# CHARACTERIZATION OF AMINO ACID TRANSPORTER FUNCTION IN *ARABIDOPSIS THALIANA*

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of JUSTIN JOE FOSTER find it satisfactory and recommend that it be accepted.

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Chair

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CHARACTERIZATION OF AMINO ACID TRANSPORTER FUNCTION IN *ARABIDOPSIS THALIANA*

Abstract

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Amino acids are the main transport form of organic nitrogen in plants and their import into plant cells is predicted to be by plasma membrane transport proteins. This research analyzed the roles of the amino permease AtAAP1 as well as AtLHT amino acid transporters in Arabidopsis with an emphasis on amino acid uptake by the roots and transport within flowers. Expression of *AtAAP1* and *AtLHT6* was localized to root cells, including root hairs. Furthermore, the transporters were targeted to the plasma membrane, indicating a function in nitrogen import into cells. Transport studies were performed with *aap1* and *lht6* seedlings and showed reduced amino acid uptake in mutants compared to wild type. Moreover, *aap1* mutants could grow on media containing amino acid concentrations that are toxic to wild type, and plants overexpressing *AAP1* displayed increased uptake of amino acids. Together, these data demonstrate that transporters are important for root amino acid acquisition and that

genetic manipulation of root located amino acid import systems results in alteration of nitrogen uptake.

When analyzing the expression of LHT amino acid transporters it was found that LHTs are strongly expressed in flowers. *AtLHT2*, *AtLHT4*, *AtLHT5*, and *AtLHT6* promoter-*GUS* studies were performed, and dependent on the transporter, GUS staining was localized in the tapetal cells of the anther, germinating pollen, pollen tubes, stigma or transmitting tissue of the pistil. Subcellular localization of AtLHT:GFP fusion proteins showed that all LHT transporters are targeted to the plasma membrane, suggesting that the LHTs function in the import of nitrogen into floral tissues, thereby supplying sufficient nutrients for reproductive success. Localization of *AtLHTs* expression was also determined throughout the plant using promoter-*GUS* studies and histochemical detection displayed tissue- and cell-specific expression patterns. Furthermore, AtLHTs were expressed in yeast cells and *Xenopus* oocytes to determine their substrate selectivity, but none of the LHTs showed transport function in these heterologous systems. For future studies homozygous *lht* mutants were identified, crosses were performed with selected mutants to produce double mutants and *AtLHT4*, *5* and *6 antisense* lines were produced.

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# Chapter 1

# Introduction

## 1. 1. Nitrogen uptake and partitioning in plants

A plant is a complex meshwork that must interact precisely to respond to internal needs and environmental stimuli. Nitrogen balance and distribution are important components of this network. Nitrogen is taken up by the roots mostly in the inorganic forms nitrate or ammonium. The inorganic nitrogen is assimilated into amino acids in the roots or transported via the xylem to leaves where they are then converted to amino acids (Lam et al. 1995; Bush 1999; Fig. 1a-b). Following assimilation in source organs (e.g. mature leaves), amino acids can be transiently stored or translocated via the phloem to sink organs (e.g. flowers and seeds), where they are needed for growth. Besides uptake of inorganic N the roots can also directly take up amino acids. The amino acids in the roots are transported to source organs through the xylem and retranslocated by the phloem to sinks. In addition, amino acids can be transferred between the xylem and phloem enhancing the ability of the plant to quickly distribute amino acids (Pate et al. 1979; Pate 1980; Layzell and La Rue 1982).

Long distance transport through the phloem occurs by bulk flow (Lalonde et al. 2003). The mechanism for transport in the phloem is based on Münch's pressure-flow hypothesis (Münch 1930), where differences in osmotic pressures dictate the movement of the phloem sap (Lalonde et al. 2003). Assimilates are loaded into the phloem in source organs and unloaded out of the phloem in sink

organs creating a large differences in the osmotic pressures. Water enters the phloem in source organs and moves the phloem sap from source organs with excess assimilates to sink organs.

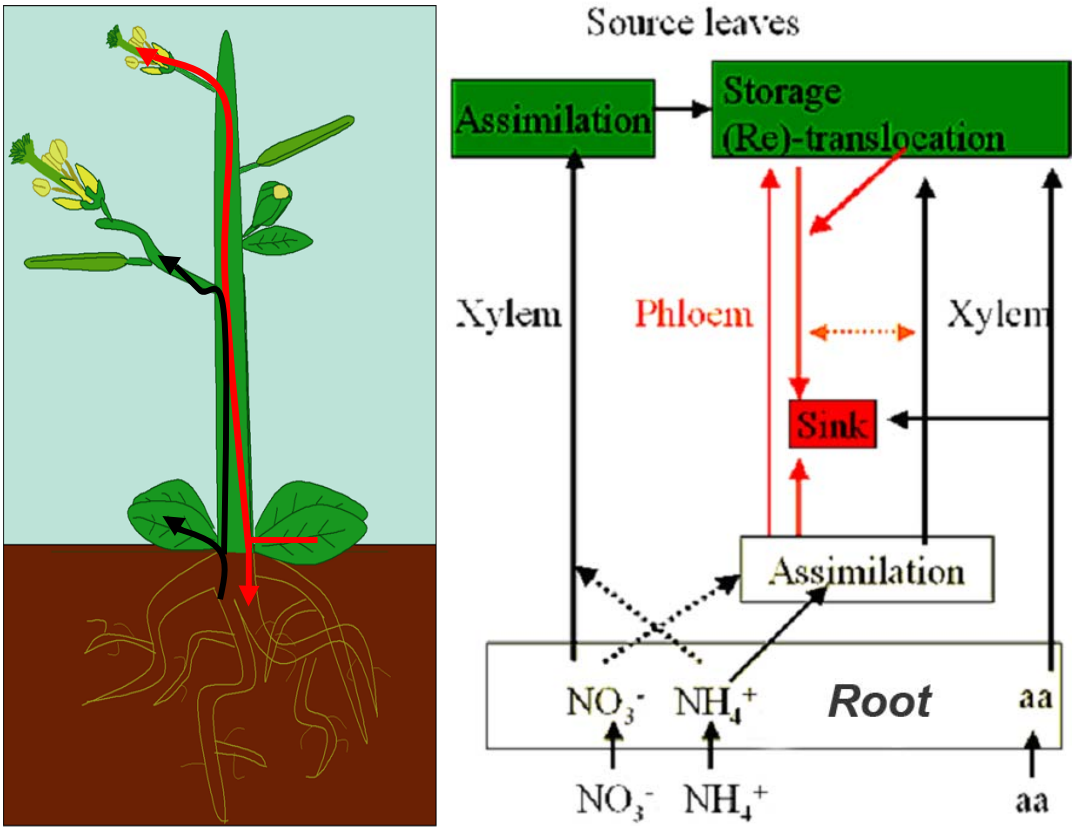


**Figure 1**. Overview of nitrogen assimilation and long distance transport in plants. **a**, Plant model describing xylem (black arrows) and phloem (red arrows) transport. **b**, Schematic diagram of nitrogen assimilation in roots and leaves, and long-distance transport (xylem, black; phloem, red arrows) of amino acids from source to sink.

### 1. 2. Phloem loading and unloading

The phloem consists of sieve elements (SE) and companion cells (CC) and loading of assimilates into the phloem can occur in both cell types (Oparka

and Turgeon 1999). However in most species phloem constituents seem to be loaded into the CC via a symplasmic or apoplasmic pathway (Turgeon et al. 1993; Oparka and Turgeon 1999; Lalonde et al. 2003). In symplasmic phloem loading species the CC is connected to the surrounding cells via plasmodesmata (Turgeon et al. 1993; Oparka and Turgeon 1999). In these species symplasmic loading is thought to occur by the "polymer trap" model. For example sucrose is thought to move through the plasmodesmata into the CC where the sucrose is then turned into raffinose and stachyose. These sugars are too large to move back through the plasmodesmata, so they are trapped and accumulate in the SE-CC complex. In contrast, apoplasmic phloem loading occurs in many species and is characterized by the SE-CC complex being symplasmically isolated from the surrounding cells (Oparka and Tugeon 1999; Lalonde et al. 2003). Solutes are released into the apoplast and are imported into the CC via membrane transporters. The transport of solutes into the SE-CC complex increases the solute concentration and forms the osmotic pressure that is needed for bulk flow from source to sink.

Phloem unloading can occur symplasmically or apoplasmically depending on the species and sink organ (Oparka and Turgeon 1999; Lalonde et al. 2003). In most cases the phloem constituents are unloaded via plasmodesmata into sinks where they are utilized. In some sink organs there are unloading steps that have no functional plasmodesmata so apoplasmic unloading must occur. Furthermore it has been shown that plasmodesmal frequency and/or conductivity can change during sink development, which causes a shift from apoplastic to

symplasmic or symplasmic to apoplasmic movement of molecules (Viola *et al*. 2001; Kwiathkowska 2003).

### 1. 3. Partitioning to and transport into sink organs

The transport of amino acids into sinks, such as developing flowers or seeds, is important for their growth and development. Large amounts of amino acids are required to sustain high levels of protein synthesis during flower and subsequent seed development (Spencer et al. 1980; Krichevsky et al. 2007). For instance, the tip growth of a pollen tube occurs at a rapid pace and relies on specific proteins, such as pectin methylesterases, cellulose synthases and GTPases (Krichevsky et al. 2007). Therefore, the role of transport proteins in directing the flow of amino acids to specific cell types for protein synthesis might be important. In addition, some sinks have an apoplasmic step in partitioning of nutrients that would necessitate amino acid membrane transporters. For example, the developing embryo is separated from the maternal seed coat by an apoplasmic space and as a result membrane transporters are needed for import of nutrients into the embryo (Patrick and Offler 2001).

### 1. 4. Physiological analysis of amino acid transport

The concentration of amino acids within a plant varies depending on the species, organ types, cell types and subcellular compartments (Schobert and Komor 1990; Riens et al. 1991; Winter et al. 1992; Schwacke et al. 1999). For example, concentrations of amino acids in flowers are relatively high compared

to other organs and the spectrum of amino acids within the flower can vary depending on the tissue type (Schwacke et al. 1999; Palanivelu et al. 2003). Stamen have the highest concentration of free amino acids and the majority of this resides in the pollen in the form of proline (Schwacke et al. 1999).

Phloem and xylem are tissue types found in all organs and in most species amino acid concentrations in the phloem are higher than in the xylem (Lam et al. 1995). The dominant form of amino acids transported in the phloem is dependent on the species (Winter et al. 1992; Girousse et al. 1996). For instance, Asn is the main amino acid in the phloem of *Medicago sativa*, whereas in *Hordeum vulgare* it is Glu. In addition, the composition of amino acids in the phloem can fluctuate depending on light and dark cycles (Winter et al. 1992; Lam et al. 1995). Likewise, amino acids concentrations are different within individual subcellular compartments (Riens et al. 1991; Winter et al. 1992). In spinach meshophyll cells the concentration of amino acids are higher in the stroma then in the cytosol, whereas in barley cells the concentrations were similar in the stroma and cytosol (Riens et al. 1991; Winter et al. 1992). However, in both species the vacuole had the lowest concentration of amino acids.

Differences in amino acid combination and concentration in organs, tissue and cells leads to the prediction that transport systems are facilitating the partitioning of amino acids within and between cells and over long distances. Initial physiological studies on amino acid transport used suspension cell cultures, membrane vesicles and plant roots (Wyse and Komor 1984; Li and Bush 1990; de Jong and Borstlap 2000; Lipson and Näsholm 2001). These

studies indicated that multiple transport systems are involved in amino acid transport in plants. For example, amino acid transport in sugarcane suspension cells and plasma membrane vesicles of sugar beet leaf demonstrate that there are separate transport systems for neutral, basic and acidic amino acids (Wyse and Komor 1984; Li and Bush 1990). In addition, transport studies in plant roots and sugar beat leaf plasma membranes also point to high and low affinity transport systems (Li and Bush 1990; Lispon and Näsholm 2001). These studies were informative but were unable to analyze individual amino acid transporters, therefore molecular techniques were utilized to decipher the role of each transporter.

## 1. 5. Molecular analysis of amino acid transporters

### 1. 5. 1. Transport of amino acids across membranes

Amino acid transport across membranes is predicted to occur through proteins that transport amino acids by passive or secondary active mechanisms (Rentsch et al. 1998; Rochat, 2001) (Fig. 2a, b). Passive transport is energy independent and amino acids move down the concentration gradient through carriers within the membrane (Fig. 2a). To transport amino acids against their concentration gradient a secondary active transport mechanism is used (Fig. 2b). The plant amino acid transporters characterized so far use a proton gradient, built up by $H^+$ ATPase, to power the movement of amino acids across the

membrane (Fischer et al. 2002). The amino acid and proton are then symported through the membrane transporter against the amino acid concentration.

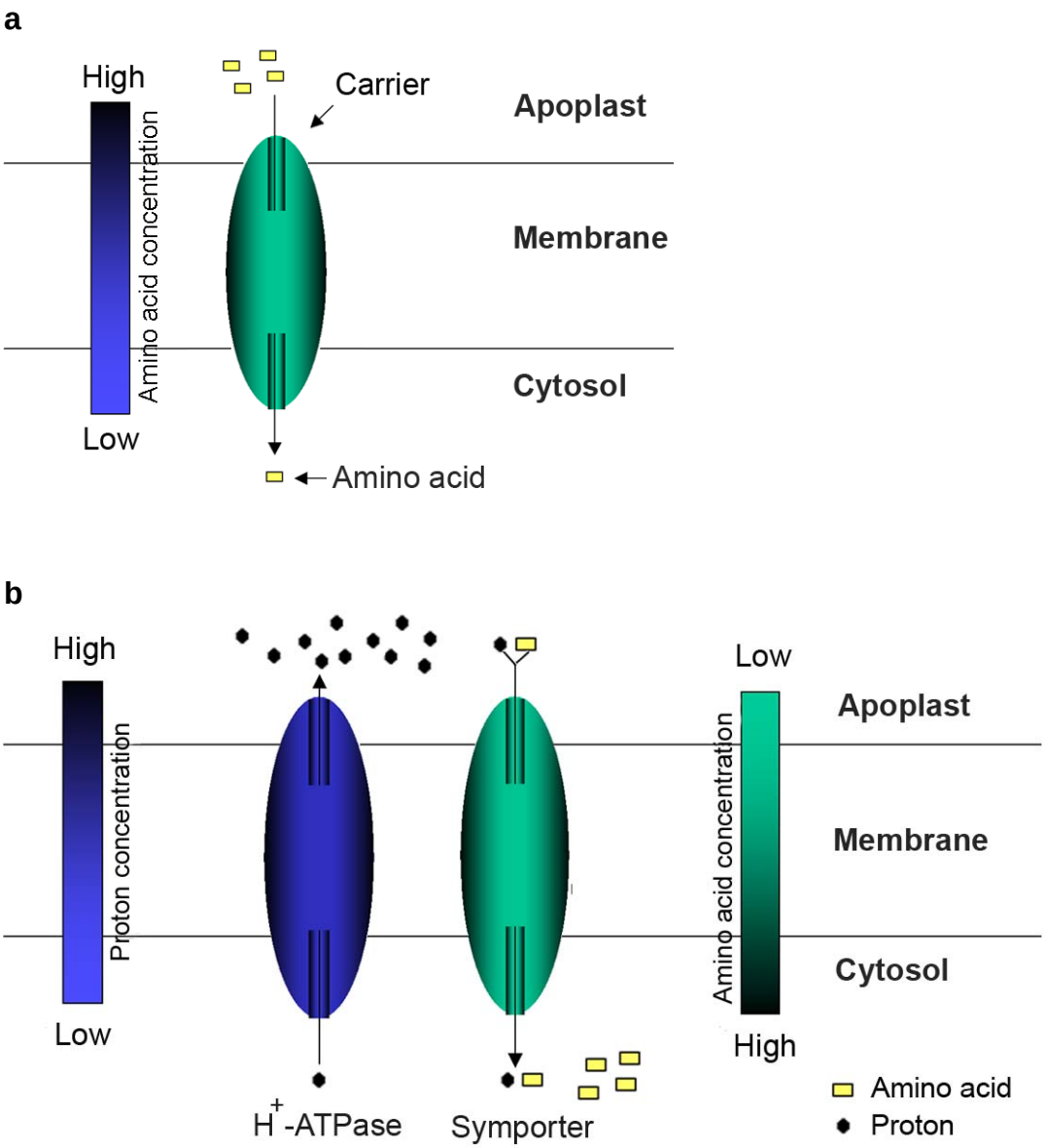


Figure 2. Schematic overview of amino acid transport across the plasma membrane by (**a**) passive and (**b**) active transport. **a**, Amino acid transport down its concentration gradient by a carrier. **b**, Amino acid transport against it's

concentration gradient by a proton amino acid symporter. The proton gradient is built up by a $H^+$ ATPase.

More than 50 amino acid transporters are predicted to be present in *Arabidopsis thaliana* and are separated into two superfamilies: the APC (Amino acid, Polyamine and Choline) and ATF (Amino acid Transporter Family) superfamilies (Fischer et al. 1998; Ortiz-Lopez et al. 2000; Wipf et al. 2002).

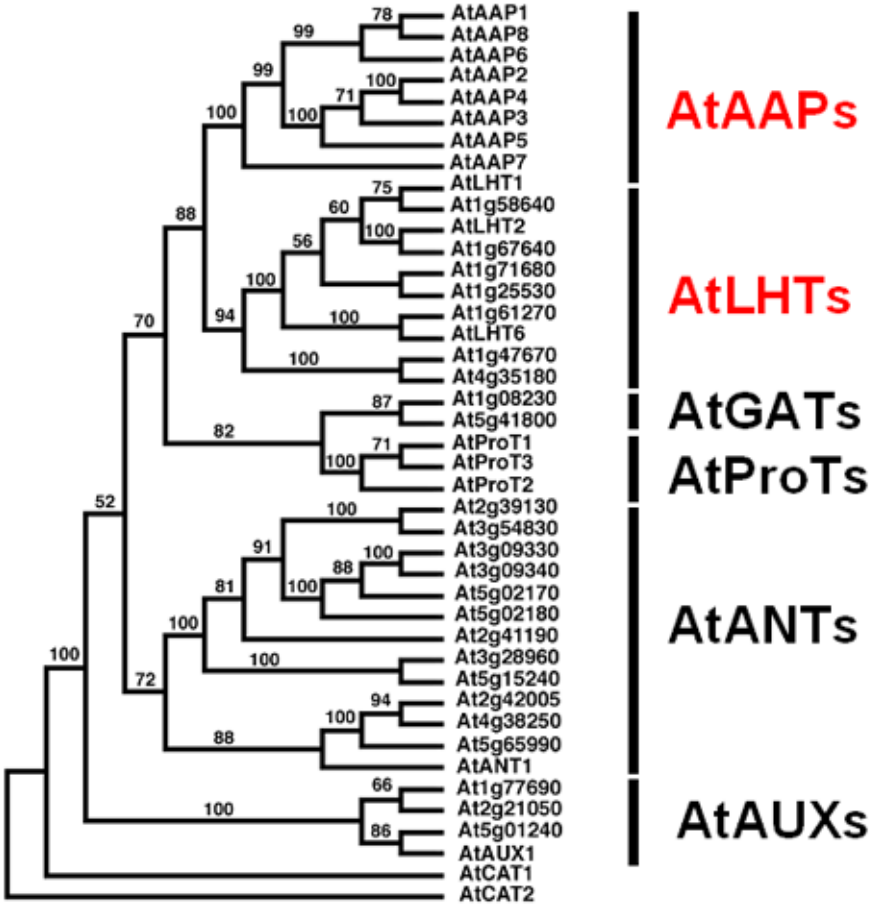


Figure 3. Phylogentic analysis of the *Arabidopsis thaliana* ATF amino acid transporter superfamily based on their protein sequence. CAT1 and CAT2 were

used as out-groups. For accession numbers see http://aramemnon.botanik.uni-koeln.de.

### 1. 5. 2. *The APC amino acid superfamily*

The APC superfamily is comprised of the LATs (L-type amino acid transporters) and the CATs (cationic amino acid transporters) (Frommer et al. 1995; Wipf et al. 2002; Su et al. 2004; Hammes et al. 2006). While in mammals the LATs have been shown to be heteromeric amino acid transporters, none of the plant LATs have been characterized.

AtCAT1 (AAT1) was the first CAT isolated (Frommer et al. 1995) and while cellular localization in Arabidopsis was not resolved, *AtCAT1* promoter-*GUS* in tobacco indicated expression was mostly found in the vasculature throughout the plant. *AtCAT5* was expressed in leaf edges and roots, whereas *AtCAT6* expression was localized to germinating seeds, side root primordia, root tips, siliques and in nematode root knots (Su et al. 2004; Hammes et al. 2006). Furthermore, AtCAT5 was localized to the plasma membrane, while AtCAT6 was targeted to the plasma membrane and ER. Biochemical studies determined that AtCAT1 and AtCAT5 transport basic amino acids with a high affinity. In contrast, AtCAT6 was shown to preferentially transport large neutral and cationic amino acids with a moderate affinity (Hammes et al. 2006).

### 1. 5. 3. The *ATF amino acid superfamily*

The ATF superfamily consists of the AAP, LHT, ProT, GAT, AUX and the ANT families (Wipf et al. 2002; Rentsch et al. 2007) (Fig. 4). Within this group

the ANTs with 13 members are the largest family, but only one ANT has been characterized so far. *AtANT1* was shown to be expressed in all organs and transport aromatic neutral amino acids and arginine (Chen et al. 2001).

The ProT family has three members that preferentially transport compatible solutes, such as proline and glycine betaine (Rentsch et al. 1996; Breitkreuz et al. 1999; Grallath et al. 2005). Furthermore, all three members were localized to the plasma membrane of protoplasts, but had unique expression patterns (Grallath et al. 2005). *ProT1* was expressed in vascular tissue throughout the plant. *ProT2* had strongest expression in roots and could be induced in leaves by wounding or salt stress. *ProT3* was found in the epidermal cells of leaves, sepals and anthers. The closely related GATs transport GABA with a high affinity and molecules with similar structure, such as 7-aminoheptanoic acid (Meyer et al. 2006). The AtGAT1 protein was localized to the plasma membrane of protoplasts. In addition, real-time RT-PCR revealed strongest expression of *GAT1* was found in flowers and was induced in wounded or senescing leaves.

The AUX family has four members and at least AtAUX1 was shown to transport auxin with a high affinity (Yang et al. 2006). Furthermore, AtAUX1 was localized to the plasma membrane of protophloem cells in roots, which is in line with the lack of gravitropism in plants with mutated *AtAUX1* (Bennett et al. 1996; Swarup et al. 2001).

The following research involves members of the AAP and LHT families. In *Arabidopsis thaliana* there are eight members within the AAP family that have

been characterized using heterologous expression in *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes and it was shown that they transport a broad spectrum of amino acids with a moderate affinity (Hsu et al. 1993; Kwart et al. 1993; Frommer et al. 1993; Fischer et al. 1995; Boorer et al. 1996; Boorer and Fischer 1997; Hirner et al. 1998; Fischer et al. 2002; Okumoto et al. 2002; Okumoto et al. 2004). When expressed in Xenopus oocytes AAPs transport acidic and neutral amino acids, while the affinity for the single substrates varies (Frommer et al. 1993; Fischer et al. 1995; Fischer et al. 2002; Lee et al. 2007).

The expression patterns differed among the AAPs. *AtAAP1* was found in developing seeds and root tips, while *AtAAP2* was expressed in the vasculature of roots, cotyledons and developing siliques (Hirner et al. 1998; Lee et al. 2007). *AtAAP3* was detected in the phloem of roots, but was also expressed in the vasculature of anthers, cotyledons and major veins of mature leaves (Okumoto et al. 2004). RNA levels for *AAP4* were shown to be highest in stems and leaves, whereas transcripts of *AAP5* were found in source leaves, stems, flowers and weak expression in roots and siliques (Fisher et al. 1995). *AAP6* was expressed in the xylem parenchyma throughout the plants, whereas *AAP8* was seen in early developing seeds and the vasculature of siliques (Okumoto et al. 2002).

The LHT amino acid transporter family from *A. thaliana* consists of 10 members of which only two have been studied, LHT1 and LHT2 (Chen and Bush 1997; Lee and Tegeder 2004; Hirner et al. 2006; Svennerstam et al. 2007). *LHT1* is expressed in lateral roots, leaves, stems, sepals and pedicels (Hirner et al. 2006), whereas *LHT2* transcripts were detected in flower buds specifically in

the tapetum, which provides the developing pollen with nitrogen and other compounds (Lee and Tegeder 2004). Both LHT1 and LHT2 transport neutral and acidic amino acids with a high affinity (Lee and Tegeder 2004; Hirner et al. 2006).

### 1. 5. 4. *Functional analysis of amino acid transporters in plants*

As described earlier there are a lot of amino acid transporters predicted, but only a few functional studies in plants have been performed. The first characterization of an amino acid transporter *in planta* was performed by repression of a leaf expressed *StAAP1* in potato plants using an *antisense* approach (Koch et al. 2003). The transgenic potato lines showed a reduction in tuber amino acid content compared to wild type, indicating a role of StAAP1 in long distance transport from leaves to tuber tissue.

Recently the function of individual transporters in amino acid uptake by the roots has been examined. Mutants of *AtCAT6*, an amino acid transporter expressed in the roots, were unable to grow on 2.5 mM Gln as sole nitrogen source indicating a role of this transporter in the acquisition or usage of Gln (Hammes et al. 2006). In a growth screen, with Arg as sole nitrogen source and amino acid T-DNA insertion lines, *aap5* mutants showed reduced growth (Svennerstam et al. 2008). Furthermore, when exposed to Arg concentrations that are toxic to wild type the *aap5* mutants survived, indicating involvement of AtAAP5 in Arg uptake.

In another study the role of *AtLHT1* in amino acid uptake by the roots was investigated, because it was expressed in side roots of young seedlings and in the root tips of older plants (Hirner et al. 2006). Analysis of *AtLHT1* T-DNA insertion lines showed a reduction in the uptake of Asp, Glu and Gln compared to wild type (Hirner et al. 2006; Svennerstam et al. 2007), indicating that AtLHT1 plays a role in the acquisition of amino acids from the rhizosphere. In addition, the *lht1* mutants demonstrated decreased biomass compared to wild type when grown on 5 mM Glu, Asp and GABA due to insufficient acquisition of these amino acids. Furthermore, when *AtLHT1* was overexpressed the plants showed improved growth on 5 mM Asp and increased uptake of Asp, Glu and Gln compared to wild type.

Besides a role in the uptake of amino acids by the roots, Amino acid transporters are predicted to be important for partitioning in sinks. *AtAAP8* was localized to the seed during early seed development and to the phloem of siliques during later stages (Okumoto et al. 2002). To test the role of AtAAP8 in siliques *aap8* mutants were analyzed and the results showed a reduction in acidic amino acids in young siliques and a 50% decrease in the number of mature seeds.

While most of the amino acid transporter studies were performed in Arabidopsis another study analyzed the transport of amino acids to seeds by overexpressing *VfAAP1* in *Pisum sativum* (Pea) and *Vicia narbonensis* under control of the legumin B4 storage protein promoter (Rolletscheck et al. 2005;

Weigelt et al. 2008). The results show that by overexpressing amino acid transporters in seeds storage proteins can be increased.

### 1. 6. Aim of this research

The acquisition of nitrogen and amino acid partitioning within the plant is important for plant growth and development. The aim of this study was to characterize the role of amino acid transporters in nitrogen uptake by the roots and import into sinks, specifically flowers.

The following research is assembled into four chapters pertaining to amino acid transport in *Arabidopsis thaliana*. The first research chapter, chapter 2, describes the characterization of AtAAP1 function in the uptake of amino acids by the roots. Chapter 3 analyzes the role of AtLHT6 in the acquisition of amino acids and investigates the effects of AtAAP1 overexpression on amino acid uptake. Chapter 4 describes the isolation and expression of *AtLHT* transporters (*AtLHT2*, *AtLHT4*, *AtLHT5* and *AtLHT6*) in flowers and discuses their potential functions. Finally, chapter 5 provides initial characterization of members of the AtLHT family using expression analyses and transport studies in heterologous systems. Furthermore, T-DNA insertion lines were identified to homozygosity and *antisense* lines were produced for analysis of AtLHT4, AtLHT5 and ATLHT6 transporter function.

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# Chapter 2

# AAP1 transports uncharged amino acids into roots of *Arabidopsis*

## 2. 1. Abstract

Amino acids are available to plants in some soils in significant amounts, and plants frequently make use of these nitrogen sources. The goal of this study was to identify transporters involved in the uptake of amino acids into root cells. Based on the fact that high concentrations of amino acids inhibit plant growth, we hypothesized that mutants tolerating toxic levels of amino acids might be deficient in the uptake of amino acids from the environment. To test this hypothesis, we employed a forward genetic screen for Arabidopsis thaliana mutants tolerating toxic concentrations of amino acids in the media. We identified an Arabidopsis mutant that is deficient in the amino acid permease 1 (AAP1, At1g58360) and resistant to 10 mM phenylalanine and a range of other amino acids. The transporter was localized to the plasma membrane of root epidermal cells, root hairs, and throughout the root tip of Arabidopsis. Feeding experiments with [14C]-labeled neutral, acidic and basic amino acids showed significantly reduced uptake of amino acids in the mutant, underscoring that increased tolerance of aap1 to high levels of amino acids is coupled with reduced uptake by the root. The growth and uptake studies identified glutamate, histidine and neutral amino acids, including phenylalanine, as physiological substrates for AAP1, whereas aspartate, lysine and arginine are not. We also demonstrate that AAP1 imports amino acids into root cells when these are supplied at ecologically relevant concentrations. Together, our data indicate an

important role of AAP1 for efficient use of nitrogen sources present in the rhizosphere. This work has recently been published (Lee *et al.*, 2007). Contributions made by this thesis are the initial toxicity screens, as well as uptake studies with radiolabled amino acid under ecologically relevant concentrations and by hydroponics.

## 2. 2. Introduction

Nitrogen is essential for plant growth and development, and nitrate or ammonium are the predominant nitrogen sources that are taken up from the rhizosphere to be assimilated in the roots or shoots to amino acids and other nitrogenous compounds important for plant metabolism or product storage. However, published results indicate that organic nitrogen, such as amino acids, may also play an important role in plant nitrogen nutrition in nature. It was found that a large variety of plants grown in natural ecosystems obtain amino acids from the soil (Chapin *et al.,* 1993; Kielland, 1994; Raab *et al.,* 1996, 1999; Schmidt and Stewart, 1999; Schobert and Komor, 1989). Analysis of different soils, including those of tropical or boreal environments, revealed that amino acid levels can be 100 μM or higher, supporting the importance of organic nitrogen uptake for plant nutrition (Chapin *et al.,* 1993; Henry and Jefferies 2003; Kielland, 1994; Näsholm *et al.,* 1998; Raab *et al.,* 1999). While a broad spectrum of amino acids is generally found in the soil, concentrations and compositions of amino acids vary considerably and seem to be dependent on the soil type, vegetation and climate (for review see Lipson and Näsholm, 2001).

Uptake of amino acids by the plant root might occur by both passive and active transport mechanisms. Passive uptake by bulk flow and diffusion probably involves

carriers or channel like pores, while active import of the organic nitrogen into the root cell is likely mediated by proton-coupled amino acid transporters in a secondary active transport step (Bush, 1993, 1999; Delrot *et al.,* 2001; Fischer *et al.,* 2002). In roots, amino acids can be transported either symplasmically or apoplastically along the cell walls. If transport occurs via the apoplast, amino acids need to be loaded into the symplast before reaching the Casparian strip of the endodermis, which blocks apoplastic passage (see Figure 8). After passing the endodermis, the nitrogen is transported to the vascular cylinder and translocated in the xylem with the transpiration stream to the shoot (Schobert and Komor, 1990). In the case of symplasmic transport, amino acid uptake might take place into the cortex and endodermis cells following apoplastic transport or directly at the root epidermis. *Arabidopsis* has both non-root hair and root hair-bearing epidermis cells (Dolan, 2001; Dolan *et al.,* 1993), and the root hairs provide an enormous surface area for uptake. Import of amino acids into the root can also occur via the root cap cells that are located at the tip of the root (Barlow, 2002). In this study, we address the question of how amino acids are taken up from the rhizosphere, and which transporters might be involved in this process.

