# IDENTIFICATION AND CHARACTERIZATION OF AMINO ACID EXPORTERS IN *Arabidopsis thaliana*

A Dissertation

by

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# ABSTRACT

Amino acids are the major form of nitrogen (N) transported within the plant body, and their transport between different plant tissues through the xylem and the phloem is indispensable for optimal growth. Such transport processes rely on critical import and export steps at the cellular membranes, mediated by transporters. Many amino acid importers and their roles in the plant body have previously been discovered, providing not only a better understanding of amino acid transport mechanisms but also tools to improve N use efficiency of crops. However, amino acid exporters are not well studied due to a lack of efficient techniques for their identification. The overall goal of this Ph.D. was to advance our understanding of amino acid export in plants by 1) developing methods in *Saccharomyces cerevisiae* that allow a faster identification of putative amino acid exporters and 2) investigating the roles of the identified amino acid exporters using genetic tools available in *Arabidopsis thaliana*.

The studies presented here revealed several novel amino acid exporter proteins, Usually Multiple Acids Move In and Out Transporters (UMAMIT) 14, UMAMIT24 and UMAMIT25, which mediated amino acid export when expressed in yeast, with little to no import activities. In plants, UMAMIT14 functions synergistically with previously characterized amino acid facilitator UMAMIT18 to mediate phloem unloading of shoot-derived amino acids. Knockout lines for these genes did not show any deleterious effects on yield; however the plants had reduced amino acid transfer from the shoot to the roots, as well as from roots to the growth medium. On the other hand, UMAMIT24 and

UMAMIT25 are expressed in various seed tissues during embryogenesis, and are responsible for amino acid transport to the seed. An increased expression of UMAMIT24 and 25 using their own promoters increased the seed yields, whereas ectopic expression of UMAMIT transporters caused a stress response linked with an amino acid imbalance. The results suggest that optimizing key amino acid export steps in plants could benefit crop production, whereas excessive amino acid export can cause deleterious effects on plant growth.

# DEDICATION

This dissertation is dedicated to my family, my friends, my American and French colleagues, and my committee.

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# NOMENCLATURE

| | |
|---|---|
| Ala | Alanine |
| Arg | Arginine |
| Asn | Asparagine |
| Asp | Aspartic acid |
| C | Carbon |
| Cit | Citrulline |
| $CO_2$ | Carbon dioxide |
| Cys | Cysteine |
| GABA | γ-aminobutyric acid |
| Glu | Glutamate |
| Gln | Glutamine |
| Gly | Glycine |
| His | Histidine |
| Ile | Isoleucine |
| Leu | Leucine |
| Lys | Lysine |
| Met | Methionine |
| N | Nitrogen |
| $NH_4^+$ | Ammonium |
| $NO_2^-$ | Nitrite |

| | |
|---|---|
| $NO_3^-$ | Nitrate |
| Orn | Ornithine |
| Phe | Phenylalanine |
| Pro | Proline |
| Ser | Serine |
| Thr | Threonine |
| Trp | Tryptophan |
| Tyr | Tyrosine |
| Val | Valine |

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# CHAPTER I

# INTRODUCTION AND LITERATURE REVIEW

## 1.1. The central role of amino acids in the nitrogen cycle

### *1.1.1. Nitrogen acquisition*

Plants are sessile organisms, and need to constantly adapt to environmental constraints to develop and reproduce. Although plants are able to produce organic carbon from $CO_2$ through photosynthesis, all other mineral nutrients need to be acquired from the soil through the roots. Nitrogen (N), which is required for producing many important macromolecules such as proteins and nucleic acids, is almost always limiting in the soil, mostly due to a slow mineralization by the soil microbial community (Vitousek and Howarth, 1991; Menge *et al.*, 2012). Therefore, plants have developed sophisticated strategies in order to acquire sufficient N from the soil for optimal growth.

Soil N is found in various forms, including inorganic $NO_3^-$ and $NH_4^+$, as well as organic forms, such as amino acids, nucleic acids, peptides and other complex forms of N. Of these different forms, $NO_3^-$ and $NH_4^+$ are considered the primary sources of N for land plant species (Marschner, 2011). The uptake of $NO_3^-$ and $NH_4^+$ into plant roots is largely mediated by members of the nitrate transporter (NRT) family and ammonium transporter (AMT) family, respectively (Doddema and Telkamp, 1979; Filleur and Daniel-Vedele, 1999; Rawat *et al.*, 1999; Wang *et al.*, 2012; Von Wittgenstein *et al.*, 2014). Both families of transporters contain multiple high- and low-affinity transport systems, and their activities are regulated at the transcriptional and post-transcriptional

levels (reviewed in Jacquot *et al.*, 2017). In particular, N status of plants has a profound impact on the activities of NRT and AMT transporters. Indeed, both families are subjected to induction under low-N condition and/or re-supply of the substrate (Loqué and von Wirén, 2004; Medici and Krouk, 2014), and feedback repression from N containing molecules such as amino acids (reviewed in Miller *et al.*, 2007).

In addition to inorganic N, some organic forms such as amino acids can be found in higher concentrations in the soil under specific conditions (e.g. boreal forest). Accordingly, some trees and herbaceous species are able to take up amino acids such as Gly and Glu from the rhizosphere (Nasholm *et al.*, 1998; Persson *et al.*, 2003; Metcalfe *et al.*, 2011).

### *1.1.2. Nitrogen assimilation*

Once taken up by the roots, both $NO_3^-$ and $NH_4^+$ are assimilated within the plant body. The location within the plant of N assimilation depends on the plant species and growth conditions. In *Arabidopsis thaliana* (Arabidopsis), $NO_3^-$ absorbed by the roots is mostly transferred to the leaf and reduced into $NO_2^-$ in the cytosol by nitrate reductase. $NO_2^-$ is further reduced in the chloroplast by nitrite reductase into $NH_4^+$, then assimilated in the chloroplast by the Glutamine Synthase / Glutamine Oxoglutarate Aminotransferase (GS/GOGAT) cycle into the amino acids Gln and Glu (Masclaux-Daubresse *et al.*, 2010). Alternatively, $NH_4^+$ ions absorbed into the roots, or derived from symbiosis in legumes, are thought to be assimilated locally by the GS/GOGAT cycle into Gln and Glu, as high concentrations of $NH_4^+$ causes cytosolic pH disturbance and depolarizes the membrane (Kretovich, 1965; Barker *et al.*, 1966; Lorenz, 1976;

Kronzucker *et al.*, 2001; Masalkar *et al.*, 2010). Beginning with Gln and Glu, sequential transamination reactions result in the generation of other amino acids which are highly compartmentalized within the cell (Figure 1, Miflin, 2014). For example, chloroplasts are responsible for the biosynthesis of many amino acids such as branched chain and aromatic amino acids, whereas Pro and Arg metabolism occurs in the mitochondria (Figure 1). Similar to the genes involved in N acquisition and assimilation, amino acid metabolism and transport are tightly regulated (Pratelli and Pilot, 2014).

### *1.1.3. The role of amino acids in plants at the cellular level*

Aside from their apparent roles as the building blocks for protein synthesis, free amino acids play a number of critical roles within plant cells. As such, the level of amino acids, which is determined by the corrective activities of metabolism and transport, has a profound effect well beyond cellular protein synthesis.

#### **1.1.3.1. Substrate for metabolites synthesis**

Gln, Glu and Gly are the substrates for the biosynthesis of purines and pyrimidins and their derivatives such as flavins (Stasolla *et al.*, 2003). Amino acids are also the precursors of various secondary metabolites; phenylpropoanoids and lignin synthesis from Phe, glutathione from Glu, Cys and Gly, polyamines from various amino acids, especially Orn. In addition, they are the precursors of vitamins such as folate from Glu, and biotin from Ala and Met (Alscher, 1989; Slocum and Flores, 1991; Hanson and Gregory Iii, 2002; Vanholme *et al.*, 2010; Alban, 2011; Fraser and Chapple, 2011).

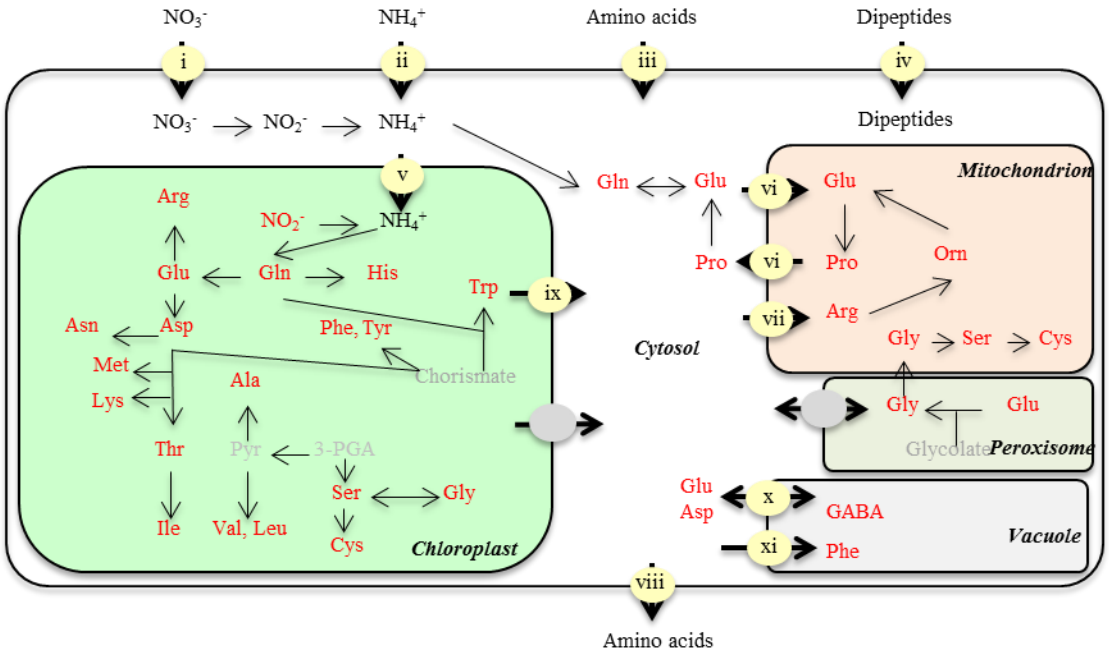


**Figure 1: Nitrogen assimilation and amino acid biosynthesis in plant cells.** The major biosynthetic pathways and their subcellular localizations are shown. Amino acids are shown in red. Grey molecules are not part of the N cycle. Known transporters are also shown in yellow circles. I) NRT/NPFs, ii) AMTs, iii) AAPs, LHTs, CATs, ProTs, GABATs, UMAMITs, iv) POT/NPFs, v) NITRs, vi) Glutamate/Proline exchangers, vii) BACs, viii) UMAMITs, ix) PhpCAT, x) SlCAT, xi) PhCAT ( Chapter I section 2). Yet unknown transporters are indicated in gray circles. Pyr, pyruvate; 3-PGA, 3-phosphoglycerate. Figure modified and reprinted with permission from Okumoto and Versaw (2017).

Additionally, Trp, Phe and Met are precursors for the synthesis of the hormones Indole-3-Acetic (IAA or auxin), salicylic acid (SA) and ethylene, respectively (Wang *et al.*, 2002; Zhao, 2012; Khan *et al.*, 2015). For hormones such as auxin, jasmonic acid and ethylene, conjugations with various amino acids represent an important mechanism for hormone activation/deactivation (Sembdner *et al.*, 1994; LeClere *et al.*, 2002; Fonseca *et al.*, 2009; Wasternack and Kombrink, 2009). Finally, some of the species-

specific secondary metabolites, such as glucosinolates found in the Brassicales order are also synthesized from various amino acids (Ravanel *et al.*, 1998).

#### 1.1.3.2. Stress tolerance

Several amino acids are involved in the response against a variety of stresses. Singh *et al.*, (1972) were the first to report that drought tolerance of various barley cultivars was correlated with the Pro accumulation by the plants during drought imposition. Many studies later showed that Pro accumulates during many other stresses, such as heavy metal, osmotic, heat and biotic stresses (Saradhi, 1991; Balibrea *et al.*, 1997; Rai, 2002; Cecchini *et al.*, 2011). Although some exceptions have been reported, Pro accumulation positively correlates with drought tolerance in many species (reviewed in Kishor *et al.*, 2014). In addition to Pro, accumulation of non-proteinogenic amino acids such as GABA, Cit, and an amino acid derivative glycine betaine is also correlated with osmotic stress tolerance (Kawasaki *et al.*, 2000; Bouché and Fromm, 2004; Chen and Murata, 2008; Daşgan *et al.*, 2009; Kusvuran *et al.*, 2013). The exact mechanism through which these molecules function in a wide range of stress tolerance is not well understood. Interestingly, Pro, Cit, GABA and glycine betaine share two properties: i) they all dissolve in water at a high concentration (high millimolar) and are innocuous to, or protective of protein functions and ii) they function as scavengers for reactive oxygen species (ROS) (Shelp *et al.*, 1999; Akashi *et al.*, 2001; Chen and Dickman, 2005; Liang *et al.*, 2013). Therefore, it has been suggested that they play dual roles as osmolyte and ROS scavengers under stress.

#### 1.1.3.3. Signaling

Amino acids are the first molecules in which inorganic N is incorporated into the reduced C generated from photosynthesis, and their levels generally reflect various factors that affect N and C availability within the cell (Lam *et al.*, 1998; Krapp *et al.*, 2011; Suzuki *et al.*, 2012;Fukushima and Kusano, 2014; Sato and Yanagisawa, 2014). Amino acids also induce massive transcriptional changes in the genes involved in N acquisition, metabolism, and remobilization when applied externally (Lam *et al.*, 1998; Oliveira and Coruzzi, 1999; Gutiérrez *et al.*, 2008), resulting in a long term developmental reprogramming (Walch-Liu *et al.*, 2005; Forde, 2013). Due to these observations, it has been proposed that amino acids function as metabolic signals in plants (Forde and Lea, 2007; Forde and Roberts, 2014; Häusler *et al.*, 2014). In addition, some specific amino acids function as signaling molecules under stress conditions. For instance, pipecolic acid, a non-proteinogenic circular amino acid produced by degradation of Lys, is synthesized as a result of pathogen infection, and promotes the development of SA-mediated defense response at the site of infection and at the whole plant level (Návarová *et al.*, 2012). Likewise, β-aminobutyric acid accumulates under a wide variety of stress conditions (Thevenet *et al.*, 2017) and induces resistance against necrotrophic pathogens (Ton and Mauch-Mani, 2004). Corroborating these observations, various mutations which induce amino acid imbalance cause strong stress phenotypes (Pilot *et al.*, 2004; Hirner *et al.*, 2006b; Yang *et al.*, 2014) through activation of SA pathway (Liu *et al.*, 2010), which will be further detailed in chapter IV.

## 1.2. Amino acid transport across the plasma membrane

### *1.2.1. Definition of amino acid import and export*

Amino acid transporters can be divided into importers, exporters and facilitators and many transporter families include members located at the plasma membrane, and / or organelle membranes. When a transporter is delivered to a membrane, the topology is expected to remain in such a way that the same side of the protein always faces the cytosol. Therefore, import will be defined as a catalyzed transport from the extracytosolic compartment (from the apoplasm or the inside of an organelle) to the cytosol, export as the opposite, and facilitation as a passive transport in or out across a membrane down the concentration gradient. Amino acid transporters listed below will be referred to accordingly as importers, exporters and facilitators.

### *1.2.2. Amino acid importers*

Earlier studies using radiolabeled amino acids and membrane vesicles from various plant tissues revealed multiple amino acid transport systems within the plant cell membrane (Bush and Langston-Unkefer, 1988; Li and Bush, 1990). The observed amino acid import activities were electrogenic and sensitive to protonophores, suggesting that amino acid importers are energized by the proton-gradient. Closely following these experiments, the first plant amino acid importer was isolated through its ability to complement the growth of *Saccharomyces cerevisiae* strains lacking endogenous amino acid transporters on media containing amino acid as the sole source of N (Hsu *et al.*, 1993; Frommer *et al.*, 1993). Such screens identified multiple families of transporters from Arabidopsis and other plants, and their primary sequences lead to the discovery of

homologs in many additional species. The majority of amino acid importers characterized so far are proton/ amino acid symporters as the earlier studies have predicted, with a few exceptions (Wipf *et al.*, 2002; Rentsch *et al.*, 2007; Yang *et al.*, 2010; Tegeder, 2012).

There are 10 superfamilies of amino acid importers in the animal, fungi, and plant kingdoms. The sodium-dicarboxylate symporter (SDS) superfamily, the neurotransmitter superfamily (NTS), the Cationic Amino acid Transporter (CAT) and Heteromeric Amino acid Transporter (HAT) superfamilies, the type 1 phopshate transporter family, the Vesicular Inhibitory Amino Acid Transporter Family (VIAAT), the Proton/ Amino Acid Transporter Family (PAT), the Sodium Coupled Neutral Amino Acid Transporter Family (SNAT), the amino acid-polyamine-choline (APC) transporter superfamily, the Amino Acid Transporter superfamily 1 (ATF1), and the major facilitator superfamily (MFS) (Müller *et al.*, 2006). Plant amino acid importers have been identified so far in the APC and ATF superfamilies (Rentsch *et al.*, 2007).

The ATF superfamily contains the largest number of plant amino acid transporters: amino acid permeases (AAPs), lysine histidine transporters (LHTs), proline transporters (ProTs), GABA transporters (GATs), and aromatic and neutral amino acid transporters (ANTs). Although the primary sequences of these transporters are related, there are substantial differences in the substrate specificities. AAPs and LHTs generally show broad specificities (Fischer *et al.*, 2002; Hirner *et al.*, 2006a), whereas ProTs, GATs and ANTs have narrower substrate ranges (Chen *et al.*, 2001; Grallath *et al.*, 2005; Meyer *et al.*, 2006). ATF family also contains auxin resistant

transporters (AUXs) which transports auxin, the plant hormone structurally related to Trp (Péret *et al.*, 2012).

APC is the second largest gene family containing characterized plant amino acid transporters. It includes members of the CAT, and the Bidirectional Amino Transporters (BAT) families. CATs have been described as high and moderate affinity importers for cationic and neutral amino acids, respectively (Frommer *et al.*, 1995; Su *et al.*, 2004a; Verrey *et al.*, 2004; Hammes *et al.*, 2006). Currently, there is only one member of the BAT family characterized as an amino acid transporter, but it was shown to be a facilitator (see below).

### *1.2.3. Amino acid exporters and facilitators*

Although amino acid export from source cells is clearly required for long-distance amino acid transport (see section 3), the properties of cellular export machineries have lagged behind for several reasons. Firstly, the amino acids that have been secreted from the plant cells can be re-imported through the amino acid importers, making the interpretation of data difficult. Secondly, labeling either the intracellular or extracellular amino acids at the beginning of the experiment allows the estimation of export and import activities, respectively, however the detection becomes challenging for the amino acids that are not secreted in large amounts or the ones metabolized intracellularly. Lastly, some tissues in which a high amino acid export activity is predicted (e.g. xylem parenchyma cells, see Section 3.4) is inaccessible for experiments that require isolation and feeding of amino acids. Therefore, physiological characterization of amino acid export has only been conducted for the tissues with (i)

easy access and (ii) relatively high amino acid export activity compared to the import activity. Such criteria are met when using seed coat tissue in legumes: by removing the embryo from growing seeds and filling the cavity with an appropriate buffer, amino acid export from the maternal tissue can be measured. Using this technique, it has been shown that seed coats are able to export amino acids such as Gln, Ala and Thr at a rate four to five times orders of magnitude greater than passive diffusion through the lipid bilayer, suggesting the presence of transporters localized at the plasma membrane of seed coat cells (De Jong *et al.*, 1997; Lanfermeijer *et al.*, 1992; Wolswinkel and De Ruiter, 1985). Further results using inhibitors that modify extracellular sulfhydryl groups, and protonophores suggest that this export was indeed facilitated by transporters, but not energized by the proton gradient across the membrane.

Although root cells are known to take up amino acids from the growth medium, net export of amino acids is observed under certain conditions. When using the isotopic labeling and pharmacological approach, it was concluded that the secretion of amino acid is selective, and not energized by the proton gradient (Lesuffleur *et al.*, 2007).

The molecular mechanisms underlying amino acid export remained unknown for almost 20 years after the first discovery of importers due to a lack of efficient techniques for their identification (Okumoto and Pilot, 2011). However, in recent years several families of transporters that can mediate amino acid export have been discovered.

### 2.3.1. Bidirectional amino acid transporters

Bidirectional Amino acid Transporter 1 (BAT1) has been characterized as an amino acid facilitator when expressed in yeast, possibly acting as an importer for Arg

and Ala, and an exporter for Lys and Glu (Dundar and Bush, 2009). In Arabidopsis, BAT1 has later been characterized as mitochondrial GABA permease, suggesting that BAT1 could be involved in amino acid transport across the mitochondrial membrane rather than in intercellular transport of amino acids (Michaeli *et al.,* 2011b).

### 2.3.2. Cationic amino acid transporters

In addition to their role in amino acid import, a few members of the CAT family have been reported to promote amino acid facilitation or export. For example, SlCAT9 was found to be expressed at the tonoplast of tomato fruits and to export Glu and Asp towards the vacuole lumen in exchange for GABA (Snowden *et al.*, 2015). In addition, two CAT members expressed in petunia flowers were shown to mediate amino acid export; PhpCAT exports aromatic amino acids towards the plastids (Widhalm *et al.*, 2015), and PhCAT2 exports Phe to the vacuole (Lynch *et al.,* 2017).

### 2.3.3. Usually multiple acid move in and out transporters

Siliques Are Red1 (SiAR1)/ Usually Multiple Acid Move In and Out (UMAMIT) 18 was the founding member of what is now known as the UMAMIT family. SiAR1/UMAMIT18 was originally discovered through its ability to mediate amino acid uptake into a yeast strain lacking endogenous amino acid transporters (22Δ8AA) (Fischer *et al.*, 2002; Ladwig *et al.*, 2012). However, when SiAR1/UMAMIT18 is expressed in wild type yeast, net amino acid uptake is reduced relative to the control, suggesting that SiAR1/UMAMIT18 mediates the leakage of amino acids imported by the yeast's endogenous amino acid importers (Ladwig *et al.,* 2012). SiAR1/UMAMIT18 is localized to the plasma membrane of Arabidopsis, making it the first amino acid

transporter that can mediate cellular export. Homology searches using the SiAR1/UMAMIT18 sequence as a query uncovered a large family of plant specific putative transporters found within different lineages (Monocots, Dicots, Conifers, Lycophytes and Bryophytes), suggesting that it is well conserved among land plants (Denancé *et al.*, 2014). Following the characterization of SiAR1 (renamed UMAMIT18), additional members of UMAMIT (UMAMIT11, UMAMIT14, UMAMIT28, and UMAMIT29) have been also shown to mediate cellular export of amino acids (Muller *et al.*, 2015). In addition, Walls Are Thin 1 (WAT1), was identified as a tonoplastic auxin transporter responsible for auxin export to the vacuole (Ranocha *et al.*, 2010). Finally, another UMAMIT member, Resistance to *Phytophtora parasitic*a 1(RTP1), was shown to mediate susceptibility to pathogens; however, biochemical function of RTP1 remains to be discovered (Pan *et al.*, 2016).

### 1.3. Amino acid transport between plant tissues

Cellular transport of amino acids mediated by the transporters described above is necessary for the proper distribution of amino acids (i) within plant tissues and (ii) in between organs through the long-distance transport systems xylem and phloem. In this section, we will describe the key transport steps leading to the local or long-distance transport of amino acids.

#### *1.3.1. Amino acid uptake and efflux in plant roots*

Amino acids can be taken up by plant roots when applied in the external medium. The uptake, observed by the accumulation of radiolabeled amino acids, was reported to be multi-phasic and dependent on the proton gradient (Soldal and Nissen, 1978; Bright

*et al.*, 1983). The collection of loss-of-function mutants in the model plant Arabidopsis enabled the testing of root amino acid uptake in the mutants of known amino acid transporters, and thus the identification of transporters that are involved in this process. For instance, the *aap1* mutant was found to be defective in importing neutral amino acid into the roots (Lee *et al.*, 2007). Other transporters have also been found to be expressed in roots and involved in amino acid uptake; AAP5 with a preference for basic amino acids (Svennerstam *et al.*, 2008), LHT1 in the uptake of Asp and Glu (Hirner *et al.*, 2006b), and CAT8 in the uptake of Gln, in an proton-independent manner (Yang *et al.*, 2010). In addition, ProT2 was found to be expressed in the root epidermis and cortex and to mediate amino acid import in yeast, suggesting its role in amino acid import in the roots (Rentsch *et al.*, 1996; Grallath *et al.*, 2005).

Despite these import activities, plant roots also secrete amino acids under certain conditions, which have been found to act as chemoattractants for microbes (Huang *et al.*, 2014a; Webb *et al.*, 2014). Amino acid secretion was initially predicted to occur through a passive process involving leakage from the root tips and the site of lateral root emergence. However, recent studies suggest that the secretion is a controlled process. For example, Chaparro *et al.*, demonstrated that amino acid secretion from Arabidopsis roots is developmentally controlled, and the temporal pattern is distinct from that of root secreted sugars (Chaparro *et al.*, 2013). Another study showed that the secretion of Trp increases when cucumber plants were treated with plant growth promoting rhizobacteria *Bacillus amyloliquefaciens*, which metabolizes Trp to IAA and promotes growth through the plant hormone (Liu *et al.*, 2017; Liu *et al.*, 2016b). Spatial patterns of secretion are

also distinct among different amino acids, as observed using amino acid sensing strains of bacteria (Jaeger *et al.*, 1999; Pini *et al.*, 2017). The molecular mechanisms for root amino acid secretion remain largely unknown. However, the work described in this Ph.D. lead to the discovery of UMAMIT14 and UMAMIT18, two amino acid exporters expressed in the root vascular tissue and indirectly responsible for secretion of shoot-derived amino acids (for more information, see chapter II).

### *1.3.2. Amino acids in the xylem*

Xylem transports water and nutrients unidirectionally by evapotranspiration through vertical files of dead cells composed of tracheids and vessel elements (Dickison, 2000). Studies using various sampling methods such as decapitation, root pressure chamber, and xylem tapping insects revealed that levels of N-containing molecules in the xylem vary within the plant species and the growth environment. For example in clover, sunflower and corn xylem, $NO_3^-$ accounts for over 50% of N content whereas amino acids do not exceed 20% (Pate, 1973). Other species such as barley and wheat contain a greater amount of amino acids in the xylem (30% and 80%, respectively) (Smirnoff and Stewart, 1985; Simpson *et al.*, 1982). In legumes under symbiosis, amines (Asn, Gln) and ureids are the main form of N transported in the xylem (McClure *et al.*, 1980).

Amino acids, either derived from *de novo* synthesis within the root or phloem-derived (see below), need to be exported from the cells within the stele to reach the xylem sap (Takano *et al.*, 2002a). Therefore, xylem loading of amino acids requires minimally one membrane exporter. Some of the UMAMIT members are expressed in the

xylem parenchyma cells, but whether they are involved in xylem amino acid loading remains unknown.

Once in the shoots, xylem-derived amino acids can be transported apoplasmically until they reach xylem parenchyma or mesophyll cells and are absorbed through amino acid importers (Alberts *et al.*, 2013). Studies using $C^{14}$-labelled amino acid fed directly to the transpiration system (xylem) of nodulating legumes revealed that the speed of xylem unloading differs between each amino acid, suggesting that the uptake process is selective to certain amino acids (McNeil *et al.*, 1979). Later studies showed that AtLHT1, which is expressed in the leaf mesophyll cells, is likely to be involved in this process. Indeed, the leaf mesophyll cells isolated from *lht1* mutants showed lower amino acid uptake activity and the leaf apoplasmic amino acid concentration was increased, likely due to the lack of amino acid import by the mesophyll cells (Hirner *et al.*, 2006b).

### *1.3.3. Amino acids in the phloem*

Contrarily to xylem, phloem is composed of living cells, which consists of sieve-elements and companion cells that form a symplasmically connected unit. The main function of phloem is to carry photosynthates and other nutrients from photosynthetically active leaves to the various sink tissues in the plant body, following the pressure gradient between source and sink (Turgeon and Wolf, 2009). Phloem sap content, sampled either by stylectomy, EDTA-facilitated exudation, or bleeding plants (especially for trees and *Ricinus communis*) revealed that molecules transported in the phloem include carbohydrates, amino acids, auxin, secondary metabolites such as glucosinolates, proteins, and RNA (Turgeon and Wolf, 2009). In non-legume plants,

amino acids are the main form of N in the phloem sap, mostly found as Glu, Gln, Asn, and Asp, a large fraction of which are synthesized and loaded within the source leaves. (Hayashi and Chino, 1986; Riens *et al.*, 1991; Winter *et al.*, 1992b; Fischer *et al.*, 1998).

Phloem loading in the leaf can be either symplasmic or apoplasmic. The apoplasmic model consists of the unloading of nutrients from the mesophyll cells, followed by their loading into the sieve elements and / or companion cells, whereas in symplasmic loading, nutrients move freely from the mesophyll cells to the phloem through plasmodesmata. So far, physiological and genetic experiments seem to suggest that apoplasmic loading of phloem is more prominent in the source leaves. Firstly, experiments using membrane-impermeant dyes or green fluorescent proteins specifically expressed in the phloem showed that the minor veins are symplasmically isolated from the mesophyll cells in mature leaves (Roberts *et al.*, 1997; Imlau *et al.*, 1999b). Secondly, experiments using protonophores on isolated sieve elements showed that phloem loading of sucrose is actively mediated by proton/sucrose transporters (Giaquinta, 1983). Finally, mutants of sugar transporters that either mediate the export from the phloem parenchyma cells or import into the phloem are defective in sugar transport to the sink tissues, which provides strong evidence for the apoplasmic mode of transport (Kühn *et al.*, 1996; Kühn *et al.*, 1997; Chen *et al.*, 2012). Therefore, it is likely that amino acid loading into the phloem follows the apoplasmic model; this would require an amino acid exporter in the mesophyll or phloem parenchyma cells, followed by import into the phloem via an amino acid importer.

The transporters responsible for amino acid export in the leaf are not well understood. UMAMIT18 and other UMAMITs expressed in the source leaf vascular tissue could be involved in this process. The exported amino acids can then be taken up by an amino acid importer expressed in the phloem. A recent study showed that AAP8 likely plays an important role in amino acid loading into the source leaf phloem (Santiago and Tegeder, 2016). Other amino acid transporters expressed in the leaf phloem (AAP2, AAP4, AAP5, ProT1, CAT6 and CAT9) could also mediate this process. (Fischer *et al.*, 1995; Rentsch *et al.*, 1996; Okumoto *et al.*, 2004b; Su *et al.*, 2004a; Hammes *et al.*, 2006; Brady *et al.*, 2007; Zhang *et al.*, 2010; Elashry *et al.*, 2013)

The mode of phloem unloading depends on sink type and developmental stage. For example, phloem unloading in meristematic vegetative tissues (root tip, shoot apices, tubers) is considered symplasmic, as there is an extensive connection between the sieve elements and the surrounding cells through plasmodesmata within these tissues (Warmbrodt, 1985; Oparka *et al.*, 1992; Bret-Harte and Silk, 1994; Schulz, 1995; Imlau *et al.*, 1999b). However, in the mature vegetative tissues such as developed roots, the unloading is apoplasmic, and requires one export step from the sieve elements / companion cells and one import step into the subsequent tissue (pericycle and / or endodermis) (Patrick and Offler, 1996). Therefore amino acid unloading into these tissues is likely to be mediated by amino acid exporters. (Okumoto and Pilot, 2011). This Ph.D. led to the discovery of UMAMIT14 and UMAMIT18, two amino acid exporters expressed in mature root phloem and pericycle, involved in root phloem unloading of amino acids (see Chapter II).

### *1.3.4. Xylem to phloem transport*

Amino acid delivery to developing sink tissues depends both on xylem and phloem, and their contribution depends upon the tissue type, species, and environmental conditions. In any given plant tissue, the amount of evapotranspiration primarily determines the amounts of solutes arriving from the xylem. Xylem and phloem flux rates are also altered by environmental conditions. For instance, a study using magnetic resonance imaging of four different plant species (poplar, caster bean, tomato and tobacco) revealed that phloem flow follows diurnal changes in two species, and xylem flow clearly changes diurnally for all species, presumably due to the lack of evapotranspiration at night (Windt *et al.*, 2006). Such changes in flux would imply that the concentrations of amino acids within the long-distance transport systems need to be altered to supply a constant amount of amino acid to the sink tissues. One of the major mechanisms for such adjustment is the transfer between the xylem and phloem. Earlier studies using the cut shoots of legumes showed that when $NO_3^-$ is fed through the xylem, $NO_3^-$ traces were also found in the fruit tip phloem sap (Pate *et al.*, 1975). Similarly, xylem-fed $^{14}C$ labelled amino acids (Asn, Gln, Asp) and their metabolites later appeared in the phloem (Pate *et al.*, 1975).

Efficient xylem-to-phloem transfer of amino acids in the source leaf through a carrier is necessary to re-route amino acids towards developing sink tissues which are less active in evapotranspiration, such as developing fruits and vegetative apices (Atkins *et al.*, 1979). Genetic resources available in Arabidopsis lead to the successful identification of some transporters involved in this process. For example, studies using

*ataap2* knockouts revealed that the partitioning of root-borne $^{14}$C-Gln to the sink leaves and siliques was decreased compared to that of the wild type. On the other hand, the content of $^{14}$C-Gln in source leaves was increased, suggesting an alteration in the xylem-phloem transfer of amino acids in the leaf (Zhang *et al.*, 2010). Likewise, xylem-to-phloem amino acid transfer was found to be decreased in an *ataap6* mutant (Hunt *et al.*, 2009).

Not only xylem-to-phloem but phloem-to-xylem transfer of amino acids is predicted to occur as well, acting to concentrate amino acids in the xylem sap that feed the growing sink organs (Jeschke and Pate, 1991; Jeschke and Hartung, 2000). According to Atkins *et al.*, phloem-fed sinks at the top of the plant are believed to have a "N-nutrition problem" (Atkins, 2000); "upper strata of leaves that supply phloem have a significant N requirement of their own, as they are still growing. Consequently, they retain a large proportion of the xylem-N they receive through transpiration and transfer little to phloem". Consequently, as the xylem sap ascends in the plant, it would become impoverished in N if there were no active enrichment in the xylem content of N. However, studies analyzing xylem saps in younger (close to the shoot apex) and older (closer to the base) stems showed the opposite is true; amino acid concentration increases as it moves closer to the shoot apex (Layzell *et al.*, 1981). It is considered that amino acid transfer from phloem to xylem along the path from source to sink tissues is responsible for such an enrichment mechanism (Atkins, 2000). This step would require at least one exporter that moves amino acid from the phloem to the apoplasm within the

stele, which is considered continuous to the xylem. The molecular mechanisms for this function are still unknown.

### *1.3.5. Amino acid transport to the seed*

The growing embryo within the seed is symplasmically separated from the maternal tissue, suggesting amino acid loading into the seed follows the apoplasmic model in these tissues: there, therefore, a need for an export step from the seed coat followed by an import step to the embryo to ensure proper seed filling during the embryogenesis. Physiological studies indicated that the unloading process is mediated by membrane transporters that are bidirectional and not proton gradient dependent, however the molecular mechanisms remained unclear until very recently. Multiple members of UMAMIT family members have been implicated in the unloading of amino acids within the developing seeds. UMAMIT11 and UMAMIT14 are expressed in the chalazal seed coat, whereas UMAMIT28 and UMAMIT29 are expressed in the endosperm and inner integument of the seed, and the loss-of-function mutants show reduced seed production (Muller *et al.*, 2015). Likewise, UMAMIT18 is expressed in the chalazal seed coat, and the loss-of-function mutant accumulates less amino acids in siliques (Ladwig *et al.*, 2012). Biochemical properties of UMAMITs (i.e. non-proton gradient dependent and bidirectional) fit the prediction of physiological studies, therefore corrective activity of UMAMITs could explain the major part of amino acid export activities from the seed coat.

Amino acids released from the maternal and/or endosperm tissue are subsequently taken up by the embryo via amino acid importers. Physiological studies

using cotyledons and cotyledon-derived vesicles showed that the import is concentrative and proton-gradient dependent, suggesting that the import is mediated by proton/amino acid symporters (Robinson and Beevers, 1981; Lanfermeijer *et al.*, 1990; De Jong and Borstlap, 2000). Some members of the AAP family were later shown to be involved in this process. AAP1 and AAP8 are expressed in Arabidopsis seeds during embryogenesis, and are responsible for neutral and acidic amino acid import to the embryo, respectively (Sanders *et al.*, 2009; Lee *et al.*, 2007; Schmidt *et al.*, 2007; Santiago and Tegeder, 2016). In addition, genome wide association studies suggested a role for CAT4 in His loading to the embryo (Angelovici *et al.*, 2016). Finally, CAT6 is expressed in developing sink tissues, and can promote amino acid transport when expressed in oocytes (Hammes *et al.*, 2006).

### 1.4. Manipulating amino acid transporters for yield increase

Earth's rising population calls for a radical increase of crop yield with respect to our current lands available for agriculture. Continued breeding effort to improve the yields and nutritional values of crops will be critical to achieve this goal. Additionally, biotechnological modification of crops remains one of the most powerful approaches, despite public acceptance issues in some countries.

