Characterizing the role in amino acid sensing and signaling of *Amino Acid Permease 1* in Arabidopsis

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(ACADEMIC ABSTRACT)

Amino acids are necessary for protein synthesis and specialized metabolism in plants. Yet very little is known about how plants sense and regulate when and where to allocate amino acids to meet the demand for nitrogen in growing tissues. In particular, while characterized in yeast and mammals, no amino acid sensor has been identified in plants. Amino Acid Permease 1 (AAP1) has been previously characterized and was shown to mediate amino acid uptake from the soil. *aap1* knockout plants and several EMS mutants affected in *AAP1* sequence display enhanced tolerance to toxic concentrations of amino acids. Yet, two of the corresponding variant proteins appear to be functional transporters, effectively dissociating amino acid transport and phenotype. To understand this apparent discrepancy, I precisely studied AAP1 localization of expression at the plant and cellular level, and in specific tissue types of the root where AAP1 function is required for the tolerance phenotype and the amino acid uptake activity. I showed that AAP1 protein is present in the endoplasmic reticulum of the cortex in wild type plants Yet, its ectopic expression in root tip and phloem increased amino acid uptake, while expression in cortex could not. This and other of my results do not support the current model of AAP1 functioning in amino acid uptake by the root. I propose that the main effect of mutations in *AAP1* is a disturbance in amino acid metabolism, possibly triggered by altered amino acid sensing. In this new model, AAP1 would be necessary for sensing amino acid status of cortex cells, possibly in the endoplasmic reticulum, and adjust amino acid metabolic activity and uptake to current availability. In effect, disruption of the sensing function, either by complete loss of AAP1 function (knockout) or by uncoupling the transport and sensing function (EMS mutants), would lead to the various characteristics of the phenotype of the *aap1* mutants I observed. My main hypothesis is that AAP1 is a transporter endowed with sensing function, i.e., an amino acid transceptor.

Characterizing the role in amino acid sensing and signaling of *Amino Acid Permease 1* in Arabidopsis

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(GENERAL ABSTRACT)

Changing environments create challenges for plants to grow under harsher, nutrient limiting conditions. Nitrogen is an essential nutrient for plant growth, used for the synthesis of amino acids and other nitrogen-containing metabolites. Amino acids are necessary for protein synthesis and other specialized metabolism – being targets for manipulation for improving agronomic traits. Protein content is a complex trait that involves many genes, possibly including amino acid transporters. In addition, the amount of nitrogen needed by and available to the plant increases or decreases depending on the environment conditions. How plants control nitrogen need and use at the molecular level is not well understood. The data presented here challenge a current model and I report how a protein (AAP1) involved in the acquisition of amino acids from the soil provides regulatory control over these processes. This valuable information is useful for better understanding how plants use nitrogen and more precise breeding methods can be used to improve traits, such as protein content in agronomically important crops.

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Chapter 1
Literature Review

While plants are able to fix carbon in a gaseous form by the process of photosynthesis, the acquisition of other nutrients such as nitrogen depends on uptake from the soil (or symbiosis for some plants). Nitrogen acquisition, assimilation and metabolism in plants is a highly-regulated process that begins with the uptake from the soil and the translocation from the roots to the shoots. Inorganic nitrogen can be imported as nitrate, which is then reduced to ammonium. The ammonium, derived from nitrate reduction or from direct uptake from the soil, is assimilated into the amino acid glutamine (Gln) by the glutamine synthase (GS) and glutamate synthetase (GOGAT) cycle. The other amino acids are synthesized using the intermediates of carbon metabolism and the nitrogen from Gln or glutamate (Glu). A large share of the amino acids are loaded into the phloem and translocated to sink tissues, where they are used for diverse functions that include protein synthesis, storage, and/ or synthesis of specialized metabolites. While essential for fine tuning nitrogen uptake and amino acid synthesis to needs and resources, the process through which plants sense and respond to both internal and external pools of amino acids is elusive. A few proteins are postulated to sense amino acids, including catalytic enzymes, transporters, and regulators (Pilot et al., 2003).

Potential Integration of the Amino Acid Signal

Root architecture, specifically branching and lateral root formation, have been shown to be controlled by the interaction of nitrate and Glu at the root tip (Zhang and Forde, 1998; Walch-Liu et al., 2006). Specifically, Glu and low nitrogen conditions cause increases in root branching and decreases in the primary root length (Zhang and Forde, 1998; Walch-Liu et al., 2006), but nitrate concentrations that exceed 10 mM do not initiate lateral root branching in Arabidopsis or barley (Drew and Saker, 1975). In these experiments, Glu caused increases in root branching when applied to the root tip, but to a lesser extent when applied to mature section of the root. The inhibitory effects on root growth and branching caused by 50 µM Glu were overcome by increasing the nitrate concentration to 0.5 mM in Arabidopsis C24 plants. These effects were even more pronounced in the Col-0 background (Walch-Liu and Forde, 2008). This suggests that both nitrogen and amino acids, specifically Glu, promote lateral root development in Arabidopsis. Potential integration of the Glu response was shown to use the MEKK 1 pathway, because the *mekk1/2/3* triple mutant did not respond to 2 mM Glu application and failed to initiate lateral roots (Forde et al., 2013).

In rice, shoot length and root growth increased with higher concentrations of Gln and Glu as the sole nitrogen source. However, neither 0.5 mM Gln nor Glu caused growth differences from rice seedlings grown in the presence of 1.43 mM NH_4NO_3 (Kan et al., 2015; Kan et al., 2017; Goto et al., 2020) Additional analysis showed that internal levels of Gln increased when N-starved plants were fed Glu or Gln, and Gln was rapidly converted to other amino acids. The application of Gln and Glu rapidly induced approximately 35 responsive genes in roots that are involved in nitrogen metabolism and signaling (Kan et al., 2015; Kan et al., 2017). Further, a systems-level genomic

approach was used to identify genes that differentially respond to inorganic and organic nitrogen (i.e., Glu or Glu-derived metabolites) (Gutierrez et al., 2008).

These data showed that roots respond both at the developmental and cellular levels to amino acids. Root architecture studies could serve as a proxy for preliminary testing the effects of specific amino acids on overall metabolism. Specifically, proteins involved in amino acid homeostasis, necessary for sensing external and internal Glu and/ or other amino acids, may cause differences in root phenotypes from wild-type plants.

Amino Acid Transporters

The movement of amino acids between organelles and cells requires several different transporters to enable the crossing of hydrophobic membranes. Amino acid transporters in plants belong to two major superfamilies: the amino acid, polyamine, choline transporter (APC) and the amino acid transport family (ATF). Within the ATF superfamily, five sub-classes have been identified: the lysine-histidine transporter (LHTs), proline transporters (ProTs), auxin transporters (AUXs), aromatic and neutral amino acid transporters (ANTs) and amino acid permeases (AAPs) (Ortiz-Lopez et al., 2000). Also, within the amino acid/ auxin permease (AAAP) family are the amino acid vacuolar transports (AVTs) that export amino acids from the vacuole to the cytosol (Okumoto and Pilot, 2011; Fujiki et al., 2017). The AAPs are the among the best described transporters in the family, with Arabidopsis *AAP1* being the first characterized amino acid transporter in plants (Frommer et al., 1993; Hsu et al., 1993). Since then, seven other AAPs have been characterized in Arabidopsis (Okumoto et al., 2002) as well as many more from other species. The AAPs are involved in different steps of the amino acid uptake and mobilization pathways. AAP1 (Lee et al., 2007) and AAP5 (Svennerstam et al., 2008) are described as participate in the uptake from the soil; AAP2 (Zhang et al., 2010) and AAP6 (Hunt et al., 2010) are involved in xylem-phloem transfer; AAP3 (Ortiz-Lopez et al., 2000) is involved in phloem loading; and AAP1 (Sanders et al., 2009) and AAP8 (Okumoto et al., 2002) have roles in seed filling, the transfer of amino acids from the mother plant to the embryo. These AAPs were shown to have different substrate specificities and affinities: AAP1, AAP6, and AAP8 selectively transport neutral amino acids - AAP6 being the only AAP capable of transporting Asp. AAP3 and AAP5 selectively transport Arg, Lys, and His (Fischer et al., 1995; Boorer et al., 1996; Fischer et al., 2002).

In rice, several AAPs are demonstrated to regulate tiller number and seed filling (Peng et al., 2014; Ji et al., 2020). Transcriptomic analysis of *Osaap1* knockout rice plants also showed that nitrogen metabolism and auxin biosynthesis signaling were disrupted (Ji et al., 2020). While AAPs in rice are not the same as Arabidopsis AAPs, this paper suggests that the AAP-family of proteins may have additional roles beyond those currently described as exclusively transporters.

Nutrient Transceptors

Several nutrient transporters have been described to sense nutrients and activate signaling cascades, in addition to a transport role; these dual function proteins are termed transceptors (Diallinas, 2017). Nutrient transceptors have been described in mammals, *Drosophila* (Goberdhan et al., 2005), yeast and plants. The General Amino Acid Permease 1 (Gap1) transporter in *Saccharomyces cerevisiae* acts as both a sensor and as a regulator of amino acid

homeostasis by rapidly inducing changes in the Protein Kinase A (PKA) pathway (Donaton et al., 2003; Kriel et al., 2011). This result was confirmed with site-directed mutagenesis of Gap1 residues involved in transport, where the amino acid transport was inhibited, but the Protein Kinase A (PKA) pathway was still activated (Donaton et al., 2003; Van Zeebroeck et al., 2014) - providing evidence that transport and signaling functions can be uncoupled. Other examples include the ammonium transporter (Mep2) (van den Berg et al., 2016) phosphorus transporter (Pho84) (Samyn et al., 2012), sulfur transporter (SUL2) (Kankipati et al., 2015) in *S. cerevisiae*, which all activated the PKA pathway upon recognition of their respective nutrients. In mammals, the Sodium-coupled Neutral Amino Acid Transporter 2 (SNAT2) senses amino acids and initiates the downregulation of the SNAT2 promoter under amino acid sufficient conditions through the activation of the JNK/ERK signaling pathways (Hyde et al., 2007). Additionally, inhibition of SNAT2 was shown to impair proper signaling of the regulatory Target of Rapamycin (mTOR) on several properties of growth (Pinilla et al., 2011; Laplante and Sabatini, 2012; Cormerais et al., 2020). The Target of Rapamycin (TOR) and Sucrose non-fermenting 1 (SNF1)-related protein kinase 1 (SnRK1) are regulatory protein complexes that integrate multiple environmental and nutrient cues to promote different aspects of plant growth (McCready et al., 2020; O'Leary et al., 2020). Gln and other nitrogen sources activated TOR-induced growth (Xiong and Sheen, 2014; Liu et al., 2021). Another amino acid transceptor, PAT1 has been shown to sense and signal amino acids in the lysosome (Goberdhan et al., 2005). The glucose transporter GLUT2 has also been characterized as a transceptor in mammals (Leturque et al., 2009).

In plants, only the Nitrate Transporter 1 (NRT1.1; NPF6;3) is characterized as a transceptor (Ho et al., 2009). There are no current reports of any amino acid transceptor in plants.

Growth on Toxic Amino Acids

Plants use amino acids for many different functions; yet the accumulation of amino acids at high levels has adverse effects on growth and development, resulting in toxicity. This can result from a loss of feedback mechanisms, particularly in the ability of the plant to repress key enzymes in amino acid synthesis leading to the accumulation of amino acids (i.e., Phe and Trp) (Yamada et al., 2008). Identifying genes involved in amino acid metabolism and amino acid regulation can be accomplished by screening mutants on toxic concentrations of amino acids. A plant germination screening on toxic concentrations of phenylalanine identified a mutant of *AAP1* (Lee et al., 2007). The report further showed that T-DNA insertions in *AAP1*, *aap1-1* and *aap1-2* provided resistance to toxic concentrations of most amino acids compared to wild-type Wassilewskija (WS) plants. Similarly, a screening for resistance to proline isolated the *pre1-1* (*proline-resistant 1-1*) mutant that contains a 19-bp deletion in Exon 1 of *AAP1*. In this case, *pre1-1* showed a ~70% increase in root length after 16 d when grown on 45 mM *L*-proline compared to Col-0 (Wang et al., 2017). In all cases, *AAP1* mRNA was not or minimally detected by RT-PCR experiments in the mutants, suggesting that loss of function contributes to these resistances.

Expression and Localization of AAP1.