Intriguingly, more than 53 amino acid transporters have been identified in *Arabidopsis* by heterologous complementation and database screening with known transporters, and the amino acid permeases (AAPs) are the best-studied organic nitrogen transporters (Fischer *et al.,* 1998; Wipf *et al.,* 2002). Heterologous expression of the plant *AAP*s in *Saccharomyces cerevisiae* mutants deficient in endogenous amino acid transport system as well as in *Xenopus* oocytes revealed that AAPs transport a wide spectrum of amino acids with low affinity (Boorer and Fischer, 1997; Boorer *et al.,*

1996; Fischer *et al.,* 1995, 2002; Frommer *et al.,* 1993; Hsu *et al.,* 1993). The amino acid permease 1 (AAP1, At1g58360; initially also called NAT1) was the first amino acid transporter isolated from plants (Frommer *et al.,* 1993; Hsu *et al.,* 1993). Transport studies in yeast cells showed that AAP1 mediates transport of neutral and acidic amino acids (Frommer *et al.,* 1993; Hsu *et al.,* 1993). In addition, electrophysiological analyses in *Xenopus* oocytes demonstrated that AAP1 preferentially transports neutral amino acids with short side chains and glutamate (Boorer *et al.,* 1996; Fischer *et al.,* 2002). While application of heterologous expression systems has allowed the characterization of the transport properties and the substrate specificity of AAP1 and other amino acid transporters, these approaches have not determined the physiological function of the transporters in plants. In potato, *antisense* inhibition of the leaf amino acid permease *AAP1* caused reduction of the pool of free amino acids in tubers (Koch *et al.,* 2003). In legumes, overexpression of *VfAAP1* in seeds leads to an increase of storage protein (Rolletschek *et al.*, 2005) and recently, using repression and overexpression analysis Hirner *et al.* (2006) have shown that *Arabidopsis* LHT1 is involved in uptake of amino acids into root and mesophyll cells. However, until now no study has resolved the substrate specificity of an amino acid transporter *in planta*.

To address the question of which transport mechanisms are involved in amino acid uptake from the soil, we set out to conduct an unbiased forward genetic screen, making use of the fact that phenylalanine is toxic to *Arabidopsis* seedlings when employed at high concentrations (Voll *et al.,* 2004). We reasoned that mutants deficient in uptake of phenylalanine from the growth media would be resistant to toxic concentrations of this amino acid. Specifically, we screened T-DNA mutagenized *Arabidopsis* populations for

mutants that tolerate high concentrations of phenylalanine in the growth medium and found that loss-of-function alleles of the amino acid transporter AAP1 conferred resistance to 10 mM phenylalanine. Here, we focus on the analysis of the physiological function of the *Arabidopsis* AAP1. We show that AAP1 is a plasma membrane transporter that functions in root epidermal cells, including root hairs and throughout the root tip (e.g. cortex and endodermis) of the main and lateral roots. We also demonstrate AAP1 function in import of amino acids into root cells and resolve the substrate specificity of AAP1 *in planta* by applying an amino acid toxicity screen and uptake studies. Together, our data show that AAP1 plays a role in root amino acid acquisition and is important to the subsequent translocation of amino acids to the shoot.

## 2. 3. Materials and Methods

### 2. 3. 1. Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were imbibed in cold water overnight at 4°C and planted in pots with a mixture of peat (60%), pumice (20%), and sand (20%). *Arabidopsis* plants were grown in an environmental chamber with a 16-hour day length with light conditions between 150-200 µmol photons $m^{-2}$ $sec^{-1}$. The day and night temperature was set to 20°C and 16°C, respectively. For *in vitro* cultures, seeds were sterilized as follows. Seeds were soaked in 50% ethanol for 1 min and then washed in 50% bleach with 0.1% Triton X-100. The seeds were rinsed five times with sterile water. *In vitro* cultures were performed on MS medium (pH 5.7) (Murashige and

Skoog, 1962) supplemented with agar (8 g $l^{-1}$), myo-inositol (100 mg $l^{-1}$), sucrose (10 g $l^{-1}$) and MES (0.5 g $l^{-1}$). *Arabidopsis* seedlings were grown in a tissue culture chamber with a day/night period of 16/8 h (20°C day and 16°C night), light intensity of 125-130 µmol photons $sec^{-1}m^{-2}$ and 50% humidity.

### 2. 3. 2. Isolation of phenylalanine tolerant AAP1 T-DNA insertion mutants

*Arabidopsis* T-DNA insertion lines from the *Arabidopsis* Knockout Facility of the University of Wisconsin (http:/www.biotech.wisc.edu/*Arabidopsis*/; Krysan *et al.,* 1999) were screened for a phenylalanine insensitive growth phenotype as described by Voll *et al.,* (2004). Genomic DNA from candidate lines that had proven positive for the phenylalanine tolerant growth phenotype in three consecutive rounds of re-screening was isolated with a urea-based protocol (Shure *et al*., 1983). Contaminating RNA was removed from the preparation by an RNase digest and the DNA was purified by phenol/chloroform and ethanol precipitation applying standard protocols. Genomic DNA fragments adjacent to the T-DNA insertion were amplified by a modified TAIL-PCR protocol derived from Liu *et al.,* (1995). The most important changes to the cited protocol were the use of arbitrary degenerated (AD) primer decamers with a 60% GC content and a two- instead of a three-step PCR amplification procedure. The primary TAIL-PCR reactions contained 20 ng template, 1U Taq polymerase (Promega, Madison, WI), Taq reaction buffer (Promega, Madison, WI), 2 mM $MgCl_2$, 50 nM AD primer (5'-AggTgACCgT-3' worked), 250nM specific primer (pSKI015-TL1, 5'-CCCCCATTTggACgTgAATgT-3', located 287bp inward from the LB) and 200 µM

dNTPs in a total volume of 50 µl. The PCR program was as follows: 94ºC, 60s – three cycles of 94ºC, 30s - 55ºC, 60s - 72ºC, 60s - 94ºC, 15s - 42ºC, 60s - 72ºC, 60s and twenty cycles of 94ºC, 15s - 57ºC, 30s - 72ºC, 30s - 94ºC, 15s - 45ºC, 30s - 72ºC, 30s and a final elongation step of 3 min at 72ºC. The secondary TAIL-PCRs contained 0.5 µl of the primary reactions as template and all constituents of the primary reactions except that 250 nM nested specific secondary primer pSKI015-TL-2 (5'-CgCCTATAAATACgACggATCg-3', 204bp inward from the T-DNA-LB) substituted for the specific primer pSKI015-TL1 employed in the primary TAIL-PCRs. The secondary TAIL-PCR program consisted of: 94ºC, 60s and twenty cycles of 94ºC, 15s - 57ºC, 30s - 72ºC, 30s - 94ºC, 15s - 45ºC, 30s - 72ºC, 30s and a final elongation step of 3 min at 72ºC. The TAIL-PCR products were separated by agarose gel electrophoresis, isolated from the gel slices with the QIAQUICK Gel Extraction Kit (QIAGEN, Hilden, Germany), ligated into pGEMT-Easy (Promega, Madison, WI) and sequenced on an ABI 3730 sequencer. The deduced insertion sites were corroborated in a standard PCR reaction employing 21mers approx. 900 bp downstream of the pSKI-TL2 primer 3'-end.

In a reverse genetic screen for *AAP* amino acid transporter mutants, a mutant line with T-DNA insertion in the *aap1* gene was identified, allowing us to test whether the phenylalanine insensitive phenotype of *aap1-2* could be confirmed. This *aap1-1* mutant was isolated by screening DNA pools of populations of ~60,000 *Arabidopsis* T-DNA insertion lines from the *Arabidopsis* Knockout Facility of the University of Wisconsin according to the facility's protocol (see http:/www.biotech.wisc.edu/*Arabidopsis*/). The location of the T-DNA (5936 nt) insertion was determined by PCR using gene specific primers (5'-CggTTgCgATggCTAgACCgATTCC-3') in combination with the left border

(LB) primer (5'-CATTTTATAATAACgCTgCggACATCTAC-3') annealing 150 nucleotides downstream of the left border of the T-DNA and sequencing. The *Arabidopsis* plants with T-DNA insertion in *AAP1* were further screened on solid MS medium (Murashige and Skoog, 1962; Weigel and Glazebrook, 2002) containing kanamycin (50 µg/ml). Kanamycin resistant plants were transferred to soil and homozygous mutants used for analysis.

### 2. 3. 3. RNA expression analysis

Total RNA was isolated from 3-week old *Arabidopsis* plants of wild-type, *aap1-1* and *aap1-2* (ecotype Wassilewskija) according Lee and Tegeder (2004), treated with DNase I (Roche, Indianapolis, IN) at 37°C for 1h, and used for Reverse Transcription-Polymerase Chain Reaction (RT-PCR) expression analysis. First-strand cDNAs of *AAP1* were synthesized at 37°C for 50 min by M-MLV reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) followed by PCR amplification of *AAP1* full-length fragments (1458 bp) using cDNA-specific primers (5'-ATgAAgAgTTTCAACACAgAAggA-3' and 5'-TCACTCATgCATgCATAgTCCgg AAggg-3'). PCR conditions were as follows: 95°C for 5 min for initial denaturation, 25 cycles of 95°C for 30 s, 58°C for 45 s, 72°C for 2 min, and 72°C for 10 min for the final extension. The experiment was repeated with an independently grown set of plants. As a control for correct RT-PCR conditions and RNA amounts, cDNA fragments of the constitutively expressed *Arabidopsis* actin gene *AtACT2* were amplified using the following primers:

5'-CCAATCgTgTgTgACAATggTACCg-3' and 5'-ggTTgTACgAC CACTggCgTACAAg-3' (An *et al.,* 1996).

### 2. 3. 4. Preparation of GFP-AAP1 fusion constructs

Six different constructs were prepared for GFP-AAP1 fusion protein studies: *GFP-AAP1* and *GFP* (control) constructs, each for expression in *Saccharomyces cerevisiae* (yeast complementation), onion cells (transient expression using particle bombardment) and *Arabidopsis thaliana* (stable transgenic plants). First, *mGFP5* cDNA with/without a stop codon was cloned into pDH51 vector containing the CaMV *35S* promoter and terminator (Pietrzak *et al.,* 1986). *mGFP5* was obtained by PCR using mgfp5er vector as template (Siemering *et al.,* 1996) and the primers 5'-CggATCCATgAgTAAAgAAggAACTTTTCACTggAg-3', 5'-CTCTAgACTATTTgTATAgTTCATCCATgCCATg-3', 5'-CggATCCATgAgT AAAgAAggAACTTTTCACTggAg-3', and 5'-CTCTAgATTTgTATAgTTCATCCATgC CATg-3', respectively, and cloned into pGEMT-Easy (Promega, Madison, WI). The *mGFP5* fragment (*Bam*HI/*Xba*I) was then transferred into pDH51 (Pietrzak *et al.,* 1986), yielding the intermediate construct pDHG52 (CaMV *35S* pro-*GFP*stop-*35S*term/pDH51) or pDHG62 (CaMV*35S*pro-*GFP*no stop-*35S*term/pDH51). The *GFP*nostop-*35S*term fragment (*Sac*I/*Bam*HI blunt) was transferred into pUC18 (*Sac*I/*Eco*RI blunt) (Yanisch-Perron *et al.,* 1985) and *AAP1*cDNA was cloned downstream of *mGFP5*no stop. The resulting construct was called *mGFP5*nostop-*AAP1-35S* term cDNA/pUC18 or pUC18.1. For expression of the GFP-AAP1 fusion protein in yeast cells, *mGFP5*nostop-

*AAP1* was excised from pUC18.1 and cloned into pDR196 (*Sma*I/*EcoR*I) (Rentsch *et al.,* 1995), yielding the final construct *PMA1* pro-*GFP-AAP1-ADH* term/pDR196. An *Eco*RI/*Hind*III *mGFP5* fragment was cloned into pDR196 for control experiments. For particle bombardment of epidermal onion cells, the *mGFP5-AAP1* fragment (*Sac*II/*Xba*I) from pUC18.1 was 'blunt-cloned' into pDHG6.2 (*Pst*I/*EcoR*I), yielding pDH51.1 or CaMV *35S* pro-*GFP-AAP1-35S* term/pDH51. pDHG52 was used in GFP control experiments. For localization of GFP-AAP1 fusion protein in *Arabidopsis*, a *Hind*III/*Asp*7001 fragment of the *AAP1* promoter (Hirner *et al.,* 1998) was transferred into pUC18.1 (*Sma*I) upstream of *mGFP5*. The *AAP1*prom-*GFP-AAP1*-term cassette was put into the blunted *Hind*III/*Eco*RI sites of pGPTV-Bar (Becker *et al.,* 1992). For the control GFP construct, the *GFP* fragment from pDHG52 was cloned (*Eco*RI/*Hind*III) into pGPTV-Bar.

### 2. 3. 5. Yeast transformation and complementation

To analyze if *GFP-AAP1* encodes a functional fusion protein, complementation experiments with the *Saccharomyces cerevisiae* strain 22574d (MAT-$\alpha$, *ura 3-1, gap 1-1, put 4-1, uga 4-1*) expressing *GFP-AAP1* were performed (Jauniaux *et al.,* 1987). The yeast cells were transformed according to Dohmen *et al.,* (1991) with *GFP-AAP1* in the yeast expression vector pDR196 as well as with *AAP1*/pFL61 and *GFP/pDR196* and the empty pDR196 vector as positive or negative controls, respectively (Fischer *et al.,* 1995). Selection for yeast growth and transporter function was carried out on nitrogen-free medium supplemented with the amino acid proline (0.3 g $l^{-1}$) as sole nitrogen

source. For non-selective conditions, minimal media was supplemented with ammonium sulfate (5 g $l^{-1}$).

### 2. 3. 6. Particle bombardment and production of transgenic Arabidopsis plants

Transient expression of *GFP-AAP1* in plant cells was performed to determine if the GFP-AAP1 fusion protein is translated/expressed and to analyze if the protein is targeted to the plasma membrane or to membranes of cellular organelles. pDHG51.1 and pDHG52 were introduced into onion (*Allium cepa*) epidermal cells by particle bombardment according to the manufacturer's protocol (Bio-Rad, Richmond, CA).

*Agrobacterium* strain GV3101 (pMP90) (Koncz and Schell, 1986) was transformed with the binary vector pGPTV-Bar (Becker *et al.,* 1992) carrying *AAP1* promoter-*GFP-AAP1* or *35S-GFP* using the freeze-thaw method (An, 1987). *Arabidopsis* plants were transformed by the flower-dip method (Clough and Bent, 1998) and transgenic plants were selected using the herbicide Finale (glufosinate-ammonium: 5.78%, other ingredient: 94.22%) (Hoechst-Roussel Agri-Vet Company, Somerville, NJ). The original solution was dissolved in water (430 µl/l) and the diluted solution was sprayed every 3 days for 2 to 3 weeks starting directly after planting the seeds of the T1 generation. To verify successful transformation, a rosette leaf of resistant plants was harvested for genomic DNA analysis. DNA was extracted according to Edwards *et al.,* (1991). For confirmation of transformation, a polymerase chain reaction (PCR) was carried out on the genomic DNA using the *bar* gene primers (5'-CTgCACCATCgTCAACCACTAC-3'

and 5'-CTgCACCATCgTCAACCACT AC-3') (Lohar *et al.,* 2001) and following standard procedures.

### 2. 3. 7. Toxicity screen and direct uptake studies with Arabidopsis seedlings grown in tissue culture

For the toxicity screen, sterilized seeds of wild-type and *aap1* mutants were plated on MS media (pH 5.7, Murashige and Skoog, 1962) containing 10 g $l^{-1}$ sucrose, 100 g $l^{-1}$ myo-inositol, 0.5 g $l^{-1}$ MES and specific concentrations of different amino acids. For amino acid uptake studies, seeds of wild-type and *aap1* mutants were placed on solid MS medium (pH 5.5) divided into six triangular sections. After 5 days, some solid media (0.5 cm width) between the sections was removed creating small 'channels'. 5 μCi of the respective [$^{14}$C]amino acid was added to 5 mL of 10 mM non-labeled amino acid solution (pH 5.5, buffered in 2.5 mM MES), which was poured into the channels between the triangular agar sections. [$^{14}$C]amino acids used were L-alanine (Ala), L-glutamine (Gln), L-glycine (Gly), L-methionine (Met), L-proline (Pro), L-tryptophan (Trp), L-phenylalanine (Phe), L-histidine (His), L-lysine (Lys), L-aspartic acid (Asp) and L-glutamic acid (Glu) (Moravek Biochemicals, Inc. Brea, CA). Cotyledonary leaves did not touch the media. After 30 min to 72 hours feeding at a day/night period of 16/8 h (24°C day and night) and light conditions between 60-70 μmol photons $m^{-2}$ $sec^{-1}$, 15-25 seedlings were harvested and rinsed in water twice. Some seedlings fed with [$^{14}$C]phenylalanine were spread on sheets of chromatography paper, placed in a plant press, dried at room temperature (RT) for 1d and then placed into a phosphor screen

cassette at RT for 15 h. The plants and their autoradiographs were scanned to obtain the respective image. HPLC analysis according to Koch *et al.* (2003) was performed with the feeding solution containing Phe to confirm that the nitrogen/label that was taken up by the plant was still in the form of Phe. Other seedlings fed with Ala, Gln, Gly, Met, Pro, Phe, Trp, His, Lys, Asp and Glu were used directly for liquid scintillation counting to quantify total amount of radioactivity present. The value obtained was normalized to amino acid uptake of wild-type, which was set to 100%.

### 2. 3. 8. Direct uptake studies with hydroponically grown Arabidopsis seedlings

A hydroponic culture system modified after Tocquin *et al.* (2003) was established. The outer surfaces of Gladware® polypropylene boxes (15 x 15 x 4 $cm^3$; Clorox, San Francisco, CA) were painted black with an oil-based enamel (Rust-Oleum spray paint; Flat protective enamel; Vernon Hills, IL) and holes (1.1cm) were cut through the lids to hold 22 micro-reaction tubes (1.5 mL) and to allow placement of an air tube and air stone into the nutrient solution for aeration of roots via a steady, gentle stream of bubbles. The caps and tips of micro-reaction tubes were cut off and the remaining 2 cm long tube was filled with 0.65% agarose. Tubes with solidified agarose were placed into holes in the lid of the container, with a piece of fiberglass screen at their bottom to prevent the agar from sliding out. Sterilized seeds were placed onto the agarose. The lid was then placed onto the container filled with modified Hoagland solution (Tocquin *et al.,* 2003) and transferred into growth chambers for approximately 4-5 weeks with 12 hours of light between 200 and 205 μmol photons $m^{-2}$ $s^{-1}$ and 70% humidity. Day and

night temperatures were 20°C and 16°C, respectively. Plants with 5 pairs of rosette leaves were transferred to modified Hoagland without nitrogen. After five days of starvation, the plants were removed from the hydroponic culture and the roots were washed in fresh nutrient solution without nitrogen and placed in microtubes containing 500 μl modified Hoagland solution (pH 5.5 buffered with 0.5 g $l^{-1}$ MES) with 2mM cold and 0.5μCi [$^{14}$C]phenylalanine (Moravek Biochemicals, Inc. Brea, CA) as sole nitrogen source. Plants were kept in light at 190-200 μmol photons $m^{-2}$ $s^{-1}$ for 30 min, then washed in modified, nitrogen-free Hoagland solution, and root and leaf tissue was harvested separately for determining the radioactivity by scintillation counting.

## 2. 4. Results

### 2. 4. 1. Identification of Arabidopsis aap1 mutants resistant to high concentrations of phenylalanine in the growth medium

A forward genetic screen for phenylalanine-insensitive growth mutants, as described by Voll *et al.,* (2004), was applied to a T-DNA mutagenized *Arabidopsis thaliana* population from the *Arabidopsis* facility at the University of Wisconsin (Krysan *et al.,* 1999). In this screening, one mutant line was found that showed a phenylalanine-resistant phenotype (Figure 1). Isolation and sequencing of the flanking regions of the T-DNA insertion identified a T-DNA located -46 to -17 nt upstream of the start codon of *AAP1* (Figure 2a). The identified mutant line was named *aap1-2*. In an earlier screen for *AAP* mutants using a reverse genetic approach and the T-DNA insertion lines from the *Arabidopsis*

facility at the University of Wisconsin, we identified another mutant allele of *AAP1*, *aap1-1,* with a T-DNA insertion in the second intron, 1733 bp downstream of the start codon of *AAP1* (Figure 2a). Both independent mutant lines were used for growth analysis on media containing phenylalanine and we found pronounced differences in growth between *aap1* mutants and wild-type seedlings (Figure 1). On 2 mM phenylalanine, development of leaves and roots was significantly inhibited in wild-type seedlings, whereas *aap1-1* and *aap1-2* displayed normal growth (Figure 1a). Higher levels of phenylalanine (10 mM) arrested germination of wild-type seeds, while this 'toxic' effect was not seen for the mutants (Figure 1b).

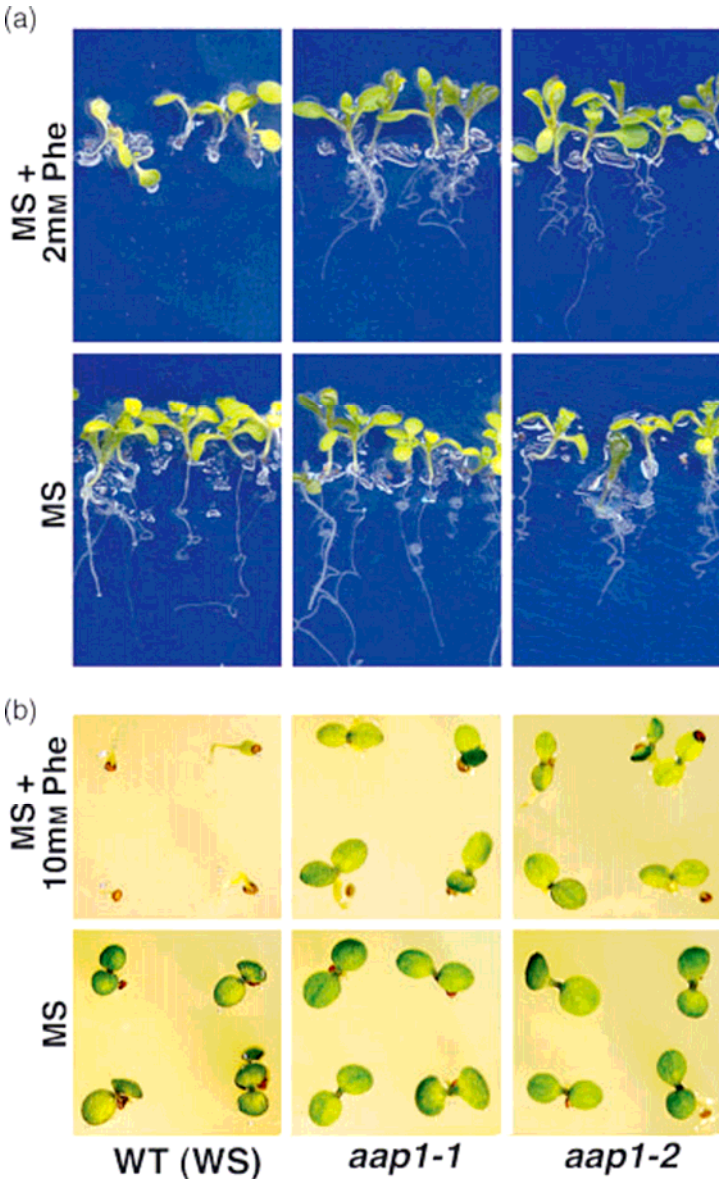


**Figure 1.** Growth of *Arabidopis thaliana* (ecotype Wassilewskija) wild-type and *aap1* plants on phenylalanine.

**(a)** Growth after 2 weeks on MS medium (Murashige and Skoog, 1962) with 2 mM phenylalanine (top) and MS medium (bottom).

**(b)** Growth after one week on MS medium with 10 mM phenylalanine (top) and MS medium (bottom).

The observed differences are most likely due to reduced phenylalanine uptake since both mutants are affected in amino acid transport. For confirmation of altered transporter expression, transcript levels of *AAP1* were determined in *aap1-1, aap1-2* and wild-type plants (Figure 2b). The T-DNA insertion in the 2nd intron in *aap1-1* completely repressed transcription of the full-length AAP1 mRNA describing *aap1-1* as a true knockout mutant. *AAP1* expression in *aap1-2* was knocked-down but still detectable. Phenotypic analyses of soil-grown *Arabidopsis* wild-type (ecotype Wassilewskija) and the two *AAP1* T-DNA insertion lines in greenhouse and growth chambers showed no obvious differences in growth rate and plant size between mutants and wild-type (Figure 2c). Equally, no phenotypic differences could be observed in tissue culture on MS media (Murashige and Skoog, 1962) (Figure 1, 2c).

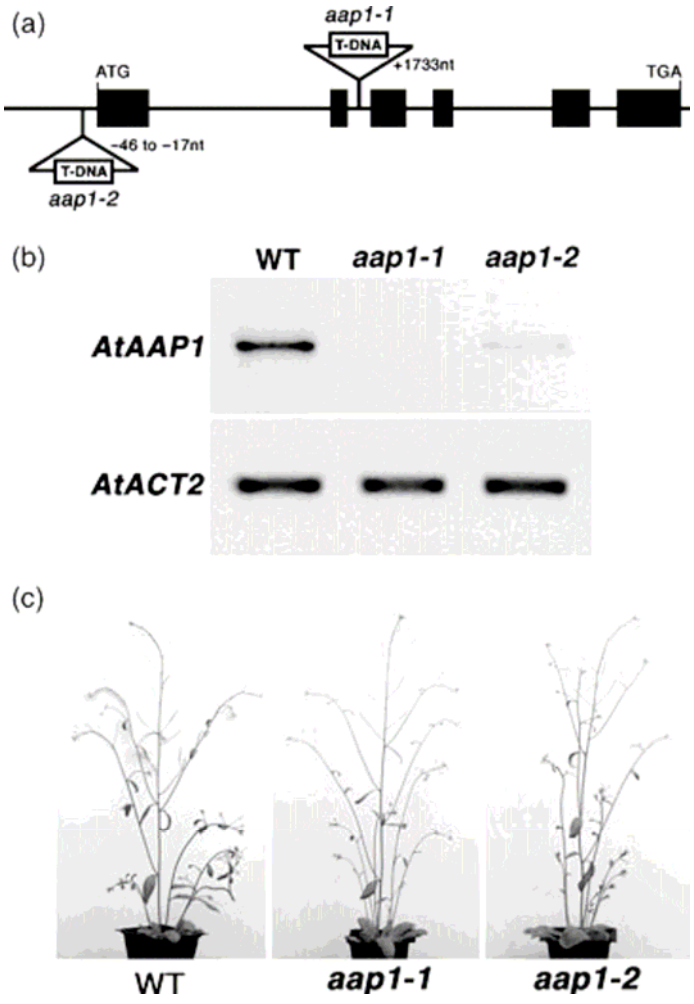


**Figure 2.** Molecular characterization of *aap1* mutants.

**(a)** Scheme of the *AAP1* gene and positions of the T-DNA insertions. The six exons of *AAP1* are boxed. The locations of the T-DNA insertion sites for *aap1-1* and *aap1-2* mutants are indicated.

**(b)** RT-PCR analysis of *AAP1* expression in 3-week-old seedlings of wild-type (Ws; Wassilewskija), *aap1-1* and *aap1-2*. *AtACT2* was amplified as a control for equal amounts of RNA.

**(c)** Phenotypical analysis of *aap1* mutants grown in the greenhouse under standard conditions.

### 2. 4. 2. AAP1 is a plasma membrane transporter and strongly expressed in both primary and lateral root tips as well as in epidermal cells of developing Arabidopsis roots

To verify that AAP1 is functioning in roots, localization studies were performed. Previous promoter-reporter gene studies have demonstrated that the Arabidopsis *AAP1* is expressed in developing seeds (Hirner *et al.,* 1998), and earlier experiments using whole mount *in situ* hybridization indicated that *AAP1* transcripts are also present in the vascular system of the cotyledons and the root elongation zone of seedlings (Kwart *et al.,* 1993). To further investigate the tissue-specific expression of *AAP1* in developing *Arabidopsis* plants and specifically in roots, additional transgenic *Arabidopsis* carrying an *AAP1*-promoter-ß-glucuronidase *(GUS)* gene construct (kindly provided by Dr. Frommer, Carnegie Institution, Stanford) were generated. Histochemical analyses of the promoter-*GUS* plants confirmed that *AAP1* is expressed in roots and cotyledons of *Arabidopsis* plants. While in young seedlings *AAP1* was only detected in the tips of the cotyledons (Figure 3a), in 14 days-old seedlings weak *AAP1* expression was found in the major veins of the cotyledon vasculature (Figure 3b). With respect to roots, in 4-day-old seedlings staining was found exclusively in the epidermal cells, in non-root hair and root hair developing cells (outer layers), and throughout the primary root tip, including the root cap, in the zone of cell division and elongation (Figure 3a, c and d). The same expression pattern, as well as staining in root hairs, was observed in 14-day-

old plants in both the primary and lateral root tips (Figure 3b, e, f and g). A closer look at the stained root tip showed *AAP1* expression in root cap cells, as well as in sloughed-off cap cells (Figure 3f). Cross section through the developing root tip prior development of the root hairs demonstrated strong staining of non-root hair and root hair epidermal cells (Costa and Dolan, 2000; Dolan *et al.,* 1993) (Figure 3f). *AAP1* expression was also seen in the cortical cells, endodermis and the vascular cylinder of the root cell division and elongation zone.

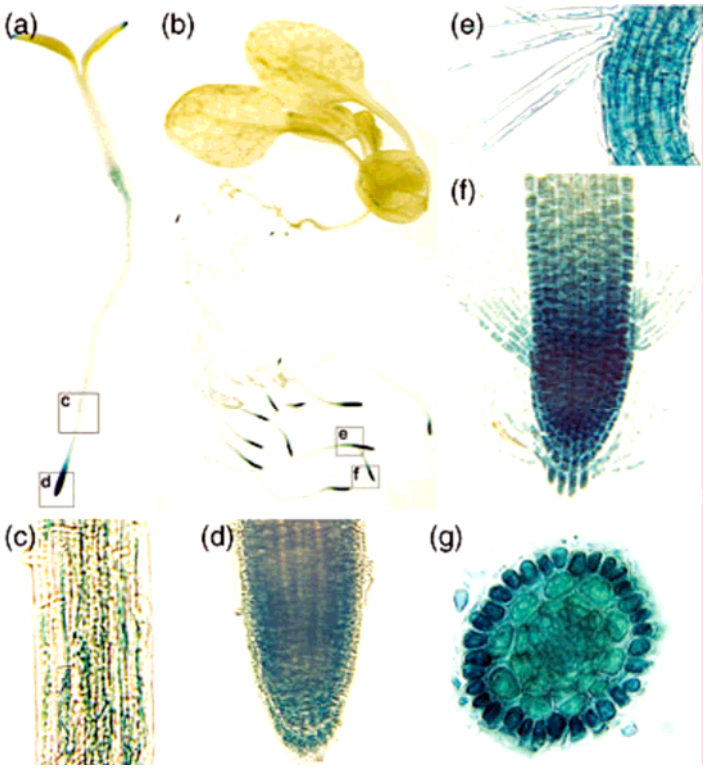


**Figure 3.** *AAP1*-promoter-*GUS* studies in developing *Arabidopsis* plants.

**(a)** 5-day-old and **(b)** 14-day-old plants grown in soil.

**(c)** GUS staining in the epidermal cells of the primary root and **(d)** primary root tip of a 5-day-old seedling grown on media.

**(e)** GUS staining of root epidermal cells and root hairs. **(f)** Stained root tip releasing root border cells **(g)** Transverse section of a root prior to the development of root hairs.

To resolve the cellular and subcellular function of AAP1 in plants, green-fluorescence (GFP)-AAP1 protein localization studies were performed. To test whether the *GFP-AAP1* gene construct encoded a functional transporter, it was first expressed in the yeast mutant 22574d that is deficient in proline transport. The yeast cells were plated on minimal media with proline as sole nitrogen source (Figure 4a). Cells expressing the empty vector failed to grow, while expression of GFP-AAP1 fusion protein restored growth of yeast cells, indicating that AAP1 retains functional transport properties when fused to GFP (Figure 4a). These results are consistent with studies by Chang and Bush (1997) showing that AAP1 (NAT2) with either N- or C-terminal myc extension remained fully active.