Recently, amino acid transport has been identified as an important target for improving Nitrogen Use Efficiency (NUE) and yields. Earlier observations in various cultivars of mustard and corn revealed a direct correlation between the amount of amino acids found in the phloem and the protein content in seeds (Lohaus *et al.*, 1998; Lohaus and Moellers, 2000). This suggested that the long-distance transport of amino acid is

crucial for yield establishment. In accordance with this hypothesis, a recent study identified OsAAP6, AAP family member from rice, as the causative mechanisms for the Quantitative Trait Loci (QTL) that controls NUE and grain protein content in rice. OsAAP6 is strongly expressed in the vascular tissues of roots and leaves as well as in the seed endosperm, and *OsAAP6* expression is positively correlated with grain protein content found in rice (Peng *et al.*, 2014a).

Loss-of-function mutants available in Arabidopsis provided further link between amino acid transport activities and agronomically important traits such as seed yield and content. Decreased activity of AtAAP8 in Arabidopsis results in a reduced phloem amino acid loading and reduction in the sink tissues development (Santiago and Tegeder, 2016; Santiago and Tegeder, 2017). In addition, the knockout lines of AtAAP2 in Arabidopsis also had a decreased amino acid content in the phloem sap, as well as less N and proteins in seeds (Zhang *et al.*, 2010). However, more seeds were produced by *aap2* plants and they were richer in fatty acids, suggesting that phloem amino acid content affects traits beyond N metabolism (Zhang *et al.*, 2010). Correctively, these studies suggested that manipulation of amino acid transport could be an effective way to modify the traits such as NUE and seed protein content.

Currently, increasing amino acid importer activities in key tissues has been proven to be a very efficient technique for yield improvement. For example, in a recent study an additional copy of AAP member from pea (*Pisum sativum* L.), *PsAAP1* was expressed in pea under the control of *AtAAP1* promoter. The protein localized in the sieve element-companion cell complexes of the leaf phloem and in the epidermis of the

seed cotyledons (Zhang *et al.*, 2015). In the transgenic lines, amino acid loading to the phloem and seed yield were strongly increased, adding to the increasing body of evidence for the importance of phloem amino acid content and the sink strength of seeds (Zhang *et al.*, 2015; Perchlik and Tegeder, 2017). The same group showed that an increased transport activities for S-adenosylmethionine and ureids, achieved by expressing respective transporters from another organism, enhances seed development and seed protein content (Tan *et al.*, 2010; Carter and Tegeder, 2016).

Few studies so far report the potential of using amino acid exporters to manipulate agronomically important traits. The discovery of amino acid exporters has lagged behind that of importers by almost 20 years; hence candidate genes for manipulating export activities are not abundant. Traditionally, amino acid import into long-distance transport systems and sink tissues have been considered the rate-limiting steps (Tegeder, 2012). Nevertheless, some recent studies have showed that a reduction in amino acid export activities to the key sink tissues such as developing seeds result in yield losses, suggesting that an increase in the export activity could present an alternative route for crop improvment (Ladwig *et al.*, 2012; Muller *et al.*, 2015).

### 1.5. Preliminary results and objectives of this Ph.D.

The discovery of the UMAMIT family revealed a large number of family members (44 in Arabidopsis; Denance *et al.*, 2014a), most of which were uncharacterized at the beginning of this Ph.D. work. Many steps in long distance amino acid transport that require exporter activities had no amino acid exporter associated with it, therefore the characterization of UMAMITs would likely lead to a better

understanding of amino acid transport within the plant body. Additionally, the discovery of more UMAMITs with amino acid transport activities might lead to additional pathways for developing desirable agronomic traits in the future. Therefore, the overall goals of this Ph.D. were to 1) discover additional UMAMIT members with amino acid export activities and 2) understand their functions in plants.

Prior to this Ph.D., a Gln secretion assay was performed in yeast expressing putative amino acid exporters from the UMAMIT family, as well as other transporters, and Gln secretion was measured using a FRET sensor for Gln and LC-MS-MS ( Gruenwald *et al.*, 2012). UMAMIT18– expressing yeasts showed the highest secretion rates compared to the other UMAMIT genes and the control. In addition, UMAMIT14, UMAMIT25 and UMAMIT46 showed a high secretion of Gln compared to the empty vector. While this assay indicated that the expression of putative amino acid exporters in yeast enhances Gln secretion, further characterization of the activities had not been conducted yet. Therefore, the first goal of this Ph.D. was to develop techniques to measure amino acid export in yeast. Presented in Chapter II, two independent methods (retention of labeled amino acids and an amino acid secretion analysis using UPLC) have been developed. We have used these methods to successfully characterize additional UMAMIT members as candidates for amino acid export.

The second goal of this Ph.D. was to understand the functions of UMAMITs that strongly function as amino acid exporters in yeast, (UMAMIT14, UMAMIT18, UMAMIT24 and UMAMIT25: see chapter II and III) *in planta*. Detailed characterization of amino acid transport using loss-of-function lines assigned these

UMAMITs to processes which have been previously predicted to involve cellular amino acid export (e.g. phloem unloading and amino acid transport to the seed). In addition, our results showed that the upregulation of UMAMITs might be a viable mechanism for increasing crop performances.

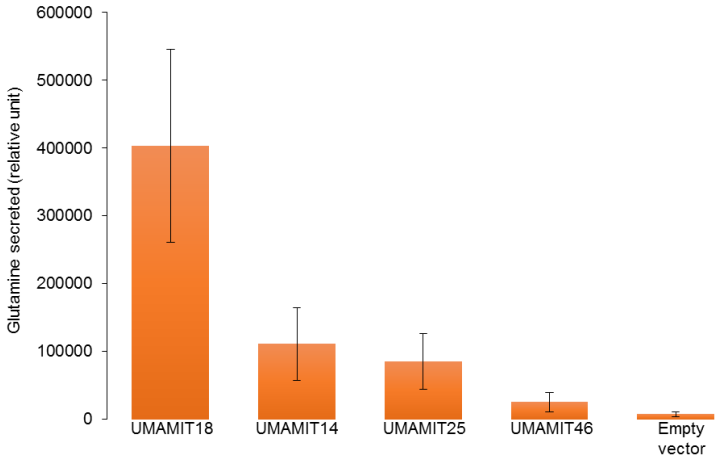


**Figure 2: Gln secretion by 22Δ8AA yeast strain expressing UMAMIT genes.** Relative unit represent the area for Gln peak detected by HPLC. Yeast 22Δ8AA cells Fischer *et al.*, 2002 expressing 33 out of 44 potentially functional UMAMIT genes were grown in a minimal medium containing $NH_4Cl$ as sole nitrogen source. After the cells reach OD600 ~0.6, the growth medium was harvested by centrifugation and added to the solution containing FRET Gln sensor protein extracted from *E.coli*.

Previous studies on either amino acid importers or the proteins that modulate amino acid export revealed that alteration in amino acid content and composition can have deleterious effects on plant performances, suggesting a link between amino acid homeostasis, sensing and stress responses (Chapter IV). Prior to this Ph.D., an examination of stress responses of plants in which the expression of amino acid exporter themselves have been induced were lacking. The third and final goal of this Ph.D. was to understand the link between amino acid export, sensing, and signaling mechanisms in

plants leading to stress responses, using plants over-expressing UMAMIT transporters (UMAMIT14, UMAMIT18, UMAMIT23, UMAMIT24, and UMAMIT25) in Arabidopsis.

# CHAPTER II

# UMAMIT 14 IS AN AMINO ACID EXPORTER INVOLVED IN PHLOEM UNLOADING IN ARABIDOPSIS ROOTS[*]

## 2.1. Abstract

Amino acids are the main form of nitrogen transported between the plant organs. Transport of amino acids across membranes is mediated by specialized proteins: importers, exporters, and facilitators. Unlike amino acid importers, amino acid exporters have not been thoroughly studied, partly due to a lack of high-throughput techniques enabling their isolation. Usually Multiple Acids Move In and out Transporters 14 (UMAMIT14) from Arabidopsis shares sequence similarity to the amino acid facilitator Silique Are Red1 (UMAMIT18), and has been shown to be involved in amino acid transfer to the seeds. We show here that *UMAMIT14* is also expressed in root pericycle and phloem cells and mediates export of a broad range of amino acids in yeast. Loss-of-function of *UMAMIT14* leads to a reduced shoot-to-root and root-to-medium transfer of amino acids originating from the leaves. These fluxes were further reduced in an *umamti14 umamit18* double loss-of-function mutant. This study suggests that UMAMIT14 is involved in phloem unloading of amino acids in roots, and that

[*] Reprinted with permission from "UMAMIT14 is an amino acid exporter involved in phloem unloading in Arabidopsis roots" by Julien Besnard, Réjane Pratelli Chengsong Zhao, Unnati Sonawala, Eva Collakova, Guillaume Pilot and Sakiko Okumoto, 2016. Journal of Experimental Botany, Volume 67, Issue 22, Page range 6385-6397, Copyright [2016] by Oxford University Press.

UMAMIT14 and UMAMIT18 are involved in the radial transport of amino acids in roots, which is essential for maintaining amino acid secretion to the soil.

## 2.2. Introduction

In addition to their role as primary metabolites, amino acids are one of the main nitrogenous compounds transported by xylem and phloem between the plant organs (Pate, 1976). In most species, Gln and Asn are the predominant amino acids found in the xylem sap, while all amino acids are transported in the phloem sap (Bollard, 1960; Winter *et al.*, 1992a; Lohaus *et al.*, 1994; Lam *et al.*, 1995; Lohaus and Moellers, 2000; Pilot *et al.*, 2004). Amino acid translocation through the plant requires crossing membranes at multiple locations. In roots, xylem loading requires membrane export from the stele cells into the xylem sap. In shoots, apoplasmic phloem loading from the leaf parenchyma requires at least one export and one import step across membranes. By their nature, amino acids are either zwitterions or have a net positive or negative charge, requiring transporters to cross the hydrophobic lipid bilayer at the experimentally observed rates (De Jong *et al.*, 1997).

Amino acid transport across membranes is mediated by importers, exporters, and facilitators. Identified plant amino acid importers belong to the families of Amino Acid Permease (AAP), Aromatic and Neutral Transporter (ANT), Cationic Amino acid Transporter (CAT), Lys His Transporter (LHT), Pro Transporter (ProT), and GABA transporter (GAT) (Pratelli and Pilot, 2014). Many of them were isolated by functional complementation of yeast strains lacking endogenous amino acid importers, based on the fact that rescued import function enables growth on media with amino acids as a sole

nitrogen source (reviewed in Ortiz-Lopez *et al.*, 2000; Wipf *et al.*, 2002; Rentsch *et al.*, 2007; Pratelli and Pilot, 2014). Several of these importers are involved in long-distance transport of nitrogen. For instance, AtAAP2 is responsible for xylem-phloem amino acid exchange (Zhang *et al.*, 2010), AtAAP8 is involved in seed filling of amino acids during embryogenesis (Schmidt *et al.*, 2007), and AtLHT1 is involved in amino acid uptake by roots and import from the apoplasm into mesophyll cells (Hirner *et al.*, 2006a;Svennerstam *et al.*, 2008; Svennerstam *et al.*, 2011). The lack of expression of any of these importers has an impact on plant growth, development or yield, emphasizing the importance of amino acid importers in nitrogen long-distance transport.

On the contrary, in spite of physiological evidence for amino acid export activity, very few exporters have been identified due to a lack of efficient screening techniques (Okumoto and Pilot, 2011). The first report on a molecular mechanism regulating amino acid export came from the study of Arabidopsis *Glutamine Dumper* 1 (*AtGDU1*). AtGDU1 is not a transporter, but its over-expression triggers Gln secretion from the hydathodes, suggesting that it functions as a positive regulator of amino acid exporters (Pilot *et al.*, 2004; Pratelli *et al.*, 2010). Bidirectional Amino Acid Transporter 1 (AtBAT1) has been shown to mediate import of GABA, Arg and Ala, and export of Lys and Glu at the mitochondrion membrane (Dundar and Bush, 2009; Michaeli *et al.*, 2011a). Siliques Are Red1 (SiAR1) has been characterized as a bi-directional amino acid transporter responsible for amino acid accumulation in developing siliques (Ladwig *et al.*, 2012). SiAR1/UMAMIT18 is a member of the Usually Multiple Acids Move In and out Transporters family (UMAMIT, also called *Medicago truncatula* Nodulin 21

[MtN21] (Denance *et al.*, 2014b), belonging to the Drug/Metabolite Transporter superfamily. Walls Are Thin1 (WAT1/UMAMIT05) has been shown to be a tonoplastic transporter for auxin, a molecule with a chemical structure similar to Trp (Ranocha *et al.*, 2013). UMAMIT14 has recently been shown to mediate both import and export of amino acids, and to be involved in amino acid loading in seeds (Muller *et al.*, 2015). Here we report the functional characterization of UMAMIT14 as an amino acid exporter in yeast and its involvement in unloading amino acids from the phloem in roots.

## 2.3. Material and methods

### *2.3.1. Plant culture*

Arabidopsis plants (Col-0) were grown in long days (16 h light at 50 $\mu mol.m^{-2}.s^{-1}$ at the soil surface, 50% humidity, and 22°C) or short days (12 h light, 35 $\mu mol.m^{-2}.s^{-1}$ at the soil surface, 35% humidity, and 22°C) in soil composed of a 2/1 ratio Sunshine Mix™ / Vermiculite. Plants were watered with 0.15 $g.L^{-1}$ MiracleGro™ fertilizer (24/8/16, N/P/K) three times a week. Arabidopsis plants were grown in hydroponic conditions as described (Pratelli *et al.*, 2016). For growth *in vitro*, Arabidopsis seeds were sowed on "J medium" (2 mM $KNO_3$, 1 mM $CaSO_4$, 1 mM $KH_2PO_4$, 0.5 mM $MgSO_4$, 50 µM NaFeEDTA, 50 µM $H_3BO_3$, 12 µM $MnCl_2$, 1 µM $CuCl_2$, 1 µM $ZnCl_2$, 30 nM $(NH_4)_6Mo_7O_{24}$; Lejay *et al.*, 1999) supplemented with 20 mM $KNO_3$ in 0.8% agar with pH adjusted to 5.8 with KOH. For hygromycin selection, J medium was supplemented with 20 $\mu g.ml^{-1}$ hygromycin and for kanamycin selection, with 50 $\mu g.ml^{-1}$ kanamycin and 30 mM sucrose. Plants were transformed using the floral dip method using *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Clough and Bent, 1998).

The *umamit14* T-DNA line SALK_037123 (*umamit14-1*) was obtained from the Arabidopsis Biological Resource Center (Alonso *et al.*, 2003), and the T-DNA insertion was confirmed by PCR (Appendix 1). To generate the complemented line, *umamit14-1* was transformed with *UMAMIT14* promoter-*UMAMIT14 cDNA-Venus*. Nitrogen starvation experiments were performed as described in Appendix 2.

### *2.3.2. DNA constructs*

*UMAMIT14* promoter (-1903 bp upstream from ATG) or *UMAMIT14* promoter and genomic sequence were PCR-amplified from Col0 genomic DNA. The PCR fragments were cloned into pDONRZeo (Life Technologies, USA). The *UMAMIT14* promoter or *UMAMIT14* genomic sequence were recombined into the destination vectors pWUTkan2 or pPWGYTkan respectively, derivatives of pJHA212K (Yoo *et al.*, 2005; Appendix 3), generating the plasmids used for *UMAMIT14 promoter-GUS* studies and transient expression of *35S:UMAMIT14-GFP* in Arabidopsis cotyledons. To generate *UMAMIT14* promoter-*UMAMIT14* cDNA-*Venus*, *UMAMIT14* cDNA without the stop codon was amplified by RT-PCR and cloned into a modified pENTR1A vector containing Venus (Price *et al.*, 2013). *UMAMIT14* cDNA-*Venus* sequence was transferred into a modified pPWYTkan (pJHA212K-derived), in which *35S* promoter was replaced with *UMAMIT14* promoter (Appendix 3). For yeast uptake studies, *UMAMIT14* cDNA was cloned into pDONRZeo vector and was transferred to the yeast expression vectors pDR196-Ws (Loque *et al.*, 2007), pAP-Ws, and pAP-Ws-AAP3. For the empty vector controls, the gateway cassettes from pDR196-Ws, pAP-Ws and pAP-Ws-AAP3 were removed by Gateway cloning with a pDONR vector containing only a

stop codon between the attL sites (Appendix 4). All entry clones were sequenced prior to use. Sequence information for all vectors is available upon request. Primers used for cloning and qRT-PCR are listed on Appendix 5.

### *2.3.3. Analytical methods*

Shoots and roots of five week-old Arabidopsis grown in hydroponic conditions were harvested and frozen in liquid nitrogen, lyophilized and pulverized. Total free amino acids were extracted by adding 400 µl of chloroform and 10 mM HCl (1:1 mixture) to 0.5-2 mg of plant tissue in a tube containing of 0.2 nmol of dry norvaline. The aqueous phase was collected, the organic phase was re-extracted with HCl and chloroform, and the supernatants were pooled. Phloem sap was obtained as described in Corbesier *et al.* (2001) from the leaves of five-week-old Arabidopsis plants grown in soil in long days. Amino acids were analyzed via Ultra Performance Liquid Chromatography (UPLC; Waters, USA), as described in Collakova *et al.*, 2013). Amino acid content in root and shoot samples was normalized against dry weight and norvaline content. Amino acid content in phloem exudates was normalized against $K^+$ content, determined using an Inductively Coupled Plasma Atomic Emission Spectrophotometer (Analytical instruments, USA). Carbon and nitrogen contents of 2 mg dry seeds were measured using the dry combustion method with a CE Instruments NC 2100 elemental analyzer (ThermoQuest, Italy) at the Duke Environmental Stable Isotope Laboratory, Duke University, NC. Seed protein extraction was performed as described by Gallardo *et al.* (2002 ) using 1 mg of seeds, and proteins were quantified by the Bradford assay (Bradford, 1976).

### *2.3.4. Phloem transfer and seedling secretion assays*

For the shoot-to-root transfer assays, five week-old Arabidopsis plants grown in hydroponic conditions were removed from the tip boxes, and sink leaves, defined as leaves with a surface area <25% of the largest leaf, were removed (Appendix 6). This largest leaf was then cut around the mid vein and dipped into a 1.5 ml tube containing 1.5 ml of J medium with either 2 mM sucrose + 2 mM Gln + [$^{3}$H]Gln, or 2 mM sucrose + [$^{14}$C]sucrose with a final specific activity of 24.4 kBq.µmol-1 in the uptake solution for Gln or sucrose. Roots were dipped in an adjacent 1.5 ml tube containing 1.5 ml of J medium. After 4 h, the fed leaf, shoots, roots and medium bathing the roots were harvested separately. Shoots and roots were dried, weighed and bleached in 500 µl 5% NaClO. Radioactivity in shoots, roots, and root bathing medium was counted using a LS 6500 Multipurpose scintillation counter (Beckman Coulter, USA). To analyze the amino acids secreted from Arabidopsis seedlings, 10 Arabidopsis seeds were germinated in 24 well-plates containing 1 ml per well of J medium supplemented with 20 mM $KNO_3$ and 30 mM sucrose, with pH adjusted to 5.8 with KOH. After two weeks, the medium was replaced with 1 ml of fresh medium and plants were grown for three more days. The medium was harvested, lyophilized and resuspended in 300 µl UPLC-grade water, and amino acid content was analyzed by UPLC as described above. Content was normalized using plant dry weight.

### *2.3.5. Yeast-based assays*

GNP1 (YDR508C) and AGP1 (YCL025C) were sequentially deleted from the genome of 22Δ8AA (Fischer *et al.*, 2002) using the loxP-kanMX-loxP disruption

cassette (Guldener *et al.*, 1996) to create 22Δ10α. Uptake of radiolabeled amino acids was performed according to Su *et al.* (2001 ). For the yeast secretion assay, cells were grown for 22 h in a minimal medium (Jacobs *et al.*, 1980; Jacobs *et al.*, 1980), the OD was recorded, the medium was separated from the cells by filtration and clarified on a 10 kDa exclusion membrane. Amino acid content of the medium was determined by UPLC as described above.

### *2.3.6. Uptake in planta*

Two week-old Arabidopsis plants were grown on a vertical plate containing half strength MS medium supplemented with 30 mM sucrose in 1% agar. Roots and shoots were separated and each sample was transferred into 1 ml half strength MS medium supplemented with 30 mM sucrose for five hours under agitation at 300 rpm. Uptake lasted 10 min in a solution containing 1 ml of half strength MS medium supplemented with 30 mM sucrose and 2 mM glutamine + [$^{3}$H]Gln with a final specific activity of 37 kBq.µmol$^{-1}$. Efflux was performed as described by Pratelli *et al.*, 2010) for 20 min. Radioactivity was then measured for each root, shoot, root bathing and shoot bathing media.

### *2.3.7. RNA extraction and qRT-PCR*

RNAs were extracted using the RNAeasy plant kit (Qiagen, USA) according to the manufacturer's recommendations. Two µg of total RNA were used for cDNA synthesis with random primers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). qRT-PCR was performed using SyBR® Green PCR Master

Mix in a 7500 Real Time PCR System (Applied Biosystems, USA) according to manufacturer's recommendation.

### *2.3.8. GUS assay and cross sections*

GUS assays were performed on two week-old Arabidopsis seedlings or six week-old flowers as described by Martin *et al.* (1992 )**.** Stained roots were fixed in 5% glutaraldehyde overnight, followed by dehydration in increasing concentrations of ethanol (30, 50, 60, 70, 80, and 90%, one hour each). Histochemical GUS analysis was performed by embedding the stained roots into Technovit 7100 resin (Kulzer, Germany) following the manufacturer's recommendation and slicing the tissues to 1 µm sections using a Leica® Ultracut UCT microtome. The sections were stained with periodic acid (0.5%) and Schiff reagent (5 mM basic fuchsin, 20 mM anhydrous sodium metabisulfite in 0.1 mM HCl).

### *2.3.9. Arabidopsis transient expression and confocal microscopy*

Arabidopsis transient expression was performed according to Wu *et al.,* (2014) using *Agrobacterium* strain C58C1 (pCH32) co-transformed with pPWGYTkan containing *UMAMIT14* genomic sequence without the stop codon, and a mCherry-expressing vector. After co-incubation, plants were imaged using a Zeiss® LSM 880 confocal laser scanning microscope. Images were taken using wavelengths appropriate for mCherry (543 Ex/600-650 Em), chlorophyll A (405 Ex/650-700 Em) and GFP (488 Ex/490-535 Em). Images obtained were merged using ZEN® software (Zeiss).

### *2.3.10. Statistical analyses*

One-way ANOVA in conjunction with Tukey's test, or t-tests were used to determine significant differences ($p<0.05$) between samples in JMP (SAS, USA).

### *2.3.11. Accession numbers*

UMAMIT14: AT2G39510 ; UMAMIT18/SiAR1: AT1G44800

## 2.4. Results

Plant amino acid transporters have typically been characterized by expression in yeast strains, particularly 22Δ8AA, lacking eight plasma membrane amino acid transporters (Fischer *et al.*, 2002). 22Δ8AA is unable to grow on media containing Asp, Glu, Pro, Citrulline and GABA as the sole nitrogen source at the supplied concentration. The main limitation of this strain is the low number of amino acids on which it can be tested for growth. We improved this strain by deleting two more transporters, Glutamine Permease 1 (GNP1) and Asparagine Glutamine Permease 1 (AGP1), shown to be necessary for transporting Gln and Thr (Velasco *et al.*, 2004; Couturier *et al.*, 2010), yielding strain 22Δ10α. 22Δ10α was unable to grow on any proteinogenic amino acid or GABA except for Arg as the sole nitrogen source (Appendix 9). Because Lys, His, and Cys do not support growth of the parental wild type strain 23344c and hence 22Δ10α, they are not usable for complementation assays at the tested concentrations. The import of radiolabeled amino acids (Gln, Ala, Pro, and Leu) into 22Δ8AA and 22Δ10α transformed with the empty vector was also compared over time. The background import for Gln and Ala by 22Δ10α was lowered compared to 22Δ8AA, but not for Pro and Leu

(Appendix 10). This strain therefore provides a better tool for transport assays when compared to 22Δ8AA.

### *2.4.1. UMAMIT14 functions as an amino acid exporter in yeast cells*

The previously cloned 44 Arabidopsis *UMAMIT* cDNAs (Jones *et al.*, 2014) were expressed in 22Δ10α, and the cells were screened for Gln secretion (Okumoto, unpublished data). Increased Gln secretion from cells expressing UMAMIT14 suggested that the protein was endowed with an amino acid exporter activity. This result is in good agreement with Müller *et al.* (2015), which reported an increased export of [$^{14}$C]Gln from *Xenopus* oocytes expressing UMAMIT14. We sought to develop a system using 22Δ10α cells that allows facile detection of amino acid export activities. We reasoned that, similar to UMAMIT18 (Ladwig *et al.*, 2012), expressing an amino acid exporter would decrease the accumulation of amino acids taken up by yeast cells. Wild type cells, harboring all amino acid importers, were used by Ladwig *et al.* (2012), with the caveat that heterologous expression of an exogenous transporter might disturb the expression/activity of endogenous yeast amino acid transporters, several of which are regulated by amino acid levels at the transcriptional and post-transcriptional levels (Stanbrough and Magasanik, 1995; Didion *et al.*, 1996; Springael and Andre, 1998). To circumvent this problem, UMAMIT14 was co-expressed with the plant amino acid importer AAP3 (Fischer *et al.*, 1995), which is unlikely to be regulated by the general amino acid control or the nitrogen catabolite repression systems of yeast. The expression of AAP3 was driven by the Alcohol Dehydrogenase (ADH1) promoter (Ruohonen *et al.*, 1995), which is also unlikely to be regulated by amino acid levels. 22Δ10α cells

expressing AAP3 showed a linear import of Gln, Pro, Ala, Leu, Lys and Asp, which was significantly higher than that of cells transformed with the empty vector (Figure 3; Fischer *et al.*, 2002). When AAP3 was co-expressed with UMAMIT14, cells accumulated less Gln, Pro, Ala, Leu, and Lys than 22Δ10α cells expressing only AAP3 (Figure 3). Asp accumulation was not statistically different between these cells. The decreased amino acid accumulation was not due to lower expression of AAP3, as tested by qRT-PCR (data not shown), while an effect on protein content/activity cannot be excluded. Similar results were obtained using 22Δ8AA with Pro (Appendix 11). Expression of UMAMIT14 in 22Δ10α did not increase import of Pro (Appendix 11), Gln, Ala, Leu, Lys and Asp compared to the empty vector (data not shown), nor complemented yeast growth on any amino acid supplied at 3 mM as the sole nitrogen source (data not shown). These results suggest that UMAMIT14 functions as an exporter in 22Δ10α.

To confirm this hypothesis, UMAMIT14- expressing cells were grown in the presence of ammonium, and let secrete amino acids in the liquid medium for 22 h, similarly to previous assays (Velasco *et al.*, 2004; Ladwig *et al.*, 2012). The content of Gln+Arg, Ala, Glu, Ser, Gly, Asn, Pro, Thr, Val, His, Ile, Leu and Phe was increased by expression of UMAMIT14 compared to cells expressing the empty vector (Figure 4).

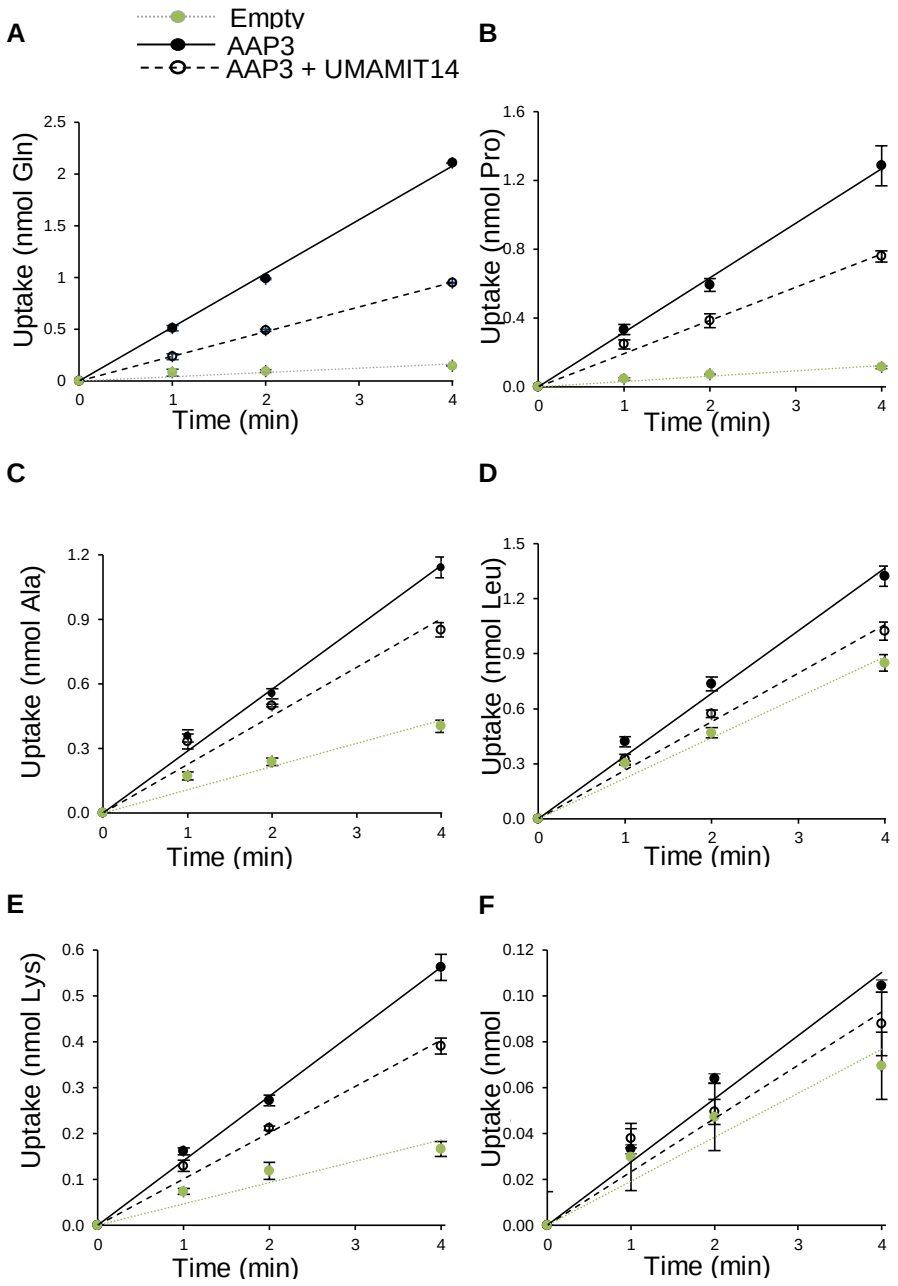


**Figure 3: Uptake of radiolabeled amino acids by 22Δ10*α* yeast cells.** Empty: cells transformed with the empty vector pDR196-Ws from which the gateway cassette has been removed; AAP3: cells expressing AAP3; AAP3 + UMAMIT14: cells co-expressing AAP3 and UMAMIT14 carried on a single vector. Uptake was examined for 2 mM of Gln (A), Pro (B), Ala (C), Leu (D), Lys (E) or Asp (F). Error bars show standard deviation (n=3 technical replicates). Reprinted from Besnard *et al*., (2016).

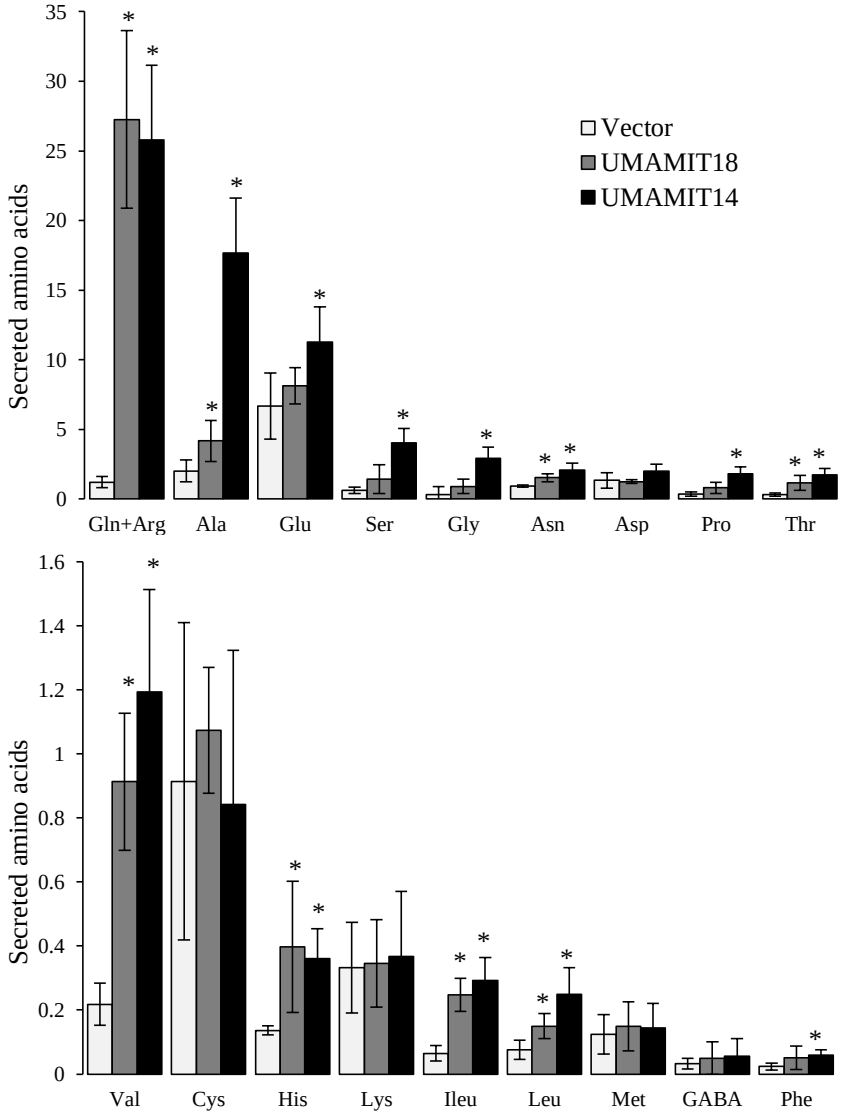


**Figure 4: Secretion of amino acids in the medium by 22Δ10α cells.** Cells transformed with the empty vector pDR196-Ws from which the gateway cassette has been removed, or containing *UMAMIT14* or *UMAMIT18*. Cells were grown for 22 h in liquid medium and amino acid composition of the medium was determined by UPLC. The Arg peak could not be resolved from the large Gln peak in UMAMIT14 and UMAMIT18 samples so these amino acids are presented by a single bar. For the control, Gln and Arg could be resolved and Gln represents ~50% of the bar height. Error bars correspond to standard deviation (n=4 biological replicates). Significant differences compared to the empty vector ($p<0.05$) are indicated by a star according to t-test. Reprinted from Besnard *et al*., (2016).

The results obtained with UMAMIT18 were comparable to those described by Ladwig *et al.* (2012). Therefore the secretion assays showed that UMAMIT14 behaves as an amino acid exporter in yeast cells under the conditions tested.

Amino acid transport specificity of UMAMIT14 and UMAMIT18 cannot be directly interpreted from these results because the activity/expression of each protein in yeast may be different. To circumvent this problem, the content of secreted amino acids was normalized to the amino acid levels for the empty plasmid control and to the total amount of secreted amino acids (Appendix 12). Gln, Val and Ile represented a larger proportion of the secreted amino acids for UMAMIT18 compared to UMAMIT14. Ala, Glu, Ser, Gly, Asp, Pro and Leu represented a larger part of the secreted amino acids for UMAMIT14 compared to UMAMIT18, suggesting that the amino acid specificity of the two transporters is different.

### *2.4.2. UMAMIT14 is a plasma membrane protein expressed in root phloem and pericycle*

The expression pattern of *UMAMIT14* was determined by a promoter-GUS approach in Arabidopsis. *UMAMIT14* promoter activity was detected in the pericycle and phloem cells of roots (Figure 5A and D, Figure 6B) and in the stamen filaments (Figure 5B). Extending GUS staining reaction revealed a weak activity of *UMAMIT14* promoter in veins of young cotyledons (Figure 5C) but not in mature leaves. In the wild type, *UMAMIT14* mRNA accumulated 15 times more in roots than in leaves (Table 1), consistent with the promoter-GUS assay results.