The localization of *AAP1* expression has been studied with northern blot analysis, *in situ* hybridization and promoter-GUS assays. Northern analyses detected *AAP1* mRNA in flowers and developing siliques, but not in roots (Kwart et al., 1993; Guo, 2004) . *In situ* hybridization of whole

Arabidopsis seedlings showed that *AAP1* transcript accumulates in the vascular system of the cotyledons, as well as in the elongation zone behind the root tip (Kwart et al., 1993; Guo, 2004). It is interesting to note that the results from these various studies do not completely agree with one another.

Fusions of the AAP1 promoter to a GFP:AAP1 construct showed localization of the GFP to the root epidermis and cortex (Lee et al., 2007). Using an indirect approach, *AAP1p:GUS*, staining was observed in the root, yet also appears to be expression in the developmental zone of the root tip, lateral root buds, and quiescent center (Chen, 1997). It should be noted that the characterization of *AAP1* was done in three different ecotypes in these studies, expression similarities exist between studies done in C24 (Kwart et al., 1993) and Col-0 (Chen, 1997; Wang et al., 2017), but differ from the expression pattern found in the WS ecotype (Lee et al., 2007). The different results could also be due to differences in the lengths of the promoter region used. The only reports of *AAP1* expression in the root cap using the promoter-GUS approach is from (Lee et al., 2007), although the staining does not yield clear cell-type, tissue-specific localization. Generally, the consensus is that *AAP1* is expressed in the stems, cotyledons, and the leaves. Expression in the roots is not preferred and needs to be further studied, despite growing evidence in publicly available transcriptomic data sets that highest expression of *AAP1* mRNA is in cortex, followed by endodermis and phloem tissues (Cartwright et al., 2009; Jean-Baptiste et al., 2019). *AAP1* localization is ubiquitous throughout the embryo, with particular expression in the parenchyma and the outer epidermis (Sanders et al., 2009). In addition, expression appears to be developmentally regulated, occurring in rapidly-growing roots and in maturing embryos, particularly in the cotyledons (Chen, 1997; Hirner et al., 1998). *AAP1* is also expressed early in young and developing siliques - specifically in days 5-9 of development, but not in the vascular tissues of the silique (Chen, 1997; Hirner et al., 1998), and then dissipates, seen only at low levels in the mature silique envelope (Wang et al., 2017), potentially suggesting an early role in transporting amino acids to the seed during filling.

The sub-cellular localization of AAP1 was tested in onion cells (Lee et al., 2007) and Arabidopsis protoplasts (Wang et al., 2017); both studies used confocal microscopy to conclude that AAP1 is present at the plasma membrane. In whole Arabidopsis plants, fluorescence of AAP1:GFP fusions was reported at the plasma membrane of the root epidermis and root cap, but only weak signals were observed in the root tip and root hairs (Lee et al., 2007). We felt that the images in both publications appear slightly fuzzy and blurry, suggesting that AAP1 is also expressed elsewhere, maybe in the ER, similar two rice AAP homologs (Taylor et al., 2015). All of these reports of for the sub-cellular localization have used the cDNA but use of the genomic GFP-AAP1 fusions under the control of the native *AAP1* promoter have not been reported. If the proteins from the genomic DNA are arrested in the ER, this may suggest another layer of regulation, previously unreported.

Functional Characterization of AAP1

AAP1 protein is composed of 11 transmembrane domains. The orientation of the protein in the plasma membrane was confirmed biochemically using protease digestion and immuno-precipitation of AAP1 with a Myc tag fused either the N or C-terminus in COS-I cell (Chang, 1998).

This study showed that the N and C-termini are on opposite sides of the plasma membrane, with the N-terminus in the cytosol and the C-terminus is on the extracellular face of the membrane (Chang and Bush, 1997; Chang, 1998).

Using *S. cerevisiae* 22574d, which contains mutations in general amino acid, Pro and γ-aminobutric acid (GABA) permease genes, Frommer *et al.,* (1993) showed that expression of the *AAP1* cDNA was able to restore Pro uptake. Similarly, the growth of the JT16 strain, which lacks His permease (*hip1*) and His biosynthesis (*his4*) genes, limiting growth on low concentrations of His (down to 130 μM), could also be complemented with *AAP1* cDNAs (Hsu et al., 1993). Competition assays for *L*-[^{14}C] Pro against each of the amino acids showed that AAP1 expressed in both strains showed preference for transport of Phe, Met, Cys, and Ala in addition to Gln and Glu (Frommer et al., 1993; Hsu et al., 1993). Studies with *Xenopus* oocytes showed that AAP1 also transports neutral amino acids with short side chains (Fischer et al., 1995; Boorer et al., 1996; Chang and Bush, 1997), and that transport requires the proton-motive force. Site-directed mutagenesis of the H47 and H337 amino acid residues confirmed that transport is a proton-coupled import of amino acids (Chen, 1997). Saturation kinetics for AAP1 indicate that AAP1 transports in the low-to-medium affinity range (>0.5 mM) (Boorer et al., 1996).

AAP1 Mediates Amino Acid Uptake in plants

AAP1 can transport amino acids in heterologous systems, but the results of uptake measurements between T-DNA mutants and the wild-type plants varies according to the authors. Feeding experiments with 2 mM [^{14}C] Phe showed that 5-day-old seedlings and 3-week-old plants had marked decreases in uptake in the mutants compared to wild-type WS plants as early as 12 h, but no significant differences were observed at 30 min (Lee et al., 2007). I found that the issue with these Phe uptake results is because the uptake and amino acids levels in the roots were actually the same, but the translocation of Phe to the shoots was altered. This would suggest that AAP1 is involved in translocation of amino acids to the shoot, perhaps in addition to a role in uptake from the soil. This does not fit the model in which AAP1 is only expressed in the root epidermis and only involved in uptake from the medium. Similar uptake rates between the mutant and the wild-type were observed for Pro and His (Foster, 2008). Another study compared the uptake of Gln, Glu, Asp, Lys, and Arg in *aap1-3* mutants to wild-type Col-0 and no differences were found for all tested amino acids at low concentrations - these were likely below the physiological range for AAP1 uptake activity (Svennerstam et al., 2011). Overexpression analyses strengthen the relationship between AAP1 activity and amino acid uptake by the root: the overexpression of *AAP1* increased uptake of [^{14}C]Phe, His, and Pro, and also inhibited growth relative to the wild-type plants when grown on 500 μM Phe (Foster, 2008). In another study, overexpression of *AAP1* led to increased uptake of the alanine-chlorantraniliprole conjugate, while no effect was observed in *aap1-3* null mutants (Ren et al., 2019). All of this suggests that AAP1 can take up amino acids in plant cells; by increasing the expression of *AAP1*, there is more protein that is able to take up more of the amino acids.

Regulation of the Expression of AAP1

AAP1 mRNA accumulation is regulated by several factors, including light, and the levels of carbohydrates, hormones, nitrogen, and amino acids themselves. When dark-adapted plants were exposed to white light, *AAP1* mRNA content was induced, peaking after 3 h before decreasing. The effects of 30 mM sucrose and 30 mM glucose had an additive effect on *AAP1* induction in the dark-adapted plants when exposed to light (Guo, 2004). This latter work suggested that that induction appears to depend on the specific C:N ratios and not one specific factor (Guo, 2004). Further, *AAP1* mRNA increased following the application of 50 mM KNO_3 to nitrogen starved plants (Hwang et al., 1997; Guo, 2004) and microarray data show that Glu and Gln elicit stronger gene responses than nitrate in shoots (Guo, 2004). In all three treatments, AAP1 was the only amino acid transporter induced by all three nitrogen sources. This suggests that amino acids may have a stronger regulatory effect on the expression of *AAP1* than inorganic nitrogen. Still further, sequence analysis of the *AAP1* promoter identified four abscisic acid (ABA) response elements (ABRE) located 123 bp upstream from the transcription start site (Hirner et al., 1998; Lee, 2005). RNA gel-blot analysis of 7-day-old soil grown plants sprayed with 100 µM ABA showed that *AAP1* transcript levels increased as early as 4 h post-treatment (Lee, 2005). Similarly, 40 µM ABA caused an increase in *AAP1* mRNA accumulation beginning at 2 h post application (Wang et al., 2017).

There are still questions regarding how *AAP1* expression is actually regulated, if amino acids directly regulate expression, and how they are sensed. Altogether, this suggests that the regulation of *AAP1* expression may be more complicated than simple responses to ABA or nitrate. Rather, a complex regulatory network that involves signals from both nitrate, sugars, and amino acids could control *AAP1* mRNA accumulation. Potential post-transcriptional and post-translational regulation remains to be explored.

Preliminary Data from the Pilot Lab

Regulation of Amino Acid Metabolism - Putative Amino Acid Sensing

A collaboration between the Pilot and Voll labs, showed that *pig1-1* is a mutation in *AAP1*, suggesting that *AAP1* plays a role in regulating amino acid metabolism. The screening of ethyl methanesulfonate (EMS)-treated Arabidopsis seeds performed by (Voll et al., 2004) showed that the *phenylalanine insensitive growth1* (*pig1*) mutants, contrary to the wild-type were able to germinate and develop on 10 mM Phe. Further, the contents of most major amino acids were increased between 1.4 and 2.3 fold in the mutants, with Phe, Gln, and Asn accounting for about 85% of the differences observed between the *pig1-1* and wild-type plants (Voll et al., 2004). Interestingly, neither Phe uptake nor root-to-shoot translocation were affected in the *pig1-1* plants. The authors showed that the overall metabolism of *pig1* mutants was disturbed compared to that of wild-type plants, leading to enhanced degradation of Phe upon uptake, putatively leading to Phe tolerance (Voll et al., 2004). A T-DNA mutant (SAIL 871 C03, Columbia) referred to as *aap1-4* was used as the loss-of-function mutant in the characterization of *AAP1*.

Current Model of AAP1 Function

The current model is that AAP1 is a low-to-medium affinity, broad specificity amino acid / H^+ symporter located in the plasma membrane of the epidermis of the mature root, mediates the uptake of amino acids from the soil and transfer to the embryo. According to this model, the resistance of *aap1* knockout mutants to toxic concentrations of amino acids is due to reduced amino acid uptake from the medium (Lee et al., 2007). The work presented herein was to resolve the expression pattern of *AAP1* in Arabidopsis and define a consensus for location and function and both a tissue-specific and subcellular level. Lastly, if AAP1 is unique in that the protein is sensing and signaling to maintain nitrogen levels, a mechanism would need be elucidated. This dissertation challenges this model proposed by Lee et. al., (2007) by testing the hypothesis that AAP1 is involved in sensing and regulatory functions related to nitrogen metabolism (Fig. 1).

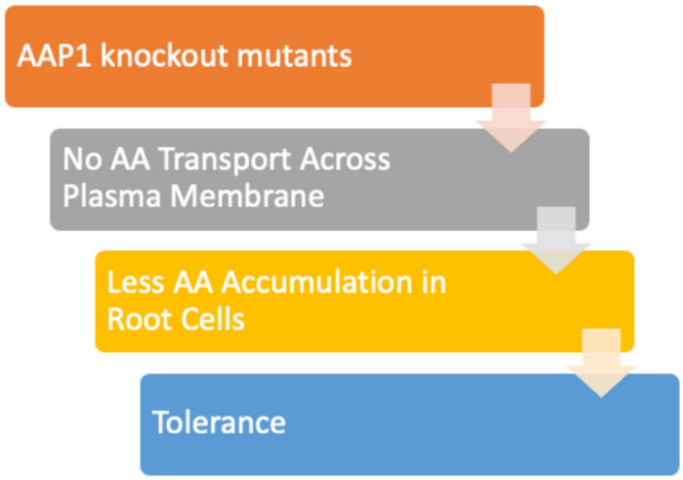


Figure 1. Model established by Lee et. al., 2007 describing how *AAP1* is responsible for mediating uptake of amino acids and the association with tolerance.

Chapter 2
AAP1 Expression and Localization Studies

As outlined in Chapter 1, the expression and localization of *AAP1* reported in the literature does not agree on the tissue-specific localization of *AAP1*, lacking consensus on the data obtained from promoter, transcriptomics, and protein localization analyses (Kwart et al., 1993; Cartwright et al., 2009; Taylor et al., 2015; Wang et al., 2017; Denyer et al., 2019; Jean-Baptiste et al., 2019; Zhang et al., 2019). Each of these reports showed the expression of *AAP1* in different tissues, potentially because these studies were performed in different ecotypes, namely Ws, C24, and Col-0. This raises questions about the tissue-specific expression of *AAP1* in plants; particularly in which tissue is *AAP1* expressed and which lab group is correct in describing the localization. Based on the results of long term uptake assay of labeled Phe, in which *aap1-1* and wild-type plants (WS) showed different root-to-shoot translocation of radioactivity, Foster (2008) hypothesized that AAP1 participates in loading root vascular tissues with amino acids rather than uptake from the soil. This hypothesis contradicts the main model established by Lee et al., (2007), and raises a question about the localization of the expression of AAP1, and if it directly participates in root uptake of amino acids.

