-1*, showing a significant 25% decrease. In a ^{14}C-Leu uptake study using leaf protoplasts, the mutant *lat4-1* showed a significantly higher accumulation of ^{14}C- Leu compared to WT (Figure 2.8B). WT cells accumulated 43.55pmol.10^5cells.hr^{-1} while *lat4-1* cells accumulated 52.74pmol.10^5cells.hr^{-1}, showing a significant 21% increase. In terms of the accumulation per area of cells, the average area ($A= 4\pi.r^2$; r = ½ diameter of a cell) of the protoplasts was 0.817 $\mu m^2.cell^{-1}$ and 0.453 $\mu m^2.cell^{-1}$ for WT and *lat4-1* cells, respectively, with a 44.5% decreased area per cell in the *lat4-1*. The average accumulation was 53.3 pmol and 116.4 pmol per $10^5 \mu m^2$ area of WT and *lat4-1* cells, respectively, a 118% increase in accumulation in the *lat4-1* protoplast cells compared to WT.

2.3.10. The mutant *lat4-1* shows increased free AA accumulation in the leaves with a corresponding decrease in the stem

An analysis of free AAs was performed in leaves, stem, and siliques of *lat4-1* plants compared to WT grown on fertilized soil under normal growth conditions. This study was conducted to investigate if AA translocation from the leaves to the siliques via the stem is altered in the mutant *lat4-1*. In mature rosette leaves, young stem, and green siliques, the mutant *lat4-1* showed a 17%, 18% and 42% increase in free AA concentration, respectively, compared to WT (Figure 2.9). In the *lat4-1* rosette leaves, concentration of Asp was significantly increased with a corresponding significant decrease in the concentrations of Asp and Glu in the stem compared to WT (Figure 2.9A, B).

2.3.11. Loss of function of the LAT4 changes the free AA-pool in seedlings grown under osmotic stress

The homozygous *lat4-1* mutant along with WT was grown on a complete MS medium containing various concentrations of mannitol to impose osmotic stress. The mutant *lat4-1* showed reduced growth compared to WT (Figure 2.10). Since *lat4-1* showed reduced growth compared to WT under normal growth conditions (Figure 2.4D, E), this growth variation under osmotic stress may not be a result of decreased osmotic stress tolerance in the *lat4-1* mutant compared to WT. Free AAs were analyzed in seedlings of *lat4-1* and WT grown with or without 150mM mannitol in the growth medium. In the MS medium without mannitol, *lat4-1* seedlings contained 195% more free AAs compared to WT (Table 2.2). Concentrations of most AAs were higher in *lat4-1* seedlings with noticeable higher concentrations of Asn (417%), Gln (214%), His (157%), Arg (377%), Ile (109%), Leu (1542%), Lys (180%), and Hydroxy-proline (Hyp) (110%). Concentrations of Ala, Gly, Thr, and Asp were decreased in the *lat4-1* seedlings compared to WT. In the presence of 150mM of mannitol in the growth medium, the free AA concentration in WT seedlings was increased by 436%, while in *lat4-1* it was increased by 132% compared to the same genotype grown in the absence of mannitol. In the presence of 150mM of mannitol, there was a 10% decrease in the total concentration of free AAs in *lat4-1* seedlings compared to WT. The concentration of Asn, Ser, Arg, Lys, Val, Ile, Phe, and Hyp was decreased by 20-50% each, while Tyr and Trp remained undetected in *lat4-1* seedlings. In contrast, the concentration of Gly, His, Ala, Met, and Leu increased. This study demonstrated that lacking the functional LAT4 increases the free AA content in seedlings grown under normal conditions. Under osmotic stress, while the free AA content in WT seedlings increases by 436%, it did not increase proportionally in *lat4-1*. Instead, *lat4-1* showed a 10% decrease in the total free AA content compared to WT under osmotic stress.

2.3.12. Heterologous expression of the *LAT4* in yeast promotes increased resistance to exogenous AA toxicity compared to the *AAP6*

The yeast strain 22Δ8AA, lacking eight AATs and unable to grow on Arg, Asp, Glu, Citrulline, GABA, or Pro as the sole N source (Fischer et al., 2002), was transformed with the *LAT4* in a yeast expression vector pDR196. The yeast strain, 22Δ8AA, complemented with an empty pDR196 vector or *AAP6* was used as negative and positive controls respectively. After three days of incubation, the LAT4 did not promote better growth of the yeast cells compared to the empty vector on various acidic, basic, or neutral AAs or polyamine (Putrescine) at 5mM concentration (Figure 2.11A). On plates with Ile, Glu, and Arg, the AAP6 mediated a reduced growth of the yeast cells compared to the empty vector. AAs at 5mM concentration was shown to inhibit growth of yeast cells complemented with the bi-directional AA transporter, BAT1 (Dundar and Bush, 2009). In order to test whether the LAT4 can inhibit growth at higher concentrations of AAs, another growth study was conducted using 5mM and 25mM of Ile in the growth medium. Ile was selected over other AAs because it inhibited growth of yeast cells complemented with the AAP6 at 5mM concentration (Figure 2.11A). The mutant yeast strain was capable of using Ile as a source of N. In the presence of 25mM Ile in the growth medium, none of the three genotypes grew (data not shown). The 25mM Ile plate was supplemented with 5mM Ala as a non-toxic AA. Over the period of seven days of incubation in both 5mM and 25mM Ile, the LAT4 mediated slow growth compared to the empty vector, while it permitted better growth on levels of Ile that were toxic to the strain with AAP6 (Figure 2.11B).

2.4. Discussion

2.4.1. LAT4 is a tissue-specific transporter

Quantitative RT-PCR detected *LAT4* transcript in all organs with a higher abundance in the rosette leaves compared to the average expression in the whole plant (Figure 2.2A). Promoter: *GUS* analysis supported the qRT-PCR data showing the GUS activity under the *LAT4* promoter in all organs (Figure 2.2B-P).

At the seedling stage, GUS activity in the cotyledon leaves (Figure 2.2B) suggested expression of the *LAT4* in these tissues. Cotyledons are a major source of storage AAs for early seedling growth (Lamont and Groom, 2002 and references therein). LAT4 may have a role in mobilizing storage-N in the form of AAs, from the cotyledon to the growing root and shoot apex. At the seedling stage, strong GUS activity was present in the roots (Figure 2.2B) and it was also observed in the mature roots (Figure 2.2C). While a cross section of roots at the seedling stage (Figure 2.2 E) showed a GUS stain throughout the roots with a relatively darker stain near the epidermal layer, the mature root cross section showed GUS activity only in the epidermis of the lateral roots (Figure 2.2D). In root epidermis, the LAT4 may have a role in importing AAs from growth medium or exporting AAs into the root zone. While plants can import AAs from the growth medium as a source of N, plants also export excess AAs in to the root zone and import them back subsequently in order to reduce toxicity or to adjust AA homeostasis within the plant (Juma and McGill, 1986). As well, plants maintain a nutrient-rich environment in the root zone through exporting sugars and AAs to promote microbial activities. AAs are the second largest component after sugar in the root exudates (Juma and McGill, 1986). It indicates a necessity for plants to have both AA importers and exporters in the root.

Tissue-specific expression analysis of the *LAT4* in leaves showed GUS activity in the mesophyll cells (Figure 2.2G, H) where it may have a role in importing AAs from the leaf apoplasm into the mesophyll cells or exporting AAs from the mesophyll cells into the apoplasm for apoplastic phloem loading. In the stem cortex, GUS activity was observed in the green cortical cells around the stomata cells (Figure 2.2K, L). Green chlorenchyma cells in the twigs and branches in trees play important roles in photosynthesis (overviewed in Wittmann et al., 2001) through providing up to 15% of the photosynthetic surface of a tree (Schaedle et al., 1968) and up to 45% of the total tree chlorophyll (Kharouk et al., 1995). The photosynthetic activity of the green cells in the cortex of a young stem in Arabidopsis has not been studied extensively. The anatomical distribution of stomata and guard cells in the young stem epidermis and the presence of

chlorophyll-containing cells in the cortex around the stomata indicate their participation in photosynthesis. These cells can be both sources and sinks of AAs. The LAT4 in the green tissues in the stem cortical cells may have a role in importing or exporting AAs to and from these cells.

In the reproductive organ, GUS activity indicated *LAT4* expression in the green sepals in the flower (Figure 2.2M) and green carpel cells in the developing siliques valve (Figure 2.2N-P). At post-fertilization stage, green carpel cells in the silique valve of Arabidopsis, obtain distinctive features with well-defined stomata in the epidermal cells for gaseous exchange and three layers of mesophyll tissues comprising photosynthetic capacity (Dinneny and Yanofsky, 2004). Thus, green carpel cells are a source and sink of AAs. In these cells, the LAT4 may have a role in importing or exporting AAs.

2.4.2. LAT4 mediates mobilization of AAs

In planta characterization of the LAT4 using a T-DNA insertion line (Figure 2.4A-C) showed reduced growth of *lat4-1* compared to WT under optimum growth conditions in a peat-based soil (Figure 2.4D). This peat-based soil contains N both in organic and inorganic forms and the *LAT4* is expressed in the roots (Figure 2.2 B-E). Reduced growth in the *lat4-1* seedlings can be due to a reduced N acquisition from the soil through the roots or a defective N distribution within the plants. However, when grown in the presence of NO_3^- as the sole source of N, mutant *lat4-1*seedlings showed reduced growth (Figure 2.4E; Figure 2.5; Figure 2.6). In the presence of NO_3^- in the growth medium, growth reduction caused by *lat4-1* may not be due to the reduced N acquisition. Thus, the LAT4 in the roots may not be involved in AA uptake. This growth reduction might be due to a defective distribution of organic N in the plant. A similar growth reduction was observed for the Lysine/Histidine-specific transporter, LHT1, T-DNA knockout line, which was attributed to a defect in importing AAs from the leaf apoplasm into the mesophyll cells (Hirner et al., 2006). A subsequent study of the *lat4-1* seedlings grown in the presence of exogenous Leu, Ile, or Val showed that lacking the functional LAT4 causes increased toxicity to the seedlings compared

to WT (Figure 2.5A, B). If the LAT4 played a role in AA acquisition from the growth medium, *lat4-1* was likely to provide increased tolerance to exogenous AA-toxicity, whereas the mutant *lat4-1* showed increased sensitivity to 2mM Leu, Ile, or Val. This can be the result of an increased accumulation of AAs in a particular organ or organelle in the seedling. This study indicated that loss of the LAT4 function did not render *lat4-1* seedlings defective in acquisition or accumulation of AAs; it may have caused seedlings to be defective in mobilizing AAs from a certain organ or organelle. Dual-labeled Leu-uptake studies conducted using both plate-grown (Supplementary Figure 2.1A) and soil-grown (Figure 2.7A-F; Supplementary Figure 2.1D) seedlings and mature plants (Supplementary Figure 2.1C) provided evidence that *lat4-1* plants were not defective in Leu uptake from the growth medium. Instead, these studies consistently showed a higher accumulation of ^{14}C-(Leu), ^{3}H-(Leu), or ^{14}C-(Glu) in *lat4-1* compared to WT. A protoplast uptake study using ^{14}C-Leu provided evidence that the absence of a functional LAT4 in the *lat4-1* leaf mesophyll cell protoplasts did not make them defective in ^{14}C-Leu uptake, rather, mutant cell protoplasts accumulated significantly more ^{14}C- compared to WT (Figure 2.8B), indicating that LAT4 may have a role in mobilizing AAs from these cells.

Heterologous expression in yeast cells demonstrated a transport property for the LAT4 different from AAP6, which is an AA importer (Okumoto et al., 2002). While the AAP6 completely inhibited the growth of the yeast cells at a toxic concentration of Ile, LAT4 mediated increased resistance compared to the AAP6 but had a slower growth rate compared to the empty vector (Figure 2.11B).

2.4.3. LAT4 mediates mobilization of AA under low C conditions

Micro array analysis published in the database suggested that the *LAT4* is induced in dark (http://bbc.botany.utoronto.ca/efp/), which refers to a low C condition. The promoter analysis suggested a predominant expression of the *LAT4* in green photosynthetic tissues (Figure 2.2), which are the sites of C-fixation. These data indicated that the AA-export activity of the LAT4 might be influenced by the cellular C content in those tissues. Thus, mutant *lat4-1* seedlings were

characterized under low C conditions. While the *lat4-1* seedlings showed a significant reduction in both biomass and root length compared to WT in the presence of a C supplement (1% sucrose) in the growth medium, a further reduction in both biomass and root length in *lat4-1* was observed in the absence of sucrose (Figure 2.6A-C), suggesting that the role of LAT4 is enhanced under a low C condition. Dual-labeled Leu uptake studies also demonstrated that the accumulation of both ^{14}C-(Leu) and ^{3}H-(Leu) is significantly different between WT and *lat4-1* when seedlings are treated overnight in the dark (Figure 2.7A-F), supporting the theory that the AA-export activity of the LAT4 is induced in the dark.

The analysis of free AA content in the root and shoot tissues in seedlings grown on plates without a C-supplement showed that *lat4-1* seedlings accumulated a significantly increased concentration (173%) of free AAs in the shoot with a corresponding decrease (80%) in the root compared to WT (Table 2.1). In the absence of a C source in the growth medium, the primary assimilation of inorganic N and the synthesis of AAs in the root will depend on the supply of photosynthetically fixed C from the shoot. In plate-grown seedlings that are less efficient in photosynthesis and depend largely on a sucrose supplement, the absence of sucrose in the growth medium may cause C-deficiency in the root and thus inefficiency in AA synthesis in the roots.

2.4.4. The role of LAT4 in mobilizing AAs from the leaves to the siliques

In planta studies using whole plants and single cells (protoplasts) and heterologous studies in yeast suggested that LAT4 may mediate AA export. Together with the predominant expression of *LAT4* in the leaves, the role of the LAT4 in exporting AAs from the leaves to the reproductive sinks was hypothesized and investigated. The translocation of free AAs from the leaves to the siliques via the stem was measured through an organ-specific free AA analysis. In soil-grown mature plants, *lat4-1* showed a significantly increased accumulation of Asp in the leaves with a corresponding significantly decreased concentration of Asp and Glu in the stem compared to WT (Figure 2.9A, B). This

study was consistent with the protoplast uptake analysis where leaf mesophyll cells in *lat4-1* were defective in releasing AAs (Figure 2.8B). However, free AA analysis in the green and developing siliques showed 42% increased concentration of AAs in the *lat4-1* siliques compared to WT (Figure 2.9C). The mutant *lat4-1* may be defective in releasing AAs from the green carpel cells where the *LAT4* showed specific expression (Figure 2.2O, P). Accumulation of AAs in the carpel cells may result in a higher concentration of free AAs in the siliques. The partitioning of free AAs into storage protein in the seeds does not occur until AAs from the source tissues are delivered into the embryo via the endosperm. Sanders et al. (2009) demonstrated that knocking out the AAP1, which mediates import of AAs into the embryo, caused a higher concentration of free AAs in mature and dry seeds (Sanders et al., 2009). Free AA content may not be an indication of the total N content in the seeds. An analysis of the total N content in *lat4-1* seeds will be useful to resolve the role of the LAT4 in delivering AAs to the siliques.

2.4.5. Additional perspectives about the role of LAT4

A phylogenetic analysis of the APC superfamily members of human, yeast and Arabidopsis, coupled with a secondary structure analysis, showed that the Arabidopsis LAT family is closer to human LATs (Wipf et al., 2002). Subsequent phylogenetic analysis of the APC superfamily members of animal, plant, fungi, bacteria, archaea and lower eukaryotes, showed that the Arabidopsis LATs belong to the PHS (Polyamine H^+-Symporter) family separate from the cluster of animal LATs (Okumoto and Pilot, 2011) and closer to the rice OsPUT1 that was shown as a polyamine uptake transporter in heterologous systems (Mulangi et al., 2012). However, the OsPUT1 showed up to 40% reduction in Polyamine (Spermidine) uptake in the presence of Met, Asn, or Gln as a competitive substrate in the uptake solution (Mulangi et al., 2012), suggesting that the uptake of the polyamine or AA are two aspects of the same transporter. The Arabidopsis LAT4 did not mediate polyamine (Putrescine) uptake in the heterologous system (Figure 2.11A), while it showed variation in growth in the presence of AAs as a source of N (Figure 2.11A, B). *In planta* studies demonstrated the activity of the LAT4 in releasing AAs, while polyamine transport activity remains to be tested. Due to the

closer sequence identity of Arabidopsis LATs with the OsPUT1, the possibility that the LAT4 may mediate both polyamine and AA transport in Arabidopsis is reasonable.

Table 2.1. Free AA analysis in the roots and shoots of *lat4-1* seedlings in comparison with WT grown in the presence or absence of 1% sucrose in the growth medium.

	0.5xMS with 1% sucrose						0.5xMS with 0% sucrose					
	Shoot(nmol.g^{-1}FW)			Root (nmol.seedling^{-2})			Shoot(nmol.g^{-1}FW)			Root (nmol.2seedling^{-1})		
Free AA	WT	*lat4-1*	% Change	WT	*lat4-1*	% Change	WT	*lat4-1*	% Change	WT	*lat4-1*	% Change
Asp	1635.98	2369.75	44.85	9.67	8.72	-9.90	1230.56	934.05	-24.10	4.13	0.22	-94.72
Glu	4086.46	3753.03	-8.16	32.20	26.89	-16.47	2252.21	3107.20	+37.96	11.78	0.45	-96.16
Asn	247.24	243.41	-1.55	7.40	5.42	-26.71	138.43	322.83	+133.20	2.60	0.28	-89.18
Ser	1842.05	1379.72	-25.10	18.98	12.85	-32.32	581.71	3187.72	+447.99*	5.26	0.96	-81.67
Gln	2126.76	1739.75	-18.20	45.52	32.53	-28.55	722.59	1268.02	+75.48	15.65	1.46	-90.66
His	39.59	37.63	-4.96	0.90	0.72	-20.13	32.67	68.49	+109.65	0.39	0.04	-90.46*
Gly	242.60	124.99	-48.48	2.74	1.98	-27.64	55.70	900.14	+1516.10	1.61	0.80	-50.07
Thr	583.26	466.22	-20.07	8.63	6.06	-29.84	192.27	825.91	+329.56*	2.81	0.35	-87.68
Arg	42.42	46.74	10.18	1.01	0.77	-23.49	41.87	134.07	+220.18*	0.53	0.13	-75.85
Ala	497.98	442.68	-11.10	5.13	3.82	-25.54	256.64	902.32	+251.58*	2.45	0.49	-79.84
Tyr	28.40	34.98	23.15	0.71	0.59	-16.07	43.37	225.40	+419.69*	0.45	0.24	-46.84*
Cys	4.81	10.45	117.23	0.19	0.15	-21.39	17.43	160.11	+818.34**	0.21	0.15	-25.69
Val	1785.28	2201.67	23.32	42.28	33.96	-19.69	2119.88	8235.10	+288.47*	25.66	8.11	-68.40
Met	22.81	28.10	23.21	0.43	0.34	-22.37	34.82	131.32	+277.13*	0.27	0.12	-56.36*
Trp	15.80	19.46	23.13	0.24	0.21	-10.52	20.89	97.51	+366.83*	0.20	0.08	-57.50*
Phe	39.98	36.13	-9.63	0.58	0.53	-8.44	42.17	247.73	+487.52**	0.34	0.19	-44.75
Ile	50.29	54.91	9.19	1.55	1.21	-22.08	56.07	301.07	+436.94*	0.84	0.29	-65.79*
Leu	48.85	57.11	16.92	2.10	1.77	-15.87	52.71	294.73	+459.19*	1.09	0.25	-77.36
Lys	26.16	39.96	52.77	0.81	0.67	-16.58	121.68	773.71	+535.84	0.85	0.71	-16.49
Total	13366.72	13086.71	-2.09	181.08	139.19	-23.13	8013.68	22117.43	176.00*	77.11	15.32	-80.13*

*, P-value <0.05; **, P-value <0.01;***, P-value <0.001; Root and shoot of two-week-old seedlings were separated prior to extracting AAs. Each data is an average of three biological replications with two seedlings per replication.

Table 2.2. Free AA analysis in *lat4-1* seedlings in comparison with WT grown with or without 150mM mannitol in the growth medium to impose osmotic stress.

Free AAs (nmol.g^{-1}DW)	0mM Mannitol			150mM Mannitol		
	WT	*lat4-1*	% change in *lat4-1*	WT	*lat4-1*	% change in *lat4-1*
Asp	6090	2813	-54	6885	6017	-13
Glu	6987	11514	+65	18988	15708	-17
Asn	18298	94632	+417	113114	89669	-21
Ser	20741	24119	+16	15685	9527	-39
Gln	96685	303687	+214	514881	486651	-5
His	1172	3013	+157	6178	6311	+2
Gly	2695	2419	-10	3299	7482	+127
Thr	5343	4759	-11	9257	8075	-13
Arg	14804	70552	+377	80878	57880	-28
Ala	6207	3678	-41	3674	4058	+10
Tyr	425	708	+67	1751	0	-100
Val	1687	2425	+44	3623	2788	-23
Met	285	319	+12	1424	5249	+269
Trp	126	234	+86	757	0	-100
Phe	1163	1281	+10	2404	1813	-25
Ile	678	1415	+109	2606	1717	-34
Leu	1375	22578	+1542	13776	16635	+21
Lys	1252	3502	+180	5341	3790	-29
Hyp	5874	12350	+110	32247	25740	-20
Total	191890	565996	+195	836767	749109	-10

For each genotype, seedlings from three plates were pooled prior to extracting AAs due to the poor growth in the presence of 150mM mannitol.

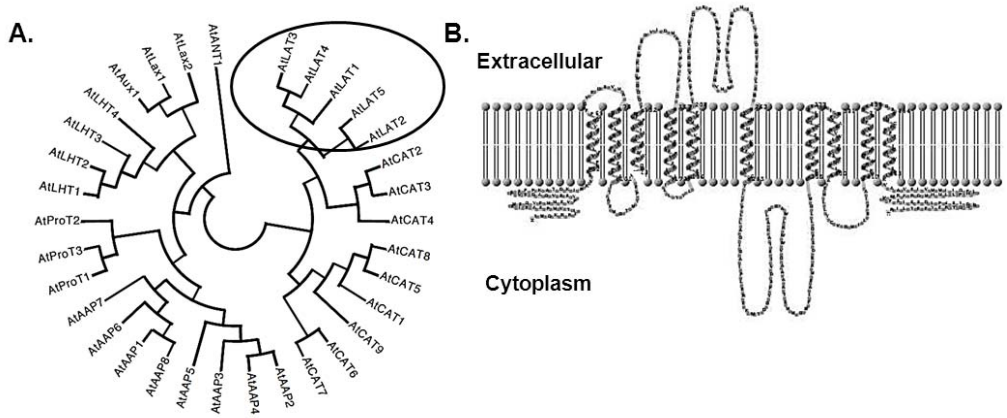


Figure 2.1. Phylogenetic and *in silico* analysis of the LAT4 in *Arabidopsis thaliana*.

A. A phylogenetic comparison of the LAT family with other well-studied AATs classified under ATF1 and APC superfamilies in *Arabidopsis thaliana* shows the LAT family as a separate cluster in the APC superfamily. LATs have been shown in the small circle. This tree shows only grouping and does not represent the distance.

B. A cartoon representation of the TMHMM prediction shows 10 transmembrane domains with both C- and N-termini in the cytoplasmic side. The transmembrane domain prediction for the LAT4 was performed using the TMHMM v.2.0 online prediction tool. The cartoon representation was produced using TMRPres2D.

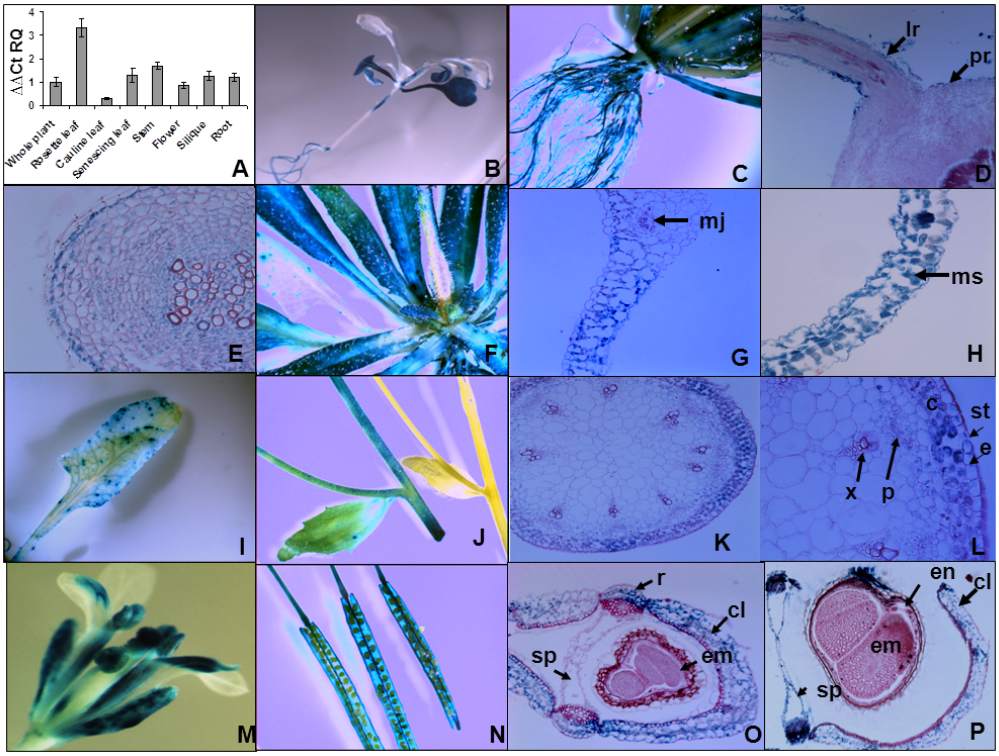


Figure 2.2. Organ and tissue specific expression analysis of the *LAT4* through qRT-PCR and P_{LAT4}: GUS analysis shows predominant expression of the *LAT4* in photosynthetically active tissues.