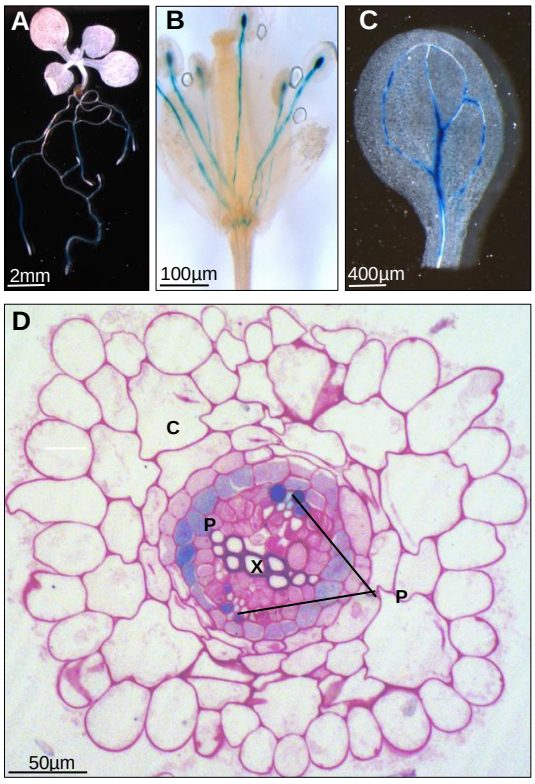


**Figure 5: Localization of UMAMIT14 expression in Arabidopsis.** A-D: histochemical analysis of GUS activity from Arabidopsis plants expressing *UMAMIT14promoter:GUS*. GUS staining in root of two week-old seedling (A), a flower from a 35 day-old plant (B), and a two week-old cotyledon (C) are shown. (D) A cross section of a two week-old root in the maturation zone. Cell walls were stained in pink by the Schiff reagent. C: Cortex, Ph: Phloem, P: Pericycle, X: Xylem. Tissues were stained for GUS activity at 37°C for 16 hours for A and 30-45 min for B-D. E-H: Subcellular localization of UMAMIT14-GFP in epidermal cells of two week-old Arabidopsis cotyledons. (E) UMAMIT14-GFP, (F) mCherry, (G) chlorophyll A, (H) merged. Reprinted from Besnard *et al*., (2016).

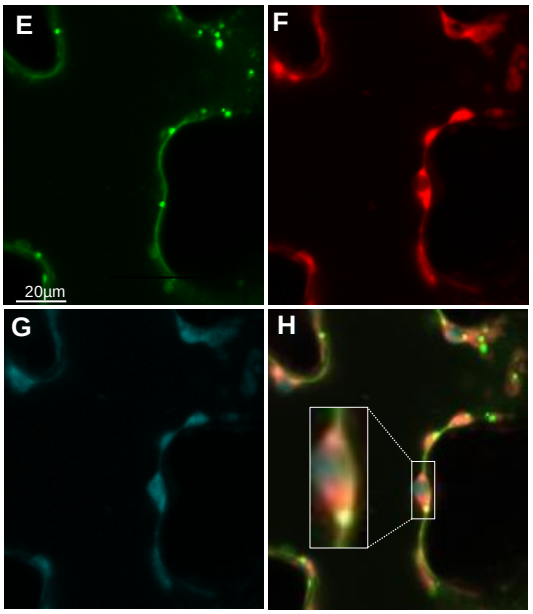


**Figure 5: Continued**

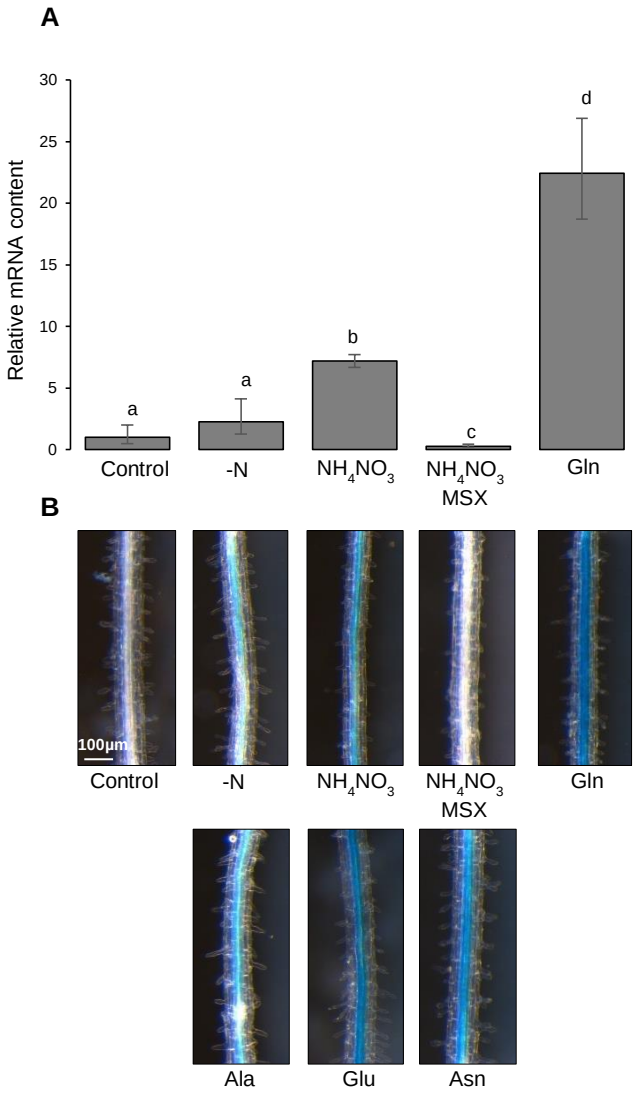


**Figure 6: UMAMIT14 transcript levels in plants grown in different nitrogen conditions.** Treatments are described in Appendix 2. (A) *UMAMIT14* mRNA levels were determined by qRT-PCR and normalized against *ACTIN2* mRNA levels. Data show fold change relative to the non-treated control plants (control). Error bars correspond to standard deviation (n=3 biological replicates). Significant differences ($p<0.05$) are indicated by different letters according to one way ANOVA in conjunction with Tukey's test. (B) GUS activity in the roots of *UMAMIT14promoter:GUS* plants subjected to the same treatments as in (A), and addition of Ala, Glu, and Asn (20, 20 and 10 mM respectively). Results from one representative GUS line among four that produced similar results are shown. Reprinted from Besnard *et al*., (2016).

**Table 1: UMAMIT14 mRNA accumulation in five-week-old Arabidopsis plants grown in hydroponic conditions.** *UMAMIT14* mRNA levels were determined by qRT-PCR and normalized against *ACTIN2* mRNA accumulation. *UMAMIT14* mRNA levels are expressed relative to the wild type (WT) root sample. Significant differences ($p<0.05$) are indicated by different letters according to one way ANOVA in conjunction with Tukey's test (2 biological replicates). Reprinted from Besnard *et al*., (2016).

| Genotype | Roots | Leaves |
|---|---|---|
| WT | 1 (a) | 0.062 (b) |
| *umamit14-1* | 0.079 (b) | 0.041 (b) |
| *umamit14-1 umamit18-1* | 0.057 (b) | 0.342 © |
| *umamit14-1:UMAMIT14* | 49.3 (d) | 0.036 (b) |

Subcellular localization of UMAMIT14 was determined by transiently expressing a *35S:UMAMIT14-GFP* construct in Arabidopsis cotyledon cells. UMAMIT14-GFP was almost exclusively localized at the plasma membrane, with some additional fluorescence in intracellular structures (Figure 5E-H), in good agreement with Müller *et al.* (2015).

*2.4.3. UMAMIT14 expression is regulated by organic nitrogen in roots*

Publicly available transcriptome data from Arabidopsis suggested that *UMAMIT14* expression is regulated by nitrogen status (Krouk *et al.*, 2010; Patterson *et al.*, 2010; Ruffel *et al.*, 2011). In particular, Patterson *et al.* (2010) showed that *UMAMIT14* mRNA accumulation is increased by nitrate and ammonium treatments. Interestingly, this increase is suppressed by the glutamine synthetase inhibitor methionine sulfoximine (MSX), suggesting that metabolites downstream from glutamine synthetase are responsible for it (Patterson *et al.*, 2010). To further test the effect of the

plant nitrogen status on *UMAMIT14* expression, Arabidopsis seedlings were starved for nitrogen for 24 h and then supplied with various inorganic and organic nitrogen sources. After four hours of incubation *UMAMIT14* transcript levels were seven and 24 times higher in plants incubated with ammonium nitrate and Gln, respectively, than in the control (Figure 6A). At the same time, *UMAMIT14* transcript levels were 10 times lower in ammonium nitrate plus MSX treated plants than in the control (Figure 6A). To test if the increase could result from change in expression pattern, *UMAMIT14* promoter activity was studied in the same conditions using four independent *UMAMIT14* promoter-GUS lines. *UMAMIT14* promoter activity correlated well with the abundance of *UMAMIT14* transcripts (Figure 6B), with no change in localization of the activity in roots: the promoter was always active in vascular tissues, without visible staining in the cortex or epidermis. Application of Glu, Asp, and Ala was also tested, and resulted in a similar increase in promoter activity (Figure 6B). *UMAMIT14* gene expression was thus increased in the stele by organic nitrogen sources, but not by inorganic ones.

### *2.4.4. umamit14 and umamit18 mutants are specifically affected in transfer of amino acids from the shoots to the roots*

To characterize the role of UMAMIT14, *umamit14-1* loss-of-function line was isolated from the SALK collection (SALK_037123; Alonso *et al.*, 2003; Appendix 1), which showed over a 10-fold reduction in *UMAMIT14* mRNA accumulation (Table 1). Because UMAMIT14 and UMAMIT18 expression overlaps in root pericycle and their biochemical functions are similar, *umamit14-1* was crossed with *umamit18-1* (Ladwig *et al.*, 2012) to generate a double homozygous mutant *umamit14-1 umamit18-1*. The

*umamit14-1* mutant and the double mutant grew similarly to the wild type in soil (Appendix 1, Appendix 13). Total free amino acid content was similar in roots and shoots for the mutants and the wild type (Appendix 14), while the contents of Ala and Asp were slightly increased in roots of *umamit14-1* and the double mutant (Appendix 15).

Disruption of *UMAMIT14* and *UMAMIT18* is expected to affect amino acid transport. While it might not change the amino acid content at the organ level, it could change amino acid translocation by the long-distance transport systems. Because *UMAMIT14* promoter was active in the root phloem and pericycle cells, we hypothesized that UMAMIT14 is involved in unloading amino acids from the phloem in roots. A shoot-to-root translocation assay was designed, in which a leaf was fed radiolabeled Gln, and the transfer of the radioactivity to the rest of the rosette, roots and medium was measured after four hours (Appendix 6). Radiolabel translocation from the fed leaf to the roots and from the roots to the medium was reduced approximately by half in *umamit14-1* compared to the wild type. This decrease was stronger in the *umamit14-1 umamit18-1* double mutant than in *umamit14-1*, to approximately 1/3 of wild type level (Figure 7A). The same trend was observed for the radioactivity secreted into the medium: secretion was reduced from ~6% in the wild type to ~2% and ~1% in the single and double mutants, respectively. Total Gln absorbed by the fed leaf was similar between the mutants and the wild type, showing that the decreased transfer to the roots is not due to less Gln absorbed by the mutants (Appendix 7).

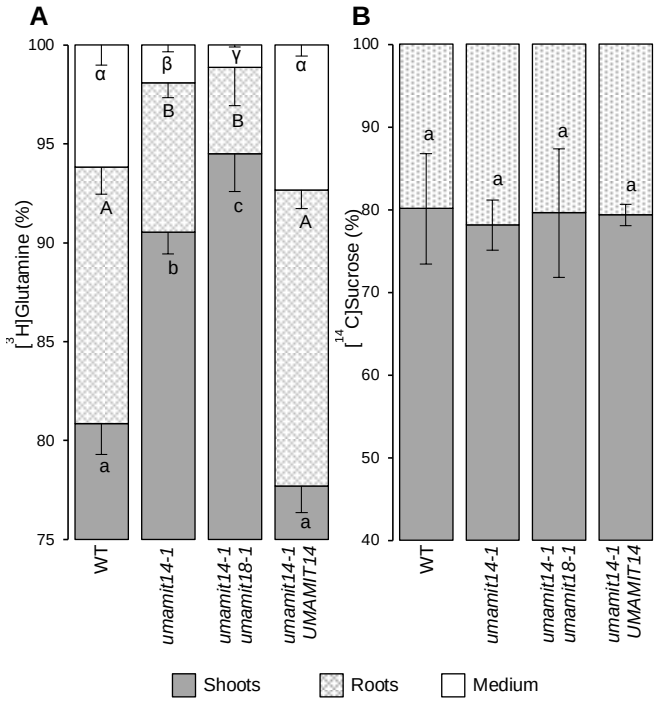


**Figure 7: Phloem transfer assay.** Distribution of the radioactivity absorbed as [$^{3}$H]Gln (A) and [$^{14}$C] sucrose (B) in shoots, roots, and medium of plants after four hours of feeding. One mature leaf of five week-old plants grown in hydroponics was soaked in J medium containing labeled compounds. The fed leaf was removed from the shoots prior to counting. Significant differences ($p<0.05$) are indicated by different letters according to one way ANOVA in conjunction with Tukey's test (n=3 biological replicates). Reprinted from Besnard *et al*., (2016).

To confirm that the observed decrease was due to the disruption of *UMAMIT14*, the *umamit14-1* mutant was complemented with the *UMAMIT14* cDNA fused to the fluorescent protein *Venus* (Nagai *et al.*, 2002) under the control of its native promoter, which restored *UMAMIT14* expression as confirmed by qRT-PCR (Table 1). Gln translocation in the shoot-to-root assay in the complemented line was identical to the wild type (Figure 7A).

To test if decreased amino acid translocation to the roots resulted from a decrease in phloem translocation rate and/or in amino acid concentration in the phloem, leaf phloem exudates from the *umamit14-1* and *umamit18-1* single and double mutants were collected and analyzed. No difference in amino acid composition was observed between wild type plants and the three mutants (Table 2).

**Table 2: Amino acid composition and $K^+$ content of phloem exudates of five-week-old Arabidopsis plants grown in soil in in long day conditions.** Amino acid composition and $K^+$ content of phloem exudates of five-week-old Arabidopsis plants grown in soil in in long day conditions. No significant difference was found according to one-way ANOVA in conjunction with Tukey's test ($p<0.05$, 3 biological replicates). Reprinted from Besnard *et al*., (2016).

| | Amino acids (nmol/mg $K^+$) | | | | | | | | | | | | | | | $K^+$ (nmol.mg$^{-1}$ DW) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ala | Asn | Asp | GABA | Gln | Glu | Gly | His | Ile | Leu | Phe | Pro | Ser | Thr | Val | Total | |
| WT | 18.0 | 11.6 | 46.4 | 46.3 | 28.4 | 34.2 | 4.3 | 2.4 | 3.9 | 5.2 | 3.2 | 3.8 | 19.5 | 14.7 | 6.3 | 248.3 | 0.43 |
| *umamit14-1* | 17.0 | 15.3 | 53.0 | 31.3 | 40.2 | 54.1 | 4.9 | 3.0 | 5.6 | 6.9 | 4.1 | 4.0 | 20.8 | 16.3 | 7.9 | 284.5 | 0.33 |
| *umamit18-1* | 20.4 | 13.2 | 47.8 | 57.4 | 33.2 | 37.3 | 6.6 | 3.5 | 4.0 | 5.7 | 3.4 | 5.6 | 23.0 | 18.3 | 6.9 | 286.4 | 0.46 |
| *umamit14-1 umamit18-1* | 22.4 | 11.4 | 42.7 | 44.3 | 29.0 | 34.2 | 7.6 | 3.1 | 3.9 | 5.3 | 3.3 | 5.5 | 25.3 | 17.4 | 6.5 | 262.0 | 0.39 |

Phloem exudation rate was estimated by measuring $K^+$ release from the leaves during this experiment, and no difference was found between the genotypes (Table 2). To further confirm these results, the shoot-to-root translocation assay was performed using radiolabeled sucrose as a proxy for phloem transfer rate. Total absorption by the fed leaf and translocation of the radioactivity to the roots was identical between the wild type and the *umamit* mutant lines (Appendix 8 and Figure 7B), indicating that phloem translocation is not affected by the mutations. These results suggest that the *umamit* mutations specifically affect translocation of amino acids from the shoots to the roots, and not phloem sap flow rate.

Decrease of amino acid supply to the roots could possibly decrease root growth under low nitrogen availability. To test this hypothesis, wild type and *umamit14-1* plants were grown under high and low nitrate regimes. No sugar was added to the medium to limit nitrate assimilation in roots (Reda, 2015). Under such conditions, root growth was expected to largely depend on the amino acids supplied from the shoots. As expected, wild type plants showed a slower root growth in low concentrations of nitrate than in high nitrate, but for a given nitrate regime, root length of the wild type and the mutant were similar (Appendix 16), showing that in these conditions loss-of-function of *UMAMIT14* did not affect root growth.

### *2.4.5. Secretion of amino acids in the medium is affected in the umamit14-1 umamit18-1 single and double mutants*

The shoot-to-root transfer assay showed that less radioactivity is secreted by mutant roots into the medium compared to the wild type. Since plants secrete a variety

of organic molecules into their environment in addition to amino acids (Chaparro *et al.*, 2013), we sought to test if this label was borne by amino acids or other compounds, which could be produced from the absorbed [$^{3}$H]Gln. Whole seedlings were grown in liquid medium for three days and the amino acid composition of the medium was analyzed. *Umamit14-1, umamit18-1* and *umamit14-1 umamit18-1* secreted respectively 25%, 33%, and 72% less amino acids in the medium than the wild type (Figure 8 and Appendix 17).

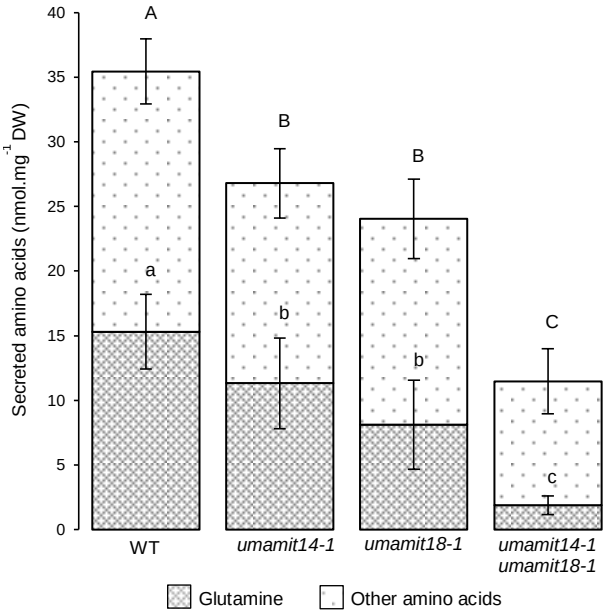


**Figure 8: Glutamine and other amino acids secreted by the wild type, umamit14 and umamit18 mutants.** Plants were grown for two weeks in liquid J medium supplemented with 20 mM $KNO_3$ and 30 mM sucrose. The medium was then replaced with fresh J medium and collected after three days for analysis. Significant differences ($p<0.05$) are indicated by different letters according to one-way ANOVA in conjunction with Tukey's test (n=6 biological replicates). Contents in individual amino acids from the same dataset are presented in Table S5. Amino acid content were normalized against the dry weight of seedlings in each sample. Reprinted from Besnard *et al*., (2016).

Gln was the most abundant amino acid secreted by the wild type (47%) and *umamit14-1* (46%), but its abundance was lower for *umamit18-1* (38% of the wild type) and for the *umamit14-1 umamit18-1* double mutant (21% of the wild type) (Appendix 17). This result suggests that most of the radioactive compounds secreted in the medium in the shoot-to-root translocation assay correspond to amino acids. To specifically test if amino acid secretion by roots is affected by the loss of function of *UMAMIT14* and *UMAMIT18*, both of which are expressed in the root stele, [$^3$H]Gln efflux from shoots and roots were measured independently. [$^3$H]Gln efflux from of *umamit14-1*, *umamit18-1*, and *umamit14-1 umamit18-1* roots were decreased compared to the wild type, while [$^3$H]Gln efflux from the shoots remained similar (Figure 9).

## 2.5. Discussion

### *2.5.1. UMAMIT14 mediates amino acid export in yeast*

Two complementary yeast assays were performed to confirm and complete the biochemical properties of UMAMIT14 described by Muller *et al.*, 2015. Export activity was first measured in the yeast strain 22Δ10α where UMAMIT14 was co-expressed with AAP3, a well-characterized amino acid importer. Amino acid uptake was lower in cells co-expressing AAP3 and UMAMIT14, compared to the cells expressing AAP3 alone (Figure 3). In the secretion assay, UMAMIT14-expressing yeast cells were let secrete amino acids in the medium (Figure 4). 22Δ10α cells expressing UMAMIT14 secreted more than four times as much amino acids than control cells.

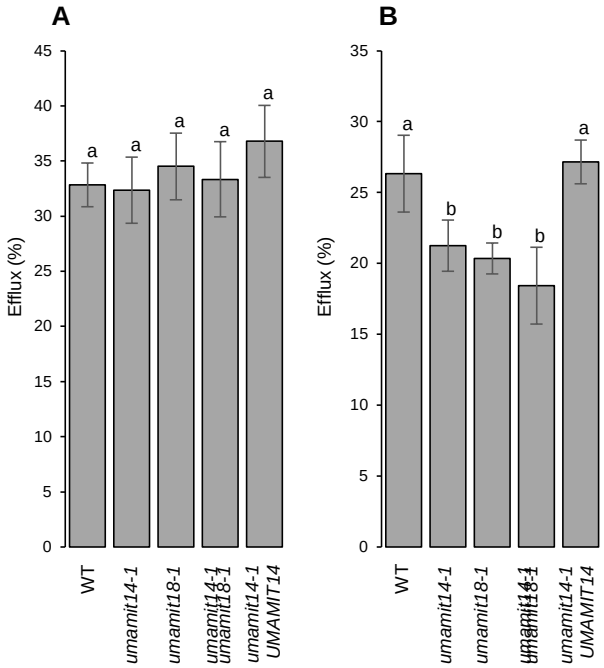


**Figure 9: Efflux of [$^3$H]Gln by two week-old Arabidopsis seedlings roots and shoots.** Uptake and efflux were performed for 20 and 10 minutes respectively, using 2 mM Gln. Efflux from shoots (A) and roots (B) are is expressed as a percentage of total uptake. Error bars correspond to standard deviation (n=4 biological replicates). Significant differences ($p<0.05$) are indicated by different letters according to one way ANOVA in conjunction with Tukey's test. Reprinted from Besnard *et al*., (2016).

These two assays, enabling measurement of export activity over different time periods, demonstrated that UMAMIT14 displays an amino acid export activity, in good accordance with the results from Müller *et al.* (2015), and reminiscent of UMAMIT18 (Ladwig *et al.*, 2012), UMAMIT11, UMAMIT28, and UMAMIT29 (Muller *et al.*, 2015). Contrary to the report of Müller *et al.* (2015), no import was detected in yeast cells expressing UMAMIT14 when tested by complementation or uptake assays (Appendix 11 and data not shown). It should be noted that Müller *et al.* (2015) found a *K*m value of ~15 mM for import by UMAMIT14 and that high substrate concentrations

(up to 100 mM) were used in their assays, compared to 2 mM in our study, likely explaining the different results. Whether UMAMIT14 displays a similar *K*m for export of cytosolic amino acids remains to be determined.

Assuming that cytosolic amino acid content is similar in yeast cells expressing UMAMIT14 or UMAMIT18, comparing the compositions of amino acids secreted in the medium suggests that UMAMIT14 and UMAMIT18 do not have the same amino acid specificities. UMAMIT14 promotes secretion of a wider spectrum of amino acids compared to UMAMIT18 (Appendix 12). The range of amino acids secreted and the activity to reduce the AAP3-mediated amino acid accumulation (Gln, Pro, Ala, Leu and Lys; Figure 3) suggest that, similar to UMAMIT18, UMAMIT14 is a broad specificity amino acid exporter.

### *2.5.2. Possible function of UMAMIT14 in phloem unloading in roots*

Publicly available transcriptomic data (Mustroph *et al.*, 2009), promoter-GUS and qRT-PCR results (Figure 5, Table 1) indicate that UMAMIT14 is expressed in roots in addition to seeds. Subcellular localization and histochemical analysis studies showed that UMAMIT14 is mainly expressed at the plasma membrane and in root pericycle and phloem cells (Figure 5) in addition to being expressed in seeds (Muller *et al.*, 2015). Because pericycle cells are involved in both xylem and phloem functions (Parizot *et al.*, 2012), we first hypothesized that UMAMIT14 was involved in the transport steps leading to root-to-shoot translocation of amino acids. Root-to-shoot translocation of amino acids first involves export from xylem parenchyma and pericycle cells for loading the xylem. Roles for pericycle-expressed transporters in xylem loading have been

previously reported for multiple solutes such as potassium, boron and auxin (Gaymard *et al.*, 1998; Takano *et al.*, 2002b; Kamimoto *et al.*, 2012). If UMAMIT14 or UMAMIT18 were involved in loading amino acids in the xylem, a decrease in xylem amino acid content or transfer rate would have been expected in the *umamit* knockout lines. The amino acid content of the xylem sap in *umamit14-1* and *umamit14-1 umamit18-1* was not significantly different compared to the wild type (Appendix 18), nor was the translocation of root-fed amino acids to the shoots (Pratelli *et al.*, 2016). While not supporting an essential role for UMAMIT14 and UMAMIT18 in this process, these results do not exclude their involvement alongside additional transporters. Indeed, publicly available microarray data show that other members of the UMAMIT family (e.g. UMAMIT10, 11, and 17) are highly expressed in root pericycle (Long *et al.*, 2010). Unloading shoot-derived amino acids from the symplasmically isolated phloem into the root stele (Oparka, 1990; Imlau *et al.*, 1999a) requires amino acid exporter activity (Figure 10). Amino acids can then be taken up from the apoplasm by importers into stele cells or be transferred to the xylem towards the shoots (Jeschke and Hartung, 2000). Functional properties and expression of UMAMIT14 in the root phloem cells points towards a role in phloem unloading in roots, a hypothesis supported by our results, which show that less amino acid coming from the shoots are unloaded in root tissues. The analysis of UMAMIT14 expression in seeds led to the hypothesis for a role in unloading amino acids from the phloem to the surrounding tissues (Muller *et al.*, 2015).

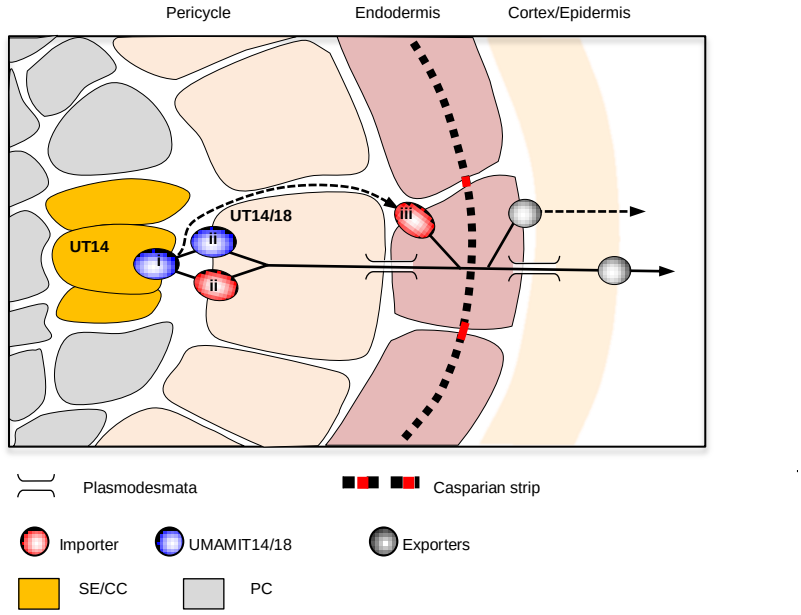


**Figure 10: Model of radial transport of amino acids in the mature root.** Amino acids coming from the shoots are exported from the sieve elements and companion cells (SE/CC) of the phloem to the stele apoplasm by an exporter (i), possibly UMAMIT14. In the apoplasm, amino acids are taken up by pericycle cells by an importer, or by the import ability of UMAMIT14 and UMAMIT18 (ii). Alternatively, amino acids can be taken up from the stele apoplasm by endodermis cells (iii), bypassing the pericycle uptake. Amino acids can be further exported to the rhizosphere from the endodermis, cortex and/or epidermis cells by presently uncharacterized exporters. Symplasmic and apoplasmic routes are indicated by solid and dotted lines respectively. Reprinted from Besnard *et al*., (2016).

UMAMIT14 thus appears to be an important phloem unloader, both in roots and seeds. Despite a decrease in phloem amino acid unloading in *umamit14-1* roots, root growth was not significantly affected under the conditions tested (Appendix 16). Nevertheless, the function of UMAMIT14 might become more prominent in other growth conditions. It is noteworthy that organic nitrogen sources applied after a nitrogen starvation period induce the expression of UMAMIT14 (Figure 6). Nitrogen resupply after starvation has been shown to increase leaf amino acid content within three hours (Balazadeh *et al.*, 2014). Shortly after amino acid levels increase in shoots, amino acids are likely translocated to the roots to support growth and cell division in roots. In such a scenario, increase in the expression of UMAMIT14 would enable higher amino acid unloading into the root tissues. Whether the *umamit14* mutant responds differently from the wild type under such specific growth conditions remains to be determined.

*2.5.3. Altered shoot-to-root translocation of amino acids decreases secretion*

Amino acid export from roots measured by amino acid secretion (Figure 8) and labeled amino acid efflux (Figure 9) was decreased in the *umamit14* and *umamit18* mutants. Based on these results, we suggest the following model that explains the *umamit14 and umamit18* phenotypes observed in roots (Figure 10): (1) Altered phloem unloading of amino acids in *umamit14* mutant increases the local concentration of amino acids in the root phloem (2) Decreased amino acid transport at the pericycle in *umamit14 and umamit18* mutants slows down the radial transfer of amino acids from the stele to the peripheral cell layers. (3) Decreased amino acid transfer to the peripheral cell layers in *umamit14* and *umamit18* mutants decreases the secretion mediated by other amino

acid exporters located in the cortex and epidermis. Interestingly, several *UMAMIT* genes are expressed in the cells that are in direct contact with the rhizosphere; for example, UMAMIT04 is expressed in root hairs and atrichoblasts, and UMAMIT06, 37 and 42 are expressed in trichoblasts (Brady *et al.*, 2007). These transporters could mediate the final steps of amino acid secretion out from the root. It should be noted that in the shoot-to-root transfer experiment using [$^{3}$H]Gln, the ratio between Gln retained in the root and Gln secreted into the medium remained similar between the wild type and the mutants (Figure 7A). This result suggests that the amino acid efflux capacity from the peripheral cell layers is not affected by the *umamit14* and *umamit18* mutations.

It is well documented that roots secrete various compounds including amino acids into the rhizosphere, which in turn affects the composition of microbial community (Huang *et al.*, 2014b). While root secretion causes net losses of assimilated carbon and nitrogen, root secreted organic molecules serve important functions such as attracting growth promoting microorganisms, increasing accessibility to mineral nutrients, and regulating defense against pathogens (Dakora and Phillips, 2002; Huang *et al.*, 2014b). For example, it has been shown that amino acids are chemotactic attractants for a wide range of soil-borne bacteria (Simons *et al.*, 1997; Oku *et al.*, 2012; Hida *et al.*, 2015 Webb *et al.*, 2016) and a recent study further suggested that Trp secreted from roots positively regulates auxin production from growth-promoting bacteria, (Liu *et al.*, 2016a) which in turn promotes plant growth.

Little is known about where amino acid secretion happens along the length of the root. However, a study using a Trp-sensing bacterial strain showed that Trp secretion

from the roots of *Avena barbata* is greatest at 12 to 16 cm from the root tip, in contrast to sucrose that is secreted mostly from the root tip (Jaeger *et al.*, 1999). In Arabidopsis, secretion of amino acids from roots peaked at a much later stage (28-31 days) compared to that of sugars (7-10 days), also consistent with the secretion from well-developed root sections (Chaparro *et al.*, 2013). Secretion of shoot-derived compounds from a mature section of roots would involve unloading from the phloem, importing into the stele and pericycle cells, radial transfer to the endodermis and cortex through the symplasmic route, and export into the rhizosphere (Figure 10). Results from the shoot-to-root translocation assay suggested that UMAMIT14 and UMAMIT18 are involved in the translocation of phloem-derived amino acids within roots and ultimately their secretion into the rhizosphere (Figure 8). Whether the altered amino acid secretion profiles from *umamit* lines impacts the root microbiome remains to be determined.

The role of the UMAMIT14 and UMAMIT18 in the pericycle in relation to the unloading of amino acids to the root tissues is not clear. The pericycle layer is highly connected to the endodermis via plasmodesmata (Ma and Peterson, 2001), suggesting that most solutes will be transferred between these cell layers via the symplasm. Moreover, pericycle cells have been described to be important for transfer of solutes towards the xylem sap via membrane export (see above), and, to our knowledge, not for uptake from the stele parenchyma. It is nevertheless possible that UMAMIT14 and UMAMIT18, for which import activity has been detected in specific conditions (Ladwig *et al.*, 2012; Muller *et al.*, 2015), are responsible for some of the import from the stele apoplasm to the pericycle. The concentration of amino acids in the xylem sap is about 5

mM (Appendix 18; Pilot et al., 2004; Zhang et al., 2010), which may be similar to the concentration of amino acids in the stele apoplasm. In this concentration range, both UMAMIT14 and UMAMIT18 are able to import amino acids into the cytosol, and could then be involved in the radial transfer of amino acids to the root periphery. Additional studies would be needed to test this hypothesis, and to determine the exact roles of UMAMIT14 and UMAMIT18 in pericycle cells.

*2.5.4. Roles of UMAMIT14 and UMAMIT18 in long distance amino acid transport*

In the *umamit14-1* single mutant, translocation from shoots to roots and root amino acid secretion were reduced compared to the wild type. Considering the localization of UMAMIT14 in root phloem and pericycle, the simplest hypothesis to explain the decreased secretion from the roots to the medium in the *umamit14* mutants is that amino acids accumulate in the root phloem and stele due to the decreased export from the phloem to the peripheral layers. Although such a change might increase the amino acid content of the phloem and the stele, it is unlikely that it will affect the overall amino acid content in roots: the decrease in amino acid secretion in *umamit14-1* (estimated at 2.9 nmol.mg $DW^{-1}.day^{-1}$, based on the data shown in Figure 8 if secretion rate from roots is similar to that from whole seedlings) is small compared to the total root amino acid content (393.8 nmol.mg $DW^{-1}$). An increase in the root phloem amino acid concentration might trigger a change in flux so that a larger portion of the amino acids delivered from the leaf phloem are redirected to the xylem before they reach the root, changing the partitioning of amino acids derived from the source leaves (Figure 7). It is noteworthy that stem/node regions (which were excluded from phloem sap

collection) are highly vascularized and are the site for extensive nutrient exchange between phloem and xylem (Jeschke *et al.*, 1987). We hypothesize that in *umamit14-1* more amino acids are retained within the root phloem and/or the stele due to the lack of exporter activities, and that the resulting increase in root phloem amino acid level triggers a change in amino acid partitioning, potentially within the stem/node region.

In almost all cases, the *umamit14-1 umamit18-1* double mutant showed an enhanced phenotype compared to the *umamit14-1* mutant: less amino acid transferred to the roots and less amino acid secreted by the root. In good agreement with their overlapping expression in root pericycle and their comparable, albeit distinct, functional properties UMAMIT18 and UMAMIT14 appear to have similar roles in roots. Unlike UMAMIT14, UMAMIT18 is also expressed in leaf veins (Ladwig *et al.*, 2012), suggesting that it has other roles in transport of amino acids in the plant. Nevertheless, this role does not seem to affect the composition or velocity of the leaf phloem (Table 2), suggesting that the enhanced effect of the *umamit14 umamit18* double mutation in shoot-to-root translocation is solely due to UMAMIT18's role in the root pericycle.

# CHAPTER III

# UMAMIT24 AND UMAMIT25 ARE ARABIDOPSIS AMINO ACID EXPORTERS INVOLVED IN TRANSFER OF AMINO ACIDS TO THE SEED

## 3.1. Abstract

Phloem-derived amino acids are the major source of nitrogen supplied to developing seeds. Amino acid transfer from the maternal to the filial tissue requires at least one cellular export step from the maternal tissue prior to the import into the symplasmically isolated embryo. Some members of Usually Multiple Acids Move In an out (UMAMIT) family (UMAMIT11, UMAMIT14, UMAMIT18, UMAMIT28 and UMAMIT29) have previously been implicated in this process. Here we show that additional members of UMAMIT family, UMAMIT24 and UMAMIT25, also function in amino acid transfer in developing seeds. Using a recently described yeast-based assay allowing screening of amino acid exporters, we showed that UMAMIT24 and UMAMIT25 promote export of a broad range of amino acids in yeast. In plants, UMAMIT24 and UMAMIT25 are expressed in distinct tissues within developing seeds; UMAMIT24 is mainly expressed in the chalazal seed coat whereas UMAMIT25 is expressed in the endosperm cells. Seed amino acid content of *umamit24* and *umamit25* knockout lines were both decreased during embryogenesis compared to the wild type, but recovered in the mature seeds without any deleterious effect on yield. The results suggest that UMAMIT24 and UMAMIT25 function in the export of amino acid from the maternal tissue.