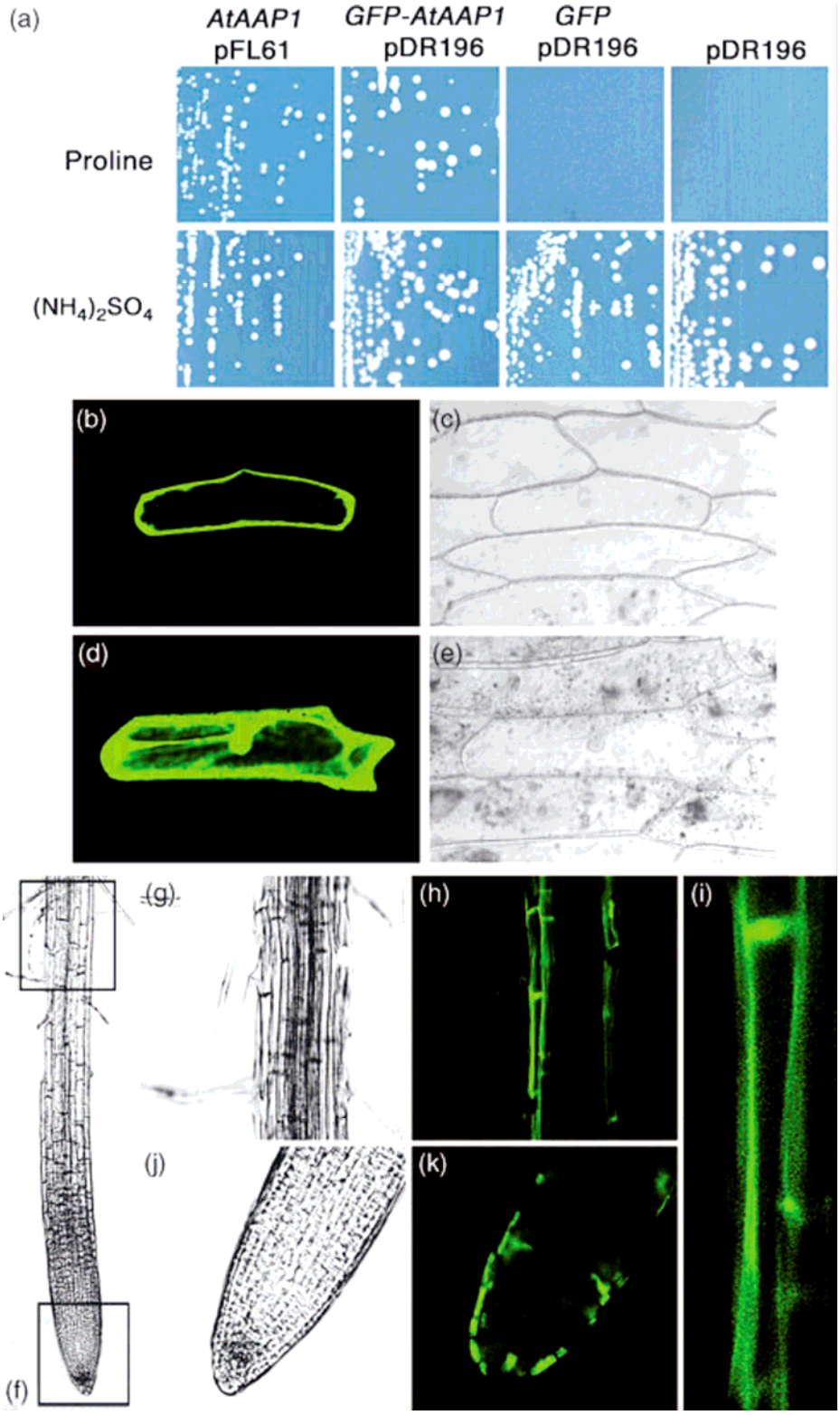
(a)
AtAAP1
pFL61
GFP-AtAAP1
pDR196
GFP
pDR196
pDR196
Proline
(NH4)2SO4
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)

**Figure 4.** Functional complementation of a yeast mutant by **(a)** GFP-AAP1 fusion protein and cellular localization of the GFP-AAP1 fusion protein in **(b-e)** onion epidermal cells and **(f-j)** *Arabidopsis* roots.

**(a)** Yeast complementation by GFP-AAP1 fusion protein. Proline-uptake deficient yeast mutant (22574d) was transformed with *GFP-AAP1* in pDR196. *AAP1* in pFL61 and *GFP* in pDR196, and empty vector pDR196 were used as controls for growth under selective condition. Minimal media was supplemented with proline (0.3 g $l^{-1}$) as a sole N source (top image) or ammonium sulfate (5 g $l^{-1}$) (bottom image).

**(b+d)** Confocal and **(c+e)** light transmission image showing GFP fluorescence emitted from onion epidermal cell bombarded with **(b+c)** *35S:GFP-AAP1* and **(d+e)** *35S:GFP* as a control.

**(f, g, j)** Light transmission and **(h, i, k)** confocal image of **(f)** a root, **(g+h)** part of the root maturation zone, **(i)** root epidermal cell and **(j+k)** root tip of a transgenic *Arabidopsis* plant expressing *AAP1:GFP-AtAAP1.*

To analyze whether AAP1 is targeted to the plasma membrane or to membranes of subcellular compartments, *GFP-AAP1* under the control of CaMV *35S* promoter (CaMV *35S*:*GFP-AAP1*) was introduced into onion (*Allium cepa*) epidermal cells by particle bombardment. The transient expression of the fusion protein in the onion cells showed that green fluorescence was restricted to the plasma membrane (Figure 4b and c), whereas in control cells expressing CaMV *35S*:*GFP*, GFP was detected throughout the cell/cytoplasm (Figure 4d and e). These data indicate that AAP1 is a plasma membrane protein involved in uptake of amino acids.

To investigate AAP1 protein localization in *Arabidopsis*, plants were transformed with the *GFP-AAP1* fusion construct under the control of the endogenous promoter (*AAP1*-promoter:*GFP-AAP1*). Visualization of GFP using confocal laser scanning microscopy revealed fluorescence in the plasma membrane of the root epidermis and in the outer cell layer of the root cap. (Figure 4f-j). Very weak fluorescence seen throughout the root tip and in root hairs could not be captured in our confocal image. Promoter-reporter gene studies and GFP-AAP1 protein localization suggest that AAP1 facilitates uptake of amino acids into root cells for transfer of amino acids to the vasculature.

### 2. 4. 3. aap1-1 *and* aap1-2 *seedlings show reduced uptake of [$^{14}$C]phenylalanine compared to wild-type*

To analyze if the differences in growth of wild-type and mutants on high concentrations of phenylalanine (see Figure 1) are linked to differences in acquisition of the amino acid by the seedlings, uptake studies with [$^{14}$C]phenylalanine were performed (Figure 5). 5-day-old seedlings of wild-type, *aap1-1* and *aap1-2* were fed with labeled phenylalanine over a period of 72 hours (Figure 5a). Clear differences in uptake were found 24 h after phenylalanine feeding, and these differences were even more pronounced after 48 and 72 h with higher uptake rates of phenylalanine in wild-type than mutant seedlings. No differences in phenylalanine uptake were observed between the *aap1-1* and *aap1-2* mutant lines. Since the seedlings were grown axenically, it is unlikely that [$^{14}$C]phenylalanine was metabolized to another nitrogen compound and taken up.

Nevertheless, we determined [$^{14}$C]-label in the feeding solution by HPLC and confirmed that all the nitrogen in solution was in the form of phenylalanine (data not shown). For visual verification of differences in amino acid uptake between wild-type and mutants, seedlings were exposed to a phosphor screen after 48 h feeding with 2 mM [$^{14}$C]phenylalanine. As presented in Figure 5b, autoradiographs of seedlings showed much stronger labeling in wild-type, specifically in cotyledonary leaves, in comparison to *aap1-1* and *aap1-2* (Figure 5b).

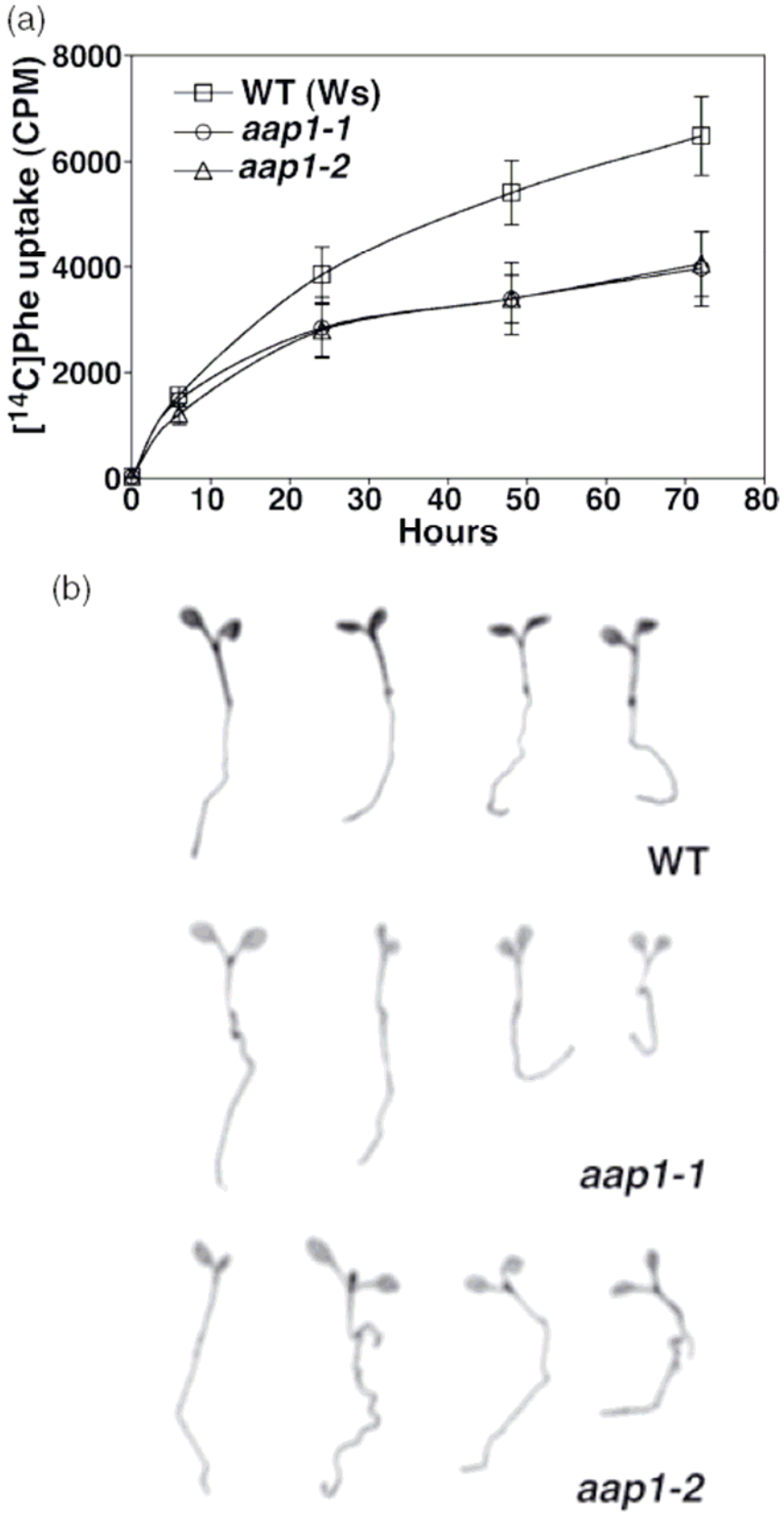
(a)
8000
6000
4000
2000
0
[14C]Phe uptake (CPM)
WT (Ws)
aap1-1
aap1-2
0
10
20
30
40
50
60
70
80
Hours
(b)
WT
aap1-1
aap1-2

**Figure 5.** Uptake of [$^{14}$C] phenylalanine in *Arabidopsis* wild-type, *aap1-1* and *aap1-2* seedlings.

**(a)** Time-dependent uptake of [$^{14}$C]phenylalanine in wild-type (ecotype Wassilewskija; WS), *aap1-1* and *aap1-2* mutants.

**(b)** Autoradiographs. Accumulation of radiolabel in wild-type, *aap1-1* and *aap1-2* seedlings after [$^{14}$C]phenylalanine feeding for 48 hours

### 2. 4. 4. aap1 mutants survive on toxic levels of a broad spectrum of amino acids

For further analysis of AAP1 function in plants and resolution of the substrate specificity of this transporter *in planta*, wild-type and *aap1* mutant seeds were plated on media supplemented with different protein amino acids. The amino acid concentrations chosen for this experiment are 'toxic' for wild-type as demonstrated by inhibition of seed germination or a strong reduction in growth. However, toxic levels could not be established for aspartate and glutamate (Figure 6). When cultured on media supplemented with neutral amino acids, clear differences in growth could be observed between wild-type and *aap1* plants (Figure 6). Since growth of *aap1-1* and *app1-2* plants is similar on all amino acids, we show only the result for *aap1-1* plants. In contrast to wild-type, *aap1* seeds germinated and seedlings developed on high levels of most neutral amino acids. When grown on the basic amino acids arginine and lysine, wild-type and *aap1* seeds failed to germinate (lysine) or seedling growth discontinued (arginine) but on histidine, *aap1* seedlings showed much stronger growth compared to wild-type. Overall, growth of *aap1* plants on toxic concentrations of amino acids points

to a reduction in uptake of a broad spectrum of amino acids in the mutants and a function of AAP1 in transport of a wide range of amino acids.

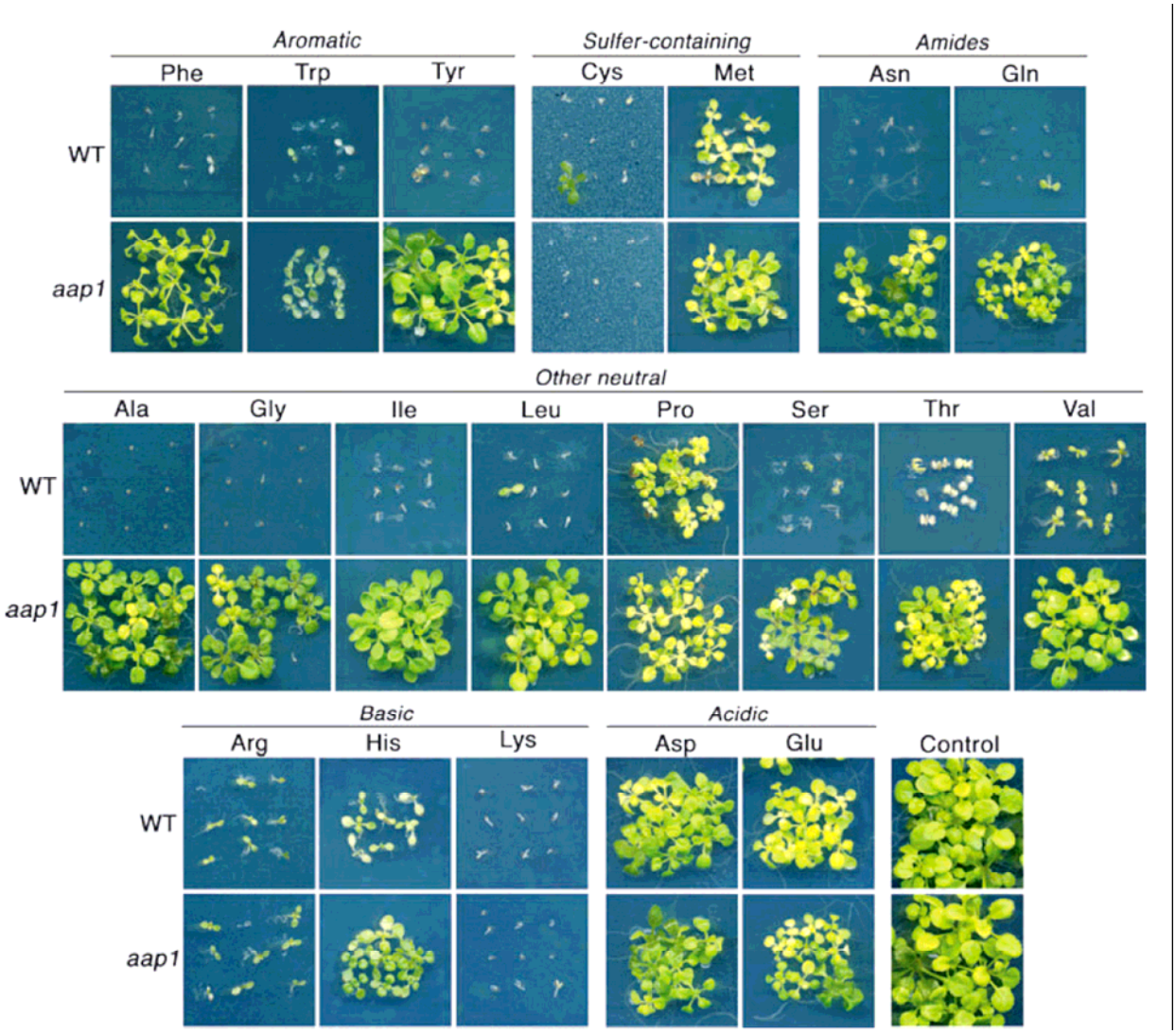


**Figure 6**. Growth of wild-type and *aap1* plants on medium with high levels of protein amino acids. Seedling growth on MS medium (Murashige and Skoog, 1962) with 2 mM tyrosine (Tyr) or lysine (Lys), 4 mM leucine (Leu) or methionine (Met), 6 mM valine (Val), 10 mM phenylalanine (Phe), isoleucine (Ile), serine (Ser), threonine (Thr) or tryptophan (Trp), 20 mM histidine (His), 25 mM cysteine (Cys) or glycine (Gly), 50 mM asparagine (Asn) or aspartate (Asp), and 100 mM glutamine (Gln), alanine (Ala), proline

(Pro), arginine (Arg) or glutamate (Glu). Generally, L-amino acids were used. As control of growth, wild-type (ecotype Wassilewskija; WS) and *aap1* mutant plants were grown on MS medium only.

Neither wild-type nor mutant grew on cysteine media, in which crystallization was observed over time. This thiol-group-containing amino acid is highly reactive and can be oxidized in aqueous or nutrient solutions within minutes, resulting in cystine or other reaction products (Darkwa *et al.,* 1998; Luo *et al.,* 2005). Therefore, the toxic effect is probably not caused by cysteine but by uptake of high amounts of oxidized cysteine (cystine). In fact, when analyzing a 10 mM cysteine solution with the Ellman's reagent (Ellman, 1959; Riddles *et al.,* 1983), we found that 40% of the cysteine is oxidized within 30 min after exposing the solution to room temperature (data not shown). In the case of proline, no differences in growth between mutants and wild-type were found. This can be explained by a high tolerance of plant cells to proline, which functions as a compatible solute (Delauney and Verma, 1993; Hasegawa *et al.,* 2000).

### 2. 4. 5. aap1 mutants show reduced uptake of neutral amino acids, histidine, and glutamate

Direct uptake studies with [$^{14}$C]-labeled amino acids were performed to further resolve AAP1 substrate specificity *in planta* (Figure 7). 5-day-old wild-type, *aap1-1* and *aap1-2* seedlings were fed with 10 mM of neutral (Gln, Gly, Met, Pro, Phe, Trp), basic (His, Lys) or acidic [$^{14}$C]amino acids (Asp, Glu) (Figure 7a). The uptake was measured as total

amount of radioactivity. The value was normalized to amino acid uptake of the wild-type, which was set to 100%. Both *aap1-1* and *aap1-2* showed a 30-50% reduction in uptake for all neutral amino acids tested, including aromatic amino acids and amides (Figure 7a). While not clearly resolved in the toxicity screen, the uptake experiments demonstrate a reduction in proline acquisition by the mutants. Studies with basic amino acids are also consistent with the growth test showing a reduced uptake of histidine for the mutants but no differences between wild-type and *aap1* seedlings in lysine acquisition. And finally, while toxic concentration for glutamate and aspartate could not be established in the growth screen, uptake studies with the acidic amino acids show a reduction in uptake of glutamate but not aspartate by the mutants, indicating that glutamate is transported by AAP1. Overall, the results demonstrate that *in planta* AAP1 transports histidine, glutamate and neutral amino acids.

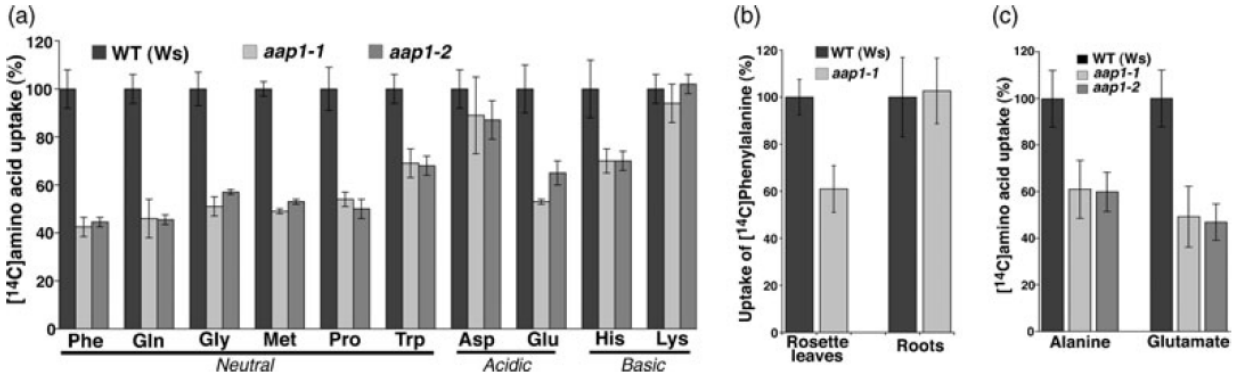


**Figure 7.** Uptake of [$^{14}$C]amino acids in *Arabidopsis* wild-type and *aap1-1* and *aap1-2* plants grown in tissue culture. Uptake of radiolabeled amino acids was measured by scintillation counting and normalized to uptake of wild-type, which was set to 100%.

**(a)** The uptake studies with 5-day-old seedlings and 10 mM of neutral, acidic or basic amino acids. Results are the mean of five replicates per experiment ± SE. A minimum of three experiments were performed.

**(b)** Uptake of [$^{14}$C]phenylalanine with hydroponically grown, 3-week-old wild-type and *aap1-1* plants. Studies were performed with 2 mM of [$^{14}$C]phenylalanine over 30 min. Results are the mean of at least 10 replicates per experiment ± SE. A minimum of three experiments were performed.

**(c)** Uptake studies with 150 μM of alanine and glutamate over 48 hours. Results are the mean of 15 replicates per experiment ± SE. Three experiments were performed.

The uptake studies were performed over 48 hours and the observed differences in accumulation of label in mutant and wild-type could also reflect effects of metabolism (e.g. feed-back regulation) on transport over this time period. To confirm that the differences in accumulation of label in mutant and wild-type are directly related to AAP1 function, uptake studies with 3-week-old (6 rosette leaves), hydroponically grown *aap1-1* and wild-type plants using 2 mM phenylalanine were performed for a duration of 30 min. The results are consistent with the long-term uptake studies (48 h) showing a reduction in label accumulation in *aap1* mutants, specifically in leaves, of about 40% (Figure 7b).

Finally, to analyze the importance of AAP1 function under ecologically relevant amino acid concentrations, uptake studies were performed with 150 μM alanine and glutamate (Figure 7c). These amino acids are found in high concentrations in soils of many ecological systems (Kieland, 1994; Raab *et al.,* 1996, 1999). In addition,

heterologous expression in *Xenpous* oocytes has shown that AAP1 transports alanine and glutamate with higher affinity than most of the other amino acids (Boorer *et al.,* 1996; Fischer *et al.,* 2002; see also Table 1). Compared to wild-type, *aap1* mutants show a reduction in uptake of [$^{14}$C]alanine and [$^{14}$C]glutamate by ca. 40 and 50%, respectively, clearly indicating that AAP1 is important for amino acid acquisition under ecological concentrations.

**Table 1** Comparison of transport function of AAP1 in *Xenopus* oocytes, *Saccharomyces cerevisiae* and Arabidopsis plants

| Amino acids tested | | $K_{0.5}$ (mM) oocytes [a] | $K_m$ (mM) yeast | AAP1 transport in plants |
|---|---|---|---|---|
| Neutral | Alanine | 0.6 ± 0.04 | 0.29 ± 0.042[b] | Yes |
| | Valine | 3.5 ± 0.6 | nd | Yes |
| | Tryptophan | 0.6 ± 0.1 | nd | Yes |
| | Asparagine | 7 ± 1 | nd | Yes |
| | Glutamine | 1.2 ± 0.1 | nd | Yes |
| | Proline | 1.9 ± 1 | 0.06[c] | Yes |
| Acidic | Glutamate | 0.80 ± 0.03 | nd | Yes |
| | Aspartate | >50 | 0.77[d] | No |
| Basic | Histidine | 6.8 ± 0.3 | nd | Yes |
| | Lysine | >50 | nd | No |

[a]Boorer *et al.* (1996), Fischer *et al.* (2002).
[b]Hsu *et al.* (1993).
[c]Frommer *et al.* (1993).
[d]Okumoto *et al.* (2002).
nd, not determined.

## 2. 5. Discussion

It has been predicted that in a range of ecosystems, amino acid uptake by the root plays an important role in plant nutrition (Chapin *et al.,* 1993; Henry and Jefferies, 2003; Kielland, 1994; Näsholm *et al.,* 1998; Raab *et al.,* 1999). Molecular studies on amino acid transport have mostly concentrated on *Arabidopsis* and the AAPs (amino acid permeases), with 8 members being the best-investigated transporters (for review, see Liu and Bush, 2006; Wipf *et al.,* 2002; Tegeder and Weber 2006). Northern-blot analyses and promoter-reporter gene studies revealed that the AAPs have distinct expression patterns, indicating that the various transporters fulfill specific functions within the plant (Fischer *et al.,* 1995, 1998; Hirner *et al.,* 1998; Kwart *et al.*, 1993; Okumoto *et al.,* 2002, 2004; Tegeder *et al.,* 2000). While individual AAPs (*AAP2* and *AAP3*) are expressed in roots, their expression seems to be restricted to the vascular tissue, and knock-down of gene expression of *AAP3* had no effect on uptake of amino acids (Okumoto *et al.,* 2002). Knock-down of the expression of an amino acid transporter from the LHT family (*LHT1*) resulted in a reduction in uptake of amino acids of 85% when 5 mM of glutamine or glutamate were supplied (Hirner *et al*., 2006). Since LHT1 is expressed in root cells as well as in leaf mesophyll cells and other plant tissues, the observed reduction in amino acid acquisition in *lht1* mutants indicates a LHT function in root uptake but also points to a more general role of this transporter in amino acid partitioning throughout plant tissue (Hirner *et al*., 2006). The studies presented here show that the AAP1 amino acid transporter is localized in root cells, were it functions in amino acid uptake to finally provide the shoot with the nitrogen compounds.

### 2. 5. 1. *AAP1* functions in amino acid uptake by root cells

In mature plants, AAP1 has been localized to flowers and developing seeds, where it is predicted to be involved in transport of amino acids into *Arabidopsis* for development and storage protein accumulation. (Fischer *et al.,* 1995; Frommer *et al.,* 1993; Hirner *et al.*, 1998; Kwart *et al.,* 1993). Using *in situ* RNA localization it was also shown that AAP1 is expressed in the vascular system of seedling cotyledons, where it is probably involved in transfer of leaf amino acids to developing parts of plant. In addition, AAP1 expression was found in the root elongation zone of seedlings (Kwart *et al.,* 1993). Localization studies presented here revealed that AAP1 is a plasma membrane protein that, during plant development, is also expressed throughout the root tip in both cell division and elongation zones, and in the epidermal root cells, which cover the entire root with the exception of the root cap. The epidermal cell layer of the maturation zone consists of hairless as well as hair-bearing epidermal cells (Costa and Dolan, 2000; Dolan *et al.,* 1993), and AAP1 expression was reported in the epidermis, including the root hairs that substantially increase the surface area of the root for more efficient uptake (Gilroy and Jones, 2000; Peterson and Farquhar 1996). The strong expression in the outer cell layers of the whole root tip and in the root epidermis points to a role of AAP1 in amino acid uptake at the plant-soil interface via both epidermis and root cap cells for transfer to the vascular xylem (Figure 8). Since AAP1 is expressed throughout the root cap and the root cap cells become symplasmically isolated as the root ages (Zhu *et al.,* 1998), AAP1 might maintain the translocation to the vasculature and/or

direct the amino acids to the apical meristem, for example for auxin biosynthesis and root development (Ljung *et al.,* 2005). Localization of AAP1 in border cells of the root cap could additionally support import of amino acids into these cells that ultimately separate from the root cap periphery for exudation of organic compounds into the rhizosphere, for example to protect the root from pathogen infection or to stimulate beneficial associations (Bais *et al.,* 2006; del Campillo *et al.,* 2004; Hawes *et al.,* 2000; Phillips *et al.,* 2004). AAP1 expression was also found in the root cortex and endodermis of the root tip (our studies; see also Kwart *et al.,* 1993). Generally, for root to shoot transfer of nitrogen, apoplastically transported amino acids need to be loaded into the root cells/symplast latest at the endodermis in order to pass the Casparian strip, which presents an apoplastic barrier (Figure 8). AAP1 localization throughout the root tissue of the division and elongation zones indicates that AAP1 facilitates uptake of amino acids into root cells along the amino acid transport path in order to direct the nitrogen to the vasculature for long distance transport to the shoot.

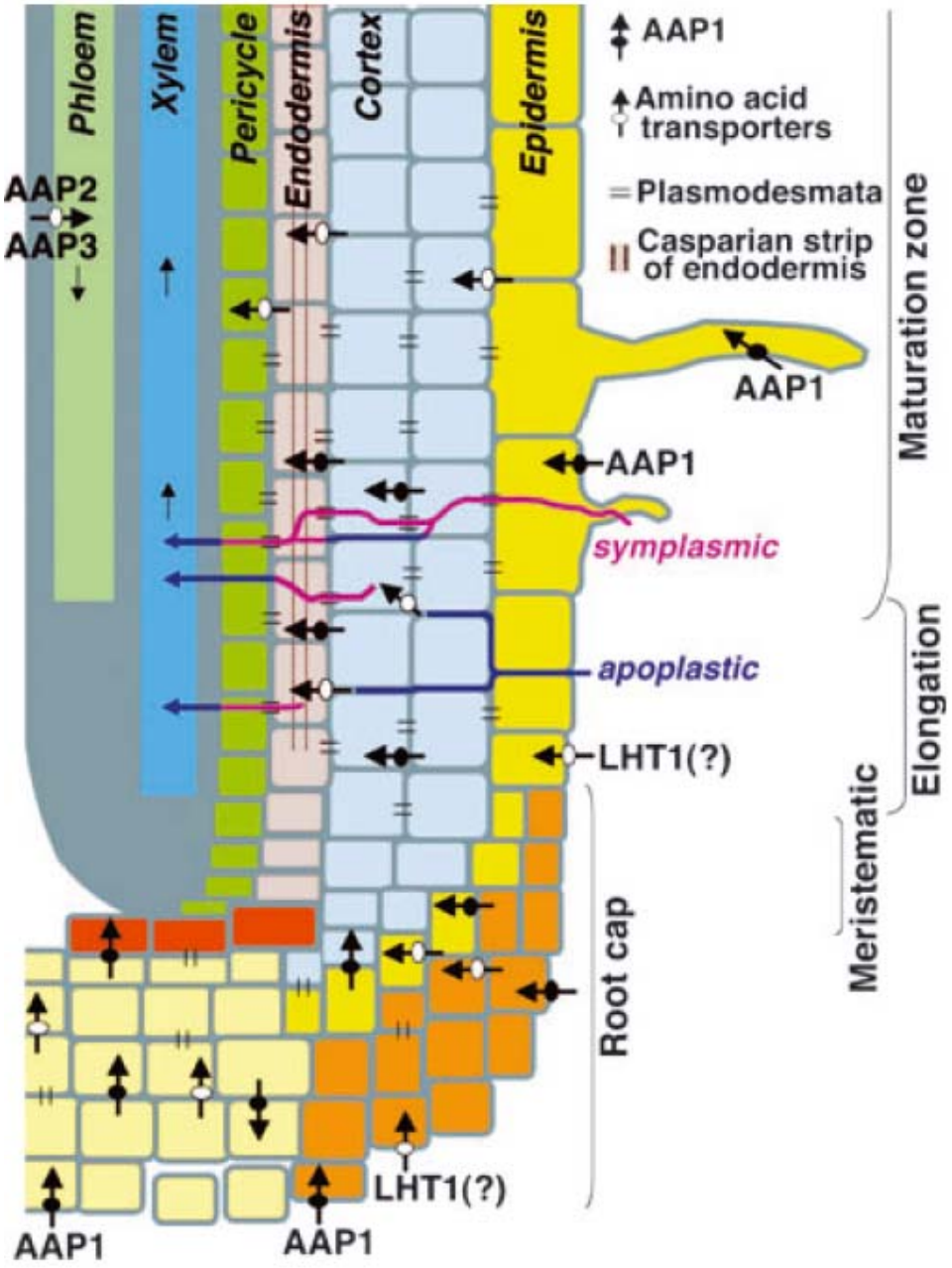


**Figure 8.** Model of amino acid transport in roots and the role of AAP1. Amino acid transport in roots may occur apoplastically until the organic nitrogen compounds reach the Casparian strip of the endodermis, which blocks apoplastic flow. For passage, the organic nitrogen has to be loaded into the symplast, presumably by transporters including AAP1, which are located in membranes of the root cortex and/or endodermis

cells. After passing the endodermis, the amino acids are transported to the vascular cylinder and translocated in the xylem with the transpiration stream to the shoot. Amino acids are also predicted to be loaded into the phloem to supply the developing root tips with nitrogen. AAP2 and AAP3 are probably responsible for this loading step (Hirner *et al.,* 1998; Okumoto *et al.,* 2004). In addition, transport of amino acids can be via the symplast after import into the root epidermis cells, root hairs and root cap cells, respectively. Our studies indicate that AAP1 is involved in this process. Other amino acid transporters might also function in amino acid uptake via the epidermis and root cap, such as LHT1 (Hirner *et al.,* 2006).