Foster (2008) also proposed that *AAP1* changes the expression and activity of other amino acid transporters since the uptake between the *aap1* knockout plants and wild-type was the same in the roots, hinting toward a regulatory role for AAP1 protein. The first part of this chapter describes efforts to understand how localization in specific tissues contributes to the function of *AAP1* in the plant. The second part describes efforts to test if the expression of other AAPs and LHT1 proteins under the control of the *AAP1* promoter can complement AAP1 function in the loss-of-function mutant. Answering these questions would define where AAP1 is active, and if its activity is shared by other members of the sub-family and other less-related amino acid transporters.

Results

Study of the Expression of AAP1 at the Promoter, mRNA, and Protein Levels

The *AAP1* promoters used in the previously published articles were all of different lengths (from 2.1 to 2.5 kbp; (Hirner et al., 1998; Wang et al., 2017), so a multiple sequence alignment was used to compare a 3.5 kbp region of the promoter from C24 and Col-0. The reference sequence length was chosen at 3.5 kbp upstream of the ATG to completely cover the sequence of two promoters used in our lab (2.8 and 3.25 kbp). The alignment showed that the 2.1 kb promoter from C24 used by Hirner et. al., (1998) and Lee et. al., (2007) contained four single nucleotide polymorphisms (SNPs) different from the Col-0 sequence (Supplemental Fig. 1).

To resolve the discrepancies in the localization of the *AAP1* promoter activity, a construct containing 2.8 kbp region upstream from the ATG of *AAP1* isolated from Col-0 was fused to the u*idA* from *Escherichia coli* encoding a β-glucuronidase (GUS) gene and expressed in *Arabidopsis thaliana Col-0* (Fig. 2). Arabidopsis plants expressing the promoter-GUS fusion showed staining

in the veins of the cotyledon. Only one line out of 11 showed staining in the vascular tissues of the roots, similar to Wang et al. (2017), but staining was never observed in the root tip (Fig 3A).

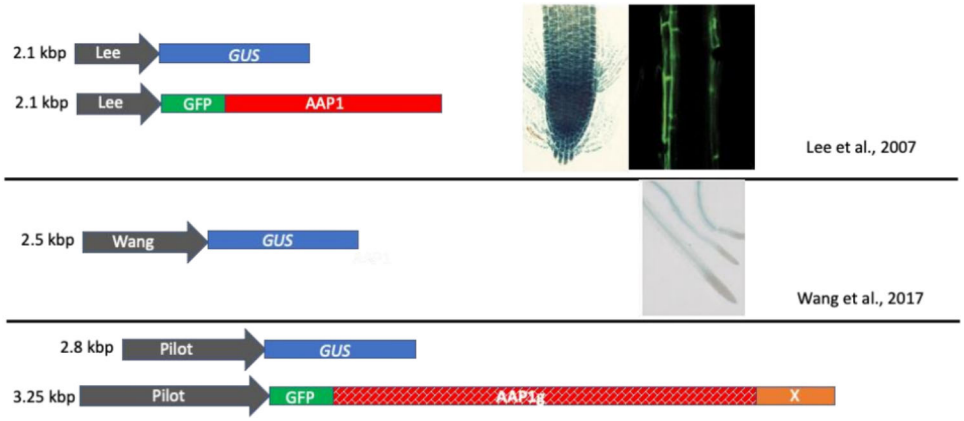


Figure 2. Constructs used to study the localization of *AAP1* expression in Arabidopsis. Top: Constructs used by Lee et al. (2007) from C24 plants showing root tip and epidermis localization. Middle: Construct used by Wang et al., (2017) showing localization in the root vasculature. Bottom: Constructs generated by me.

The localization of the expression of the AAP1 protein in *Arabidopsis* roots was explored using transgenic plants expressing GFP:AAP1 fusion proteins under the control of a 3.25 kbp AAP1 promoter region (AAP1p-GFP-AAP1g). Images taken with a confocal fluorescent microscope showed that the signal was present along the mature root, but only repeatable with treatment by ABA (Fig. 3C).

To localize the AAP1 protein at the subcellular level, a YFP:AAP1 fusion protein encoded by cDNA was transiently expressed in *N. benthamiana* epidermal cells. The YFP signal accumulated in the endoplasmic reticulum (ER) (Fig. 3B) similar to the AAP1 homolog from rice, *OsAAP1* (Taylor et al., 2015). Localization at the plasma membrane claimed by (Lee et al., 2007) and demonstrated for *OsAAP1* could not be confirmed by our approach. To test whether AAP1 would also accumulate in the ER in Arabidopsis roots, the previously used AAP1p-GFP-AAP1g expressing plants were studied. The GFP signal was also present in the ER (window) in cortex cells (Fig. 3C-E).

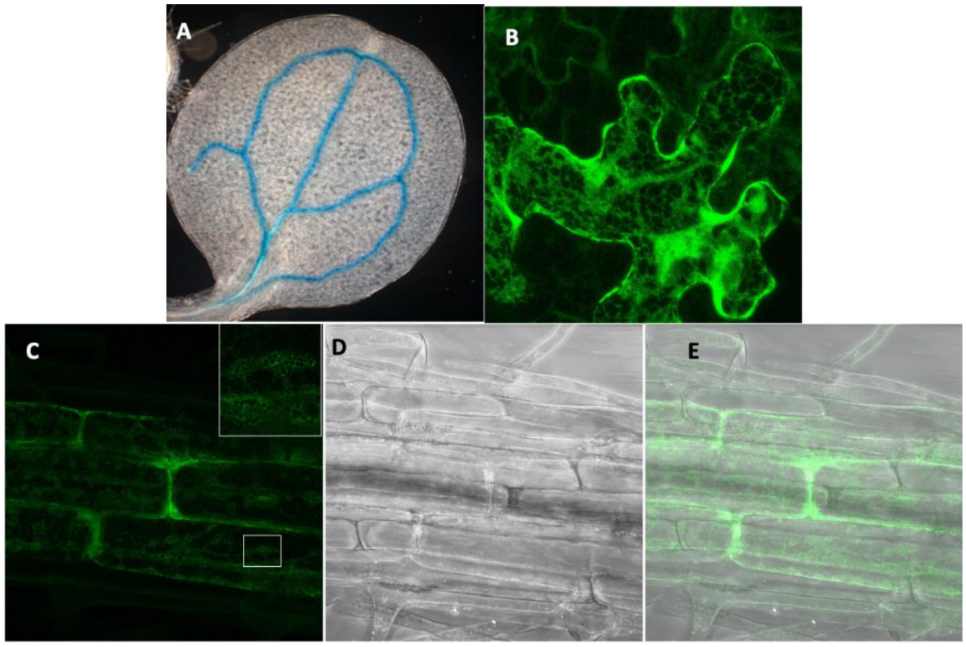


Figure 3. Localization of *AAP1p* activity in Arabidopsis, and of AAP1 protein in Arabidopsis and *N. benthamiana*. (A) Histochemical staining of 10-day old Col-0 seedlings expressing *AAP1p:GUS*. 5X Magnification. (B) Projection of Z-stacks for *N. benthamiana* leaf pavement cell expressing YFP:AAP1. (C-E) 10-day old Arabidopsis root cortex cells expressing AAP1p:GFP:AAP1g treated by 100 µM ABA for 24 h (D) Projection of Z-stacks (insert showing ER localization), (D) Bright Field, (E) Merger of C and D.

Ectopic Expression of AAP1 in Root tip and Phloem Complement the Knockout Mutant

In parallel to the study of the expression of AAP1 at the organ and sub-cellular levels described above, I wanted to identify the tissues where AAP1 needed to be expressed to be physiologically active. This was prompted by the above-mentioned discrepancies in the reported localization of AAP1 expression. To answer this question, *AAP1* gDNA sequence was placed under the control of tissue-specific promoters, active in the three main tissues where *AAP1* expression has been observed: root tip (*TMO7*, *AGP30*, *PMEI* promoters), cortex/epidermis (*UMAMIT04*, *UMAMIT31*, *UMAMIT32* promoters), and phloem (*SUC2*, *AKT2*, *CoYMV* promoters). These nine constructs were introduced in the *aap1-4* mutant. If AAP1 needed to be expressed in a specific tissue to be active, the *aap1-4* plants expressing *AAP1* in this tissue would have a wild-type phenotype. The most obvious phenotype of the *aap1-4* knockout mutant is tolerance to amino acids, compared to the wild-type. If the phenotype of the *aap1*-4 mutant is complemented by expression of any of these constructs, the plants should be susceptible to toxic concentrations of amino acids, similar to the wild-type. *AAP1* expressed under the control of the root tip promoters *TMO7* and *AGP30*, and the phloem promoters *SUC2* and *CoYMV* restored amino acid susceptibility to the *aap1-4* mutant plants. *AKT2p* is a weak promoter (Pilot et al., 2003), and only partially complemented the *aap1-4* mutant. However, the three cortex/epidermis-specific promoters

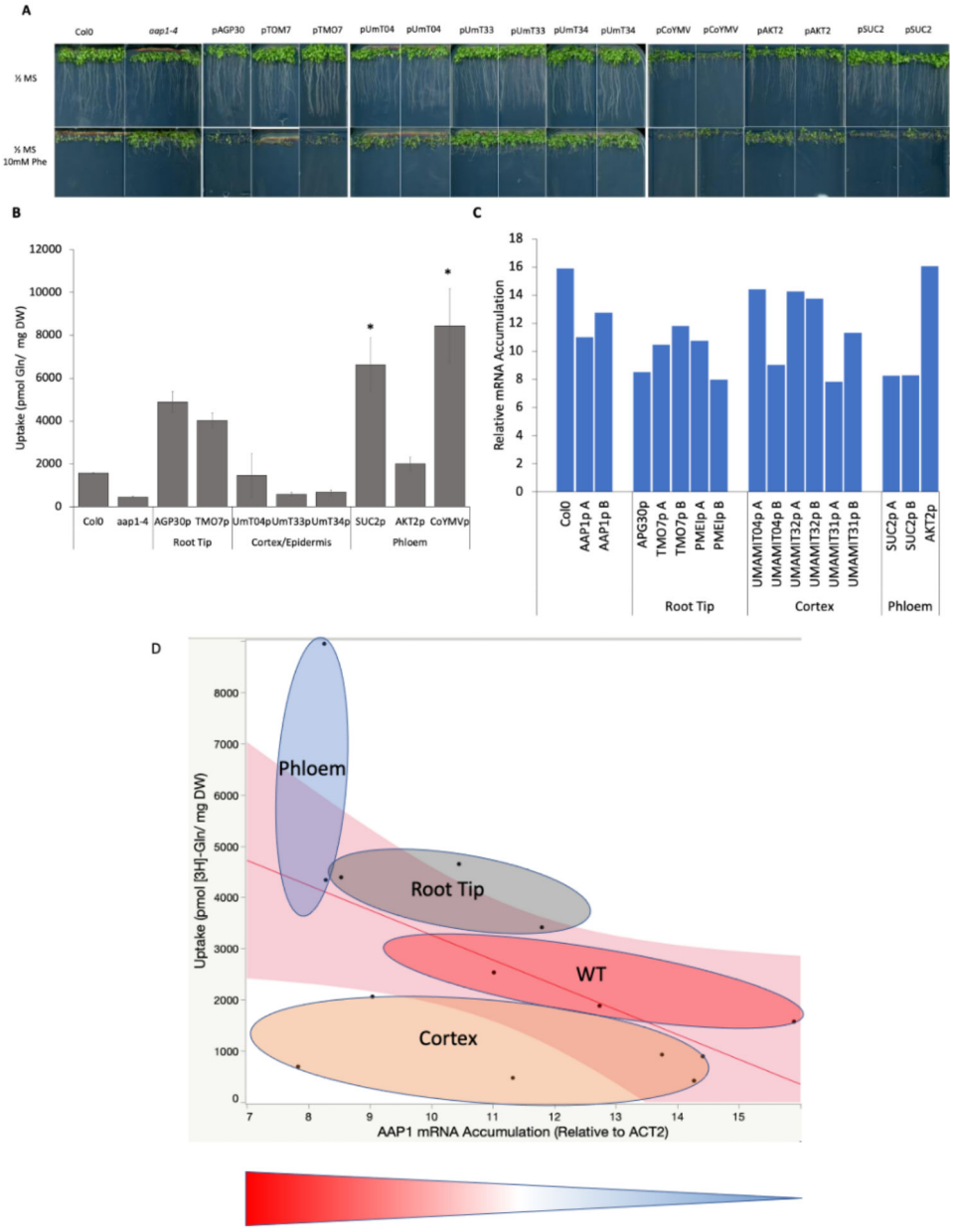
(*UMAMIT04, UMAMIT31,* and *UMAMIT32*) did not fully restore the susceptibility to amino acids. It should nevertheless be noted that the *UMAMIT04p:AAP1g* construct slightly reduced amino acid tolerance of *aap1-4* (Fig. 4A).