## 3.2. Introduction

Transfer of nutrients from the mother plant to the reproductive tissues is critical to ensure proper seed development. In most plant species, phloem-derived amino acids are the main form of nitrogenous compounds transported to the seeds and stored as proteins, which are necessary for seed metabolism during seed maturation and germination (Atkins *et al.*, 1979; Pate *et al.*, 1975). The quantity of amino acids delivered through the phloem to the developing seed correlates with seed protein and lipid content, suggesting that phloem amino acid concentration is an important factor in determining seed quality traits such as protein content (Lohaus and Moellers, 2000; Saravitz and Raper, 1995). Manipulation of phloem amino acid content through loss-of-function mutations and enhanced gene expression also affects seed protein content and yield, supporting the importance of phloem-derived amino acids in determination of yield (Santiago and Tegeder, 2016; Santiago and Tegeder, 2017; Zhang *et al.*, 2010).

Once delivered to the reproductive tissues through the phloem, amino acids are released from the mother tissue before they are taken up by the symplasmically isolated daughter tissues via $H^+$/amino acid symporters (Sanders *et al.*, 2009; Schmidt *et al.*, 2007; Tan *et al.*, 2008; Tegeder, 2014; Zhang *et al.*, 2015). Studies using the "empty seed coat" technique in legumes revealed that amino acids are released from the seed coat by a transporter-mediated mechanism that is not dependent on a proton gradient (De Jong *et al.*, 1997). For a long time, the molecular mechanism for this amino acid transport remained unknown (Okumoto and Pilot, 2011). However, several members of Usually Multiple Acid Move In and out Transporters (UMAMIT) family mediate

proton-gradient insensitive amino acid transport and represent a potential mechanism for release of amino acids from the mother tissues to the developing seeds. Indeed, several UMAMIT proteins have been suggested to play a role in the seed loading process: UMAMIT18 is expressed in the chalazal seed coat within Arabidopsis seeds during early stage of embryogenesis, and the loss of function mutant accumulates less amino acids in the developing siliques (Ladwig *et al.*, 2012). Likewise, UMAMIT11, UMAMIT14, UMAMIT28 and UMAMIT29 are expressed within developing seeds and are involved in amino acid loading in siliques during early stages of embryogenesis (Muller *et al.*, 2015). For each of the UMAMIT proteins found so far to be expressed within the seed, the phenotype caused by a single loss-of-function mutation is relatively benign (*i.e.* no loss of seed viability), suggesting the involvement of multiple amino acid exporters, including previously uncharacterized UMAMIT, in amino acid transport to the embryo.

In this study, we report the role of two additional members of UMAMIT family in Arabidopsis, UMAMIT24 and UMAMIT25 in the seed filling process. The expression patterns of UMAMIT24 and UMAMIT25 differ during seed filling stage, suggesting that they play distinct roles in seed filling of amino acids during embryogenesis.

## 3.3. Material and methods

### *3.3.1. Plant culture*

Arabidopsis (Col-0) plants were grown in long days (16 h light at 125 µmol $m^{-2}$ $s^{-1}$, 60 % humidity, 23/18°C day/night in SUN GRO SUNSHINE$^{v}$LC1 GROWER MIX). Plants were watered with 0.15 g $l^{-1}$ MiracleGro fertilizer (24:8:16, N:P:K) three times a week. For kanamycin selection, seeds were sowed on MS medium (half strength Murashige and Skoog medium supplemented with 30 mM sucrose and 0.8 % agarose with pH adjusted to 5.8 with KOH) containing 50 µg $ml^{-1}$ kanamycin. Hygromycin selection was performed as described by Harrison *et al*. (2006) using MS medium complemented with 20 µg $ml^{-1}$ hygromycin. Kanamycin and hygromycin resistant plants were transferred in the long days conditions described above after one week of selection. Plants were transformed using the floral dip method using *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Clough and Bent, 1998). The *umamit24* T-DNA line GABI_012H03 (*umamit24-1*) was obtained from the GABI-Kat III (Bielefeld, Germany). The *umamit25* T-DNA line SALK_140423 (*umamit25-1*) was obtained from The Arabidopsis Biological Resource Center (Alonso *et al.*, 2003). Both T-DNA insertions were confirmed by PCR (Appendix 19) and qPCR (Appendix 20). To generate the complemented lines, *umamit24-1* and *umamit25-1* were transformed with *UMAMIT24* promoter–*UMAMIT24* gDNA-GFP or *UMAMIT25* promoter–*UMAMIT25* gDNA-GFP, respectively. To generate the UMAMIT24 and UMAMIT25 lines used for the analysis of spatiotemporal expression of UMAMIT24-GFP and UMAMIT-25GFP, wild type (WT) plants were transformed with *UMAMIT24* promoter–*UMAMIT24*

gDNA-GFP or *UMAMIT25* promoter–*UMAMIT25* gDNA-GFP, respectively. To generate the overexpressor lines used for subcellular localization of UMAMIT25-GFP, WT plants were transformed with 35S:*UMAMIT25*-GFP.

### *3.3.2. DNA constructs*

The *UMAMIT24* promoter (2546 bp upstream from ATG) and gDNA or *UMAMIT24* gDNA were PCR-amplified from Col-0 gDNA. The PCR fragments were cloned into pDONRZeo (Life Technologies, USA). The *UMAMIT24* promoter–*UMAMIT24* gDNA or *UMAMIT24* gDNA were recombined into the destination vectors pWGkan2 or pPWGYTkan, respectively, derivatives of pJHA212K (Yoo *et al.*, 2005). pWGKan2 carrying *UMAMIT24*promoter–*UMAMIT24* gDNA was used to generate the lines used to visualize spatiotemporal expression of UMAMIT24-GFP, as well as the *umamit24-1/*UMAMIT24 complemented line. The same cloning steps applies for UMAMIT25 using *UMAMIT25* promoter (2963 bp upstream from ATG) – *UMAMIT25* gDNA or *UMAMIT25* genomic DNA, as well as for the generation of *umamit25-1/*UMAMIT25 complemented line. For yeast uptake studies, *UMAMIT24* cDNA or *UMAMIT25* cDNA were cloned into the pDONRZeo vector and transferred to the yeast expression vectors pDR196-Ws (Besnard *et al.*, 2016; Loque *et al.*, 2007). All entry clones were sequenced prior to use. Sequence information for all constructs is available upon request. Primers used for cloning and qRT-PCR are listed in Appendix 21.

### *3.3.3. Analytical methods*

Acquisition and harvesting of siliques at 7, 10 and 14 days after pollination (DAP) was performed as described in Appendix 22. Free amino acids were extracted by

adding 200 µl of chloroform and 10 mM HCl (1:1 mixture) to 1–2 mg of lyophilized plant tissue, and the aqueous phase was collected. Samples were derivatized using o-phthalaldehyde (OPA, Agilent #5061-3335) and 9-fluoromethyl-chloroformate (FMOC, Agilent #5061-3337) and the derivatized amino acids were separated by reverse phase HPLC using an Agilent 1260 liquid chromatograph. Amino acids were detected by fluorescence using an in-line fluorescence detector (G1321B). Protein extraction and quantification was performed as described in Gallardo *et al.* (2002) using 2 mg of lyophilized plant tissue. The nitrogen content of seed protein was deduced using the average nitrogen content of nine proteins belonging to the two major families of seed storage proteins (napins and cruciferins) in Arabidopsis (17.7%). Pericarp nitrogen content was calculated using a commonly used conversion factor for plant tissues (16%) (Jones, 1941).

### *3.3.4. Glutamine and sucrose transfer assay in isolated siliques*

Three siliques were excised from the plant 10 DAP (Appendix 22), aligned and cut using a razor blade so that the pedicels all have a length of 5 mm. The three siliques were transferred into a 0.25 ml PCR tube with their pedicel in contact with 5 µl of a solution containing of 20 mM sucrose and 2 mM Gln. At the beginning of the experiment, the solution was replaced with 5 µl of 20 mM sucrose and 2 mM Gln with 5% and 10% isotopic excess of $U^{13}C$ sucrose and $U^{15}N$ Gln, respectively, (or no isotopic excess for the negative controls) and the PCR tube was closed. Four hours later, the solution was removed from the PCR tube, the pedicel was excised from each silique, and the three siliques were lyophilized. The seeds were separated from the silique pericarp

tissue (defined here as the silique minus the seeds), and $\delta^{13}$ C, $\delta^{15}$ N, % N and % C seed content were determined in the Stable Isotopes for Biosphere Science (SIBS) Laboratory, Texas A&M University, using a Costech Elemental Combustion System coupled to a Thermo Scientific Delta V Advantage stable isotope mass spectrometer and Conflo IV in continuous flow (helium) mode. Isotopic enrichment was determined by subtracting the $^{13}$C and $^{15}$N content of each line's negative control from the $^{13}$C and $^{15}$N of the samples.

### *3.3.5. Yeast secretion assay*

Determination of the amount of amino acids secreted in the medium by yeast expressing the UMAMIT proteins was performed as described in Besnard *et al.* (2016).

### *3.3.6. RNA extraction and qRT-PCR*

RNA was extracted according to Downing *et al.* (1992) using ~25 mg of fresh seeds. Samples of 2 µg of total RNA were used for cDNA synthesis with random primers using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). qRT-PCR was performed using SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix in a CFX96 Touch Real-Time PCR machine (Biorad) according to manufacturer's recommendations.

### *3.3.7. Transient expression in Arabidopsis*

Proteins were transiently expressed in Arabidopsis according to Wu *et al.* (2014) using Agrobacterium strain C58C1 (pCH32) transformed with pPWGYTkan containing the *UMAMIT24* genomic sequence without the stop codon, and the binary vector ER-RB

CD3-960 containing HDEL-mCherry coding sequence for endoplasmic reticulum (ER) visualization (Nelson *et al.*, 2007).

### *3.3.8. Fluorescence and confocal microscopy imaging*

Images and Z-stacks of UMAMIT24-GFP and UMAMIT25-GFP seeds were taken using a Zeiss LSM 880 confocal laser scanning microscope. Fluorescence of siliques used to visualize spatiotemporal expression of UMAMIT24-GFP and UMAMIT25-GFP was checked using an Olympus SZXZRFL3 stereomicroscope attached with the Olympus DP71 digital camera (Olympus America, NY, USA). Subcellular localization of UMAMIT24-GFP or UMAMIT25-GFP was imaged using an Olympus FV1000 confocal microscope equipped with an UPLSAPO 60x/1.2 water immersion objective. All images were taken using wavelengths as follows: GFP (Ex. 488 nm, Em. 500-530nm), mCherry (Ex. 543 nm, Em. 565-615 nm), chlorophyll (Ex. 488 nm, Em. >650 nm), or Syto82 (Ex. 543nm, Em. 565-615) and merged using ZEN software (Zeiss), Olympus Fluoview (Olympus) and ImageJ.

### *3.3.9. Statistical analyses*

T-tests were performed in Microsoft Excel. One-way ANOVA in conjunction with Tukey's test were used to determine significant differences between samples in JMP (SAS, USA). PCA analyses were performed using Stats package in R (Venables and Ripley, 2002).

### *3.3.10. Accession numbers*

Accession numbers for UMAMIT24 (AT1G25270) and UMAMIT25 (AT1G09380) have been assigned by The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/).

## 3.4. Results

### *3.4.1. UMAMIT24 and UMAMIT25 function as amino acid exporters in yeast cells.*

Using a yeast strain 22Δ10α which lacks 10 of the endogenous amino acid transporters (Besnard *et al.*, 2016), we have screened 44 UMAMITs from Arabidopsis that are capable of exporting Gln into the growth medium (Okumoto, unpublished). This screen identified UMAMIT25 as a potential amino acid exporter, and phylogenetic analysis revealed two closely related paralogs, UMAMIT23 and UMAMIT24 (Denancé *et al.*, 2014). Publicly available microarray data showed that UMAMIT23, UMAMIT24 and UMAMIT25 are almost exclusively expressed in the developing siliques and seeds (Schmid *et al.*, 2005), in which amino acid exporters play an important role in nitrogen transfer to the filial tissue. Therefore, we analyzed the functions of these three transporters in detail in yeast. In order to examine whether these three UMAMITs also function as exporters, (Besnard *et al.*, 2016; Fischer *et al.*, 1995) they were expressed in the *Saccharomyces cerevisiae* 22Δ10α strain (Besnard *et al.*, 2016). The cells were grown for 24 h in a medium lacking amino acids with ammonium as the sole nitrogen source, and the amino acid content in the medium was determined. If any of the UMAMIT genes encode an amino acid exporter, the amino acid content in the medium from the cells expressing these genes is expected to be higher than for control cells

carrying the empty vector (Besnard *et al.*, 2016; Ladwig *et al.*, 2012; Velasco *et al.*, 2004). Cells expressing UMAMIT24 or UMAMIT25 showed a significantly higher secretion of most of the detectable amino acids compared to control cells (Figure 11).

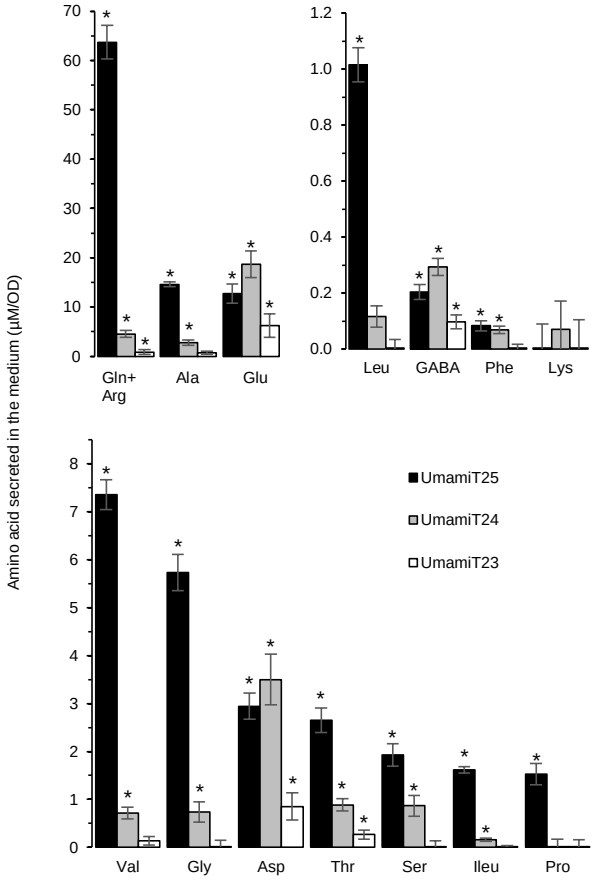


**Figure 11: Secretion of amino acids in the medium by UMAMIT-expressing yeast cells.** 22Δ10α cells were transformed with the empty vector pDR196-Ws from which the gateway cassette has been removed, or containing *UMAMIT25*, *UMAMIT24* or *UMAMIT23*. Cells were grown for 22 h in liquid medium, then amino acid composition of the medium was determined by UPLC. The Arg peak could not be resolved from the large Gln peak in samples so these amino acids are presented by a single bar. Concentrations found in the medium were divided by the OD of the culture, and the value obtained for the empty vector was subtracted. Error bars correspond to standard error (n=4 biological replicates). Significant difference ($p<0.01$) compared to the empty vector are indicated by a star according to t-test.

UMAMIT25 expressing cells secreted more than 5 times as much Gln, Val, Ile, Gly and Leu (47x, 10.5x, 9x, 5.2x, 5x, respectively) as control cells, while UMAMIT24 expressing cells secreted amino acids to a lesser degree but significantly higher than control cells (between 5.4x for Gln and less than 2.5x for the other amino acids relative to the control cells). UMAMIT23 expressing cells never secreted more than 2.8 time amino acids than control cells. Total detected amino acid secretion from yeast expressing the empty vector was 26 ± 3 mM, which was significantly lower compared to the amino acid secreted by the yeast expressing UMAMIT23, UMAMIT24 and UMAMIT25 (35 ± 4 mM, 59 ± 6 mM, and 142 ± 9 mM respectively; $p<0.05$ according to t test with n=4). The three UMAMITs expressed in yeast act as broad amino acid exporters, with a preference for Gln. When 22Δ10α growth was tested on media containing amino acid as the sole nitrogen source, none of the three proteins could complement the transport defect of the yeast cells (data not shown), suggesting that none of them are able to mediate amino acid import. Because of the low activity of UMAMIT23, this gene was not included in further study. Uptake of radiolabeled Gln, Trp, His, Pro, or Met was measured on 22Δ10α cells expressing UMAMIT24 or UMAMIT25. Neither cells showed a significant import of any of the amino acid tested compared to the cells expressing the empty vector (data not shown).

### *3.4.2. UMAMIT24 and UMAMIT25 are expressed in distinct cell types in developing seeds*

Publically available microarray data suggested that both UMAMIT24 and UMAMIT25 are expressed in seed and silique tissues six DAP, and show low expression

in vegetative tissues (Schmid *et al.*, 2005). To identify the cell types that express UMAMIT24 and UMAMIT25 within the silique, UMAMIT24 or UMAMIT25 were tagged with the GFP at the C-terminus and expressed under the control of their native promoter (*UMAMIT24promoter:UMAMIT24-GFP* and *UMAMIT25promoter:UMAMIT25-GFP*). Siliques were collected between 4 and 14 DAP and GFP fluorescence was visualized in dissected siliques. UMAMIT24-GFP expression became visible at six DAP in the chalazal seed coat of developing seeds with little to no expression in the embryo, seed coat, or endosperm (Figure 12A), whereas control plants did not show any fluorescence in the seeds (Appendix 23). The dissection of seeds at 14 DAP confirmed the exclusive expression of UMAMIT24 in the chalazal seed coat (Figure 12B) with a weak fluorescence found in the embryo, most likely due to auto fluorescence (Appendix 23). In addition, UMAMIT24 expression was detected in the pericarp tissues appearing at 12 DAP (Figure 12). Confocal microscopy revealed that UMAMIT24-GFP expression in seeds was restricted to the chalazal seed coat (see Supplemental video 1). In Arabidopsis plants carrying *UMAMIT25promoter:UMAMIT25-GFP* construct, GFP fluorescence was visible six DAP in the endosperm of developing seeds (Figure 13A) with little to no signal in the embryo (Figure 13B). Similar to UMAMIT24, UMAMIT25-GFP expression was detected in the pericarp tissues 12DAP. Confocal microscopy of UMAMIT25-GFP expressing seeds at 12 DAP revealed that cells of the outer integument did not express UMAMIT25-GFP, whereas cells surrounding the embryo showed a strong expression of

UMAMIT25-GFP (Supplemental video 2), suggesting that UMAMIT25 is expressed in the endosperm of Arabidopsis seeds.

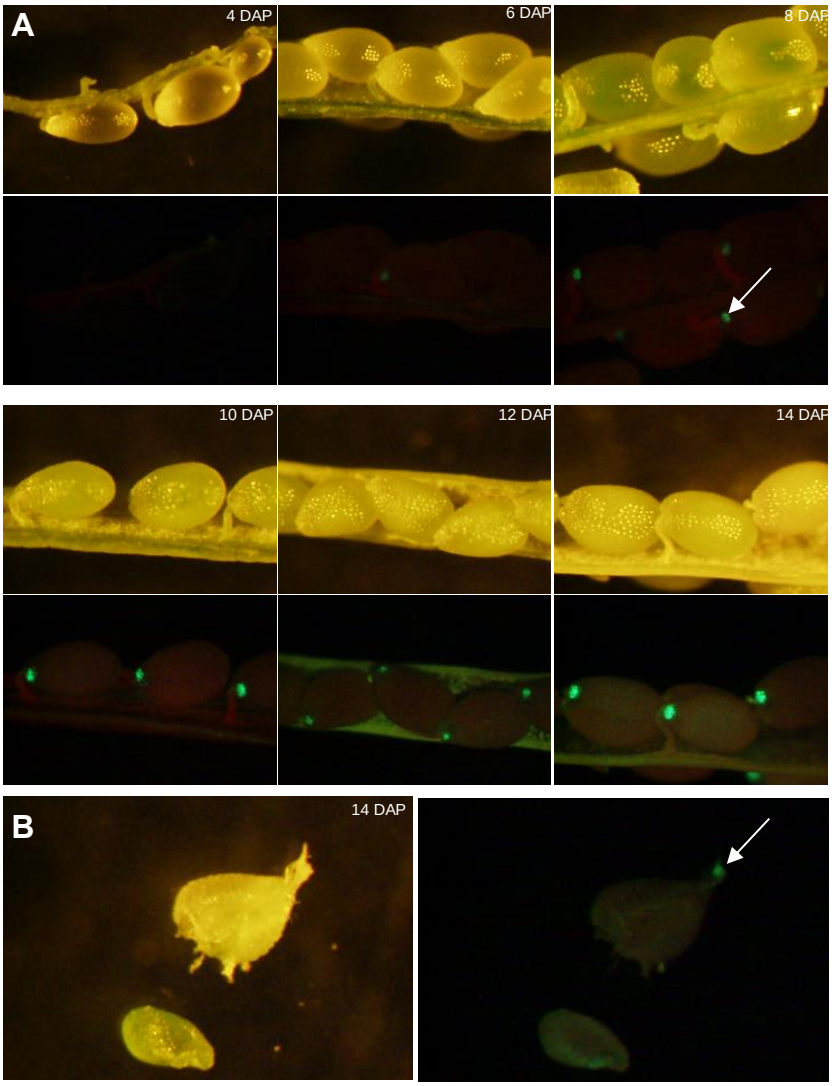


**Figure 12: Localization of UMAMIT24 in Arabidopsis seeds.** A: Silique with valves removed. B: dissected seeds revealing the embryo (bottom left of the picture) and the seed coat. White arrowhead points to the chalazal seed coat. Seeds were observed under bright light (top rows) or under GFP-exciting wavelength (bottom rows). DAP: days after pollination.

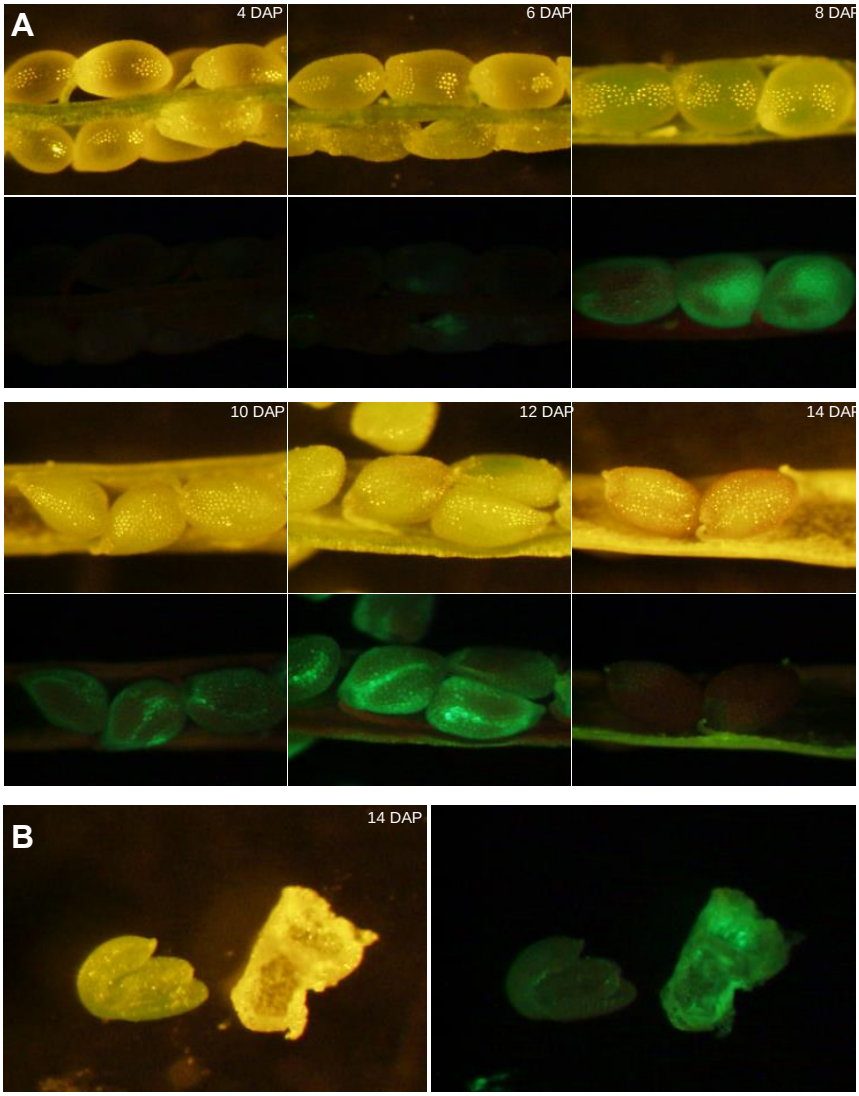


**Figure 13: Localization of UMAMIT25 in Arabidopsis seeds.** A: Silique with valves removed. B: dissected seeds revealing the embryo (bottom left of the picture) and the seed coat. Seeds were observed under bright light (top rows) or under GFP-exciting wavelength (bottom rows). DAP: days after pollination.

### *3.4.3. Subcellular localization of UMAMIT24 and UMAMIT25*

Previously characterized UMAMIT members include transporters localized at the plasma membrane (Besnard *et al.*, 2016; Ladwig *et al.*, 2012; Muller *et al.*, 2015), tonoplast (Ranocha *et al.*, 2010; Ranocha *et al.*, 2013) and the ER membrane (Pan *et al.*, 2016). To determine the subcellular localization of UMAMIT24 a construct harboring UMAMIT24-GFP under the control of the CaMV35S promoter were transiently expressed in Arabidopsis cotyledons using the Agrobest method (Wu *et al.*, 2014). UMAMIT24-GFP was localized to a net-like structure resembling the ER (Figure14A). Co-expression of UMAMIT24-GFP with an ER lumen marker (mCherry-HDEL; Figure14B; Nelson *et al.*, 2007) showed a large overlap of the GFP signal with mCherry-HDEL. The GFP signal did not overlap with the ER bodies but surrounded them, suggesting that UMAMIT24-GFP mainly localizes at the ER membrane (Figure14E). Similar results were obtained using UMAMIT24 fused to the GFP tag at the N-terminus in cotyledons (Appendix 24), or C-terminus in root cells (Appendix 25). When UMAMIT25-GFP was overexpressed in Arabidopsis hypocotyl cells, the GFP signal was localized almost exclusively at the plasma membrane (Figure14G-J). These data suggest that subcellular localization of UMAMIT24 and UMAMIT25 differ, with UMAMIT24 localizing mainly to the ER membrane and UMAMIT25 to the plasma membrane.

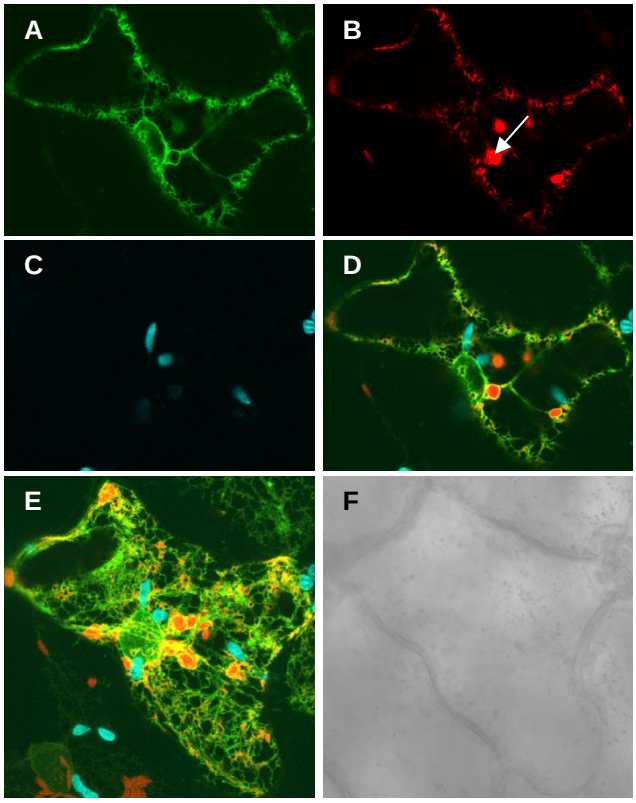


**Figure 14: Subcellular localization of UMAMIT24-GFP and UMAMIT25-GFP in one week-old seedlings.** (A-F) Cotyledons co-transformed with 35S:UMAMIT24-GFP and HDEL-mCherry. (A) GFP, (B) mCherry, (C) chlorophyll, (D) merged, I Z-Stack of approximately 75% of the cell displayed in A-D and (F) bright field. White arrowhead points to ER body. (G-J) Hypocotyls transformed with 35S:UMAMIT25-GFP. (G) GFP, (H) chlorophyll, (I) merged and (J) bright field.

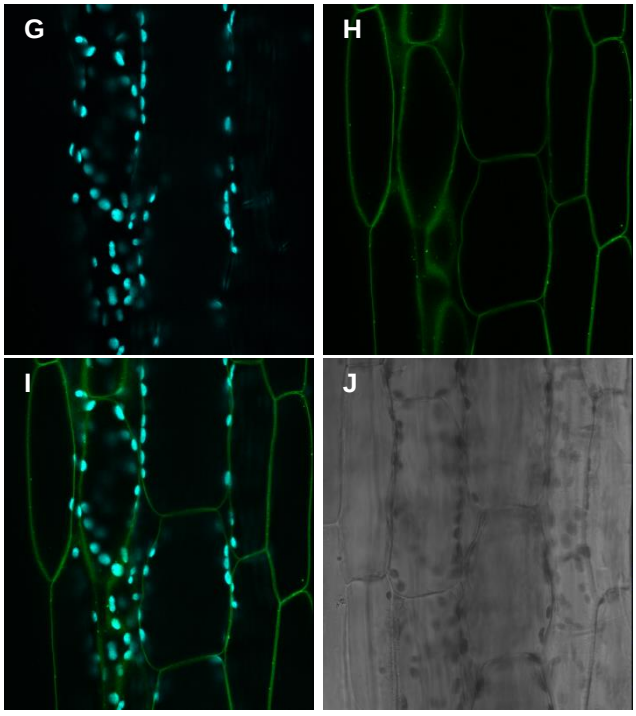


**Figure 14: Continued**

*3.4.4. Increase in UMAMIT24 and UMAMIT25 expression increases the seed yield*

In order to understand UMAMIT24 and UMAMIT25 functions, a T-DNA insertion line for each gene has been isolated (*umamit24-1* and *umamit25-1*; Appendix 19). qRT-PCR showed that the T-DNA insertion decreased the gene expression level in both cases, to ~4% of the WT for *umamit24-1*, and to undetectable levels for *umamit25-1* (Appendix 20). In order to confirm that any phenotype was due to the lack of these UMAMIT genes, each T-DNA insertion line was complemented with an additional copy of the respective UMAMIT gene expressed under its native promoter (*umamit24-1/*UMAMIT24, *umamit25-1/*UMAMIT25). Both lines expressed the introduced gene at a higher level than that of the WT (14.8 fold for *umamit24-1/*UMAMIT24, 2.3 fold for *umamit25-1/*UMAMIT25, Appendix 20).

Since both UMAMIT24 and UMAMIT25 show amino acid export activities in yeast and are expressed in the developing seeds, seed number and mass produced per plant were measured for each of the mutants and complemented lines. No difference was observed between the two *umamit* lines and the WT. However, an increase in seed number and seed mass for both of the complemented lines compared to the WT was observed (Figure 15). Weight for 100 seeds was slightly smaller for the *umamit25-1* and *umamit24-1/*UMAMIT24 lines compared to the WT (Appendix 26). The percentages of carbon and nitrogen in seeds were analyzed in all the lines, and *umamit24-1/*UMAMIT24 seeds contained less nitrogen (4.82 %) compared to all the other lines (between 5.1 to 5.4 %; Appendix 26). Finally, no significant differences were observed

for the seed germination rate of all lines tested in long days conditions (>90%, data not shown).

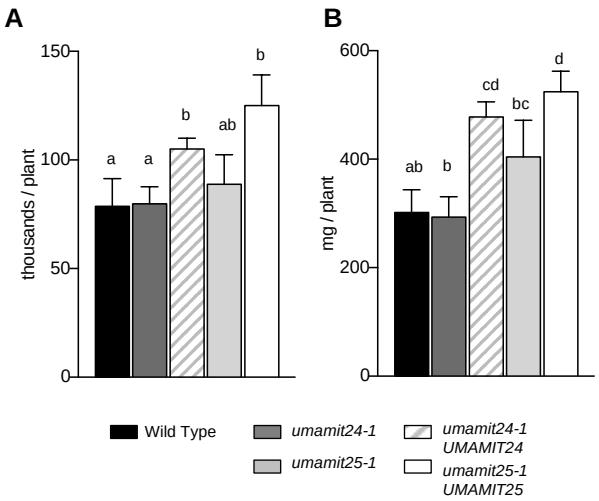


**Figure 15: Seed yield.** A. Number of seeds produced per plant. B. Mass of seeds per plant. Error bars correspond to standard deviation (n=4). Significant differences ($p<0.05$) are indicated by different letter according to one way ANOVA in conjunction with Tukey's test.

### *3.4.5. The expression levels of UMAMIT24 and UMAMIT25 affect amino acid levels in the fruit tissues*

To investigate further the potential roles of UMAMIT24 and UMAMIT25 in reproductive tissues, amino acid and protein contents were analyzed for the WT, the *umamit* mutants and the complemented lines during the course of embryogenesis. To distinguish the effects of UMAMIT activities on amino acid accumulation in the silique from the effects on amino acid transfer to the seed, the developing seeds were separated from the pericarp (defined here as the silique minus the seeds; Appendix 22). This experiment was performed at 7, 10, and 14 DAP, since UMAMIT24 and UMAMIT25

are both actively expressed during this period (Figure 12 and Figure 13). Estimated nitrogen in seed proteins accumulated quickly between 7 and 14 DAP, increasing nearly five times between the two time points. However, nitrogen in seed amino acids was only a small portion of total nitrogen at all time points (Appendix 27). Nitrogen in both proteins and amino acids decreased between 10 and 14 DAP in the pericarp tissue (Appendix 27). Further analysis revealed that, in both *umamit24-1* and *umamit25-1* seeds, amino acid content at 14 DAP was significantly lower than that of WT, which was partially restored in the complemented lines (Figure16A). These differences did not persist in the mature stage, at which none of the lines displayed any significant difference from the WT (Figure 16A). The decreases in amino acid content at 14 DAP in both *umamit* mutants were also observed in the pericarp tissues, which were restored to WT levels in the complemented lines (Figure 16B). Although seed protein content was unchanged in between WT and the knockout lines at all the stages tested, seed protein content in the complemented line *umamit24-1*/UMAMIT24 was higher at 10 DAP (Figure 16C). Interestingly, the seed protein content of this complemented line did not increase further after 10 DAP. In addition, amino acid content at 10 DAP was lower in *umamit24-1/*UMAMIT24 seeds (Figure 16A) and pericarp tissues (Figure 16B). A PCA analysis revealed that the amino acid composition of *umamit24-1/*UMAMIT24 pericarp tissues resembled that of other lines at 14 DAP, suggesting that pericarp maturation might be accelerated in the *umamit24-1/*UMAMIT24 complemented line (Appendix 28).

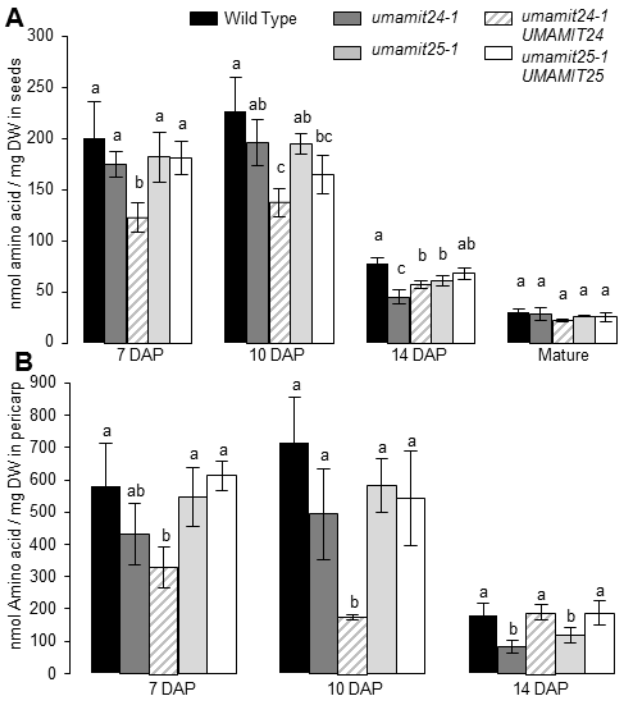


**Figure 16: Protein and amino acid contents in seeds and pericarp tissue.** Plants were grown side by side in soil under long day conditions for five weeks. One biological replicate represents seeds or pericarp tissue from two siliques. Amino acid content in seeds (A) and pericarp tissue (B) or protein content in seeds (C) and pericarp tissue (D). Samples were harvested at day 7, 10, 14 DAP or at mature stage. Error bars correspond to standard deviation ($n \geq 3$ biological replicates). Significant differences ($p<0.05$) are indicated by different letter according to one way ANOVA in conjunction with Tukey's test. Contents in individual amino acids from the same dataset are presented in Appendix 29 and Appendix 30.