Overall, localization of AAP1 in the different root cells clearly points to a function of AAP1 in amino acid import into the root. This is supported by our growth and uptake studies with *aap1*. The mutants tolerate toxic concentrations of amino acids and showed a reduction in amino acid uptake of up to 50%, depending on the amino nitrogen compound. Interestingly, analysis of amino acid uptake in seedlings and in 3-weeks old plants (see Figure 7b) showed differences in accumulation of label in leaves between mutants and wild-type, but levels of radiolabel in roots were similar. The reason for the lack of differences in accumulation of [$^{14}$C]-label in the roots after 30 min of feeding is unclear, but different scenarios can be envisaged. (i) Most of the radiolabel measured in the root is present in the apoplast, and the apoplastic concentrations are similar for wild-type and mutant since these simply depend on amino acid concentrations in the feeding solution versus root apoplast and the diffusions gradient. Amino acids that are loaded into the symplast are immediately transferred to the xylem and transported with the

transpiration stream to the upper part of the plant. Since loading into the symplast is reduced in *aap1* mutants, less label is transferred to, and accumulated in, the leaves. (ii) Amino acids are only exported out of the roots when certain amino acid concentrations are exceeded. In our uptake experiments, the root amino acid pool is 'filled' and the excess of amino acids is transported out of the root. Again, since the overall uptake of amino acids into root cells is reduced in *aap1* mutants, smaller amounts of the nitrogen compounds will be distributed to the shoot. Further analyses are needed to test these hypotheses, e.g. determining apoplastic and symplasmic amino acid concentrations in the root.

A reduction in amino acid uptake of up to 50% in *aap1* mutants implies that at least 50% of the amino acid import into the root occurs via other transport mechanisms. They might involve other amino acid transporter proteins that are located in the root epidermis/root hairs or in the root cortex, endodermis and stele for uptake of apoplastically transported amino acids and transfer to the xylem (Figure 8). Surprisingly, with a 50% reduction in amino acid uptake, we failed to see phenotypic differences when *aap1* and wild-type plants are grown in soil, hydroponic culture or on solid media containing different amino acids with varying concentrations (10 μM - 5 mM, data not shown). It could well be that disruption of AAP1 function might be balanced over time by the activity of other (induced) amino transporters expressed in the roots (for review see Liu and Bush, 2005). In contrast, studies done with *lht1* mutants with re-expressed LHT1 in green tissue displayed a reduction in growth on 5 mM asparagine compared to wild-type (Hirner *et al.,* 2006). These differences might be due to differences in substrate specificity and affinity as well as timing and location of transporter expression.

Further experiments need to be performed to resolve the cellular localization and regulation of function of AAP1, LHT1 and other root-located amino acid transporters.

### 2. 5. 2. AAP1 *transports neutral, uncharged amino acids in planta*

Physiological studies were performed using T-DNA insertion lines to resolve which amino acids are transported by AAP1 *in planta*. Such an analysis of the substrate specificity of an amino acid transporter in plants is novel. Up to now substrate specificity of amino acid transporters has only been analyzed using biochemical studies with sugar cane suspension cultures and plasma membrane vesicles from sugar beet (Li and Bush, 1990, 1991, 1992; Weston et al., 1995, Wyse and Komor, 1984) and by heterologous expression of transporters in yeast cells and *Xenopus* oocytes (Boorer *et al.,* 1996; Fischer *et al.,* 1995, 2002; Frommer *et al.,* 1993; Hirner *et al.,* 2006; Hsu *et al.,* 1993; Lee and Tegeder, 2004). Amino acids and their toxic analogs have been used previously to identify mutants defective in the regulation of amino acid metabolism, and to screen for amino acid transport mutants (Heremans *et al.,* 1997; Verbruggen *et al.,* 1996; Voll *et al.,* 2004). Resistance of *aap1* mutants to toxic concentrations of neutral amino acids strongly indicates that a 'knock-out' or knock-down of *AAP1* resulted in decreased accumulation of amino acids in the seedling due to reduced uptake. To confirm this prediction and to analyze if glutamate and aspartate, for which the toxicity screen could not be established, are transported by AAP1, direct uptake studies with [$^{14}$C]amino acids were performed. Compared to wild-type seedlings, mutants showed a strong reduction in uptake of neutral amino acids (see Figure 4 and 7), confirming that

AAP1 transports neutral amino acids. Interestingly, while no differences in aspartate uptake were observed, glutamate acquisition is strongly reduced in *aap1* mutants, clearly demonstrating a role of AAP1 in glutamate transport. This *in planta* data fits with the transport studies done in AAP1 expressing yeast cells showing that aspartate, in contrast to glutamate, is a weak competitor for proline uptake (Kwart *et al.,* 1993). *AAP1* expression in *Xenopus* oocytes also demonstrated that AAP1 transports glutamate but not aspartate (Boorer *et al.,* 1996, Fischer *et al.,* 2002, Table 1). The transport of glutamate was pH-dependent (decreasing with increasing pH), leading to the conclusion that glutamate ($pK_R$=4.4) is transported in its protonated, zwitterionic form (Boorer *et al.,* 1996; Fischer *et al.,* 2002). Concerning the differences in AAP1 transport of the two acid amino acids, Boorer and Fischer (1997) suggest that the $\gamma$-carboxylate of aspartate prevents substrates binding to the transporter. Their electrophysiological studies show that the addition of one carbon, resulting in a glutamate, leads to dramatic increase of AAP-mediated glutamate transport (Boorer and Fischer, 1997; Boorer *et al.,* 1996; Fischer *et al*., 2002).

With respect to basic amino acids, the growth screen demonstrates that high concentrations of the basic amino acids arginine and lysine are toxic for both wild-type and *aap1* seedlings (see Figure 6). However, histidine seemed to be different in that *aap1* seedlings grew better on high histidine levels than wild-type. These data were confirmed by uptake studies with [$^{14}$C]-labeled basic amino acids. While *aap1* seedlings showed a reduction in histidine uptake, no differences were observed for lysine, indicating that histidine represents a substrate for AAP1. The results are consistent with AAP1 transport studies in *Xenopus* ooctyes and can be explained by the fact that at

pH 5.5 about 100 % of the arginine and lysine carry positive net charges, but ca. 25% of the histidine is zwitterionic with no net charge (Boorer and Fischer 1997; Fischer *et al*., 2002). Overall, our work demonstrates the first analysis of the substrate specificity of an amino acid transporter *in planta,* showing that neutral amino acids as well as histidine and glutamate, presumably in their uncharged form, are transported by AAP1. These data are consistent with biochemical studies using plasma membrane vesicles of sugar beet that revealed the existence of transport systems for neutral amino acids in plants (Li and Bush 1990, 1991, 1992). It is also noteworthy that the analysis of AAP1 transporter function in plants correlates with results obtained from expression of *AAP1* in yeast or oocytes (Table 1). This is of relevance, since it confirms the importance of heterologous expression systems for analysis of substrate specificity of plant transporters.

### 2. 5. 3. Ecological relevance of AAP1

Biochemical analyses of AAP1 kinetics in yeast and oocytes indicate that this transporter probably represents a low affinity system (Boorer *et al.,* 1996; Fischer *et al.,* 1995, 1998, 2002; Kwart *et al.,* 1993; Table 1). The direct uptake of amino acids from the soil solution by plant roots has been an ecologically critical issue, and the studies presented here demonstrate that AAP1 contributes up to 50% to the amino acid uptake when the organic nitrogen is offered in concentrations of 2 and 10 mM, respectively. Measurement of the availably of amino acids in soils is complex due to soil characteristics (e.g. particle size, cation exchange capacity, water availability etc.),

microbial activity and competition between microbes and plants or between plants (for review see Lipson and Näsholm 2001). In certain ecosystems like the arctic tundra, boreal forest and alpine areas, concentrations of free amino acid in soil solutions might range from 1-100 μM but can also be above 150 μM, and these amino acids are utilized as an important nitrogen source by a range of plant species (Chapin *et al.,* 1993; Henry and Jefferies 2003; Kielland, 1994; Näsholm *et al.,* 1998; Raab *et al.,* 1999). However, analysis of soil organic nitrogen levels is dependent on the extraction methods, and the concentrations measured might be far below what is available to the plant (for review, see Lipson and Näsholm 2001). While a range of amino acids is generally found in the soil solution, glutamate, alanine and glycine often dominate (Kieland, 1994; Raab *et al.,* 1996, 1999). Our uptake studies with alanine and glutamate using *aap1* and wild-type seedlings showed that AAP1 functions at amino acid concentrations of 150 μM, which strongly supports the importance of AAP1 in organic nitrogen acquisition under ecologically relevant amino acid concentrations. Future growth and uptake studies with the *aap1* mutants and 'knock-out' lines of other root-located amino acid transporters (e.g. LHT1), as well as with their double or triple mutants, using amino acids at soil concentrations are necessary to further dissect the importance of transport proteins in amino acid acquisition and to resolve differences in their substrate specificity *in planta*.

In conclusion, we show AAP1 function in amino acid uptake by the root and demonstrate that neutral amino acids, glutamate and histidine are transported by AAP1. Direct uptake studies at low concentrations of alanine and glutamate suggest that AAP1 is important for organic nitrogen acquisition under eco-physiological conditions. And

finally, while these data present the first analysis of the substrate specificity of an amino acid transporter *in planta*, they also correlate with the expression studies previously done in yeast and *Xenopus* oocytes. This is encouraging with respect to usage of heterologous expression systems for functional analysis of plant metabolite transporters.

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# Chapter 3

# Transporter function in amino acid import into root cells

## 3. 1. Abstract

This research analyzed the role of the *Arabidopsis thaliana* AtLHT6 transporter in amino acid uptake by the roots. Using a promoter-GUS approach, expression of *AtLHT6* was localized throughout the root, including root epidermis and root hairs. Transport studies with *lht6* mutant seedlings and radiolabeled amino acids were performed and showed reduced amino nitrogen uptake in mutants compared to wild type. It was further tested if import of amino acids into the root can be increased by overexpressing *AtAAP1*, an Arabidopsis transporter with a broad substrate selectivity, in the root epidermis and hairs. *AtAAP1*promoter-*AtAAP1*cDNA-*GFP* Arabidopsis seedlings were analyzed and they displayed an increased uptake of specific amino acids. Furthermore, the *AAP1* overexpressing lines were more sensitive to phenylalanine, which is toxic to the plant at high levels, due to increased uptake. Together, our localization and transport studies with mutant and overexpressing lines demonstrate that amino acid transporters are important for organic nitrogen acquisition from the rhizophere. It was further discovered that genetic manipulation of root located amino acid import systems leads to alteration of nutrient uptake from the soil.

## 3. 2. Introduction

The acquisition of nitrogen is a vital step in the growth and development of plants. Recent research has demonstrated that free amino acids are a major source of soil nitrogen for plants in some ecosystems (e.g. boreal forest) (Näsholm et al. 1998; Streeter et al. 2000; Lipson et al. 2001; Henry and Jefferies 2003; Weigelt et al. 2005). Furthermore, studies analyzing amino acid uptake by plant roots reveal that multiple transport systems are involved in the acquisition of amino acids (for review see Lipson and Näsholm 2001).

In *Arabidopsis thaliana* CAT6, LHT1, AAP1 and AAP5 transporters have been analyzed for their role in amino acid uptake by the roots. *AtCAT6* was localized to root tips and side root primordial and analysis of *cat6* mutant seedlings showed reduced growth on Gln compared to wild type, indicating an inability to acquire or utilize Gln (Hammes et al. 2006).

*AtLHT1* belongs to the LHT amino acid transporter family and heterologous expression in *Saccharomyces cerevisiae* (yeast) demonstrate that it transports neutral and acidic amino acids with a high affinity (Wipf et al. 2002; Hirner et al. 2006; Rentsch et al. 2007). Expression analysis localized *AtLHT1* to the side roots of young seedlings and in the root tips of older plants, but the transporter was also strongly expressed in mesophyll cells of leaves (Hirner et al. 2006). Analysis of *lht1* seedlings showed reduced growth on Glu, Asp and GABA as sole nitrogen source compared to wild type (Hirner et al. 2006;

Svennerstam et al. 2007). Furthermore, uptake studies with *lht1* seedlings revealed reduced acquisition of Asp, Glu and Gln and *AtLHT1* overexpression lines demonstrated improved growth on amino acid containing media, as well as increased uptake of amino acids.

Two members of the AAP family, AtAAP1 and AtAAP5 seem to be involved in amino acid acquisition (Lee et al. 2007; Svennerstam et al. 2008). While tissue specific expression of *AtAAP5* was not resolved, analysis of *aap5* mutant seedlings revealed decreased uptake of Arg and Lys compared to wild type (Svennerstam et al. 2008). AtAAP1 was analyzed with growth studies using *aap1* mutants and toxic amino acid concentrations as well as direct uptake studies with radiolabled amino acids and *aap1* seedlings (Lee et al. 2007). The results demonstrated that AtAAP1 functions in the transport of neutral amino acids and Glu into the roots. These results are also in agreement with previously performed transport studies using AtAAP1 in heterologous systems (Hsu et al. 1993; Kwart et al. 1993; Boorer et al. 1996).

Uptake of nutrients from the soil occurs mainly via root epidermal and root hair cells. While *AtAAP1* is expressed in these cells and might be directly involved in amino acid uptake, expression of *AtCAT6* and *AtLHT1* are in other root tissues and may play an indirect role in amino acid acquisition (Hammes et al. 2006; Hirner et al. 2006). Here we analyze another amino acid transporter, *AtLHT6*, which is localized to root cells responsible for nutrient uptake. We show *AtLHT6* expression in epidermal and root hair cells of young seedlings as well as

throughout the side roots of older plants. Furthermore, an *AtLHT6* T-DNA insertion line is investigated with respect to changes in amino acid uptake.

We also investigate if amino acid uptake can be increased by targeted overexpression of an amino acid transporter to root cells important for amino acid acquisition. *AtAAP1*-promoter-*AtAAP1*cDNA-*GFP* transgenic plants from Lee et al. (2007) displayed increased uptake of specific amino acids.

## 3. 3. Materials and Methods

### 3. 3. 1. Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Columbia plants were grown in soil consisting of peat (60%), pumice (20%) and sand (20%), and in environmentally controlled growth chambers with 16 h light at 150–200 μmol photons $m^{-2}$ $s^{-1}$, 50% humidity and temperatures of 18˚C in light and 16˚C in dark. For *in vitro* cultures Arabidopsis seeds were sterilized and placed on MS media (pH 5.5; Murashige and Skoog 1962) supplemented with agar (8 g $l^{-1}$), myo-inositol (100 mg $l^{-1}$), sucrose (10 g $l^{-1}$) and 2-(N-morpholine)-ethane sulphonic acid (MES; 0.5 g $l^{-1}$) and were grown in tissue culture chambers at 20˚C, 50 % humidity and 16 h light at 125-130 μmol photons $m^{-2}$ $sec^{-1}$.

### 3. 3. 2. RNA expression analysis

Plant organs including roots, rosette leaves, cauline leaves, sink leaves, stems, buds, flowers and siliques were collected from mature *Arabidopsis thaliana* plants. Furthermore, 6-day-old seedlings of wild type *A. thaliana*, *AtLHT6* and *AtAAP1* T-DNA insertion lines (*lht6-1, aap1-3)*, *lht6-1/LHT6* complementation line and the *AtAAP1* overexpressing lines (*AtAAP1* OE-1, *AtAAP1* OE-2 and *AtAAP1* OE-3*)* were collected*.* Total RNA from whole plant organs and seedlings was then isolated according to Pélissier and Tegeder (2007). First strand cDNA synthesis was carried out using the isolated RNA, M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo d(T) primers. PCR (polymerase chain reaction) was performed with the cDNA and gene specific primers. The primers used for organ specific expression of *AtLHT6* were: 5'-CTTAAgTgCACTgggTgA AATgg-3' and 5'-CATgTTggACCACCAACTATTTgg-3' (2) 5'-CTgATCTCgACAAgTAgTTgTAgg-3’ and 5'-ATggCgggAATCCCAgATCATATCC-3' (62˚C). The primers used to determine *AtLHT6* expression in wild type, *lht6-1* and *lht6-1*/*LHT6* seedlings were: 5'-gCAACggTTCgATAggTACC-3' and 5'-CATCggATggTAAACCgTAg-3'. To determine *AtAAP1* expression in *aap1-3*, *AtAAP1* OE-1, *AtAAP1* OE-2 and *AtAAP1* OE-3 seedlings PCR was performed using the genes specific primers: 5'-gCTAgATCTTAgTgTATCCTg-3' and 5'-ATggTCgAgAgAAgCgTACC-3'. As a control for even amounts of cDNA templates PCR was also performed with the *AtACT2* primers: 5'-

CCAATCgTgTgTgACAATggTACCg-3' and 5'-ggTTgTACgACCACTggCgTACAAg-3' (An et al., 1996).

### 3. 3. 3. Construct preparation and plant transformation

The *AtLHT6* promoter and cDNA of *AtLHT6* was isolated as described in Foster et al. (2008), see chapter 4. PCR was performed using Arabidopsis BAC (bacterial artificial chromosome) clone F28J7.9 as template and promoter-specific oligonucleotide primers 5'-TggTATAAACCAggTTATTgg-3' and 5'-TgCAAACTTAggAATgTTgTg-3'. Both the promoter and cDNA of *AtLHT6* was transferred into the TA-cloning vector pGEM®-T Easy (Promega, Madison, WI, USA). The *AtLHT6* promoter-GUS construct was prepared as described in Foster et al. (2008) (see chapter 4). To build the *LHT6* promoter/*LHT6* cDNA construct for complementation of the *lht6-1* mutant, the *AtLHT6* cDNA in pGEM®-T Easy was excised with *Not*I, blunted and cloned into the *Spe*I/blunt site of the pGEM®-T Easy vector carrying the *AtLHT6* promoter. The *LHT6* promoter/*LHT6* cDNA in pGEM®-T Easy was then cut with *Nco*I blunt/*Pst*I and transferred into the *Sma*I *Pst*I site of the binary vector pTKAN derived from pPZP212 (Hajdukiewicz et al, 1994). The vector was kindly provided by Karin Schumacher, ZMBP, Tübingen, Germany. The *AtLHT6* cDNA/*AtLHT6* promoter in pTkan was used for transformation of the *lht6-1* mutant line with *Agrobacterium tumefaciens* strain pGV3101 (Koncz and Schell 1986) and via floral dip method (Clough and

Bent 1998). Transgenic *AtAAP1*promoter/*GFP*/*AtAAP1*cDNA plants were obtained by Lee et al. (2007) (also see chapter 2).

### 3. 3. 4. Histochemical analysis of *LHT6*-promoter-*GUS* lines

Seedlings and whole plant organs of *LHT6*-promoter-*GUS* (β-*glucuronidase*) lines were placed in GUS staining solution containing 2 mM X-Gluc substrate (5-bromo-4-chloro-3- indoxyl-beta-D-glucuronide; cyclohexyl ammonium salt; Gold Biotechnology, St. Louis, MO, USA), 10 mM EDTA (ethylenediaminetetraacetic acid, pH 8), 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 0.1% Triton-X and 100 mM $PO^4$ buffer (pH 7) followed by vacuum infiltration for 15 min. The seedlings and whole plant organs were then incubated overnight at 37˚C and cleared using 95% ethanol. Some tissue samples were embedded in London Resin White Acrylic (Ted Pella Inc., Redding, CA, USA) according to Pélissier et al. (2004) and sectioned using a Reichert Ultracut R microtome (Leica, Vienna, Austria). Whole seedlings were analyzed with a stereoscopic light microscope (Wild, HeerBrugg, Switzerland), while roots hairs and sections of roots were viewed using a compound light microscope (Leitz, Wetzlar, Germany).

### 3. 3. 5. Isolation of T-DNA insertion lines

The *LHT6* T-DNA insertion line (SALK_049092.50.70) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA,

http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm). The line was screened for homozygous T-DNA insertions by PCR using T-DNA specific 5'-TggTTCACgTAgTgggCCATCg-3' and *LHT6* specific 5'-CTTAAgTgCACTgggTgAAATgg-3' primers. The *AAP1* T-DNA insertion line (Sail_871_C03) was acquired from SAIL (Syngenta Arabidopsis Insertion Library) collection via the Arabidopsis Biological Resource Center (Columbus, OH, USA) and screened for homozygosity by PCR using T-DNA specific 5'-TTCATAACCAATTCTCgATACAC-3' and *AAP1* specific 5'-ATggTCgAgAgAAgCgTACC-3' primers. The PCR products from both T-DNA insertion lines were cloned into the vector pGEM®-T Easy (Promega, Madison, WI, USA) and sequenced to verify exact location of the T-DNAs.

### 3. 3. 6. Uptake studies with Arbdidopsis seedlings

Uptake of amino acids was analyzed with seedlings of wild type, *lht6-1* mutant, *lht6-1* complementation line, *aap1-3* mutant and *AtAAP1* overexpression lines grown on MS media (pH 5.5 Murashige and Skoog 1962) with agar (8 g $l^{-1}$), myo-inositol (100 mg $l^{-1}$), sucrose (10 g $l^{-1}$) and MES (0.5 g $l^{-1}$), as described in Lee et al. (2007) (See chapter 2). After 4 days of seedlings growth channels were cut into the media and radiolabled [$^{14}$C] amino acids (Moravek Biochemicals, http://www.moravek.com) at 2 μCi, along with 5 ml of non-labeled amino acids, at concentrations of 2 mM or 150 μM in 2.5 mM MES buffer at a pH of 5.5, were filled into the channels. After 48 hrs, seedlings were collected, rinsed (twice) in

water and their radioactivity was counted using liquid scintillation counter machine. The counts per minute were normalized to wild type, which was set at a 100%.

### 3. 3. 7. Growth studies with seedlings grown in tissue culture with low or high levels of amino acids.

In previous studies concentrations of amino acids that are toxic to wild type *A. thaliana* were established (see Lee et al. 2007) (chapter 2). Growth on high (toxic) levels of amino acids was performed using MS media (pH 5.5 Murashige amd Skoog 1962) with agar (8 g $l^{-1}$), myo-inositol (100 mg $l^{-1}$), sucrose (10 g $l^{-1}$), MES (0.5 g $l^{-1}$) and varying concentrations of protein amino acids. Seeds from wild type (Col), *lht6-1*, *lht6/LHT6* and *aap1-3* were sterilized, placed on the plates with high concentrations of amino acids that are toxic to wild type and grown vertically. To test the sensitivity of wild type and *AtAAP1* overexpression lines to phenylalanine (Phe), seedlings were grown vertically on MS media suplemented with agar (8 g $l^{-1}$), myo-inositol (100 mg $l^{-1}$), sucrose (10 g $l^{-1}$), MES (0.5 g $l^{-1}$) and Phe (500 mM).

Growth of wild type (Col), *lht6-1*, *lht6/LHT6*, *AtAAP* OE and *aap1-3* seedlings on low levels of amino acids was carried out using two different growth media without nitrogen. The first media was a modified MS with 440 mg/L$CaCl_2$ $2H_2O$, 370 mg/L $MgSO_4$ $7H_2O$, 170 mg/L $KH_2PO_4$, 6.2 mg/L $H_2BO_3$ (Boric acid), 16.8 mg/L $MnSO_4$ $1H_2O$, 8.6 mg/L $ZnSO_4$ $7H_2O$, 0.83 mg/L KI, 0.25 mg/L

$NaMoO_4$ $2H_2O$, 0.025 mg/L $CoCl_2$ $2H_2O$, 0.025 mg/L $CuSO_4$, 37.3 mg/L NaEDTA, 27.8 mg/L $FeSO_4$ $7H_2O$, 0.25 mg/L nicotinic acid, 0.25 mg/L Pyrodoxine HCl, 0.5 mg/L Thiamine HCl, 100 mg/L myo-Inositol, 1% sucrose, 0.5 g/L MES (pH5.5) and 0.8% agar. The second media was from Okamoto et al. (2006), which contained 1 mM $KH_2PO_4$, 0.5 mM $MgSO_4$, 0.25 mM $CaSO_4$, 20 μM Fe-EDTA, 25 μM $H_3BO_3$, 2 μM $ZnSO_4$, 2 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $(NH_4)_6Mo_7O_{24}$ 0.5% (w/v) sucrose and 0.5 g/L of MES (pH 5.7) and 0.7% (w/v) agarose. To each media Ala, Asp, Gly, Glu, His or Phe was added at varying concentrations (10 μM, 50 μM, 100 μM 150 μM, 300 μM 500 μM and 1 mM) along with 0, 150 or 300 μM nitrate or 0, 150 or 300 μM of ammonium.

## 3. 4. Results

### 3. 4. 1. *LHT6* is strongly expressed in young seedlings and lateral roots of older plants

To determine organ-specific expression of *AtLHT6* RT-PCR was performed using RNA from different *A. thaliana* organs and 6-day-old seedlings (Fig. 1). The expression profile shows high levels of *AtLHT6* transcripts in roots and 6-day-old seedlings, as well as slightly lower expression in rosette leaves, buds and flowers. To examine localization of *AtLHT6* expression histochemical analysis using *AtLHT6*-promoter-*GUS* lines was performed. GUS staining was found in the roots of young seedlings, including root hairs and the lateral roots of growing

mature plants (Fig. 2a-e). Furthermore, cross sections through GUS-stained roots and microscopic analysis using dark field imaging revealed *AtLHT6* expression within all root cells (Fig. 2d red crystals). In addition, a patchy staining pattern was detected on the rosette leaves of some mature transgenic lines (see chapter 5 Fig. 7) and within flowers (see chapter 4 Fig. 4).

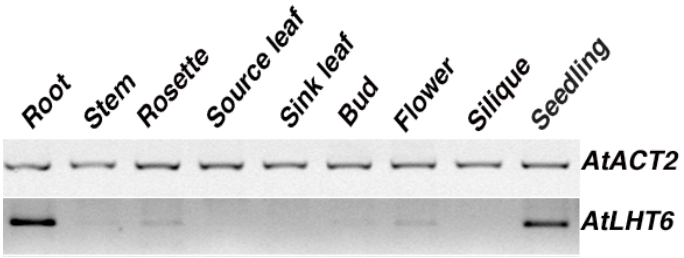


**Figure 1**. Expression of *LHT6* in different organs of *A. thaliana*. Expression of *AtACT2* was used as a control for equal concentrations of cDNAs in each organ.

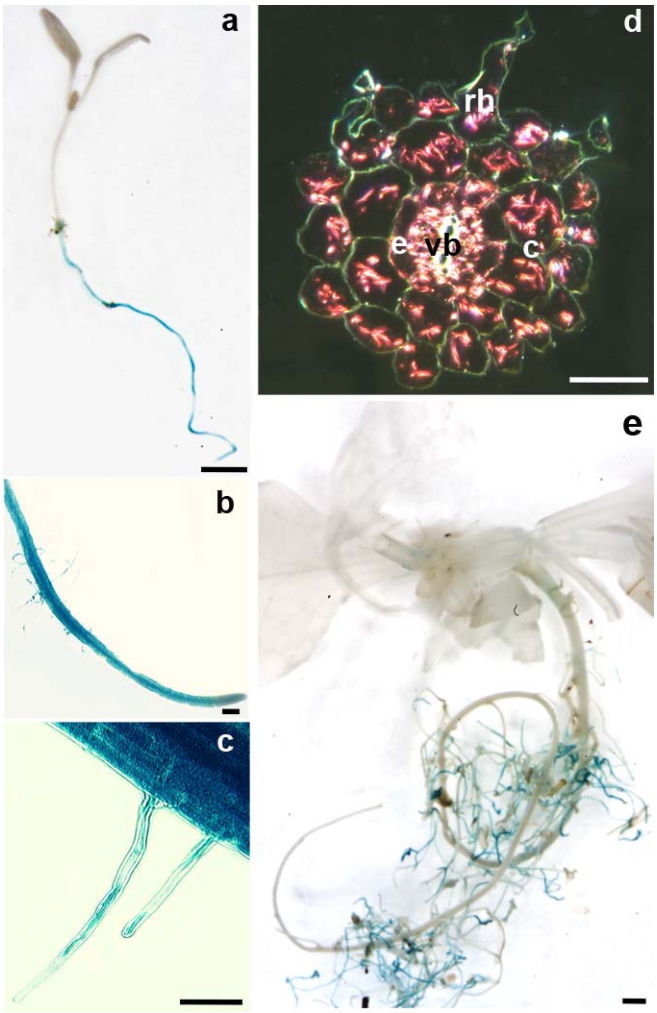


**Figure 2**. *LHT6*-promoter-*GUS* studies in Arabidopsis plants. **a-c,** Gus staining in a 5-day-old seedling. **d,** Dark field micrograph of a root cross section of a 5-day-old seedling. **e,** Gus staining in 3 week old plants. Staining can be seen throughout the root of the 5-day-old seedling including root hairs (**a**-**d**). *LHT6* expression is also seen in the side roots of 3-week-old plants (**e**). *e* endodermis, *rh* root hair, *vb* vascular bundle. Bars = 500 μm (**a, e**), 100 μm (**b**), 50 μm (**c**) and 25 μm (**d**).

### 3. 4. 2. *AtLHT6* expression in *lht6* seedlings

The *AtLHT6* T-DNA insertion line (*lht6*) was screened to homozygosity using PCR. The PCR product using an *AtLHT6* gene specific primer and a T-DNA left border (LB) primer was sequenced, and it was verified that the insertion is within the third exon 1114 bp downstream of the ATG (Fig. 3a). Furthermore, *AtLHT6* transcript levels were analyzed in wild type, *lht6* and *lht6/LHT6* seedlings using RT-PCR (Fig. 3b). Expression of *AtLHT6* in *lht6/LHT6* was similar to wild type, whereas in the *lht6* mutant no transcripts could be detected.

### 3. 4. 3. *lht6-1* seedlings show a reduced uptake of $^{14}$C labeled acidic amino acids and alanine

To determine the physiological role of *AtLHT6 in planta*, uptake studies using the *AtLHT6* T-DNA insertion line and [$^{14}$C] amino acids were performed. Furthermore, because there was only one T-DNA insertion line for *AtLHT6,* the *lht6* mutant was complemented with *AtLHT6* promoter-*AtLHT6* cDNA to restore function and to verify that potential changes in uptake of amino acids are due to the knockout of *AtLHT6*. Uptake of radiolabled Glu was reduced in *lht6* seedlings compared to wild type, whereas the *lht6/LHT6* lines showed similar uptake as wild type (Fig. 4a). The uptake of other radiolabled amino acids at concentrations of 2 mM was analyzed and results show a reduction in the uptake of aspartate (20%), glutamate (25%) and alanine (30%) in *lht6* seedlings,

compared to wild type (Fig. 4b). Uptake of wild type and *lht6*/*LHT6* seedlings were similar for all amino acids (Fig. 4b).

Uptake studies with wild type, *lht6* and *lht6/LHT6-2* seedlings were also performed using amino acids that are present in many soils, specifically, Ala or Glu (Kieland 1994; Raab et al, 1996; Raab et al, 1999). Furthermore, these studies were performed at amino acid concentrations of 150 μM that can be found in some soil types. Compared to wild type, uptake of Ala and Glu was reduced in *lht6-1* by 25 % and 20% respectively (Fig. 4c). The lht6/LHT6 showed uptake similar to wild type, further demonstrating *AtLHT6* plays a role in amino acid uptake at ecologically relevant concentrations (Fig. 4c)

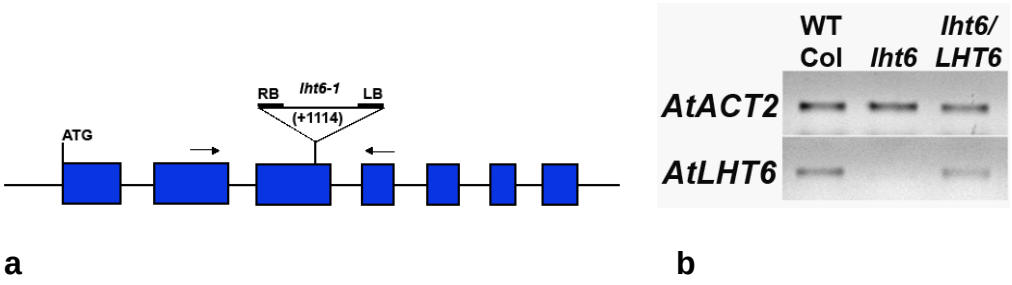


**Figure 3**. **a,** Schematic diagram of the T-DNA insertion within *AtLHT6*. The boxes represent exons whereas the lines denote introns. Arrows indicate primers used for screening to homozygosity. **b,** *AtLHT6* and *AtACT2* expression analysis of wild type, *lht6* mutant, and lht6/*LHT6* seedlings using RT-PCR.