It was possible that the ability of the various constructs to complement the *aap1-4* mutant was simply caused by promoter strength and not tissue-specific activity. This could translate into the stronger promoters leading to higher *AAP1* mRNA content, higher amino acid uptake, and finally complementation (susceptibility).

AAP1 Expression in Root tip and Phloem Promote Uptake

To test this hypothesis, *AAP1* mRNA accumulation was measured in roots of the lines generated above. *AAP1 mRNA* levels were increased in all lines, between 1 and 250-fold compared to the wild-type, in general irrespective of the tissue-specific localization (Fig. 4C). The greatest accumulation of *AAP1* mRNA occurred when *AAP1* was expressed under the control of the *AGP30* and *SUC2* promoters (250-fold increase over the wild-type). The lines were also assayed for Gln uptake. Gln was used for uptake as this amino acid was shown to be transported by AAP1 (Boorer et al., 1996; Fischer et al., 2002). The uptake correlated with the complementation study, where the uptake by plants expressing AAP1 under the control of the *TMO7* and *AGP30* promoters increased by 2- and 1.5-fold in the root tip, respectively. Similarly, the uptake by AAP1 under control of the *SUC2* and *CoYMV* promoters significantly increased ($p<0.05$) Gln uptake, by 3- to 4-fold, respectively, compared to Col-0 (Fig. 4B). *aap1-4* plants expressing *AAP1* in the cortex/epidermis did not show uptake significantly different from either Col-0 or *aap1-4* plants. It thus appears that increased uptake correlated poorly with increased expression of *AAP1* at a tissue-specific level (Fig. 4C,D). In addition, it remained puzzling that expressing *AAP1* solely in root tip or in phloem had such a strong effect on uptake by the whole root. This is particularly interesting, given that the assay was developed to measure uptake only by the mature root and not by the root tip (placed over parafilm to exclude area elongation zone and below, as well as the hypocotyls). These data suggest that AAP1 is not directly responsible for uptake of amino acids by the roots.

I hypothesized that the ectopic expression of *AAP1* alters the root metabolism, to which the cells respond by changing the expression of other transporters. The correlation between *AAP1* mRNA accumulation and uptake is positive, but the uptake is dependent on the tissue type (Fig. 4D) To test these possibilities, the mRNA accumulation of two amino acids transporters (LHT1 and AAP6), and of two nitrogen transporters (NPF6.3 and AMT1;1 - known to respond to nitrogen availability) was measured in the transgenic plants. *LHT1* mRNA accumulation was higher in all of the transgenic plants than in *aap1-4,* while *AAP6* mRNA accumulation decreased. The mRNA accumulation of *NPF6.3* or *AMT1;1* did not show a tissue-specific change (Fig. 4E).


A
Col0
aap1-4
pAGP30
pTOM7
pTMO7
pUmT04
pUmT04
pUmT33
pUmT33
pUmT34
pUmT34
pCoYMV
pCoYMV
pAKT2
pAKT2
pSUC2
pSUC2
½ MS
½ MS 10mM Phe
B
Uptake (pmol Gln/ mg DW)
Col0
aap1-4
AGP30p
TMO7p
UmT04p
UmT33p
UmT34p
SUC2p
AKT2p
CoYMVp
Root Tip
Cortex/Epidermis
Phloem
C
Relative mRNA Accumulation
Col0
AAP1p A
AAP1p B
APG30p
TMO7p A
TMO7p B
PMEIp A
PMEIp B
UMAMIT04p A
UMAMIT04p B
UMAMIT32p A
UMAMIT32p B
UMAMIT31p A
UMAMIT31p B
SUC2p A
SUC2p B
AKT2p
Root Tip
Cortex
Phloem
D
Uptake (pmol [3H]-Gln/ mg DW)
Phloem
Root Tip
WT
Cortex
AAP1 mRNA Accumulation (Relative to ACT2)

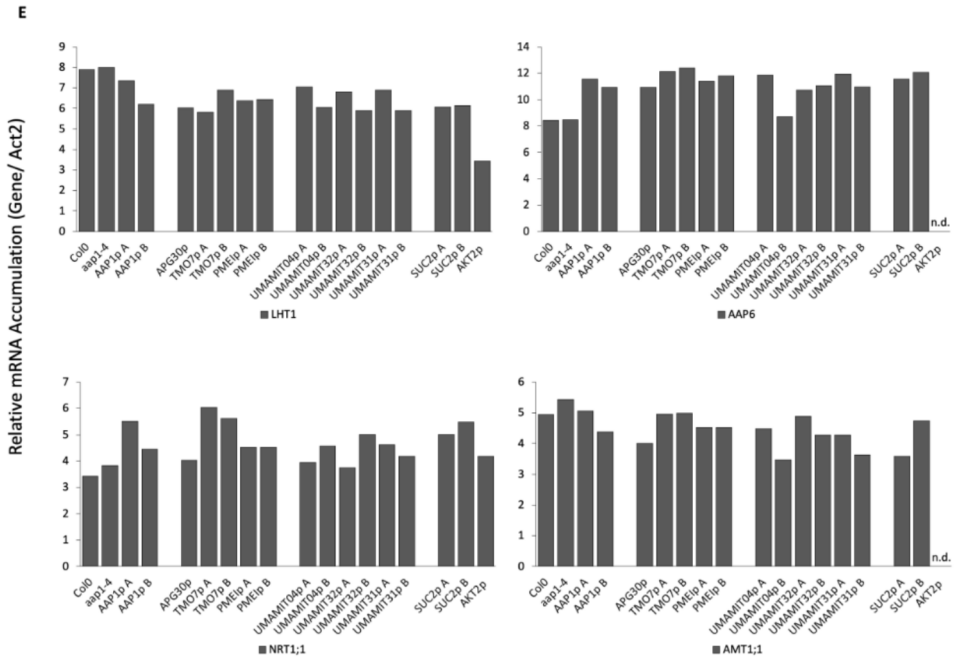


Figure 4. Complementation of the *aap1-4* plants with *AAP1* under the control of tissue-specific promoters. (A) Tolerance assay of 14-day old Arabidopsis plants grown on ½ MS ± 10 mM Phe. (B) Uptake of 1 mM ^{3}H-Gln by whole plants expressing *AAP1* under the control of a tissue-specific promoter. Each bar represents the mean ± SE of three biological replicates from two independent lines, asterisks represent significance from Col-0 using Dunnett post hoc test. (C) *AAP1* mRNA accumulation by *aap1-4* plants expressing *AAP1* under the control of a tissue-specific promoter relative to *AtACTIN2.* (D) Correlation between *AAP1* mRNA accumulation and uptake by *aap1-4* plants expressing tissue-specific *Pro:AAP1* (E) Relative mRNA accumulation of *LHT1, AAP6, NPF6.3,* and *AMT1;1* relative to *AtACTIN2* for two independent lines. Lower values correspond to increased accumulation.

LHT1 and other AAPs and do not Complement AAP1 Function

Another approach to characterize the correlation between amino acid uptake and the amino acid tolerance of *aap1-4* plants was to test whether expressing other amino acid transporters under the control of *AAP1* promoter would complement the *aap1-4* phenotype. I chose the best characterized amino acid transporters from Arabidopsis (i.e., AAP2, 3, 4, 6, 8, and LHT1), whose amino acid specificity and affinity are known. Amino acid tolerance to 10 mM Phe and Gln uptake were measured for several transformants for each construct. None of the AAPs or LHT1 were able to substitute for AAP1 in their ability to complement the *aap1-4* plants: only the plants expressing *AAP1p:AAP1*were susceptible to Phe (Fig. 5A). Similarly, only the *AAP1p:AAP1* plants showed an uptake similar to the wild-type. Interestingly, the *AAP1p:GUS* plants in the Col-0

background, imported less Gln than untransformed Col-0 from previous studies, as the GUS protein may affect the accumulation of wild-type AAP1 protein. This suggests that AAP1 has a unique function that is not shared by any of the amino acid transporters tested (Fig. 5B).

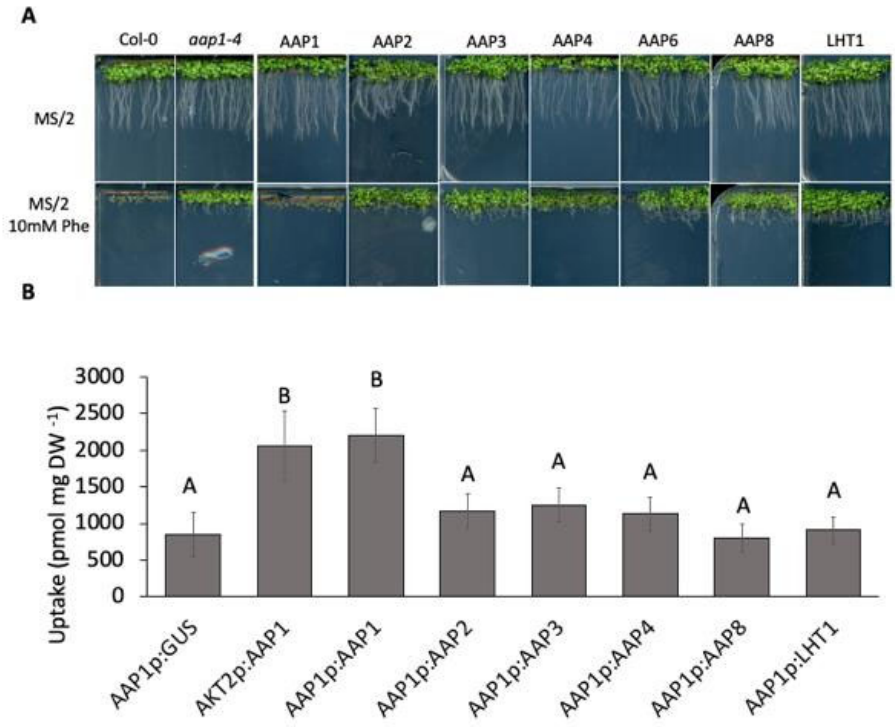


Figure 5. Complementation of *AAP1* by other AAPs and LHT1. (A) Complementation assay of 14-day old Arabidopsis plants grown on ½ MS ± 10 mM Phe. (B) Root length uptake of 1 mM 3-H Gln by *aap1-4* null mutants expressing *AAPs* and *LHT1* under control of the *AAP1p*. Plants expressing *AKT2p:AAP1* were used as a wild-type since previous studies showed Gln uptake at the same level as Col-0. Each bar represents the mean ± SD of three biological replicates from two independent lines.

Discussion

The work from this chapter aimed at clarifying the expression pattern of AAP1 in the root and testing its direct involvement in amino acid uptake. The model established by Lee et al., (2007) posited that AAP1 was expressed in the root epidermis and root tip where it mediates amino acid uptake; loss of AAP1 function reduces uptake, decreasing the intracellular content of toxic amino acid and enabling the plant to grow in these conditions. This model was weakened by the disagreements between the reported expression patterns obtained by different approaches: transcriptomic data detected *AAP1* mRNA in cortex cells (Cartwright et al., 2009; Mustroph et al.,

2009; Denyer et al., 2019; Zhang et al., 2019); my promoter-*GUS* assays did not replicate staining seen by (Lee et al., 2007) or (Wang et al., 2017) who observed expression in root tip, epidermis or phloem; in-situ localization detected AAP1 mRNA in the root tip (Kwart et al., 1993). Work with the 2.8 kb promoter of *AAP1* showed that expression is very weak and not seen in the roots in agreement with results from Guo (2004), which agrees with Northern blot analyses that showed AAP1 is not expressed in the roots (Kwart et al., 1993). Noteworthy, the expression pattern of *AAP1p:GUS* in Figure 2 more closely resembles that of Figure 7D from Kwart et al., (1993) and Wang et al., (2007), regarding expression in leaf vasculature and flowers (Supplemental Fig. 2).