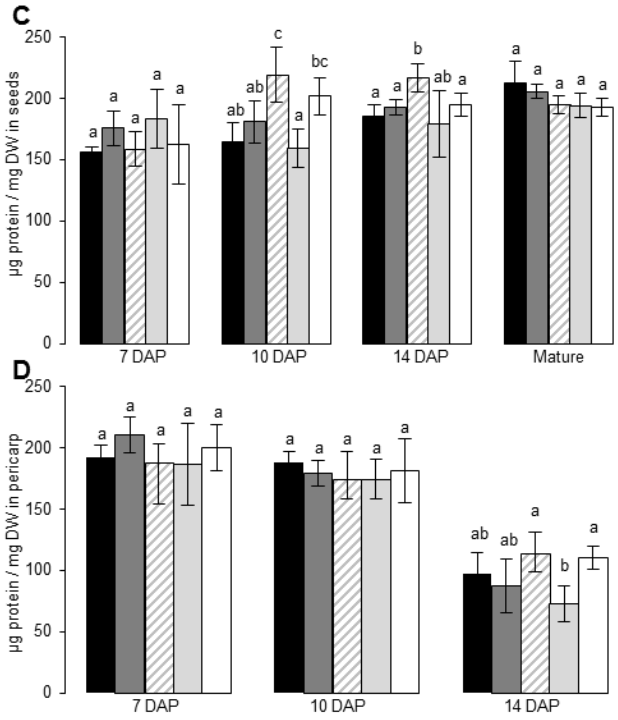


**Figure 16: Continued**

### *3.4.6. Amino acid transfer to the seeds was reduced in the umamit25-1 mutant*

Amino acid content reduction in the *umamit* mutants either could result from a decrease in amino acid transport into the seeds or from an indirect effect related to a perturbation in amino acid metabolism. In order to measure amino acid transport to the seeds directly, the transfer of $^{15}$N-labeled Gln into the seeds was measured. In this assay, a solution containing $^{15}$N-Gln and $^{13}$C-sucrose was fed to the siliques for four hours, followed by silique dissection and quantification of $^{15}$N and $^{13}$C enrichment in seeds. Siliques at 10 DAP were used for this experiment, since nitrogen loading in seeds is still ongoing at that time compared to 14 DAP (Appendix 27 and data not shown). The $^{15}$N/$^{13}$C ratio was reduced in the *umamit25-1* plants compared to the WT (Figure 17), suggesting that amino acid transport into the seeds is affected in the *umamit25-1* mutant. A similar, non-statistically significant trend was noted for the *umamit24-1* mutant.

## 3.5. Discussion

### *3.5.1. Proposed roles of UMAMIT24 and UMAMIT25 during seed development*

We have shown here that UMAMIT24 and UMAMIT25, previously uncharacterized members of the UMAMIT family, function as amino acid exporters when expressed in yeast (Figure 11). These results are in good accordance with previous studies reporting amino acid export activities for other UMAMIT genes (Besnard *et al.*, 2016; Ladwig *et al.*, 2012; Muller *et al.*, 2015). In addition, we did not observe an import activity for either UMAMIT24 or UMAMIT25 for Gln, Trp, His, Pro, or Met under the conditions tested (data not shown).

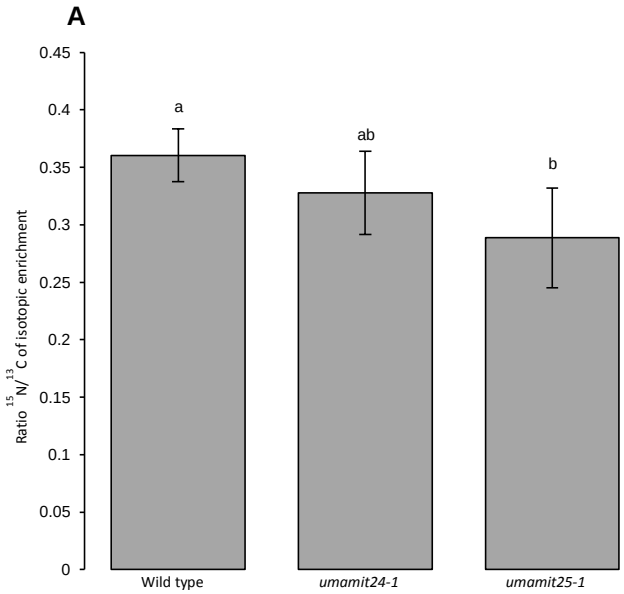


**Figure 17: Glutamine and sucrose transfer assay in isolated siliques.** $^{15}N/^{13}C$ ratio of isotopic excess found in seeds at 10 DAP. Siliques were excised 10 DAP, and transport was measured according to the experimental design presented in methods. One biological replicate correspond to three siliques worth of seeds coming from the same plant. $^{15}N$ and $^{13}C$ enrichment have been calculated against the non-labelled sample control. Error bars correspond to standard deviation ($n \geq 3$ biological replicates). Significant differences ($p<0.05$) are indicated by different letters according to one way ANOVA in conjunction with Tukey's test.

UMAMIT24-GFP mainly localized to the ER membrane in plant cells (Figure 14; Appendix 24 and 25). The functional significance of amino acid transport activity across the ER membrane is not understood in plants. However, other plant amino acid transporters that localize at least partially to the ER membrane have been reported in the past (Okumoto *et al.*, 2004a; Peng *et al.*, 2014b). These transporters might mediate transport across the ER to transport amino acids derived from endocytosis or protein degradation within endomembrane systems. Alternatively, this observed localization might be artefactual due to the expression system used: exit of UMAMIT24 from the ER may require an accessory protein ({Palacín, 2004 #427; Rosas-Santiago *et al.*, 2015;

Rosas-Santiago *et al.*, 2017; Ruggiero *et al.*, 2008) or a modifying protein (Popov-Celeketic *et al.*, 2016) present at too low amounts in those cells where the tagged transporter is expressed. A correlation between the activity of an ER-localized amino acid transporter and amino acid uptake at the plant level has previously been reported for *Oryza sativa* Amino Acid Permease 6 (OsAAP6;Peng *et al.*, 2014b), suggesting that the protein is also active at the plasma membrane. While most of the fluorescence of the UMAMIT24-GFP protein localized to the ER, it is thus possible that, similar to OsAAP6, a small fraction is addressed to the plasma membrane. UMAMIT24 is expressed in chalazal seed coat and in the pericarp (Figure 12). The chalazal seed coat is symplasmically connected with the funicule phloem and represents the entry point of metabolites to the seed. In young seeds, the chalazal seed coat is further symplasmically connected with the outer and inner tegument cells constituting the seed coat, and this connection starts to decrease around stage 5 during embryogenesis (*i.e.* 6 DAP) (Stadler *et al.*, 2005). Interestingly, the onset of UMAMIT24 expression coincides with this decrease in symplasmic connectivity. Therefore, UMAMIT24 might be involved in amino acids unloading once the chalazal seed coat becomes symplasmically isolated. The idea that metabolite transport across chalazal seed coat requires membrane carriers is substantiated by previous studies showing that (i) mRNA population within this tissue is enriched in the expression of genes involved in transport processes (Becker *et al.*, 2014), and (ii) transporters for other molecules such as sugars (Chen *et al.*, 2015), sulfate (Zuber *et al.*, 2010) and phosphate (Vogiatzaki *et al.*, 2017) are expressed within chalazal seed coat. Moreover, some of the previously characterized UMAMITs which

are involved in amino acid transport to the silique (UMAMIT11, UMAMIT14, and UMAMIT18) are expressed in this tissue as well (Ladwig *et al.*, 2012; Muller *et al.*, 2015).

UMAMIT25 is localized at the plasma membrane and is expressed in the endosperm as well as the pericarp (Figure 13 and Figure 14). During Arabidopsis seed development, the endosperm transitions through three stages; syncytial, cellularization, and cellular (Becker *et al.*, 2014). During the cellularization stage, cell walls gradually develop around free nuclei to form endosperm cells. This transition happens three to four DAP, shortly before the onset of UMAMIT25 induction. UMAMIT25 could be involved in the amino acid export from mature endosperm cells, which consists the last step of nutrient transport to the developing embryo, along with UMAMIT28, whose expression was detected first at the cellularizing endosperm (Muller *et al.*, 2015). Expression of both genes was also detected in the pericarp, but our analysis could not resolve in which tissue (Figure 12 and Figure 13). This suggests an additional role for these proteins, potentially in amino acid transport from the pericarp tissue to the developing seeds.

### *3.5.2. Loss of the expression of UMAMIT24 or UMAMIT25 affects amino acid transfer to the seed*

Seed yields of *umamit24-1* and *umamit25-1* were not different from the WT, although a slight decrease in the size of *umamit25-1* seeds was observed (Appendix 26 and Figure 15). Nevertheless, amino acid levels were lower in both seed and pericarp tissues in both of the *umamit* lines at 14 DAP, corresponding to the late seed filling stage, and the difference was at least partially complemented by adding another copy of

the genes (Figure 16). At 10 DAP, the previous time point studied, the transfer of amino acids from the silique to the seed was reduced in the *umamit25-1* mutants (Figure 17). Between 10 and 14 DAP, rapid protein degradation is happening in the pericarp (Figure 16 and Appendix 27), much of which are presumably transported to the seeds as amino acids. The loss of UMAMIT24 and UMAMIT25 function affecting the transfer of amino acids from the pericarp to the seed could explain the decreased amino acid content observed in the seeds. It is possible that this decrease in amino acid transfer feeds back to the pericarp, decreasing protein degradation and/or amino acid transfer from the mother tissue, and hence free amino acid content. This feedback would be reminiscent of the effect observed in the shoot-to-root transfer of amino acid the in *umamit14* and *18* loss-of-function plants (Besnard *et al.*, 2016). The fact that this does not translate into a decrease in mature seed protein content suggests that the effect is counter balanced by a slightly longer filling period, which could not be measured by our 3-time point time course. A study on two different canola varieties revealed that pod walls can serve as a reservoir for nitrogen for seeds, especially under low nitrogen conditions where up to 70% of the nitrogen found in seeds came from the pod walls (Girondé *et al.*, 2015). Similarly, we could hypothesize here that during Arabidopsis embryogenesis, the nitrogen found in the pericarp is remobilized in the form of amino acids which are exported to the seeds by UMAMIT24 and UMAMIT25 starting at 12DAP.

We summarize the roles of UMAMIT24 and UMAMIT25 in the model presented in Figure 18. UMAMIT24 is involved in the transport from the chalazal seed coats to the surrounding cells, whereas UMAMIT25 is involved in the unloading of amino acids

from the endosperm. The lack of these activities results in a decreased amino acid unloading to the seed tissue, which is reflected in the decrease in seed amino acid content at 14 DAP. The decrease in the unloading also results in the slower amino acid transfer from the mother tissue, which affects the amino acid content in the pericarp tissue. The extent to which the lack of UMAMIT24 and UMAMIT25 activities in the pericarp is contributing to this effect remains to be elucidated.

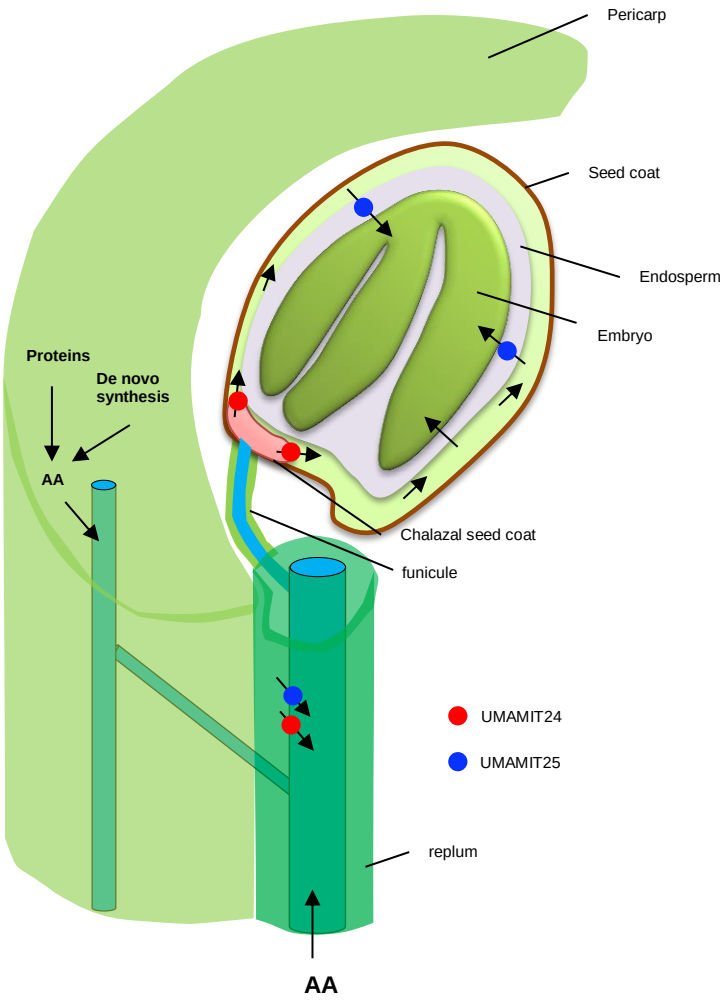


**Figure 18: Model of amino acid transport into the seed by UMAMIT24 and UMAMIT25.** Amino acids are delivered from the mother plant to the growing embryo through the funicule, then unloaded from the chalazal seed coat. UMAMIT24 is involved in this process. Once out of the chalazal seed coat, amino acids are transported radially through the integument cells, then delivered to the endosperm, and finally to the embryo. UMAMIT25, acting in the endosperm cells, unloads amino acids out of those cells, which then become available for import by the embryo. Loss of function of UMAMIT25 reduces the amino acid unloading process, and decreases amino acid transfer from the rest of the plant body to the developing seed.

# CHAPTER IV

# USE OF AMINO ACID EXPORTERS TO UNDERSTAND THE SIGNALING FUNCTION OF AMINO ACIDS

## 4.1. Introduction

In addition to their role in the biosynthesis of important molecules, such as DNA, proteins, and secondary metabolites, amino acids are known to function as signaling molecules through various pathways to report N-status and trigger appropriate metabolic and cellular responses. In prokaryotes, PII like proteins can sense N-status through amino acid levels; PII conformation reversibly toggles between non-uridylated and uridylated forms in the presence of Gln or its precursor, 2-oxoglutarate (2-OG), respectively. Non-uridylated PII proteins promotes responses under N sufficiency conditions (e.g. amino acid synthesis, repression of $NH_4^+$ transport) while the uridylated form triggers those promoted under N deficiency (e.g. N fixation), making PII the master regulator of the N status (Dixon and Kahn, 2004).

In eukaryotes, distinct pathways communicate N sufficiency and deficiency signals, both of which involve amino acids. The evolutionarily conserved Target Of Rapamycin Complexes (TORCs) are cytosolic kinases that are conserved among fungi and metazoans, and are activated in the presence of amino acids such as Gln, Leu and Arg (Nakajo *et al.*, 2004; Fumarola *et al.*, 2005; Kingsbury *et al.*, 2015; Shimobayashi and Hall, 2016). TORC activation is critical for promoting biosynthetic pathways (e.g. protein synthesis) and repressing catabolism (e.g. autophagy) under N-sufficient

conditions. Another cytosolic kinase, General Control non de-Repressible 2 (GCN2), is important for sensing N deficiency and is also conserved in fungi and metazoans. GCN2 does not sense amino acids *per se*, however it is activated when bound to de-acetylated tRNAs, which are more abundant when the cell is deprived of amino acids. Activated GCN2 represses protein synthesis by deactivating eukaryotic initiation factor 2α (eIF2α) through phosphorylation (Chantranupong *et al.*, 2015). Therefore, TORC and GCN2 monitor the cytosolic amino acid sufficiency and deficiency, respectively.

Extracellular amino acids are also important signaling molecules within the central nerve systems in animals. Glu, GABA, Gly, and Asp released from presynaptic membrane through exocytosis are perceived by the receptors located in the postsynaptic membrane and alter their ionic conductivity, hence functioning as neurotransmitters (Kandel *et al.*, 2000).

In recent years, homologs of proteins involved in the pathways described above have been found in plants, and some plant-specific roles of amino acids in signaling have also been elucidated. The plant counterpart of bacterial PII protein is a chloroplastic protein, as befits its prokaryotic origin (Forchhammer and Lüddecke, 2016). It has been show that plant PII protein directly interacts with acetylglutamate kinase (NAGK) which is involved in Arg biosynthesis and regulates its activity (Ferrario-Méry *et al.*, 2005; Llácer *et al.*, 2010). The Arabidopsis genome encodes only one copy of a PII homolog, and the loss-of-function mutant does not show a strong phenotype, suggesting that unlike the bacterial PII, plant PII proteins are not the master regulator of N metabolism.

TORC and GCN2 seem to play important roles in plants for sensing N status. The plant TOR has now been well characterized; it acts as an integrator of multiple signaling networks to control processes such as translation activation, cell division, and N assimilation, which promotes growth (Xiong and Sheen, 2014; Dobrenel *et al.*, 2016). However, the mechanism through which amino acid levels alters TORC activity is different in plants. The proteins that senses amino acids and activate TORC in yeast and mammals are lacking in plants (Rexin *et al.*, 2015). A very recent study showed that the metabolites involved in the biosynthesis of Cys, rather than Cys itself, regulate both TORC and GCN2 activities (Dong *et al.*, 2017). The activation of TOR-related pathways depends upon the amount of the sulfur precursors for Cys synthesis, whereas a reduction in O-acetylserine levels, a non-proteinogenic amino acid which is the carbon and nitrogen backbone for Cys synthesis, activates GCN2 (Dong *et al.*, 2017). In another study, it was shown that β-aminobutyric acid (BABA), a stress-induced amino acid and an analog to Asp, can competitively inhibit the binding of Asp to aspartyl-tRNA synthetase and mimic amino acid depletion, which triggers GCN2-dependent eIF2α phosphorylation and growth arrest (Luna *et al.*, 2014). These results show that TORC regulation through amino acids utilizes a different pathway in plants compared to fungi and metazoans, whereas the activation of GCN2 through unloaded tRNAs might be conserved among plants, fungi, and mammals.

Glutamate receptor-like proteins (GLRs), mammalian homologs of which sense extracytosolic amino acids, are also found in plants. They are located in different cell membranes: the plasma membrane, the inner and outer chloroplast membranes, and the

mitochondrion (reviewed in Weiland *et al.*, 2016). Studies using electrophysiology and calcium imaging revealed that plant GLRs can be activated by at least 12 out of the 20 proteinogenic amino acids (Qi *et al.*, 2006; Stephens *et al.*, 2008; Vincill *et al.*, 2012; Tapken *et al.*, 2013). Diverse functions have been associated with the role of GLRs with the use of knockouts or overexpressor lines. These studies implicated GLRs in various processes, such as abscisic acid (ABA) synthesis, stomatal closure (Kang *et al.*, 2004), root branching and maintenance of the primary root meristem, (Walch-Liu *et al.*, 2006; Vincill *et al.*, 2013), gravitropism (Miller *et al.*, 2010), pollen tube signaling (Michard *et al.*, 2011), and establishment of a defense response (Kwaaitaal *et al.*, 2011; Vatsa *et al.*, 2011). In particular, plant GLRs seem to mediate long distance signaling transmission; in the Arabidopsis double mutants for GLRs *glr3.3 glr3.6*, distal leaves from an original wounded leaf displayed retarded induction of jasmonate-zim domain 10, a marker of the jasmonate pathway leading to defense responses (Mousavi *et al.*, 2013). In a more recent study using the same mutant, it was suggested that GLRs send an electrical current through the phloem upon wounding to the unwounded tissue, which triggers the defense responses (Hedrich *et al.*, 2016). It is suggested that a disruption of amino acid content in the apoplasm caused by a stress affects the activity of GLR, which triggers the propagation of the defense response. Therefore, extracellular amino acid could be functioning as Damage Associated Molecular Patterns (DAMPS) (Forde and Roberts, 2014).

These well-conserved sensors for intracellular and extracellular amino acid levels in plants suggest that an alteration of amino acid homeostasis via amino acid transport

could trigger the pathways associated with TOR, GCN2, PII and/or GLRs activation. Indeed, several independent studies in which the transport of amino acids has been enhanced or altered seem to confirm this possibility. A study using knockout lines for the amino acid importer LHT1 revealed that amino acid contents within the tissue and the extracellular fluid were both altered, and these mutants showed a constitutive stress response (Hirner *et al.*, 2006a). Later, another study confirmed that the stress observed was linked to amino acid homeostasis imbalance, and that the mutants were resistant to a broad spectrum of pathogens in a salicylic acid (SA) dependent manner (Liu *et al.*, 2010). Similarly, CAT1 overexpression in plants caused a stunted phenotype, associated with increased SA levels, resistance against *Pseudomonas synringae*, and the upregulation of genes associated with the development of systemic acquired resistance (pathogen related genes or *PR*) (Yang *et al.*, 2014). Similar observations were reported upon over-expression of Glutamine Dumper 1 (GDU1), a single-transmembrane protein. Plants over-expressing GDU1 showed a constitutive stress phenotype (Pilot *et al.*, 2004) similar to *lht1* knockouts and CAT1 overexpressor mutants. It was later shown that GDU1 overexpression stimulates non-selective amino acid export systems (Pratelli *et al.*, 2010) which requires GDU1 interaction with an ubiquitin ligase Loss of GDu1D 2 (LOG2) (Yu, 2015; Guerra *et al.*, 2017).

For a long time, it was not possible to stimulate amino acid export activity by directly altering the expression of amino acid exporters due to the lack of understanding of amino acid export molecular mechanisms (Okumoto and Pilot, 2011). During this Ph.D., we have discovered that a family of transporters, namely Usually Multiple Acid

Move In and Out transporters (UMAMITs), contains putative amino acid exporters such as UMAMIT14, UMAMIT18, UMAMIT23, UMAMIT24 and UMAMIT25 (for more information, see chapter I II and III). The goal of this study was to investigate the effects of overexpressing UMAMITs genes on plant development, growth, and reproduction to further examine the link between amino acid transport and stress responses. The results presented here show strong evidence that amino acid export activity positively correlates with stress response, most likely due to the establishment of a constitutive stress response via a SA-dependent pathway.

## 4.2. Material and methods

### *4.2.1. Plant culture*

Arabidopsis plants (Col-0) were grown on soil in long days (16 h light at 50 $\mu mol.m^{-2}.s^{-1}$ at the soil surface, 50% humidity, 22°C) in soil composed of a 2/1 ratio Sunshine Mix™ / Vermiculite in 6x6x5 cm pots. Plants were watered 0.15 g/L MiracleGro™ fertilizer (24/8/16, N:P:K) three times a week. For kanamycin selection, seeds were sowed on half-strength MS medium (½ Murashige and Skoog salt supplemented with 30 mM sucrose and 0.8% agarose with pH adjusted to 5.8 with KOH) containing 50 µg/ml kanamycin. Kanamycin resistant plants were transferred to the long day conditions described above after one week of selection. For *in vitro* growth, Arabidopsis seeds were sowed in 100x15 mm petri dishes containing MS medium. Wild type Arabidopsis plants were transformed using the floral dip method using *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Clough and Bent, 1998) to

generate the overexpressor lines for UMAMIT14, UMAMIT18, UMAMIT23, UMAMIT24 and UMAMIT25.

### *4.2.2. DNA constructs*

*UMAMIT14*, genomic sequence (from ATG to stop codon) was PCR-amplified from Col-0 genomic DNA with primers adding *attb1* and *attb2* cassettes flanking the genomic region. The PCR fragments were cloned into pDONRZeo vector using BP clonase II (Life Technologies, USA), and all entry clones were sequenced prior to use. *UMAMIT14*, genomic sequence was transferred to the destination vector pPWYTkan using LR clonase II (Life Technologies, USA) to generate the UMAMIT overexpressor lines *35S:UMAMIT14*. This vector is a derivative of pJHA212K (Yoo *et al.*, 2005; R. Pratelli and G. Pilot, unpublished data, Appendix 13). The same cloning steps apply to UMAMIT18, UMAMIT23, UMAMIT24 and UMAMIT25. List of primers used for cloning is available in Appendix 31.

### *4.2.3. Analytical methods*

Leaves of five week-old Arabidopsis grown under long day conditions were harvested and frozen in liquid nitrogen, lyophilized and ground with a 4 mm glass bead in a 2 ml microtube. Total free amino acids were extracted by adding 400 µl of chloroform and 10 mM HCl (1:1 mixture) to 0.5-2 mg of plant tissue in a tube containing of 0.2 nmol of dry norvaline. The aqueous phase was collected, the organic phase was re-extracted with HCl and chloroform, and the supernatants were pooled. Amino acids were analyzed by Ultra Performance Liquid Chromatography (UPLC; Waters, USA; Collakova *et al.*, 2013), after derivatization with the AccQ-Tag Chemistry

Kit following the manufacturer's recommendations, and injection of 0.5 µl of the derivatized extract. Amino acid content in the phloem exudates was normalized against $K^+$ content, determined using an Inductively Coupled Plasma Atomic Emission Spectophotometer (Analytical instruments, USA). Leaf amino acid content was normalized against dry weight and norvaline content. C and N content of 2 mg dry seeds was measured using the dry combustion method with a CE Instruments NC 2100 elemental analyzer (ThermoQuest, Italy; at the Duke Environmental Stable Isotope Laboratory). For protein extraction, 100 mg of fresh leaves were ground in 250 mM Tris pH 8.5, 25 mM EGTA, 30% Sucrose, 5 mM DTT, 10% c0mplete™ proteinase inhibitor cocktail (Roche, USA). Seed protein extraction was performed as described by (Gallardo *et al.*, 2002b) using 1 mg of mature Arabidopsis seeds. Protein quantification was then performed using a Bradford assay (Bradford, 1976).

### *4.2.4. Protein electrophoresis and western blot*

Ten µg of extracted leaf proteins were denatured at 50°C for 15 min in a ratio 1:1 v/v of loading buffer (62.5 mM Tris-HCl pH 6.8 adjusted with KOH, 2.7 M glycerol, 150 µM bromophenol blue and 70 mM SDS). Proteins were separated by SDS-PAGE with the Mini-Protean® system (Biorad, USA) according to manufacturer's protocol, into a Mini-Protean® TGX™ gel 4-20% (Biorad, USA). Proteins were then transferred to a Hybond ECL™ (GE Healthcare, UK) nitrocellulose membrane using Mini Trans-Blot® Cell, following manufacturer's recommendations. UMAMIT14-c-myc was detected using an anti-cmyc rabbit polyclonal IgG (Santa Cruz Biotechnology, clone sc-789; 1/4000) and an anti-rabbit IgG (conjugated to horseradish peroxidase; 1/10,000).

Antibodies were detected by reaction with the ECL™ Prime Kit (GE Healthcare, UK) using manufacturer's recommendations.

### *4.2.5. RNA extraction and qRT-PCR*

RNA was extracted using the RNAeasy plant kit (Qiagen, USA) according to the manufacturer's recommendation. Two µg of total RNA was used for cDNA synthesis with random primers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The list of the genes and primers used for qRT-PCR are described in Appendix X. qRT-PCRs were performed using SyBR® Green PCR Master Mix in a 7500 Real Time PCR System (Applied Biosystems, USA) according to manufacturer's recommendation.

### *4.2.6. [$^3$H]Gln uptake in planta*

Uptake of [$^3$H]Gln was performed according to Pratelli *et al.,* (2010) using two-week-old plants grown *in vitro* in a solution containing 1 ml half strength MS medium supplemented with 2 mM glutamine + [$^3$H]Gln with a final specific activity of 37 kBq.µmol$^{-1}$. Efflux was performed as described by Pratelli *et al.*, (2010). Radioactivity was then measured for each plant sample and bathing media using a LS 6500 Multipurpose scintillation counter (Beckman Coulter, USA)

### *4.2.7. Sporangiophore assay and trypan blue staining for Hyaloperonospora arabidopsidis infection*

The sporangiophore assay was performed using *Hyaloperonospora 99rabidopsidis* isolate Noco2 on 12-day-old seedlings. Trypan staining was performed to visualize hyphal growth and cell death on samples collected at 7 dpi (days post

inoculation). Both, the sporangiophore assay and trypan staining were performed as described in McDowell et al.(2011).

#### *4.2.8. Statistical analyses*

One-way ANOVA in conjunction with Tukey's test, or t-tests were used to determine significant differences ($p<0.05$) between samples in JMP (SAS, USA).

## 4.3. Results

If UMAMIT14 is able to export amino acids in plants, its over-expression is expected to mimic the *gdu1-1D* phenotype, characterized by enhanced amino acid export (Pilot *et al.*, 2004; Pratelli *et al.*, 2010; Besnard *et al.*, 2016). Transgenic Arabidopsis lines were constructed that over-express *UMAMIT14-cmyc* under the control of the 35S promoter.

The observed phenotype was indeed reminiscent of *gdu1-1D*, and the severity of the phenotype observed with these over-expressor lines was positively correlated with the level of c-myc-tagged UMAMIT14 protein (Figure 19). A representative line, *35S:UMAMIT14-4*, which accumulated around 130 (in roots) and 11,000 times (in leaves) more *UMAMIT14* mRNA than the wild type, was chosen for further analysis (Table 3).


**Figure 19: Phenotype of the 35S:UMAMIT14 lines and corresponding western blot.** (A) Phenotype of three week-old wild type and 35S:UMAMIT14 lines grown in soil in long day conditions. Each plant corresponds to an independent transformation event. (B) Western blot targeting UMAMIT14 tagged with c-myc. Each lane contained 10 µg of proteins extracted from wild type and 35S:UMAMIT14 leaves. Arrowhead indicates 40 kDa.

**Table 3: *UMAMIT14* mRNA accumulation in wild type and 35S:UMAMIT14 roots and leaves.** *UMAMIT14* mRNA levels were determined by qRT-PCR and normalized against *ACTIN2* mRNA accumulation. *UMAMIT14* mRNA levels are expressed relative to the wild type (WT) root sample, set at 1. Significant differences ($p<0.05$) are indicated by different letters according to one way ANOVA in conjunction with Tukey's test (2 biological replicates).

| Genotype | Roots | Leaves |
|---|---|---|
| WT | 1 ± 0.22(a) | 0.062 ± 0.003 (b) |
| 35S:*UMAMIT14-4* | 134 ± 15 (c) | 670 ± 133 (d) |

Under long day conditions, *35S:UMAMIT14-4* displayed a shorter life cycle, reduced plant size, biomass, and total number of seeds, siliques, and seeds per silique compared to the wild type. While protein, nitrogen and carbon contents were identical between the two lines, seeds from the over-expressor were ~50% heavier than wild type seeds (Table 4).

**Table 4: Characteristics of nine-week-old Arabidopsis plants grown in soil under long day conditions.** Biomass represents all plant tissue collected from the aerial parts, minus the seeds. Significant differences ($p<0.05$) are indicated by different letters according to one-way ANOVA in conjunction with Tukey's test (n=4 biological replicates).

| | Plant size (cm) | Biomass (mg) | Weight of 100 seeds (mg) | Number of seeds (thousands) | Number of seeds per silique | %C in seeds | %N in seeds | Seed protein content ($\mu g.mg^{-1}$ DW) |
|---|---|---|---|---|---|---|---|---|
| WT | 36.5 (a) | 657 (a) | 2.08 (a) | 134 (a) | 49.8 (a) | 55.9 (a) | 4.82 (a) | 134.5 (a) |
| 35S:*UMAMIT14-4* | 23 (b) | 240 (b) | 3.2 (b) | 36 (b) | 23.3 (b) | 54.7 (a) | 4.85 (a) | 145.3 (a) |

Although total free amino acid content in the leaves of the over-expressor was not different from the wild type, the relative content of some amino acids was changed: Ala and Gln contents decreased 1.5 and 3 times respectively in the *35S:UMAMIT14-4* line compared to the wild type, while His, Ser and Thr contents increased 1.7, 1.7 and 2 times respectively (Figure 20).

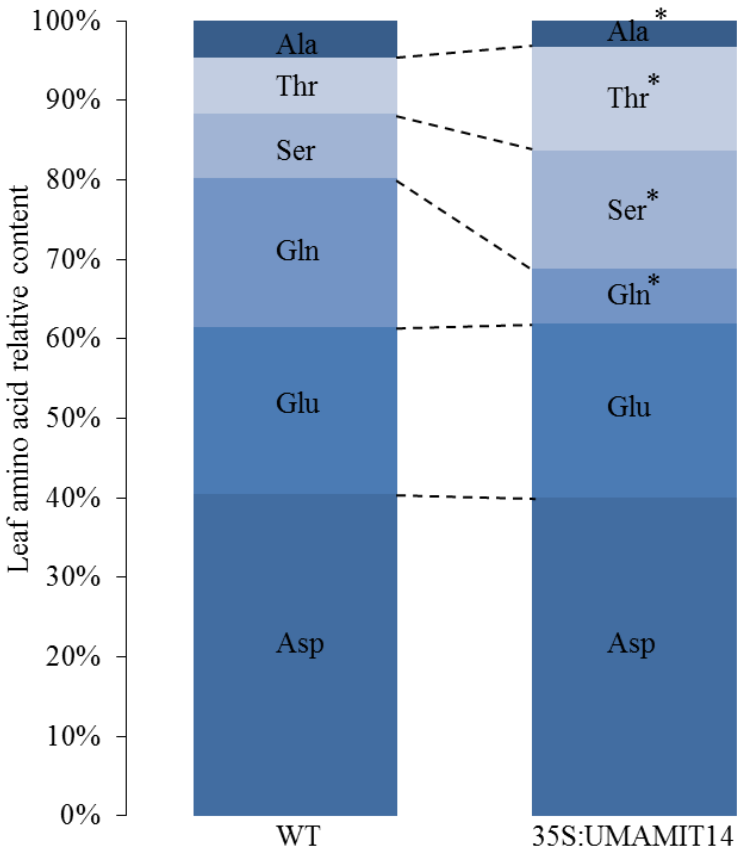


**Figure 20: Leaf amino acid composition of five week-old wild type and 35S:UMAMIT14 plants.** Sum of Ala, Thr, Ser, Gln, Glu and Asp represent 88% of total amino acids in the wild type leaf. Significant differences are indicated by a star according to t-test ($p<0.05$ with $n=4$). Total amino acid content in the leaf of wild type and overexpressor line were 111.7 and 110.9 nmol / mg DW, respectively.

If the over-expressor *35S:UMAMIT14-4* plants have increased amino acid export activities, it is expected that they will secrete more amino acids, reminiscent of the *gdu1-1D* phenotype (Pilot *et al.*, 2004). Amino acid transport was measured in wild type, over-expressor *35S:UMAMIT14-4*, knockout line *umamit14-1*, as well as the double knockout *umamit14-1 umamit18-1*, immersed in a liquid medium, a condition which has been used previously to study the amino acid uptake and efflux in the wild type and

*gdu1-1D* transport (Pilot *et al.*, 2004; Pratelli *et al.*, 2010). The *gdu1-1D* mutant, used as a positive control, showed the expected changes in both uptake and efflux (Yu *et al.*, 2015). Surprisingly, no difference in uptake or export was detected between *35S:UMAMIT14-4* and the wild type (Figure 21). As observed in a previous study, neither uptake nor efflux were changed for the knockout lines compared to the control (Besnard *et al.*, 2016; Figure 21).

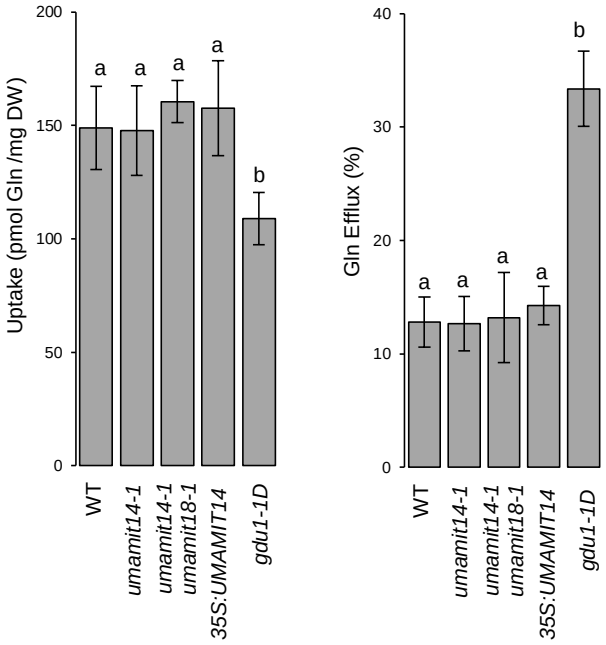


**Figure 21: Uptake and efflux of [$^3$H]Gln by Arabidopsis seedlings.** Plants were grown *in vitro* for one week, then transferred into liquid J medium with 1% sucrose for four days. Uptake and efflux were performed for 20 minutes each. (A) Gln uptake using 2 mM Gln. (B) Gln efflux, expressed as a percentage of total uptake. Error bars correspond to standard deviation (n=4 biological replicates). Significant differences ($p<0.05$) are indicated by different letters according to one way ANOVA in conjunction with Tukey's test

Expression of *PR1*, a gene induced by salicylic acid (SA) (Malamy *et al.*, 1990), was previously shown to be induced in *gdu1-1D*, and the SA-mediated stress response was suggested to be the underlying cause of the growth defect of this mutant (Liu *et al.*, 2010). *PR1* mRNA levels increased by 47± 20 fold ($p<0.05$) in the *35S:UMAMIT14-4* plants compared to the wild type, suggesting that *35S:UMAMIT14-4* plants display a constitutive SA-mediated stress response. We reasoned that this line would then be resistant to biotrophic pathogens, whose growth is decreased by plant synthesizing higher SA levels (McDowell *et al.*, 2011; Wei *et al.*, 2015). Resistance of over-expressor *35S:UMAMIT14-4* plants to the biotrophic pathogen *Hyaloperonospora 105rabidopsidis* (Hpa) was then tested, and found to be much higher than the wild type, with no hyphae developing in the mutant leaves, and high level of cell death (Figure 22). These results suggest that over-expression of *UMAMIT14* disrupts amino acid homeostasis leading to a constitutive immune response.