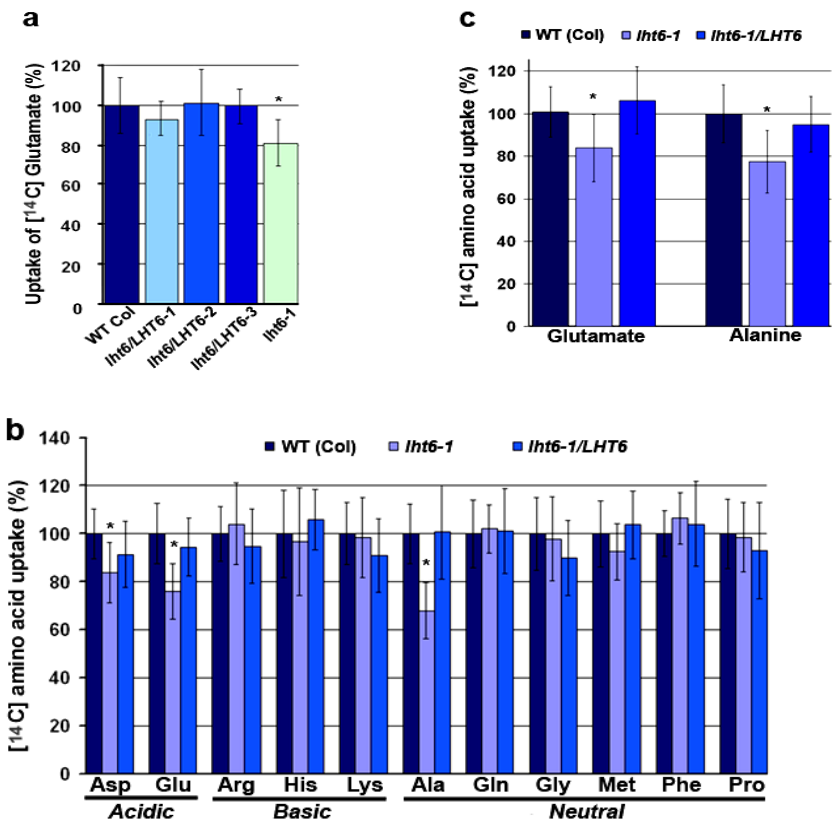


**Figure 4**. Uptake studies using *lht6-1, lht6/LHT6* and wildtype seedlings and $^{14}$C-labeled amino acids. *lht6/LHT6* complementation lines were produced by transforming the *lht6-1* Arabidopsis mutant with a *AtLHT6* promoter -*AtLHT6* cDNA construct to restore transporter function. Uptake of radiolabeled amino acids was measured by scintillation counting and normalized to uptake of wild type, which was set to 100%. **a,** Uptake studies with 2 mM Glu and 6-day-old seedlings of wild type, *lht6*-1 and three *lht6/LHT6* lines. **b,** Uptake studies with 6-day-old seedlings and amino acid concentrations of 2 mM. **c,** Uptake studies with 6-day-old seedlings and 150 μM Glu or Ala.

### 3. 4. 4. Growth of *lht6-1* on high or low concentrations of amino acids was similar to wild type

In previous studies amino acid concentrations that are toxic for wild type Arabidopsis plants were established (see Lee et al. 2007; chapter 2). Since uptake studies with *lht6* mutants showed reduced uptake for specific amino acids compared to wild type, we also tested if *lht6* seedlings survive toxic levels of amino acids. Seedlings of wild type, *lht6-1* and *lht6*/*LHT6* were grown on media plates with high concentrations of Phe (10 mM), Met (4 mM), Ala (100 mM), Gln (100 mM), Glu (50 mM), His (20 mM) and Lys (2 mM). The results show that no difference in growth for *lht6-1*, *lht6/LHT6* and wild type on all amino acids and concentrations tested (Fig. 5), indicating that amino acids are still taken up when offered at high concentrations, probably by other amino acid transporters located in the root epidermis, such as AtAAP1 (Lee et al. 2007; Chapter 2).

To analyze if AtLHT6 is important at low amino acid concentrations *lht6*, *lht6*/*LHT6* and wild type were grown on low levels of amino acids as sole nitrogen or nitrate or ammonium were added (Table 1). No difference in growth was seen between *lht6-1*, *lht6/LHT6* and wild type when cultured on any nitrogen containing media (amino acid with/without inorganic N) (Fig. 6).

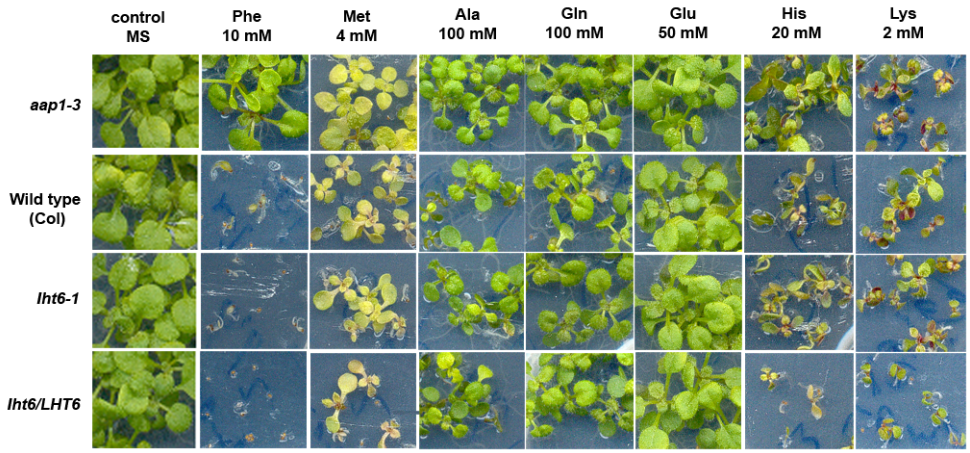


**Figure 5**. Seedlings grown on MS media (control) and MS media supplemented wtih high concentrations of various amino acids.

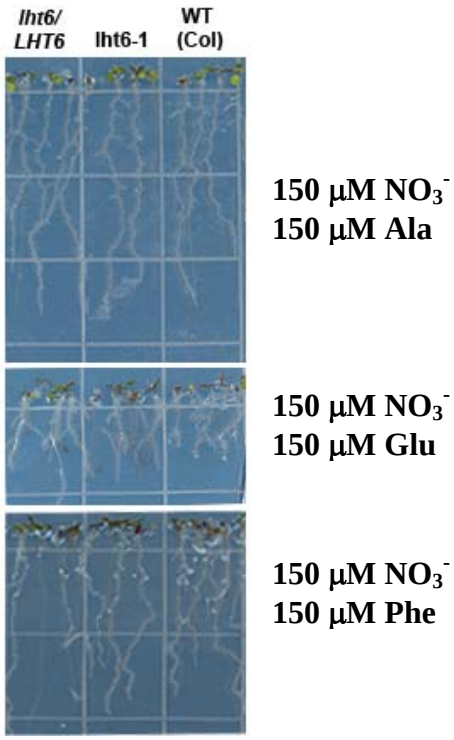


**Figure 6**. Growth of 14-day-old seedlings on media containing low concentrations of amino acids and nitrate.

**Table 1**. Nitrogen sources and concentrations used for growth analysis of *lht6*, *lht6*/*LHT6* and wild type plants.

| | **Without $NO_3^-$ or $NH_4^+$** | **$NO_3^-$ (μM)** | | **$NH_4^+$ (μM)** | |
|---|---|---|---|---|---|
| | | **150** | **300** | **150** | **300** |
| **Ala (μM)** | 150<br>300 | 150<br>300 | 150<br>300 | 150<br>300 | 150<br>300 |
| **Glu (μM)** | 150<br>300 | 150<br>300 | 150<br>300 | 150<br>300 | 150<br>300 |
| **Phe (μM)** | 150<br>300 | 150<br>300 | 150<br>300 | 150<br>300 | 150<br>300 |
| **Gly (μM)** | 150<br>300 | 150<br>300 | 150<br>300 | 150<br>300 | 150<br>300 |

### 3. 4. 5. Seedlings of *AAP1* overexpressors show an increase in uptake of $^{14}C$ labeled His, Phe and Pro and they are more sensitive to Phe toxicity

Previous results demonstrated that AAP1 is functioning in the uptake of amino acids in the Wassilewskija ecotype. The *aap1* mutants in the Wassilewskija background showed a reduction in the uptake of Glu, His and neutral amino acids compared to wild type. In this study an *AtAAP1* T-DNA insertion line in the Columbia background was acquired and screened to homozygosity. The T-DNA insertion was verified to be located in the first exon, 278 bp downstream of the ATG by isolating and sequencing the LB flanking region of the T-DNA (Fig. 7a). RT-PCR expression of *AtAAP1* in wild type, *aap1-3* revealed no expression of *AtAAP1* in *aap1-3* (Fig. 7b). Uptake studies with radiolabled aap1-3 and wild type seedlings indicated reduced uptake for Glu, His and neutral amino acids, which is consistent with *aap1-1* and *aap1-2* (Fig. 8a-b).

We further tested the effect of *AtAAP1* overexpression on amino acid uptake. Three *AAP1* overexpression (OE) lines with the *AtAAP1*promoter/*GFP*/*AtAAP1*cDNA construct demonstrate an increase in uptake of Phe (Fig. 8a). Furthermore, uptake studies with seedlings and varying radiolabled amino acids show an increase in the acquisition of His (25%) Phe (20%) and Pro (20%) for the *AAP1* OE line compared to wild type (Fig. 8b). Growth of *AAP1* OE seedlings was inhibited more than wild type when grown on 500μM Phe (Fig. 8c). Growth of all three *AAP1* OE lines is reduced compared to wild type indicating they may take up more Phe.

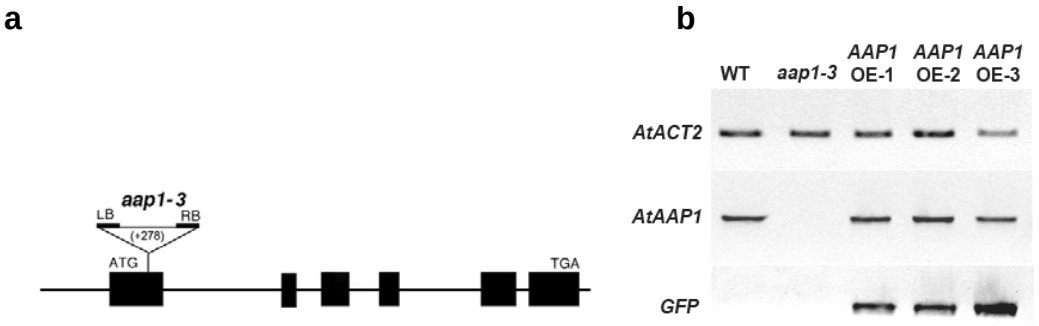


**Figure 7**. **a,** Schematic diagram of T-DNA insertion within *AtAAP1*. The exons are boxed and the introns are represented by lines. **b,** RT-PCR of *AtAAP1* and *AtACT2* expression in seedlings of wild type, *aap1-3* mutant, and *AtAAP1* overexpression lines.

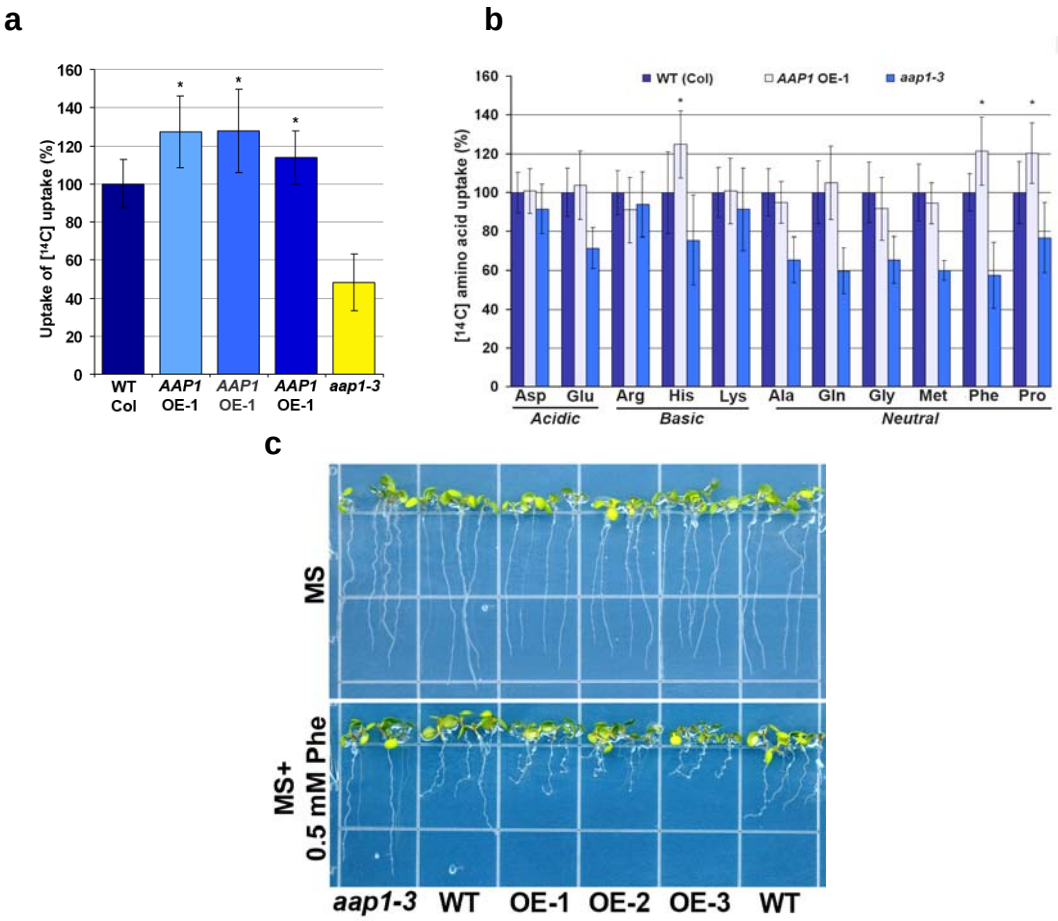


**Figure 8**. Uptake and growth studies using *aap1-3, AAP1* overexpressors and wild type seedlings. **a,** Uptake studies with 2 mM Phe and 6-day-old seedlings of wild type, *aap1-3* and three *AAP1* overexpression lines. **b,** Uptake studies with 6-day-old seedlings and different amino acids at concentrations of 2 mM. Uptake of radiolabeled amino acids was measured by scintillation counting and normalized to uptake of wild type, which was set to 100%. **c,** Seedlings of wild type, *aap1-3* and *AtAAP1* overexpression line seedlings after 10 days of growth on medium containing 0.5 mM Phe.

## 3. 5. Discussion

### 3. 5. 1. AtLHT6 functions in the uptake of acidic amino acids and alanine

Previous studies showed that AtLHT6 is localized to the plasma membrane of onion epidermal cells and is expressed in floral tissues (Foster et al. 2008) (see chapter 4). Analysis of *AtLHT6* expression also showed that the transporter is present in roots of young seedlings and mature plants (Fig. 1). The expression of *AtLHT6* in root epidermal cells including root hair cells is similar to *AtAAP1* (Lee et al. 2007; chapter 2). In addition *AtLHT6* is expressed in the cortex cells, this expression pattern clearly pointed to a function in amino acid uptake from the soil, as well as import of apoplastic amino acids into root cells, which was confirmed by uptake studies with radiolabled amino acids (see below). This expression pattern differs from other amino acid transporters expressed in the roots. *AtAAP2* and *AtAAP3* showed expression only in the vasculature of roots, whereas *AtCAT6* is localized to the tip of roots (Hirner et al. 1998; Okumoto et al. 2004; Hammes et al. 2006). *AtCAT6* T-DNA insertions lines demonstrated growth inhibition on Gln, which might be due to reduced uptake of amino acids, but most probably is caused by other transport related processes in the root tip. , In another study *aap5* mutant plants displayed reduced uptake of Arg and Lys, but since the localization of the transporter in root tissue is unknown, it might be directly or indirectly affected (Fischer et al. 1995; Svennerstam et al. 2008).

Recent work on *AtLHT1* revealed that this transporter is expressed in side roots of young seedlings and root tips, but also in mesophyll cells. Uptake of amino acids in *lht1* seedlings was reduced, which could be directly related to AtLHT1 activity in root cells or indirectly to changes in import processes of AtLHT1 in the leaves.

Uptake studies with radiolabled amino acids revealed acquisition of Glu, Asp and Ala for *lht6* seedlings compared to wild type (Fig. 4a, b). In lht6/LHT6 amino acid uptake was similar to wild type, confirming that the "knockout" of *AtLHT6* is causing the reduction in amino acid uptake (Fig. 4a, b). Previous studies showed that AtLHT1 and AtLHT2 transport acidic and neutral amino acids when expressed in yeast transport mutants (Lee and Tegeder 2004; Hirner et al. 2006). Furthermore, the *lht1* mutant lines were shown to have reduced uptake of acidic amino acids and Gln compared to wild type, although other amino acids were not tested. The transport of acidic amino acids by AtLHT6 *in planta* is consistent with the substrate selectivity of the other AtLHTs studied so far. Interestingly, Ala was the only neutral amino acid that showed reduced uptake by *lht6* seedlings. This might point to differences in the substrate selectivity between AtLHT6 compared to AtLHT1 and AtLHT2 (Lee and Tegeder 2004; Hirner et al. 2006). On the other hand AtLHT6 might transport other neutral amino acids but this may be compensated by other transporters in *lht6* seedlings.

In certain soils Glu and Ala are dominant forms of amino acids (Henry and Jefferies 2003). *lht6* seedlings were exposed to 150 μM of Glu or Ala,

concentrations that are found in some soils (Chapin et al. 1993; Raab et al. 1996). Our studies show a reduction in uptake of Glu and Ala at low concentrations when comparing *lht6* and wild type seedlings demonstrating that AtLHT6 is important under ecologically relevant amino acid concentrations. To determine if the decreased uptake of amino acids will affect growth, *lht6* seedlings were grown on plates containing media with low concentrations of amino acids. The growth of *lht6* was comparable to wild type seedlings (Fig. 6). Similarly, when exposed to toxic amino acid concentrations, no difference was observed in growth by *lht6* and wild type plants (Fig. 5). The lack of growth differences between *lht6* and wild type seedlings, which might be caused by the activity of other amino acid transporters in the mutant, for example through upregulation of *AtAAP1* that is expressed in root epidermal cells (Lee et al. 2007; chapter 2). Furthermore, since AtLHTs are predicted to be high affinity transporters, so AtLHT6 might not play a role in uptake when the root is exposed to high amino acid concentrations, as done in the toxicity screen.

### 3. 5. 2. Overexrepssion of *AAP1* in root epidermal cells leads to increased amino acid uptake

Increasing uptake of amino acids could be beneficial to plant growth and development. Furthermore, enhanced acquisition in agricultural crops may reduce the usage of N fertilizer, consequently reducing the negative impact N fertilization on the environment, as well as production energy and costs (Tilman

1999). Recently, *AtLHT1* has been overexpressed in Arabidopsis under control of the CaMV *35S* promoter and transgenic plants showed an increase in amino acid uptake (Hirner et al. 2006; Forsum et al. 2008). However, a better strategy might be to express amino acid transporters in tissues or cells that are important for uptake, specifically root hair and epidermal cells. The *AtAAP1* promoter has recently been shown to target expression to these cells as well as to the root tip (Lee et al. 2007). In addition, *aap1* mutants showed a reduction in the uptake of Glu and neutral amino acids compared to wild type verifying its role in amino acid acquisition. The reduction in a wide spectrum of substrates for *aap1* mutants suggested that *AtAAP1* is a good candidate for increasing amino acid uptake by overexpression. Therefore *AtAAP1*p-*AtAAP1*cDNA-*GFP* plants (Lee et al. 2007) were used for transport studies, and seedlings showed an increase in His, Phe and Pro uptake compared to wild type (Fig. 8a, b). The elevated acquisition in the overexpressor was confirmed by growth of seedlings on high levels of Phe. Transgenic plants displayed growth inhibition compared to wild type, caused by increased uptake of Phe and its inhibitory effect on amino acid metabolism in plant cells (Fig. 8c).

Our previous work showed that AtAAP1 transports His, Phe and Pro, but also the other neutral amino acids and Glu (Lee et al. 2007; Chapter 2). The increased uptake of only His, Phe and Pro in the overexpressor could be due to changes in expression of other amino acid transporters being caused by increased *AtAAP1* expression or alteration of single amino acids within the cell that might serve as signals. In addition, the construct used for the

overexpression approach of *AtAAP1* is fused to *GFP* and the translated GFP protein could alter the transport properties of AtAAP1. Further growth analysis with the overexpression lines, as described for Phe, might be needed to confirm if *AtAAP1* overexpression truly affects only His, Phe and Pro uptake. Nevertheless, these data clearly demonstrate that amino acid acquisition from the rhizosphere can be increased by overexpressing a transporter responsible for nutrient uptake in the root cells.

### 3. 6. References


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# Chapter 4

# Distinct expression of members of the LHT amino acid transporter family in flowers indicates specific roles in plant reproduction

## 4. 1. Abstract

Sexual plant reproduction necessitates proper development of pollen, pollen germination and tube growth through various tissues of the pistil, the female organ of the flower. Finally, sperm cells are released to fertilize the female gametophyte. These processes require high metabolic activities of all tissues involved and rely on the delivery of nitrogen assimilates for success. However, transporters mediating nitrogen fluxes are mostly unknown. The presented work provides an expression analysis of members of the LHT amino acid transporter family in relation to pollen development and pollen-pistil interaction. Expression of *Arabidopsis LHT*s was analyzed during flower development and the location of LHT function resolved by transporter-GFP and promoter-*GUS* studies. GFP-LHT localization in onion cells indicates that all LHTs analyzed are targeted to the plasma membrane. We further showed that *LHT*s are expressed in anthers and male gametophytes where they are proposed to function in transport of amino acids for pollen development and maturation. Expression in germinating pollen, pollen tubes and transmitting tissue of the pistil points to a role of LHTs in support of the fertilization process. Overall, our study suggests that LHT function

in flowers is cell or tissue specific, developmentally regulated and highly coordinated between male and female tissues.

## 4. 2. Introduction

In angiosperms, flowers serve an essential role in sexual reproduction and flower development is a dynamic process that requires the coordination of many tissue types to achieve reproductive success. It is well accepted that establishment of floral structures, pollination of the stigma and fertilization of the ovule require high levels of nitrogen (N) assimilates but related transport processes for (N) metabolites within the floral tissues are less understood. Amino acids are necessary for development including anther, pollen and pistil growth and to guarantee synthesis of large amounts of protein for pollen tube elongation for successful fertilization and seed set (Bhadula and Sawhney 1991; Wang et al. 1993; Cheung et al. 1995; Sanchez et al. 2004; Dong et al. 2005; Krichevsky et al. 2007). While a large number of amino acid transporters are present in plants (more than 50 putative transporters alone in *Arabidopsis*; see Rentsch et al. 2007) only a few have been localized to reproductive structures (Lalanne et al. 1997; Schwacke et al. 1999; Lee and Tegeder 2004; Grallrath et al. 2005; Hammes et al. 2006). However, RNA expression data indicate that far more amino acid transporters play a role in the reproductive process (Honys and Twell 2004; Pina et al. 2005; Schmid et al. 2005; Toufighi et al. 2005; Bock et al. 2006; Liu and Bush 2006; see also http://www.ncbi.nlm.nih.gov/geo/). For example, analysis of the *Arabidopsis*

pollen transcriptome using micro-arrays suggests that members of the LHT amino acid transporter family are functioning in male gametophyte development (Toufighi et al. 2005; Bock et al. 2006). Based on the expression analyses and studies done previously in our group (Lee and Tegeder 2004) we aimed to further understand the role of LHT transporters in flowers and to resolve their localization during flower development.

Up to now, localization of expression has only been determined for two *Arabidopsis LHT*s. *LHT1* is expressed in roots and mesophyll cells, and *LHT2* was localized to the tapetum of anthers (Chen and Bush 1997; Lee and Tegeder 2004; Hirner et al. 2006; for review see also Liu and Bush 2006; Rentsch et al. 2007). These LHTs display substrate selectivity for a broad range of amino acids and were shown to transport neutral and acidic amino acids with high-affinity, while their affinity for basic amino acids is much lower (Chen and Bush 1997; Lee and Tegeder 2004; Hirner et al. 2006). Here, we use RNA expression and promoter-*GUS* studies as well as analysis of LHT-GFP fusion protein localization to demonstrate a role of *Arabidopsis* LHTs in reproduction. This work provides evidence that in reproductive floral organs *LHT* transporters are (1) highly tissue or cell-specifically expressed, (2) developmentally regulated and that (3) their expression in male and female tissue is tightly coordinated. The expression patterns and strict coordination of *LHT* expression point to a role of these transporters in reproductive success.

## 4. 3. Materials and methods

### 4. 3. 1. Plant materials and growth conditions

*Arabidopsis thaliana* L. ecotype Columbia seeds were imbibed at 4˚C overnight and sown in soil consisting of peat (60%), pumice (20%) and sand (20%). Plants were grown in environmentally controlled growth chambers with 16 hrs light at 150-200 μmol photons $m^{-2}$ $sec^{-1}$, 50 % humidity and temperatures of 18˚C in light and 16˚C in dark.

### 4. 3. 2. RNA expression analysis

Buds and flowers of 6 week-old *Arabidopsis* plants were harvested at different developmental stages according to Smyth et al. (1990) and RNA was extracted from buds of stage 8-10 ([I], see Fig. 2) and stage 11-12 [II] as well as from flowers (stage 13-15 [III]) following the procedure of Pélissier and Tegeder (2007). First strand synthesis was carried out using the isolated RNA, M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo d(T) primers. PCR (polymerase chain reaction) was performed on first strand cDNAs with two sets of gene specific oligonucleotide primers for each *LHT* transporter, and *ACT2* primers were used as control for even amounts of cDNA templates. The primers sets were: *LHT2* (1) 5'-ATggggAACAgTgAAATgTCAgC-3' and 5'-CggAAgCAAAgATCATgATCC-3' (annealing temperature of 60˚C), (2) 5'-

ATggggAACAgTgAAATgTCAgC-3' and 5'-CCATCCgAgATTggACATAgc-3' (60˚C); *LHT4* (1) 5'-ATggACgAAAgACCCgAgACAgl-3' and 5'-TTAgTTAggCggCTTgAAgAAC-3' (60˚C), (2) 5'-gAgTgAACCAgAAgTCgAAggC-3' and 5'-gAAATTCAgTCgACgATgCCATC-3' (60˚C); *LHT5* (1) 5'-CATgACCTTCTTTgCACC-3' and 5'-AATggCgATAAgCCAgATG-3' (50˚C), (2) 5'-CATgACCTTCTTTgCACC-3' and 5'-TCAAgAgAAgAATTCgTAgTTTTTgg-3' (60˚C); *LHT6* (1) 5'CTTAAgTgCACTgggTgAAATgg-3' and 5'-CATgTTggACCACCAACTATTTgg-3' (65˚C), (2) 5'-CTgATCTCgACAAgTAgTTgTAgg-3' and 5'-ATggCgggAATCCCAgATCATATCC-3' (62˚C). Some PCR products were cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA) and sequenced to confirm amplification and expression of the specific *LHT* transporter.

### 4. 3. 3. *LHT-GFP* construct preparation and onion cell bombardment

*LHT6* was isolated by PCR approach using a seedling cDNA library from *Arabidopsis thaliana* ecotype (Minet et al. 1992) and the primers 5´-AtggCgggAATCCCAgATCATATCC-3´ and 5'-TCAgTgTTTAggTAAgTTgCATC-3´. *LHT4* and *LHT5* cDNAs were obtained from first strand cDNAs (described earlier) of flower buds from *Arabidopsis thaliana* ecotype Columbia using PCR with the following primers: *LHT4* 5´-ATggACgAAAgACCCgAgACAg-3´ and 5´-TTAgTTAggCggCTTgAAgAAC -3´; *LHT5* 5´-ATggAgAAAAgTCAATCgTCTCC-3´ and 5´-TCAAgAgAATTCGTAgTTTTTgg-3´. The PCR products were cloned

into plasmid vector pGEM®-T Easy (Promega, Madison, WI, USA) and sequenced.

*LHT5* and *LHT6* were then amplified using PCR and transferred into the *Bgl*II site of pUC18-spGFP6 (Meyer et al. 2006). *LHT2* PCR products (Lee and Tegeder 2004) were cloned into the *Xba*I site of pUC18-GFP5Tsp (Meyer et al. 2006). Primers were the following: *LHT2* 5´-gCTCTAgggAACAgTgAAATgTCAgC-3´ and gCTCTAgACTAAgAgACCTTgTAggTC-3´; *LHT5* 5´-gAAgATCTTATggAgAAAAgTCAATCgTC-3´ and 5´-gAAgATCTTCAgAgAAgAATTCgTAgTTTTTg-3´; *LHT6* 5´-gAAgATCTTATggCgggAATCCCAgATC-3´ and 5´-gAAgATCTTCgTgTTTAggTAAgTTgCATC-3´. Primers used for *LHT5* and *LHT6* amplification carried *Bgl*II restriction sites and *LHT2* primers contained *Xba*I sites.

*LHT4* cDNAs were amplified using primers 5´-ATggACgAAAgACCCgAgACAg-3´ and 5´-TTAgTTAggCggCTTgAAgAAC -3´, and cloned into pCR8/GW/TOPO (Invitrogen, Carlsbad, CA, USA). *LHT4* cDNAs were then transferred into the binary vector pMDC83 (Curtis and Grossniklaus 2003) using LR clonase (Invitrogen, Carlsbad, CA, USA).

Transient expression of GFP5-LHT2, LHT4-GFP6, LHT5-GFP6 and LHT5-GFP6 fusion proteins was performed in onion (*Allium cepa)* epidermal cells using particle bombardment (PDS-1000; Bio-Rad, Hercules, CA, USA) with

1.1-$\mu$m tungsten particles and 1,350 rupture discs according to the manufacturer's protocol (http://www.bio-rad.com). Images were captured with a Zeiss LSM 510 META confocal microscope (Thornwood, CA, USA).

### 4. 3. 4. Isolation of *LHT* promoters, *GUS* construct preparation and plant transformation

*LHT* promoters were isolated by PCR approach using *Arabidopsis* BAC (Bacterial Artificial Chromosome) clones as template (*LHT2*, F21J9.6; *LHT4*, F16N3.4; *LHT5*, F12A21.22; *LHT6*, F28J7.9; see http://www.ncbi.nlm.nih.gov/) and promoter specific oligonucleotide primers. For isolation of the promoter the DNA sequence between the specific *LHT* transporter (start codon) and the stop codon of the gene upstream of the transporter was used. Dependent on the promoter, 1733 bp *(LHT2*, At1g24400), 1325 bp fragments *(LHT4*, At1g47670), 1014 bp *(LHT5*, At1g67640), and 617 bp (*LHT6*, At3g01760) fragments upstream of the start codon of the respective transporter were isolated (Fig. 1). Primers were the following: *LHT2* 5'-CCATgATAgTCCAgAATCCAT-3' and 5'-ATCTCCTTgTgATATAATCgAg-3'; *LHT4* 5'-ATgTCATgTCCTTgTAgCTTC-3' and 5'-TggAgCAggTgAAgAgTA-3'; *LHT5* 5'-ATTTTATATTgAACATgAAAA-3' and 5'-CTTATTTTggCTTTgAAg-3'; and *LHT6* 5'-TggTATAAACCAggTTATTgg-3' and 5'-TgCAAACTTAggAATgTTgTg-3'. The PCR products were cloned into pGEM®-T Easy (Promega, Madison, WI, USA) and sequenced. The promoters were excised using *Not*I, then the *Not*I site was blunted, and the fragment

subcloned into the *Sma*I site of the binary vector pGPTV-BAR (Becker et al. 1992) upstream the *uidA* gene. The promoter-*GUS* constructs in the binary vectors were used for transformation of *Arabidopsis* ecotype Columbia with *Agrobacterium tumefaciens* strain pGV3101 (Koncz and Schell 1986) and via floral dip method (Clough and Bent 1998). For selection of transgenic *LHT*-promoter-*GUS* plants for glufosinate resistance, a solution (430 $\mu$l $L^{-1}$) of the herbicide Finale® (5.78% glufosinate-ammonium, 94.22% other ingredients; Hoechst-Roussel Agri-Vet Company, Somerville, NJ, USA) was sprayed on soil-grown plants every 3 days for the first 2-3 weeks following germination. Seeds were collected for continued screening until homozygous lines were produced.

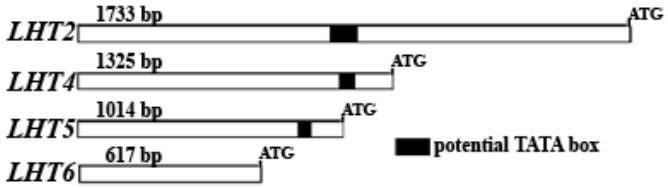


**Figure 1.** Length of *Arabidopsis LHT* promoters used for the promoter-*GUS* studies. While no specific promoter motifs could be identified when using PLACE, the Database of Plant Cis-acting Regulatory DNA Elements (http://www.dna.affrc.go.jp/PLACE/), a potential TATA box for initiation of transcription was found for most *LHT* promoters

### 4. 3. 5. Histochemical analysis of *LHT*-promoter-*GUS* lines

Buds and flowers of the transgenic *LHT*-promoter-*GUS* plants were collected and placed in GUS staining solution containing 2 mM X-Gluc substrate (5-Bromo-4-chloro-3-indoxyl-Beta-D-Glucuronide; cyclohexyl ammonium salt; Gold Biotechnology, St. Louis, MO, USA), 10 mM EDTA (ethylenediaminetetraacetic acid, pH 8), 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 0.1% Triton-X and 100 mM $PO_4$ buffer (pH 7) followed by vacuum infiltration for 15 min. The buds and flowers were then incubated overnight at 37˚C and cleared using 95% ethanol. Some tissue samples were embedded in London Resin White Acrylic (Ted Pella Inc., Redding, CA, USA) according to Pélissier et al. (2004) and sectioned using a Reichert Ultracut R microtome (Leica, Vienna, Austria). Whole organs were analyzed with a stereoscopic light microscope (Wild, HeerBrugg, Switzerland), while sections were viewed using a compound light microscope (Leitz, Wetzlar, Germany).