A. The quantitative RT-PCR shows a higher abundance of the *LAT4* in rosette leaves. The relative quantitation was calculated using an average expression of the *LAT4* in the whole plant as a calibrator and *EF1α* as an endogenous control. The error bar represents the average difference between the RQ_{min} and RQ_{max} of three replications.

B. The GUS activity under the *LAT4* promoter in a three-week old seedling shows staining in cotyledon leaves, young stem, and root.

C-E. The GUS stain is visible on the surface of a mature root (C). The longitudinal section of a lateral root branched from the primary root shows GUS

activity in the epidermis (D).The cross section of the lateral root shows GUS activity in the epidermis (E).

F-I. There is a dark blue stain in the mature rosette leaves (F). The cross section of a leaf shows absence of GUS activity in the major vein (G). There is a dark blue stain in the leaf mesophyll cells (H). There are sporadic stains in the senescing leaf (I).

J-L. A blue stain is present on the stem surface with a light stain in the cauline leaf (J). The young stem cross section shows GUS activity in the cell layer in the cortex concentrating around the stomata (K). This is a closer view of GUS staining in the cortical cells around the stomata (L).

M-P. In the flower, a dark blue GUS stain is present in the sepals, stigma, and anthers (M). Developing siliques show a stain in the siliques valve (N). The cross section of a developing silique shows GUS activity specifically in the green carpel cells in the siliques valve (O, P).

cl, Carpel; c, collenchyma; e, Epidermis; em, Embryo; en, Endosperm; lr, Lateral Root; mj, Major Vein; ms, Mesophyll cell; pr, Primary root; p, Phloem; r, Replum; sp, Septum; st, Stomata, x, Xylem.

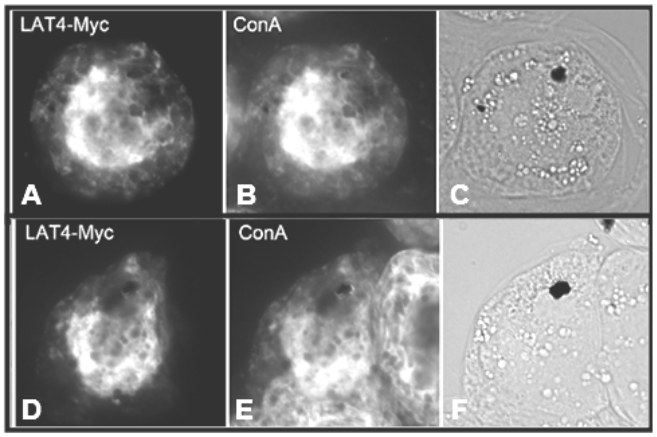


Figure 2.3. Subcellular localization study of the LAT4

A, D. Epifluorescence micrographs specific to Alexa-488, the fluorescent probe attached to the secondary anti-Myc antibody, show c-Myc tagged LAT4 localized in the endoplasmic reticulum in a tobacco bright yellow-2 (BY-2) cell.

B, E. Epifluorescence micrographs specific to Alexa-594 attached to ConcanavalinA, an ER- specific marker stain, visualize the endoplasmic reticulum in the same cell as in (A and C).

C, F. This shows a corresponding differential interference contrast (DIC) image for each cell.

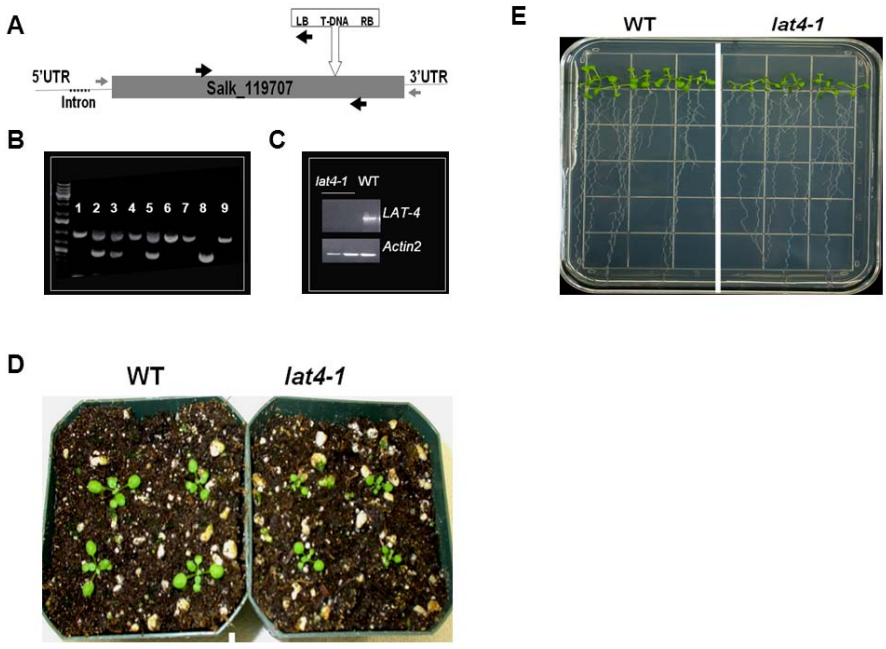


Figure 2.4. Isolation of a homozygous T-DNA insertion line knocking out the *LAT4,* and characterization under normal growth conditions. The mutant shows reduced growth under optimum growth conditions.

A. This shows the position of T-DNA in the exon of the *LAT4* gene. Black and bold arrows indicate the position of primers used in three-primer PCR. Grey and thin arrows indicate the position of primers used in RT-PCR.

B. The three-primer PCR shows WT (lane 1, 4, 6, 7 and 9), heterozygous (lane 2, 3 and 5), and homozygous (lane 8) plants for T-DNA insertion in *LAT4* locus in the Salk_119707 line.

C. RT-PCR shows an absence of *LAT4* transcript in the Salk_119707 line.

D. Under normal growth parameters, the homozygous *lat4-1* mutant shows reduced shoot growth compared to WT in fertilized soil.

E. In a complete 0.5xMS medium with 1% sucrose under optimum growth parameters, the mutant showed reduced shoot growth compared to WT. Photographs were taken when the seedlings were two weeks old. Each growth condition had three or more replications.

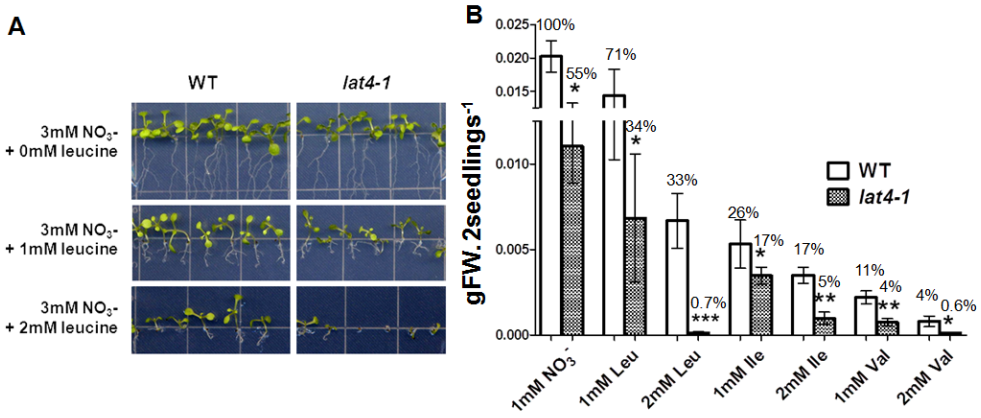


Figure 2.5. Growth studies on plates with variable doses of AA supplement in the growth medium along with nitrate show the *lat4-1* mutant to be more sensitive to exogenous Leu, Ile, and Val compared to WT.

A. Mutant *lat4-1* seedlings are more sensitive to exogenous Leu compared to WT. The variable concentration of L-Leu was added to a 0.5xMS medium with 3mM nitrate as the source of N. Plates were incubated under optimum growth parameters. Photos were taken when the seedlings were two weeks old. Each growth condition had three or more replications. The experiment was repeated more than twice with similar results.

B. The quantitative representation shows that the *lat4-1* mutant seedlings experienced a significant growth reduction compared to WT in presence of a variable concentration of Leu, Ile, or Val, as shown in the figure. The percent of growth was calculated using the WT growth on 1mM nitrate as 100%. The error bar represents the SD of three to five biological replications with two seedlings per replication. *, P-value <0.05; **, P-value <0.01; ***, P-value <0.001)

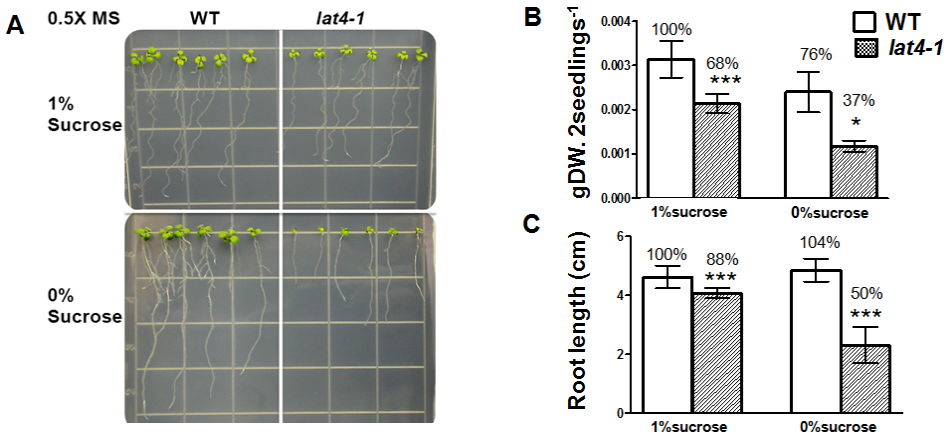


Figure 2.6. In the absence of a C supplement in the growth medium, the *lat4-1* mutant shows a drastic reduction in both root and seedling growth compared to WT.

A-C. The mutant shows significant reduction in total biomass and root length compared to WT in presence of 1% sucrose in the 0.5x MS medium. In the absence of a sucrose supplement, both the root length and biomass of mutant seedlings show a further decrease compared to those in presence of sucrose.

The percent of root or shoot biomass was calculated by arbitrarily using the average growth obtained by the WT in presence of 1% sucrose as 100%. Plates were incubated under 20/4 hrs of a light (~250µE.m^{-2}.sec^{-1}) and dark regime. Each growth condition had four or more plate replications. The root length measurement and plate photos were taken when the seedlings were nine days old, with ten replications for each. For the dry weight of biomass, two-week-old seedlings were dried at 70^{o}C for two hrs before weight was taken. The error bar represents a SD of four replications with two seedlings per replication. (*, P-value <0.05; **, P-value <0.01; ***, P-value <0.001).

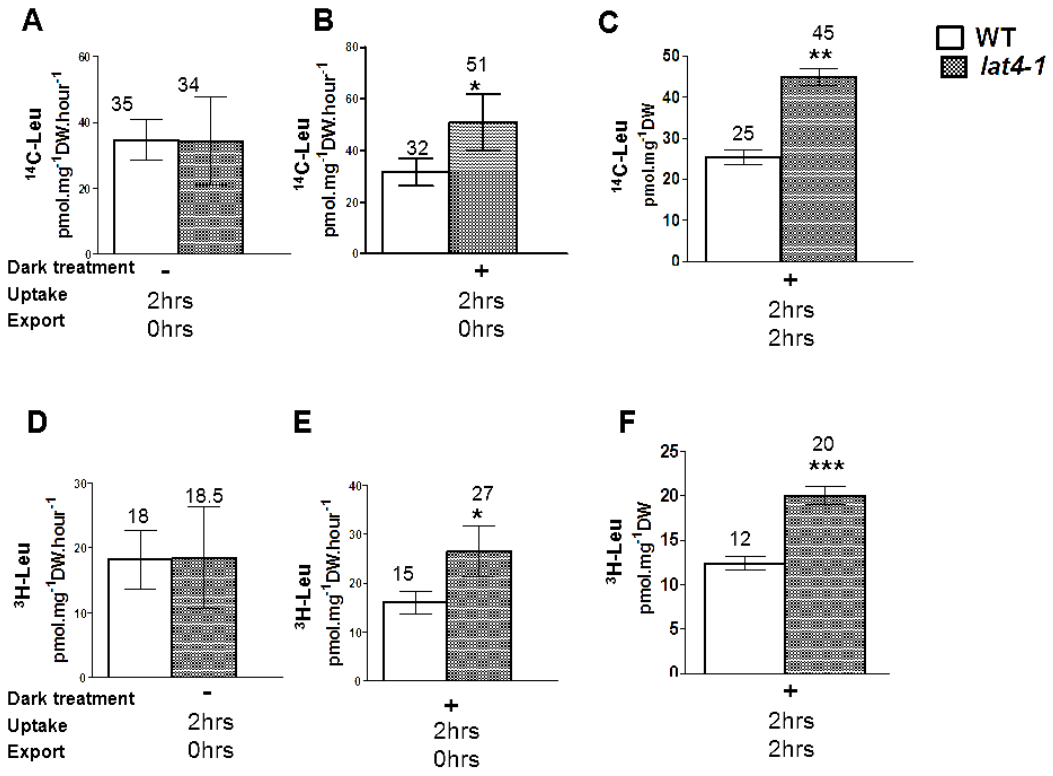


Figure 2.7. Dual-labeled Leu uptake studies show a higher accumulation of both ^{14}C- and ^{3}H- in *lat4-1* compared to WT under low carbon conditions.

A. Seedlings exposed to light for six hrs post night time show no variation in the accumulation of ^{14}C- between WT and *lat4-1* after two hrs of uptake.

B. Seedlings, kept in soil in the dark for 24 hrs, show a significantly higher accumulation of ^{14}C- in *lat4-1* compared to WT after two hrs of uptake.

C. Dark treated seedlings (as in B), after two hrs of uptake followed by two hrs of export, show significantly higher accumulations of ^{14}C- in *lat4-1* compared to WT.

D. Seedlings exposed to light for six hrs post night time show no variation in the accumulation of ^{3}H- between WT and *lat4-1* after two hrs of uptake.

E. Seedlings, kept in soil in the dark for 24 hrs, show a significantly higher accumulation of ^{3}H- in *lat4-1* compared to WT after two hrs of uptake.

F. Dark treated seedlings (as in E), after two hrs of uptake followed by two hrs of export, show a significantly higher accumulation of ^{3}H- in *lat4-1* compared to WT.

Soil-grown one-month-old seedlings were used in all studies. As the uptake medium, a mixture of ^{14}C-Leu and ^{3}H-Leu was used with added cold Leu to a final concentration of 10μM in a half-strength liquid MS medium without N and sucrose. The carboxyl group (^{14}C-) or the amino-group (^{3}H-) of L-Leu was radioactively labeled. For all studies, only roots were dipped in the uptake medium. Seedlings were removed from the uptake medium after two hrs, rinsed three times in sterile water, and dried at 70^{o}C overnight before being weighed. For export, seedlings removed from the uptake medium after two hrs were dipped (root only) in a 0.5xMS medium (without N or sucrose or Leu) for two hrs. Radioactivity in the whole seedling was counted in a liquid scintillation counter. A "+" or "-" sign indicates the presence or absence of dark treatment. The error bar represents the SD of three biological replications. *, P-value <0.05; **, P-value <0.01; ***, P-value <0.001; hrs, hrs.

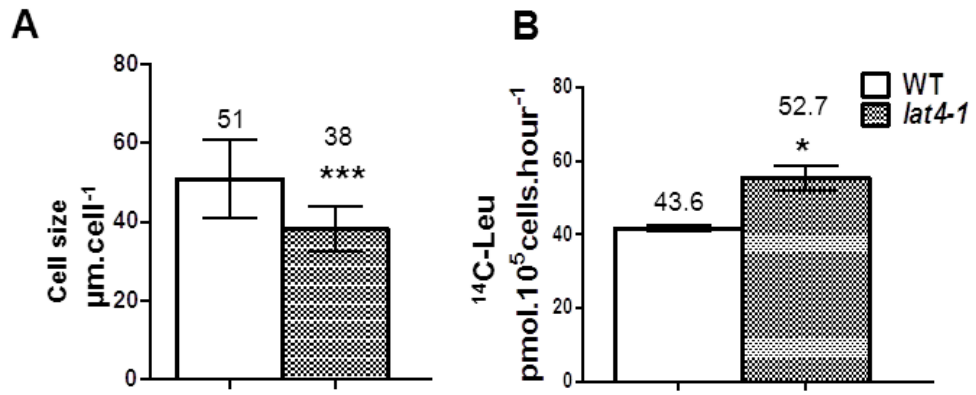


Figure 2.8. Size and AA uptake analysis using leaf mesophyll protoplasts of the homozygous *lat4-1* mutant in comparison with WT.

A. The diameter measurement shows a significantly smaller size of leaf protoplasts in l*at4-1* compared to WT. The error bar represents SD.

B. Leaf protoplasts of *lat4-1* show a significantly higher accumulation of ^{14}C-Leu compared to WT. The error bar represents the SD of three to five replications. *, P-value <0.05; **, P-value <0.01; ***, P-value <0.001.

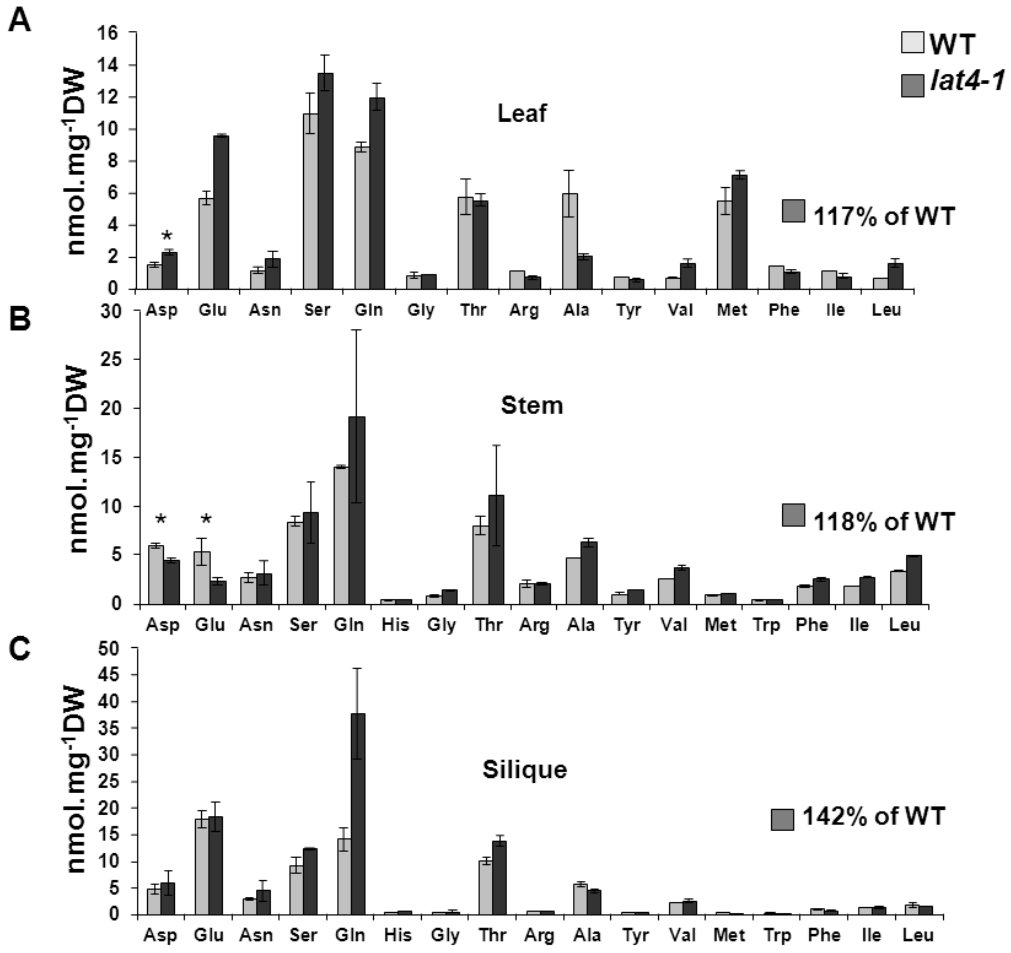


Figure 2.9. Organ-specific free AA analysis.

An analysis of the free AAs in leaf (A), stem (B), and silique (C) of *lat4-1* in comparison with WT shows an overall higher abundance of free AAs in *lat4-1* compared to WT. For each organ, the total amount of detected AAs in the WT was arbitrarily taken as 100% and % change in the mutant was calculated accordingly. The error bar represents SD of three biological replications. *, P-value <0.05.

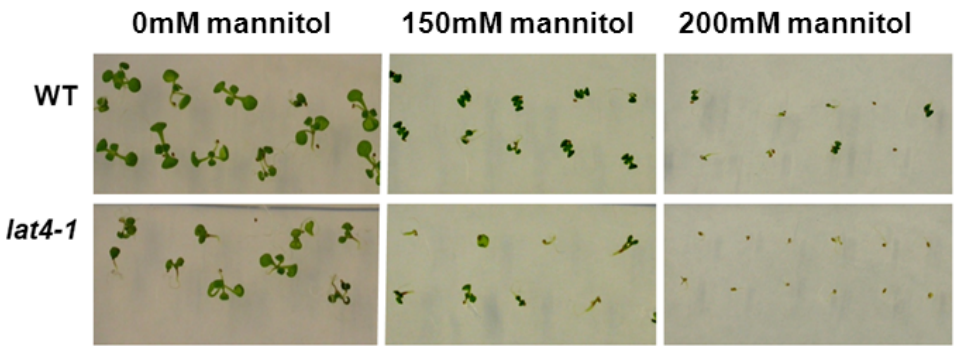


Figure 2.10. Conditional growth study of the LAT4 under osmotic stress.

The mutant *lat4-1* shows reduced growth under osmotic stress compared to WT. Variable concentrations of mannitol were added to a full-strength MS medium (1% sucrose) to impose osmotic stress. Plates were incubated under optimum growth conditions. Both genotypes were grown on the same plate with three or more plate replications for each concentration of mannitol.

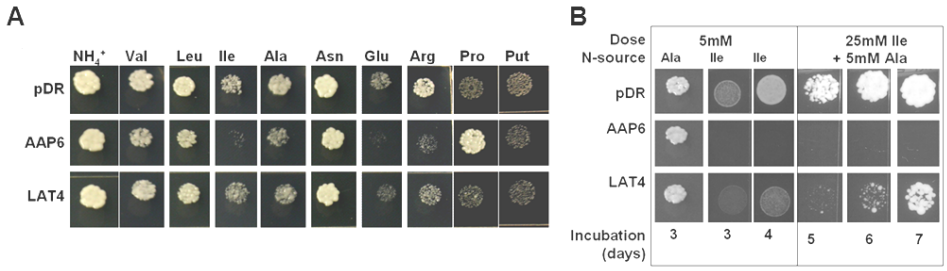
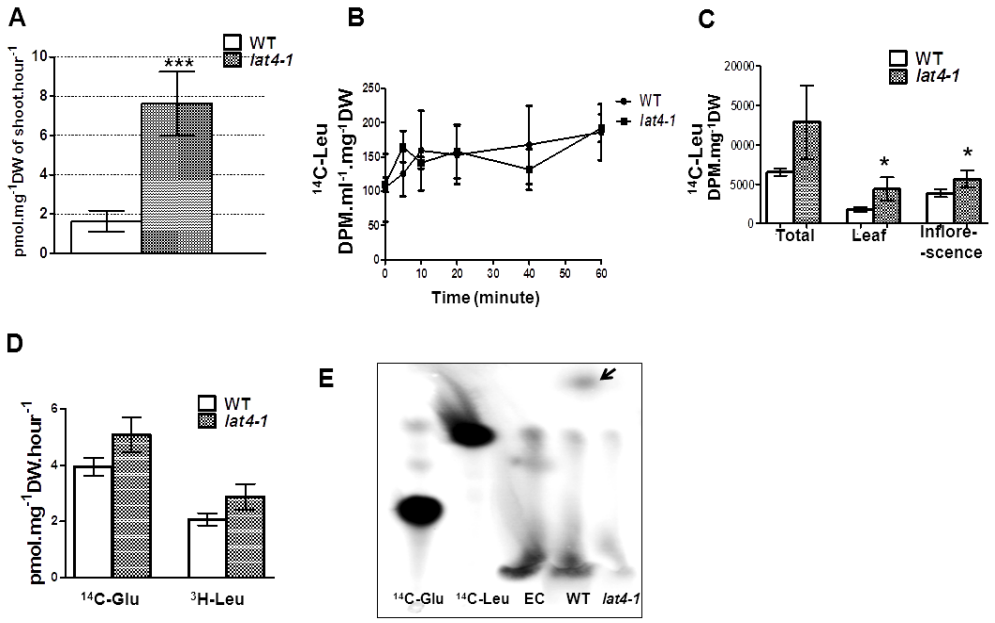


Figure 2.11. Heterologous expression analysis of the LAT4 in a unicellular yeast strain.

A. The 22Δ8AA yeast strain complemented with the LAT4 did not promote better growth compared to the 22Δ8AA strain containing a pDR empty vector on plates containing 5mM of various AAs and a polyamine (Putrescine) after three days of incubation. The AAP6 mediated reduced growth compared to the empty vector on plates containing Arg, Glu, and Ile.

B. Yeast cells containing LAT4 showed an increased resistance to external AA toxicity compared to AAP6. While LAT4 mediated slow growth compared to the empty vector pDR- on both moderate (5mM) and extreme high (25mM with 5mM Ala supplement) concentration of Ile, AAP6 completely inhibited growth on both concentrations of Ile over the period of seven days of incubation. One hundred cells were plated for each genotype and plates were incubated at 30^{o}C. Controls and an experimental group were grown on the same plate with three or more replications for each genotype per growth condition.