Previous studies from our group and others indicate that in addition to UMAMIT14, other UMAMITs (UMAMIT18, UMAMIT24 and UMAMIT25) function as amino acid exporters (Ladwig *et al.*, 2012; Muller *et al.*, 2015; Besnard *et al.*, 2016). If the phenotypic changes observed in *UMAMIT14* over-expressing plants are due to its increased amino acid export activity, a similar phenotype would be observed by over-expressing other *UMAMIT* genes with amino acid exporter activities. Therefore we overexpressed other UMAMIT transporters, known to export amino acids in yeast. In addition, we also over-expressed *UMAMIT23* as a negative control, which is closely

related to *UMAMIT24* but does not show strong amino acid exporter activities (Chapter III).

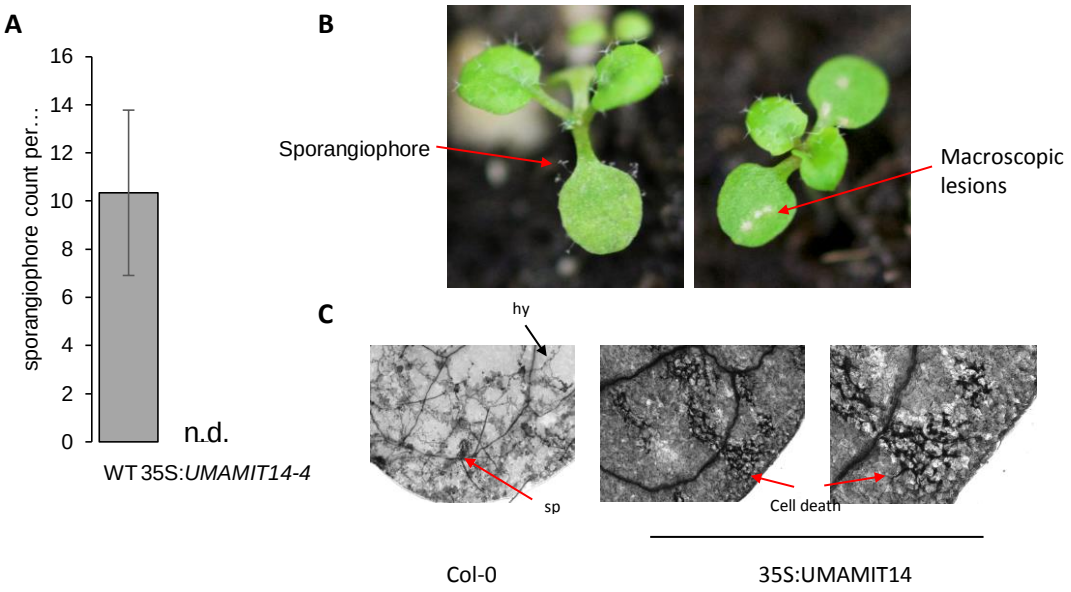


**Figure 22: Response of WT and 35S:UMAMIT14-4 to *Hyaloperonospora arabidopsidis* infection.** (A) Sprorangiophore counts on 11-days-old cotyledons. The cotyledons were inoculated with $5x10^4$ spores per ml and counted after seven day post inoculation. Nd; no sporangiophores were detected on cotyledons. (B) Col-0 (left) and 35S:UMAMIT14 (right) inoculated with *Hyaloperonospora arabidopsidis* (Noco2), 7 days post inoculation. Col-0 cotyledons display asexual reproductive structures (sporangiophores) whereas 35S:UMAMIT14 cotyledons display macroscopic lesions indicating cell death/ necrosis. (C) Trypan blue-stained cotyledons of Col-0 (left) and 35S:UMAMIT14 (center and right) inoculated with *Hyaloperonospora arabidopsidis* (Noco2), 7 days post inoculation. Hyphal growth (hy) and sporangiophores (sp) can be observed in Col-0 cotyledons whereas cell death is apparent in 35S:UMAMIT14 cotyledons.

*UMAMIT18*, *UMAMIT24* and *UMAMIT25* over-expressing lines showed stunted phenotypes reminiscent of 35S:*UMAMIT14-4*. They were smaller in stature and flowered earlier compared to the WT (Figure 15). However, an overexpression of *UMAMIT23* did not result in stunted phenotypes (Figure 23). These results support the

hypothesis that an excess amino acid export activity caused the stress phenotypes observed in *35S:UMAMIT14-4* plants.

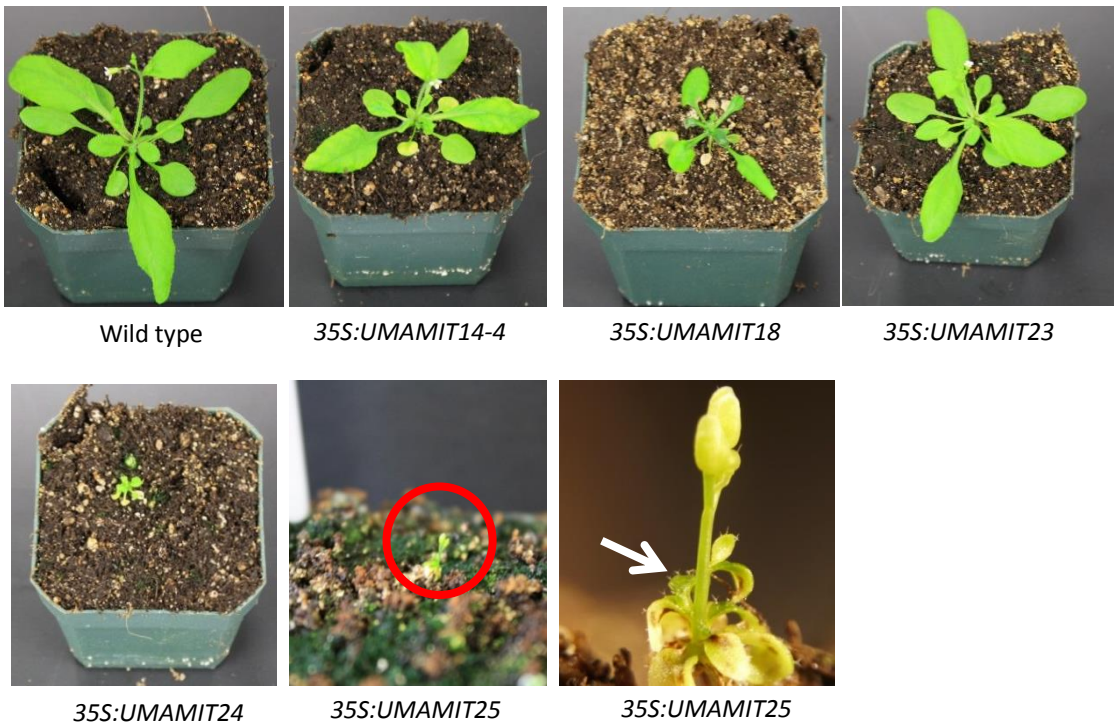


**Figure 23: Phenotypes of 5 week-old UMAMIT overexpressor lines.** Arrowhead points at a trichome. A representative picture was chosen from 8 different plants per line displaying a similar phenotype

### 4.4. Discussion

In the present study, it has been shown that over-expressing four independent UMAMITs that function as amino acid exporters results in a stunted growth phenotype (Figure 23). Further studies of one such over-expressor line, *35S:UMAMIT14-4*, revealed an alteration of several physiological traits such as decrease in biomass (Table 4) and leaf amino acid composition (Figure 20). *UMAMIT14* over-expression also resulted in an upregulation of a marker gene responding to the SA-dependent signaling

pathway, *PR1* (Malamy *et al.*, 1990; Van Loon and Van Strien, 1999) and an increased resistance to a pathogen, (Figure 22), both hallmarks of elevated biotic stress response.

The results from our study are in agreement with previous reports in which the mis-regulation of amino acid transport leads to a stress response. The exact mechanism through which amino acid imbalance causes such stress responses remains to be elucidated. However, some evidence suggests that the control of metabolite transport is important for the survival of pathogens. Within plant leaves, the plant-pathogen interaction often happens in the apoplasm; pathogens feed on the metabolites found in the apoplasm, while they inject effector molecules into the cell to compromise plant defense mechanisms (Giraldo and Valent, 2013). One such class of targets are the sugar transporters at the cellular membrane. Several members of the Sugar Will Eventually bE Transported (SWEET) gene family, which mediate sugar efflux from plant cells, are upregulated directly by bacterial effector molecules (Chen *et al.*, 2010; Chen, 2014). Conversely, the plant's defense mechanism increases sugar uptake from the apoplasm. A recent study revealed that flagellin-sensitive 2 (FLS2), a plant receptor which recognizes bacterial flagellin and initiates defense responses, and its co-receptor BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1), interacts with an Arabidopsis sugar transporter 13 (STP13) and activates the transporter activity to promote sugar uptake from the apoplasm (Yamada *et al.*, 2016). Therefore, it is tempting to hypothesize that amino acid transporters are targeted by the pathogen in a similar manner. Indeed, some pathogens were also found to enhance apoplastic amino acid content by increasing apoplastic protease activities (Solomon and Oliver, 2001).

Elevated amino acid levels caused by the pathogen infection could in turn activate the biotic defense responses of the plant. The site of such amino acid imbalance detection is currently not understood. Since proteins belonging to the GLR family are capable of detecting extracellular amino acids and elevating intracellular $Ca_2^+$ concentration, they could therefore be involved in this process (Forde and Roberts, 2014). Alternatively, the exporters' activity could change intracellular amino acid concentrations and lead to the repression and activation of TOR and GCN2 pathways, respectively, resulting in retarded growth. The results presented here do not permit us to identify which of these systems were triggered by the overexpression of UMAMITs. Genetic studies combining UMAMIT over-expressing mutants and loss-of-function mutations or RNAi in putative amino acid sensing pathways such as GLRs, GCN2, TOR proteins would be helpful in identifying the pathway leading to the stress responses.

Surprisingly, the *35S:UMAMIT14* overexpressor lines did not secrete more amino acids compared to the wild type plants. There are several possible explanations for this phenomenon. Firstly, amino acid importer activities might be altered during stress responses, similar to the case found in sugar transporters (Yamada *et al.*, 2016; Williams *et al.*, 2000). It is possible that such a mechanism also happens for amino acids, which would explain why *35S:UMAMIT14-4* plants do not secrete less amino acids compared to the control. Another possibility is an effect of intracellular amino acid concentrations. In *UMAMIT14* over-expressing plants, intracellular concentrations were decreased for Gln (the amino acid tested for secretion; Figure 21) and Ala, two major amino acids that are secreted from plants and are also preferred substrates of

UMAMIT14 (Chapter II). Therefore, the secreted amount could have been affected simply by a decreased intracellular substrate concentration.

In this study, *35S:UMAMIT23* was the only overexpressor line which did not display a stunted phenotype. There are two possible explanations. 1) UMAMIT23 does not transport amino acids, thus does not trigger stress responses. When expressed in yeast, UMAMIT23 was the weakest transporter when compared with UMAMIT24 and UMAMIT25 (see chapter III). The UMAMIT family contains transporters for non-amino acid substrate (i.e. auxin Ranocha *et al.*, 2013). Interestingly plants expressing *UMAMIT23Promoter:UMAMIT23-GFP* all had an early bolting time compared to the WT (data not shown), suggesting that the preferred substrate of UMAMIT23 might not be amino acids but hormones. 2) A subcellular localization study of UMAMIT23-GFP fusion showed that UMAMIT23 did not localize to the plasma membrane (data not shown), which means that UMAMIT23 in both yeast and plants could localize on another membrane, promoting cellular rather than intercellular amino acid trafficking.

### 4.5. Future work and perspectives

Although we have demonstrated in this study the link between amino acid export activity and stress responses, many questions remain unanswered. Previous studies and work reported in this Ph.D. have demonstrated that in yeast, the amino acid export rate of UMAMIT14, UMAMIT18, UMAMIT23, UMAMIT24 and UMAMIT25 differ, and that their specificity for each amino acid seems different as well (Ladwig *et al.*, 2012 and chapter II, III). In future work, it would be interesting to link the severity of the phenotype observed in each overexpressor line with (i) amino acids levels found in the

apoplasm (ii) SA-levels and SA-related defense response gene *mRNA* levels (iii) defense against pathogens, in order to identify the correlation between the level of single or certain classes of amino acids and resulting stress response.

Constitutive enhancement of amino acid export activities caused pleiotropic effects on metabolism, biomass production and stress responses. Therefore, a simple over-expression approach does not allow for a dissociation of the primary effects of an increase in amino acid export and subsequent plant response defense mechanisms. Inducible expression of exporters such as UMAMIT14 (i.e. tissue-specific or inducible expression) and genetic analysis of mutants that uncouple amino acid imbalance and stress phenotype (Yu *et al.*, 2015) would be necessary to further understand and dissect the connection between amino acid balance and stress responses. Recent studies using an inducible system for GDU1 showed that amino acid export activity, caused by GDU1 overexpression, precedes stress-related phenotypes (Yu, 2015). Similar studies using inducible lines for various UMAMITs genes would be of great interest to confirm these results. So far, we have successfully isolated inducible lines for UMAMIT14, UMAMIT18, UMAMIT23, UMAMIT24 and UMAMIT25, and tested their inducible trait with a GUS assay (data not shown). These lines will allow further understanding and separation of the sequences of events that occur upon induction (e.g. increased UMAMIT expression levels, changes in amino acid levels within the tissue and its apoplasm, changes in SA levels, expression of marker genes for SA-mediated stress response, and resistance to pathogens upon induction). Comparison between studies

using inducible UMAMIT lines and GDU1 lines would also be useful in identifying core pathways affected by amino acid export increase.

# CHAPTER V

# CONCLUSIONS

Before this study began, little was known on amino acid export mechanisms in plants, due to a lack of techniques for amino acid exporters identification. Using two novel techniques allowing the measurement of amino acid export in yeast, we have confirmed that some members of the UMAMIT family promote amino acid export when expressed in yeast, UMAMIT14 UMAMIT18 UMAMIT24 and UMAMIT25, which in plants are all involved in the long distance transport of amino acids. *umamit14* and *umamit18* losses-of-functions did not have any impact on the plant statute or yield, but plants did not properly transfer amino acids from the shoots to the roots. As a result, amino acid secretion in the medium was decreased. This raises the question of what is the consequence of a reduced secretion of amino acid by plants on the diversity of the root microbiome. *umamit24* and *umamit25* losses-of-function led to a decreased amount of amino acids transferred to the seeds during embryogenesis. This difference was gone in the mature seeds, suggesting the existence of compensatory mechanisms allowing the plant to restore the lack of nitrogen lost during seed filling. Reminiscent to the manipulation of amino acid importers, we have showed here that increasing amino acid export activity (UMAMIT25) led to a gain in yield. However, this induction needs to be tissue and time specific, as a uncontrolled expression of amino acid exporters can cause deleterious effects on plant statue, due to the establishment of a constitutive stress response. Future studies combining the manipulation of amino acid importers and

exporters at the same time could be of great interest to increase plant nitrogen use efficiency and yield in the agronomical context of the next 50 years.

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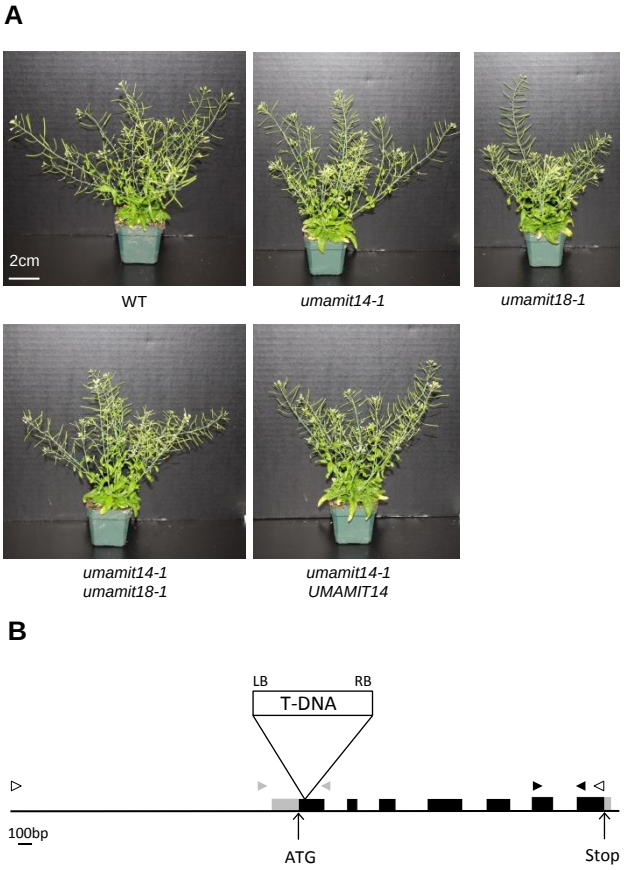
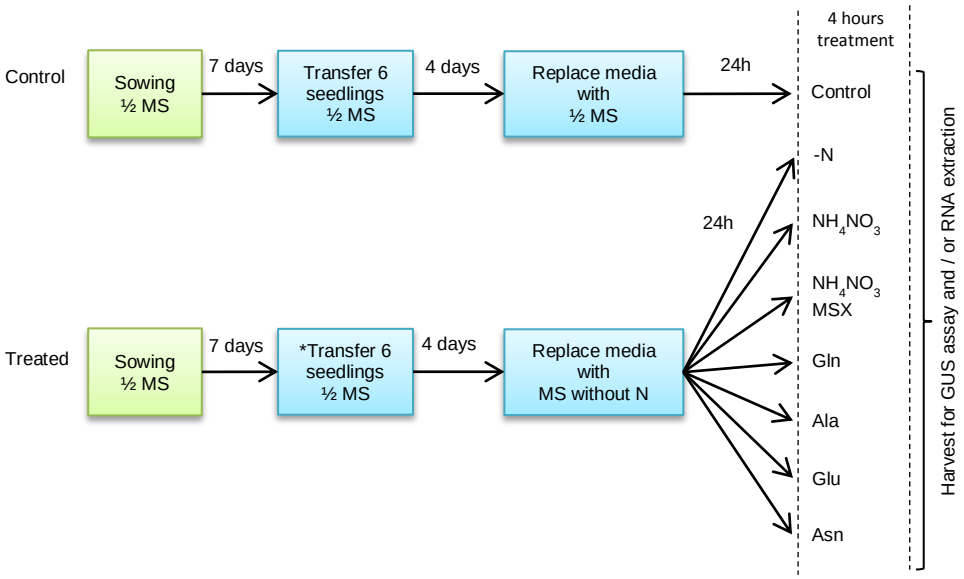
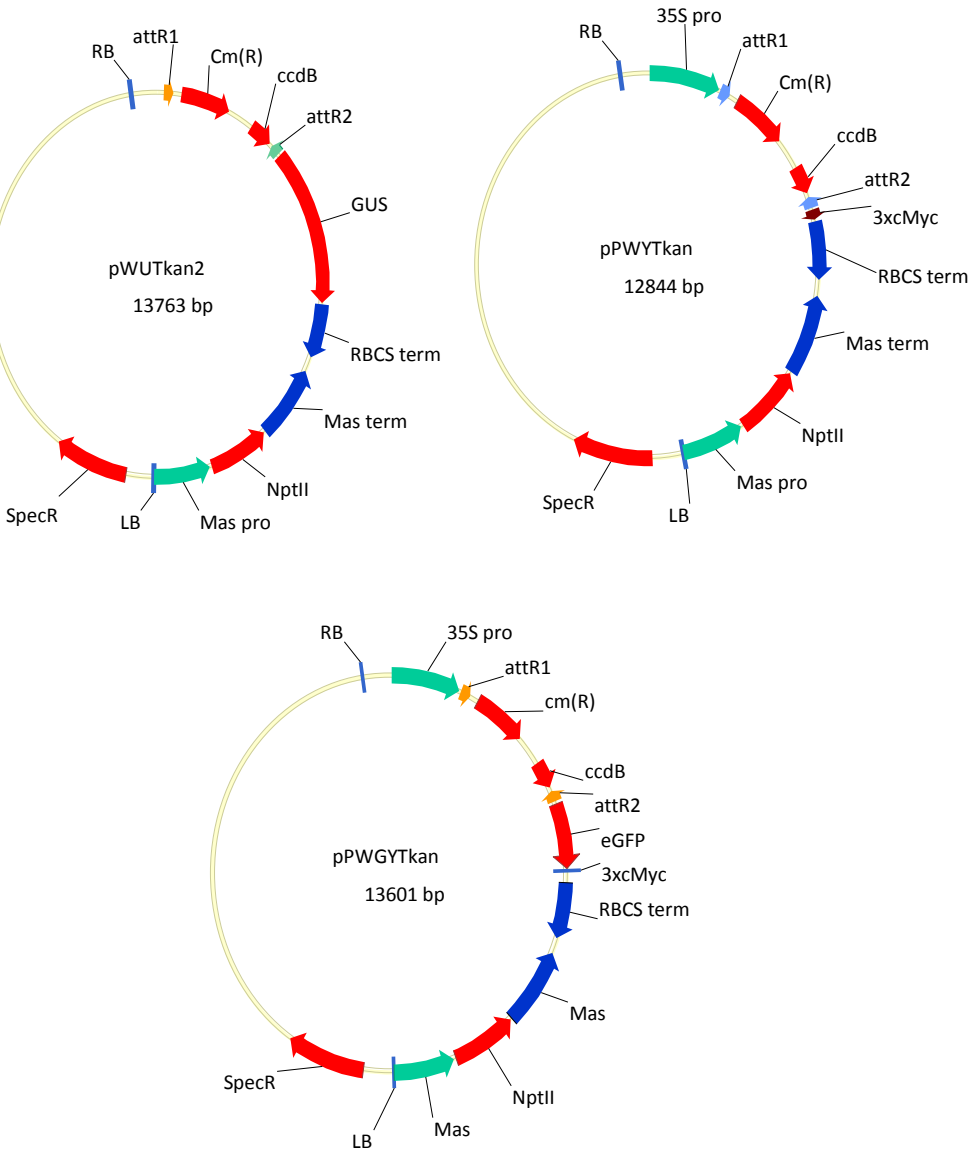
# APPENDIX



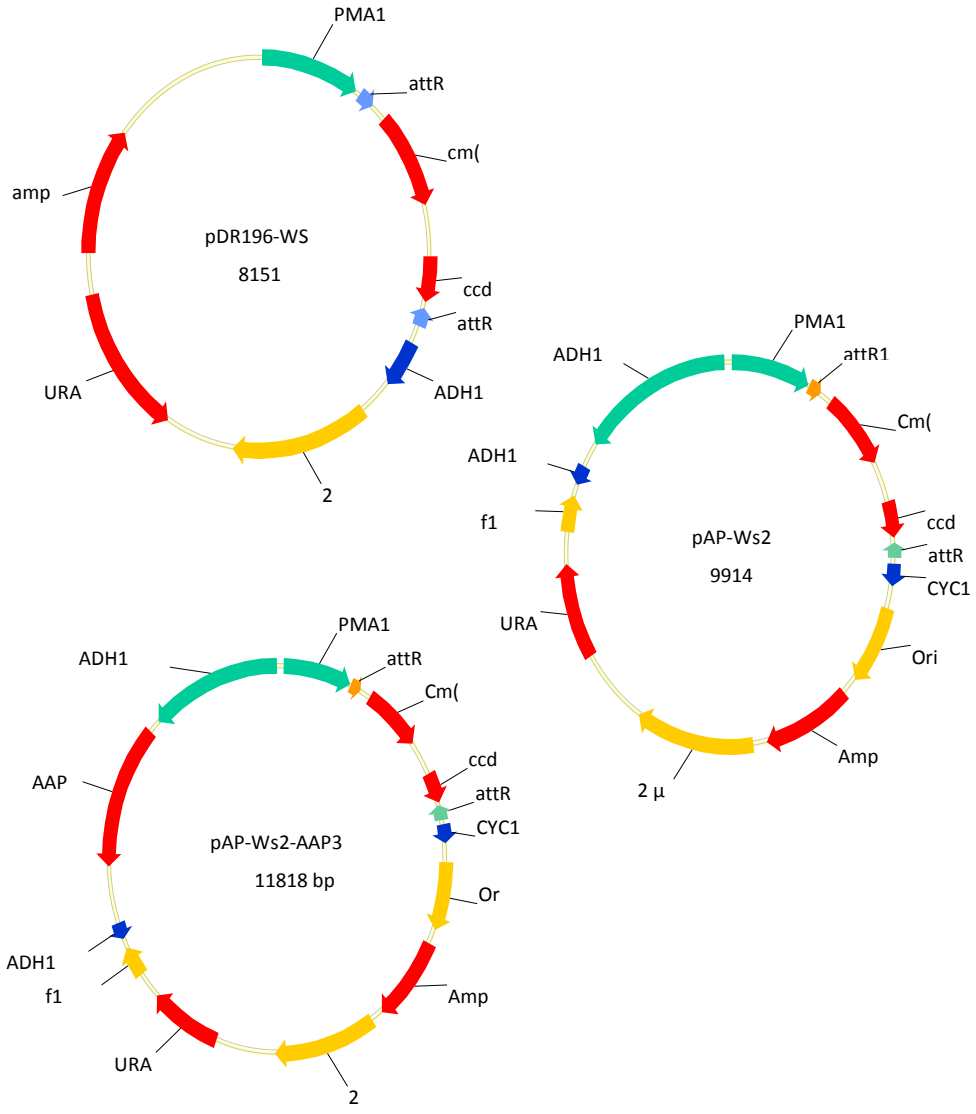

Appendix 1. Phenotype of *umamit14-1* and *umamit18-1* mutants and location of the T-DNA insertion in *umamit14-1*. (A) Phenotypes of six-week-old wild type and *umamit* mutants grown in long day conditions. (B) Location of the T-DNA insertion in *umamit14-1*. Grey and black boxes represent untranslated and translated regions of exons, respectively. Closed arrowheads represent the positions of forward and reverse primers used for qRT-PCR. Open arrowheads represent the positions of the primers used for creation of the complemented line. Grey arrowheads represent the position of the primers used for genotyping the T-DNA insertion.




Appendix 2. Nitrogen starvation and recovery experiment setup. These conditions were used to obtain the results shown on Figure 4. Green boxes and blue boxes represent solid and liquid media, respectively. ½ MS: Half strength Murashige and Skoog media. MS without N: full strength Murashige and Skoog media without nitrogen. Control treatment: ½ MS. MSX: methionine sulfoximine used at 1mM. For liquid growth conditions and treatments, plants were grown in 1 mL of liquid medium in a 24-well plate. All nitrogenous treatments were applied at 10 mM except Ala and Glu (20 mM), to match the nitrogen molarity of the other compounds used.



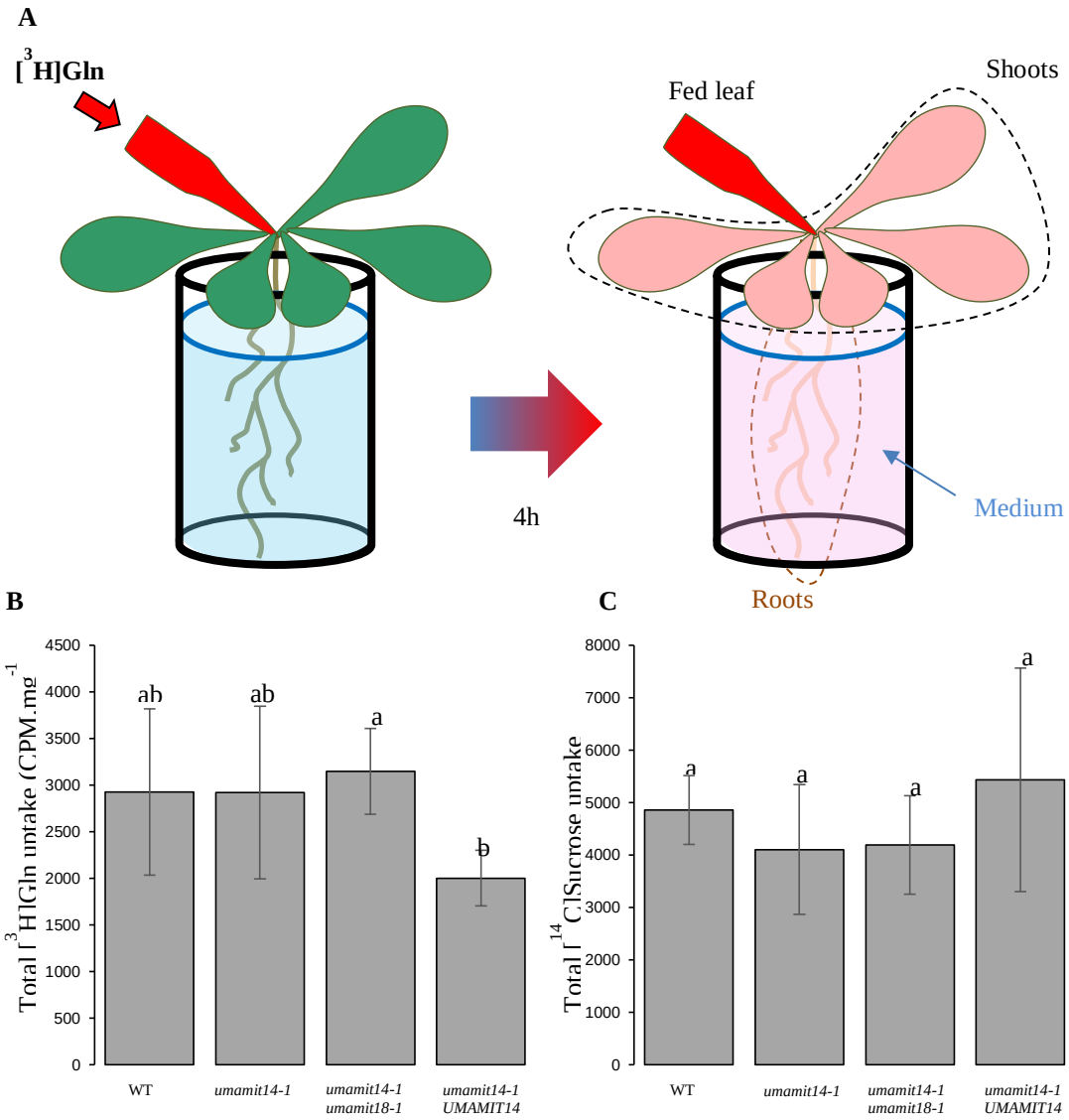

Appendix 3. Maps of vectors pWUTkan2, pPWYTkan and pPWGYTkan used for creating promoter-GUS, UMAMIT14promoter-UMAMIT14cDNA-venus, and gene-GFP fusion, respectively. 35S pro: CaMV 35S promoter; attR1 and attR2: gateway recombination sites; ccdB: suicide gene; Cm(R): chloramphenicol resistance gene; cMyc: cMyc epitope; eGFP: enhanced GFP gene; GUS: uidA from *E. coli*; LB: T-DNA left border; Mas term: mannopine synthase terminator; Mas pro: mannopine synthase promoter; NptII: neomycin phosphotransferase; RB: T-DNA right border; RBCS term: Rubisco terminator from pea; SpecR: spectinomycin resistance gene.




Appendix 4. Maps of yeast vectors pDR196-Ws, pAPWs2-AAP3 and pAP-Ws used for expression in yeast. 2µ: 2µ replication origin for yeast; AAP3: Arabidopsis amino acid permease 3; ADH1 pro and term: alcohol dehydrogenase promoter and terminator; AmpR: ampicillin resistance gene; attR1 and attR2: gateway recombination sites; ccdB: suicide gene; Cm(R): chloramphenicol resistance gene; CYC term: terminator of the cytochrome C gene; f1 origin: replication origin for the f1 phage; Ori: replication origin in E. coli; PMA1 pro: plasma membrane ATPase promoter; URA3: uracil selection marker.

Appendix 5. Primers used for cloning and qRT-PCR. Primers sequences are given from 5' to 3'. Underlined lower case represents bases added for the creation of *attB1* and *attB2* in forward and reverse primers, respectively.

| Name | Type | Sequence |
|---|---|---|
| UMAMIT14 promoter | Forward | acaagtttgtacaaaaaagcaggcttcCACTAAAATATTTACATTGACC |
| | Reverse | accactttgtacaagaaagctgggtCTCATGTTTTTAAAGCCATATCTCAACG TAG |
| UMAMIT14 promoter (for modifying pPWYTkan) | Forward | GAGAAGATCTCTCGAGGTACAAAAAAGCAGGCTGGCACTAAA ATATTTACATTGACC |
| | Reverse | TATAGAGCTCTGTTTTTAAAGCCATATCTCAACGTAG |
| UMAMIT14 genomic | Forward | acaagtttgtacaaaaaagcaggcttcGATATGGCTTTAAAAACATGGAAG |
| | Reverse | accactttgtacaagaaagctgggtCTCAGACTGATTCATTGGTGTTAGGCCT |
| UMAMIT14 CDS | Forward | gacaagtttgtacaaaaaagcaggcttcAGATATGGCTTTAAAAACATGGAAG |
| | Reverse | gaccactttgtacaagaaagctgggtACYAGACTGATTCATTGGTGTTAGGCC T |
| UMAMIT14 CDS (venus fusion) | Forward | GAGAGTCGACACCATGGCTTTAAAAACATGGAAG |
| | Reverse | ATATAGCGCTTGCGACTGATTCATTGGTGTTAGG |
| UMAMIT18 CDS | Forward | gacaagtttgtacaaaaaagcaggcttcAATAAAGATGAAAGGTGGAAGCATG |
| | Reverse | gaccactttgtacaagaaagctgggtACYAGGTACTGGTAACCACACCGTTAG T |
| UMAMIT14 T-DNA detection | Forward | CTATCAAAATTGCATTACTTAGTGG |
| UMAMIT14 T-DNA detection | Reverse | GGCGATACGACGCTAGAACGTGAG |
| Actin2 qPCR | Forward | GGTAACATTGTGCTCAGTGGTGG |
| | Reverse | AACGACCTTAATCTTCATGCTGC |
| UMAMIT14 qPCR | Forward | ATGGTCATTGTTGCGATCTT |
| | Reverse | TGGTTCGTCTTTGCTTTTTC |



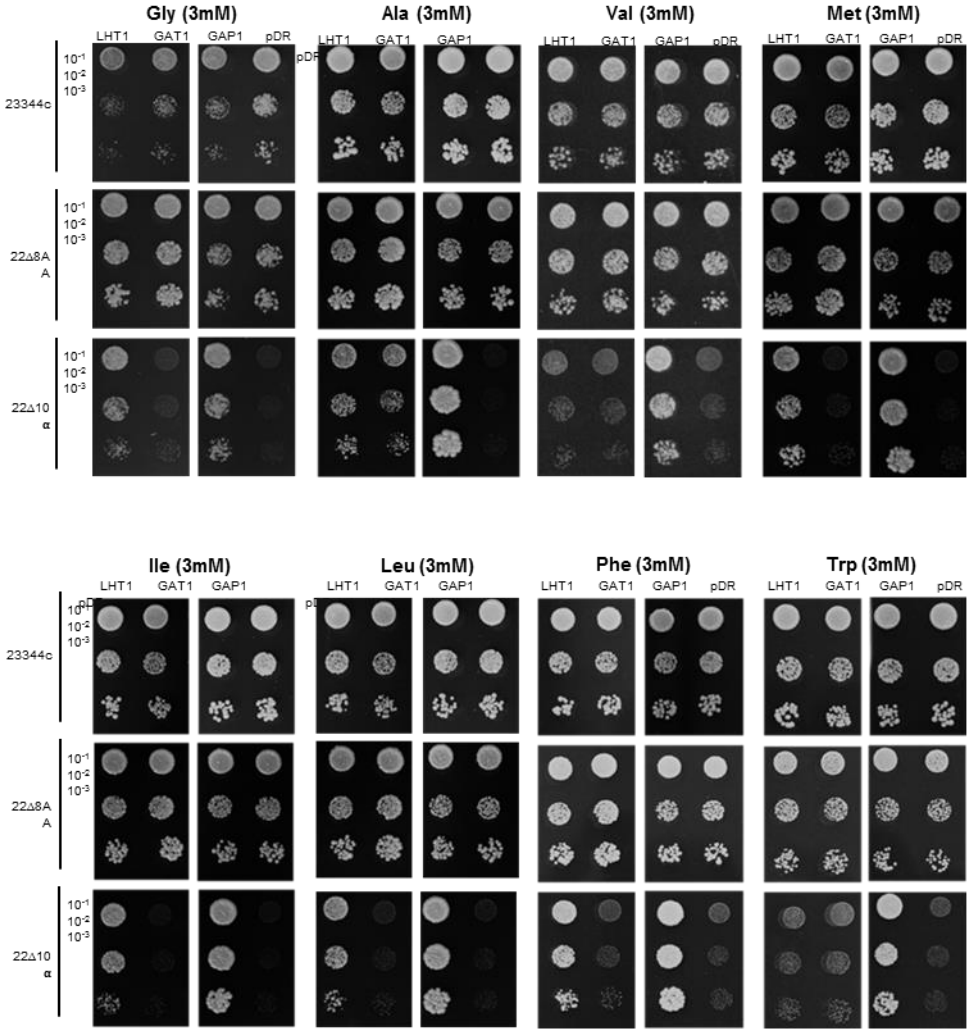

Appendix 6. Phloem transfer assay. (A) Set up. The names of the different samples correspond to the legend of Figure 7. (B) Glutamine and (C) Sucrose total uptake by plants. CPM: count per minute. Total uptake is the sum of CPM found in each sample for a given plant (Fed lead, shoots, roots and medium) divided by the plant dry weight. Significant differences ($p<0.05$) are indicated by different letters according to one way ANOVA in conjunction with Tukey's test (n=3 biological replicates). Raw data of the phloem translocation assay are presented in Appendix 7 and Appendix 8.