In the contrary, the use of a larger construct potentially containing more regulatory elements (promoter+introns+downstream region) of the AAP1 gene was used to localize the fluorescently labeled AAP1 protein. This construct (AAP1p-GFP-AAP1g-AAP1t) led to the expression of GFP-AAP1 in root cortex cells, in perfect agreement with three independent transcriptomics approaches. I thus believe that AAP1 is expressed in root cortex cells, rather than any other cell type. Yet, I had to treat the plants with ABA, previously shown to enhance AAP1 expression (Wang et al., 2017), to detect any fluorescence in roots (See also Chapter 4). Whether AAP1 is expressed in cortex cells solely under stress conditions remains to be determined. The discrepancy between the GUS and GFP data were surprising: no GUS signal was ever detected in roots, even after ABA treatment. The main differences between the two constructs are the length of the promoters (3.25 vs 2.8 kbp) and the fact that the GFP construct contains all introns of the gene. It is possible that the first intron (large for a typical Arabidopsis gene, 1.2 kb) contains regulatory signals necessary for promoting transcription. It is important to note that none of the previous publications has used a similar construct to study AAP1 expression.

The fact that amino acid uptake is not drastically enhanced by expression of AAP1 in cortex, compared to root tip or phloem raises the question of the role of AAP1 in cortex cells. Furthermore, the AAP1 protein did not localize to a great extent (if any) at the plasma membrane, where it would be expected if it has a major role in uptake from the soil solution. It is instead found in the ER, in good agreement of orthologs from rice (Taylor et al., 2015).

Finally, I showed that close homologs (AAP6 and AAP8) or more distant amino acid transporters (AAP2, AAP3, AAP4 and LHT1) were unable to complement AAP1 function when expressed under the control of AAP1 promoter. Two hypotheses can explain this result: (1) these proteins are lacking a specific function of AAP1, important in cortex cells to lead to increased uptake and amino acid susceptibility that is not ability to transport amino acids; (2) the promoter region of AAP1 does not contain all regulatory elements to express a downstream coding sequence to the correct tissue and level necessary to carry on the transport function, as suggested above. These data thus question the validity of the current model for the role of AAP1 in amino acid uptake, and drove me into postulating alternating hypotheses, that I tested in later chapters.

Chapter 3
Characterization of the AAP1-EMS mutants

A forward genetic screening performed before I started in the Pilot Lab led to the isolation of several Ethyl methanesulfonate (EMS)-mutagenized mutants that were tolerant to toxic concentrations of amino acids. Four of the mutants contained nonsense mutations in *AAP1* (not shown), while four contained missense mutations in *AAP1*: S87F (*aap1-5*), G212D (*aap1-6*), G251E (*aap1-7*), and A417T (*aap1-8*). Getting amino acid tolerant mutants bearing mutations in *AAP1* was not surprising, since loss-of-function of *AAP1* (i.e., the *aap1-1* and *aap1-2* mutants) had previously been shown to be tolerant to amino acids (Lee et al., 2007).

My results presented in Chapter 2 question the model for the role of AAP1 in amino acid uptake from the soil, which was established by the characterization of transgenic *aap1* knockout mutants. Missense mutations have the potential to affect specific functions of the protein (*e.g.,* activity, stability, localization, etc.), which can enable studying them independently and provide more details about the physiological role of the corresponding gene. I reasoned that precisely characterizing the effect of each of these mutations would help us testing the model established by (Lee et al., 2007).

The functional properties of each of the variant proteins was tested in yeast and in plants, in order to answer the question whether the mutations resulted in loss-of-function mutations - this would explain the amino acid tolerance of the corresponding mutant plants. The sub-cellular localization, the amino acid uptake activity of the proteins and the phenotype of the mutant plants were also carefully examined.

Results

Localization of the Protein Variants

A 3-D and an expanded model of the AAP1 protein were constructed by a collaborator of the Pilot lab (Dr. Ariela Vergara Jaque, University of Talca, Chile) by homology modeling using models from crystallized *E. coli* proteins from the ATF superfamily as templates (unpublished results). Two of the identified mutants, aap1-7 and aap1-8, show amino acid substitutions in the same transmembrane domain as the predicted substrate binding site of the protein (areas marked in black), while the aap1-6 and aap1-5 mutations are in other transmembrane domains (Fig. 6). The 3-D models shows that two of the mutations are embedded in the plasma membrane while two of the amino acid residues are facing toward the binding pocket.

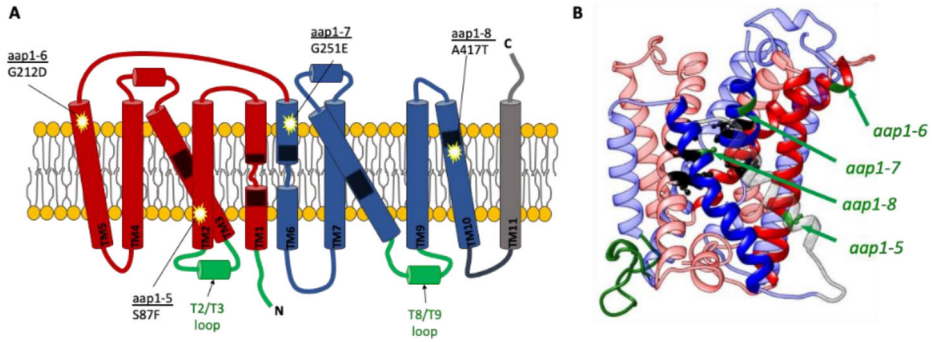


Figure 6. Identification of the missense mutations in the AAP1 protein model. A) Open model, B) 3-D model. Blue and red transmembrane helices correspond to the two inverted sub-domains forming the AAP1 protein; N-terminus and the larger cytosolic loops are in green; the last transmembrane helix and the C-terminus are in grey. Black shading represents the position of the residues predicted to bind the amino acid substrate, based in the *E. coli* proteins used as templates for the homology modeling.

aap1 EMS-mutant Plants Tolerate toxic concentrations of Amino Acids

The four mutants *aap1-5, -6, -7* and *-8* (thereafter called EMS mutants) were initially selected for growth in media containing toxic concentration of Phe and Val. To test that these mutants were tolerant to toxic concentrations of multiple amino acids, the *aap1-4* knockout mutant, Col-0, and each of the EMS mutant plants were grown on ½ MS supplemented with the toxic concentrations of amino acids similar to Lee et al. (2007). All *aap1* mutants were tolerant to amino acids (Fig. 7A). While fresh weights of the plants were less affected by amino acids than the wild-type (Fig. 7B), *aap1-6* was developing the least.

In a different experiment, I tested if two genes that have been linked to Glu sensing and metabolism, namely Glutamate Receptor (GLR) and MAP kinase kinase (MEK) are involved in amino acid tolerance (Forde et al., 2013; Goto et al., 2020). Loss-of-function mutant plants for these genes, the *glr3.3* knockout and *mek1/2/3* triple knockout, were grown on ½ MS supplemented 2.5, 5, or 10 mM Phe. Neither the *glr3.3* nor the *mek1/2/3* plants tolerated the Phe (see Fig. 7C for the results of 5 mM Phe), showing that, contrary to *AAP1*, tolerance to amino acids is not mediated by these two genes or the pathways they are involved in.

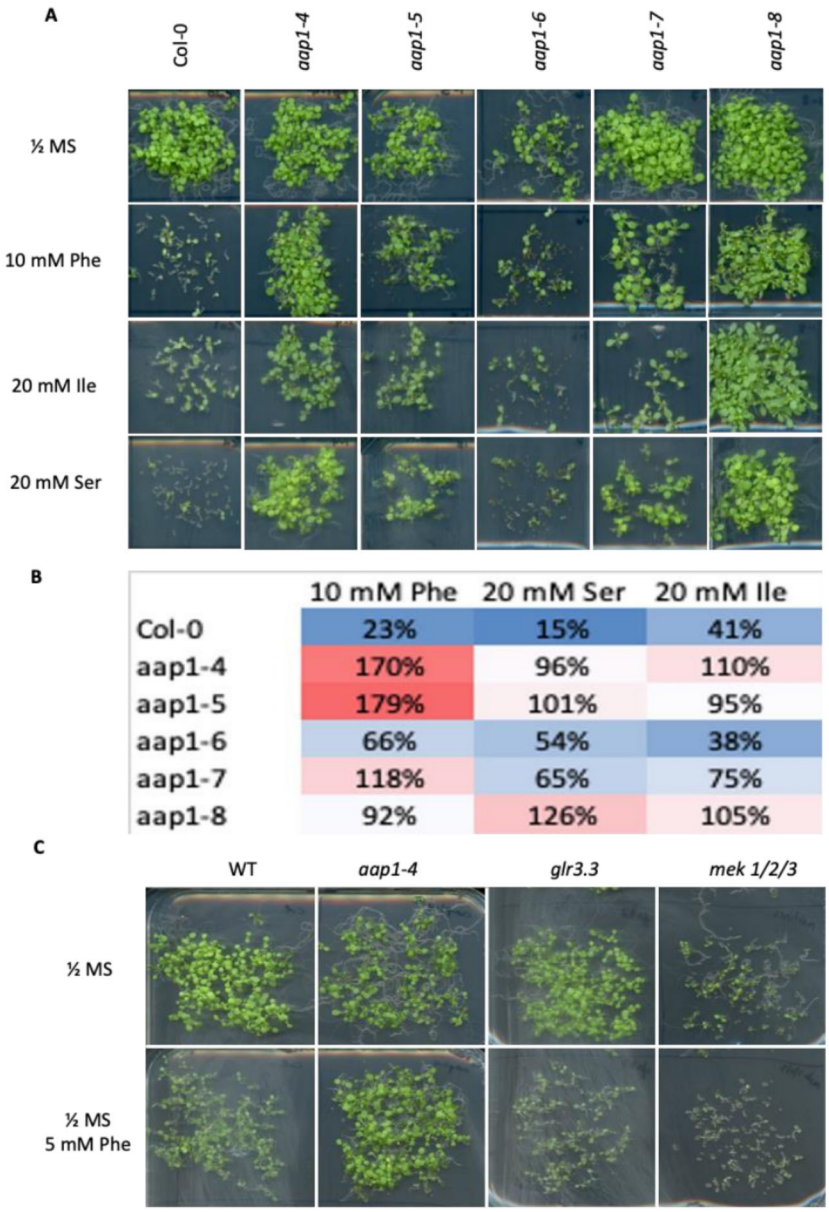


Figure 7. Tolerance of *aap1* mutants to amino acids. A) Plants were grown on ½ MS medium with toxic concentrations of amino acids for 14 days. B) Percent of fresh weights for 10 plants grown on amino acids compared to plants grown on ½ MS medium from A. C) Tolerance of *glr3.3* and *mek1/2/3* plants to 5 mM Phe.

A wild-type AAP1 Construct Complements the aap1 *Mutants*

Since the four mutants come from EMS mutagenesis, it can be expected that they contain several hundred-point mutations in their genome. To test that the amino acid tolerance phenotype results from the mutations in *AAP1*, and not from another gene, an *AAP1p:AAP1g* construct was expressed in *aap1-4, -5, -6, -7, -8* and Col-0 plants. Transformants were grown on a medium supplemented with 10 mM Phe to test for their resistance. In each case, the mutants expressing *AAP1p:AAP1g* were susceptible to 10 mM Phe, similar to wild-type (Col-0), proving that expressing the wild-type *AAP1* gene complements the phenotype of each of the mutants (Fig. 8).

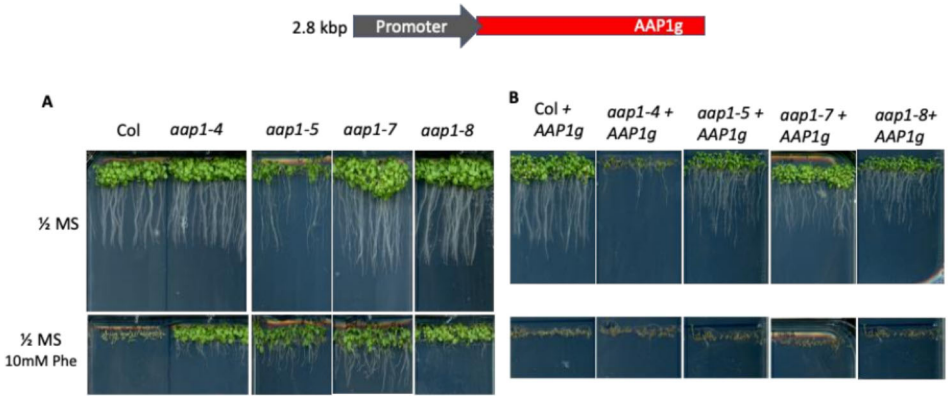


Figure 8. Amino acid tolerance assay of *aap1* mutants expressing *AAP1p:AAP1g* WT-AAP1. A) Amino acid tolerance of *aap1* mutants to 10 mM Phe. Plants were grown for 10 days on ½ MS ± 10 mM Phe. B) *aap1* EMS-mutants expressing *AAP1p:AAP1g* grown on ½ MS ± 10 mM Phe for 10 days. The transformed *aap1-4* plants expressing *AAP1p:AAP1g* germinated poorly on ½ MS, (Fig. 6B), but no plant displayed tolerance to 10 mM Phe.