Supplementary Figure 2.1. ^{14}C-Leu uptake and export studies using plate-grown seedlings or mature plants.

A. The ^{14}C-Leu uptake study shows a higher accumulation in *lat4-1* compared to WT.Three-week-old seedlings grown on a 0.5x MS medium were used in the uptake study. Seedlings grown on 100mm X 100mm square plates in a vertical orientation were transferred to a liquid MS medium and acclimatized overnight at room temperature. Seedlings were then grown on a 0.5xMS without N overnight. Nitrogen-starved seedlings were used in the uptake study. ^{14}C-Leu was added to a 50 ml uptake medium to a final concentration of 1µM without any cold Leu. Seedlings, removed from the uptake medium after the respective time point, were rinsed three times in sterile water for a total of 10 minutes. Samples were dried at 70^{o}C for two hrs before their weight was taken. Radioactivity was counted in a liquid scintillation counter. The error bar represents SD of six biological replications with two seedlings per replication. ***, P-value <0.001.

B. The ^{14}C-Leu export study shows no significant variation between WT and *lat4-1* mutant. Plate-grown seedlings were starved for light and N for overnight before use. Both uptake and export were carried out at RT and normal light for one hr. Roots were rinsed with sterile water for five minutes before being

dipped into the export medium. The error bar represents SD of three biological replications with three seedlings per replication.

C. A long-term ^{14}C-Leu accumulation study using plate-grown mature plants shows a significantly higher accumulation of ^{14}C- in *lat4-1* leaves and inflorescence compared to WT. Plants were grown on plates up to the flowering stage. The ^{14}C-Leu dissolved in sterile water was pipetted equally to the roots of all plants. Radioactivity in the organs was counted eight days after the ^{14}C-Leu application. The error bar represents the SD of four replications with two plants per replication. *, P-value <0.05.

D. Mutant *lat4-1* seedlings show a higher accumulation of both ^{14}C-Glu and ^{3}H-Leu compared to WT. The study was carried out as described in Figure 5C, D using a mixture of ^{14}C-Glu and ^{3}H-Leu. No cold AAs were added in this study. The error bar represents SD of three biological replications.

E. A TLC analysis of AA extracted from seedlings after four hrs of ^{14}C-Leu uptake shows that a noticeable part of ^{14}C- in WT ran faster (indicated by a black arrow) than ^{14}C-Leu, while it does not appear to be the case for the *lat4-1* sample. Soil-grown one-month-old seedlings, kept overnight in the dark, were used in the ^{14}C-Leu uptake study. AAs (crude) were extracted from leaves immediately after uptake using a methanol and chloroform extraction buffer. Seedlings not used in ^{14}C-Leu uptake were used as extraction control (EC) with ^{14}C-Leu added during grinding. Samples, dried in the speed-vac and re-suspended in 70% ethanol, were loaded on a TLC plate and run for two hrs. ^{14}C-Leu and ^{14}C-Glu were loaded as a control. The TLC plate was exposed to a phosphorus storage screen for two weeks at -80^{o}C before scanning the screen using an FLA-5000 image reader. EC, Extraction control.

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3. Knocking out the L-type Amino acid Transporter 5 (LAT5) changes the seed N content and abiotic stress tolerance in Arabidopsis

3.1. Introduction

Due to a high rate of transpiration in the leaves, amino acids (AAs) from the roots are translocated primarily to the leaves, which then serve as a secondary source of AAs for the reproductive sinks, specifically the seeds (Tegeder and Rentsch, 2010). AAs from the roots can also be translocated directly to the green photosynthetic tissues in the reproductive organs, which are likely to have a high rate of transpiration due to the presence of stomata. The translocation of AAs to the seeds directly from the roots or via the leaves is regulated by the amino acid transporters (AATs).

The AATs involved in the export of AAs from the root symplasm into the xylem apoplasm have not been identified to date. However, a lysine-histidine-specific AAT, LHT1, was identified in the leaf mesophyll cell plasma membrane (PM) that plays a role in importing AAs from the apoplasm into the leaf mesophyll cells (Hirner et al., 2006). AAs from the leaf mesophyll cells need to be loaded into the phloem to be translocated to the seeds. While AATs with net export capacity in the mesophyll cell PM have not been identified to date, based on expression analysis, Arabidopsis AAP5 (Brady et al., 2007; Fischer et al., 1995), CAT6 (Hammes et al., 2006), CAT9 (Su et al., 2004), and ProT1 (Grallath et al., 2005; Rentsch et al., 1996) have been speculated to be involved in loading AAs from the apoplasm into the phloem minor vein. AAs loaded into the phloem are often transferred to the xylem for upward translocation to the seeds. In Arabidopsis, the expression of AAP2 (Hirner et al., 1998; Zhang et al., 2010) and AAP6 (Hunt et al., 2010; Okumoto et al., 2002) along the vascular transport strand in the stem indicated their involvement in the active exchange of AAs between the xylem and the phloem.

While a number of AATs have been identified in Arabidopsis, in the AA translocation route from the root to the seeds or from the leaves to the seeds, their

role in the seed N content have not been tested except for the AAP2. Arabidopsis AAP2 is involved in phloem loading and delivering AAs to the seeds; thus, knocking out AAP2 resulted in a reduced N content in the seeds (Zhang et al., 2010). Expression analysis identified the Arabidopsis L-type amino acid transporter 5 (*LAT5*; At3g19553) expressed in the roots, leaves, siliques, and in the stem vascular transport strand, suggesting an important role for this AAT in plants. The role of LAT5 in the AA translocation and seed N content is studied and discussed in this chapter.

In addition to the net supply of AAs from mature source tissues to the developing sink tissues, an important aspect of AA-transport is to provide the appropriate proportional distribution of free AAs throughout the plant. Free AAs in plants play important roles as regulatory components in many cellular and physiological processes. In roots, free AAs play a role in regulating inorganic N uptake from the soil (Lee et al., 1992; Muller et al., 1995; Rawat et al., 1999). Recent studies demonstrated that the AA homeostasis in plants plays an important role in abiotic stress tolerance (Bonner et al., 1996; Martino et al., 2003; Sharma and Dietz, 2006; Wu et al., 2010) and disease resistance (Song et al., 2004; van Damme et al., 2009; Liu et al., 2010).

Homeostasis of AAs requires antiporters, exchangers, or bi-directional facilitators to mediate the proportional distribution of AAs without changing the net flux of the total free AAs across the membrane. To date, most characterized AATs in plants showed H^+-coupled secondary active transport that allows the AA-transport towards the H^+ concentration gradient (overviewed in Bush, 1993; Wipf et al., 2002). Thus, bi-directional facilitators, exchangers, or antiporters are less understood in the AA-distribution pathway in plants.

In animals, LATs function as AA-exchangers in association with a glycoprotein (Mastroberardino et al., 1998; Pineda et al., 1999; Rossier et al., 1999) and play a role in maintaining AA homeostasis (Verrey et al., 2000). Animal LATs mediate the exchange of intracellular AAs for extracellular AAs at

a 1:1 stoichiometry without changing the net flux across the membrane (Meier et al., 2002). Experiments discussed in this chapter showed a bi-directional AA transport capacity for the Arabidopsis LAT5.The role of Arabidopsis LAT5 in AA homeostasis was investigated in this chapter.

3.2. Materials and methods

3.2.1. Computer-aided Analysis

The *in silico* analysis of the LAT5 protein sequence was carried out as described in Chapter 2. The LAT5 amino acid sequence was taken from the Arabidopsis membrane protein database (http://aramemnon.botanik.uni-koeln.de/).

3.2.2. Construction of vectors for yeast study, promoter analysis and sub-cellular localization

The vector construction for the subcellular localization, yeast complementation, and promoter-reporter analysis was carried out as described in Chapter 2. The primer sequences used to clone the *LAT5* ORF for the sub-cellular localization study are as follows: forward primer

(5'- CTGTCGGTGATATTTGTTTGTCTAGAATGGGTGAAG -3') and reverse primer (5'-CACACTTTTGTTGGTGGGGACCATGGATGGGAGA -3').For the yeast complementation study, the *LAT5* ORF was amplified using the following primers: forward (5'-GAATTCCTCTCTGTCTCTCTGTCGGTGA-3') and reverse (5'- GTCGACTGCGCTTTTAAAGACTTGTGC-3').

The promoter region (1288 bp upstream from start codon) was PCR amplified using *SalI* and *NcoI* sites added to the 5'-end of forward (5'-GTCGACTGCGAGATATCGGACATCATA-3') and reverse (5'-AACCATGGCTTAGAGCTGTTTTCATCG-3') primer respectively.

3.2.3. Quantitative RT-PCR

The organ specific quantitative RT-PCR was carried out as described in Chapter 2. Synthesized cDNA was used to amplify Arabidopsis Actin2 to be used as an endogenous control. A TaqMan Gene Expression Assay (assay ID: At02254880_gI for *LAT5*; assay ID: At02335270_gH for Actin2) was used in PCR amplification. To detect the change in the *LAT5* expression in response to salt stress, the total RNA was extracted from multiple seedlings of WT grown in the presence of various concentrations of NaCl. Preparation of the PCR template from total RNA and the PCR program was the same as described in Chapter 2.

3.2.4. GUS Histochemical Analysis for organ- and tissue-specific expression of the *LAT5*

GUS staining and microscopy was performed as described in Chapter 2.

3.2.5. Sub-cellular localization

The transient expression of c-Myc epitope-tagged *LAT5* in tobacco bright yellow-2 (BY-2) suspension cells and fluorescence microscopy was done in the Mullen lab at the University of Guelph, as described in Gidda et al. (2009). See Chapter 2 for details.

3.2.6. Isolation of a *lat5-1* knockout mutant

The homozygous T-DNA insertion line (Salk_007135c) with a knockout in *LAT5* was obtained from the SALK collections (Alonso et al., 2003) at the Arabidopsis Biological Resource Centre. The T-DNA insertion in the second exon of *LAT5* was primarily confirmed through the PCR screening scheme described in the Salk webpage (http://signal.salk.edu/). One primer specific to the left border sequence of T-DNA, LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3'), and two primers specific to the *LAT5* sequence (reverse primer 5'-TGACTCAGGTACAAACCCTCC-3' and forward primer 5'-ATATTTGATTCCCCTCATGGC-3') were used in the PCR screen. The position of the T-DNA in the *LAT5* locus (At3g19553) was confirmed through aligning the gene-specific primers designed by the SIGNAL iSECT tool

(http://signal.salk.edu/tdnaprimers.2.html) and sequencing product produced using the T-DNA left-border-specific primer. Reverse Transcriptase (RT)-PCR, using UTR-specific primers, confirmed the absence of the *LAT5* transcript in the Salk line (Figure 3.4C).

3.2.7. *In planta* radio-labeled Leu and Glu uptake study

A ^{14}C- and ^{3}H-Leu uptake analysis was carried out using homozygous *lat5-1* seedlings. Seedlings grown on soil or on plates (on 0.5x MS medium containing 5mM NO_3^-, 1% sucrose, pH 5.7) as described in Chapter 2 were used in the uptake studies. The soil-grown seedlings, which had been grown normal or treated in the dark for 24 hrs, were removed from the soil, rinsed, and cultured on plates with sterile water for 3-4hrs at 4^{o}C temperature before using in the uptake study. The plate-grown seedlings, grown in 100mm X 100mm square plates in vertical orientation, were transferred to a liquid medium (0.5xMS, 1% sucrose, pH 5.7) and acclimatized overnight at RT. The seedlings were then transferred to a starvation medium (0.5xMS without N, 1% sucrose, pH 5.4) and grown in the dark for 24 hrs. For the uptake study using mature plants, soil-grown dark treated or untreated plants at the reproductive stage were removed from the soil and washed before being used in the study. In all studies (unless otherwise mentioned), ^{14}C-Leu and/or ^{3}H-Leu were added to the uptake medium (liquid MS without N and C, pH 5.4) with cold Leu added to a final concentration of 10µM. The ratio of radio-labeled and cold Leu and the concentration and specific activity of the stock was the same as described in Chapter 2. For the mixed uptake study, an equal mixture of ^{14}C-Glu and ^{3}H-Leu was used without any cold AA in the uptake medium. In the ^{14}C-Glu, the label was in the α-carbon and the specific activity and the concentration of the stock was 260mCi/mmol and 0.1mCi/ml respectively. Seedlings/plants were removed from uptake medium after the respective time point, and rinsed three times in sterile water for a total of 10 minutes. During uptake or rinsing, only roots were dipped in the solutions to avoid surface contamination of radio-activity in the shoots. Samples were dried at 70^{o}C overnight and weighed. Samples were digested with bleach and prepared for

counting with HIONIC FLUOR (Perkin Elmer). Radioactivity was counted in DPM in a liquid scintillation counter (Beckman). Conversion of DPM into pmol was calculated as described in Chapter 2.

For the export study, plate-grown or soil-grown seedlings, starved overnight for light and N (only for plate grown seedlings), were fed with ^{14}C-Leu. Feeding seedlings with ^{14}C-Leu was carried out as described above. Both uptake and export were carried out at RT for one to two hours. For export studies, roots of the seedlings removed from the uptake medium were rinsed with sterile water for five minutes before being placed in the export medium. Half-strength liquid MS without N and C was used as an export medium. At each respective time point, 50µl of export solution was transferred to a glass scintillation vial to measure the radioactivity that the seedlings released.

For the ^{14}C-Leu uptake study using leaf protoplasts, the proptoplasts were prepared using leaves of a similar size and age from two-month-old plants grown under 8/16 hrs of a light/dark regime. The plants were kept in the dark for 24 hrs before the rosette leaves were collected. Leaf digestion was also carried out in the dark. Protoplast preparation was performed using the protocol described in Yoo et al. (2007). For size analysis, photos of cells were taken using a ZEISS Scope A1 light microscope attached to an optronics camera. All photos were taken under the 20x objective lens. The cell size was measured using PictureFrame (TM) Software Application 2.3. The software program was calibrated using a stage micrometer under the 20x objective lens prior to measuring the diameter of the cells. For the ^{14}C-Leu uptake study, cells of each genotype were counted under a haemocytometer and the final concentration was adjusted to $4x10^5$cells.ml^{-1}. An MMG (4mM MES, 0.4M Mannitol and 15mM $MgCl_2$, pH 5.6) solution with ^{14}C-Leu and cold Leu at a final concentration of 10µM was used as an uptake medium. After one hr of uptake under light, cells were rinsed with an MMG solution before radioactivity was counted. For rinsing, equal volume of fresh MMG solution was added to each vial, centrifuged and allowed the cells to settle

in the bottom, and carefully discarded the supernatant using a micro-pipette. The rinsing step was repeated three times.

For all studies, seeds of both genotypes were planted at the same time on the same flat with the exact same treatment of soil, water, fertilizer, and other growth parameters. For all studies, the data analysis and significance test were performed using PRISM.

3.2.8. Analysis of free AAs through HPLC

Free AA extraction and analysis through HPLC was performed as described in Chapter 2.

3.2.9. Analysis of the total N content in mature seeds

Mature and dry seeds harvested from *lat5-1* and WT plants grown under normal conditions with similar treatment of fertilizers and water were weighed using a Mettler Toledo XP56 Delta Range Microbalance (readability up to 10μg). For the analysis of total N and C content, nine sample replications were prepared for each genotype with seeds from three different plants with 100 mature and dry seeds per replication. A CE440 Elemental Analyser (Exeter Analytical Inc., North Chelmsford, MA, USA) was used to analyse the carbon (CO_2), hydrogen (H_2O), and nitrogen (N) content in the organic and inorganic compounds. The combustion of weighed samples was carried out in pure oxygen under static conditions at 975^{o}C. Helium was used to carry the combustion products through the analytical system. Products of combustion were passed over suitable reagents in the combustion tube to assure complete oxidation. In the reduction tube, oxides of N were converted to molecular N at 690^{o}C, and the residual oxygen was removed. Samples were passed through a thermal conductivity detector. The percentage of carbon and nitrogen were calculated in each sample by dividing the total mass of N or C by the weight of 100 seeds.

3.2.10. Plant growth, transformation, and data analysis

The Columbia-0 ecotype of Arabidopsis was used in all studies. All plants (unless otherwise mentioned) were grown in well aerated soil under 16/8 hrs of a light/dark regime at ~170μE light intensity, 60% R/H and 23^{o}C. The soil type and fertilizer regime was the same as described in Chapter 2.The transformation for producing a transgenic Arabidopsis line with P_{LAT5}:*GUS* was carried out using a modified floral dip transformation protocol described by Clough and Bent (1998). Transformants were screened on a 0.5x MS medium containing 25 $\mu g.ml^{-1}$ hygromycin and confirmed by PCR using *GUS* specific primers. For phenotypic studies under abiotic stresses, seedlings were grown on soil or a half- or full-strength MS medium as specified for each experiment (1% sucrose, 1% agar, pH-5.7) with variable sources and concentrations of N, NaCl, and mannitol. All data were analyzed using PRISM (unless otherwise stated). A two-way T-test was performed for the statistical significance.

3.2.11. Heterologous expression of the *LAT5* in yeast

The yeast strain 22Δ8AA (Fischer et al., 2002) was transformed with three independent cDNA clones of *LAT5* driven by the yeast *Plasma membrane ATPse 1* promoter in the pDR196 yeast expression vector (Rentsch et al., 1995). Similar procedure was followed as described in Chapter 2 to prepare the cell culture, and same controls were used. The cells were grown on a minimal BA medium containing $(NH_4)_2SO_4$ (control), Val, Leu, Ile, Ala, Asn, Glu, Arg, and Pro at a 5mM concentration as the sole source of N. In square plates, each genotype had three replications with 100 cells per replication. Plates were incubated at 30^{o}C for three to five days prior to taking photos.

3.3. Results

3.3.1. *In silico* analysis

The Arabidopsis LAT5 is an integral membrane protein with a molecular weight of 52.8kDa and 12 transmembrane domains with both N- and C-termini in

the cytoplasmic side (Figure 3.1). An NCBI conserved domain search identified a putative amino acid permease domain conserved in the LAT5 protein sequence. A motif search identified one N-glycosylation site and four protein kinase C phosphorylation sites in the N-terminal half of the LAT5 protein sequence. The Arabidopsis LAT family shares sequence similarity with the putative AA/polyamine transporters in other plant species such as *Oryza sativa*, *Vitis vinifera*, *Zea mays*, *Populus trichocarpa,* and *Brachypodium distachyon* (http://aramemnon.botanik.uni-koeln.de/). Arabidopsis LAT5 shares ~ 50% sequence identity with the characterized rice Polyamine Uptake Transporter 1 (OsPUT1; Os02g47210) at the AA level. A PSI-BLASTp search in the NCBI non-redundant protein databank identified putative *Ricinus communis* neutral AA transporter (Accession: XP_002527494.1), *Populus trichocarpa* neutral AA transporters (Accession XP_002335345.1; XP_002300260.1; XP_002313902.1), and a *Medicago truncatula* neutral AA transporter (Accession: XP_003610625.1) that share >70% sequence similarity with Arabidopsis LAT5. A PSI-BLASTp search in the Protein Data Bank (http://www.pdb.org/) identified *Escherichia coli* AdiC (GI: 289526962), the Arginine/Agmatine antiporter that shares 23% sequence identity with LAT5.

3.3.2. *LAT5* shows relatively higher abundance in leaves, stem, and siliques with expression in the phloem

The public database Arabidopsis eFP Browser (At-TAX) showed *LAT5* expression in the leaves, stem, flower, whole seedlings, and shoot apex. The Arabidopsis eFP Browser (tissue -specific) showed *LAT5* expression in the leaf mesophyll and guard cells and stem epidermis (http://bbc.botany.utoronto.ca/efp/). A promoter analysis using P_{LAT5}:*GUS* (*ß-glucuronidase*) was carried out to examine the organ- and tissue-specific expression pattern. At the seedling stage, GUS histochemical analysis showed blue stain in the whole seedling (Figure 3.2A, B). At the mature stage, the GUS stain was observed in all organs including the root (Figure 3.2C), leaf (Figure

3.2F-H), flower (Figure 3.2M), and silique (Figure 3.2N). In the leaf and flower, GUS staining showed a pattern indicating the expression in vascular tissues.

Transgenic Arabidopsis plants expressing P_{LAT5}: *GUS* were further studied to elucidate the tissue-specific expression pattern of the *LAT5*. Microtome sections of GUS stained leaves, stem, root, and siliques were examined under a light microscope. In the mature root cross section, no stain was observed in the primary root while in the lateral root, a blue stain was present in almost all tissues (Figure 3.2D, E). In the cross section of a rosette leaf, GUS activity was observed in the leaf mesophyll cells and minor veins (Figure 3.2I, J). While no noticeable GUS stain was observed on the surface of the stem (Figure 3.2H), the cross section and longitudinal section of the stem showed GUS activity specifically in the phloem tissue (Figure 3.2K, L). In the whole silique, a GUS stain was present in the fruit carpel with a pattern along the vasculature (Figure 3.2N). Cross sections of a developing silique showed a blue stain in the vascular tissues within the replum and in the auxiliary vascular tissues in the siliques valve (Figure 3.2O, P).

Organ-specific quantitative RT-PCR was performed to see the relative abundance of *LAT5* transcript in various organs such as root, stem, rosette leaf, senescing leaf, cauline leaf, flower, and silique. In support of the promoter analysis, *LAT5* transcripts were detected in all organs with relatively higher abundance in the leaves, stem, and silique (Figure 3.2Q).

3.3.3. Subcellular localization: with a Myc epitope in the C-terminus, LAT5 remains in the ER

Multiple prediction programs (predisi; PredSL; SignalP; suba) suggested that LAT5 is localized to the secretary pathway. The subcellular proteomic database (http://suba.plantenergy.uwa.edu.au/) and Wolf PSORT (http://wolfpsort.seq.cbrc.jp/) predicted LAT5 localization to the PM or chloroplast membrane. With a Myc epitope in the C-terminus, *LAT5* was transiently expressed in tobacco BY-2 cells to see the intra-cellular localization of

this transporter. In these cells, *LAT5* was mis-localized in the endoplasmic reticulum (ER) (Figure 3.3A). The correct DNA sequence of the *LAT5* in the expression vector was sequenced and analyzed through a six-frame translation. It is possible that the c-Myc epitope is blocking the ER-retrieval signal peptide, preventing it from interacting with other factors or from properly folding. A similar result was obtained for the LAT1 (Chapter 4) and LAT4 (Chapter 2), suggesting that the C-terminal end of the Arabidopsis LAT proteins have a role in the proper folding of these transporters. It would be useful to test this hypothesis by repeating the study with the epitope in the N-terminus.

3.3.4. Identification of a homozygous *lat5-1* mutant that shows reduced growth under normal growth conditions

The Arabidopsis Salk line (Salk_007135c) with a T-DNA insertion in the second exon of the *LAT5* (At3g19553) locus (Figure 3.4A) was received from the Salk Collections (Alonso et al., 2003) at the Arabidopsis Biological Resource Centre. The mutant line, homozygous for T-DNA insertion, was confirmed through three-primer PCR (Figure 3.4B) recommended by the SALK Institute (http://signal.salk.edu/). The position of T-DNA was confirmed using gene-specific primers in a three-primer-PCR analysis and then by aligning the sequencing product produced using the T-DNA-specific primers. The absence of the *LAT5* transcript in the Salk_007135c line was confirmed through reverse transcriptase PCR (Figure 3.4C). The homozygous line containing the T-DNA insertion in the *LAT5* locus was designated as *lat5-1* in the subsequent studies.

Homozygous *lat5-1* mutant plants showed reduced leaf width under normal growth conditions on soil (Figure 3.4D, F, G). While the average number of leaves per plant in *lat5-1* did not differ from the WT (Figure 3.4E), the width of rosette leaves in *lat5-1* were significantly smaller compared to that of the WT (Figure 3.4F). A single leaf biomass of *lat5-1* was also significantly reduced compared to the WT (Figure 3.4G). The flowering time, and the initiation of inflorescence and maturity showed no noticeable difference between the two

genotypes. No noticeable growth variation was observed in the length or growth of inflorescence between the WT and *lat5-1* plants.

3.3.5. Loss of function of the LAT5 shows increased sensitivity to exogenous Leu

Large neutral AAs such as Leu, Ile, and Val are capable of being toxic and reducing growth when supplied as an exogenous source of N at higher concentrations. Thus, Leu was used to screen the mutant *lat5-1* for AA-uptake capacity under the hypothesis that if LAT5 mediates Leu uptake, mutant *lat5-1* seedlings will show an increased tolerance to Leu toxicity. While *lat5-1* showed a 13% growth reduction on 1mM NO_3^-, in the presence of 1mM Leu, the *lat5-1* seedlings showed a significant 62% growth reduction compared to the WT (Figure 3.5A, B). In the presence of 2mM L-Leu, WT seedlings showed a drastic reduction in growth while the *lat5-1* seeds barely germinated and showed a significant 96% growth reduction compared to the WT in the same growth medium.

A free AA analysis was carried out to investigate the effect of Leu on *lat5-1* and WT seedlings grown in the presence or absence of Leu in the growth medium. No significant variation was detected in the total content or composition of free AAs in *lat5-1* seedlings compared to the WT grown on 1mM NO_3^- as a source of N (Table 3.1). In the presence of 1mM Leu in the growth medium, the concentration of Leu in the endogenous free AA pool in both the WT and *lat5-1* was significantly higher than that without Leu, making up 76% and 77% of the total free AAs detected, respectively. However, there was no significant variation in the accumulation of Leu between the WT and *lat5-1* seedlings.