Appendix 7. CPM and dry weight of samples used in the shoot-to-root transfer assay using [$^{3}$H]Gln.

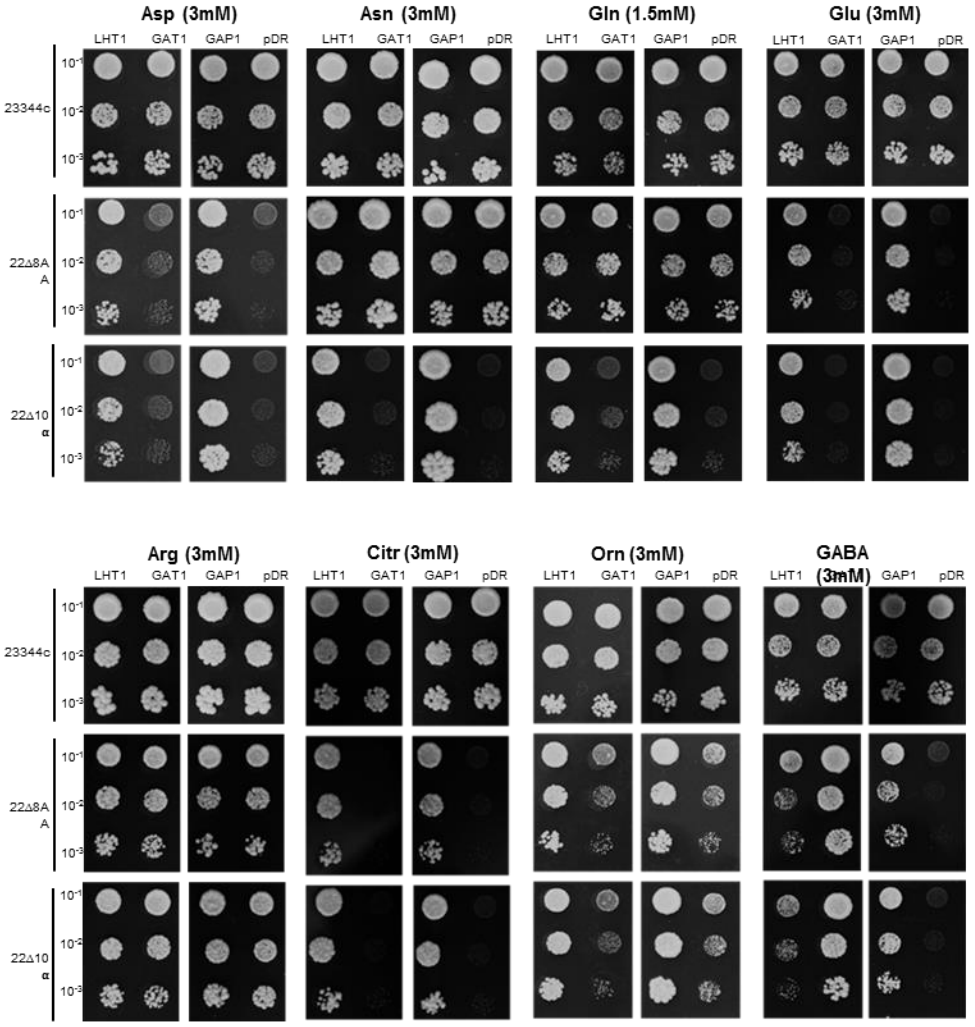
| | Sample | CPM | | | | | Dry weight (mg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Fed leaf | Shoot | Root | Medium | Total | Fed leaf | Shoot | Root | Total |
| | 1 | 6908 | 716 | 24 | 88 | 7648 | 0.6 | 2.71 | 0.58 | 3.89 |
| WT | 2 | 11967 | 2680 | 259 | 185 | 14907 | 0.4 | 2.88 | 0.72 | 4 |
| | 3 | 11000 | 1702 | 88 | 156 | 12791 | 0.35 | 3.36 | 0.44 | 4.15 |
| | 1 | 9648 | 1884 | 81 | 36 | 11613 | 0.81 | 2.75 | 0.89 | 4.45 |
| *umamit14-1* | 2 | 8101 | 1588 | 61 | 41 | 9750 | 0.47 | 2.88 | 1.1 | 4.45 |
| | 3 | 13739 | 2803 | 138 | 51 | 16681 | 0.31 | 2.45 | 1.45 | 4.21 |
| | 1 | 9374 | 3378 | 54 | 41 | 12807 | 0.71 | 2.72 | 1.19 | 4.62 |
| | 2 | 6877 | 3620 | 114 | 41.8 | 10611 | 0.29 | 1.9 | 1.34 | 3.53 |
| *umamit14-1 umamit18-1* | 3 | 11742 | 3726 | 126 | 40.8 | 15595 | 0.33 | 2.97 | 0.96 | 4.26 |
| | 4 | 7623 | 2361 | 43 | 31 | 10027 | 0.34 | 2.24 | 0.97 | 3.55 |
| | 1 | 5729 | 1480 | 174 | 126 | 7383 | 0.62 | 2.44 | 1.01 | 4.07 |
| *umamit14-1 UMAMIT14* | 2 | 7836 | 1098 | 144 | 112 | 9078 | 0.34 | 3.45 | 1.14 | 4.93 |
| | 3 | 9343 | 953 | 77 | 89 | 10373 | 0.77 | 2.81 | 0.84 | 4.42 |

Appendix 8. CPM and dry weight of samples used in the shoot-to-root transfer assay using [$^{14}$C]sucrose. n.d. means the radioactivity found in the sample was similar to the background.

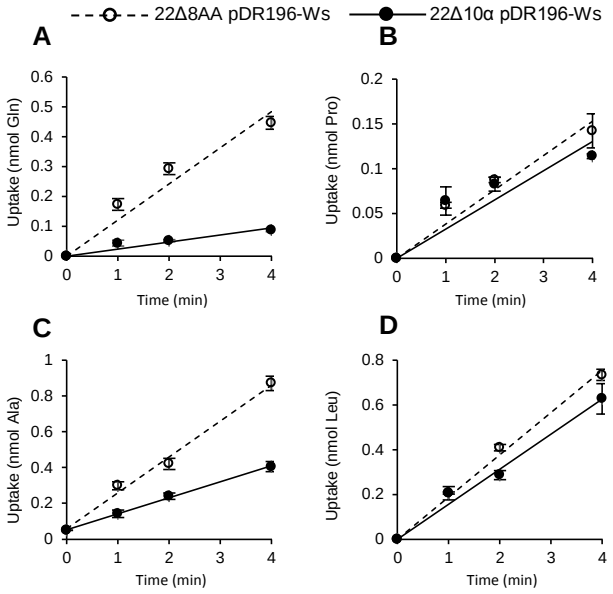
| | Sample | CPM | | | | | Dry weight (mg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Fed leaf | Shoot | Roots | Medium | Total | Fed leaf | Shoots | Roots | Total |
| WT | 1 | 5618 | 8982 | 3355 | n.d. | 17955 | 0.35 | 2.85 | 1.07 | 4.27 |
| | 2 | 8205 | 11955 | 1958 | n.d. | 22118 | 0.42 | 3.16 | 0.98 | 4.56 |
| | 3 | 8496 | 12928 | 2915 | n.d. | 24339 | 0.27 | 3.52 | 0.62 | 4.41 |
| *umamit14-1* | 1 | 6806 | 4677 | 1574 | n.d. | 13057 | 0.37 | 3.4 | 0.7 | 4.47 |
| | 2 | 5552 | 8440 | 2281 | n.d. | 16273 | 0.51 | 2.72 | 0.84 | 4.07 |
| | 3 | 11804 | 6834 | 1626 | n.d. | 20264 | 0.44 | 2.67 | 0.65 | 3.76 |
| *umamit14-1 umamit18-1* | 1 | 6707 | 9296 | 1625 | n.d. | 17628 | 0.48 | 2.56 | 0.83 | 3.87 |
| | 2 | 4679 | 4364 | 1805 | n.d. | 10848 | 0.41 | 2.27 | 0.79 | 3.47 |
| | 3 | 5687 | 9341 | 1926 | n.d. | 16954 | 0.36 | 2.21 | 0.9 | 3.47 |
| *umamit14-1 UMAMIT14-1* | 1 | 5743 | 8070 | 2193 | n.d. | 16006 | 0.43 | 2.97 | 0.85 | 4.25 |
| | 2 | 4589 | 10473 | 2482 | n.d. | 17544 | 0.37 | 2.7 | 0.67 | 3.74 |
| | 3 | 9563 | 11386 | 3097 | n.d. | 24046 | 0.33 | 2.33 | 0.41 | 3.07 |



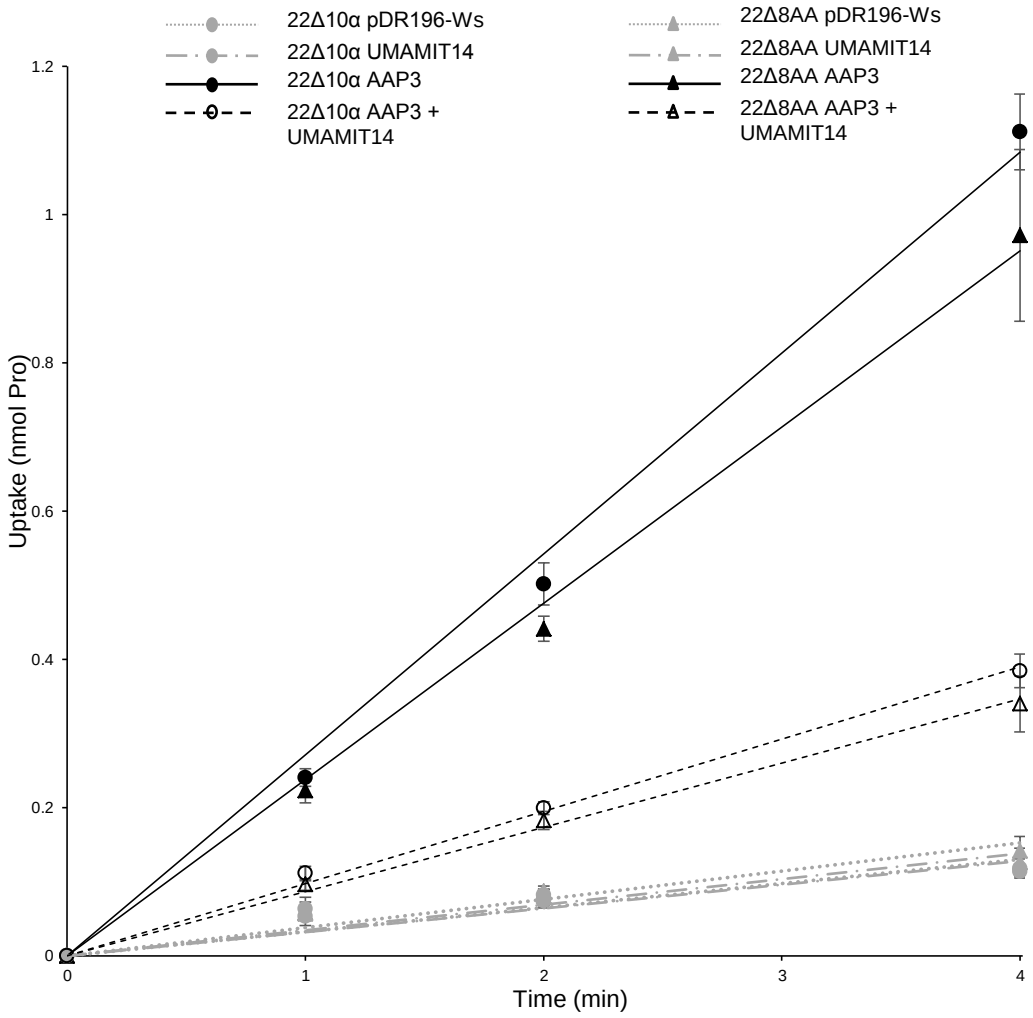

Appendix 9. Yeast growth complementation assay on media containing amino acids as the sole nitrogen source. The parental strain 23344c, 22Δ8AA and 22Δ10α were transformed with the vector pDR196-Ws containing the cDNAs of LHT1 (Lysine Histidine Transporter1; AT5G40780), GAT1 (GABA Transporter1; AT1G08230), GAP1 (General Amino acid Permease1; YKR039W) and the empty pDR196 vector. Yeast cells were grown overnight in selective medium, brought to OD=0.1, and diluted to OD=0.01 and 0.001. Drops of 4 µl were aligned on minimum media containing the mentioned concentration of amino acids. Pictures were taken after 5 days of growth at 30°C.




Appendix 9. Continued




Appendix 10. Uptake of radiolabeled amino acids by 22Δ8AA and 22Δ10α yeast cells expressing pDR196-Ws empty vector from which the gateway cassette has been removed. Uptake was measured with 2 mM Gln (A), Pro (B), Ala (C) or Leu (D). n=3 technical replicates.



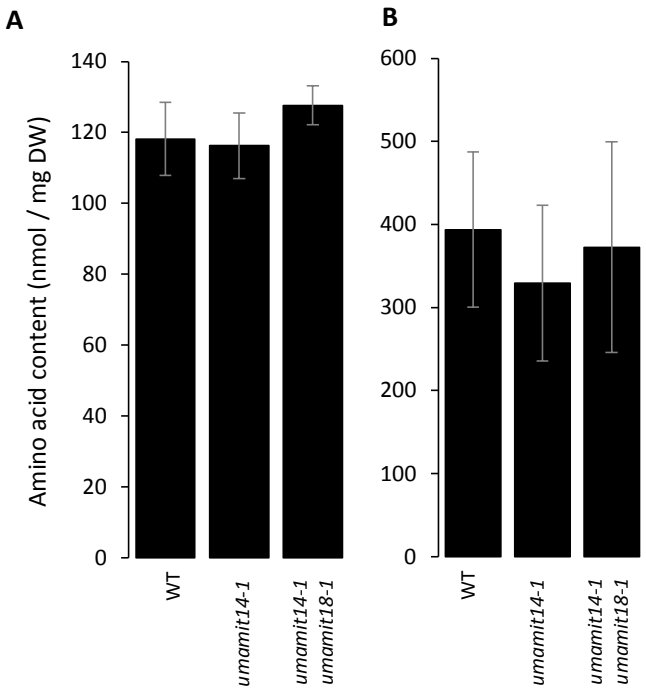

Appendix 11. Uptake of radiolabeled Pro by 22Δ8AA and 22Δ10α yeast cells. pDR196-Ws: cells transformed with empty pDR196-Ws, UMAMIT14: cells expressing UMAMIT14, AAP3: cells expressing AAP3, AAP3 + UMAMIT14: cells co-expressing AAP3 and UMAMIT14 carried on a single vector. Uptake was examined for 2 mM Pro (n=3 replicates).

Appendix 12. Amino acid contents represented in Figure 2 were used. The average of content obtained for the empty plasmid was subtracted to the content of each biological replicates of UMAMIT14 and UMAMIT18. Each value, which represents amino acid secretion above the background level, was divided by the sum of these normalized values, and expressed as a percentage. Stars indicate significant difference between UMAMIT14 and UMAMIT18 results (t-test; p-value<0.5). GABA: gamma-amino-butyric acid.

| | Gln+ Arg* | Ala* | Glu* | Ser* | Gly* | Asn | Asp* | Pro* | Thr | Val* | Cys | His | Lys | Ile* | Leu* | Met | GABA | Phe |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UMAMIT18 | 77.8 | 5.8 | 3.7 | 1.8 | 1.8 | 1.7 | -0.4 | 1.1 | 2.3 | 2.0 | 0.5 | 0.7 | 0.0 | 0.5 | 0.2 | 0.0 | 0.0 | 0.06 |
| UMAMIT14 | 43.3 | 27.6 | 7.7 | 5.9 | 4.6 | 2.0 | 1.1 | 2.5 | 2.5 | 1.7 | 0.0 | 0.4 | 0.0 | 0.4 | 0.3 | 0.0 | 0.0 | 0.06 |

Appendix 13. Characteristics of nine-week-old Arabidopsis plants grown in soil in long day conditions. Biomass represents all plant tissue collected from the aerial parts, minus the seeds. Significant differences ($p<0.05$) are indicated by different letters according to one-way ANOVA in conjunction with Tukey's test (n=4 biological replicates).

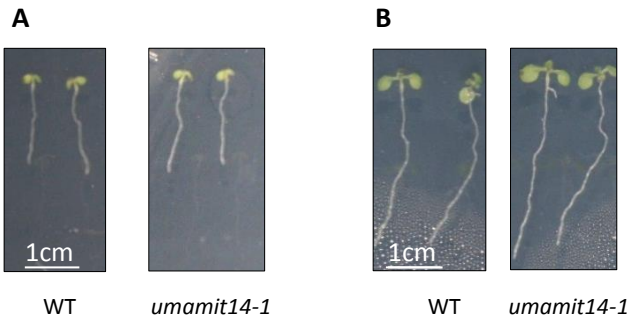
| | Plant size (cm) | Biomass (mg) | Weight of 100 seeds (mg) | Number of seeds (thousands) | Number of seeds per silique | %C in seeds | %N in seeds | Seed protein content ($\mu g.mg^{-1}$ DW) |
|---|---|---|---|---|---|---|---|---|
| WT | 36.5 (a) | 657 (a) | 2.08 (a) | 134 (a) | 49.8 (a) | 55.9 (a) | 4.82 (a) | 134.5 (a) |
| *umamit14-1* | 35.5 (a) | 400 (ab) | 2.31 (a) | 165 (a) | 39.7 (a) | 55.2 (a) | 4.72 (a) | 152.5 (a) |
| *umamit18-1* | 28.2 (ab) | 400 (ab) | 1.97 (a) | 118 (a) | 44.5 (a) | 54.4 (a) | 4.58 (a) | 170.6 (a) |
| *umamit14-1* *umamit18-1* | 27.6 (ab) | 396 (ab) | 2.15 (a) | 194 (a) | 41.4 (a) | 54.9 (a) | 4.50 (a) | 124.3 (a) |
| *umamit14-1* *UMAMIT14* | 32.5 (a) | 800 (a) | 2.26 (a) | 111 (a) | 45.4 (a) | 55.3 (a) | 4.98 (a) | 137.2 (a) |




Appendix 14. Amino acid content in (A) shoots and (B) roots of five week-old Arabidopsis plants grown in hydroponic conditions. Error bars correspond to standard deviation (n=3 biological replicates). No significant differences were found according to one-way ANOVA in conjunction with Tukey's test ($p<0.05$). Contents in individual amino acids from the same dataset are presented in Table S4.

Appendix 15. Amino acid content in shoots and roots of five-week-old Arabidopsis plants grown in hydroponic conditions. Values are expressed in nmol.mg$^{-1}$ DW. Significant differences ($p<0.05$) are indicated by different letters according to ANOVA in conjunction with Tukey's test (n=4 biological replicates). n.d.: not detected.

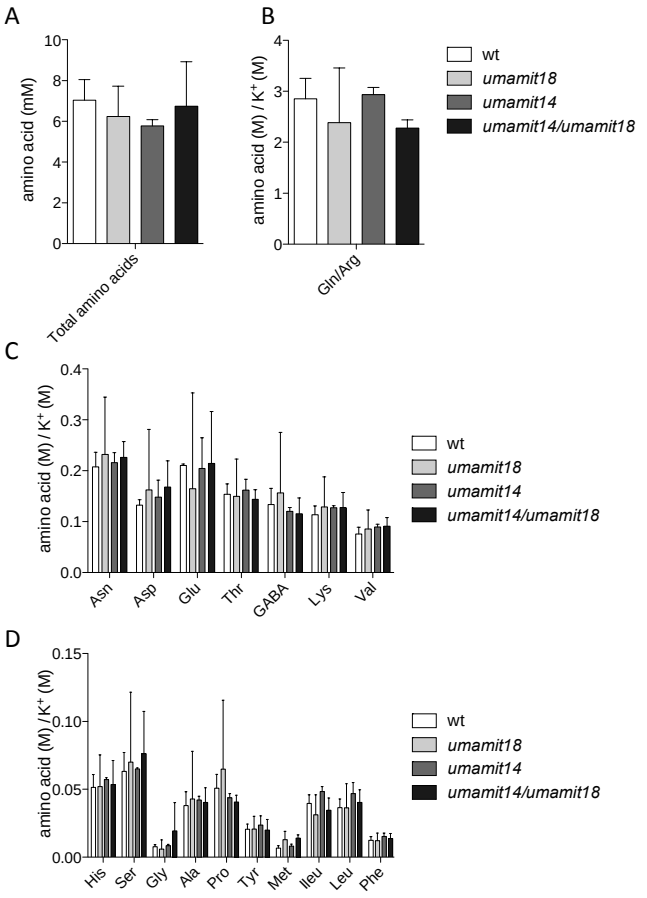
| | Genotype | Ala | Asn | Asp | GABA | Gln | Glu | Gly | His | Ile | Leu | Phe | Pro | Ser | Thr | Val | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Shoots | WT | 1.24 (a) | 6.20 (a) | 9.29 (a) | 0.78 (a) | 22.6 (a) | 10.6 (a) | 18.0 (a) | 1.32 (a) | 1.88 (a) | 2.56 (a) | 1.96 (a) | 2.55 (a) | 30.5 (a) | 6.16 (a) | 2.61 (a) | 118.1 (a) |
| | *umamit14-1* | 1.53 (a) | 6.32 (a) | 11.1 (a) | 0.46 (b) | 22.2 (a) | 12.2 (ab) | 15.0 (a) | 1.24 (a) | 1.79 (a) | 2.33 (a) | 1.77 (a) | 2.02 (a) | 29.8 (a) | 6.07 (a) | 2.52 (a) | 116.2 (a) |
| | *umamit14-1 umamit18-1* | 2.81 (b) | 7.29 (a) | 12.8 (b) | 0.61 (a) | 23.3 (a) | 14.8 (b) | 15.5 (a) | 1.22 (a) | 1.63 (a) | 2.35 (a) | 1.85 (a) | 1.89 (a) | 33.4 (a) | 5.53 (b) | 2.53 (a) | 127.6 (a) |
| Roots | WT | 121 (a) | 48.2 (a) | 19.3 (a) | 28.2 (a) | 47.1 (a) | 62.1 (a) | 4.92 (a) | n.d. | 2.40 (a) | 4.39 (a) | 0.92 (a) | 9.12 (a) | 27.2 (a) | 13.1 (a) | 5.45 (a) | 393.8 (a) |
| | *umamit14-1* | 38.5 (b) | 50.4 (a) | 25.1 (ab) | 19.5 (a) | 65.3 (a) | 53.3 (a) | 4.31 (a) | n.d. | 3.02 (ab) | 6.43 (a) | 1.53 (a) | 10.9 (a) | 33.3 (a) | 11.0 (a) | 6.87 (a) | 329.3 (a) |
| | *umamit14-1 umamit18-1* | 59.8 (ab) | 60.1 (a) | 29.4 (b) | 18.1 (a) | 71.7 (a) | 56.2 (a) | 4.15 (a) | n.d. | 4.71 (b) | 5.79 (a) | 1.25 (a) | 13.1 (a) | 29.9 (a) | 11.5 (a) | 6.91 (a) | 372.6 (a) |



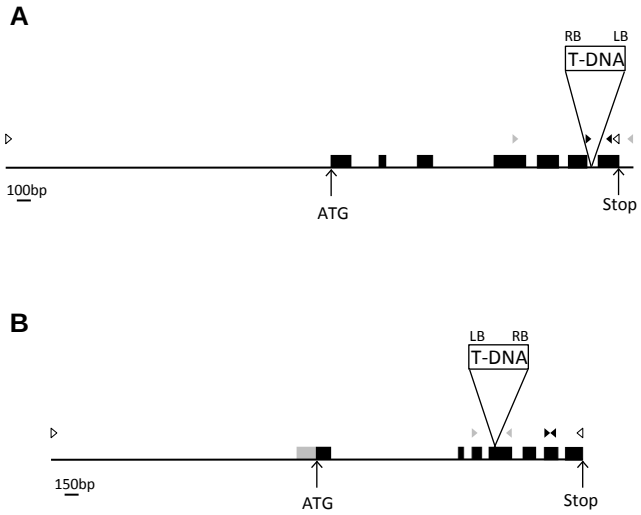

Appendix 16. Root length of wild type and *umamit14-1* grown under different nitrogen regimes. One-week-old Arabidopsis seedlings grown vertically on solid J medium without sugar, containing 0.1 (A) or 10 mM $KNO_3$ (B).

Appendix 17. Amino acid secreted by two-week-old Arabidopsis plants. The data are the ones used in Figure 6. Plants were grown for two weeks in liquid J medium supplemented with 20 mM $NH_4NO_3$; the medium was replaced with fresh J medium and collected after three days for analysis. Values are expressed in nmol.mg$^{-1}$ DW. Significant differences ($p<0.05$) are indicated by different letters according to one-way ANOVA in conjunction with Tukey's test (n=6 biological replicates).

| | Ala | Asn | Asp | Gaba | Gln | Glu | Gly | His | Ile | Leu | Phe | Ser | Thr | Val | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild Type | 1.26 (a) | 7.74 (a) | 1.03 (a) | 1.64 (a) | 15.3 (a) | 3.16 (a) | 3.23 (a) | 0.28 (a) | 0.12 (a) | 0.14 (a) | 0.15 (a) | 0.80 (a) | 0.46 (a) | 0.50 (a) | 35.43 (a) |
| *umamit14-1* | 1.09 (a) | 5.83 (b) | 0.86 (a) | 0.98 (b) | 10.4 (b) | 2.57 (b) | 2.45 (b) | 0.21 (b) | 0.08 (b) | 0.09 (b) | 0.10 (a) | 0.56 (b) | 0.32 (b) | 0.11 (a) | 26.80 (b) |
| *umamit18-1* | 0.79 (b) | 6.49 (a) | 0.85 (a) | 1.11 (b) | 8.11 (b) | 2.48 (b) | 2.67 (b) | 0.29 (a) | 0.07 (b) | 0.08 (b) | 0.16 (a) | 0.57 (b) | 0.30 (b) | 0.10 (b) | 24.04 (b) |
| *umamit14-1 umamit18-1* | 0.34 (c) | 3.07 (d) | 0.71 (b) | 0.47 (b) | 1.90 (c) | 1.74 (c) | 2.32 (b) | 0.29 (a) | 0.02 (c) | 0.02 (c) | 0.13 (a) | 0.32 (c) | 0.12 (c) | 0.03 (c) | 11.47 (c) |




Appendix 18. Amino acid levels in the xylem sap of WT and *umamit* mutants. (A) Total amino acid concentrations. (B) to (D) Concentrations of individual amino acids, normalized by the $K^+$ concentrations. Gln/Arg (B); Asn, Asp, Glu, Thr, GABA, Lys, Val (C); and His, Ser, Gly, Ala, Pro, Tyr, Met, Ile, Leu, Phe (D). Xylem sap was extracted from decapitated 55-day-old plants grown in short day conditions. Error bars correspond to standard deviation (n=3 biological replicates). No significant difference was found according to one way ANOVA in conjunction with Tukey's test ($p<0.05$).



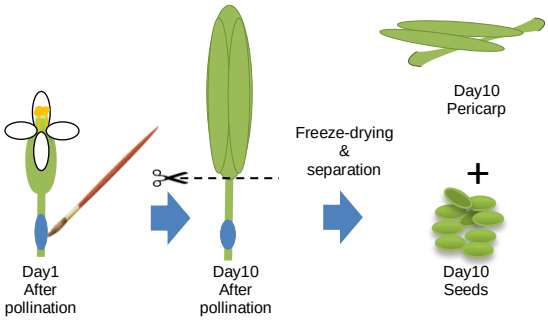

Appendix 19. Location of the T-DNA insertion in *umamit24-1* and *umamit25-1*. (A) and (B) location of the T-DNA insertion in *umamit24-1* and *umamit25-1,* respectively. Grey and black boxes represent untranslated and translated regions of exons, respectively. Closed arrowheads represent the positions of forward and reverse primers used for qRT-PCR. The forward primer used for UMAMIT24 qRT-PCR amplification was specific to the c-DNA at the transition exon 6 – exon 7. Open arrowheads represent the positions of the primers used for creation of the complemented line. Grey arrowheads represent the position of the primers used for genotyping the T-DNA insertion.

Appendix 20. *UMAMIT24* and *UMAMIT25* mRNA expression levels in 14 day-old developing seeds. Means and standard deviations are displayed (n=3). Significant differences ($p<0.05$) are indicated by different letters according to one way ANOVA in conjunction with Tukey's test. N.D. no amplification was detected. *UMAMIT24* and *UMAMIT25* mRNA levels obtained by RT-qPCR have been normalized against Actin 8 and are relative to wild type expression.

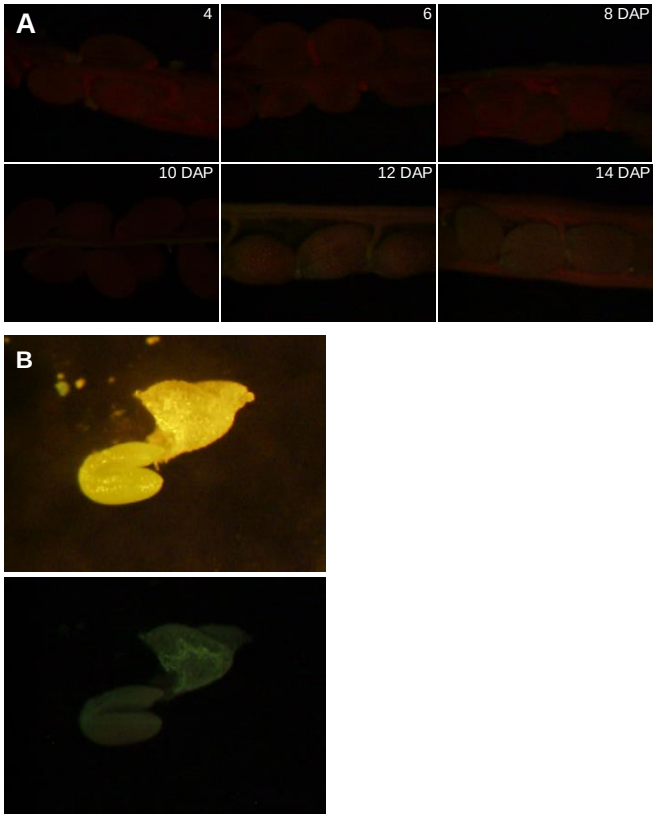
| | *UMAMIT24* | | *UMAMIT25* |
|---|---|---|---|
| Wild type | 1<br>± 0.13 a | Wild type | 1<br>± 0.06 a |
| *umamit24-1* | 0.043<br>± 0.03 b | *umamit25-1* | n.d. |
| *umamit24-1*<br>UMAMIT24 | 14.79<br>± 7.5 c | *umamit25-1*<br>UMAMIT25 | 2.30<br>±0.032 b |

Appendix 21. Primers used for cloning and qRT-PCR. Primer sequences are given from 5' to 3'. Underlined bases were added for the creation of *attB1* and *attB2* in forward and reverse primers, respectively.

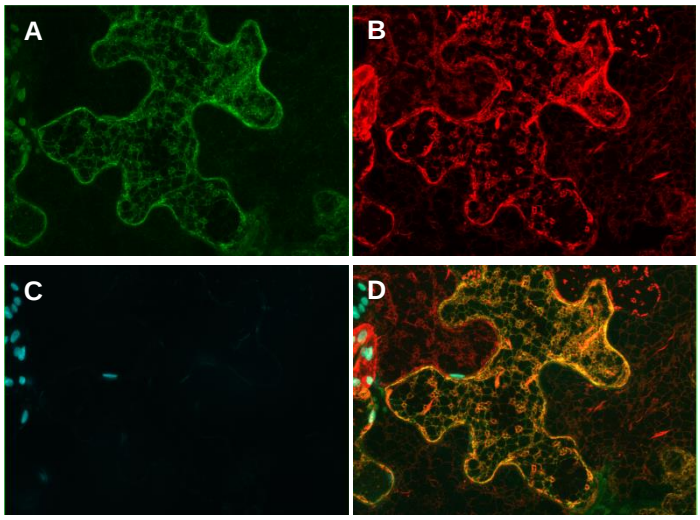
| Name | Sequence |
|---|---|
| UMAMIT24 promoter (Forward) | GGACAAGTTTGTACAAAAAAGCAGGCTTCAGTTCATCTTCTGCAGAACGACCAA |
| UMAMIT24 end of gDNA (Reverse) | GGACCACTTTGTACAAGAAAGCTGGGTCGGGGACATCTCTATTTACTGATGAAAGATT |
| UMAMIT25 promoter (Forward) | GGACAAGTTTGTACAAAAAAGCAGGCTTCGCTCGGGATTTGAAATGGAGGA |
| UMAMIT25 end of gDNA (Reverse) | GGACCACTTTGTACAAGAAAGCTGGGTCAGGCGATGTAGACCTTGTGGAACC |
| UMAMIT23 cDNA expression in yeast (Forward) | GACAAGTTTGTACAAAAAAGCAGGCTCAGAAATGAAAGATATAACGGCAATG |
| UMAMIT23 cDNA expression in yeast (Reverse) | GACCACTTTGTACAAGAAAGCTGGGTACYAAGGGACATTTGTACTTAATGTTGG |
| UMAMIT24 cDNA expression in yeast (Forward) | GACAAGTTTGTACAAAAAAGCAGGCTCAGGAGAAATGAAGAGTGTAGTTGCA |
| UMAMIT24 cDNA expression in yeast (Reverse) | GACCACTTTGTACAAGAAAGCTGGGTACYAGGGGACATCTCTATTTACTGATGAA |
| UMAMIT25 cDNA expression in yeast (Forward) | GACAAGTTTGTACAAAAAAGCAGGCTCAGAGATGGCTAAATCAGATATGTTGC |
| UMAMIT25 cDNA expression in yeast (Reverse) | GACCACTTTGTACAAGAAAGCTGGGTACYAAGGCGATGTAGACCTTGTGG |
| Actin 8 qRT-PCR (Forward) | GTGTCTGGATTGGTGGTTCTATCC |
| Actin 8 qRT-PCR (Reverse) | GCCTTAGAGATCCACATCTGCTG |
| UMAMIT24 qRT-PCR (Forward) | TTTGGGAAGTATAATCGGTGCC |
| UMAMIT25 qRT-PCR (Reverse) | AAAGATTGCCAAGGTCCAGTTCT |
| UMAMIT25 qRT-PCR (Forward) | TGGGCTATGCAGAGGAAAGGTC |
| UMAMIT25 qRT-PCR (Reverse) | CCACAAGTGCTGATCCCATAAACG |



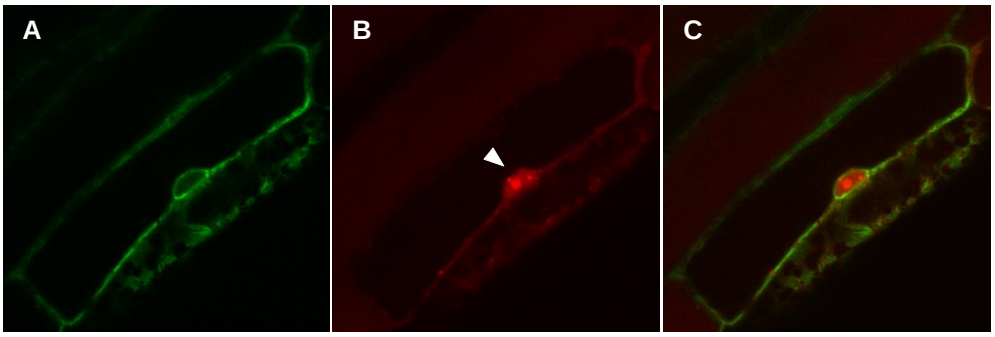

Appendix 22. Acquisition of 7, 10 or 14 day-old silique experimental procedure. The example above is given for day 10 after pollination. A newly opened flower was considered day 1 after pollination and its pedicel was painted. Nine days later (day 10 after pollination), the pedicel was removed, the siliques were freeze-dried and the seeds were separated from the pericarp (defined here as the silique minus the seeds). In case of the glutamine and sucrose transfer assay in isolated siliques, the silique right on top and right underneath the painted silique were used to avoid potential interference caused by the paint. All labeling and harvesting happened during early afternoon.