## 4. 4. Results

### 4. 4. 1. *LHT*s are expressed during flower development

In previous studies we localized *LHT2* expression to the tapetum of *Arabidopsis* anthers and microarray expression data indicated that other LHTs are also expressed in floral tissue (see http://aramemnon.botanik.uni-koeln.de/). Based on these data, expression of specific *LHT* amino acid transporters in developing

Arabidopsis flowers was analyzed by RT-PCR approach and confirmed that *LHT 2*, *4*, *5*, and *6* are present in the floral organs (Fig. 2). Expression of the transporters was then resolved during flower development using young and further developed buds (Fig. 2 [I and II]), respectively as well as mature flowers (Fig. 2 [III]). The results show that expression of all tested *LHT*s is developmentally regulated. *LHT2* and *LHT4* transcripts are high in buds and low(er) in mature flowers. *LHT5* transcripts are strongly increased in flowers compared to buds and expression of *LHT6* peaks in developed buds.

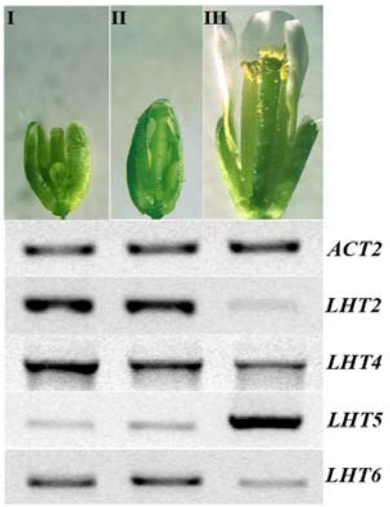


**Figure 2.** RNA expression of *LHT*s during development of *Arabidopsis* flowers. Expression of *LHTs* was determined at three stages of flower development using an RT-RCR approach. The stages were defined according to Smyth et al. (1990): (I) flower stage 8-10, (II) stage 11-12, and (III) stage 13-15. *ACT2* expression was analyzed as a control for equal concentrations of cDNAs used as template. *LHT* expression levels are dependent on the developmental stage of the flower

### 4. 4. 2. *LHT*s are expressed in anthers and during pollen development

Localization of *LHT* expression was resolved using promoter-*GUS* studies in *Arabidopsis*. 15 transgenic lines per promoter-*GUS* construct were examined and staining patterns were consistent for the specific constructs and lines. Analysis of *LHT4*-promoter-*GUS* lines demonstrated that *LHT4* is expressed in anthers and that its expression is developmentally regulated (Fig. 3). Expression was induced at flower bud stage 9 (Smyth et al. 1990) and was highest at stage 11 (Fig. 3a-d). GUS staining decreased during flower maturation and was not found at flower stage 15 (Fig. 3e-g). Light microscopy with whole anthers was used to resolve *LHT4* expression further. *GUS* staining revealed that in developing buds and anthers (flower stage 9/anther stage 6-7; Sanders et al. 1999) *LHT4* is expressed throughout the anther tissue with strongest staining in the developing microspores (Fig. 3h, j). As flowers develop *LHT4* expression is restricted to the tapetum cell layer that surrounds the developing microspores within the loculus (Fig. 3i, k).

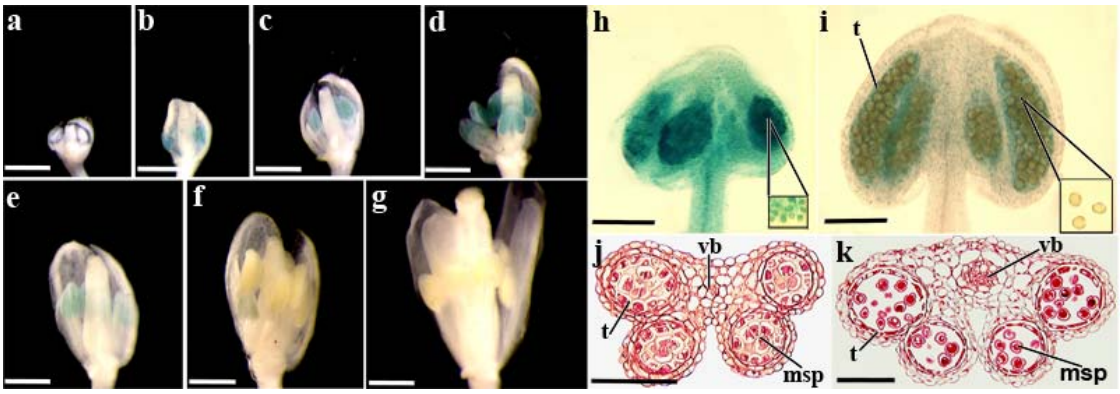


**Figure 3**. *LHT4*-promoter-*GUS* studies during flower development.

**a-g**, GUS staining at developmental stages of flowers. Stages were defined according to Smyth et al. (1990): **a**, flower stage 8; **b**, stage 9; **c**, stage 10; **d**, stage 11; **e**, early stage 12; **f**, late stage 12/early stage 13; **g**, stage 15. **b-d**, Strong GUS staining was seen in anthers of flower stage 9-11. **h-i**, Light micrograph of GUS staining at (**h**) anther stage 6-7 (Sanders et al. 1999)/flower stage 9 and (**i**) anther stage 10-11/flower stage 12. **h**, GUS staining was seen throughout the anther including the microspores. **i**, GUS staining was localized to the tapetal cells surrounding the microspores within the loculus. **j-k**, Light micrograph of anther cross sections stained with safranin-O at anther stages; **j**, stage 6 and **k**, stage 10. ta, tapetum; vb, vascular bundle; msp, microspore. *Bars* = 500 μm (**a-g**), 100 μm (**h-k**).

*LHT6* expression was found throughout anther development with location of expression being dependent on the developmental stage of the flower (Fig. 4a-g). At early flower stages staining was detected throughout the anthers (Fig. 4a-b). As flowers develop *LHT6* expression in anthers becomes localized to the tapetal tissue (Fig. 4c-d) and is absent in anthers at late flower stages, when the tapetum is degraded (Fig. 4e-g).

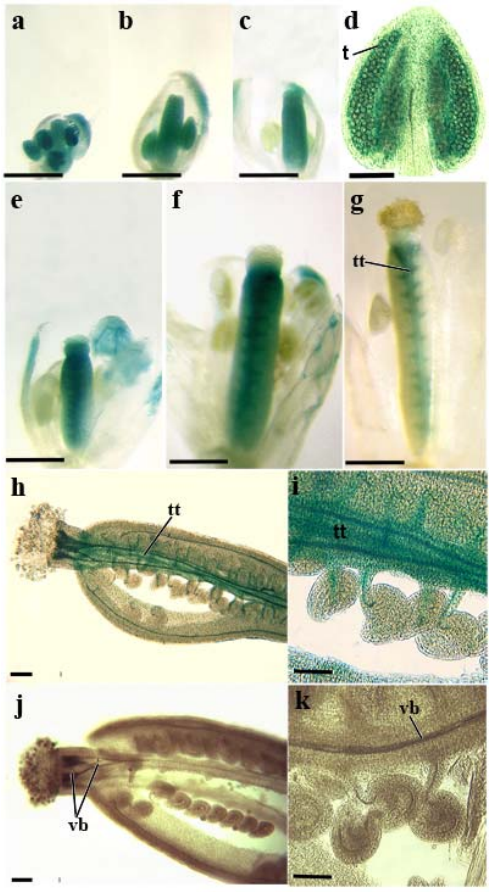


**Figure 4.** *LHT6*-promoter-*GUS* studies during flower development.

**a-c** and **e-g**, GUS staining at developmental stages of flowers. **a**, flower stage 9; **b**, stage 10; **c**, stage 11; **e**, late stage 12; **f**, stage 13 and **g**, stage 15 (Smyth et

al. 1990). **a-b**, *GUS* staining was seen throughout anther tissue. **c**-**d**, *GUS* staining is restricted to tapetal cells. **d**, Light micrograph of anther from flower bud shown in **c**. **a-c** and **e-f**, Strong staining is evident throughout the pistil. **g**, Staining within the pistil is localized to the transmitting tissue of the ovary. **h-i**, Light micrograph of pistil from flower of stage 15 (**g**) shows GUS staining in the transmitting tissue of the ovary. Pistil was crushed with microscopic cover glass. **j-k**, Crushed pistil of a non-transgenic flower of stage 15; darker coloured lines show the vasculature. ta, tapetum; tt, transmitting tissue; vb, vascular bundles. *Bars* = 500 μm (**a-g**), 100 μm (**h-k**).

*LHT2* has recently been localized to the tapetum tissue in mature anthers (Lee and Tegeder 2004), and using promoter-*GUS* studies this localization pattern was confirmed (Fig. 5). *LHT2* expression in the tapetum was induced at flower stage 10 and was then seen throughout anther development (see weak staining of anthers in Fig. 5b-d). Higher magnification imaging resolved that in developing flower buds *LHT2* expression is restricted to the tapetum (Fig. 5g and h). Interestingly, a switch of GUS staining could be observed when the tapetum degrades and *LHT2* expression was reported in mature pollen (Fig. 5e-f and i-j).

GUS staining was not found in buds of *LHT5*-promoter GUS plants (data not shown). However, staining was observed in mature flowers (see below and Fig. 6).

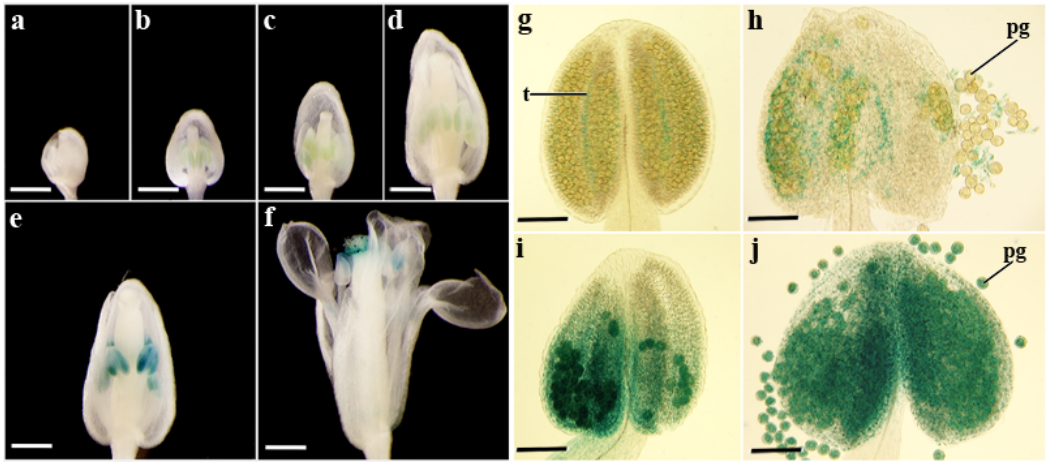


**Figure 5.** *LHT2*-promoter-*GUS* studies during flower development

**a-f**, GUS staining at developmental stages of flowers. Stages were defined according to Smyth et al. (1990). **a**, flower stage 9; **b**, stage 10; **c**, stage 11; **d**, early stage 12; **e**, late stage 12/early stage 13 and **f**, stage 15. **b-d** and **g-h**, GUS staining in the tapetum of anthers. **e**-**f** and **i-j**, GUS staining of mature and germinating pollen. **g, i**, Light micrograph of GUS staining in anther. **h, j,** Light micrographs of anthers shown in **g** and **i** after crushing with a microscopic cover glass. **g-h**, anther stage 11 (Sanders et al. 1999)/early flower stage 12 and **i-j**, anther stage 12/late flower stage 12. **g-h**, GUS staining in the tapetum but not in pollen grains. **i-j**, GUS staining in mature pollen. ta, tapetum; pg, pollen grain. *Bars* = 500 μm (**a-f**), 100 μm (**g-j**).

### 4. 4. 3. *LHT*s are expressed in germinating pollen, pollen tubes and in the transmitting tissue of the pistil

Pistils of fertilized flowers of *LHT*-promoter-*GUS* plants were analyzed and blue staining was seen in pollen germinating on the stigma of *LHT2*-promoter *GUS* lines (Fig. 5f). *LHT5*-promoter-*GUS* studies showed *LHT5* expression in pollen, pollen tubes and pistil tissues (Fig. 6). GUS staining was observed in pollen grains germinating on the surface of the stigma (Fig. 6a). Expression of *LHT5* in pollen tubes and pistil tissues could be resolved by analyzing hemizygous and homozygous promoter-*GUS* lines. In hemizygous lines, *LHT5* expression was be detected in pollen tubes growing through the stigma into the transmitting tissue of the style and ovary and into the ovules (Fig. 6b-e). The pollen tubes burst within the ovules releasing blue stain. In homozygous lines, GUS staining was found in pollen tubes, stigma and transmitting tissue of the pistil (Fig. 6f-k). Cross sections of GUS stained pistils and analyses using dark field imaging further confirmed expression of *LHT5* throughout the stigma and style tissues (Fig. 6g-i). In the ovary, GUS staining (red crystals in dark field) was restricted to the transmitting tract with strongest staining in the septum epidermis cells, the funiculus and the cells surrounding the medial vascular bundles (Fig. 6j-k).

*LHT6* was also expressed in pistil tissue (see Fig. 4). GUS staining was found throughout the pistils of buds (Fig. 4a-f), while in mature flowers, *LHT6* expression in pistils is restricted to the transmitting tract of the ovary (Fig. 4g-k).

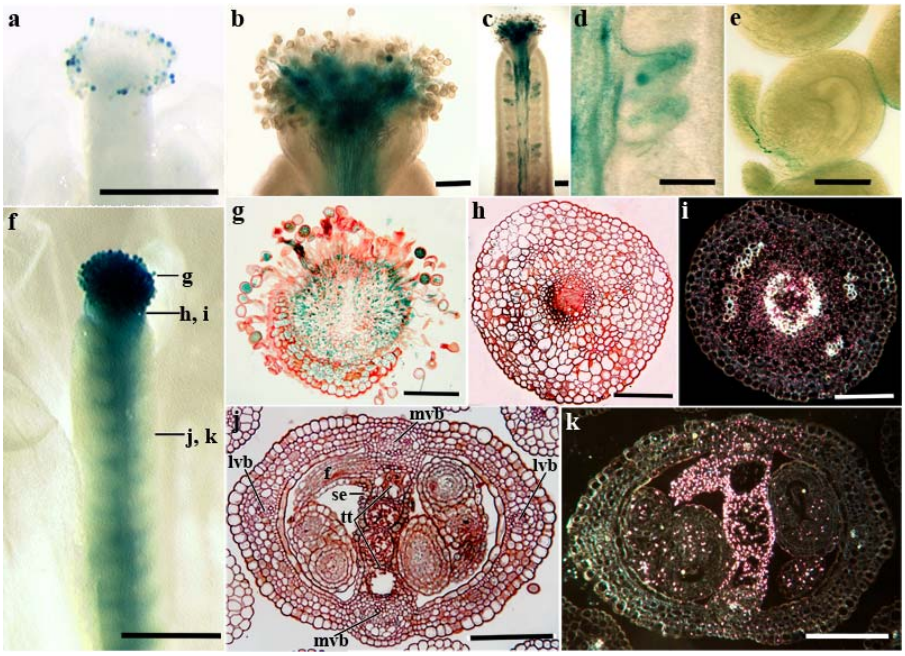


**Figure 6.** *LHT5*-promoter-*GUS* studies in flowers.

**a-k**, GUS staining at developmental stages of flowers according to Smyth et al. (1990). Gus staining of **a, b**, germinating pollen on the stigma; **b**, pollen tubes growing through the stigma and style; **c**, pollen tubes growing through the pistil to the ovules; **c-e**, pollen tubes growing into ovules and **f**, GUS staining within the pistil including style and ovary. **g-k**, Cross sections of GUS stained pistils. **g**, Light micrograph of the stigma stained with safranin-O; *LHT5* expression is reported throughout the stigma tissue as well as in germinating pollen grains and pollen tubes. **h**, Light micrograph of cross section of a style stained with safranin-O. **i**, Dark field micrograph of cross section of the style in **h**; GUS staining is shown as red crystals and found throughout the style tissue. **j**, Light micrograph of cross section of the ovary stained with safranin-O. **k**, Dark field micrograph of cross section of the ovary in **j**. fu, funiculus; lvb, lateral vascular

bundles; mvb, medial vascular bundles; se, septum epidermis; tt, transmitting tissue. *Bars* = 500 μm (**a, f**), 100 μm (**b-e** and **g-k**).

### 4. 4. 4. LHTs are targeted to the plasma membrane

Onion cell bombardment with transporter-*GFP* constructs has been frequently used to resolve protein localization to cellular membranes (see for example Murata et al. 2006; Latz et al. 2007; Sivitz et al. 2007; Pineros et al. 2008). In this study, onion epidermal cells were bombarded with vectors containing *GFP-LHT2*, *LHT4-GFP*, *LHT5-GFP*, *LHT6-GFP* or *GFP* only. Using confocal microscopy we found that all LHT transporters are localized to the plasma membrane (Fig. 7). Further, LHT4-GFP localization seems to be similar to what has been shown for some K+ channels (see Latz et al. 2007) and suggests that LHT4 might also be present in the tonoplast (Fig. 7 c-d). However, this needs to be confirmed using protoplasts or stable transgenic plants.

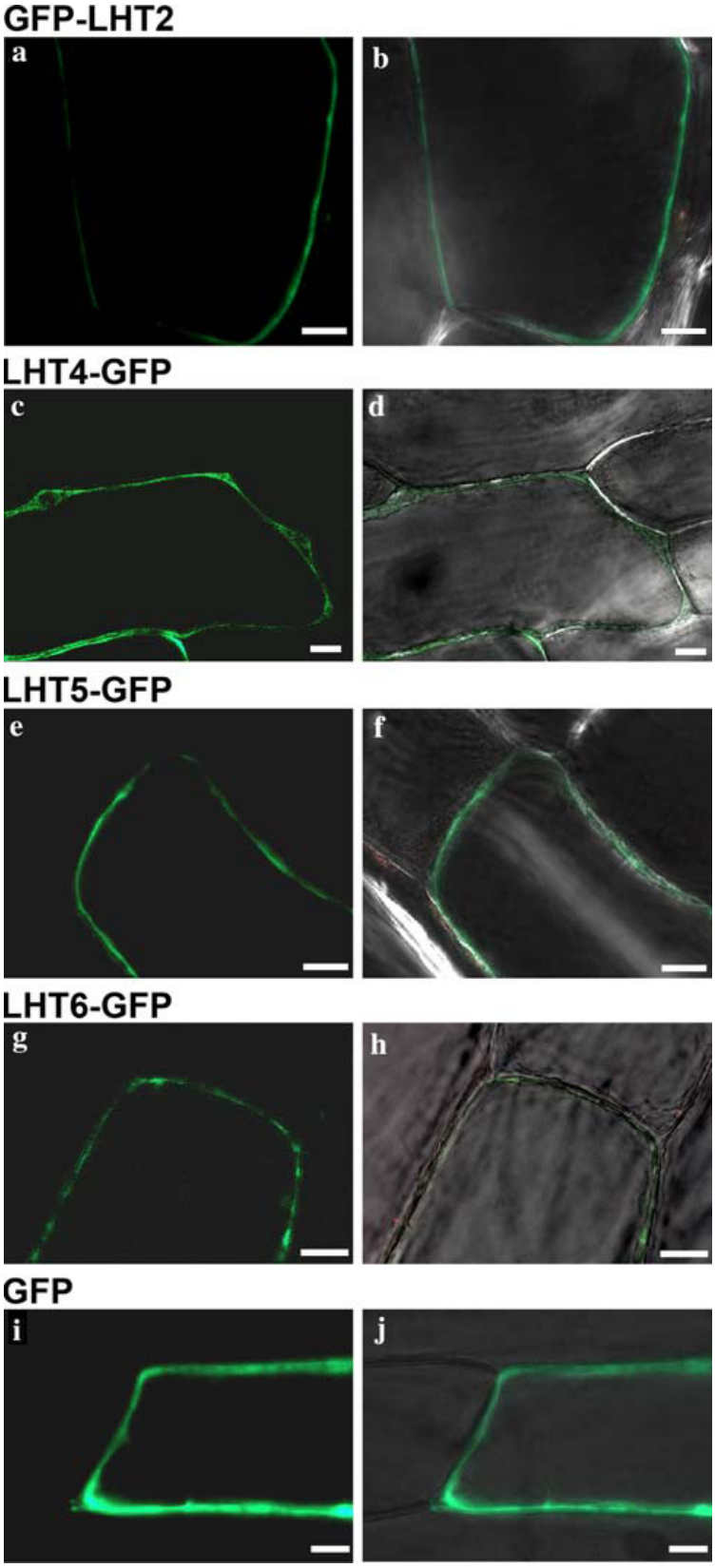
GFP-LHT2
a
b
LHT4-GFP
c
d
LHT5-GFP
e
f
LHT6-GFP
g
h
GFP
i
j

**Figure 7.** Membrane localization of LHT proteins in epidermal onion cells. (**a, b**) GFP5-LHT2, (**c, d**) LHT4-GFP6, (**e, f**) LHT5-GFP6, (**g, h**) LHT6-GFP6 and (**i, j**) GFP6. Detection of GFP fluorescence is shown in the first column (**a, c, e, g, i**). The second column (**b, d, f, h, j**) shows an overlay of bright field and fluorescence images in the first column. All LHTs are localized to the plasma membrane. LHT4 seems also to be localized to the tonoplast. *Bars* = 20 μm

## 4. 5. Discussion

Reproductive flowers represent a strong sink for N that is predominantly delivered to the floral organs as amino acids. A broad spectrum of amino acids is present in flowers although the concentrations of the single amino nitrogen compounds vary dependent on the plant species and tissue analyzed (Krogaard et al. 1983; Evans et al. 1987; Chiang and Dandekar 1995; Xie et al. 1997; Schwacke et al. 1999; Palanivelu et al. 2003). Microarray expression data have shown that *LHT* amino acid transporters are highly expressed in flowers or pollen (Honys and Twell 2004; Pina et al. 2005; Schmid et al. 2005; Toufighi et al. 2005; Bock et al. 2006; see also http://www.ncbi.nlm.nih.gov/geo/) where they are predicted to function in delivery of organic nitrogen within the floral tissue. These expression data are generally informative, but only localization studies can provide information on the location of transporter expression and function. We isolated the promoters of *LHT* amino acid transporters, which are expressed in floral organs, and performed promoter-reporter gene studies. Our

expression analyses by *LHT*-promoter-*GUS* studies were consistent with the *LHT* RNA expression analyses done in flowers (see Fig. 2-6; Honys and Twell 2004; Pina et al. 2005; Schmid et al. 2005; Toufighi et al. 2005; Bock et al. 2006; see also http://www.ncbi.nlm.nih.gov/geo/), but most importantly, the *GUS* experiments demonstrate that expression of *LHT*s in flowers is tissue and cell specific, and highly coordinated.

Onion cell bombardment has commonly been used to determine protein/transporter localization to the plasma membrane (Murata et al 2006; Sivitz et al. 2007; Pineros et al. 2008) and was also applied in our studies (Fig. 7). All LHT transporters analyzed seem to be localized to the plasma membrane indicating that they are involved in cellular import of amino acids. LHT4 appears to be also localized to the tonoplast (see Fig. 7 c-d and Latz et al. 2007) where it might function in vacuolar amino acid transport processes.

In early anther development initial cell expansion and nutrient acquisition are critical for creating the proper environment for growth of male gametophytes. *LHT4* and *LHT6* are expressed throughout the anther tissue (including the microspores) at these initial stages where they might be involved in amino acid supply for growth. The developing pollen grains are symplastically isolated from the sporophytic tissue and are dependent on the nourishing tapetum, a cell layer that secrets nutrients, proteins and other compounds for production of fertile pollen (Taylor et al. 1998; Sanders et al. 1999; Wilson et al. 2001; Lévesque-Lemay et al. 2003; Yang et al. 2003). Import of large amounts of amino acids

into the tapetal cells is needed for synthesis of these proteins and metabolites (Dickinson and Lewis 1973; Steer 1977; Bhadula and Sawhney 1991; Hess and Hesse 1994; Platt et al. 1998). In addition, amino acids transported into the tapetum are released into the anther locule and taken up by the growing male gametophyte (Clément et al. 1998). Localization of *LHT2, LHT4* and *LHT6* (Lee and Tegeder 2004; see Fig. 3, 4 and 5) in tapetal cells strongly suggests LHT function in amino nitrogen transport processes in the tapetum in support of pollen development.

Following the breakdown of the tapetum tissue, pollen maturation proceeds and simultaneously *LHT2* expression switches from tapetum cells to pollen. In *Nicotiana sylvestris,* tomato, and *Arabidopsis NsAAP1* (which is according to our sequence analysis a member of the LHT and not the AAP family; data not shown), *LeProT1* and *AtCAT6,* respectively have also been localized to pollen (Lalanne et al. 1997; Schwacke et al. 1999; Hammes et al. 2006), indicating that LHTs and other transporters are important for uptake of amino acids by the pollen. This is in agreement with a broad spectrum of amino acids present in anthers and pollen grains and the high amounts of ribosomes and tRNAs found in pollen (Sangwan 1978; Horner and Pratt 1979; Zhang et al. 1982; Krogaard et al. 1983; Peña-Valdivia 1999; Schwacke et al. 1999).

Following germination, *Arabidopsis* pollen tubes enter the wall of the stigma cells and grow through the extra cellular matrix (ECM) of the style tissue (Elleman et al. 1992). It is well accepted that pollen tubes use exogenous amino

acids for synthesis of protein (Zhang et al. 1982) and large amounts of serine, glutamine and asparagine have been found in *Nicotiana alata* styles most probably in support of the fast tube growth (Tupy 1961). The majority of tube growth is sustained by uptake of nutrients from the extra cellular matrix (ECM) of pistil transmitting tissue (Labarca and Loewus 1973; Knox 1984). *LHT5* is expressed in pollen tubes growing through the transmitting tract of the style and ovary until they reach the ovules and release the sperm cells, and it might function in acquisition of amino acids for tube elongation.

The transmitting tissue consists of secretory cells in which large amounts of proteins such as transmitting tissue-specific proteins (TTS) are synthesized to attract pollen tubes and accelerate their growth (Wang et al. 1993; Cheung et al. 1995; Cheung 1996). *LHT5* and *LHT6* are expressed in the transmitting tissue and are probably involved in delivery of amino acids for synthesis of these proteins (see Fig. 4 and 6). In addition, it has been shown for *Arabidopsis* pistils that an increasing gradient of γ-amino butyric acid (GABA) exists from the stigma to the micropyle probably stimulating pollen tube elongation (Palanivelu et al. 2003). Amino acid transport processes in the transmitting tract mediated by LHT5 and LHT6 could therefore also be important for the production of GABA.

After emerging from the transmitting tract the pollen tubes reach the septum, where the ovule is attached (Lennon et al. 1998; Palanivelu and Preuss 2006). They then grow up the funiculus or immediately turn to finally enter the micropyle for release of the sperm cells (Lord and Russell 2002). *LHT5* and *LHT6*

expression in the cells of the septum epidermis and funiculus indicate transporter function in support of pollen tube nutrition, or to prevent leakage of the amino acids out of these tissues.

Overall, *LHT* amino acid transporters show distinct and highly regulated expression in floral organs indicating a role for reproduction. However, expression data using micro-arrays and other approaches indicate that besides the LHTs, other amino acid transporters are present in flowers, where they are expected to accommodate transport of a wide spectrum of amino acids into or within cells of reproductive structures. Much work is needed (e.g. localization studies and mutant analysis) to further dissect the importance of the single amino acid transporters in sexual plant reproduction.

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# Chapter 5

# Characterizing LHT amino acid transporters from *Arabidopsis thaliana*

## 5. 1. Abstract

This research focuses on characterizing members of the LHT amino acid transporter family in *Arabidopsis thaliana.* Localization of *AtLHT2*, *AtLHT4*, *AtLHT5*, *AtLHT6* and *AtLHT7* expression was determined throughout the plant using RNA expression and promoter-*GUS* studies. The *AtLHT* displayed transporter specific expression patterns, and some *AtLHTs* showed cell-specific expression for example in guard cells of photosynthetic tissues (*AtLHT5)* and trichomes (*AtLHT2* and *AtLHT6),* suggesting transporters function in amino acid uptake in these cell types. *AtLHT4* and *AtLHT7* were localized to the vasculature of roots and leaves, probably involved in phloem loading. Furthermore, AtLHT4, 5, 6 and 7 were expressed in yeast cells and *Xenopus* oocytes to determine their substrate selectivity, but none of the AtLHTs showed transport function in these heterologous expression systems. For future studies, homozygous *lht2, lht4, lht7* and *lht8* mutants were identified, crosses were performed with selected mutants to produce double mutants and *AtLHT4*, *AtLHT5* and *AtLHT6* antisense lines were produced.

## 5. 2. Introduction

In *Arbadopsis thaliana* more than 50 amino acid transporters are predicted (Wipf et al. 2002; Rentsch et al 2007; see chapter 1). In this study members of the LHT amino acid transporter family were analyzed, which consists of 10 members in Arabidopsis. Up to now only three AtLHT transporters have been characterized, AtLHT1, AtLHT2 and AtLHT6 (Lee and Tegeder 2004; Hirner et al. 2006; Svennerstam et al. 2007; see also chapter 3).

*AtLHT1* expression was determined to be in lateral roots and epidermal cell layers of leaves in young Arabidopsis seedlings, whereas expression in older plants was localized to root tips and epidermal mesophyll cells in leaves (Hirner et al. 2006). Transport studies in yeast indicate that AtLHT1 is a high affinity transporter for neutral and acidic amino acids. *Atlht1* mutant plants displayed reduced growth on media with aspartate and glutamate, as well as decreased uptake of Glu, Asp and Gln compared to wild type. Further transport studies with *lht1* mesophyll protoplasts showed a decrease in uptake of radiolabeled Pro. In addition, concentrations of amino acids within the *lht1* leaves plants were increased in comparison to wild type, probably due to amino acid accumulation in the mesophyll apoplast. Together these data indicated a role of AtLHT1 in root uptake and import into mesophyll cells.

*AtLHT2* was localized to the tapetum of anthers using RNA *in situ* hybridization and *in situ* RT-PCR methods (Lee and Tegeder 2004). Functional analysis of AtLHT2 using yeast transport mutants illustrated that it transports proline and aspartate with a high affinity. Furthermore, competition studies in yeast showed other neutral and acidic amino acids are potential substrates for AtLHT2. Currently analyses of AtLHT2 function *in planta* are not available.

The expression of *AtLHT6* was localized to the roots of young seedlings and lateral roots of mature plants (see chapter 3). AtLHT6 function in plants was determined using *lht6* seedlings and uptake studies with radiolabled amino acids with wild type, *lht6-1* and *lht6-1* complementation. They demonstrated that alanine and acidic amino acids are substrate for AtLHT6 and that it is involved in the uptake of amino acids by the roots.

In this chapter further analysis of members of the LHT family were performed. Previous research localized AtLHT2, 4, 5 and 6 to the plasma membrane and examined their expression patterns in floral tissues (Foster et al. 2008; see chapter 4). Here the expression of *AtLHT2, 4, 5, 6, 7* in other organs and subcellular localization of AtLHT7 was examined. Attempts to characterize AtLHT4, 5, 6 and 7 function using heterologous expression systems, and the identification and production of *lht* mutants and *antisense* lines are described.

## 5. 3. Materials and methods

### 5. 3. 1. Plant materials and growth conditions

*Arabidopsis thaliana* L. ecotype Columbia seeds were imbibed at 4˚C overnight and sown in soil consisting of peat (60%), pumice (20%) and sand (20%). Plants were grown in environmentally controlled growth chambers with 16 h light at 150-200 μmol photons $m^{-2}$ $sec^{-1}$, 50 % humidity and temperatures of 18˚C in light and 16˚C in dark. Kanamycin selection (50 μg $ml^{-1}$) of transgenic lines was performed using *in vitro* cultures with half strength MS media (pH 5.7; Murashige and Skoog 1962) supplemented with agar (9 g $l^{-1}$), myo-inositol (100 mg $l^{-1}$), sucrose (10 g $l^{-1}$) and 2-(N-morpholine)-ethane sulphonic acid (MES; 0.5 g $l^{-1}$). Plants were grown in environmentally controlled tissue culture chambers at 20˚C, 50 % humidity and with 16 h light at 125-130 μmol photons $m^{-2}$ $sec^{-1}$. Kanamycin resistant plants were transferred to soil after 3 weeks of sterile culture. For selection of transgenic *LHT*-promoter-*GUS* plants for glufosinate resistance, a solution (430 μl $L^{-1}$) of the herbicide Finale® (5.78% glufosinate-ammonium, 94.22% other ingredients; Hoechst-Roussel Agri-Vet Company, Somerville, NJ, USA) was sprayed on soil-grown plants every 3 days for the first 2-3 weeks following germination. Seeds were collected for continued screening until homozygous lines were produced.