Uptake and Efflux of Amino Acids are Altered in EMS-mutants

To test if these mutations affected the amino acid transport in Arabidopsis plants, uptake of amino acids by whole plants was measured. All of the EMS-mutants, except *aap1-6* showed lower Gln uptake than Col-0. The uptake of Gln by *aap1*-8 mutants was decreased relative to the wild-type when supplied at 10 µM, which is ~100 times below the average Km for amino acids (0.5-1.5 mM) for AAP1 (Boorer et al., 1996; Fischer et al., 2002) (Fig. 9A,C). This is the only line that showed a difference in uptake in the high affinity range. Interestingly, the *aap1-8* plants effluxes were 1.5-2 times that of the wild-type and *aap1-4* plants (Fig. 9B,D). The transport of other amino acids was also measured (Fig. 9E) - Leu and Met uptake was increased in the *aap1-8* plants compared to the wild-type and *aap1-4*, while Pro efflux was increased, similar to Gln (Fig. 9F). This shows that while the aap1-6 and aap1-8 proteins are functional in yeast, the corresponding mutants show amino acid transport defects in Arabidopsis plants.

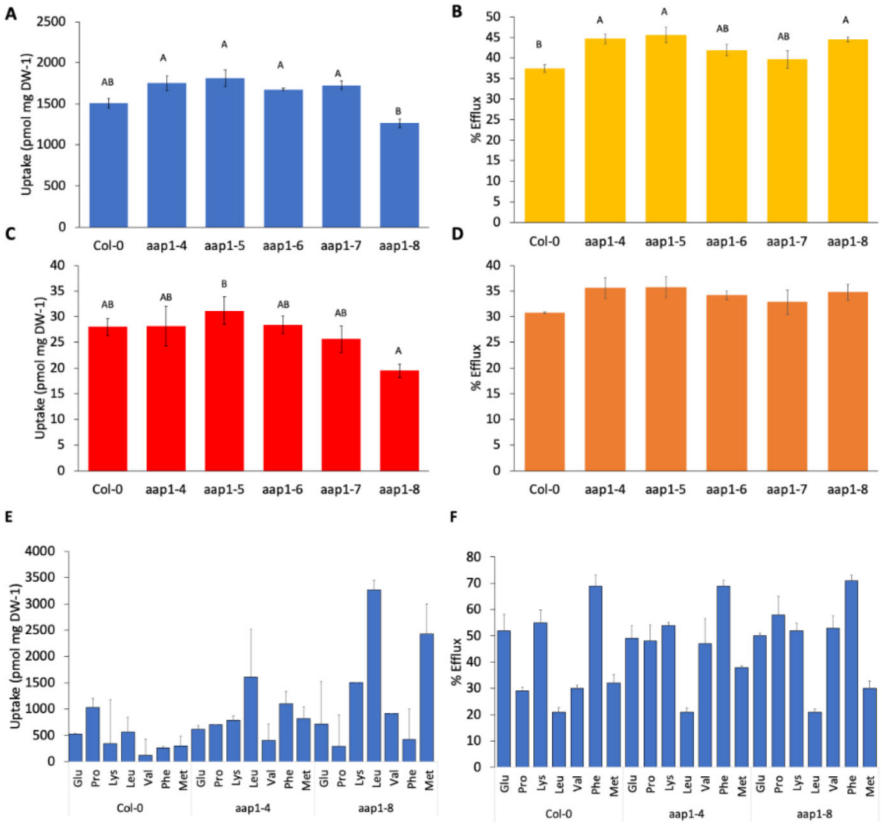


Figure 9. Amino acid uptake activity of the *aap1* mutant plants. (A, C) Whole plant assay for amino acid uptake. (B, D) Whole plant amino acid efflux. Efflux is expressed as the percentage released compared to the total amount taken up. (A, B) 1 mM Gln. (C,D) 10 µM Gln. (E, F) Col-0, aap1-4 and aap1-8 uptake of amino acid supplied at 1 mM. Each bar represents the mean ± SE of three biological replicates. One-way ANOVA with Tukey HSD comparison for each treatment ($p<0.05$). Letters indicate significant differences.

Sub-cellular Localization of the AAP1 *Protein Variants*

In Chapter 2, AAP1 in both Arabidopsis and *N. benthamiana* was shown to localize at the ER membrane. An argument could be made that the four missense mutations affect protein stability or localization and are *bone fide* loss-of-function mutations. The accumulation level and the localization of the EMS-mutant proteins were determined by fusing them to the YFP (YFP:AAP1) and co-expressing them with the wild-type AAP1 fused to the mCherry (AAP1:mCherry) in *N. benthamiana*. Projection Z-stacks showed that both YFP and mCherry co-localized perfectly to the ER for each of the combinations (Fig. 10), indicating that aap1-5, -6, -7, and -8 proteins co-localized perfectly with AAP1:mCherry. This shows that the mutations do not affect location and/or stability of the mutant proteins, at least in this heterologous expression system.

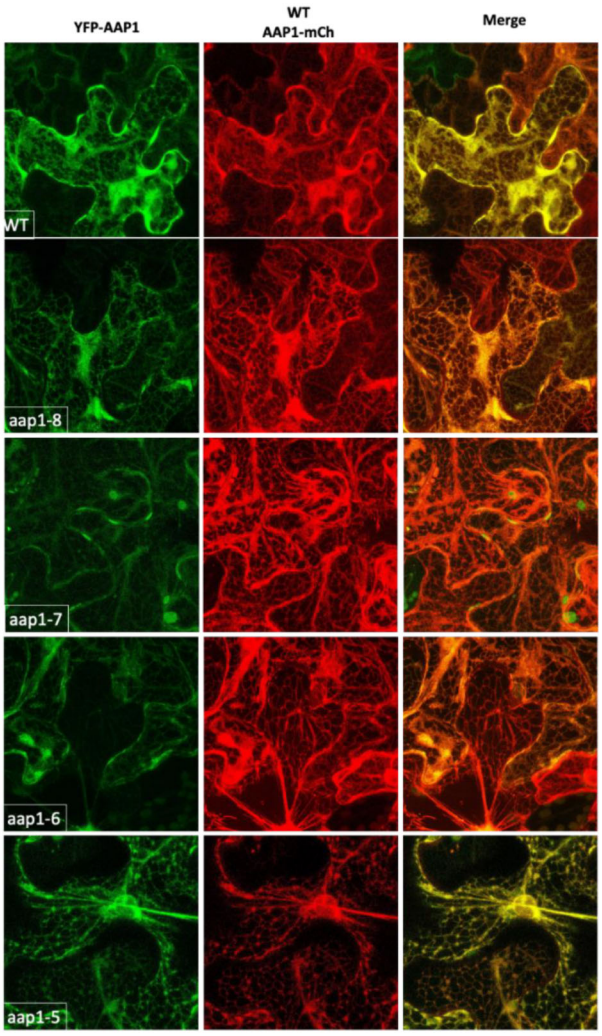


Figure 10. Co-localization of the aap1 variant protein with the wild-type AAP1 protein. Optical projections of *N. benthamiana* cells expressing aap1 mutant proteins aap1-5, -6, -7 and -8 fused with the YFP, with wild-type AAP1:mCherry. White boxes in each row indicate the mutant protein fused to YFP.

aap1-6 *and* aap1-8 *are Functional Transporters in Yeast*

Successful accumulation of the proteins in tobacco does not necessarily means that they are functional transporters. To characterize the transport properties of the four mutant proteins, the corresponding cDNAs were first expressed in 22Δ10α yeast strain, which lacks 10 of the endogenous amino acid transporters and is unable to grow on media where amino acids are the sole nitrogen source (Besnard et al., 2016). Fiona Harris, an undergraduate researcher in the Pilot lab, showed that the aap1-6 and aap1-8 proteins both transported amino acids at, or above, wild-type levels (Fig. 11) for several amino acids. In addition to growth assays, uptake studies showed that aap1-6 and aap1-8 variants are able to transport Gln, Leu, and Pro (Fig. 11B), whereas aap1-5 and aap1-7 did not support growth or import amino acids.

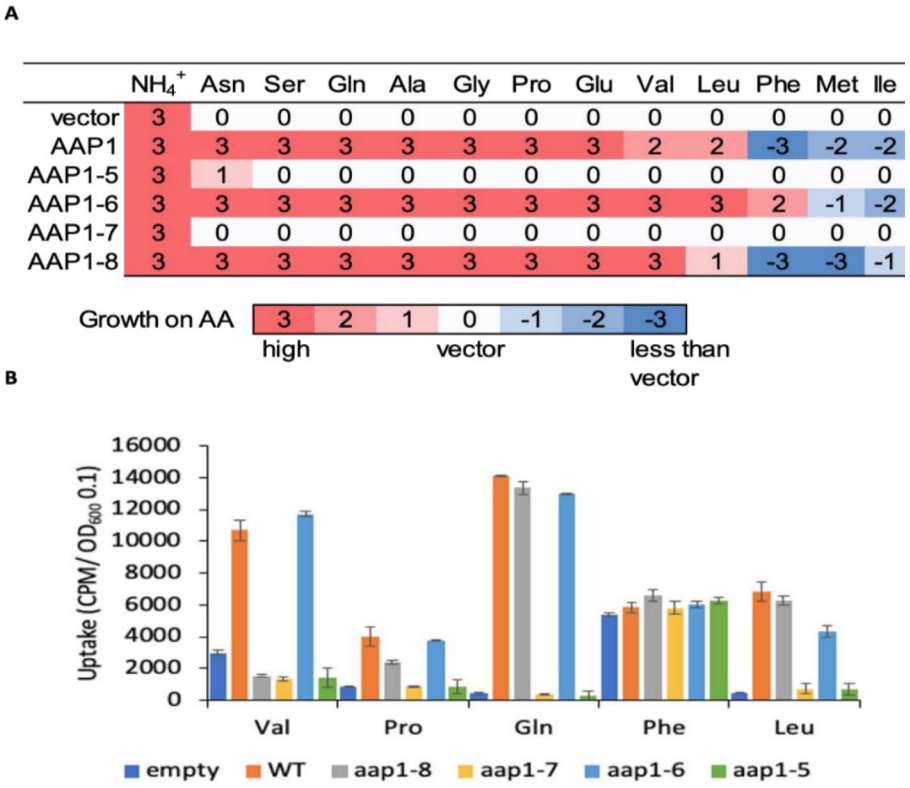


Figure 11. Uptake by 22Δ10α yeast expressing *aap1* mutant cDNAs. A) Growth of 22Δ10α expressing aap1 variants on amino acids as sole nitrogen source. B) Comparison of amino acid uptake of the four mutant proteins to the wild-type AAP1, when expressed in 22Δ10α. Amino acids were supplied to yeast at a concentration of 1 mM each, and accumulation was determined after 3 min. Each bar represents the mean ± SD of three biological replicates.

Overexpression of AAP1 has been shown to increase uptake of chlorantraniliprole-alanine (Ren et al., 2019) in Arabidopsis. I used an over-expression system to study the transport properties of the aap1 variant proteins. For this purpose, an inducible system was used to avoid the selection bias that may have arisen when using a constitutive over-expression approach (e.g., lethality may have happened at high *AAP1* expression levels, which would have selected for lower-expressing lines). The dexamethasone (DEX) inducible system (Rutherford et al., 2005), which has been previously employed in the Pilot lab, was used to express each of the variant proteins. The induction of the aap1 variant proteins in 4C-S7 plants led to increased uptake and decreased efflux for the aap1-6 and aap1-8 proteins but not for the aap1-5 and aap-7 proteins, in good agreement with their functional properties measured in yeast (Fig. 12A,B). These data suggest that the aap1-6 and aap1-8 proteins are functional not only in yeast but also in plants.