Appendix 23. Wild type fluorescence of Arabidopsis silique. A: silique with valves removed under GFP-exciting wavelength. B: dissected seeds revealing the embryo (bottom left of the picture) and the seed coat. For panel B, seeds were observed under bright light (top row) or under GFP-exciting wavelength (bottom row). DAP: day after pollination.



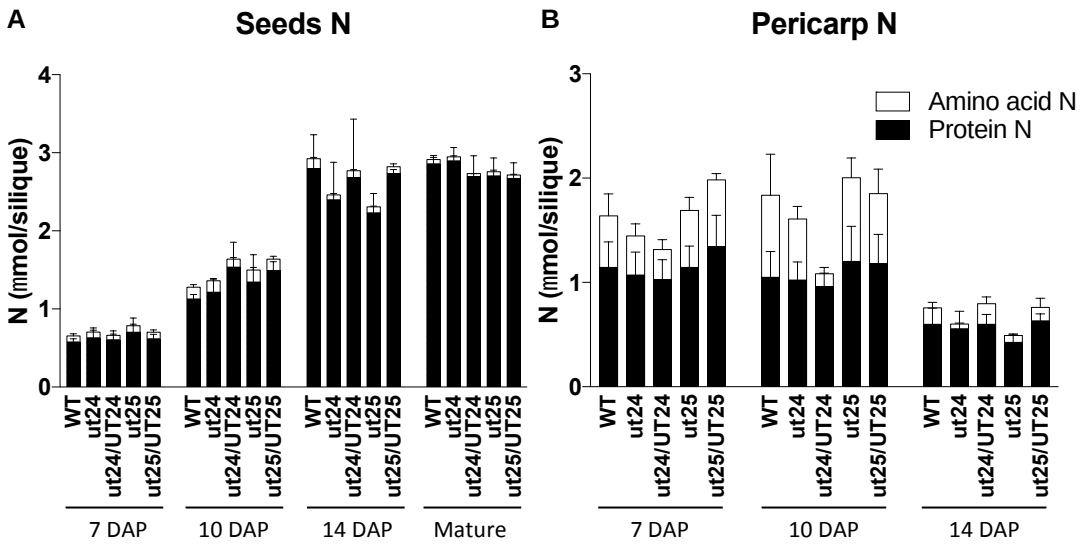

Appendix 24. Ectopic expression of 35S:GFP-UMAMIT24 in Arabidopsis cotyledons. Z-Stacks of approximately 75% of the cell are displayed. (A) GFP, (B) HDEL-mCherry, (C) chlorophyll and (D) merged.



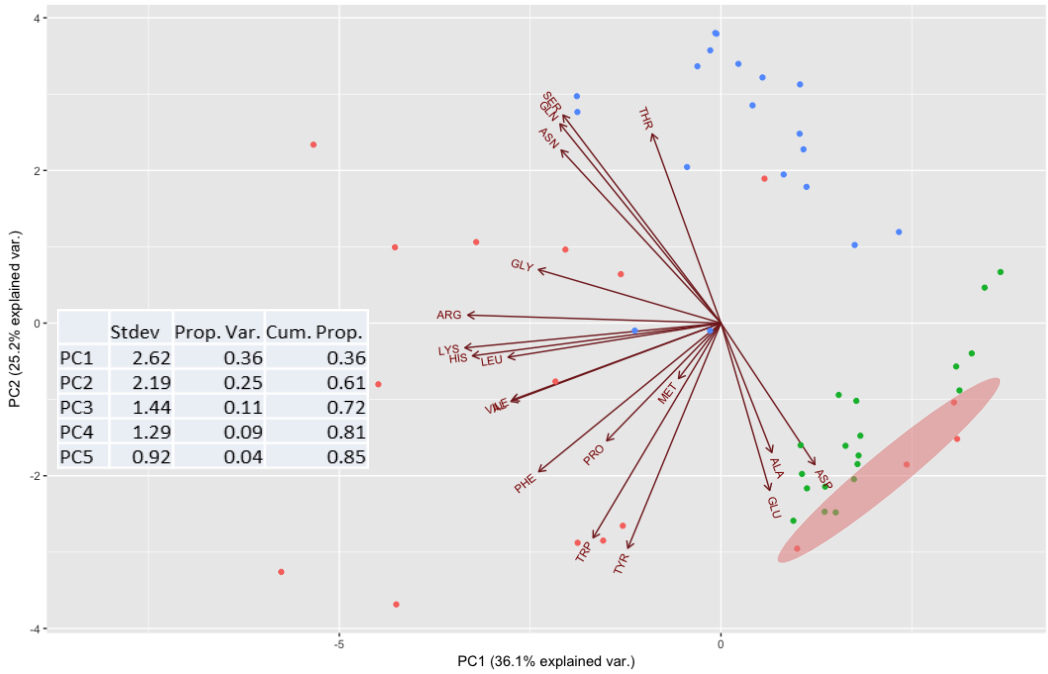

Appendix 25. Ectopic expression of 35S:UMAMIT24-GFP in Arabidopsis roots. (A) GFP, (B) Syto82® labelling the DNA and (C) merged. White arrowhead points to the nucleus.

Appendix 26. Plant and seed biomass obtained on plants at maturity. Dead biomass includes the primary root and all aerial parts, except the seeds. Means and standard deviations are displayed with n=4. Significant differences ($p<0.05$) are indicated by different letters according to one way ANOVA in conjunction with Tukey's test.

| | Dead Biomass (g) | Seed number (thousands) | Seed mass (mg) | Weight of 100 seeds (mg) | Seed %C | Seed %N |
|---|---|---|---|---|---|---|
| Wild type | 1.07 ± 0.07 a | 78.7 ± 12.7 a | 301.6 ± 42.3 ab | 2.60 ± 0.11 a | 55.9 ± 0.72 a | 5.40 ± 0.17 a |
| *umamit24-1* | 1.1 ± 0.17 a | 79.8 ± 7.91 a | 293.3 ± 37.5 b | 2.74 ± 0.39 ab | 56.3 ± 0.73 a | 5.34 ± 0.23 a |
| *umamit24-1* UMAMIT24 | 1.01 ± 0.01 a | 105 ± 5.07 b | 477.5 ± 28.2 cd | 2.21 ± 0.08 b | 56.3 ± 0.42 a | 4.82 ± 0.02 b |
| *umamit25-1* | 1.04 ± 0.03 a | 88.8 ± 13.6 ab | 404 ± 67.7 bc | 2.20 ± 0.07 b | 55.9 ± 0.66 a | 5.13 ± 0.18 a |
| *umamit25-1* UMAMIT25 | 1.22 ± 0.13 a | 125 ± 14.2 b | 524 ± 38.1 d | 2.38 ± 0.17 ab | 56.5 ± 0.80 a | 5.10 ± 0.20 a |




Appendix 27. Accumulation of nitrogen in proteins and amino acids in seed and pericarp tissues. (A) Nitrogen in proteins and amino acids in seeds at 7, 10, 14 DAP and mature seeds. (B) Nitrogen in proteins and amino acids in the pericarp tissues at 7, 10 and 14 DAP. Nitrogen in seed and pericarp proteins was estimated as described in the material and methods. Amino acid contents were derived from the data represented in Table S4 and S5. Closed and open bars represent nitrogen from proteins and amino acids, respectively. WT, wild type; ut24, *umamit24-1*; ut24/UT24, *umamit24-1*/UMAMIT24; ut25, *umamit25-1*; and ut25/UT25, *umamit25-1*/UMAMIT25 lines.




Appendix 28. PCA analysis of amino acid content in the pericarp tissue. Amino acid content of pericarp tissue (presented in Table S5) has been analyzed. The data points at 7, 10, and 14 DAP are marked with blue, red and green, respectively. The four points representing 10 DAP *umamit24-1/*UMAMIT24 are marked with an ellipse (probability = 0.68). Inset: the loadings of PCA analysis. Stdev, standard deviation; Prop. Var, proportion of variance; Cum.Prop, cumulative proportion.

Appendix 29. Amino acid content in 7, 10, 14 day-old and mature seeds. Values are expressed in nmol per µg of dry weight. Each sample corresponds to two siliques worth of seeds coming from the same plant. Means and standard deviations are displayed with n=3. Significant differences ($p<0.05$) are indicated by different letter according to one way ANOVA in conjunction with Tukey's test.

| Day 7 | ASP | GLU | ASN | SER | GLN | HIS | GLY | THR | ALA | ARG | TYR | VAL | MET | TRP | PHE | ILE | LEU | LYS | PRO | Sum |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type | 5.43 ± 0.74 a | 22.8 ± 1.36 a | 7.85 ± 2.08 a | 48.6 ± 8.28 a | 30.7 ± 12.2 a | 1.47 ± 0.18 a | 13.4 ± 7.55 a | 7.77 ± 1.66 a | 9.09 ± 0.71 a | 6.71 ± 1.49 a | 1.94 ± 0.13 a | 5.78 ± 0.17 a | 13 ± 1.17 a | 0.76 ± 0.12 a | 3.28 ± 0.05 a | 4.08 ± 0.26 a | 6.68 ± 0.21 a | 5.09 ± 0.02 a | 5.65 ± 1.57 a | 200 ± 35.8 a |
| *umamit24-1* | 6.14 ± 0.94 a | 22.3 ± 2.99 a | 11.2 ± 4.45 a | 37.8 ± 7.92 ab | 32.8 ± 9.05 a | 0.89 ± 0.28 bc | 5.95 ± 1.77 ab | 8.22 ± 1.94 a | 8.65 ± 1.59 a | 6.65 ± 1.47 a | 1.67 ± 0.5 a | 5.61 ± 1.38 a | 5.02 ± 0.86 b | 0.5 ± 0.17 ab | 2.68 ± 0.74 ab | 3.37 ± 0.89 ab | 6.2 ± 1.73 a | 2.47 ± 1.28 b | 6.96 ± 1.76 a | 175 ± 12.6 a |
| *umamit24-1* UMAMIT24 | 5.66 ± 0.71 a | 20.7 ± 1.34 a | 8.09 ± 1.35 a | 21.7 ± 4.17 b | 13.9 ± 1.97 a | 0.63 ± 0.15 c | 3.49 ± 0.36 b | 7 ± 1.52 a | 6.7 ± 0.88 a | 3.1 ± 1.09 b | 1.26 ± 0.24 a | 4.06 ± 0.84 a | 7.04 ± 1.39 b | 0.4 ± 0.08 b | 2 ± 0.32 b | 2.42 ± 0.59 b | 4.47 ± 0.85 a | 2.31 ± 0.77 b | 7.77 ± 3.16 a | 122 ± 14.3 b |
| *umamit25-1* | 6.35 ± 0.52 a | 23.3 ± 4.8 a | 8.16 ± 1.03 a | 41.8 ± 14.6 a | 38.4 ± 17.1 a | 1.08 ± 0.18 ab | 6.99 ± 3.48 ab | 6.17 ± 1.14 a | 8.56 ± 0.77 a | 5.58 ± 0.94 ab | 1.71 ± 0.38 a | 5.99 ± 0.97 a | 6.01 ± 1.27 b | 0.52 ± 0.1 ab | 2.62 ± 0.64 ab | 3.42 ± 0.71 ab | 6.31 ± 1.26 a | 2.32 ± 1.01 b | 6.45 ± 4.72 a | 182 ± 24.9 a |
| *umamit25-1* UMAMIT25 | 5.86 ± 0.42 a | 22.4 ± 2.7 a | 10.5 ± 3.58 a | 36.5 ± 5.3 ab | 43.3 ± 19.2 a | 1.05 ± 0.13 abc | 5.69 ± 0.72 ab | 6.78 ± 0.92 a | 8.53 ± 0.66 a | 5.03 ± 0.86 ab | 1.7 ± 0.36 a | 5.55 ± 0.68 a | 7.24 ± 1.31 b | 0.51 ± 0.09 ab | 2.69 ± 0.47 ab | 3.26 ± 0.39 ab | 6.1 ± 0.95 a | 3.11 ± 0.77 ab | 5.19 ± 1.77 a | 181 ± 16.4 a |

| Day 10 | ASP | GLU | ASN | SER | GLN | HIS | GLY | THR | ALA | ARG | TYR | VAL | MET | TRP | PHE | ILE | LEU | LYS | PRO | Sum |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild Type | 5.25 ± 1.06 a | 21 ± 2.02 a | 12.2 ± 3.37 a | 73.2 ± 33.9 a | 25.7 ± 3.85 a | 1.11 ± 0.03 a | 8.11 ± 1.99 a | 7.87 ± 0.64 a | 11.4 ± 2.72 a | 10.9 ± 1.57 a | 3.13 ± 0.71 a | 8.08 ± 1.57 a | 7.81 ± 0.29 a | 0.85 ± 0.25 a | 5.65 ± 1.38 a | 6.27 ± 1.39 a | 12.2 ± 2.93 a | 7.77 ± 1.61 a | 5.26 ± 1.48 a | 234 ± 36.2 a |
| *umamit24-1* | 5.34 ± 0.7 a | 17.9 ± 4.97 a | 24.2 ± 10.7 a | 43 ± 14.1 ab | 30.1 ± 9.39 a | 1.18 ± 0.39 a | 5.09 ± 2.36 ab | 6.79 ± 0.9 a | 8.42 ± 3.57 a | 9.42 ± 2.93 a | 2.17 ± 1.52 a | 5.6 ± 3.14 a | 7.58 ± 1.96 a | 0.5 ± 0.33 a | 4 ± 2.82 a | 4.25 ± 2.74 a | 8.45 ± 6.04 a | 7.04 ± 3.71 a | 4.89 ± 2.63 a | 196 ± 22.5 ab |
| *umamit24-1* UMAMIT24 | 6.55 ± 1.9 a | 23.1 ± 2.02 a | 13.4 ± 4.76 a | 16.3 ± 3.55 b | 20.8 ± 4.36 a | 0.61 ± 0.38 a | 2.96 ± 0.57 b | 5.68 ± 1.19 a | 7.99 ± 1.99 a | 4.63 ± 1.09 b | 1.64 ± 0.86 a | 4.14 ± 1.81 a | 7.41 ± 0.33 a | 0.46 ± 0.26 a | 2.96 ± 1.54 a | 3.28 ± 1.57 a | 6.24 ± 3.38 a | 4.77 ± 2.11 a | 3.8 ± 1.19 a | 137 ± 13.7 c |
| umamit25-1 | 6.18 ± 1.89 a | 20 ± 5.31 a | 18.5 ± 8.86 a | 39.1 ± 22.2 ab | 32.3 ± 15.4 a | 0.99 ± 0.24 a | 5.61 ± 2.21 ab | 6.77 ± 1.13 a | 9.48 ± 2.45 a | 8.83 ± 2.05 ab | 2.49 ± 1.26 a | 6.43 ± 2.98 a | 7.2 ± 1.04 a | 0.78 ± 0.42 a | 4.34 ± 2.15 a | 4.76 ± 2.34 a | 9.41 ± 4.79 a | 5.9 ± 2.39 a | 5.78 ± 3.18 a | 195 ± 9.67 ab |
| *umamit25-1* UMAMIT25 | 6.42 ± 1.81 a | 20 ± 4.28 a | 12.1 ± 5.69 a | 33.4 ± 24 ab | 22.5 ± 10.7 a | 1.11 ± 0.29 a | 3.98 ± 0.7 b | 5.84 ± 0.98 a | 8.76 ± 4.41 a | 7.13 ± 2.1 ab | 2.22 ± 1.73 a | 5.24 ± 3.36 a | 8.07 ± 0.98 a | 0.74 ± 0.63 a | 3.93 ± 3.01 a | 4.11 ± 2.87 a | 8.19 ± 6.28 a | 6.48 ± 4.2 a | 4.47 ± 2.41 a | 164 ± 19.1 bc |

| Day 14 | ASP | GLU | ASN | SER | GLN | HIS | GLY | THR | ALA | ARG | TYR | VAL | MET | TRP | PHE | ILE | LEU | LYS | PRO | Sum |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type | 4.11 ± 0.6 ab | 10.3 ± 0.66 a | 6.03 ± 4.7 a | 4.56 ± 0.61 a | 7.03 ± 0.92 ab | 0.77 ± 0.06 a | 2.81 ± 0.13 a | 2.78 ± 0.19 ab | 7.76 ± 0.65 a | 4.54 ± 0.55 a | 1.61 ± 0.31 a | 3.23 ± 0.59 a | 4.58 ± 0.47 a | 0.38 ± 0.08 a | 2.91 ± 0.69 a | 2.66 ± 0.5 a | 5.78 ± 1.36 a | 3.44 ± 0.59 a | 2.44 ± 0.34 a | 77.9 ± 5.36 a |
| *umamit24-1* | 2.98 ± 0.81 b | 9.89 ± 1.67 a | 1.66 ± 1 a | 3.51 ± 0.7 a | 3.92 ± 1.22 c | 0.53 ± 0.15 b | 2.19 ± 0.34 b | 1.83 ± 0.21 c | 4.8 ± 1.07 b | 1.55 ± 0.59 c | 0.74 ± 0.38 b | 1.53 ± 0.83 b | 2.66 ± 0.22 d | 0.23 ± 0.12 ab | 1.16 ± 0.56 b | 1.21 ± 0.67 b | 2.02 ± 1.25 b | 1.35 ± 0.79 b | 0.94 ± 0.31 b | 44.8 ± 6.78 c |
| *umamit24-1* UMAMIT24 | 3.79 ± 0.55 ab | 8.86 ± 0.44 a | 2.91 ± 1.74 a | 3.55 ± 0.88 a | 7.81 ± 1.73 ab | 0.57 ± 0.05 ab | 2.15 ± 0.14 b | 2.16 ± 0.54 bc | 6.49 ± 2.16 ab | 3.74 ± 0.82 a | 0.84 ± 0.31 b | 1.7 ± 0.64 b | 3.31 ± 0.27 cd | 0.2 ± 0.06 b | 1.39 ± 0.56 b | 1.38 ± 0.55 b | 2.67 ± 1.19 b | 1.87 ± 0.48 b | 1.66 ± 0.35 ab | 57.1 ± 3.65 b |
| *umamit25-1* | 3.24 ± 0.1 b | 9.91 ± 0.9 a | 2.01 ± 0.46 a | 4.81 ± 0.21 a | 5.69 ± 0.43 bc | 0.6 ± 0 ab | 2.73 ± 0.22 a | 2.8 ± 0.15 ab | 5.96 ± 0.34 ab | 2.11 ± 0.39 bc | 1.27 ± 0.23 ab | 2.52 ± 0.51 ab | 3.5 ± 0.35 bc | 0.31 ± 0.06 ab | 2.16 ± 0.52 ab | 2.06 ± 0.5 ab | 4.21 ± 1.04 ab | 2.54 ± 0.5 ab | 2.34 ± 0.38 a | 60.8 ± 4.58 b |
| *umamit25-1* UMAMIT25 | 4.63 ± 0.41 a | 10.8 ± 1.22 a | 2.14 ± 0.19 a | 3.48 ± 0.16 a | 8.85 ± 1.99 a | 0.71 ± 0.12 ab | 2.31 ± 0.3 ab | 2.94 ± 0.23 a | 6.51 ± 0.55 ab | 3.54 ± 0.78 ab | 1.26 ± 0.2 ab | 2.47 ± 0.52 ab | 4.34 ± 0.53 ab | 0.28 ± 0.05 ab | 2.2 ± 0.44 ab | 2.12 ± 0.4 ab | 4.22 ± 0.85 ab | 2.76 ± 0.56 ab | 2.44 ± 0.93 a | 68.1 ± 5.77 ab |

| Mature | ASP | GLU | ASN | SER | GLN | HIS | GLY | THR | ALA | ARG | TYR | VAL | MET | TRP | PHE | ILE | LEU | LYS | PRO | Sum |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type | 1.75 ± 0.85 a | 7.02 ± 2.44 a | 5.55 ± 0.71 a | 3.04 ± 1.5 a | 1.18 ± 0.65 a | 0.52 ± 0.05 a | 2.1 ± 0.63 a | 0.58 ± 0.11 a | 1.13 ± 0.13 a | 4.17 ± 1.95 a | 0.17 ± 0.03 a | 0.61 ± 0.02 a | 0.3 ± 0.03 a | 0.14 ± 0.08 a | 0.54 ± 0.08 a | 0.3 ± 0 a | 0.26 ± 0.02 a | 0.51 ± 0.14 a | 0.79 ± 0.1 a | 30.75 ± 3.08 a |
| *umamit24-1* | 1.91 ± 0.37 a | 6.82 ± 1.01 a | 5.7 ± 1.22 a | 2.47 ± 1.42 a | 1.42 ± 0.85 a | 0.44 ± 0.09 ab | 1.54 ± 0.61 a | 0.6 ± 0.13 a | 0.98 ± 0.27 a | 3.4 ± 1.65 a | 0.18 ± 0.02 a | 0.59 ± 0.14 a | 0.26 ± 0.08 a | 0.13 ± 0.04 a | 0.51 ± 0.08 a | 0.29 ± 0.06 a | 0.24 ± 0.06 a | 0.48 ± 0.13 a | 0.56 ± 0.14 a | 28.6 ± 6.1 a |
| *umamit24-1* UMAMIT24 | 2.31 ± 0.13 a | 7.92 ± 0.21 a | 3.33 ± 0.1 b | 1.15 ± 0.06 a | 0.31 ± 0.05 a | 0.27 ± 0.01 c | 1.11 ± 0.19 a | 0.41 ± 0.04 a | 0.92 ± 0.07 a | 1.39 ± 0.2 a | 0.18 ± 0 a | 0.5 ± 0.04 a | 0.24 ± 0 a | 0.16 ± 0.01 a | 0.43 ± 0.03 a | 0.28 ± 0.03 a | 0.19 ± 0.02 a | 0.3 ± 0.01 a | 0.36 ± 0.37 a | 21.85 ± 0.88 a |
| *umamit25-1* | 1.69 ± 0.17 a | 6.87 ± 0.77 a | 5.34 ± 0.42 ab | 1.57 ± 0.07 a | 1.23 ± 0.11 a | 0.42 ± 0.07 abc | 1.39 ± 0.22 a | 0.65 ± 0.01 a | 0.75 ± 0.02 a | 3.11 ± 0.44 a | 0.17 ± 0.01 a | 0.61 ± 0.05 a | 0.27 ± 0.01 a | 0.07 ± 0.01 a | 0.44 ± 0.02 a | 0.31 ± 0.01 a | 0.27 ± 0.01 a | 0.47 ± 0.09 a | 0.43 ± 0.12 a | 26.15 ± 0.94 a |
| *umamit25-1* UMAMIT25 | 2.03 ± 0.43 a | 7.2 ± 0.96 a | 4.37 ± 1.16 ab | 1.68 ± 0.7 a | 0.88 ± 0.49 a | 0.34 ± 0.06 bc | 1.53 ± 0.54 a | 0.49 ± 0.11 a | 0.95 ± 0.18 a | 2.71 ± 1.37 a | 0.19 ± 0.02 a | 0.54 ± 0.07 a | 0.28 ± 0.04 a | 0.13 ± 0.02 a | 0.47 ± 0.04 a | 0.29 ± 0.07 a | 0.24 ± 0.03 a | 0.41 ± 0.11 a | 0.67 ± 0.51 | 25.5 ± 4.31 a |

Appendix 30. Amino acid content in 7, 10 and 14 day-old pericarp tissues. Values are expressed in nmol per µg of dry weight. Each sample corresponds to two siliques worth of pericarp tissue coming from the same plant. Means and standard deviations are displayed with n=3. Significant differences ($p<0.05$) are indicated by different letter according to one way ANOVA in conjunction with Tukey's test.

| Day 7 | ASP | GLU | ASN | SER | GLN | HIS | GLY | THR | ALA | ARG | TYR | VAL | MET | TRP | PHE | ILE | LEU | LYS | PRO | Sum |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type | 7.19 ± 0.42 a | 20 ± 2.23 a | 26.5 ± 10 a | 117 ± 8.31 a | 247 ± 91.9 ab | 4.63 ± 0.76 a | 20.1 ± 9.67 a | 25.8 ± 2.38 a | 5.56 ± 0.92 a | 66.1 ± 13.6 a | 0.55 ± 0.08 a | 3.74 ± 0.53 a | 4.6 ± 2.29 a | 0.5 ± 0.11 a | 1.16 ± 0.23 a | 3.21 ± 0.47 a | 4.81 ± 2 a | 4.04 ± 0.86 a | 14.8 ± 3.53 a | 578 ± 135 a |
| *umamit24-1* | 9.85 ± 2.57 a | 18.7 ± 7.21 a | 31.5 ± 10.7 a | 77.7 ± 17.3 ab | 174 ± 56.2 bc | 1.77 ± 0.96 b | 3.51 ± 1.82 b | 28.2 ± 8.12 a | 4.95 ± 0.68 a | 47.2 ± 13 ab | 0.44 ± 0.13 a | 3.21 ± 0.37 a | 2.23 ± 0.2 a | 0.34 ± 0.17 a | 0.64 ± 0.12 ab | 2.13 ± 0.52 a | 1.74 ± 0.49 b | 2.16 ± 0.79 b | 19.4 ± 7.64 a | 430 ± 94.9 ab |
| *umamit24-1* UMAMIT24 | 18.8 ± 2.06 b | 30.7 ± 4.64 a | 25.2 ± 6.1 a | 59 ± 15 b | 74 ± 22 c | 1.75 ± 0.48 b | 2.53 ± 2.35 b | 36.4 ± 5.27 a | 5.94 ± 0.97 a | 30.9 ± 13.6 b | 0.54 ± 0.2 a | 4.03 ± 1.21 a | 3.29 ± 1.23 a | 0.29 ± 0.18 a | 0.5 ± 0.19 b | 2.59 ± 0.98 a | 1.47 ± 0.39 b | 1.78 ± 0.86 b | 29.2 ± 8.07 a | 329 ± 63.3 b |
| *umamit25-1* | 10.9 ± 3.51 a | 22.8 ± 8.55 a | 31.5 ± 12.2 a | 87.2 ± 28.6 ab | 263 ± 48 ab | 3.19 ± 0.59 ab | 5.8 ± 5.72 b | 25.5 ± 6.06 a | 5.95 ± 1.69 a | 59.2 ± 17.9 ab | 0.57 ± 0.38 a | 4.31 ± 1.41 a | 1.83 ± 1.08 a | 0.33 ± 0.33 a | 0.72 ± 0.4 ab | 2.86 ± 1.58 a | 2.5 ± 1.57 ab | 3.08 ± 0.79 ab | 14.8 ± 6.46 a | 547 ± 92.2 a |
| *umamit25-1* UMAMIT25 | 11.1 ± 1.75 a | 19.4 ± 4.89 a | 40.6 ± 12 a | 105 ± 19.8 a | 306 ± 37.3 a | 2.62 ± 1.79 ab | 3.32 ± 0.65 b | 28.4 ± 2.14 a | 4.84 ± 0.85 a | 59.4 ± 14.6 ab | 0.42 ± 0.05 a | 3.51 ± 0.29 a | 3.78 ± 0.76 a | 0.32 ± 0.1 a | 0.64 ± 0.18 ab | 2.26 ± 0.29 a | 1.74 ± 0.45 b | 3 ± 0.13 ab | 14.4 ± 6.55 a | 612 ± 45.4 a |

| Day 10 | ASP | GLU | ASN | SER | GLN | HIS | GLY | THR | ALA | ARG | TYR | VAL | MET | TRP | PHE | ILE | LEU | LYS | PRO | Sum |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type | 7.21 ± 0.89 a | 24.4 ± 3.49 a | 36.9 ± 18.4 a | 97.9 ± 13.3 a | 242.8 ± 99.6 a | 9.67 ± 0.68 a | 26 ± 14.7 a | 22.5 ± 2.76 a | 5.4 ± 0.76 a | 161 ± 25.8 a | 1.01 ± 0.33 a | 5.54 ± 0.88 a | 2.71 ± 0.86 a | 1.99 ± 1.05 a | 2.85 ± 1.6 a | 3.93 ± 0.94 a | 12.5 ± 8.5 a | 20.2 ± 4.16 a | 31.5 ± 8.87 a | 716 ± 140 a |
| *umamit24-1* | 12.2 ± 3.08 ab | 27 ± 8.26 a | 28.1 ± 7.23 ab | 57.2 ± 26 ab | 134.4 ± 78.3 ab | 5.6 ± 1.23 b | 6.01 ± 7.77 ab | 14.1 ± 3.65 b | 6.01 ± 1.74 a | 140 ± 34.6 a | 0.81 ± 0.32 a | 4.35 ± 1.44 a | 2.7 ± 2.38 a | 1.54 ± 0.87 a | 1.86 ± 1.2 a | 3.21 ± 1.34 a | 7.38 ± 6.34 a | 12.1 ± 6.65 a | 28.4 ± 5.11 a | 494 ± 140 a |
| *umamit24-1* UMAMIT24 | 20 ± 1.64 b | 37 ± 1.36 a | 8.6 ± 2.61 b | 17.1 ± 2.57 b | 14.77 ± 3.17 b | 1.94 ± 0.62 c | 1.2 ± 0.19 b | 10.4 ± 2.38 b | 10.6 ± 1.78 b | 18.1 ± 7.43 b | 0.67 ± 0.28 a | 3.61 ± 0.79 a | 3.46 ± 1.75 a | 1.06 ± 0.79 a | 0.7 ± 0.24 a | 2.44 ± 1.06 a | 1.83 ± 0.6 a | 1.8 ± 0.44 b | 19.8 ± 4.76 a | 175 ± 7.67 b |
| *umamit25-1* | 15.3 ± 8.93 ab | 39 ± 8.91 a | 35 ± 11.7 a | 55.8 ± 30.6 ab | 193 ± 69.2 a | 7.57 ± 1.21 ab | 9.52 ± 14.5 ab | 14.4 ± 2.74 ab | 4.16 ± 1.43 a | 147 ± 13.2 a | 1 ± 0.57 a | 4.96 ± 1.36 a | 2.66 ± 1.43 a | 1.9 ± 0.86 a | 2.23 ± 1.16 a | 3.52 ± 1.19 a | 5.02 ± 4.35 a | 14.4 ± 5.41 a | 24.6 ± 3.49 a | 582 ± 83.9 a |
| umamit25-1 UMAMIT25 | 15.6 ± 4.36 ab | 31.4 ± 4.2 a | 31.3 ± 15.3 ab | 49.2 ± 20 ab | 176.1 ± 88.3 ab | 6.56 ± 2.65 ab | 3.8 ± 1.7 ab | 17.3 ± 4.91 ab | 5.75 ± 1.67 a | 129 ± 31.9 a | 0.89 ± 0.4 a | 5.95 ± 2.22 a | 3.14 ± 1.7 a | 1.42 ± 0.56 a | 1.56 ± 0.5 a | 4.15 ± 1.73 a | 11.36 ± 3.23 a | 12.4 ± 3.52 a | 35.5 ± 15.8 a | 543 ± 145 a |

| Day 14 | ASP | GLU | ASN | SER | GLN | HIS | GLY | THR | ALA | ARG | TYR | VAL | MET | TRP | PHE | ILE | LEU | LYS | PRO | Sum |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type | 16.4 ± 1.61 a | 34.7 ± 1.17 a | 5.76 ± 0.7 a | 13.93 ± 1.81 a | 12.6 ± 6.13 ab | 2.78 ± 0.1 a | 3.25 ± 0.15 a | 11.7 ± 3.72 a | 6.79 ± 0.72 a | 29.8 ± 17.2 ab | 1.01 ± 0.14 a | 3.29 ± 0.29 a | 3.33 ± 0.32 a | 1.34 ± 0.29 ab | 1.51 ± 0.27 a | 2.03 ± 0.12 a | 3.29 ± 0.09 a | 2.04 ± 0.43 a | 25.1 ± 5.78 ab | 180. ± 36. a |
| *umamit24-1* | 9.69 ± 2.32 b | 27.4 ± 2.86 b | 2.13 ± 0.66 c | 7.27 ± 1.51 b | 4.04 ± 2.85 b | 0.75 ± 0.4 b | 3.36 ± 0.95 a | 4.06 ± 1.2 b | 5.64 ± 3.9 a | 1.19 ± 1.54 b | 0.81 ± 0.5 a | 2.06 ± 0.84 b | 2.78 ± 2.38 a | 0.76 ± 0.45 b | 1.3 ± 0.81 a | 1.33 ± 0.72 a | 2.37 ± 1.24 a | 1 ± 1.09 a | 4.9 ± 2.28 c | 82.9 ± 19.3 b |
| *umamit24-1* UMAMIT24 | 15.1 ± 1.04 a | 32.2 ± 0.97 ab | 5.91 ± 1.28 a | 12.6 ± 1.59 ab | 13.7 ± 1.67 ab | 3.43 ± 0.26 a | 2.64 ± 0.1 a | 11.93 ± 2.38 a | 5.31 ± 1 a | 44.5 ± 25.5 a | 0.89 ± 0.07 a | 2.96 ± 0.41 ab | 2.41 ± 1.28 a | 1.52 ± 0.56 ab | 1.48 ± 0.47 a | 1.89 ± 0.3 a | 2.77 ± 0.58 a | 2.33 ± 1.14 a | 26.15 ± 4.72 ab | 189. ± 22.2 a |
| *umamit25-1* | 14.2 ± 1.57 a | 30.7 ± 4.11 ab | 3.08 ± 1.43 bc | 10.8 ± 2.4 ab | 5.48 ± 1.67 b | 1.25 ± 0.54 b | 3.32 ± 0.56 a | 8.03 ± 2.28 b | 5.94 ± 1.41 a | 2 ± 2.43 b | 0.9 ± 0.25 a | 2.54 ± 0.58 ab | 3.27 ± 0.37 a | 1.35 ± 0.36 ab | 1.47 ± 0.7 a | 1.66 ± 0.49 a | 2.68 ± 0.8 a | 1.06 ± 0.6 a | 18.41 ± 7.67 b | 118. ± 22 b |
| *umamit25-1* UMAMIT25 | 14.9 ± 1.44 a | 29.6 ± 3.73 ab | 5.1 ± 1.46 ab | 11.1 ± 0.64 a | 17.9 ± 9.56 a | 2.84 ± 1.12 a | 3.05 ± 0.49 a | 12.3 ± 1.91 a | 5.86 ± 0.63 a | 35.2 ± 19.3 ab | 0.97 ± 0.01 a | 2.82 ± 0.22 ab | 3.41 ± 0.06 a | 1.72 ± 0.12 ab | 1.2 ± 0.38 a | 1.75 ± 0.09 a | 2.67 ± 0.15 a | 1.38 ± 0.28 a | 33.3 ± 8.29 a | 187 ± 36.9 a |

Appendix 31. Primers used for cloning and qRT-PCR. Primer sequences are given from 5' to 3'. Underlined bases were added for the creation of *attB1* and *attB2* in forward and reverse primers, respectively.

| Name | Sequence |
|---|---|
| UMAMIT14 beginning of gDNA (Forward) | ACAAGTTTGTACAAAAAAGCAGGCTTCGATATGGCTTTAAAAACATGGAAG |
| UMAMIT14 end of gDNA (Reverse) | ACCACTTTGTACAAGAAAGCTGGGTCTCAGACTGATTCATTGGTGTTAGGCCT |
| UMAMIT18 beginning of gDNA (Forward) | GACAAGTTTGTACAAAAAAGCAGGCTCAATAAAGATGAAAGGTGGAAGCATG |
| UMAMIT18 end of gDNA (Reverse) | GACCACTTTGTACAAGAAAGCTGGGTACYAGGTACTGGTAACCACACCGTTAGT |
| UMAMIT23 beginning of gDNA (Forward) | GGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAAGATATAACGGCAATGGTGGT |
| UMAMIT23 end of gDNA (Reverse) | GGACCACTTTGTACAAGAAAGCTGGGTCAGGGACATTTGTACTTAATGTTGGG |
| UMAMIT24 beginning of gDNA (Forward) | GGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGAGTGTAGTTGCAATGGTGGC |
| UMAMIT24 end of gDNA (Reverse) | GGACCACTTTGTACAAGAAAGCTGGGTCGGGGACATCTCTATTTACTGATGAAAGATT |
| UMAMIT25 beginning of gDNA (Forward) | GGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCTAAATCAGATATGTTGCCGTT |
| UMAMIT25 end of gDNA (Reverse) | GGACCACTTTGTACAAGAAAGCTGGGTCAGGCGATGTAGACCTTGTGGAACC |
| Actin 8 qRT-PCR (Forward) | GTGTCTGGATTGGTGGTTCTATCC |
| Actin 8 qRT-PCR (Reverse) | GCCTTAGAGATCCACATCTGCTG |
| PR1 qRT-PCR (Forward) | GCGGTAGGCGTAGGTCCCA |
| PR1 qRT-PCR (Reverse) | CGCCAGACAAGTCACCGCTA |