In presence of 2mM Leu in the growth medium, WT and *lat5-1* seedlings contained 71% and 69% Leu in the total free AA pool respectively, while *lat5-1* accumulated 18% more Leu and 22% more total free AA compared to the WT (Table 3.1). In the presence of 2mM Leu, *lat5-1* showed an overall increase in the concentration of most AAs, with a significant increase in Tyr (40%) and Lys

(162%) respectively, compared to the WT. In the presence of 1mM or 2mM Leu in the growth medium, the total free AA concentration in the WT increased by 400% and 619% respectively compared to the WT grown on 1mM NO_3^-, while it increased in *lat5-1* by 404% and 822% respectively compared to *lat5-1* on 1mM NO_3^-.

3.3.6. ^{14}C- and ^{3}H-Leu uptake studies: *lat5-1* accumulates less radionuclide in normal conditions and more when treated in the dark

In an *in planta* AA-uptake study using soil-grown seedlings, a mixture of ^{14}C-Leu and ^{3}H-Leu was used to trace both the -NH_2-group and the C-skeleton in the uptake study. Leu was used due to its ability to cause hypersensitivity in *lat5-1* seedlings (Figure 3.6). Mutant *lat5-1* seedlings showed a significant increased (53%) accumulation of ^{14}C- compared to the WT when treated in the dark for 24 hrs prior to performing the study. In seedlings not treated in the dark, *lat5-1* showed a 43% decrease in ^{14}C- compared to the WT (Figure 3.6A, B). Similar results were obtained for ^{3}H-Leu. While *lat5-1* seedlings showed a decrease (45%) in ^{3}H-Leu accumulation, dark treated *lat5-1* seedlings showed a significant increase (56%) in ^{3}H-Leu, compared to the corresponding WT (Figure 3.6D, E).

The same study in Figure 3.6 B and E was conducted again with two hrs of uptake followed by two hrs of export to investigate if the magnitude of accumulation in *lat5-1* compared to WT changes when seedlings were allowed to export for an extended period. Dark-treated seedlings subjected to two hrs of uptake followed by two hrs of export showed a significant 283% increased accumulation of ^{14}C- and ^{3}H- respectively in *lat5-1* seedlings compared to WT (Figure 3.6C, F).

Another ^{14}C-Leu uptake study was conducted with dark-treated and N-starved seedlings. With the same study as in Figure 3.6B, N-starvation was added to investigate if starvation in both N and C changed the accumulation in *lat5-1* compared to the WT. In this study, *lat5-1* showed a significant increase of ^{14}C- compared to the WT (43%, Figure 3.6H), which was similar to Figure 3.6B and E.

In a separate study using soil-grown dark-treated seedlings, a mixture of ^{14}C-Glu and ^{3}H-Leu was used to investigate if *lat5-1* shows a higher accumulation for both Glu and Leu. Mutant *lat5-1* accumulated significantly higher levels of ^{14}C- (Glu) and ^{3}H- (Leu) compared to WT (125% and 152% respectively; Figure 3.6G).

To elucidate the AA-transport activity of LAT5 at a cellular level, a ^{14}C-Leu uptake study was conducted using leaf mesophyll cell protoplasts produced from soil-grown, dark-treated plants. Mutant *lat5-1* cells accumulated a significant 6% more ^{14}C- compared to WT (Figure 3.6I).

To investigate the Leu export activity of LAT5, repeated studies were conducted using dark-treated or untreated soil-grown and plate-grown seedlings. No study showed any significant export of ^{14}C-Leu in either of the two genotypes over time. Figure 3.6J shows the result for the export study conducted using plate-grown dark-treated seedlings where the radio-activity detected in the export medium did not show a consistent increasing trend over time for either of the two genotypes.

3.3.7. Knocking out the LAT5 changes free AA profile in leaves, stem, and siliques with increased N content in the seeds

An analysis of free AAs was performed in the leaf, stem, and silique of *lat5-1* and WT plants grown on fertilized soil under normal growth conditions. In total, free AA concentration decreased by 8% in leaves and increased by 24% and 62% in the stem and siliques of *lat5-1* respectively, compared to that in the WT (Figure 3.7A-C). In the *lat5-1* stem, the concentration of free Asn, Ser, and Gly increased significantly compared to the WT (Figure 3.7B). In the silique, *lat5-1* showed significantly increased concentrations of Asp, Asn, Gln, Val, and Ile compared to the WT, while Glu was not detected in the *lat5-1* siliques (Figure 3.7C). In general, the free AA profile of *lat5-1* compared to the WT was altered in both content and composition, with an increase in the concentration of most AAs in the stem and siliques.

An analysis of the seed dry weight showed a significant 7% increase in *lat5-1* compared to the WT (Figure 3.8A). An analysis of the percentage of C and N content in seeds showed that the *lat5-1* seeds contained a significant 3% increase in N and a 0.31% decrease in C compared to WT, while the total of N and C content was the same for both genotypes (Figure 3.8B).

3.3.8. Characterization of the *lat5-1* mutant under abiotic stresses

The homozygous *lat5-1* mutant was grown in the complete nutrient medium (1x MS) containing various concentrations of NaCl or mannitol to impose salt or osmotic stress. The mutant *lat5-1* seedlings showed increased salt tolerance compared to WT (Figure 3.9A), while no noticeable growth variation was observed under osmotic stress (Figure 3.9C). A quantitative RT-PCR was conducted to determine the expression level of *LAT5* in WT seedlings under various levels of salt stress. The expression level of *LAT5* was elevated up to 60% in the presence of 150mM NaCl, compared to 0mM NaCl with a gradual decreasing trend with the increase in the concentration of NaCl in the growth medium (Figure 3.9B). Overall, the transcript level of *LAT5* remained higher in the presence of NaCl compared to the absence of NaCl in the growth medium.

Free AAs were analyzed in seedlings of *lat5-1* and WT grown under salt stress and osmotic stress. In the presence of 0mM or 100mM NaCl in the growth medium, *lat5-1* seedlings showed decreased concentrations of total free AAs compared to WT (Table 3.2), although these differences were not significant. (Note: the seedlings were left on plates on the benchtop (low light intensity compared to growth chamber) over three days prior to extracting AAs, which may have changed the free AA content in these seedlings). In the MS medium without any NaCl, *lat5-1* seedlings showed significant decreased concentrations of Asn (6%), Ala (15%), Cys (15%), and Met (21%) and a significant increased concentration of Lys (39%), while the total concentration of free AA decreased by 2% compared to WT (Table 3.2). In the presence of 100mM NaCl in the growth medium, the WT and *lat5-1* showed a 3% increase and 4% decrease, respectively,

in the total content of free AAs compared to the same genotype in the absence of NaCl (Table 3.2). In the presence of 100mM of NaCl, *lat5-1* showed significant decrease in the concentration of Gln (13%), Thr (20%), Arg (10%), Cys (13%), Met (17%), Ile (14%), and Lys (32%) while the total AA concentration decreased by 9% compared to WT (Table 3.2).

In the presence of 0mM or 150mM mannitol in the growth medium, *lat5-1* seedlings showed a 20% and 43% increase respectively in the total free AA content compared to the WT (Table 3.3). In the presence of 150mM mannitol, the WT showed a 150% increase in the free AA content compared to that in the absence of mannitol, while it increased by approximately 200% for *lat5-1* (Table 3.3). In the presence of 150mM of mannitol in the growth medium, *lat5-1* seedlings showed a 151% increase in Arg and a 100% decrease in Lys content compared to WT.

The *lat5-1* mutants consistently showed an increased drought tolerance compared to WT during handling and AA uptake studies. Figure 3.9D shows a photo of plants in soil where *lat5-1* rosette leaves are turgid, while the WT plants are completely wilted after withdrawing one spell of watering (Figure 3.9D).

3.3.9. Heterologous expression of the *LAT5* in yeast does not promote growth variation compared to the controls

Yeast strain 22Δ8AA, lacking eight AATs and unable to efficiently use Arg, Asp, Glu, Citrulline, GABA, or Pro (Fischer et al., 2002), was complemented with three different cDNA clones of *LAT5*. In the yeast expression vector pDR196, *LAT5* was driven by the yeast PMA1 (*Plasma Membrane ATPase 1*) promoter. Yeast strain 22Δ8AA, complemented with an empty pDR196 vector or Arabidopsis AAP6 (At5g49630), was used as negative and positive controls respectively. After three days of incubation, LAT5 did not mediate any growth variation of the yeast cells compared to the empty vector on various acidic, basic, or neutral AAs at 5mM concentration (Figure 3.10). On plates with Ile, Glu, and Arg, AAP6 mediated reduced growth compared to the empty vector. Another

study was conducted with a higher concentration of Ile to test if LAT5 could reduce the growth of yeast cells similar to AAP6. Isoleucine was selected for its capacity to inhibit the growth of yeast cells complemented with AAP6 at a 5mM concentration. While AAP6 inhibited the growth of yeast cells in the presence of 5mM, 10mM and 25mM of Ile in the growth medium, LAT5 mediated a slow growth compared to the empty vector and increased resistance compared to AAP6 in the presence of 25mM Ile (supplemented with 5mM Ala) in the growth medium (Figure 3.10).

3.4. Discussion

3.4.1. The role of LAT5 in AA distribution in plants

The LAT5 is a putative integral membrane transporter protein (Figure 3.1) with the AA permease domain conserved in the protein sequence. The expression analysis revealed that the *LAT5* is a widely expressed AAT with expression in the phloem (Figure 3.2), indicating that the LAT5 has an important biological role in amino-N distribution in Arabidopsis. To investigate the role of the LAT5, a T-DNA insertion mutant *lat5-1* line was isolated and studied (Figure 3.4A-C). Mutant *lat5-1* showed reduced shoot growth on fertilized soil characterized by the significantly smaller size of rosette leaves (Figure 3.4F), reduced leaf biomass (Figure 3.4G) and the smaller size of leaf protoplasts (Figure 3.4H). The growth reduction produced by the *lat5-1* mutant in the presence of both organic and inorganic N in soil could be due either to a reduced N acquisition from the soil, a defect in internal AA distribution, or a cumulative effect of both. The organ-specific qRT-PCR detected *LAT5* transcript in the root (Figure 3.2Q), and the promoter analysis showed GUS activity in the root (Figure 3.2B, C). Cross sections of mature roots showed strong GUS activity throughout the lateral root tissues (Figure 3.2E), suggesting a role in the root AA-transport for the LAT5. In roots, the LAT5 may have a role in acquiring AAs from the growth medium, exporting AAs into the root zone or in inter-cellular distribution of AAs in the root. Mutant *lat5-1* seedlings in the presence of Leu in the growth medium,

showed increased toxicity to exogenous Leu characterized by the significant reduction in biomass (Figure 3.5). The biomass reduction in *lat5-1* was correlated with the increasing concentration of Leu in the growth medium, suggesting that mutant *lat5-1* was not defective in Leu acquisition from the growth medium; instead, *lat5-1* might have accumulated more Leu, causing increased toxicity. Free AAs were analyzed in these seedlings to investigate the Leu accumulation in the *lat5-1* seedlings (Table 3.1). The Leu accumulation in *lat5-1* compared to that in WT was less in the presence of 1mM Leu and more in the presence of 2mM Leu in the growth medium; and the accumulation was correlated with the total free AAs (Table 3.1). While this study did not show any clear indication about the Leu transport activity of the LAT5 in the roots, it showed that mutants are not defective in Leu uptake. An *in planta* radio-labeled Leu uptake study suggested that the LAT5 in roots mediates AA acquisition under normal conditions and AA mobilization under low C conditions (Figure 3.6A-H; Supplementary Figure 3.1). This study suggested that the transport direction of the LAT5 may reverse depending on the cellular C content.

The promoter analysis showed GUS activity in the rosette leaf mesophyll cells and minor vein (Figure 3.2 F, I, J). The organ-specific qRT-PCR indicated a higher abundance of the *LAT5* transcript in the leaves (Figure 3.2Q), suggesting that the LAT5 plays a role in the leaf AA distribution. Radio-labeled Leu and Glu uptake studies using both dark-treated and untreated mature plants did not show a significant variation in the Leu or Glu accumulation between the *lat5-1* and the WT leaves (Supplementary Figure 3.1). In a further investigation, a ^{14}C-Leu uptake study using dark-treated leaf mesophyll cell protoplasts, the *lat5-1* cells accumulated significantly more ^{14}C- compared to WT (Figure 3.6I), which is consistent with the uptake studies using dark-treated whole seedlings (Figure 3.6B, C, E, F, G, H) and mature plants (Supplementary Figure 3.1). This suggests that LAT5 mediates AA mobilization from the leaf mesophyll cells in the dark. While the AA-transport activity of the LAT5 in the leaf minor vein remains to be

studied, it can be anticipated that the LAT5 plays a role in both loading and unloading AAs in the leaf phloem.

Tissue-specific GUS activity under the *LAT5* promoter in the stem (Figure 3.2K, L) showed a GUS stain in the phloem along the vascular transport strand. In the stem phloem, the LAT5 may mediate exchange of AAs between the xylem and phloem along the translocation pathway. Depending on the bi-directional mode of transport, the LAT5 in the stem phloem may mediate export of AAs from the phloem into the xylem and import of AAs from the xylem into the phloem. The existence of an AA exchange between the xylem and phloem has been demonstrated in a number of physiological studies (Pate et al., 1975; Schobert and Komor, 1987; van Bel, 1984; Atkins, 2000).

In the flowers and siliques, the GUS stain followed a pattern along the vasculature consistent with the expression of the *LAT5* in the phloem (Figure 3.2 M, N). A cross section of siliques indicated *LAT5* expression in the funiculi phloem and in the auxiliary phloem in the carpel cells (Figure 3.2 O, P), suggesting that the LAT5 plays a role in delivering AAs into the seeds via the phloem. The Arabidopsis AAP2 showed a similar expression in the stem phloem and in the funiculi in the siliques (Hirner et al., (1998).

The role of the LAT5 in delivering AAs into the siliques from the leaves via the stem phloem was investigated by doing an organ-specific free AA analysis in the leaves, stem, and siliques (Figure 3.7). In the leaves, *lat5-1* showed a decreased concentration of free AAs, while in the stem, *lat5-1* showed an increased concentration of AAs compared to WT (-8% and +24%; Figure 3.7A, B). The AA content detected in the leaves and stem included the free AAs both in the xylem and phloem. If the LAT5 played a role in AA import into the leaf mesophyll cells, the mutant *lat5-1* is likely to acquire less AAs from the xylem translocation stream, resulting in an increased accumulation of free AAs in the xylem stream with a decreased concentration of AA in the leaves. The significant increased accumulation of Asn in the *lat5-1* stem (Figure 3.7) supports this

hypothesis, since Asn is one of the major AAs translocated from the roots to the shoots. The free AA analysis in the *lat5-1* siliques showed a 62% increase in the total AA content, with a significant increased concentration of Asp, Asn, Gln, Val, and Ile compared to WT. Since the mutant *lat5-1* caused a reduced AA import into the leaves and increased accumulation in the stem, it is likely that the green tissues in the siliques acquired more AAs from the xylem that was directly translocated from the roots. An analysis of seed weight and N content revealed that both the seed weight and total N content significantly increased in the *lat5-1* compared to WT (Figure 3.8A, B).

The analysis was compared to the similar studies published for the Arabidopsis AAP2 (Zhang et al., 2010) as *AAP2* showed similar expression pattern as *LAT5* in the stem and siliques (Hirner et al., 1998). While the AAP2 knockout mutant showed a decrease in the leaf AA content, it also showed a decrease in the total seed N content (Zhang et al., 2010). This might be due to the transport property of the AAP2 being different from the LAT5. While the AAP2 is a H^+-AA symporter (Hirner et al., 1998; Zhang et al., 2010), studies indicated a a bi-directional AA transport activity for the LAT5 modulated by the light and dark treatment (Figure 3.6; Supplementary Figure 3.1).

3.4.2. LAT5 may have a role in AA homeostasis and abiotic stress tolerance

The putative bi-directional AA transport capacity suggested a possible role for the LAT5 in the AA homeostasis. AA homeostasis plays a role in abiotic stress tolerance (Bonner et al., 1996; Martino et al., 2003; Sharma and Dietz, 2006; Wu et al., 2010). Thus, the *lat5-1* mutant line was characterized under abiotic stress conditions through analyzing the growth phenotype and free AA content. Salt stress can cause up to a 300-fold increase in the concentration of free AAs that may require cellular redistribution (Martino et al., 2003). Under salt stress, broadly specific AATs are down-regulated and specific AATs are up-regulated (Rentsch et al., 1996). Therefore, salt stress creates an ideal condition to investigate the role of the LAT5 in AA homeostasis. Since osmotic stress is

similar to salt stress in terms of AA homeostasis and it does not include the Na^+-derived ionic homeostasis, both salt and osmotic stresses were used to characterize the *lat5-1* mutant. While no noticeable growth variation was observed under osmotic stress (Figure 3.9C), *lat5-1* seedlings showed increased salt (Figure 3.9A) and drought tolerance (Figure 3.9D) compared to the WT. Transcript levels of the *LAT5* were increased in the WT seedlings grown in the presence of various concentrations of NaCl (Figure 3.9B). Free AA analysis in seedlings grown in the presence of 100mM NaCl showed a significant decrease in the concentration of Gln, Thr, Arg, Cys, Met, Ile, and Lys with a 9% decrease in the total AA content in *lat5-1* compared to WT (Table 3.2). Under the osmotic stress, *lat5-1* seedlings showed an overall increase in the free AA content compared to WT (Table 3.3). The role of the LAT5 in abiotic stress tolerance needs further studies; however, the change in the stress tolerance and in the free AA content in the *lat5-1* seedlings provides a clear indication that the LAT5 plays an important role in this process.

Table 3.1. Free AA analysis in homozygous *lat5-1* seedlings compared to WT grown on 0.5x MS medium with 1mM NO_3^- and variable concentration of L-Leucine.

1mM NO_3^-				1mM NO_3^- +1mM Leu			1mM NO_3^- + 2mM Leu		
Free AA (nmol.g^{-1}FW)	WT	*lat5-1*	%change in *lat5-1*	WT	*lat5-1*	%change in *lat5-1*	WT	*lat5-1*	%change in *lat5-1*
Asp	702	753	+7	825	866	+5	1116	1210	+8
Glu	2857	3492	+22	3764	3640	-3	3854	4867	+26
Asn	1233	1115	-10	523	496	-5	1899	2130	+12
Ser	1232	1332	+8	1350	1195	-11	2612	4437	+70
Gln	3336	2355	-29	1478	1457	-1	8278	7306	-12
His	47	36	-23	52	43	-18	160	318	+98
Gly	120	146	+21	167	157	-6	308	304	-1
Thr	397	348	-12	357	307	-14	498	610	+23
Arg	130	73	-44	387	338	-13	615	765	+24
Ala	535	522	-2	836	610	-27	861	1461	+70
Tyr	52	43	-17	123	102	-17	157	220	+40*
Val	3272	2697	-18	2653	1901	-28	3654	7545	+106
Met	56	50	-10	297	297	0	266	318	+19
Trp	50	34	-32	108	80	-26	108	197	+82
Phe	75	59	-21	265	252	-5	281	357	+27
Ile	159	109	-31	434	365	-16	723	1188	+64
Leu	159	131	-18	44514 (76% of total)	42191 (77% of total)	-5	64664	76066	+18
Lys	243	210	-14	453	329	-27	395	1037	+162*
Total	14654	13507	-8	58586	54625	-7	90772	110998	22
Total change WT Vs WT	100%			400%			619%		
Total change *lat5-1* Vs *lat5-1*		100%			404%			822%	

Free AAs were extracted from three-week-old seedlings. Each data is an average of three biological replications with two seedlings per replication. See Figure 3.5A for phenotype and growth parameters. *, P-value<0.05.

Table 3.2. Free AA analysis in the *lat5-1* seedlings compared to WT grown in the presence or absence of NaCl in the growth medium.

1xMS (1% sucrose) 0mM NaCl				1xMS (1% sucrose) 100mM NaCl		
Free AA content (nmol.g^{-1}FW)	WT	*lat5-1*	% change in *lat5-1*	WT	*lat5-1*	% change in *lat5-1*
Asp	644	574	-11	423	406	-4
Glu	1345	1438	+7	1016	1112	+9
Asn	19380	18211	-6*	16689	17236	+3
Ser	1738	1715	-1	3290	3346	+2
Gln	8639	7807	-10	10147	8778	-13**
His	576	572	-1	575	480	-17
Gly	404	396	-2	329	340	+4
Thr	395	322	-19	505	403	-20*
Arg	10160	11443	+13	10994	9945	-10**
Ala	219	186	-15*	310	315	+2
Tyr	188	184	-2	140	111	-20
Cys	305	260	-15*	289	251	-13*
Val	2223	2192	-1	2303	2096	-9
Met	51	40	-21**	53	44	-17*
Trp	275	293	+7	168	130	-23
Phe	987	1038	+5	599	474	-21
Ile	135	127	-6	128	110	-14*
Leu	822	1142	+39**	968	1023	+6
Lys	613	545	-11	603	413	-32*
Pro	4277	3791	-11	5583	3192	-43
Total	53375	52277	-2	55111	50205	-9
Total change WT Vs WT	100%			103%		
Total change *lat5-1 Vs lat5-1*		100%			96%	

Free AAs were extracted from three-week-old seedlings. Each data is an average of four biological replications with two seedlings per replication. See Figure 3.9A for phenotype and growth parameters. *, P-value <0.05; **, P-value <0.01; ***, P-value <0.001.

Table 3.3. Free AA analysis in *lat5-1* and WT seedlings grown on 1x MS medium with or without 150mM Mannitol in the growth medium to impose osmotic stress.

Free AAs ($nmol.g^{-1}DW$)	0mM Mannitol			150mM Mannitol		
	WT	*lat5-1*	% change in *lat5-1*	WT	*lat5-1*	% change in *lat5-1*
Asp	6090	8533	+40	6295	9543	+52
Glu	6987	10196	+46	11863	11639	-2
Asn	18298	20359	+11	34154	58457	+71
Ser	20741	27441	+32	17007	20662	+21
Gln	96685	107641	+11	332659	495500	+49
His	1172	1282	+9	2121	3901	+84
Gly	2695	3248	+21	6148	5795	-6
Thr	5343	6820	+28	14042	18740	+33
Arg	14804	16337	+10	10286	25804	+151
Ala	6207	7545	+22	8036	9135	+14
Tyr	425	481	+13	491	584	+19
Val	1687	1915	+14	2147	2754	+28
Met	285	315	+11	0	0	nd
Trp	126	137	+8	396	591	+49
Phe	1163	1207	+4	1110	1506	+36
Ile	678	765	+13	727	858	+18
Leu	1375	1521	+11	1024	1403	+37
Lys	1252	1391	+11	611	0	-100
Hyp	5874	7417	+26	15503	27128	+75
Pro	13571	22051	+62	48872	40538	-17
Total	205461	246603	+20	513489	734538	+43
Total change WT Vs WT	100%			250%		
Total change *lat5-1* Vs *lat5-1*		100%			298%	

For free AA extraction, three-week-old seedlings from three to four replication plates were pooled. See Figure 3.9C for phenotype and growth parameters.

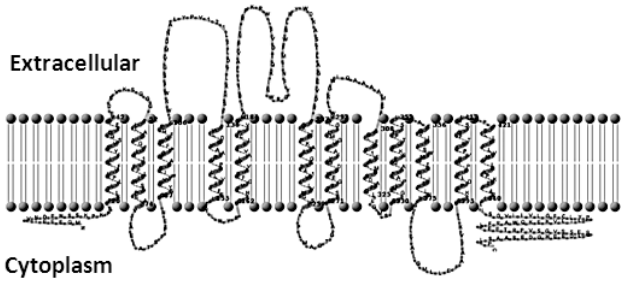


Figure 3.1: A cartoon representation of the TMHMM prediction showing 12 TMs of the LAT5 with both C- and N-terminus in the cytoplasm.

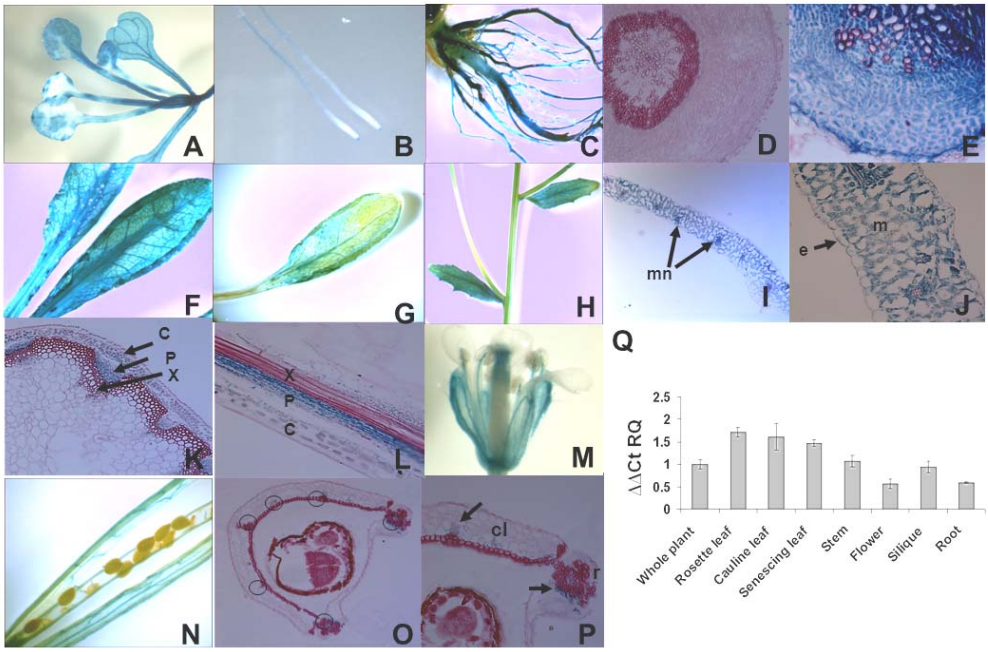


Figure 3.2. Organ- and tissue-specific expression analysis of the *LAT5*.

A. Organ-specific GUS activity under the *LAT5* promoter in three-week-old seedlings shows staining in the leaves and stem.