### 5. 3. 2. RNA expression analysis

Total RNA was isolated from different organs (roots, rosette leaves, source leaves, sink leaves, stems, buds, flowers and siliques) of *A. thaliana* plants and whole seedlings following the procedure of Pélissier and Tegeder (2007). First strand synthesis was carried out using the isolated RNA, M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo d(T) primers. PCR (polymerase chain reaction) was performed on first strand cDNAs with two sets of gene specific oligonucleotide primers for each *LHT* transporter, and *ACT2* primers were used as control for even amounts of cDNA templates. The primers sets were:

*AtLHT1* (1) 5'-gCAAgAgAAACgCCggAgATgg-3'

5' ATggTAgCTCAAgCTCCTCATg-3' (60˚C),

(2) 5'-CgCATgggCATCATCAgCAAgC-3'

5'-CgTAgAAATTACggACAAAgAACC-3' (60˚C);

*AtLHT2* (1) 5'-ATggggAACAgTgAAATgTCAgC-3'

5'-CggAAgCAAAgATCATgATCC-3' (60˚C),

(2) 5'-ATggggAACAgTgAAATgTCAgC-3'

5'-CCATCCgAgATTggACATAgc-3' (60˚C);

*AtLHT3* (1) 5'-CCgAAgggACTTATAATTgTggC-3'

5'-TTAggTgCTTTTgCAACTggAgTC-3' (60˚C),

(2) 5'-CTCATAgACATgACggCAgCTACC-3'

5'-ggACCTggAgTTgTggTgTTAATC-3'(65˚C);

*AtLHT4* (1) 5'-ATggACgAAAgACCCgAgACAgl-3'

5'-TTAgTTAggCggCTTgAAgAAC-3' (60˚C),

(2) 5’-gAgTgAACCAgAAgTCgAAggC-3’

5’-gAAATTCAgTCgACgATgCCATC-3’ (60˚C);

*AtLHT5* (1) 5’-CATgACCTTCTTTgCACC-3’ and

5’-AATggCgATAAgCCAgATG-3’ (50˚C),

(2) 5’-CATgACCTTCTTTgCACC-3’ and

5’-TCAAgAgAAgAATTCgTAgTTTTTgg-3’ (60˚C);

*AtLHT6* (1) 5’CTTAAgTgCACTgggTgAAATgg-3’

5’-CATgTTggACCACCAACTATTTgg-3’ (65˚C)

(2) 5’-CTgATCTCgACAAgTAgTTgTAgg-3’

5’-ATggCgggAATCCCAgATCATATCC-3’ (62˚C);

*AtLHT7* (1) 5’-ATgTCACCAgCCCCCTCCACgg-3’

5’-CAAgAATCgTACAAgCTCCTCC-3’ (65˚C),

(2) 5’-ATgTCACCAgCCCCCTCCACgg-3’

5’-TTAgggTCTgAAgAAgTTAgCATgC-3’ (60˚C);

*AtLHT8* (1) 5’-CgATTTggACAAgAAgCTgTTgC-3’

5’-ATgCCAAAATACCTTgATCAg-3’ (58˚C),

(2) 5’-gACATAgTCTACAACgTgACAgg-3’

5’-ggTTTCTTggAAggCACTTCAgg-3’(65˚C);

*AtLHT9* (1) 5’-CAgCTTggCTCATTgCTgCTgC-3’

5’-TTAAgCgTAgAAACTATAAgTAgAAg-3’ (65˚C),

(2) 5’-gTgCAgCTCCATgAgTgTgTACC -3’

5’-TTAAgCgTAgAAACTATAAgTAgAAg-3’ (65˚C);

*AtLHT10* (1) 5’-CTACCAATgACATgCATAACgACg-3’

5’-ATgTATATTCAgATgACAgATggAg-3’ (65˚C),

(2) 5’-gTggAgATgCATgAgATggTTCC-3’

5’-CTACCAATgACATgCATAACgACg-3’ (65˚C).

### 5. 3. 3. *GFP*-transporter construct preparation and onion cell bombardment

Subcellular localization of AtLHT2, AtLHT4, AtLHT5 and AtLHT6 was described earlier (see chapter4), but here membrane localization of AtLHT7 was determined. *AtLHT7* cDNAs were obtained using cDNAs of flower buds from *Arabidopsis thaliana* ecotype Columbia and the PCR method with the following primers: 5’-ATgTCACCAgCCCCCTCCACgg-3’ and 5’-TTAgggTCTgAAgAAgTTAgCAT-3’. The PCR products were cloned into the plasmid vector pGEM®-T Easy (Promega, Madison, WI, USA) and sequenced. *AtLHT7* cDNA in was then amplified using primers 5’-gTCACCAgCCCCCTCCACgg-3’ and 5’-TTAgggTCTgAAgAAgTTAg-3’. The product was cloned into pCR8/GW/TOPO (Invitrogen, Carlsbad, CA, USA), and subsequently transferred into the Gateway binary vector pMDC83, upstream of the *GFP* cDNA (Curtis and Grossniklaus 2003) using LR clonase (Invitrogen, Carlsbad, CA, USA).

Transient expression of the AtLHT7-GFP fusion protein was performed in onion (*Allium cepa)* epidermal cells using particle bombardment with 1.1 *μ*m

tungsten particles and 1,350 psi rupture discs according to the manufacturer's protocol (Bio-Rad, PDS-1000, Hercules, CA, USA); (http://www.bio-rad.com). Images were captured with a Zeiss LSM 510 META confocal microscope (Thornwood, CA, USA).

### 5. 3. 4. Isolation of *LHT* promoters, *GUS*-construct preparation and plant transformation

*LHT* promoters were isolated by PCR approach using *Arabidopsis* BAC (Bacterial Artificial Chromosome) clones as template (*LHT3*, F11P17.1; *LHT5*, F12A21.22; *LHT6*, F28J7.9; *LHT7*, T12J5; *LHT8*, F25A9.9, see also http://www.ncbi.nlm.nih.gov/) and promoter specific oligonucleotide primers. Construct preparation for *AtLHT2*, *AtLHT4*, *AtLHT5* and *AtLHT6* promoters is described in chapter 4. For isolation of the *AtLHT3*, *AtLHT7* and *AtLHT8* promoter, the DNA sequence between the start codon of the specific *LHT* transporter and the stop codon of the gene upstream of the transporter was used. Dependent on the promoter, 630 bp (*LHT3,* At1g61270), 2,499 bp (*LHT7,* At4g35180 ), and 2,583 bp (*LHT8,* At1g71680) fragments upstream of the start codon of the respective transporter were isolated (Fig. 1). Primers were the following:

*AtLHT3* 5'-TTgCAAAATCgTTgAgAAATTgA-3'

5'-TCCTCAAATTTATATACgCAAAA-3' (58˚C);

*AtLHT7* 5’-AAgTTggTTgCTCgTAACTgg-3’

5’-TgTATACAAAATAAACTTTATTTTCC-3’ (58˚C);

*AtLHT8* 5’-gAAATgCTCggTAgCTgAAAA-3’

5’-gCTTAATATAATgAATTAAgAgAAC-3’ (55˚C)

The PCR products were cloned into pGEM®-T Easy (Promega, Madison, WI, USA) and sequenced. The *AtLHT3* promoter was excised using *Not*I, then the *Not*I site was blunted, and the fragment subcloned into the *Sma*I site of the binary vector PGPTV-BAR (Becker et al. 1992) upstream the *uidA* gene. The *AtLHT7* promoter was excised from pGEM®-T easy and inserted into PCR2.1 using *Not*I. The promoter was then cut with *EcoR*V and *Xba*I and subcloned into the *Sma*I and *Xba*I site of the binary vector PGPTV-Kan (Becker et al. 1992). The *AtLHT8* promoter was digested with *Apa*I (blunted) and *Sal*I, and inserted into the *Sma*I and *Sal*I site of the binary vector PGPTV-Kan (Becker et al. 1992). The promoter-*GUS* constructs in the binary vectors were used for transformation of *Arabidopsis* ecotype Columbia with *Agrobacterium tumefaciens* strain pGV3101 (Koncz and Schell 1986) and the floral dip method (Clough and Bent 1998).

### 5. 3. 5. Histochemical analysis of *LHT*-promoter-*GUS* lines

Tissues of the transgenic *LHT*-promoter-*GUS* plants were collected and placed in GUS staining solution containing 2 mM X-Gluc substrate (5-Bromo-4-chloro-3-indoxyl-Beta-D-Glucuronide; cyclohexyl ammonium salt; Gold

Biotechnology, St. Louis, MO, USA), 10 mM EDTA (ethylenediaminetetraacetic acid, pH 8), 1 mM potassium ferrocyanade, 1 mM potassium ferricyanide, 0.1% Triton-X and 100 mM $PO_4$ buffer (pH 7) followed by vacuum infiltration for 15 min. The tissues were then incubated overnight at 37˚C and cleared using 95% ethanol. Whole organs were analyzed with a stereoscopic light microscope (Wild, HeerBrugg, Switzerland), whereas sections were viewed using a compound light microscope (Leitz, Wetzlar, Germany).

### 5. 3. 6. Cloning of *AtLHTs* into yeast and oocyte expression vectors

*AtLHT4, 5, 6* and *7* cDNAs were isolated and cloned into pGEM®-T Easy (Promega, Madison, WI, USA) as described in chapter 4. In addition, the *AtLHT6* genomic DNA was isolated via PCR on the BAC (Bacterial Artificial Chromosome) clone F28J7.9 using the primers 5´-ATggCgggAATCCCAgATCATATCC-3´ and TCAgTgTTTAggTAAgTTgCATC-3´ and cloned into pGEM®-T Easy (Promega, Madison, WI, USA). The DNAs were then subcloned into the yeast expression vector pDR195 (Rentsch et al., 1996) and p002, a vector used for transport studies in *Xenopus laevis* oocyte (Ludewig et al. 2002). *AtLHT5* and *AtLHT7* cDNA, as well as *AtLHT6* cDNA and genomic DNA, were excised from pGEM®-T Easyusing *Not*I and subcloned into the *Not*I site of the yeast expression vector pDR195 (Rentsch et al. 1996). *AtLHT4, 5, 6* and *7* were subcloned from pGEM®-T Easy (Promega, Madison, WI, USA) into the *EcoR*I site of the p002 vector.

### 5. 3. 7. Heterologous expression of *LHTs* in yeast

The yeast strains JT16 (*MATα, hip1-614, his4401, can1, ino1, ura3-52*; Tanaka and Fink 1985), 22574d (*MATα, ura 3-1, gap 1-1, put 4-1, uga 4-1*; Jauniaux et al. 1987), 22Δ8AA (MAT-*α*, ura3-1, gap-1, put4-1, uga4-1, can1::HisG, lyp/alp::HisG, hip1::HisG, and dip5::HisG; Tegeder et al. 2000; Fischer et al. 2002) and 22Δ6AAL (MAT-a, ura3-1, gap-1, put4-1, uga1; can1::HisG, lyp/alp::HisG, and lys2::HisG; Tegeder et al. 2000; Fischer et al. 2002) were transformed with *AtLHT 5, 6*, or *7* in pDR195 and controls *AtLHT2*, *AtAAP3*, *AtAAP3* and empty pDR195 according to Dohmen et al. (1991). Transformants were then grown on media containing His (JT16), Pro (22574d), Asp, Glut, Arg, Cit (22Δ8AA) or Lys (22Δ6AAL) as sole nitrogen source.

### 5. 3. 8. Heterologous expression of LHTs in *Xenopus laevis* oocytes

*AtLHT 4, 5, 6* and *7* in p002 were employed to synthesize cRNAs that were injected into the oocytes for electrophysiological analysis of amino acid transport by a two-electrode voltage clamp system according to Chandran et al. (2003). The transport properties of the AtLHTs were analyzed by measuring the current induced by individual amino acids. The injected oocyte was bathed in a modified ringer solution (115 mM NaCl, 2mM KCl, 1.8 mM $CaCl_2$, 5mM MES, 1 mM $MgCl_2$, pH adjusted with Tris to of 5.7 or 7). Two electrodes were clamped on opposite sides of the oocyte membrane and held a membrane potential of –40 mV using a Dagan TEV 200A amplifier. Once a steady state was reached, the

oocyte was bathed a modified ringer solution containing 3 mM of the amino acid. A voltage pulse between –157 to 57 mV for 203 ms was then applied and the currents measured. All twenty amino acids found in proteins, as well as GABA, were used for transport studies.

### 5. 3. 9. Screening of *AtLHT* T-DNA lines

Transgenic lines of *A. thaliana* with T-DNA insertions in the respective LHT gene were obtained from the Arabidopsis Biological Resource Center (ABRC) (http://signal.salk.edu), and homozygous lines were identified by performing PCR on genomic DNA with gene specific and T-DNA specific left border (LB) primers.

1. *AtLHT2* 5’-gAgAACgAgCCACATAATgC-3’
   (LB) 5’-TTCATAACCAATCTCgATACAC-3’ (55˚C);
2. *AtLHT4* 5’-TTAgTTAggCggCTTgAAgAAC-3’
   (LB) 5’-TggTTCACgTAgTgggCCATCg-3’ (63˚C);
3. *AtLHT7* 5’-CCAgAATCgTACAAgCTCCTCC-3’
   (LB) 5’-TggTTCACgTAgTgggCCATCg-3’ (65˚C);
4. *AtLHT8* 5’-CgATTTggACAAgAAgCTgTTgC-3’
   (LB) 5’-TggTTCACgTAgTgggCCATCg-3’ (63˚C);

### 5. 3. 10. Construction of *antisense* lines

*AtLHT4*, *AtLHT5* and *AtLHT6 antisense* lines were produced. Two *antisense* constructs were made for each DNA sequence from (1) the least conserved regions of the specific *AtLHT* transporter gene and (2) a region with the highest similarity to its closest related gene determined by sequence alignment using ClustalX (Thompson et al. 1994; Thompson et al. 1997; Jeanmougin et al, 1998) which might lead to repression of both transporters (for closest related see also Fig. 1 and Table 4). PCR was performed using the primers:

AtLHT4 (1) 5'-ATggACgAAAgACCCgAgACAg-3'

5'-ggTTAggAgTATggCTTgCATTg-3' (65˚C),

(2) 5'-TTAgTTAggCggCTTgAAgAAC-3'

5'-ggTTAggAgTATggCTTgCATTg-3' (63˚C);

AtLHT5 (1) 5'-ggTgCCACAACAACTAATCg-3'

5'-CATTTCCTgAggTTgTAgATgC-3 (58˚C),

(2) 5'-gAAACCCATCTggCTTATCg-3'

5'-CTTTggTTTCTTgATACAAAgCC-3' (58˚C);

AtLHT6 (1) 5'-CTgATCTCgACAAgTAgTTgTAgg-3'

5'-ATggCgggAATCCCAgATCATATCC-3' (65˚C),

(2) 5'-CATCggATggTAAACCgTAg-3'

5'-gCAACggTTCgATAggTACC-3' (55˚C).

The PCR products were cloned into pGEM®-T easy (Promega, Madison, WI, USA) in *antisense* orientation. The sequence was excised from pGEM®-T easy with *Not*I, blunted and then subcloned into the *Sma*I site of the binary vector

pBINAR (Hofgen and Willmitzer 1990). The constructs were utilized for transformation of *A. thaliana* by the floral dip method (Clough and Bent 1998). The transgenic lines were then screened for homozygosity using MS (Murashige and Skoog 1962) plates with Kanamycin (50 $\mu$g $ml^{-1}$).

## 5. 4. Results

### 5. 4. 1. Phylogenetic analysis of the LHT amino acid transporters

A phylogenetic tree was constructed using full length protein sequences of all known LHT transporters (Fig. 1). A consensus tree was created in PAUP* 4.0b10 (Swofford 2000) with maximum parsimony and a heuristic search. Bootstrap values were produced using 5000 replicates and the values >50% were recorded on the tree. The LHTs group into two main clades. The smaller clade consists of AtLHT4, 7 and two rice genes, whereas the other clade contains the remaining AtLHTs, OsHTs (rice), NsAAP1(*Nicotiana sylvestris*) (Fig. 1).

Furthermore, a phylogenetic tree of all LHT and AAP amino acid transporters was created in PAUP* 4.0b10 (Swofford 2000) with maximum parsimony and a heuristic search. Amino acid character states were analyzed using MacClade (Maddison and Maddison 2000) to determine if the positions of amino acids were conserved in different clades (Fig. 2). For example, some amino acid character states (positions) were conserved within all LHT and AAP

transporters (Fig. 2a), whereas others were conserved within the LHTs or AAPs (Fig. 2b). In addition, some clades have diverged from the other transporters, such as the small LHT clade that contains AtLHT4, 7 and two rice genes (Fig. 2c).

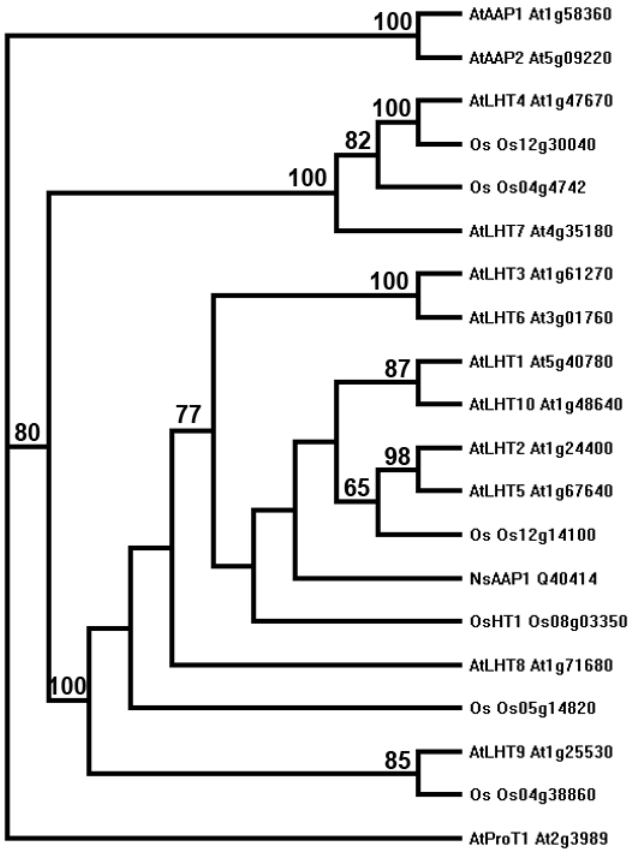
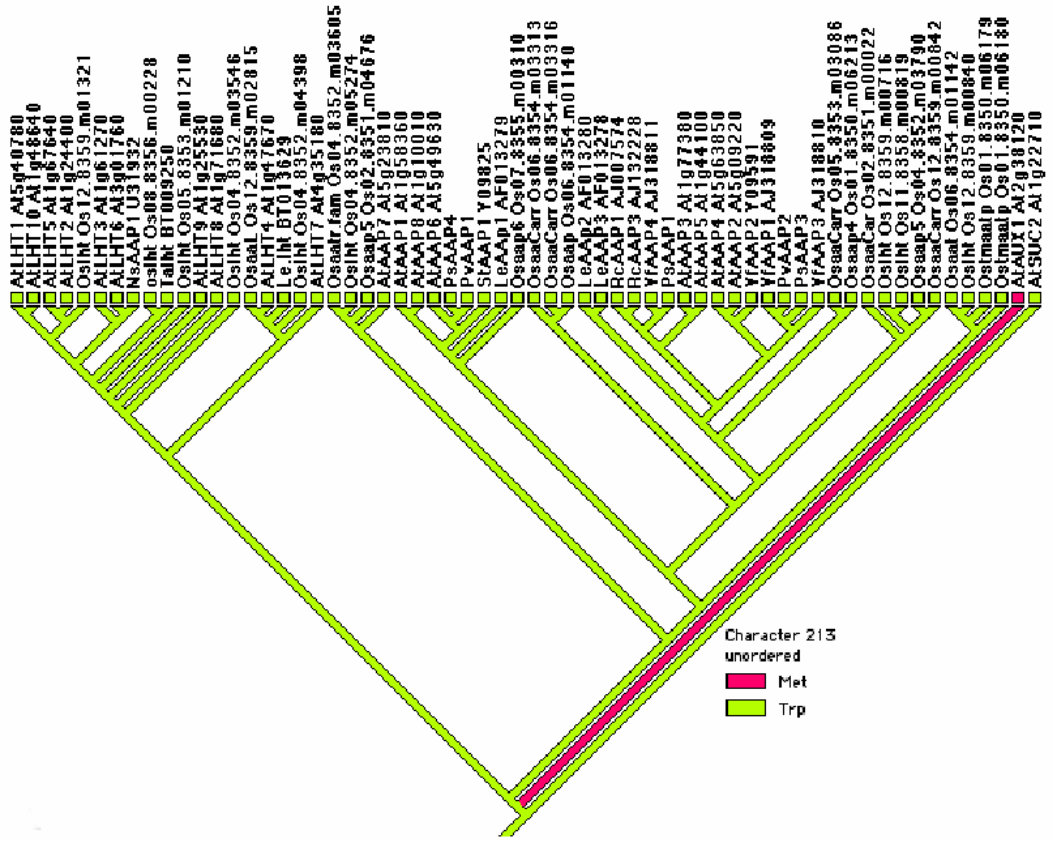



**Figure 1**. Comparison of LHT amino acid transporters.

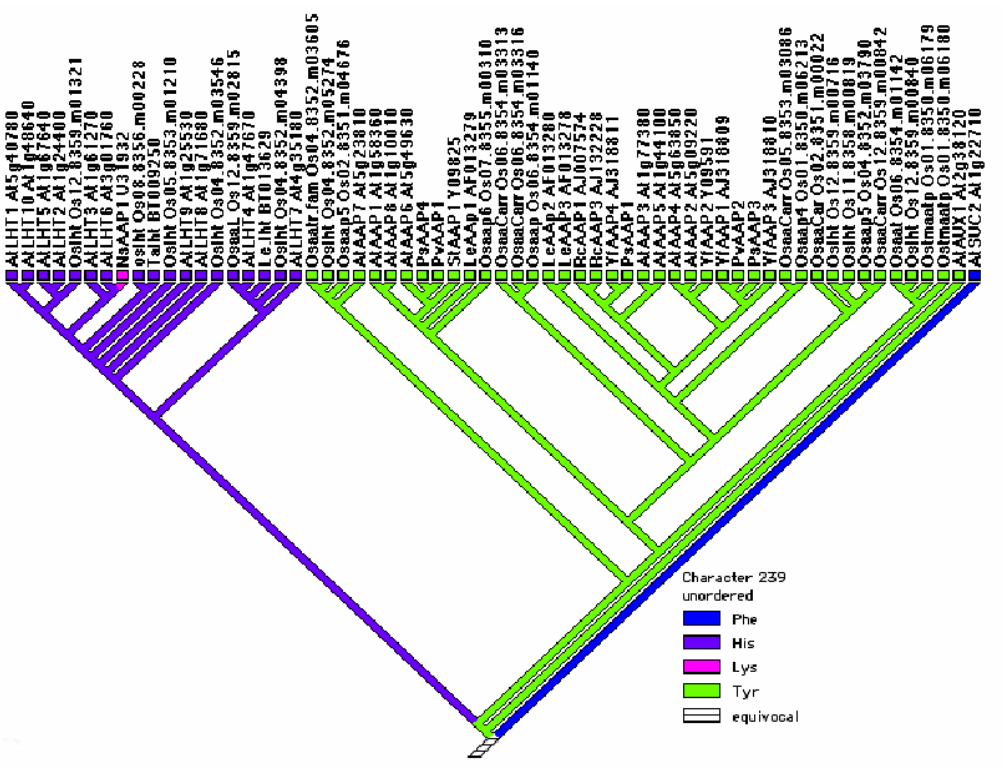
Phylogenetic analysis of LHTs from *Arabidopsis thaliana* and published LHTs from *Nicotiana sylvestris* (NsAAP1; Lalanne et al. 1997) and *Oryza sativa* (OsHT1; Liu et al. 2005) with the out groups AtAAP1 (Frommer et al. 1993), AtAAP2 (Kwart et al. 1993) and AtProT1 (Rentsch et al. 1996). Protein

sequences were obtained from the Aramemnon database (http://aramemnon.botanik.uni-koeln.de/; Schwacke et al. 2003) and were aligned using ClustalX (Thompson et al. 1997).

**a**




**b**


Character 239
unordered
Phe
His
Lys
Tyr
equivocal

**c**

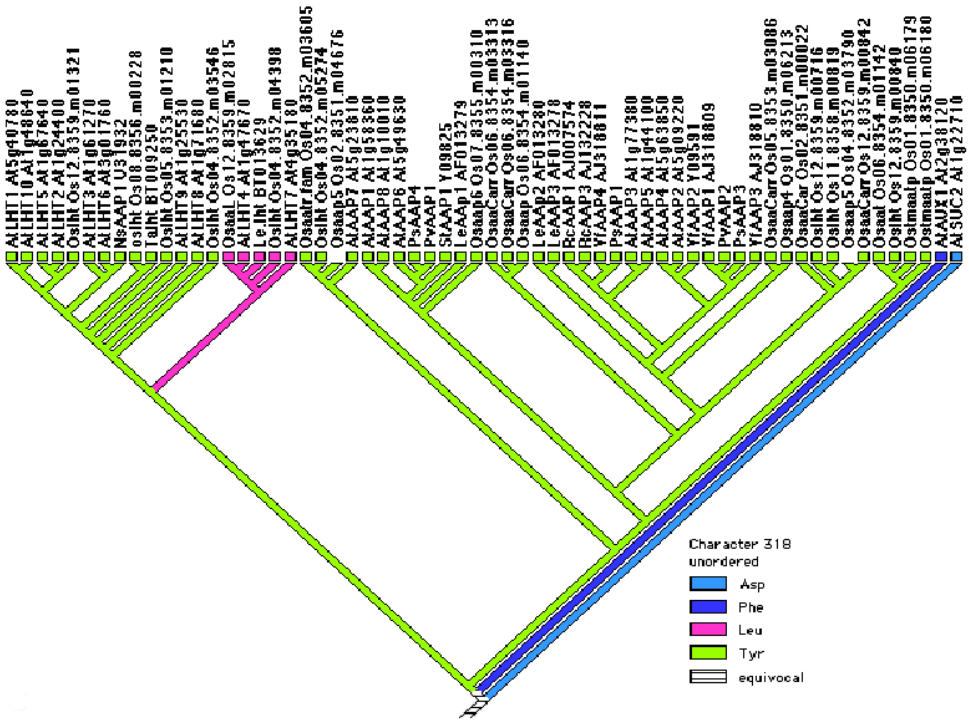

Character 318
unordered
Asp
Phe
Leu
Tyr
equivocal

**Figure 2**. Phylogenetic analysis of LHTs and AAPs using residues as character states. Protein sequences were obtained from the Aramemnon database (http://aramemnon.botanik.uni-koeln.de/; Schwacke et al. 2003) and were aligned using ClustalX (Thompson et al. 1997). A consensus tree was created in PAUP* 4.0b10 (Swofford 2000) with maximum parsimony and a heuristic search. Amino acid character states were analyzed using MacClade (Maddison and Maddison 2000) and amino acid tree data from Fig 1**.** **a**, character state 213. **b**, character state 239. **c**, character state 318

### 5. 4. 2. Organ-specific expression of the *AtLHTs*

RT-PCR was performed using two sets of primers to analyze *LHT* expression (Fig. 3). *AtLHT1, 4, 9* and *10* were expressed in all organs of mature plants, as well as seedlings. *AtLHT3* showed expression in the roots, stems and seedlings and *AtLHT6* transcripts were found in roots, stems, rosette leaves, buds, flowers and seedlings. Furthermore, *AtLHT5* was expressed in sink leaves, buds, flowers and seedlings, whereas *AtLHT8* transcripts were only present in buds and flowers. Expression of the *AtLHTs* was seen in the roots with the exception of *AtLHT5* and *AtLHT8*. Interestingly, all *AtLHTs* were expressed in the buds and flowers. A detailed analysis of *AtLHT 2, 4, 5* and *6*

expression in flowers was performed using promoter-*GUS* studies and has recently been published (Foster et al. 2008, see chapter 4).

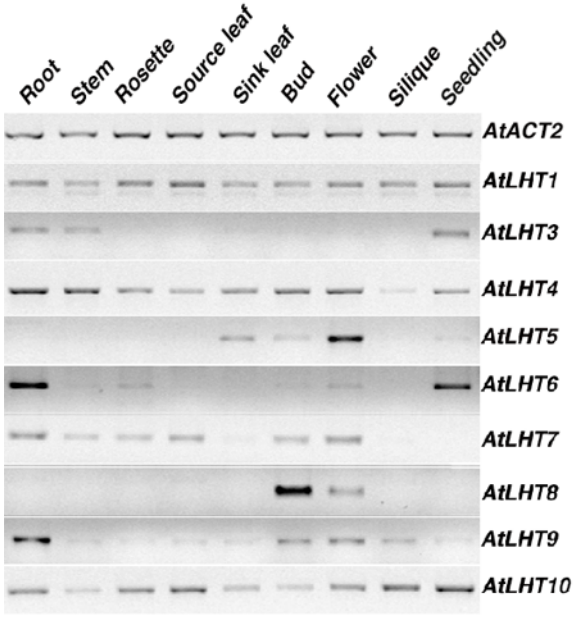



**Figure 3**. Expression of *LHT*s in different organs of *A. thaliana*.
RT-PCR was performed with RNA from the different organs and seedlings. RT-PCR with *AtACT1* was used as control for equal amounts of cDNA in each sample.

### 5. 4. 3. Tissue- and cell-specific localization of *AtLHTs*

In previous studies tissue specific expression of *AtLHT2*, *AtLHT4*, *AtLHT5* and *AtLHT6* was analyzed in flowers (Lee and Tegeder 2004; Foster et al. 2008; see also chapter 4). Here *AtLHT* promoter-*GUS* studies were used to determine tissue expression of *AtLHTs* in other organs of Arabidopsis plants. Transgenic

plants were harvested and incubated in a GUS (X-Gluc) staining solution. *AtLHT3* and *AtLHT8* promoter-*GUS* lines showed no staining in any tissue type. The RT-PCR and microarray data (see Fig. 3 and http://bar.utoronto.ca/) indicate that *AtLHT4* is expressed in roots, however only 1 of the 15 *AtLHT4*p-*GUS* lines showed staining in the root, specifically in the vasculature (Fig. 4). *AtLHT5* expression was reported to be in guard cells of photosynthetic tissues (Fig. 5). On the other hand, *AtLHT2* (Fig. 6) and *AtLHT6* (Fig. 7) were expressed in trichomes of leaves. In addition, staining was seen in a random pattern in rosette leaves of mature *AtLHT6* plants (Fig. 7). Three lines of *AtLHT7* were examined and showed staining in major and in some minor veins of leaves, but more lines need to be analyzed (Fig. 8). An overview of the tissue- and cell-specific expression of *AtLHTs* is presented in Table 1.

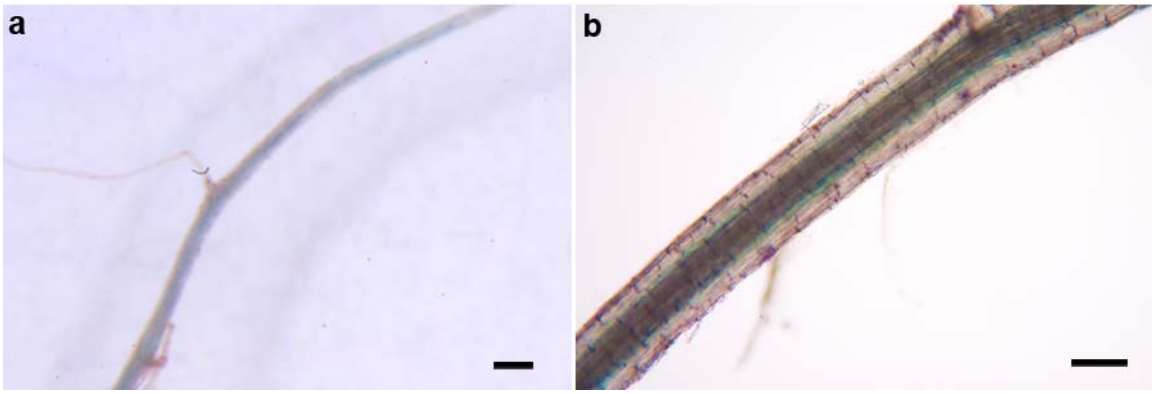



**Figure 4**. Histochemical detection of *AtLHT4*-promoter-*GUS* expression in roots. **a,** GUS staining in the main root. **b,** Is a higher magnification of (**a**) showing GUS staining in the vasculature. Bars = 2 mm (**a**), and 500 μm (**b**).