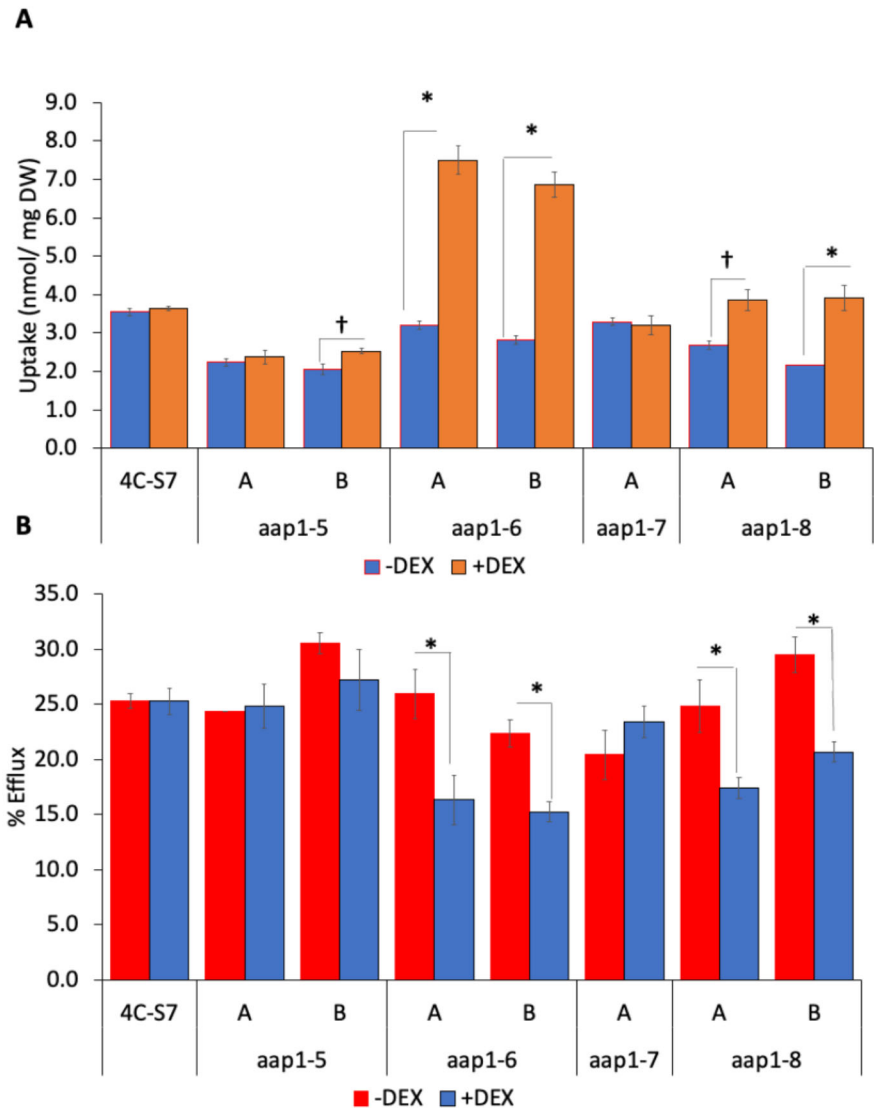


Figure 12. Amino acid uptake activity of 4C-S7 plants overexpressing the aap1 variant proteins. (A,) Whole plant assay of [3H] Gln (1mM) amino acid uptake by inducible AAP1 plants, induced (+DEX) or not (-DEX) by 30 µM dexamethasone. (B) Whole plant amino acid efflux by plants from A. Efflux is expressed as the percentage released compared to the total amount taken up. Gln (1 mM) transport of 4C-S7 are the parental control line. Each bar represents the mean ± SE of three biological replicates. Asterisks indicated significant differences ($p<0.001$) † ($p<0.05$) between lines treated by DEX, tested by paired t-Test

The aap1 *EMS-mutants Show a Disturbed Metabolism*

The *pig1-1* mutant plants (Voll et al., 2004), displaying an enhanced tolerance to Phe, described in Chapter 1, share the same mutation as the *aap1-7* plants. While studying *pig1-1*, the authors showed that the ^{14}C taken up as Phe was redistributed into cationic, anionic, or neutral metabolic fractions faster in *pig1-1* than in the wild-type. This observation led the authors to conclude that metabolism was deregulated in *pig1-1*, while Phe transport was not different between the two genotypes.

To test whether the *aap1* EMS-mutant plants studied here also display a deregulated metabolism, plants were grown on ½ MS, or 1 mM KNO_3 supplemented with 10 mM Glu. The plants were then fed [^{14}C] Glu 10 mM Glu for 4 h (pulse), then allowed to metabolize the imported amino acid for 4 h (chase). Amino acids were extracted and separated using ion-exchange columns into positive (basic), negative (acidic), and neutral fractions. The distribution of the label within the three fractions for plants grown only on ½ MS was not statistically different between each line (Fig. 13: top). When the plants were grown in the presence of 10 mM Glu, the distribution of the ^{14}C was different between the mutants and the wild-type: more label was observed in the positive fraction, and less in the negative fraction (Fig. 13: bottom), but the neutral fraction showed a redistribution of the label, where *aap1-6* was the only mutant not like wild-type. Prior exposure to Glu caused the label being incorporated more into the anionic and neutral fractions but less in the positive fraction. These data show that, similar to *pig1-1*, amino acid metabolism is affected by mutations in the *AAP1* gene.

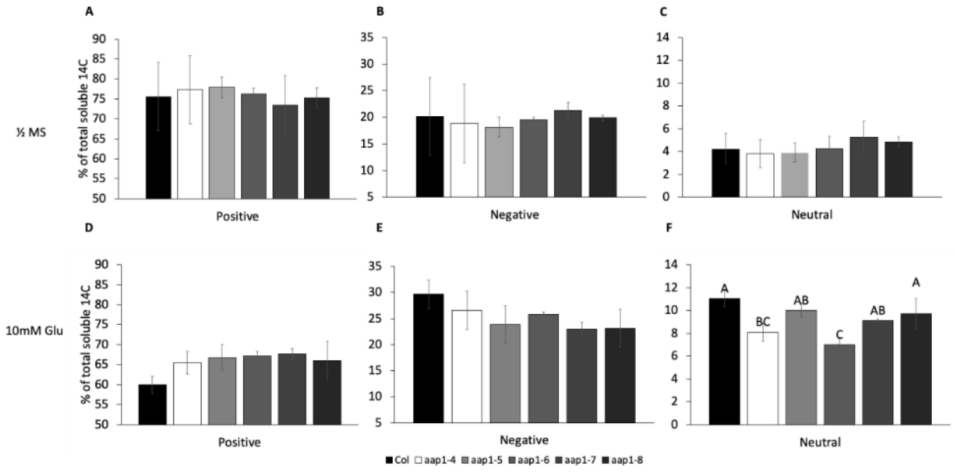
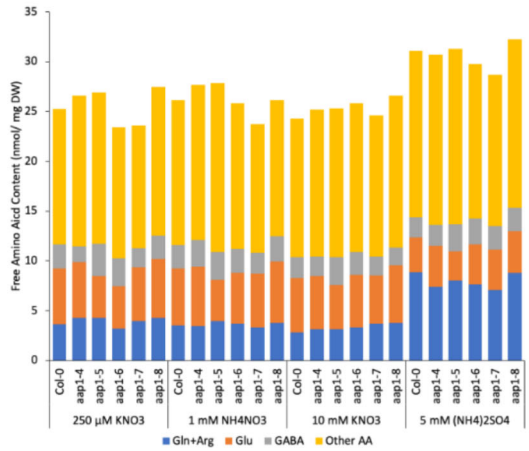


Figure 13. Partitioning of ^{14}C Glu into different fractions in the *aap1* mutant shoots. Percent of ^{14}C Glu label eluted in the positive (basic) (A, D), negative (acidic) (B, E) and neutral (C, F) fractions. Graphs A-C show the distribution of ^{14}C Glu label from plants grown on ½ MS medium, while graphs D-F show the distribution of the label by plants grown on B medium supplemented with 1 mM KNO_3 and 10 mM Glu. Each bar represents the mean ± SE of three biological replicates. Significance was determined using one-way ANOVA and Tukey HSD comparison. Different letters indicate significance differences between mutants.

Shoot Amino Acid Content is Unchanged in aap1 *EMS-mutants*

I then tested if the modification in amino acid metabolism affects amino acid content in shoots. Plants were grown in hydroponic conditions in presence of 1 mM KNO3, and then exposed to media containing various inorganic nitrogen sources and concentrations. The total free amino contents in shoots were not significantly different between any of the *aap1* mutants and the wild-type. Overall, the treatment with 5 mM $(NH_4)_2SO_4$ led to an increase in all amino acid content, noticeably Gln+Arg, but no difference was observed between any line (Fig. 14). These data showed that the altered metabolism does not translate into different accumulation of amino acids, even under different nitrogen regimes.

A



B

Treatment	Line	Gln+Arg	Glu	GABA	Other AA
250 µM KNO3	Col-0	a	ab	abc	a
	aap1-4	a	ab	c	a
	aap1-5	a	b	a	a
	aap1-6	a	b	abc	a
	aap1-7	a	ab	bc	a
	aap1-8	a	a	abc	a
1 mM NH4NO3	Col-0	a	a	a	ab
	aap1-4	a	a	a	ab
	aap1-5	a	b	a	a
	aap1-6	a	ab	a	ab
	aap1-7	a	ab	a	b
	aap1-8	a	a	a	ab
10 mM KNO3	Col-0	a	ab	a	a
	aap1-4	a	a	a	a
	aap1-5	a	b	a	a
	aap1-6	a	a	a	a
	aap1-7	a	a	a	a
	aap1-8	a	a	a	a
5 mM (NH4)2SO4	Col-0	a	ab	a	a
	aap1-4	a	ab	a	a
	aap1-5	a	ab	a	a
	aap1-6	a	b	a	a
	aap1-7	a	ab	a	a
	aap1-8	a	a	a	a

Figure 14. Amino acid content of *aap1* mutant plants in response to different nitrogen treatments. (A) Free amino acid content of four 21-day old Arabidopsis shoots grown on rock-wool in a hydroponics system with J medium supplemented with 250 µM KNO_3, 1 mM NH_4NO_3, 10 mM KNO_3, or 5 mM $(NH_4)_2SO_4$ for 7 days. Data are normalized to added norvaline, a non-proteinogenic and represented as nmol of each amino acid per mg DW. Data were the average of four replicates. (B) Results Table of One-way ANOVA with Tukey HSD comparisons between each of the mutants in a given nitrogen treatment. Each amino acid was compared separately in each treatment. Each block represents one ANOVA and blocks should not be compared. Gln + Arg, Glu were chosen as separate amino acids from the entire pool since these are the first amino acids to assimilate nitrogen from the GS/ GOGAT system. Results for GABA were shown separately as it is not a proteinogenic amino acid. Same letters indicate samples are not significantly different from one another.

Nitrogen Metabolism is Altered in aap1 Mutant Plants

Since the pulse/chase experiment showed a differences in the metabolic activity between wild-type and the mutant plants, I postulated that the expression of some genes could be different between the lines. Microarray data from Gutierrez et al. (2008) were mined for genes that were specifically induced by organic nitrogen (Fig. 15A). Analysis of genes were selected on the basis

that they responded to inorganic nitrogen, were not induced when treated by 1 mM Methyl sulfoximine (MSX - a glutamine synthetase blocker) but induced with Glu. In addition to these treatments, I was interested in the response of the *aap1* mutants to nitrogen-limiting (starvation) conditions (100 µM KNO_3). Nitrogen treatment (20 mM KNO_3, 20 mM NH_4NO_3) led to an increase in the accumulation of mRNA of three of the identified genes UKN4 , UKN5, and RZFP34. The responses were variable between the replicates, possibly because the day length was adjusted from 12 h to 16 h, and the pre-treatments were not the same in each conditions (100 µM vs. 1 mM KNO_3). The specific role *AAP1* exerts on these genes cannot really be deduced from these heat maps, which represent the relative mRNA accumulation (Fig. 15B, C). However, the response in the mutants fit neither the wild-type or *aap1-4* expression patterns. These experiments indicate that *AAP1* is exerting changes either directly or indirectly to gene expression, caused by deviations from the wild-type and *aap1-4* plants for the mRNA accumulation of UKN4, UKN5, and RZPF34. However, no direct conclusions can be made from this data on how AAP1 is working to alter the metabolism.