B. GUS activity is present in the root at the seedling stage.

C-E. In a mature root cross section, no stain is detected in the primary root (D), while the lateral root shows a blue stain almost everywhere.

F-H. Blue stain is present in the rosette leaf (F), senescing leaf (G), and cauline leaf (H).

I-J. A cross section of the rosette leaf shows GUS activity in the mesophyll cells, concentrating more in the minor veins.

K-L. A cross section of a mature stem shows GUS activity in the phloem (K). A longitudinal section of the stem confirms that a GUS stain is present in the phloem and absent in the xylem (L).

M. In the flower, GUS staining is detected in the sepal and stamen filament.

N-P. GUS activity in the silique shows the vascular pattern in the siliques valve (N). Circles in the cross section of a developing silique show the positions of the vascular tissues where GUS activity was present under the LAT5 promoter (O). A cross section of the silique shows a blue stain in the vascular tissues in the replum and in the secondary vasculature in the valve carpel cells (P).

Q. An analysis of the organ-specific *LAT5* expression through quantitative RT-PCR shows a relatively higher abundance of the *LAT5* in the leaf, stem, and silique. A relative quantitation was calculated using an average expression of the *LAT5* in the whole plant as a calibrator and Actin2 as an endogenous control. The error bar represents the average difference between the RQ_{min} and RQ_{max} of three replications.

For the GUS Histochemical assay, all samples were prepared by incubating the seedlings and matured plant in a GUS staining buffer for six hrs and overnight respectively at 37^{o}C. Samples were fixed in the FAA solution overnight before photographs were taken under a dissecting microscope. For tissue-specific expression analysis, the rosette leaf, stem, root, and silique samples were processed and embedded in wax. Thin cross sections were produced using a microtome. Samples were de-waxed and counter-stained with safranin O prior to being photographed under a light microscope. c, Cortex; cl, Carpel; e, Epidermis; mn, Minor Vein; r, Replum; P, Phloem; X, Xylem.

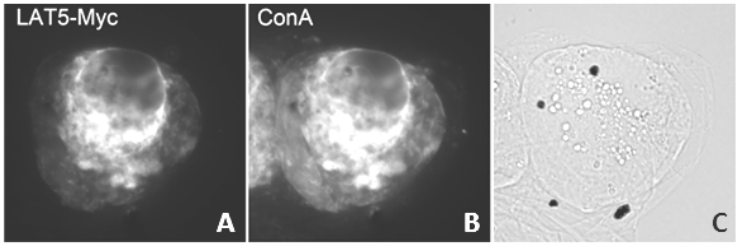


Figure 3.3. Sub-cellular localization of the LAT5.

A. The epifluorescence micrograph specific to Alexa-488, the fluorescent dye attached to the secondary anti-Myc antibody, shows the c-Myc tagged LAT5 localized in the endoplasmic reticulum in a tobacco bright yellow-2 (BY-2) cell.

B. The epifluorescence micrograph specific to the ConcanavalinA-Alexa-594 ER specific marker stain visualizes the endoplasmic reticulum in the same cell as in (A)

C. This shows the corresponding differential interference contrast (DIC) image for each cell.

The epitope-tagged *LAT5* was transiently expressed in tobacco BY-2 cells under a constitutive promoter. The transformed cells were processed for immunofluorescence staining by being incubated with a mouse anti-Myc antibody and Alexa488-conjugated goat anti-mouse antibodies. Cells were also incubated with Alexa594-conjugated Concanavalin A as a marker stain for endogenous ER.

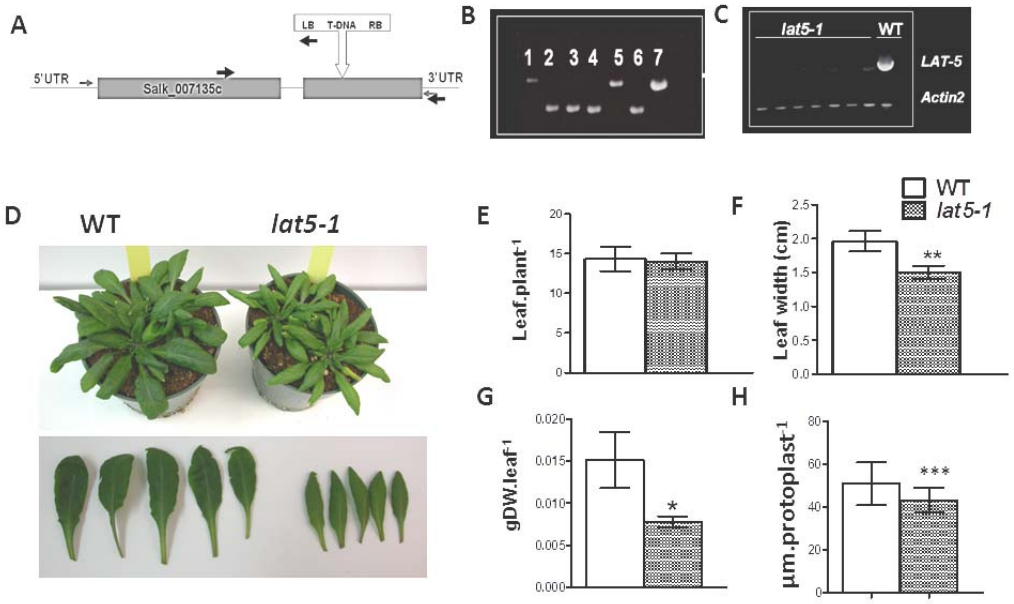


Figure 3.4. Isolation of a homozygous *LAT5* T-DNA insertion line and characterization under normal growth conditions shows reduced leaf growth in the mutant *lat5-1* plant.

A. This shows the position of theT-DNA in the second exon of the *LAT5* locus in the Salk_007135c line. The black and bold arrows indicate the position of primers used in the three-primer PCR. The grey and thin arrows indicate the position of primers used in RT-PCR to confirm the absence of the functional size of the *LAT5* transcript in the Salk line.

B. The three-primer PCR shows the WT (lane 1, 5 and 7) and homozygous (lane 2, 3, 4 and 6) plants for T-DNA insertion in the *LAT5* locus.

C. The RT-PCR shows an absence of transcript in the *lat5-1* homozygous mutant line.

D. The homozygous *lat5-1* mutant plants show reduced leaf width under normal growth condition on fertilized soil. Both genotypes were grown in the same flat with similar treatment of fertilizer and watering. Each genotype had three pots with three plants per pot. Photos were taken when the plants were five weeks old.

E. The average number of leaves per plant is not different in the *lat5-1* and the WT.

F. The width of the rosette leaves in the *lat5-1* is significantly smaller compared to that in the WT. Each bar represents an average width of five random mature rosette leaves taken from the second whorl of three different plants per genotype. The error bar represents SD. The leaf width was measured at the point where the leaf blade showed a maximum width.

G. A single leaf biomass of the *lat5-1* is significantly reduced compared to the WT. Mature rosette leaves were cut off and dried at 70^{o}C for two hrs before being weighed. The error bar represents SD of five replications.

H. The diameter of leaf mesophyll cell protoplasts in the *lat5-1* is significantly smaller compared to that of the WT. The error bar represents SD. For each genotype, the diameter of 100 cells was measured from five photographs of three individual plant protoplast cultures.

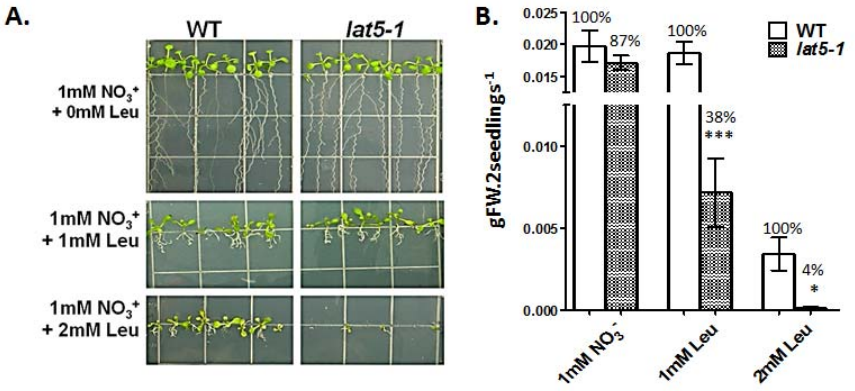


Figure 3.5. Characterization of the *lat5-1* mutant seedlings in the presence of L-Leucine in the growth medium.

A. The homozygous *lat5-1* shows an increased sensitivity to exogenous Leu compared to the WT. The variable concentration of Leu was added to 0.5xMS medium containing 1mM NO_3^- as the sole source of N. Each growth condition had three or more replications. The experiment was repeated with similar results.

B. Quantitative data shows a >95% growth reduction in the *lat5-1* seedlings compared to the WT in the presence of 2mM of Leu in the growth medium. For each growth condition, the biomass obtained by the WT was taken as 100% to calculate the relative growth of the *lat5-1*. The error bar represents SD of 3-6 biological replications with two seedlings per replication. *, P-value <0.05; ***, P-value <0.001.

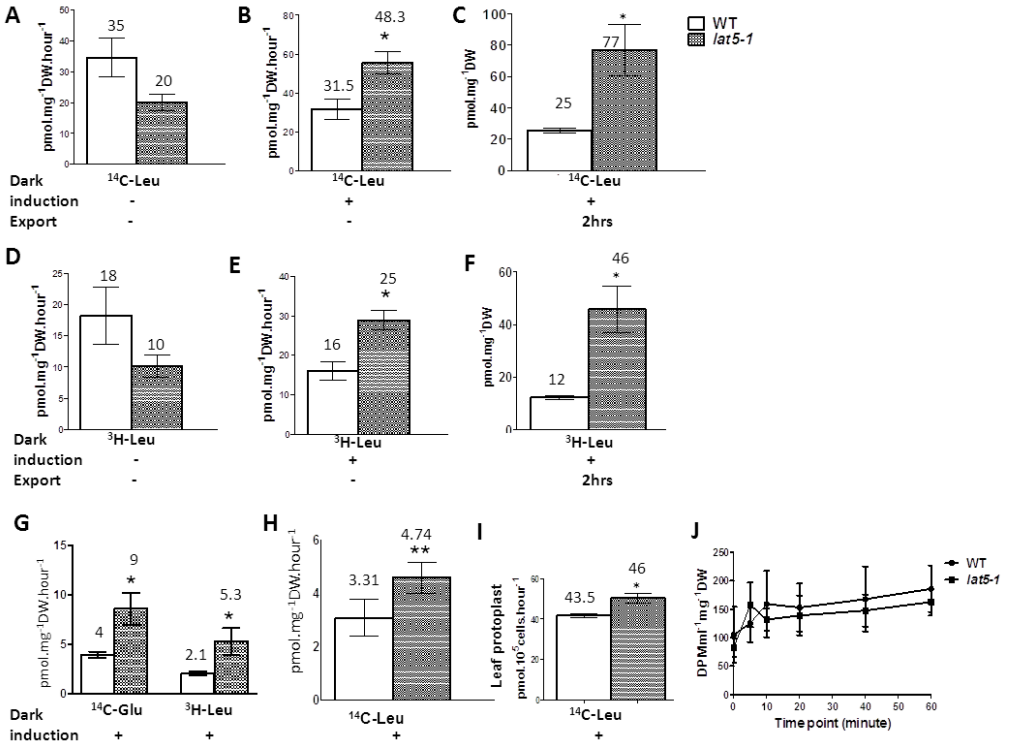


Figure 3.6. Radio-labeled AA uptake studies show a decreased accumulation in normal conditions and an increased accumulation when seedlings are treated in the dark in the *lat5-1* seedlings compared to WT.

A. In a ^{14}C-Leu uptake study, soil-grown normal seedlings show a reduced accumulation of ^{14}C- in the *lat5-1* compared to WT.

B. In a ^{14}C-Leu uptake study, soil-grown seedlings treated for 24 hrs in the dark before being used in the study show an increased accumulation of ^{14}C- in the *lat5-1* seedlings compared to WT.

C. Dark-treated seedlings (as in B), after two hrs of ^{14}C-Leu uptake followed by two hrs of export, show an increased accumulation of ^{14}C- in *lat5-1* compared to WT.

D. In a ^{3}H- Leu uptake study, soil grown normal seedlings show a reduced accumulation of ^{3}H- in *lat5-1* compared to WT.

E. In a ^{3}H- Leu uptake study, soil grown seedlings treated for 24hrs in the dark show an increased accumulation of ^{3}H- in the *lat5-1* compared to WT.

F. Dark-treated seedlings (as in E), after two hrs of ^{3}H- Leu uptake followed by two hrs of export, show an increased accumulation of ^{3}H- in the *lat5-1* compared to WT.

G. In a mixed uptake study using ^{14}C-Glu and ^{3}H-Leu, overnight dark-treated soil-grown seedlings show an increased accumulation of both ^{14}C- and ^{3}H- in the *lat5-1* compared to WT.

H. Seedlings treated in the dark and starved for nitrogen for 24 hrs show an increased accumulation of ^{14}C- in the *lat5-1* compared to WT.

I. Leaf mesophyll cell protoplasts produced from dark-treated plants show an increased accumulation of ^{14}C- in the l*at5-1* cells compared to WT.

J. The ^{14}C-Leu export study shows no significant variation between WT and the *lat5-1* mutant. Plate-grown seedlings were starved for light and N for overnight before being used. The error bar represents the SD of three biological replications with three seedlings per replication.

A "+" or "-" sign indicates the presence or absence of a treatment. The error bar represents SD of three to five biological replications. *, P-value <0.05; **, P-value <0.01; ***, P-value <0.001.

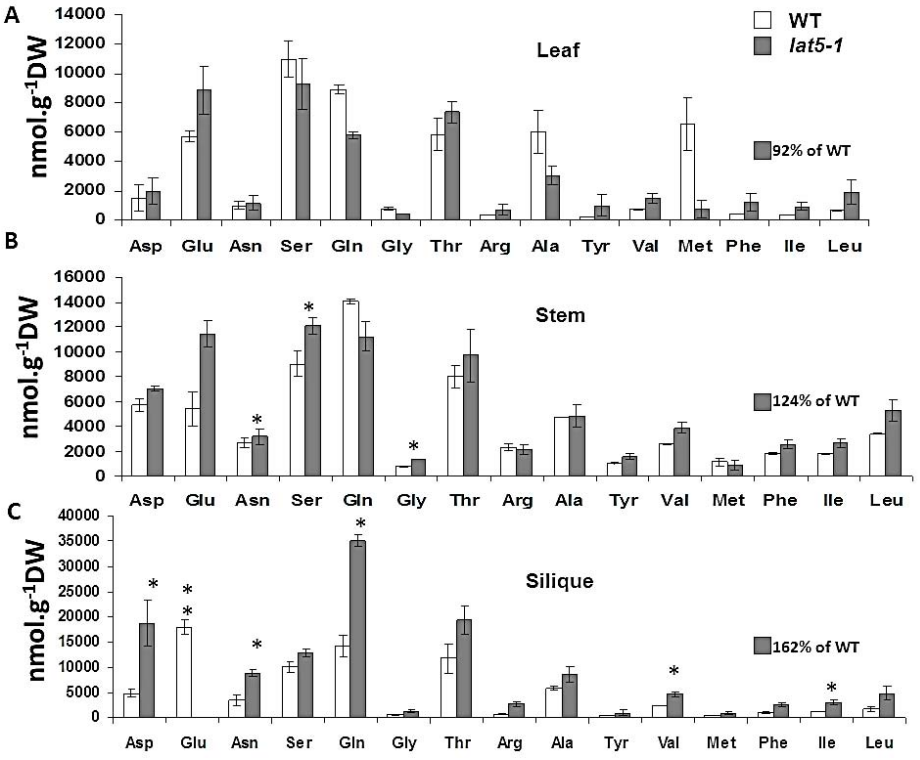


Figure 3.7. Organ-specific free AA analysis in mature plants.

A-C. The analysis of free amino acids in leaves (A), stem (B), and silique (C) shows an altered distribution in all organs of the *lat5-1* compared to the WT. The total free AA concentration increased in both the stem and silique and decreased in the leaves in the *lat5-1* compared to the WT. For each organ, the total AAs in the the WT was arbitrarily taken as 100%, and the % change in the *lat5-1* was calculated accordingly. The error bar represents the SD of three biological replications. *, P-value <0.05; **, P-value <0.01.

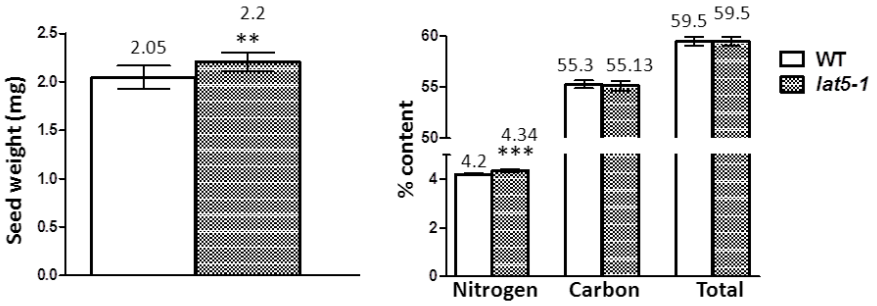


Figure 3.8. An analysis of the total C and C content in seeds.

A. The seed weight of the *lat5-1* is significantly increased compared to that of the WT. Mature and dried seeds were weighed using a delta-range microbalance. The error bar represents the SD of nine replications with 100 seeds per replication. **, P-value <0.01.

B. The percent of N content in the *lat5-1* seeds is significantly increased compared to the WT, while the variation in the C content and total N and C content is nonsignificant. The error bar represents the SD of nine replications with 100 seeds per replication. ***, P-value <0.001.

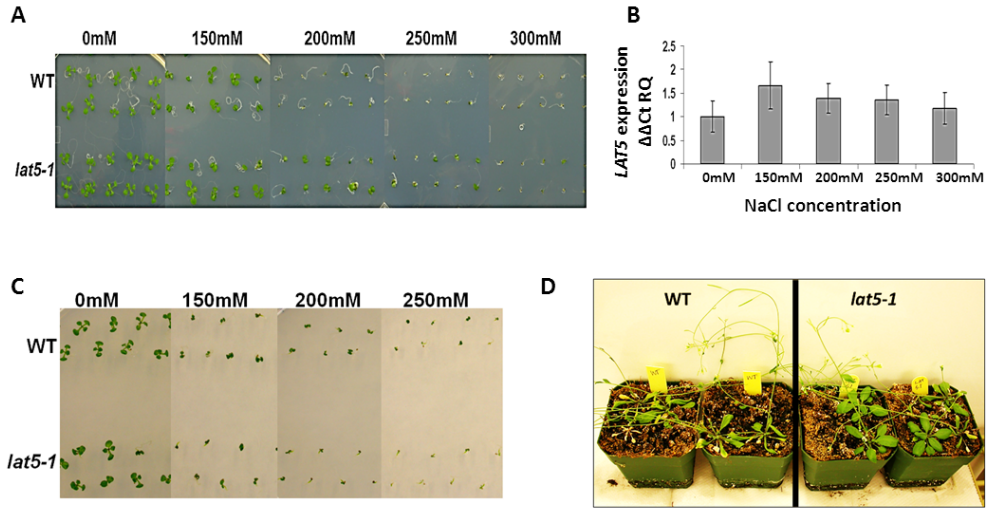


Figure 3.9. Characterization of the mutant *lat5-1* under abiotic stresses.

A. The homozygous *lat5-1* mutant shows increased salt stress tolerance compared to the WT in a full-strength MS medium containing a variable concentration of NaCl. Photos were taken when the seedlings were two weeks old. For each growth condition, both genotypes were grown on the same plate with 3-5 plate replications.

B. Quantitative (RT)-PCR shows a relatively higher abundance of the *LAT5* transcript in the WT seedlings under a variable concentration of NaCl compared to that in 0mM NaCl. The total RNA was extracted from WT seedlings shown in panel A. The expression level in 0mM NaCl was used as a calibrator and Actin2 was used as an endogenous control. The error bar shows the difference between the RQ_{min} and RQ_{max} of three biological replications.

C. The homozygous *lat5-1* mutant shows no noticeable growth variation under osmotic stress compared to the WT on a complete MS medium supplemented with a variable concentration of mannitol. Photographs were taken when the seedlings were two weeks old. Each growth condition had three or more replications.

D. On soil, the *lat5-1* plants show an increased drought-tolerance compared to the WT. Plants were grown on fertilized soil under normal growth parameters. When they were approximately one month old, one spell of watering was withdrawn to impose drought conditions. While the WT plants wilted completely, the *lat5-1* leaves remained turgid.

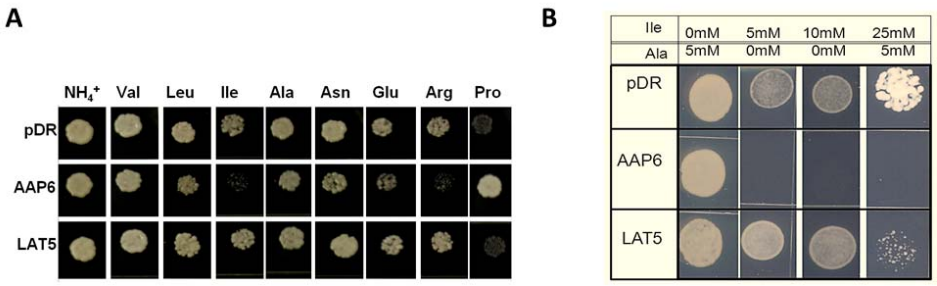
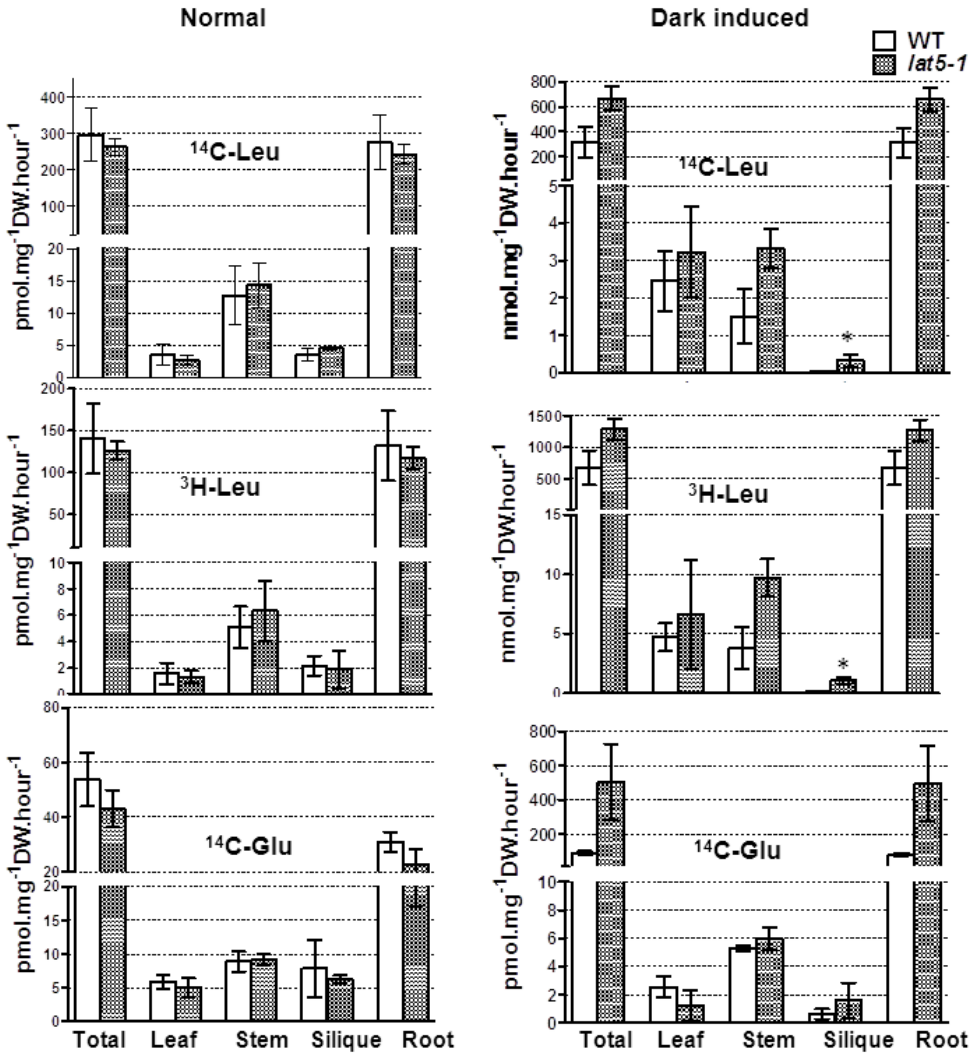


Figure 3.10. Characterization of the LAT5 in a unicellular 22Δ8AA yeast system.

A. The 22Δ8AA yeast strain that was transformed with the *LAT5* did not promote growth variation compared to the 22Δ8AA strain containing the pDR-empty vector on plates containing 5mM of various AAs. The AAP6 mediated the reduced growth compared to the empty vector on plates containing Arg and Ile.

B. In the presence of 25mM of Ile and 5mM of Ala in the growth medium, yeast cells complemented with the LAT5 show reduced growth compared to the pDR-empty vector, while the AAP6 completely inhibited the growth.

The 22Δ8AA strain complemented with a cDNA clone of the *LAT5* was grown on a minimal BA medium supplemented with a variable concentration of AAs as the sole source of N. The 22Δ8AA strain complemented with an empty vector or Arabidopsis AAP6 was used as negative and positive controls respectively. One hundred cells were plated for each genotype. The plates were incubated at 30^0C for three to five days. Each plate had six replications for each genotype.



Supplementary Figure 3.1. ^{14}C-Glu and dual-labeled Leu uptake analysis using mature plants.