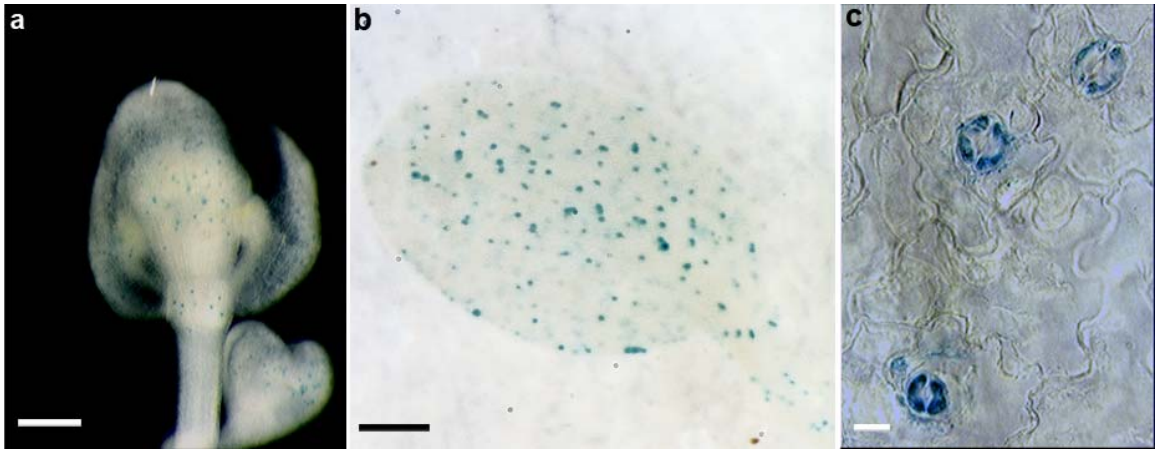



**Figure 5**. Histochemical detection of *AtLHT5*-promoter-*GUS* expression in guards cells of (**a**) buds (**b, c**) leaves. **c,** Is a higher magnification of leaves (**b**). Bars = 250 μm (**a, b**) and 10 μm (**c**).

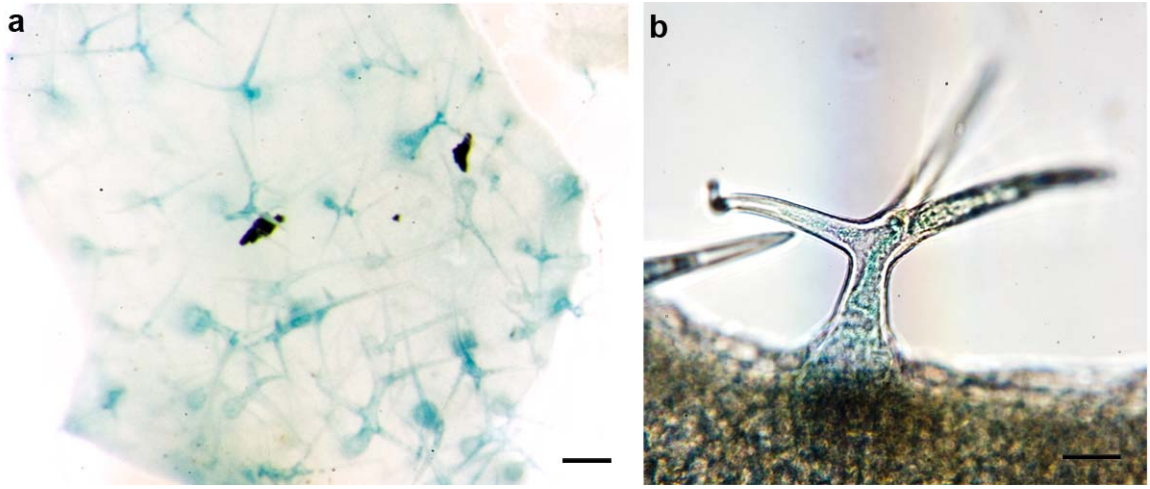



**Figure 6**. Histochemical detection of *AtLHT2*-promoter-*GUS* in trichomes. **a,** GUS staining in trichomes of young leaves. **b,** Is a higher magnification of (**a**). Bars = 200 μm (**a**) and 100 μm (**b**).

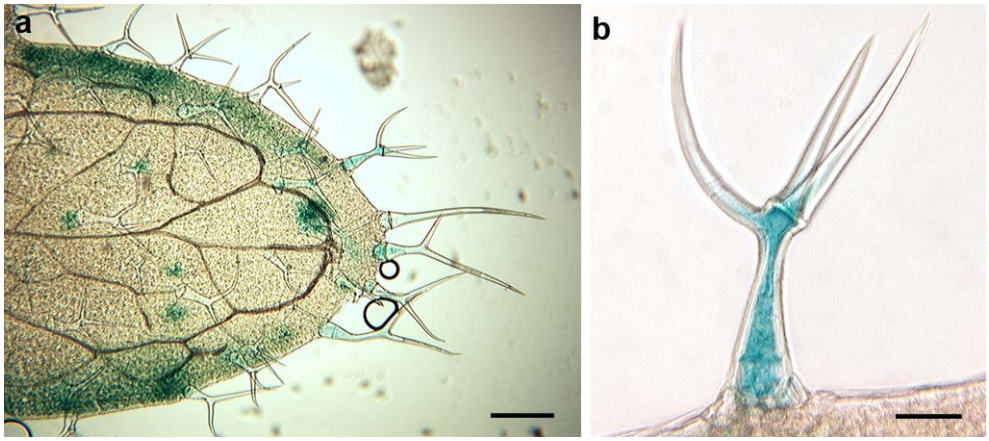



**Figure 7**. Histochemical detection of *AtLHT6*-promoter-*GUS.* **a**, Expression in leaves. **a-b,** Expression in trichomes Bars = 200 μm (**a**) and 50 μm (**b**).

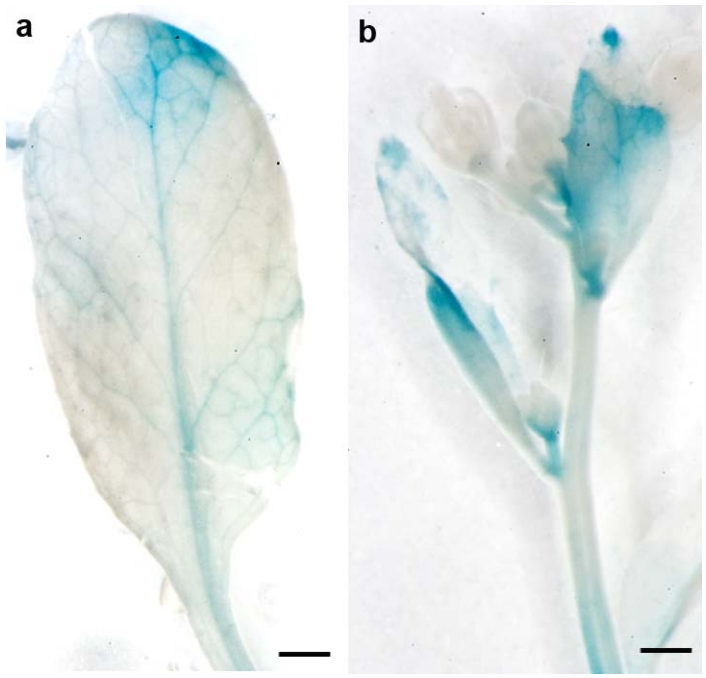



**Figure 8**. Histochemical detection of *AtLHT7*-promoter-*GUS*. **a**, Rosette leaf of a mature flowering plant. **b**, Inflorescence. Bars = 500 μm.

**Table 1**. An overview of *AtLHT* expression using promoter-*GUS* and RT-PCR studies.

| Gene | GUS expression | RNA |
|---|---|---|
| *LHT2* | Taptum of anthers, mature pollen, trichomes | Strong in buds and flowers, weak in other shoot tissues |
| *LHT3* | No GUS stain (initial prediction of start codon was incorrect) | Strong in roots and seedlings, weak in stem |
| *LHT4* | Throughout young anther, tapetum of older anthers | All tissues |
| *LHT5* | Throughout transmitting tissue of the pistil, germinating pollen grains and pollen tubes, guard cells | Strongest in flowers, weak in buds, young leaves and seedlings |
| *LHT6* | Roots of seedlings and side roots of older plants, all cells of root cross section, randomly in rosette leaves, trichomes, throughout young buds anther and pistil, tapetum of older anthers | Strongest in seedlings and roots, weaker in buds, flowers, stems, and rosettes |
| *LHT7* | Main vasculature of leaves, some areas of leaves, minor veins, mature pollen. | All tissues except siliques and seedlings |
| *LHT8* | No GUS stain | Only in buds and flowers |

### 5. 4. 4. Subcellular localization of AtLHT7

The *AtLHT7-GFP* fusion construct in pMDC was used for bombardment of onion epidermal cells and analyzed by confocal microscopy. Fluorescence of the AtLHT7:GFP fusion protein was found in the plasma membrane of onion epidermal cells (Fig. 9a), whereas the control GFP did not show specific localization (Fig. 9c).

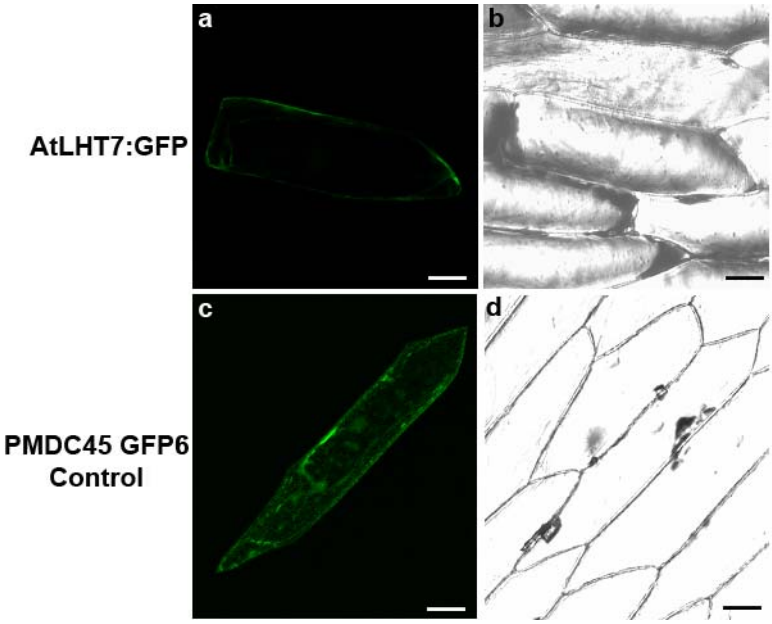



Figure 9. Subcellular localization of AtLHT7-GFP fusion protein. **a**, Particle bombardment of onion epidermal cells with *AtLHT7-GFP* under control of the *CaMV 35S*-promoter. **c**, Particle bombardment of onion epidermal cells using *CaMV 35S*-promoter-*GFP* in the PMDC45 vector (control). **b** and **d**, Bright field image of (**a**) and (**c**) respectively. Bars = 50 μm.

### 5. 4. 5. Functional characterization of AtLHT amino acid transporters using heterologous expression systems

Oocytes expressing *AtLHT4, 5, 6* or *7* were bathed in a solution with specific amino acids and the current was measured using a two-electrode-voltage clamp technique. Unfortunately, no change in current was observed for *AtLHT 4, 5, 6* or *7* with any of the amino acids tested (Fig. 10; Data only shown for *AtLHT6*).

Alternatively, heterologous complementation experiments with yeast mutants expressing *AtLHTs* were performed. *AtLHT5, 6, 6IE* (introns and exons) and *7* in pDR195 and pDR195 only were expressed in different yeast mutant strains and tested on the following minimal media with specific amino acids as sole nitrogen source: 22574d (1, 3 and 6 mM Pro), 22Δ8AA (0.5, 1, 3 and 6 mM Asp, Glu, Arg, and citrulline), 22Δ6AAL (0.015, 0.05, 0.15, 1, 3 and 8.3 mM Lys) and JT16 (0.01, 0.05, 0.15 1, 2.5 and 5 mM His). None of the *AtLHTs* were able to mediate growth of yeast cells on the specific media.

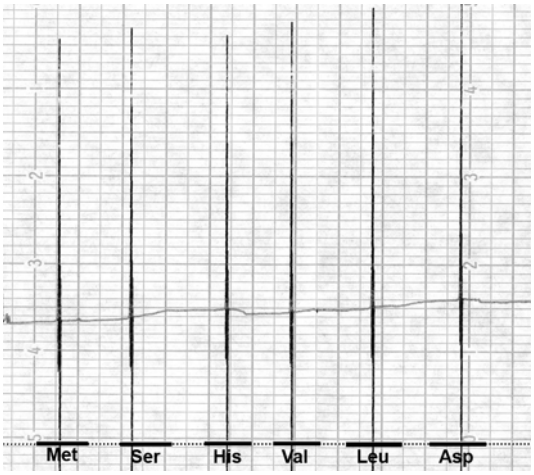
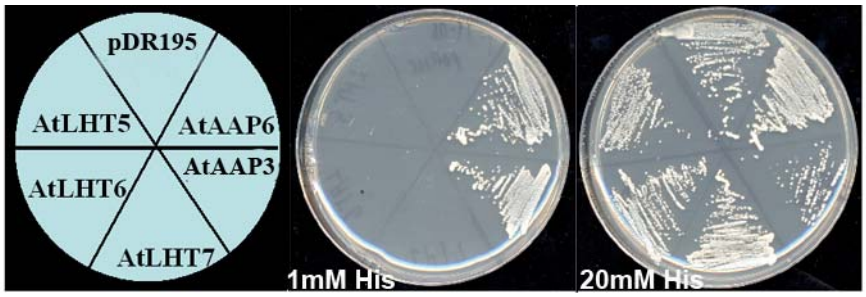
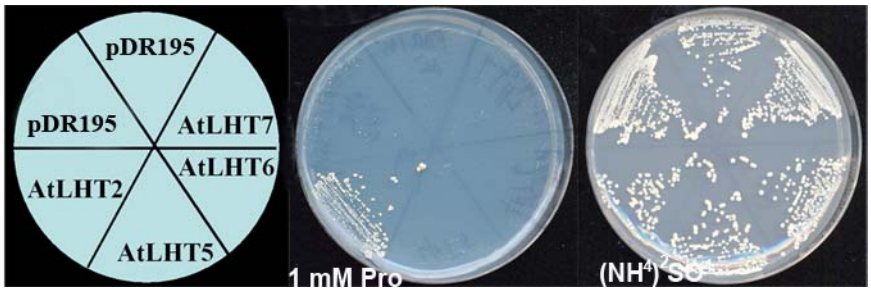



Figure 10. Electrophysiological measurements of oocytes expressing *AtLHT6*. The oocytes were bathed the buffer solution (·····) until the membrane was stabilized followed by the addition of different amino acids (——). All 20 protein amino acids and GABA were tested, but only Met, Ser, His, Val, Leu and Asp are shown here.

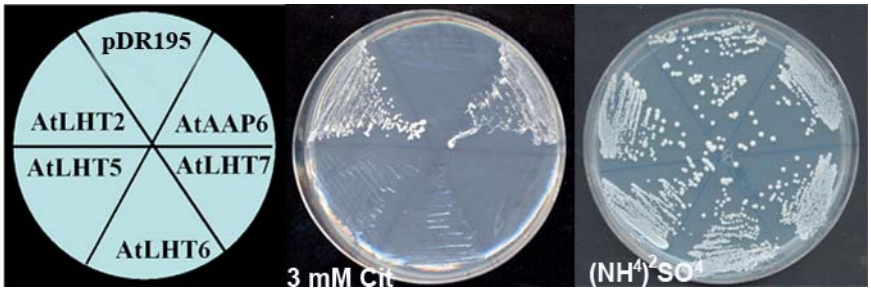
JT16




22574d




22Δ8aa




22Δ6aaL

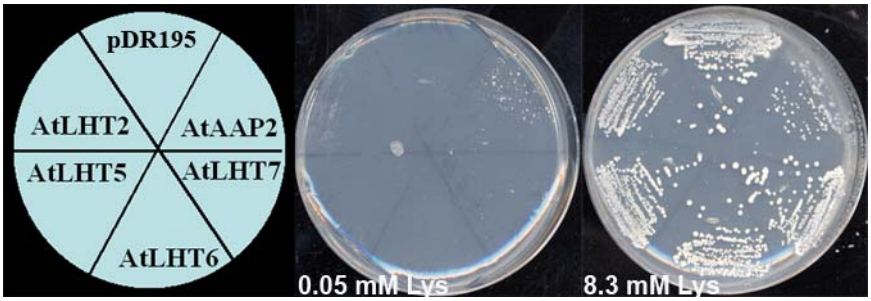



**Figure 11**. Complementation of the yeast strain JT16, 22574d, 22574d, 22Δ8aa or 22Δ6aal with *AtLHT5*, *AtLHT6*, *AtLHT6*IE and *AtLHT7* in the pDR195 vector. *AtLHT2*, *AtAAP2*, *AtAAP3* or *AtAAP6* in the pDR195 vector and the pDR195 vector were used as controls. The left images describe what plasmid was used for transformation of the yeast in each partition. The middle images show the yeast grown on selective media. The right images are yeast grown on media for a positive control.

### 5. 4. 6. Resolving the function of AtLHTs *in planta*

For analysis of LHT transporter function in plants, *LHT antisense* lines were produced and homozygous T-DNA insertion lines isolated. T-DNA insertion lines for *AtLHT1* (SALK-034566.55 and SALK-036871.47), *AtLHT2* (SAIL_222_C12 and SAIL_1143_B02), *AtLHT4* (SALK_005293.36), *AtLHT6* (SALK049092.50 and SALK_004201.48) *AtLHT7* (SALK_043013.37, SALK_027033.52 and SALK_105583.49) and *AtLHT8* (SALK_059358.40 and SALK_059360.56) were obtained and screened for homozygosity using the primers listed in the materials and methods. *AtLHT1* T-DNA lines grown in the

greenhouse displayed reduced growth compared to wild type, however none of the other homozygous lines showed obvious growth differences. *lht1* mutants were generally smaller than wild type and had reduced number and size of rosette leaves. However, we did not include this mutant in our studies since it was already being analyzed by two other research groups (Hirner et al. 2006; Svennerstam et al. 2007). None of the other mutants were studied in detail so far, with the exception *AtLHT6* (SALK049092.50) that was described in chapter 3.

Different amino acid transporter families with multiple members exist, and a knock-out in a single transporter might not lead to phenotypic changes due to compensation by other transporters of the same or other families. Therefore, double or triple mutants are often produced by crossing mutant lines of related transporters. Here crosses between *lht* mutant lines, as well as *lht* and *aap* mutant lines were performed based on the expression pattern of the specific transporters (Table 2). For example, *AtLHT6* and *AtAAP1* were crossed, because both are expressed in root epidermal cells. While the crosses were performed, they still need to be confirmed and the double mutants need to be screened for homozygosity as described in Table 3.

Since only one T-DNA insertion line was available for each *AtLHT4*, *AtLHT5* and *AtLHT6, antisense* lines were produced for these transporters (Table 4). Two *antisense* constructs were made for transporter using cDNA sequences from the least conserved regions of the specific *AtLHTs* or a region with the highest similarity to its closest related gene, which might lead to repression of

both transporters. *AtLHT4* and *AtLHT5 antisense* lines for both constructs were screened and homozygous seeds were collected.

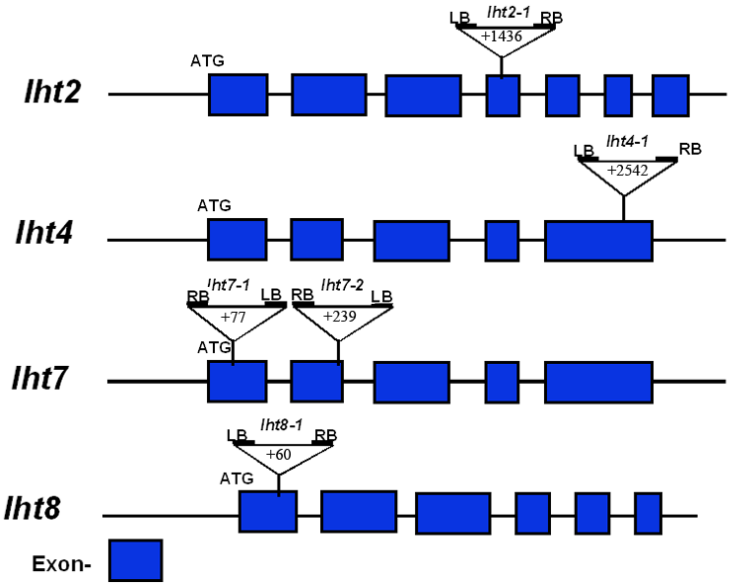



**Figure 12**. Schematic diagram of homozygous T-DNA insertions in *AtLHT* genes. The blue boxes represent exons, whereas the lines represent introns and UTRs. The position (bp) of the T-DNA insertion within the genomic sequence downstream of the start codon is marked. LB and RB refer to the left border and right border of the T-DNA insertion.

**Table 2**. An overview of the transporters, their expression pattern and the names of the homozygous T-DNA insertion lines available for each transporter. The position of the T-DNA insertion was verified for some of the mutants.

| Gene | RNA expression | GUS expression | Mutant Line | Checked for insertion postion |
|---|---|---|---|---|
| *LHT2* | flower, weak in leaves | tapetum (Lee and Tegeder 2004), mature pollen (Foster et al. 2008), trichomes | SAIL_222_C12 | Yes |
| *LHT4* | all organs | Throughout young anther, tapetum of older anther (Foster et al. 2008) | SALK_00529 | No |
| *LHT6* | root<br>rosette leaf<br>bud<br>flower<br>seedling | root epidermal, root hair, cortex and vasculature cells (chapter 3), random in rosette leaves, some trichomes (chapter 5), throughout young buds anther and pistil (Foster et al. 2008), in transmitting tissue of older pistils, tapetum of older anthers | SALK_049092 | Yes |
| *LHT7* | root<br>stem<br>rosette leaf<br>cauline leaf<br>bud<br>flower | major veins, some minor veins of leaves (Chapter 5), mature pollen | SALK_043013<br>SALK_027033 | No<br>No |
| *LHT8* | bud<br>flower | no staining | SALK_059360 | Yes |
| *AAP1* | flower and silique (Fischer et al. 1995) older siliques (Hirner et al. 1998) | seeds (Hirner et al. 1998), root tips, epidermal cells (Lee et al. 2007) | 415.9(*aap1-1*)<br>855-1(*aap1-2*)<br>M22(*aap1-3*) | Yes<br>Yes<br>Yes |
| *AAP2* | strongest in silique and stem, weak in flower, root, source leaf (Fischer et al. 1995) | vasculature of roots stem and siliques (Hirner et al. 1998) | C25<br>E27<br>F4 | Yes<br>Yes<br>Yes |
| *AAP8* | not available | young seeds (Okumoto et al. 2002), vasculature sepals and older siliques | G22<br>H3<br>I1 | Yes<br>Yes<br>Yes |

**Table 3**. T-DNA insertion line crosses and the method for screening each cross to homozygosity. Y = kanamycin resistant, Y? = partially kanamycin resistant, n = lost kanamycin resistantce, ♀ plant that was pollinated, ♂ plant that was used to pollinate

| **Crosses** | **Screening of crosses** |
|---|---|
| *AtLHT4* (Salk_00529) ♀ (Y?) X *AtLHT2* (Sail_222_c12) ♂ (Basta) | Basta |
| *AtLHT4* (Salk_00529) ♀ (Y?) X *AtLHT8* (Salk-059360) ♂ (Y) | PCR |
| *AtLHT6* (Salk_049) ♀ (n) X *AtLHT2* (Sail_222_c12) ♂ (Basta) | Basta |
| *AtLHT6* (Salk_049) ♀ (n) X *AtLHT4* (Salk_00529) ♂ (Y?) | Kan |
| *AtLHT6* (Salk_049) ♀ (n) X *AtLHT7* (Salk_027) ♂ (Y) | Kan |
| *AtLHT6* (Salk_049) ♀ (n) X *AtLHT7* (Salk_105) ♂ (Y) | Basta |
| *AtLHT6* (Salk_049) ♀ (n) X *AtLHT8* (Salk-059360) ♂ (Y) | Kan |
| *AtLHT7* (Salk_027) ♀ (Y) X *AtLHT2* Sail_222_c12) ♂ (Basta) | Basta |
| *AtLHT7* (Salk_027) ♀ (Y) X *AtLHT4* (Salk_00529) ♂ (Y) | Kan/PCR |
| *AtLHT7* (Salk_027) ♀ (Y) X *AtLHT8* (Salk-059360) ♂ (Y) | Kan/PCR |
| *AtLHT8* (Salk_059360) ♀ (Y) X *AtLHT2* Sail_222_c12) ♂(Basta) | Basta |
| *AtAAP1* (M22) ♀ X *AtAAP2* (E27) ♂ | Kan |
| *AtAAP1* (M22) ♀ X *AtLHT7* (Salk_027) ♂ | Kan |
| *AtAAP1* (M22) ♀ X *AtLHT7* (Salk_105) ♂ | Kan |
| *AtAAP2* (V6) ♀ X *AtAAP1* (415.90) ♂ | Phe |
| *AtAAP2* (E27) ♀ X *AtAAP1* (M22) ♂ | Phe |
| *AtAAP2* (C25) ♀ X *AtAAP1* (M22) ♂ | Phe |
| *AtLHT6* (Salk_049) ♀ X *AtAAP1* (M22) ♂ ** | PCR/Phe |
| *AtAAP8* (G22) ♀ X *AtAAP1* (M22) ♂ | Phe |
| *AtAAP8* (G22) ♀ X *AtAAP* 2 (E27) ♂ | Kan |

*At this time all seed available from the different crosses are heterozygous with the exception of **

**Table 4**. Overview of *AtLHT antisense* lines. Primer # indicates the lab stock number used for isolation of the *antisense* sequence.

| Gene | Primer Sequence | Primer # | (bp) | Gene repressed | Homozygous/ Heterozygous seeds | # of lines |
|---|---|---|---|---|---|---|
| *AtLHT4* | ATggACgAAAgACCCgAgACAg ggTTAggAgTATggCTTgCATTg | 250+ 450 | 1000 | *AtLHT4* | Homozygous | 12 |
| | TTAgTTAggCggCTTgAAgAAC ggTTAggAgTATggCTTgCATTg | 251+ 449 | 850 | *AtLHT4* | Homozygous | 15 |
| *AtLHT5* | ggTgCCACAACAACTAATCg and CATTTCCTgAggTTgTAgATgC | 747+ 748 | 300 | *AtLHT5* | Homozygous | 11 |
| | gAAACCCATCTggCTTATCg CTTTggTTTCTTgATACAAAgCC | 749+ 750 | 320 | *AtLHT5*+ *AtLHT2* | Homozygous | 7 |
| *AtLHT6* | CATCggATggTAAACCgTAg gCAACggTTCgATAggTACC | 663+ 666 | 380 | *AtLHT6*+ *AtLHT3* | Heterozygous | 5 |
| | CTgATCTCgACAAgTAgTTgTAgg ATggCgggAATCCCAgATCATATCC | 254+ 614 | 390 | *AtLHT6* | Heterozygous T3 seeds | 11 |

## 5. 5. Discussion

In previous work expression of *AtLHT2, AtLHT4, AtLHT5* and *AtLHT6* was localized to reproductive tissues (Foster et al. 2008; see chapter 4), as well as root tissue for *AtLHT6* (see chapter 3). *AtLHT* expression patterns were cell or tissue specific, developmentally regulated and tightly coordinated. Here, analysis of *AtLHT* expression in other organs was examined using RT-PCR and promoter-*GUS* studies. Expression of *AtLHTs* in organs revealed individual expression patterns indicating multiple functions within *A. thaliana*. A majority of the *AtLHTs* are expressed in roots and may play a role in amino acid uptake as was found for *AtLHT6* (see chapter 3). All *AtLHTs*, with exception of *AtLHT3,* were expressed

in buds and flowers, which is in line with their tissue and cellular localization (Foster et al. 2008; see chapter 4) and available micro array data (Toufighi et al. 2005; Bock et al. 2006). Some *AtLHTs* were expressed throughout the plant and may play a general role in amino acid distribution.

Expression of *AtLHT2* (Fig. 6) and *AtLHT6* (Fig. 7) was localized to trichomes where they might function in import of amino acids for glutathione synthesis (Gutiérrez-Alcalà et al. 2000; Foyer et al. 2001), enabling detoxification of heavy metals (Gotor et al. 1997; Saito 2000). In addition, *AtLHT6* was expressed randomly in leaves (Fig. 7), which indicates a role in the uptake of amino acids from the apoplast similar to *AtLHT1* (Hirner et al. 2006). *AtLHT4* and *AtLHT7* were found in the vasculature of roots (Fig. 4) and leaves (Fig. 8), respectively. Their cell-specific localization has not been resolved yet, but needs to be addressed in future studies. Other amino acid transporters have also been localized to the plant vascular system, such as *AtAAP3* (Okumoto et al. 2004) that was expressed in root phloem, and is probably involved in phloem loading and *AtAAP6* which was localized to the xylem parenchyma and might take up amino acids from the xylem apoplast (Okumoto et al. 2002). In addition, *AtLHT5* was expressed in guard cells (Fig. 5), which are symplasmically isolated from other cells (Palevitz and Hepler 1985), and require import of amino acids across the cell membrane. This transporter might import amino acids that may be needed for synthesis of large amounts of proline functioning as an osmoprotectant (Hua et al. 1997)

Previous studies demonstrated that AtLHT2, 4, 5 and 6 are localized to the plasma membrane (Foster et al. 2008; see chapter 4). Here, AtLHT7 was also shown to be plasma membrane transporter, most likely involved in import of amino acids into the cell. To determine the substrate selectivity of the AtLHTs, the transporters were analyzed using heterologous expression systems. Characterization of amino acid transporters using yeast transport mutants and Xenopus oocytes have been successful for AtLHT1, AtLHT2 and AtAAPs and they were shown to transport a wide spectrum of amino acids with varying affinities (Hsu et al. 1993; Kwart et al. 1993; Frommer et al. 1993; Fischer et al. 1995; Boorer et al. 1996; Boorer and Fischer 1997; Chen and Bush 1997; Hirner et al. 1998; Fischer et al. 2002; Okumoto et al. 2002; Lee and Tegeder 2004; Okumoto et al. 2004; Hirner et al. 2006). Unfortunately, the AtLHTs (AtLHT4, 5, 6 and 7) tested in this study were unable to complement yeast cell growth and failed to transport amino acids into yeast cells and Xenopus oocytes. While this could be due to improper folding or targeting of the transporter within the heterologous systems, it may also be that the AtLHT transporters associate with other proteins and can only function as a protein complex, as seen for the HAT amino acid transporters in mammals (Wipf et al. 2002; Verrey et al. 2004). Nevertheless, although AtLHT6 did not function in heterologous systems its role in amino acid transport could be demonstrated by analyzing mutant plants (see chapter 3).

Analyzing amino acid transporter function using gene repression has been successful in some studies (Koch et al. 2003; Hirner et al. 2006; Lee et al. 2007;

Schmidt et al. 2007; Svennerstam et al. 2007; see chapters 2 and 3) and needs to be performed in future studies to understand AtLHT function *in planta*. In this work *AtLHT* T-DNA insertion lines were screened to homozygosity and *AtLHT4*, *AtLHT5* and *AtLHT6 antisense* lines were produced. Since repression of single transporter genes do not always result in phenotypic changes, amino acid transporter mutant lines (*aap1, aap2, aap8, lht2, lht4, lht6, lht7* and *lht8*) were crossed and are available for phenotypic analysis. For example, mutants with a T-DNA insertion in *AtLHT6* (see chapter 2) and *AtAAP1* (see chapter 1), which are expressed in root epidermal cells were crossed and can be tested for effects on amino acid acquisition from the soil.

While valuable genetic material has been produced in this study (homozygous T-DNA insertion lines and antisense lines) future studies will need to focus on a comprehensive analysis of the mutant plants. For example *AtLHT5* is expressed in pollen tubes and the transmitting tissue and repression of the transporter may affect pollen tube growth. Furthermore, crosses of mutant lines could reveal severe phenotypic changes. For instance *AtLHT2* are *AtLHT4* are expressed in pollen, while *AtLHT6* is expressed in the transmitting tissue of the pistil and double mutants of *lht2*/*lht6* or *lht4*/*lht6* might be strongly affected, with respect to reproductive success. Another candidate that will be exciting to analyze in further studies is *AtLHT5* and its antisense lines. *AtLHT5* is expressed in guard cells and the membrane potential could be determined in guard cells of mutants and wild type plants using patch clamp technique (Schroeder et al. 1987) or fluorescent voltage sensitive dyes (Konrad and

Hedrich 2008). In addition, physiological studies could be performed with *AtLHT5* mutant lines, such as determining gas exchange and photosynthetic rates. In summary, valuable materials have been produced that can be used in the future for resolving amino acid transporter function in plants.

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