Soil-grown mature plants show an increased accumulation of ^{14}C-Leu, ^{3}H-Leu, and ^{14}C-Glu in the *lat5-1* when treated overnight in the dark, while plants not treated in the dark show a decreased accumulation in the *lat5-1* compared to the WT. The change was found to be consistent for ^{14}C-Leu, ^{3}H-Leu, and ^{14}C-Glu. The error bar represents the SD of the three biological replications.

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4. Over-expression of the Arabidopsis L-type amino acid transporter 1 (LAT1) changes the C/N ratio and N content in the seeds.

4.1. Introduction

Crop plants primarily use nitrate (NO_3^-) or ammonium (NH_4^+) as their source of nitrogen (N), however, some species of plants (particularly those living in cooler climates), often use a significant amount of organic N, taken up as amino acids (AAs). Soils have large reservoirs of organic N which is more stable than inorganic forms. Over 90% of the N in the surface layer of many soils occurs in an organic form (Schulten and Schnitzer, 1998), and up to 50% of the total soil N can be in the form of AAs (Senwo and Tabatabai, 1998). In nature, plants take up organic N directly from soils with very slow mineralization rates, such as the soils of boreal forests, arctic tundra or alpine regions (Meline and Nilsson, 1953; Nasholm et al., 1998). In controlled growth conditions, crop plants can also use AAs as a source of N. For example, for maize (*Zea mays*), AAs can make up to 30-90% of the total N requirement (Jones and Darrah, 1994). Rice (*Oryza sativa*) can also take up AAs at a rate comparable to or greater than inorganic N (Yamagata and Ae, 1996).

AAs, in addition to being an important source of N in the soil for many plant species, are one of the main ways in which N is distributed among the sources and sinks within a plant. Inorganic N is assimilated into AAs via the GS/GOGAT pathway, and these AAs then serve as N carriers in the translocation pathway (Lam et al., 1995), whereas AAs acquired from the soil may directly enter the translocation stream. Plants have very complex AA uptake and translocation systems involving a large number of integral membrane transporter proteins that mediate the transport and distribution of AAs from source to sink tissue. To date, 67genes have been identified in Arabidopsis that encode putative amino acid transporters (AATs) (Rentsch et al., 2007). The two major super families of AATs in Arabidopsis are: the Amino-acid Transporter Family 1 (ATF1) and the Amino-acid Polyamine Choline (APC) transporters (Fischer et al., 1998; Wipf et al., 2002). The APC super family includes Cationic Amino-acid Transporters (CATs), γ-Amino

Butyric Acid (GABA)-permeases and L-Type Amino-acid Transporters (LATs) (Fischer et al., 1998). While the function of CATs and GABA-permeases are relatively well characterized, the function of LATs remained unknown in the plant system (Wipf et al., 2002).

The functional characterization of AATs in plants is important for a variety of reasons. At a practical level, most of the research on Nitrogen Use Efficiency (NUE) has focused on the efficient use of inorganic N. However, the ability to manipulate the efficiency of a plant to use organic N in the soil may also be an important component of improving NUE. As well, many of the AATs have not been functionally characterized and therefore their role in N translocation in plants remains unclear. Most of the characterized AATs function as importers while AATs having export capacity remained to be identified in plants (Wipf et al., 2002; Lalonde et al., 2003; Pratelli et al., 2009; Okumoto and Pilot, 2011). This chapter presents the functional characterization of the Arabidopsis LAT1 (At5g05630), one of the five members of the LAT family in the APC super family of *Arabidopsis thaliana*.

4.2. Materials and Methods

4.2.1. *In-silico* analysis

The *in silico* analysis of the LAT1 protein sequence for homology search, transmembrane domain and conserved domain prediction and cartoon representation was performed as described in Chapter 2. The AA sequences of the LATs were taken from the Arabidopsis plant membrane protein database (http://aramemnon.botanik.uni-koeln.de/) and the human y+LAT1 and y+LAT2 were taken from the NCBI.

4.2.2. Construction of DNA plasmid vectors

The expression vector, containing a C-terminal Myc-epitope tagged *LAT1*, was constructed through the following steps. The *LAT1* ORF was amplified through a one-step reverse transcriptase PCR (RT-PCR) using *SuperScript III* One-Step RT-PCR with a Platinum *Taq* High Fidelity (*Invitrogen*) kit and *DNAse*-treated total RNA extracted from multiple seedlings of the WT as a template. Appropriately designed forward and reverse primers to add an *XbaI* site prior to the start codon and an *NheI* site to

replace the stop codon were used in the PCR program (see Table 4.1 for the primer sequence). The PCR product was electrophoresed in a 1.0% agarose gel and visualized with ethidium bromide to check the amplicon and then phenol: chloroform purified. The pure PCR product was sub-cloned into XbaI/NheI digested pRTL2/myc-MCS to obtain pRTL2/LAT1-myc. The final expression vector with the LAT1-myc reading frame, driven by the Cauliflower Mosaic Virus 35S promoter (CaMV35S), was confirmed through PCR, restriction analysis, and sequencing. For yeast complementation studies, the *LAT1* ORF was amplified through a one-step RT-PCR as mentioned above. Forward and reverse primers with added *EcoRI* and *SalI* sites respectively in the 3' and 5' end were used in the PCR. The purified and digested PCR product was cloned into the yeast expression vector pDR196 (Rentsch et al., 1995) between *EcoRI* and *SalI* in the MCS. The final expression vector was confirmed by PCR, restriction digestion and sequencing.

4.2.3. Quantitative RT-PCR for organ-specific expression analysis

The total RNA extraction, processing and organ specific quantitative RT-PCR (qRT-PCR) was carried out as described in Chapter 2. TaqMan Gene Expression Assay (assay ID: At02181748_sI for *LAT1*; assay ID: At02335270_gH for Actin2; assay ID: At02337969_g1 for *EF1α*) was used in PCR amplification. Actin2 was used as the endogenous control and the expression level in whole plant tissue was used as calibrator during analysis.

4.2.4. Isolation of the *LAT1* over expression line

The *LAT1* T-DNA insertion line (Salk_053859), which has been shown to over express LAT1 (See details in Section 4.3.4) was isolated from the Salk T-DNA insertion collection of the Arabidopsis Biological Resource Centre (Alonso et al., 2003), using the PCR screening scheme described in the Signal Salk webpage (http://signal.salk.edu/). One primer specific to the left border sequence of the T-DNA, LBb1, and two primers specific to the *LAT1* sequence were used to screen for plants homozygous for the T-DNA insertion in the *LAT1* locus. The position of the T-DNA in the 5'UTR (Untranslated Region) of the *LAT1* was confirmed through aligning the gene-specific primers used in

three-primer PCR (designed by the SIGNAL iSECT tool available in the Signal Salk web resource) and sequencing the product flanking the T-DNA. RT-PCR was carried out to determine the presence or absence of *LAT1* transcript in the Salk line. A quantitative RT-PCR (qRT-PCR) was carried out to confirm the over expression of *LAT1* in the T-DNA insertion line. Total RNA extracted from source leaves was used in the reaction. Primers specific to the 5' and 3' UTR were used in both RT-PCR and qRT-PCR. The *LAT4* was used as the positive control. In the qRT-PCR, 1.6µg of total RNA from each genotype was used in the first reaction vial. A serial dilution of the template was produced in the subsequent reaction vials as shown in the figure (Figure 4.3C). A one step RT-PCR kit supplemented with additional *reverse-transcriptase* enzyme was used in the RT- and qRT-PCR reactions.

4.2.5. Radio-labeled AA uptake and export analysis

For the ^{14}C-Leu uptake study 25-days old seedlings of the OXLAT1 and WT, grown on 0.5x MS (Murashige and Skoog) medium (3mM NO_3-, 1% sucrose, pH 5.7) were used. Seedlings grown on square plates in a vertical orientation were transferred to a liquid MS medium (0.5xMS, 1% sucrose, pH 5.7) and acclimatized overnight at room temperature (RT). Seedlings were then grown in a starvation medium (0.5xMS without N, 1% sucrose, pH 5.4) overnight. A 0.5x liquid MS medium containing 10µM Leu (^{14}C-Leu and cold Leu) was used as uptake medium. Seedlings removed from the uptake medium were rinsed three times in sterile water for approximately 10 minutes. Roots and shoots were cut separated and placed directly in the scintillation vial. Shoot tissues were dried at 70^{o}C for two hrs and weighed.

For the mature plant uptake studies, plants at reproductive stage were removed from the soil and washed before use. ^{14}C-Glu, ^{14}C-Leu or ^{3}H-Leu was added to the uptake medium (liquid MS without N and C, pH 5.4) with cold AA to a final concentration of 10µM. Uptake studies were carried out at room temperature and light for two hrs. Samples were dried at 70^{o}C overnight and weighed. Samples were digested with bleach and prepared with HIONIC FLUOR (Perkin Elmer) for counting the radioacitivity in DPM in a liquid scintillation counter (Beckman).

For the export study, soil grown seedlings were fed with ^{14}C-Leu for two hrs followed by a two hrs export. Feeding the seedlings with ^{14}C-Leu was carried out as described above. Roots of the seedlings were removed from the uptake medium, rinsed with sterile water for five minutes before dipping into the export medium. Half-strength liquid MS without N and C was used as the export medium. At each time point, 50µl of the export solution was transferred to a glass scintillation vial to measure the radioactivity released by the seedlings. See Chapter 2 for the detail information about the radio-labeled AA stocks, volume of the stock used, and the calculation to obtain concentration of AAs (pmol) from the DPM of radionuclide in samples.

4.2.6. Analysis of free AAs through HPLC

Free AAs were extracted using methanol: chloroform extraction buffer. Fresh samples were weighed and ground in liquid N with 1.5ml of methanol: chloroform: water (65:25:10) buffer containing 20pmol.µl^{-1} of internal standard (Norvaline). The extraction procedure was same as described in Chapter 2. Valine was suspected to co-elute with an unknown AA giving unusually higher concentration in all plant tissue samples. Thus, this AA was excluded in the data interpretation and discussion.

4.2.7. Analysis of the C and N content in mature seeds

Mature and dry seeds were harvested from the OXLAT1 and WT plants grown under normal condition. The analysis of seed weight, and total N and C content was performed as described in Chapter 3.

4.2.8. Yeast Complementation

The yeast strain 22Δ8AA (Tanaka and Fink, 1985) was transformed with three independent cDNA clones of the *LAT1* in the expression vector pDR196 (Rentsch et al., 1995). The yeast strain 22Δ8AA was also transformed with a cDNA clone of the *AAP6* in pDR195 (Rentsch et al., 1995) and an empty pDR196 expression vector. The transformation was carried out using a Yeast Transformation Kit (Sigma) as per the manufacturer's instructions. The transformants were selected on a synthetic complete (drop out) medium lacking uracil and confirmed through colony PCR using gene and vector specific primers. Liquid cultures of the PCR positive colonies were grown

until the log phase. The cell concentration was counted using a haemocytometer. Cell cultures were diluted to a concentration of 20cells.μl^{-1}. Cells were grown on BA plates containing Leu, Ile, Val, Glu, Arg, Ala and Pro at a 5 mM concentration as the sole source of N. In a square plate, each genotype had three replications with 200 cells per replication. Plates were incubated at 30^{o}C for three days prior to taking photographs. As a second step, 5mM of the same AA (dissolved in liquid BA medium with 5mM $(NH_4)_2SO_4$ without uracil) was added to each BA plate. Plates were dried under a laminar flow. The same cell cultures produced above were plated using a volume of 10 µl per replication. Each genotype had three replications.

4.2.9. Subcellular localization

For the sub-cellular localization study, tobacco BY-2 cells were transformed transiently with pRTL2/myc vector with the *LAT1* having a Myc-epitope in the C-terminus followed by immune-fluorescence micrography This experiment was conducted in the Mullen lab at the University of Guelph as described in Chapter 2.

4.2.10. Plant Growth

The Columbia-0 ecotype of Arabidopsis was used in all studies. All plants (unless otherwise mentioned) were grown in well aerated fertilized soil in a growth chamber under 16/8 hrs of light and dark regime at 23^{o}C temperature. For the phenotypic studies and AA uptake studies, seedlings were grown in 0.5xMS media (1% sucrose, 1% agar, pH 5.7) with variable sources and concentrations of N as specified in the text. See Chapter 2 for the type of soil and fertilizer used for plant growth. All data analysis and significance tests were conducted using PRISM. A two-way T-test was performed for statistical significance test.

4.3. Results

4.3.1. *In silico* analysis of the LAT1

Within the Arabidopsis family of LATs, the LAT1 is closer to the LAT3 and LAT4 with 63% and 67% sequence similarity respectively at the protein level. The LAT1 is a 490AA long protein with a 53.7KDa molecular

weight and contains no intron in the coding region. An analysis of the transmembrane (TM) domain using TMHMM v.2.0 predicted 12 TM domains in the LAT1 protein with both N- and C-termini in the cytoplasm (Figure 4.1). An NCBI conserved domain search identified putative amino acid transporter, fructose/lysine transporter, arginine/agmatine antiporter, basic amino acid/polyamine antiporter, L-type amino acid transporter, glutamate/GABA antiporter domains conserved in the LAT1 protein sequence. The Arabidopsis interactions viewer predicted the LAT1 to interact with a plasma membrane cytosolic HEAT-SHOCK PROTEIN90 (At5g52640).

4.3.2. *In silico* comparison of the Arabidopsis LATs with animal LATs

A PSI-BLASTp search was performed with the AA sequence of the Arabidopsis LAT1 against the human genome to compare Arabidopsis LATs with the well-characterized human LATs. The BLASTp search found the y+LAT1 (NP_001119578) and y+LAT2 (NP_001070253) to be the most similar transporters.. In humans, y+LATs exchange Na^+-coupled neutral AAs against cationic AAs while LATs exchange neutral AAs without any ion coupling at a 1:1 stoicheiometry (Torrents et al., 1998; Pfeiffer et al., 1999; Verrey et al., 2000). Human LATs and y+LATs have a conserved cysteine residue in the extra-cellular loop between the third and fourth TM, which has been shown to interact with a glycoprotein (Verrey et al., 2000), whereas this residue is absent in the Arabidopsis LATs. However, the Arabidopsis interaction viewer does not show an interaction of any of the LATs with a glycoprotein. The Arabidopsis LATs share <15% sequence similarity with human LATs while LATs in animal species share at least 40% sequence similarity at the protein level.

4.3.3. Expression analysis of *LAT1*: expressed in all organs with relatively higher abundance in cauline leaves

The public database, Arabidopsis eFP Browser, showed the *LAT1* expression in leaves, stem and flower. An organ specific quantitative RT-PCR was carried out to determine the relative abundance of the *LAT1* in different organs of Arabidopsis plants at mature stage. The expression level of the *LAT1* in the whole plant was set as 1.0 and the relative abundance in various organs

was based on a comparison with this level of expression. The transcript of the *LAT1* was detected in all organs with a relatively higher abundance in the green leaves, flower and silique (Figure 4.2). In the cauline leaves, the *LAT1* transcript level was 3.4 times higher compared to the average expression in the whole plant. The microarray analysis for the tissue specific expression of the *LAT1,* published in the Arabidopsis eFP Browser, showed *LAT1* expression in the mesophyll and guard cells (Yang et al., 2008), stem epidermis (Suh et al., 2005) and ovary tissues (Swanson et al., 2005). Another study reported in the same database showed that the *LAT1* is expressed in the lateral root cap under low N conditions and is suppressed in response to high N concentrations in the root zone (Gifford et al., 2008).

4.3.4. Isolation and characterization of the OXLAT1 on various sources of N: OXLAT1 shows a reduced biomass and altered free AA content

In an attempt to isolate a *LAT1* knockout mutant, only one Salk line (Salk_053859) was identified from the Arabidopsis Biological Resource Centre with the T-DNA inserted in the 5'untranslated region (UTR), 160bp upstream from the translation start codon, of the *LAT1* locus (Figure 4.3A, B). While RT-PCR showed the presence of *LAT1* transcript in the Salk line, seedlings of this line showed reduced biomass and an altered free AA content in the presence of NO_3^- or AA in the growth medium (Table 4.2). Given the results of the phenotypic study and the free AA analysis, a quantitative RT-PCR was carried out to determine if the *LAT1* transcript level was reduced in the Salk_053859 line. The Salk_053859 line showed a higher abundance of the *LAT1* transcript compared to WT (Figure 4.3C). Since the T-DNA contains a CaMV35S promoter driving the antibiotic resistance gene and is inserted upstream of the start codon (5'UTR) of the *LAT1*, it is possible that the constitutive promoter enhanced the expression of the *LAT1* gene. The Salk_053859 line appears to be an over-expressing line instead of a knock down line of the *LAT1*. This line was designated as the OXLAT1 in the subsequent studies.

In the presence of 2mM NO_3^-, Asp or Glu as the sole source of N, the OXLAT1 showed a 15%, 23.3% and 26.4% reduction in biomass compared to

WT, respectively (Table 4.2). Free AAs were extracted from seedlings to compare the AA profile between the OXLAT1 and WT seedlings. While the OXLAT1 showed a 9% reduction in the total free AA content in the presence of 2mM NO_3^-, it showed a 24% and 13% increase in the concentration of free AAs on 2mM Asp and Glu respectively compared to WT (Table 4.2). Both the WT and OXLAT1 seedlings showed an increase in the total free AA content when grown on Asp or Glu as a sole source of N (compared to seedlings grown on NO_3^-), but the change was greater in the OXLAT1. The WT seedlings showed a 65% and 128% increase in the total free AA content on Asp and Glu, respectively, compared to that grown on NO_3^- while it was 123% and 180%, respectively, in the OXLAT1 (Table 4.2). On 2mM NO_3^-, the OXLAT1 had 3% and 14% less Asp and Glu, respectively, compared to WT, while it was 10% and 25% more compared to WT when grown on the respective AA as a source of N (Table 4.2). The OXLAT1 showed a significant increased concentration of Gly (23%), Tyr (22%), Trp (19%), Ile (20%) and Pro (44%) on Asp; and Glu (25%), Asn (31%), His (31%), Met (31%) and Leu (20%) on Glu compared to WT (Table 4.2).

4.3.5. The OXLAT1 line shows an increased toxicity to exogenous leucine

The OXLAT1 seedlings were grown with WT on plates with 3mM NO_3^- and 0mM, 1mM or 2mM L-Leu as sources of N. The OXLAT1 line showed a significant reduction in biomass (61%) compared to WT in the presence of NO_3^- as a source of N (Figure 4.3D, E). Adding 1mM of Leu to the growth medium caused a further reduction in the OXLAT1 biomass while the WT showed no reduction (Figure 4.3E). In the presence of 1mM of Leu, the OXLAT1 showed a significant reduction in biomass (85%), compared to WT (Figure 4.3D, E). In the presence of 2mM of Leu, both the WT and OXLAT1 showed growth reduction; however, the OXLAT1 seeds barely germinated and showed a 94% reduction in biomass compared to WT (Figure 4.3D, E). The sensitivity of the OXLAT1 to Leu was not affected by the concentration of NO_3^- (1mM, 2mM or 3mM) supplement in the growth medium (data not shown). To determine whether the OXLAT1 acquired more Leu from the growth medium resulting in an increased toxicity, a free AA analysis was conducted on seedlings grown in the presence of 1mM of Leu.

Both the WT and OXLAT1 seedlings showed a noticeable increase in the Leu content, however, the OXLAT1 seedlings had less accumulation of Leu compared to WT (Table 4.3).

4.3.6. OXLAT1 is not affected by the low C conditions

The Arabidopsis LAT4 and LAT5 functionality had been shown to be influenced by the low C conditions. In order to test if the OXLAT1 shows growth variation under low C, seedlings were grown on a 0.5xMS medium with or without a sucrose supplement. On the complete MS medium with 1% sucrose, the OXLAT1 showed a significant reduction in biomass and root length (45% and 21% respectively) compared to WT (Figure 4.4A-C), which is consistent with the previous phenotypic studies (Table 4.2; Figure 4.3D, E). In the absence of sucrose in the growth medium, a significant reduction in biomass and root length was observed in both the WT (30%, 39%) and OXLAT1 (69%, 55%) compared to WT grown in the presence of sucrose. In the absence of sucrose, the OXLAT1 did not show a reduction in the root length, while the biomass was significantly reduced compared to WT (Figure 4.4A-C). An analysis of free AAs was carried out in the shoots and roots of seedlings grown without sucrose in the growth medium. While the OXLAT1 showed an 8% and 20% increase in the free AA-content in the shoots and roots respectively, no significant variation was observed in the content and composition of free AAs compared to WT (Table 4.4).

4.3.7. OXLAT1 shows a net reduced accumulation of ^{14}C-Leu and ^{14}C-Glu

In the presence of exogenous Leu in the growth medium, the OXLAT1 showed increased toxicity and decreased accumulation of Leu (Figure 4.3D,E and Table 4.3), while, in the presence of Asp or Glu, it showed a decreased biomass and increased accumulation of Asp and Glu compared to WT (Table 4.2). Thus both Leu and Glu were used in the *in planta* uptake studies. In the ^{14}C-Leu uptake study at the seedling stage, the OXLAT1 showed a reduced accumulation of ^{14}C- compared to WT (Figure 4.5A). Another study was conducted using mature plants to investigate the organ specific accumulation of Leu or Glu. A mixture of ^{14}C-Leu and ^{3}H-Leu was used to trace both the C-

skeleton and $–NH_2$ group of the Leu in the plant. In the ^{14}C-Leu, the radionuclide was located in the alpha-C, and in the ^{3}H-Leu, the radionuclide was located in the $–NH_2$ group. At the mature stage, the OXLAT1 showed a reduced accumulation of both ^{14}C- and ^{3}H- in the whole plant (Figure 4.5B, C). However, for specific organs, the OXLAT1 line showed a decreased accumulation of both ^{14}C- and ^{3}H- in the leaves and roots while it showed an increased accumulation in the stem and silique compared to WT. In the ^{14}C-Glu uptake study, the OXLAT1 showed a reduced accumulation of ^{14}C- in the whole plant compared to WT (Figure 4.5D). The OXLAT1 also showed a significantly decreased accumulation of ^{14}C- in the leaves and a significantly increased accumulation in the silique compared to WT (Figure 4.5D). Both Leu and Glu uptake studies showed a similar distribution of ^{14}C- and ^{3}H- in the whole plant as well as at the organ level.

4.3.8. The OXLAT1 line mobilizes more radionuclide compared to WT

A Leu export analysis was conducted to investigate whether the OXLAT1 shows an increased Leu export over time. A mixture of ^{14}C-Leu and ^{3}H-Leu was used to trace the Leu including the C-skeleton and $–NH_2$ group in the study. The OXLAT1 seedlings, previously fed with ^{14}C-Leu or ^{3}H-Leu, released more ^{14}C- and ^{3}H- compared to WT (Figure 4.6). The elevation of the intercepts for the OXLAT1 was significantly higher compared to WT for both ^{14}C- (P-value <0.001) and ^{3}H- (P-value <0.001) release.

4.3.9. OXLAT1 shows decreased N content and altered C/N ratio in seeds

The OXLAT1 plants grown on soil under optimum growth conditions and in the presence of sufficient NO_3^-, showed a significant increase in seed weight (12%) compared to WT (Figure 4.7A). The analysis of total N and C in the seeds showed a significant decrease (6%) in the N content in the OXLAT1 seeds compared to WT while the C content did not differ (Figure 4.7B). The average C/N ratio in nine replications with 100 seeds per replication was 14.01 and 13.17 in the OXLAT1 and WT respectively, showing a significant (P-value <0.001) increase in the C/N ratio in the OXLAT1 compared to WT.

4.3.10. Sub-cellular localization

The *in silico* analysis suggested that the LAT1 may localize to the PM, chloroplast membrane, or extra-cellular. With a Myc-epitope tagged in the C-terminus, the *LAT1* was transiently expressed in tobacco BY-2 cells to determine the intra-cellular localization of the translated product. The LAT1 remained in the endoplasmic reticulum (ER) in these cells (Figure 4.8A). In the neighbouring non-transformed cells, visible in Figure 4.8B but not visible in Figure 4.8A, there is no immuno-fluorescence attributable to Myc. This indicates that the indirect immuno-fluorescence technique is specific to the ectopically expressed LAT1-myc protein. In the epi-fluorescence micrographs, it seems to cause a change in the morphology of the ER, as evidenced by the difference in the ConcanavalinA-stained ER in the LAT1-Myc transformed cell compared to the neighboring non-transformed cells (Figure 4.8B). This change in the ER morphology is typical for most of the transiently expressed membrane proteins that localize temporarily to this compartment. The ER-located proteins typically possess two conserved Lysine residues at the -3 and -5 positions relative to the C-terminus (Benghezal et al., 2000; McCartney et al., 2004). A motif search of the C-terminus of LAT1 did not identify any 'di-lysine' motif. It is possible that the C-Myc epitope is blocking the ER-retrieval signal peptide, preventing it from interacting with other factors or from properly folding for further localization. As per the Arabidopsis Interactions Viewer (BAR), the LAT1 interacts with a Heat Shock Protein 90 (HS90, At5g52640) that localizes to the plasma membrane. It suggests that the LAT1 may also localize to the plasma membrane and that this HS90 may have a role in the folding and functionality of the LAT1.

4.3.11. Heterologous expression in yeast: the LAT1 mediates enhanced growth on exogenous AA toxicity

The yeast mutant strain 22Δ8AA, mutated for eight native AATs was complemented with three independent clones of the *LAT1* to elucidate the ability of this transporter to allow growth on medium containing various AAs as an N source. The 22Δ8AA yeast strain was transformed with the *LAT1* driven by the yeast *Plasma Membrane ATPase 1 (PMA1*) promoter in the pDR196 expression vector. The 22Δ8AA yeast strain, transformed with a

broadly specific AAT gene, *AAP6* (At5g49630) (Fischer et al., 2002), was used as a positive control. The negative control was the 22Δ8AA yeast strain transformed with the empty expression vector pDR196. When grown on 5mM of various AAs (Leu, Ile, Val, Ala, Glu, Arg, Pro), after three days of incubation, the LAT1 did not show any detectable growth variation compared to the negative control; however, the AAP6 mediated reduced growth on Ile and Arg compared to both the negative control and the LAT1 (Figure 4.9 left panel). Exogenous AAs at a 5mM concentration was shown to be toxic to the yeast cells complemented with BAT1 (Dundar and Bush, 2009). In order to test whether the LAT1 can cause growth inhibition at a higher concentration of AAs, as seen for the AAP6 on 5mM Ile, the concentration of AAs in the growth medium was increased to 10mM (supplemented with 5mM NH_4^+). In the presence of NH_4^+ in the growth medium, reduced growth can be attributed to AA toxicity rather than an N deficiency. On Leu and Ile, the LAT1 mediated better growth compared to AAP6 but reduced growth compared to the empty vector (Figure 4.9 right panel). In the presence of Arg, Asn and Glu at 10mM, the LAT1 mediated better growth compared to both AAP6 and the empty vector.

4.4. Discussion

4.4.1. LAT1 may have a role in mobilizing AAs

The Arabidopsis LAT1 is a putative integral membrane protein with 12 TMs with both C- and N-termini in the cytoplasm (Figure 4.1), with <15% sequence similarity at the protein level with animal LATs. The LAT1 lacks the conserved cysteine residue present in animal LATs that interact with a glycoprotein to function as an exchanger. An *in silico* comparison of the LAT1 with animal LATs indicated that the Arabidopsis LAT1 may not be a glycoprotein associated exchanger. *In planta* ^{14}C-Leu and ^{14}C-Glu uptake studies showed that the over expression of the LAT1 causes a decreased accumulation of both ^{14}C-(Leu) and ^{14}C-(Glu) (Figure 4.5) suggesting that LAT1 does not mediate AA uptake. An *in planta* ^{14}C-Leu export analysis showed an increased release of ^{14}C- (fed in the form of Leu) by the OXLAT1 seedlings compared to WT (Figure 4.6) indicating that the LAT1 played a role

in releasing AAs from these seedlings. A heterologous expression analysis showed that the LAT1 mediates an enhanced growth of the yeast cells on exogenous AAs at a toxic level (Figure 4.9) which supports the idea that the LAT1 provided a means for the yeast cells to reduce toxic effects through releasing AAs back to the growth medium. An analysis of the Arabidopsis LAT4 (Chapter 2) and LAT5 (Chapter 3) using a knockout mutant demonstrated that these LATs are different from the H^+/AA-symporters. The LAT4 mediated mobilization of AAs from source tissues (Chapter 2) while the LAT5 mediated bi-directional AA-transport (Chapter 3). An *in planta* analysis of the LAT1 using an over-expressing line thus provided a useful means to test the role of LAT1 in mobilizing AAs from seedlings.

4.4.2. The possible role of the LAT1 in AA translocation from leaves to the seeds

A microarray analysis showed predominant expression of the *LAT1* in the leaves. Quantitative RT-PCR showed a relatively higher abundance of the *LAT1* transcript in green leaves such as cauline leaves and rosette leaves (Figure 4.2). *In planta* ^{14}C-Leu (Figure 4.5B), ^{3}H-Leu (Figure 4.5C) and ^{14}C-Glu (Figure 4.5D) uptake studies using mature plants consistently showed that the overexpression of the *LAT1* causes a decreased accumulation of radionuclide (attached to Leu or Glu) in the leaves and an increased accumulation in the siliques. As the LAT1 showed a capacity to mobilize AAs (Figure 4.5 and 4.6) and is relatively abundant in the green leaves (Figure 4.2), it is possible that the OXLAT1 mediated an increased AA-release from the leaves towards the reproductive tissues. Mature seed weight analysis showed that the seed weight in the OXLAT1 is significantly increased compared to WT (Figure 4.7A). However, the total N and C analysis in the mature seeds showed a significantly decreased N content (Figure 4.7B) and an altered C/N ratio in the OXLAT1 compared to WT. As the over expression of the *LAT1* in the OXLAT1 line is driven by a constitutive promoter, the *LAT1* might be ectopically expressed in this line resulting in a reduced N content in the seeds. Identification of the tissue specific expression and analysis of a knockout mutant may contribute to elucidate the actual role of the LAT1 in seed N content.

Table 4.1 - Primers used for cloning and characterization of the *LAT1*.

Name	Analysis	Sequence
XbaIAtLAT1LP	Subcellular localization	5'CCAATTCTCGTCTAGAATG ACTGAGCTTA3'
NheIAtLAT1RP	Subcellular localization	5'TCGGTGGTCTGCTAGCCTCCATCAGGTTTG3'
EcoRIAtLAT1LP	Yeast complementation	5' GAATTCGAAGCAACTTCCCTAGCAC 3'
SalIAtLAT1LP	Yeast complementation	5' GTCGACGCATCACCATCTTCGGTGGT 3'
LBb1	Isolation of mutant	5'-GCGTGGACCGCTTGCTGCAACT-3'
AtLAT1LP	Isolation of mutant	5'-CAGCTTATGACTGGTGAAGGC-3'
AtLAT1RP	Isolation of mutant	5'TTGATTTGGTTGACGGTTCTC3'

Table 4.2. Free AA analysis in the OXLAT1 seedlings in comparison with WT grown on various sources of N.

	2mM NO_3^-			**2mM Asp**			**2mM Glu**		
	WT	OXLAT1	% change in OXLAT1	WT	OXLAT1	% change in OXLAT1	WT	OXLAT1	% change in OXLAT1
Biomass ($gFW.2sdlngs^{-1}$)	0.0235	0.020	-15	0.0095	0.0073	-23.3	0.012	0.009	-26.4
Free amino acids ($nmol.g^{-1}FW$)									
Asp	389.48	377.76	-3	1250.54	1528.18	22	1561.59	1643.86	5
Glu	1708.83	1466.36	-14	2363.90	2595.53	10	2566.15	3203.35*	25
Asn	2804.68	2445.50	-13	4028.65	5354.87	33	5832.84	7634.27**	31
Ser	402.03	319.30	-21	346.03	378.30	9	430.40	522.74	21
Gln	1139.32	976.39	-14	5557.79	6208.27	12	8514.83	9054.64	6
His	106.65	111.28	4	101.97	133.29	31	147.22	192.28*	31
Gly	116.86	126.16	8	125.35	154.48*	23	154.92	186.49	20
Thr	324.43	273.02	-16	251.00	267.05	6	388.18	431.04	11
Arg	407.52	352.79	-13	675.29	904.26	34	927.75	1273.83	37
Ala	151.15	137.25	-9	257.29	257.04	0	296.15	350.58	18
Tyr	59.88	47.75	-20	50.15	61.29*	22	60.66	77.30	27
Cys	10.79	46.34	330	25.58	36.46	43	52.78	16.44	-69
Val	3826.64	3275.12	-14	3267.21	4411.62**	35	4350.11	5596.79*	29
Met	15.33	13.42	-12	22.99	28.26	23	25.28	33.14*	31
Trp	30.99	22.90	-26	22.06	26.27*	19	24.22	32.70	35
Phe	93.41	74.84	-20	44.74	45.98	3	46.48	60.11	29
Ile	202.77	167.48	-17	129.60	155.84**	20	210.44	267.49	27
Leu	126.90	112.34	-11	118.87	138.53	17	149.04	178.71*	20
Lys	69.15	47.65	-31	73.89	63.56	-14	71.40	122.83	72
Pro	867.50	1356.26	56	2441.19	3505.46*	44	3433.62	2078.12	-39
Total	12854.29	11749.91	-9	21154.10	26254.53	24	29244.08	32956.71	13
WT vs WT	100%			165%			228%		
OXLAT1 vs OXLAT1		100%			223%			280%	

Free AAs were extracted from three weeks old seedlings. Each data is an average of three biological replications with two seedlings per replication. *, P-value <0.05; **, P-value <0.01.

Table 4.3. Free AA analysis in multiple seedlings of the OXLAT1 in comparison with WT in the presence of 1mM Leu in the growth medium.

Free amino acids (nmol.g^{-1}FW)	WT	OXLAT1	% change in OXLAT1
Asp	953	854	-10
Glu	2826	2404	-15
Asn	434	318	-27
Gln	1271	1284	+1
Arg	232	142	-39
Ala	210	240	+14
Val	55	0	-100
Ile	183	149	-19
Leu	10805	10479	-3

Free AAs were extracted from 17-days old seedlings grown in the presence of 1mM Leu. Seedlings from three plates were pooled to extract AAs.

Table 4.4. Free AA profile in the OXLAT1 seedlings in comparison with WT grown in the absence of sucrose in the growth medium.

	Shoot			Root		
	WT	OXLAT1	% change in OXLAT1	WT	OXLAT1	% change in OXLAT1
Biomass gFW.2sdlngs^{-1}	0.0055	0.0038	-30.4878			
Free AA content (nmol.g^{-1}FW)						
Asp	1704.16	1932.09	13.37	479.91	553.88	15.41
Glu	4244.28	4245.84	0.04	1635.69	1630.60	-0.31
Asn	321.95	295.78	-8.13	339.82	429.60	26.42
Ser	1939.64	1832.10	-5.54	786.86	910.10	15.66
Gln	2108.36	1647.09	-21.88	2296.85	2563.93	11.63
His	58.90	36.70	-37.68	58.12	72.67	25.05
Gly	372.31	373.24	0.25	237.94	191.51	-19.51
Thr	456.74	424.37	-7.09	399.72	520.99	30.34
Arg	77.77	78.55	0.99	84.68	97.58	15.23
Ala	765.54	804.29	5.06	378.83	430.88	13.74
Tyr	84.19	100.64	19.54	70.03	87.00	24.22
Cys	145.70	34.21	-76.52	21.42	12.70	-40.73
Val	6803.25	7347.75	8.00	5455.39	6261.39	14.77
Met	56.52	58.35	3.25	40.80	43.90	7.61
Trp	39.59	37.28	-5.82	28.19	24.15	-14.34
Phe	113.47	117.07	3.18	74.89	85.57	14.27
Ile	133.93	144.79	8.11	142.96	163.48	14.36
Leu	175.12	201.60	15.12	192.67	234.33	21.62
Lys	181.64	270.19	48.75	741.17	1160.82	56.62
Pro	4401.03	6037.06	37.17	4677.48	6214.74	32.87
Total	24184.09	26019.00	7.59	18143.39	21689.82	19.55

Free AAs were extracted from two-weeks-old seedlings. Each data is an average of three biological replications with two seedlings per replication. None of these changes were statistically significant.

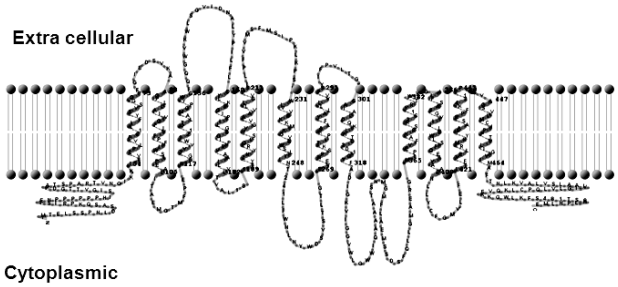

Figure 4.1. *In silico* analysis of the LAT1.

A cartoon representation of the TMHMM prediction shows 12 transmembrane domains of the LAT1 with both C- and N-terminus in the cytoplasmic side.

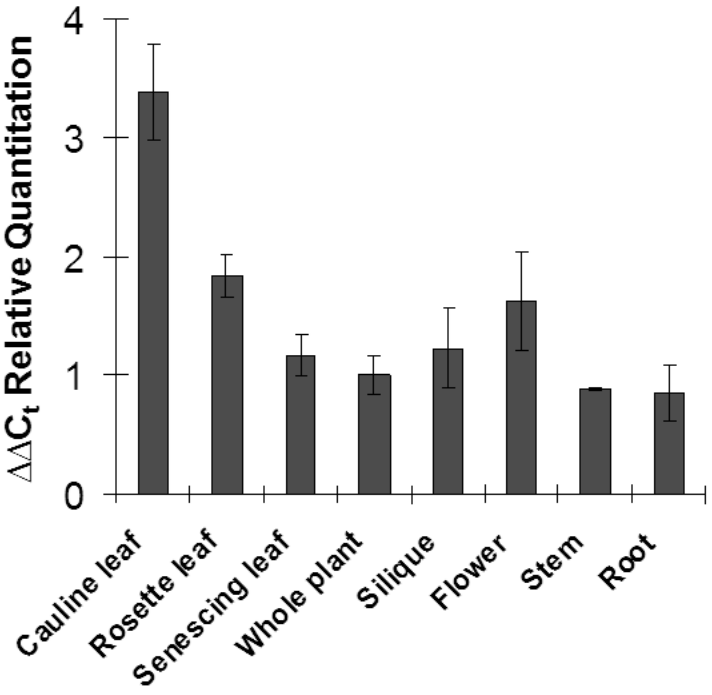

Figure 4.2. Organ-specific expression analysis of the *LAT1*.

An organ-specific qRT-PCR shows a relative abundance of the *LAT1* in various organs compared to the average expression level (arbitrarily set as 1.0) in the whole plant. DNA-free total RNA extracted from the root, stem, rosette leaf, cauline leaf, senescing leaf, flower, silique, and whole plant was used as template in the qRT-PCR. For each sample, 2.5 μg of total RNA was used in the cDNA synthesis with oligo-dT and random primers. Actin2 and EF1α were used as endogenous control. Data analysis was performed using Actin2 as the endogenous control and the average expression level in the whole plant as a calibrator. The error bar represents the SD of three replications.

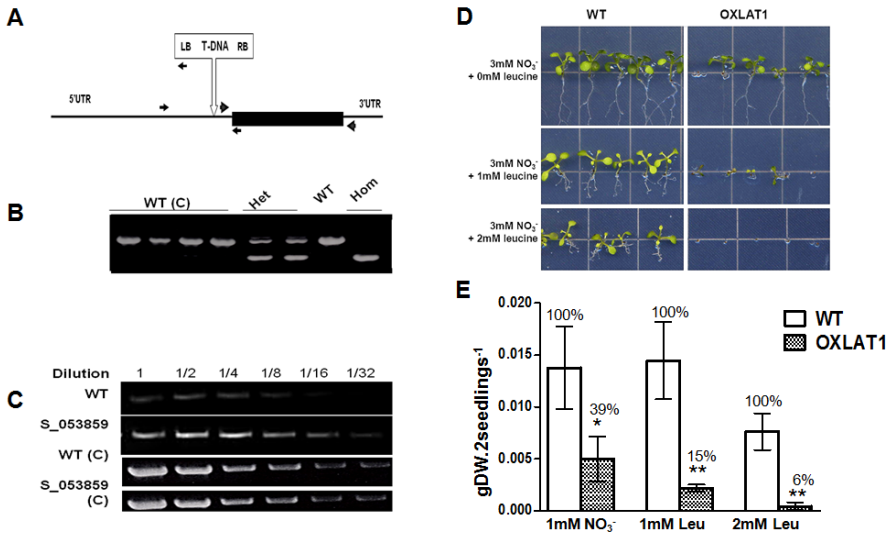

Figure 4.3. Isolation and characterization a T-DNA insertion line over-expressing *LAT1*.

A. Shows the position of T-DNA in the At5g05630 (*LAT1*) locus in the Salk_053859 line. Black and thin arrows indicate the position of the *LAT1* and T-DNA left border-specific primers in the three-primer PCR. Grey and bold arrows indicate the primer position for RT-PCR and qRT-PCR. The black bar shows the coding region.

B. Three-primer PCR produces a single product of the *LAT1* and LBb1-specific primers in the homozygous T-DNA insertion line.

C. Quantitative RT-PCR shows the presence and higher abundance of the *LAT1* transcript in the Salk_053859 line compared to the WT. The *LAT4* was used as a dilution control. *DNAse*-treated 1.6 µgs of total RNA from each genotype was used in the first reaction vial. A serial dilution of the template was performed in the subsequent reaction vials, as shown in the figure. A one-step RT-PCR kit supplemented with an additional reverse-transcriptase enzyme was used in the RT- and qRT-PCR reactions.

D. The OXLAT1 shows increased toxicity to Leu in the growth medium compared to WT. WT and OXLAT1 seedlings were grown on the same plate

under optimum growth condition on a 0.5x MS medium containing 3mM NO_3^- and variable concentration of Leu as the sole source of nitrogen. Each growth condition had three replications. Photos were taken when the seedlings were two weeks old.

E. Shows the quantitative representation of the biomass obtained by the OXLAT1 and WT seedlings in the presence of 1mM NO_3^- and variable concentration of Leu in the growth medium.

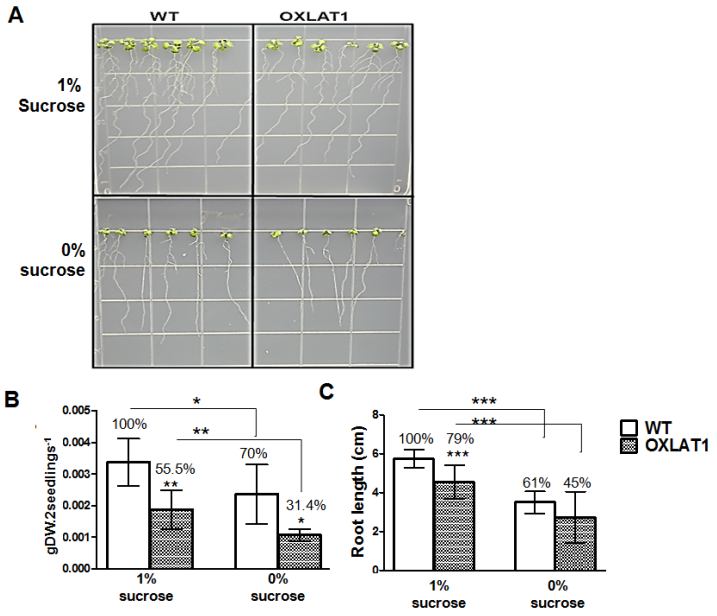

Figure 4.4. Characterization of the OXLAT1 line under low C conditions.

A. The OXLAT1 seedlings grown on a 0.5xMS medium with 2mM NO_3^- as the sole source of N with or without sucrose has shown growth variation in both root and shoot. In the presence of 1% sucrose in the growth medium, the OXLAT1 shows significantly reduced biomass and root length compared to WT. In the absence of sucrose in the growth medium, both the WT and OXLAT1 show a reduction in biomass and root length; however, the reduction in the OXLAT1 is greater compared to WT.

B. Shows the quantitative representation of the biomass obtained in the WT and OXLAT1 seedlings in the presence or absence of sucrose in the growth medium. Error bar represents SD of four replications with two seedlings each.

C. Shows the quantitative representation of the root length of WT and OXLAT1 seedlings in the presence or absence of sucrose in the growth medium. Error bar represents SD of eight replications.

Plants were grown under 16/8 hrs of light/dark regime at 23^oC temperature and 250µE.m^{-2}.sec^{-1} light intensity. For biomass, two-week old seedlings were dried at 70^oC for two hrs before being weighed. The root length was measured when the seedlings were 10-days old.

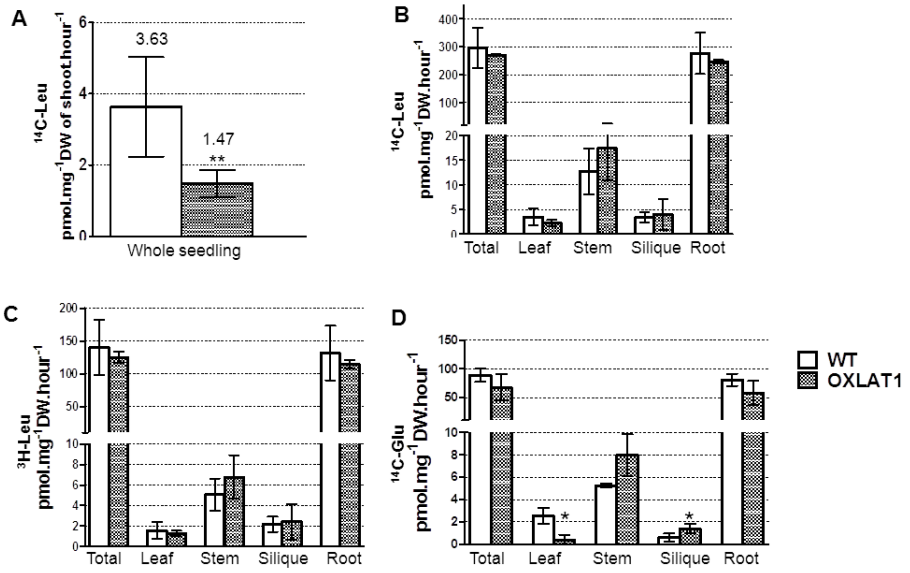

Figure 4.5. *In planta* Leu and Glu uptake studies using the OXLAT1 seedlings.

A. In a ^{14}C-Leu uptake study, the OXLAT1 seedlings show a reduced accumulation of ^{14}C- compared to WT. Twenty-five-days-old seedlings grown on a 0.5x MS medium were used in the study. The roots of overnight N-starved seedlings were dipped in the uptake medium containing ^{14}C-Leu and cold Leu to a final concentration of 10µM. The error bar represents a SD of five biological replications with two seedlings per replication.

B-C. A ^{14}C-Leu (B) and ^{3}H-Leu (C) uptake study using soil-grown mature plants showed a reduced accumulation of both ^{14}C- and ^{3}H- in the roots and leaves and an increased accumulation in the stem and siliques with a total net reduced accumulation in the whole plant compared to WT.

D. A ^{14}C-Glu uptake study using soil-grown mature plants shows a decreased accumulation of ^{14}C- in the leaves and roots, and an increased accumulation in the stem and siliques, with a net reduced accumulation in the whole plant in the OXLAT1 compared to WT.

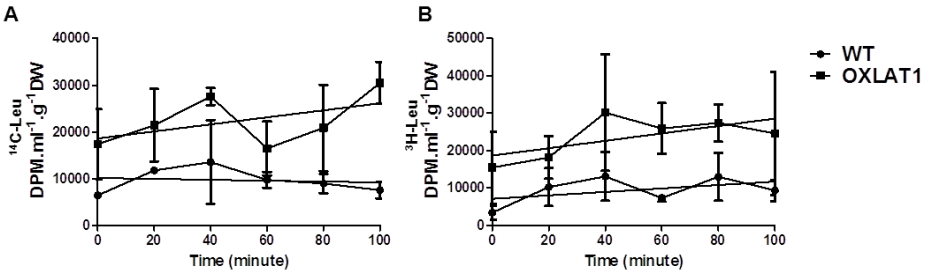

Figure 4.6. An AA export analysis using the OXLAT1 seedlings.

A. In a ^{14}C-Leu export study, the OXLAT1 seedlings released more ^{14}C- over time compared to WT.

B. Another study using ^{3}H-Leu produced result similar to that in A.

Soil-grown three-weeks-old seedlings were subjected to two hrs of ^{14}C-Leu or ^{3}H-Leu uptake followed by two hrs of export. Each data point is an average of three biological replications. The error bar represents SD.

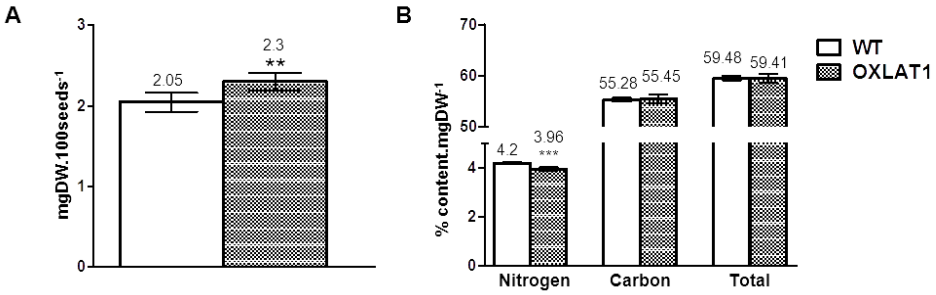

Figure 4.7. Analysis of the total C and N content in seeds.

A. The OXLAT1 seed weight was significantly increased compared to that of the WT. Mature and dried seeds were weighed using a delta-range microbalance. The error bar represents a SD of nine replications with 100 seeds per replication. **, P-value <0.01.

B. The percentage of the N and C content in the OXLAT1 seeds was significantly decreased compared to the WT while the variation in the C content and total N and C content is nonsignificant. The error bar represents a SD of nine replications with 100 seeds per replication. ***, P-value <0.001

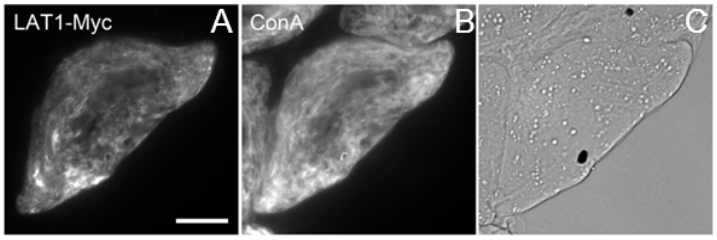

Figure 4.8. Subcellular localization study of the LAT1.

A. The epifluorescence micrograph specific to Alexa-488, the fluorescent dye attached to the secondary anti-Myc antibody, shows c-Myc tagged LAT1 localized in the endoplasmic reticulum in a tobacco bright yellow-2 (BY-2) cell.

B. The epifluorescence micrograph specific to the ConcanavalinA-Alexa-594 ER specific marker stain visualizes the endoplasmic reticulum in the same cell as in (A).

C. Shows the corresponding differential interference contrast (DIC) image for each cell.

Epitope tagged *LAT1* was transiently expressed in tobacco BY-2 cells under a constitutive promoter. Transformed cells were processed for immunofluorescence staining by incubating with mouse anti-Myc antibody and Alexa488-conjugated goat anti-mouse antibodies. Cells were also incubated with Alexa594-conjugated Concanavalin A as a marker stain for endogenous ER.

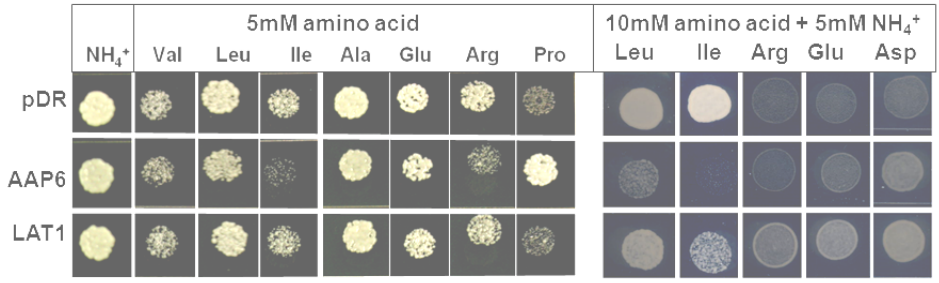

Figure 4.9. Heterologous expression analysis of the *LAT1* in yeast.

The 22Δ8AA yeast strain complemented with the LAT1 does not show any detectable growth variation compared to the pDR-empty vector on growth media containing 5mM of various AAs.

In the presence of 10mM AAs supplemented with 5mM NH_4^+, the LAT1 mediates increased growth of 22Δ8AA on Arg, Glu, and Asn compared to AAP6 and the empty expression vector (pDR). On Leu and Ile, the LAT1 mediates reduced growth compared to the empty vector but increased growth compared to AAP6. Liquid cultures of the PCR positive colonies were grown until log phase. The cell concentration was counted using a haemocytometer. Cell cultures were diluted to a concentration of 20cells.μl^{-1}. Ten µl of the cell culture for each genotype was plated in the same plate. Each genotype had three replications per plate. Plates were incubated at 30^{o}C for two to four days before photographers were to taken.

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4.6. List of links

1. Arabidopsis eFP Browser (Developmental Map At-TAX for LAT1 expression) [http://bbc.botany.utoronto.ca/efp/]

2. Aramemnon Plant Membrane Protein Database [http://aramemnon.botanik.uni-koeln.de/].

3. EMBL-EBI ClustalW online sequence alignement tool [http://www.ebi.ac.uk/Tools/msa/clustalw2/]

4. PAUP Version 4.0b10 [http://paup.csit.fsu.edu/]

5. TMHMM Server v. 2.0, Prediction of transmembrane helices in proteins [http://www.cbs.dtu.dk/services/TMHMM/]

6. High Quality Visual Representation of Transmembrane Protein models [http://bioinformatics.biol.uoa.gRTMRPres2D].

7. CLC combined work bench [http://clc-combined-workbench.en.softonic.com/]

8. National Centre for Biotechnology Information [http://www.ncbi.nlm.nih.gov/Structure/cdd/].

9. Signal Salk Institute [http://signal.salk.edu/].

10. Agilent Technologies literature library [http://www.chem.agilent.com/Library/applications/5989-6297EN.pdf]

5. General Discussion and Conclusions

The distribution of amino acids (AAs) in plants is a complex process which in Arabidopsis may involve up to 67 genes that encode putative amino acid transporters (AATs) (Rentsch et al., 2007). To date, 42 AATs (including 13 peptide transporters) have been characterized using heterologous expression systems such as *Saccharomyces cerevisiae* mutants and *Xenopus laevis* oocytes (Table 5.1; Tegeder and Rentsch, 2010). However, only 10 of them have been characterized *in planta* which has contributed in part to understanding the source-sink distribution of AAs in plants (Table 5.2; Tegeder and Rentsch, 2010). The L-type amino acid transporter (LAT) family, which includes five members in Arabidopsis, was uncharacterized prior to the beginning of this research in 2007. The Arabidopsis LAT family was initially classified and named based on similarity to the animal LATs (Wipf et al., 2002). A recent phylogenetic analysis classified the Arabidopsis LATs under the Polyamine Proton Symporter (PHS) family (Okumoto and Pilot, 2011). While the LATs in animals are glycoprotein associated exchangers of neutral AAs (Torrents et al., 1998; Pfeiffer et al., 1999; Verrey et al., 2000), members of the PHS family are thought to be polyamine transporters. At the beginning of this research, the similarity between the Arabidopsis LATs and animal LATs were analyzed *in silico* and revealed very little homology (described in Chapter IV). A BLAST search using the NCBI database identified genes homologous to the Arabidopsis LATs, with increasing numbers being identified with the growing number of sequenced genomes. Most of these homologous genes were annotated as putative AATs or transporters with unknown function. Thus, *in silico* analysis did not provide any indication about the role of these LATs in plants.

This research presents the functional characterization of three members of the Arabidopsis LAT family. To the best of my knowledge, this is the first report of the functional characterization of the Arabidopsis LAT4 and LAT5. A very recent publication (published online on April 4, 2012) demonstrated that a polymorphic variation of the LAT1 (renamed as RMV1, Resistant to

Methyl Viologen 1) shows variable paraquat tolerance in Arabidopsis (Fujita et al., 2012). This study indicated that LAT1 mediates polyamine uptake. An initial screening showed significantly increased sensitivity of mutant or over-expressing lines of LAT1, LAT4 and LAT5 to the exogenous AA toxicity. Thus, I characterized the AA transport activity of LAT1, LAT4 and LAT5 in Arabidopsis.

Characterization of the LATs in Arabidopsis will not only contribute to understanding the AA-translocation pathway in plants, but also LATs are the first identified AATs that mediated mobilization of AAs from the leaf mesophyll cells. Thus, this research contributes to an understanding of the amino-N mobilization from source leaves to the developing sinks including seeds. Considering the limitations of the heterologous or *in planta* systems alone, both systems were used in this research to elucidate the role of these LATs. However, with an increasing understanding about the nature of these transporters, more experimental questions arose that will require further study. This chapter summarizes the roles of LAT1, LAT4 and LAT5 as identified by this research.

5.1. The role of LAT4

Chapter 2 describes the functional characterization of the LAT4 in Arabidopsis. LAT4 was characterized through *in silico* studies, subcellular localization, organ and tissue specific expression, heterologous expression in yeast, and *in planta* knockout mutant analysis. LAT4 is an integral membrane protein with 10 TMs and 54.8kDa molecular weight. With a Myc epitope in the C-terminus, LAT4 was unable to localize to the membrane (Figure 2.3) which was the same for the LAT1 (Figure 4.8; Chapter 4) and LAT5 (Figure 3.3; Chapter 3). While the subcellular localization of these transporters remained unidentified, this study provides data that suggests that the C-terminal end of these LATs may have a role in the tertiary folding of these proteins or they may have a binding partner at the C-terminal end. Future experiments should be designed considering these possibilities.

In Arabidopsis, LAT4 is a widely expressed AAT in the whole plant with high levels of expression in the green tissues (Figure 2.2). A knockout mutant line *(lat4-1)* was characterized phenotypically under various growth conditions and used in radio-labeled Leucine (Leu) uptake studies. The mutant *lat4-1* showed reduced biomass and root length under normal growth condition which was further reduced when grown under low C conditions (Figure 2.6). Radio-labeled Leu transport studies showed that mutant shows significant variation in the net AA uptake activity compared to WT when seedlings are subjected to low C conditions (Figure 2.7). These studies indicated that the AA-transport activity of the LAT4 is influenced by the cellular C content, which is related to its expression in the green tissues where cellular C content varies, depending on the light/dark regime.

Heterologous expression analysis of the *LAT4* in yeast in comparison with a high affinity AA importer (AAP6) indicated that the LAT4 is different from the AAP6 (Figure 2.11). Mutant *lat4-1* seedlings showed increased sensitivity to exogenous AA toxicity, suggesting that mutants are not defective in AA accumulation (Figure 2.5). Radio-labeled Leu uptake studies showed that the mutant accumulates significantly more radionuclides (^{14}C- and ^{3}H-) compared to WT, suggesting that the LAT4 plays a role in AA release but not in AA acquisition (Figure 2.7). Radio-labeled Leu-uptake study using leaf mesophyll cell protoplasts provided evidence that the LAT4 mediates mobilization of AAs from leaf mesophyll cells (Figure 2.8). Expression analysis, together with the transport property, suggests that the LAT4 mediates mobilization of AAs from source leaves, photosynthetic cells in the young stem, and green carpel cells in the silique valve (Figure 5.1). Free AA analysis in these three organs also supported this hypothesis (Figure 2.9).

Future studies using leaf protoplasts of knockout mutant and over expressing lines of the *LAT4* would be useful to elucidate the AA-transport mechanism of the LAT4. These studies would also be useful to identify the substrate affinity and specificity of this transporter. Free AA analysis in the

leaf apoplasm and total N content in the seeds would provide additional data on the role of the LAT4 in mobilizing AAs from the leaves to the seeds.

5.2. The role of LAT5

Chapter 3 describes the functional characterization of the Arabidopsis LAT5. Similar approaches as mentioned above for the LAT4 were used to characterize the LAT5 with an additional experiment to analyze the total N content in the seeds.

LAT5 is an integral membrane protein with 52.8kDa molecular weight and 12 transmembrane domains with both N- and C-termini in the cytoplasmic side (Figure 3.1). LAT5 is a widely expressed AAT with expression in the phloem (Figure 3.2). LAT5 mediated AA-acquisition in the seedlings under normal conditions and AA mobilization from seedlings as well as from mesophyll cell protoplasts under low C conditions, suggesting a bi-directional AA transport activity for LAT5 (Figure 3.6). Loss of function of the LAT5 caused significant reduction in the size of leaf mesophyll cells and leaf width (Figure 3.4). Correlated with the reduced leaf size, free AA analysis in the leaves and stem of *lat5-1* mature plants showed a decreased AA content in the leaves with an increased accumulation in the stem (Figure 3.7A, B). These studies suggested that the major role of LAT5 is to mediate AA translocation to the leaves (Figure 5.1). In the absence of LAT5, increased accumulation of AAs in the *lat5-1* stem is likely to increase the AA translocation to the siliques. Free AA analysis in the siliques showed significantly increased concentrations of AAs in the *lat5-1* siliques compared to WT, supporting this hypothesis (Figure 3.7C), which was further confirmed through analyzing the total N-content in the seeds. Both the dry weight and total N content were significantly increased in the *lat5-1* seeds compared to WT (Figure 3.8).

Another aspect of the AA transport in plants is to maintain the AA homeostasis since free AAs play a role in biotic and abiotic stress tolerance (Bonner et al., 1996; Martino et al., 2003; Sharma and Dietz, 2006; Wu et al., 2010). Maintaining the AA homeostasis requires both cellular export and

import to achieve the proportional distribution of AAs across the membrane. While most AATs in plants were thought to be H^+-coupled AA-symporters (overviewed in Bush, 1993; Wipf et al., 2002), the LAT5 mediated a bi-directional AA transport activity suggesting a possible role in the AA homeostasis. The role of LAT5 in AA homeostasis was investigated by characterizing the *lat5-1* mutant line under salt, osmotic and drought stress conditions. The mutant *lat5-1* showed increased salt and drought stress tolerance and altered free AA content in the seedlings under salt and osmotic stresses indicating that LAT5 plays a role in AA homeostasis and abiotic stress tolerance (Figure 3.9).

5.3. The role of LAT1

Chapter 4 describes the characterization of the LAT1. Similar approaches as described above for the LAT4 and LAT5 were used to investigate the role of LAT1. However, in the absence of a knockout mutant, a LAT1 over-expressing line was used. Since both LAT4 and LAT5 were studied using knockout mutant, a LAT1 over-expressing line was considered useful and comparable to the LAT4 and LAT5 *in planta* studies for the characterization of the LATs as a family.

LAT1 is an integral membrane protein with twelve transmembrane domains and 53.7kDa molecular weight with both C- and N-terminus in the cytoplasm (Figure 4.1). Heterologous expression of the *LAT1* in yeast mediated increased tolerance to exogenous AA toxicity (Figure 4.9). Radio-labeled Leu and/or Glu uptake and export analysis showed decreased accumulation (Figure 4.5) and increased release of radionuclides (attached to the AA) by the OXLAT1 seedlings compared to WT (Figure 4.6), supporting the role of LAT1 in mobilizing AA.

The transcript of *LAT1* was detected in all organs with a higher abundance in cauline leaves and rosette leaves (Figure 4.2), suggesting a role of LAT1 in mobilizing AAs from the leaves (Figure 5.1). Radio-labeled Leu and Glu uptake studies using mature plants consistently showed reduced accumulation of radionuclides (^{14}C- and ^{3}H-) in the leaves and increased

accumulation in the siliques in OXLAT1 compared to WT (Figure 4.B-D), further supporting the role of LAT1 in mobilizing AAs from the leaves to the siliques. The analysis of mature seeds showed increased seed weight and decreased N content with an altered C/N ratio in the OXLAT1 seeds compared to WT (Figure 4.7). Since *LAT1* was over expressed under a constitutive promoter (CaMV35S) in the OXLAT1 plants, it is likely to have ectopic expression in tissues where it normally does not express. Thus, an over expression line was useful to study the role of LAT1 in mobilizing AAs from the whole seedlings; however, its role in AA distribution in plants was inconclusive.

In summary, this research characterized a novel family of AATs in Arabidopsis that mediate mobilization of AAs and contribute in amino-N partitioning among source and sink tissues. Experimental evidences suggested that this family represents AATs that are different from proton coupled symporters which makes them interesting for further characterization. Elucidating the transport mechanism and substrate preference of this family of AATs may shed light into the AA export and antiport systems that remained poorly understood in plants.

Table 5.1. Overview of genes encoding transporters for amino Acids (aa) or peptides that have been characterized using heterologous expression in *Saccharomyces cerevisiae* mutants and/or *Xenopus laevis* Oocytes, gene expression analyses, and/or localization (RNA, Protein) studies.

Functional characterization using heterologous expression systems, expression analyses and/or localization studies		
Family	Substrate	Gene and publications
ATF (or AAAP) amino acid transporter family (or amino acid/auxin permease)		
AAP amino acid permease	Neutral aa, Glu	***AtAAP1*** (NAT2), Frommer et al., 1993, Hsu et al., 1993, Boorer et al., 1996, Chang and Bush, 1997; ***AtAAP1-2***, Kwart et al., 1993, Hirner et al., 1998; ***AtAAP1-5***, Fischer et al., 1995; ***AtAAP1-6***, Fischer et al., 2002; ***AtAAP3***, Breitkreuz et al., 1999, Okumoto et al., 2004; ***AtAAP5***, Boorer and Fischer, 1997; ***AtAAP6***, Rentsch et al., 1996; ***AtAAP6, 8***, Okumoto et al., 2002; ***BnAAP1, 2, 6***, Tilsner et al., 2005; ***PsAAP1-2***, Tegeder et al., 2000, 2007; ***VfAAP1, 3, 4***, Miranda et al., 2001, 2003; ***VfAAP2, a, b, c***, Montamat et al., 1999; ***PvAAP1***, Tan et al., 2008; ***RcAAP1***, Marvier et al., 1998; ***RcAAP1-2***, Bick et al., 1998; ***RcAAP3***, Neelam et al., 1999; ***NaAAP1-3***, Schulze et al., 1999; ***PtAAP1-14***, Couturier et al., 2010a; ***StAAP1***, Koch et al., 2003
LHT 'lysine/histidine' transporter	Neutral and acidic aa, Lys and His	***AtLHT1***, Chen and Bush, 1997; Hirner et al., 2006; ***AtLHT2***, Lee and Tegeder, 2004; ***AtLHT2, 4, 5, 6***, Foster et al., 2008; ***McLHT*** (McAAT2), Popova et al., 2003; ***NsLHT*** (NsAAP1), Lalanne et al., 1997
ProT proline transporter	Pro, quaternary ammonium compounds	***AtProT1-2***, Rentsch et al., 1996; ***AtProT1-3***, Grallath et al., 2005; ***AtProT2***, Breitkreuz et al., 1999; ***AmBet/ProT1-3*** (AmT1-3), Waditee et al., 2002; ***BvBet/ProT1***, Yamada et al., 2009; ***HvProT***, Ueda et al.,

Functional characterization using heterologous expression systems, expression analyses and/or localization studies		
Family	Substrate	Gene and publications
		2001; ***HvProT2***, Fujiwara et al., 2010; ***LeProT1-3***, Schwacke et al., 1999; ***OsProT***, Igarashi et al., 2000
ANT1-like aromatic–neutral aa transporter	Neutral and aromatic aa	***AtANT1***, Chen et al., 2001
GAT γ-aminobutyric acid transporter	GABA and related compounds	***AtGAT1, AtGAT2***, Meyer et al., 2006
APC amino acid-polyamine-choline		
CAT cationic aa transporter	Neutral and cationic aa	***AtCAT1*** (AtAAT1), Frommer et al., 1995; ***AtCAT2, 3, 6, 8***, Su et al., 2004; ***AtCAT6***, Hammes et al., 2006; ***McCAT*** (McAAT1), Popova et al., 2003; ***PtCAT1-12***, Couturier et al., 2010b
BAT bidirectional aa transporter	Ala, Arg, Glu, Lys	***AtBAT1***, Dündar and Bush, 2009
OEP16 or PRAT plastid outer envelope porin of 16 kDa (or preprotein and amino acid transport)		
OEP plastid outer envelope protein	OEP16: aa, amines; OEP24: charged aa, ATP, phosphate, triosephosphate	***AtOEP16*** (1–4), Reinbothe et al., 2004a, Drea et al., 2006, Murcha et al., 2007, Philippar et al., 2007; ***PsOEP16***, Pohlmeyer et al., 1997, Linke et al., 2000; ***HvOEP16*** (PTC16), Baldi et al., 1999, Reinbothe et al., 2004a, 2004b; ***PsOEP24***, Pohlmeyer et al., 1998, Röhl et al.,1999
MCF mitochondrial carrier family		
BAC (mitochondrial) basic aa carrier	Arg, Lys, Orn, His	***AtmBAC1-2***, Catoni et al., 2003, Hoyos et al., 2003, Palmieri et al., 2006
DASS divalent anion:Na^+ symporter		
DiT (plastidic) dicarboxylate	Exchange Glu/malate	***AtDiT2.1, AtDiT2.2***, Renné et al., 2003

Functional characterization using heterologous expression systems, expression analyses and/or localization studies		
Family	Substrate	Gene and publications
transport		
PTR/NRT1 peptide transporter/nitrate transporter 1		
PTR1-like peptide transporter	di- and tripeptides (PTR1-branch and AtPTR3) His, nitrate	***AtPTR1***, Dietrich et al., 2004; ***AtPTR2*** (AtNTR1, AtPTR2B), Frommer et al., 1994, Rentsch et al., 1995, Song et al., 1996, Chiang et al., 2004; ***AtPTR3***, Karim et al., 2005, 2007; ***AtPTR5***, Komarova et al., 2008; ***HaPTR4***, Paungfoo-Lonhienne et al., 2009; ***HvPTR1***, West et al., 1998, Waterworth et al., 2000, 2005; ***NaNTR1***, Schulze et al., 1999; ***VfPTR1***, Miranda et al., 2003; ***BnNRT1;2***, Zhou et al., 1998
OPT oligopeptide transporter		
OPT oligopeptide transporter	tetra- and pentapeptides, glutathione (and GS-conjugates)	***AtOPT1-9***, Koh et al., 2002; ***AtOPT1, 3, 4, 6, 7, 8***, Stacey et al., 2006; ***AtOPT4***, Osawa et al., 2006; ***AtOPT6***, Cagnac et al., 2004, Pike et al., 2009; ***BjGT1***, Bogs et al., 2003; ***OsGT1***, Zhang et al., 2004

Arabidopsis, At *Arabidopsis thaliana*; mangrove, Am *Avicennia marina*; Indian mustard, Bj *Brassica juncea*; canola, Bn *Brassica napus*; sugar beet, Bv *Beta vulgaris*; Ha *Hakea actites*; barley, Hv *Hordeum vulgare*; tomato, Le *Lycopersicon esculentum* (= *Solanum lycopersicum*); ice plant, Mc *Mesembryanthemum crystallinum*; pitcher plant, Na *Nepenthes alata*; South American tobacco, Ns *Nicotiana sylvestris*; rice, Os *Oryza sativa*; pea, Ps *Pisum sativum*; popular, Pt *Populus trichocarpa*; common bean, Pv *Phaseolus vulgaris*; castor bean, Rc *Ricinus communis*; potato, St *Solanum tuberosum*; Faba bean, Vf *Vicia faba*. (Source: Tegeder and Rentsch, 2010).

Table 5.2. Amino acid and peptide transporters with demonstrated function *in planta* using knockout and/or overexpressing lines.

Function in plants	
Family	Gene, role or effects in transgenic plants and publications
AAP	***AtAAP1***, root uptake, seed loading, Lee et al., 2007, Sanders et al., 2009; ***AtAAP5***, root uptake, Svennerstam et al., 2008; ***AtAAP6***, phloem amino acid content, Hunt et al., 2010; ***AtAAP8***, seed development, Schmidt et al., 2007; ***StAAP1***, long-distance transport, Koch et al., 2003; ***VfAAP1***, seed size, seed protein, vegetative biomass, Rolletschek et al., 2005, Götz et al., 2007, Weigelt et al., 2008
LHT	***AtLHT1***, uptake in root and leaf mesophyll cells, Hirner et al., 2006, Svennerstam et al., 2007, 2008
ProT	***AtProT2***, uptake into roots, Lehmann and Rentsch, unpublished; ***HvProT***, growth, tissue proline levels, Ueda et al., 2008
ANT	***AtANT1***, phloem amino acids content, Hunt et al., 2006
CAT	***AtCAT6***, sink supply, Hammes et al., 2006
OEP	***AtOEP16***, role in deetiolation and NADPH:protochlorophyllide oxidoreductase A import (Pollmann et al., 2007), but not confirmed by other studies (Philippar et al., 2007; Pudelski et al., 2009)
DASS	***AtDiT2.1***, glutamate/malate exchange, Renné et al., 2003
PTR	***AtPTR1, 5***, root uptake, biomass, N content, uptake in pollen, Komarova et al., 2008; ***AtPTR2***, flowering, seed development, Song et al., 1997; ***AtPTR3***, seed germination on salt, pathogen defense, Karim et al., 2005, 2007
OPT	***AtOPT3***, seed development (Stacey et al., 2002), however, phenotype is due to a function of AtOPT3 in iron nutrition e.g. by transporting a peptide/modified peptide Fe chelator or Fe chelator complex (Stacey et al., 2008)

Arabidopsis, At *Arabidopsis thaliana*; barley, Hv *Hordeum vulgare*; potato, St *Solanum tuberosum*; Faba bean, Vf *Vicia faba*. (Source: Tegeder and Rentsch, 2010).

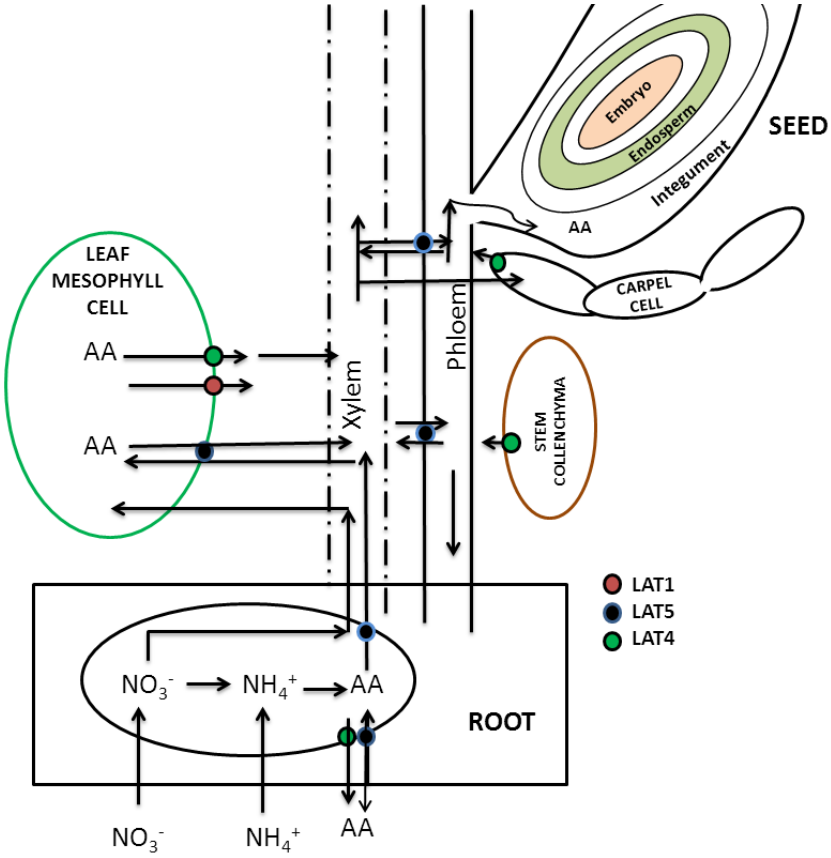

Figure 5.1. Proposed role of the LAT1, LAT4 and LAT5 in the AA translocation pathway in Arabidopsis.

In the root epidermis, LAT4 mediates release of AAs from the plants, while LAT5 may mediate both uptake and export throughout the root tissues. Both LAT4 and LAT5 mediate mobilization of AAs from the leaf mesophyll cells under low C conditions, while LAT5 also mediates AA acquisition into the mesophyll cells under normal conditions which is presumably the main role of LAT5. In the stem, LAT4 may mediate mobilization of AAs from the cortical cells, while LAT5 may mediate bi-directional transport of AAs in and out of the phloem. In the siliques, LAT4 may mediate mobilization of AAs from the green carpel cells in the valve, while LAT5 may have a role in AA-loading and unloading in the phloem. LAT1 may mediate mobilization of AAs from the leaves.

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