A

Candidate		N	N+MSX	N+MSX+Glu	
UKN2	AT2G20670	2.629	-0.808	2.200	unknown function
ASN1	AT3G47340	2.141	0.386	1.667	Asn synthetase
UKN5	AT5G19120	2.042	-0.894	1.663	aspartyl protease
RZPF34	AT5G22920	1.644	-0.697	1.459	RING finger protein
UKN4	AT3G15450	1.463	-0.102	1.417	Aluminum induced protein
BT5	AT4G37610	1.155	-0.254	1.576	BTB and TAZ domain protein
UKN3	AT1G33055	1.132	0.391	2.089	unknown protein
DMR2	AT2G33830	0.873	0.195	0.936	Domancy associated 2

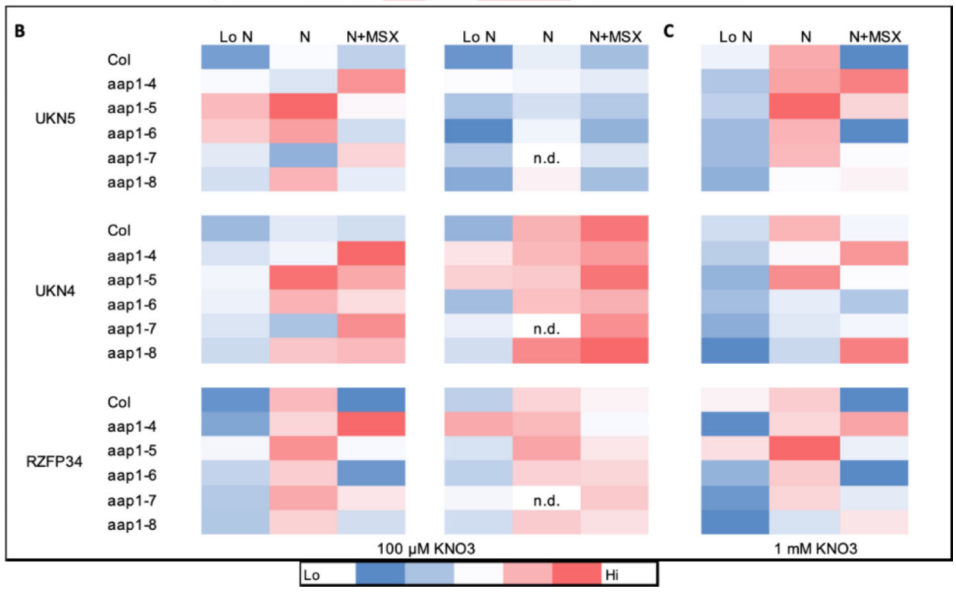


Figure 15. Effect of nitrogen treatments on mRNA accumulation of *aap1* mutant plants. (A) Microarray analysis of nitrogen and Glu responsive genes, used to select genes for qRT-PCR experiments. Genes were chosen based on response to induction by both (20 mM KNO3, 20 mM NH_4NO_3) and Glu. (B) Relative expression heat maps for qRT-PCR analysis of submerged *aap1* mutants for response to different nitrogen treatments (Lo N =100 µM KNO_3; N = 20 mM KNO_3; 20 mM NH_4NO_3; N + MSX = 20 mM KNO3, 20 mM NH_4NO_3 + 1 mM MSX). First two panels show plants starved on 100 µM for 24 h (left: response of plants on 12 h light/ 12 h dark; right: 16 h light/ 8 h dark. (C) Relative expression heat map for qRT-PCR analysis for plants nitrogen-replenished (1 mM KNO3) for 24 h, prior to treatments. Plants were grown under short-days (12 h light/ 12 h dark). No starvation (plants were given 1 mM KNO_3) treatment was applied to these plants in (C) prior to treatment with nitrogen conditions reported in (A and B). mRNA content is represented as blue for low levels and red for high levels. UKN4 (AT3G15450), UKN5 (AT5G19120), and RZFP34 (AT5G22920)

Root Architecture

Glu was shown to inhibit the elongation of the primary root and increase the number of lateral roots (Walch-Liu et al., 2006). The results above show that Glu is degraded faster in the *aap1* mutant plants than in the wild-type, which could translate into a heightened Glu tolerance of the root. Plants were grown vertically on media containing 1 mM KNO3 with or without 1 mM Glu. The main result was that growth of *aap1-8* roots was not affected by Glu, contrary to the wild-type and all other mutants (Fig. 16A). Glu tolerance of this line was studied further by using media with various NO_3^- and Glu concentrations (Supplemental Fig. 3). Specifically, *aap1-8* roots grew to the same length or longer than the wild-type plants on the same medium was supplemented with Glu and KNO_3 (Fig. 16B). However, when the NO_3^- concentration exceeded 10 mM, no differences in root length was observed between wild-type, *aap1*-4 and *aap1*-8 plants.

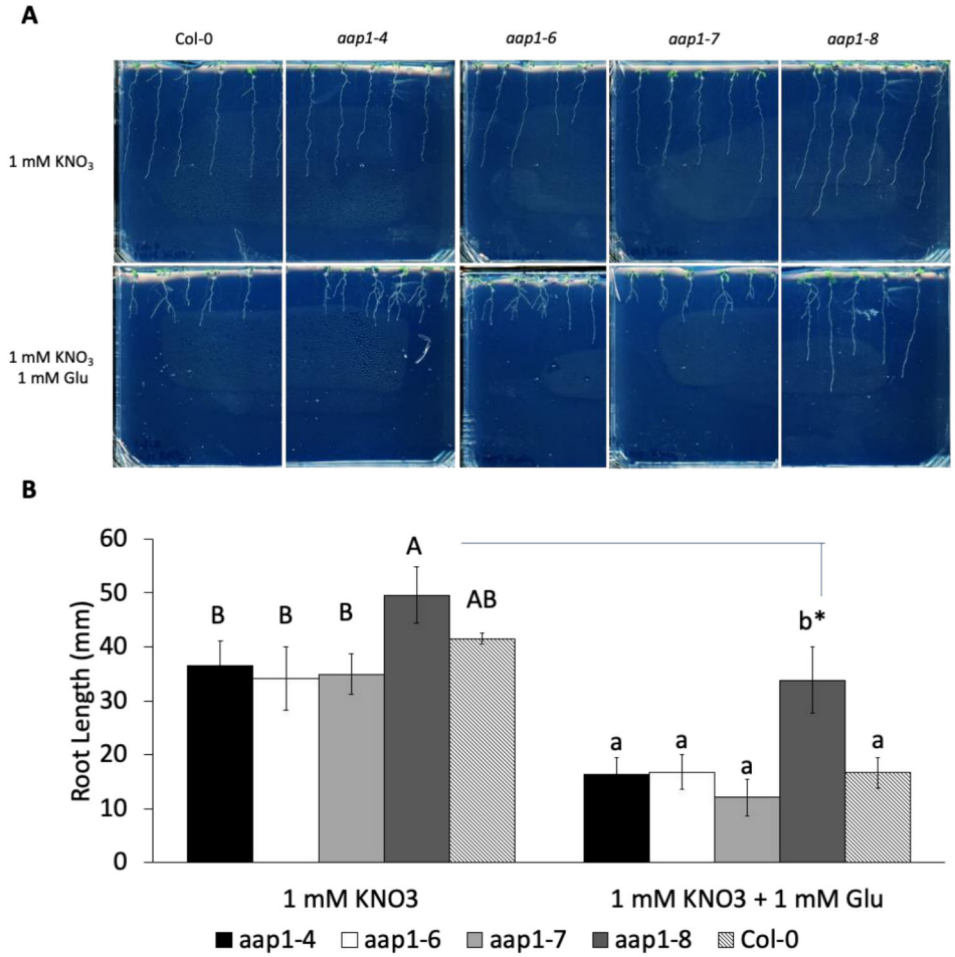


Figure 16. Root architecture of *aap1* mutants in response to 1 mM Glu. A) Root architecture of 10-day old plants grown on B medium + 1 mM KNO3 ± 1 mM Glu. B) Root length (cm) of 10-day old wild-type, aap1-4 and aap-8 plants from (A). Each bar represents the mean ± SE of three biological replicates. Significance was determined using one-way ANOVA and Tukey HSD comparison. Different letters indicate significance differences between mutants. The asterisk shows that aap1-8 is still significantly decreased with Glu treatment using a paired T-test.

Root Growth Inhibition is Less in aap1-8 *Mutants in Limiting N and Pi Conditions*

A recent publication reporting the study of NPF6.3 and the phosphate-sensing domain SPX4 showed that the two proteins interact to initiate transcription of nitrogen and phosphate responsive genes (Hu et al., 2019). Since amino acids are nitrogen-carrying molecules, I worked with members of the Gillaspy Lab to explore the potential interaction between phosphate and amino acid metabolism, since phosphate-limiting conditions showed regulation of *ZmAAP2* and *ZmLHT1* amino acid transporters (Wang et al., 2021). Differences in root length and root branching were measured to study how plants with mutations in AAP1 would lead to different responses to phosphate. When grown on medium with deplete nitrogen (100 µM KNO_3) and phosphate (100 µM KH_2PO_4) conditions, *aap1-8* mutant plants produced roots 1.5-to-2 times longer than *aap1-4* and Col-0, respectively (Fig. 17). Roots of *aap1-8* like wild-type and *aap1*-4 plants are substantially shorter in the depleted nitrogen conditions, with the *aap1-8* showing no differences in growth with or without phosphate. This suggests that the aap1-8 mutation provides some tolerance to low nitrogen, low phosphate conditions and *AAP1* might have roles that extend beyond transport.

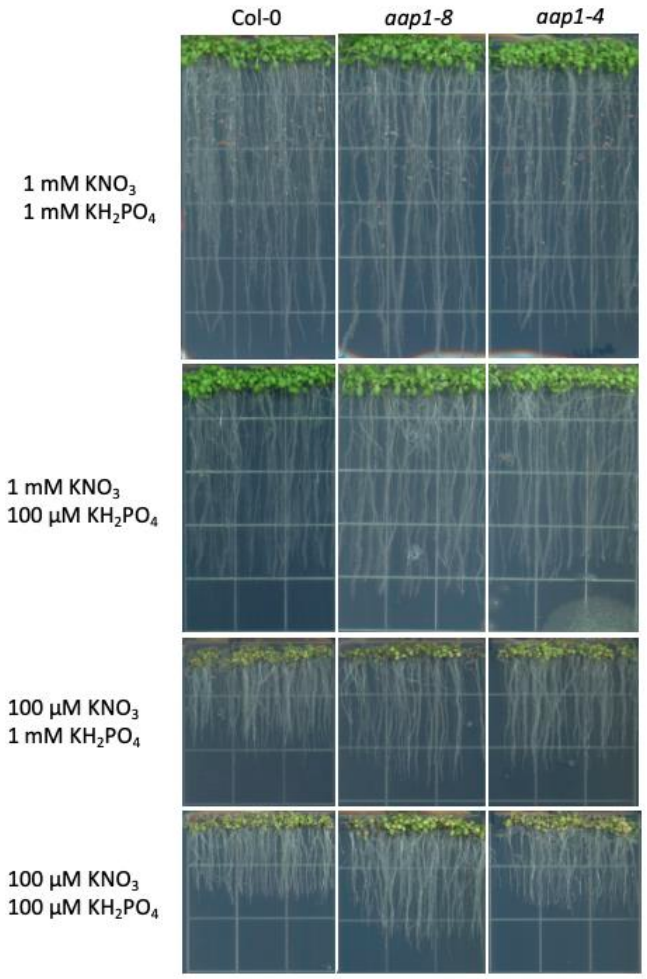


Figure 17. Response of *aap1* mutant plants root length to phosphate. Col-0, *aap1-4* T-DNA, and *aap1-8* mutants were grown on modified B medium containing different ratios of nitrogen and phosphate. Plates were grown for 14 days. Pictures were cropped; grid size is the same in all treatments.

Discussion

Amino Acid Resistance is Not Based on Lack of Transport

The work from this chapter aimed at characterizing four missense mutations in AAP1 and how each contributed to the amino acid tolerance phenotype. In the model proposed by Lee et al. (2007), *AAP1* is responsible uptake of amino acids in the epidermis cells in roots. The model argues that loss of AAP1 results in lowered uptake of amino acids by the roots, resulting in fewer amino acids in the roots; thus, providing tolerance to amino acids. The unaltered functional properties of the aap1-6 and aap1-8 variant proteins in yeast and the uptake experiments showed that both protein variants were able to transport amino acids, exposing a flaw in the model.

An Altered Signaling Mechanism?

These data showed that the tolerance phenotype is not based on transport, but possibly governed by some other mechanism, of which AAP1 is still involved. *pig1*-1 mutants, showed that amino acid metabolism for Phe is disrupted, specifically affecting translocation of amino acids from the roots to the shoots and metabolite partitioning (Voll et al., 2004). A collaboration between Drs. Voll and Pilot identified that *pig1-1* is the same mutation as *aap1-7* (unpublished data). The metabolite partitioning in the EMS-mutants was also affected as plants fed 1 mM KNO_3 + 10 mM Glu redistributed more of the ^{14}C into the negative and neutral fractions, similar to *pig1-1* plants.

Growth on Glu or KNO_3 alone did not yield differences in root length; but only when Glu was added in combination with KNO_3, did the *aap1-8* mutants displayed differences in root length. In particular, when presented with 0.1-10 mM Glu, the *aap1-8* did not display the typical sensitivity phenotype (Supplemental Fig. 3) as described by (Walch-Liu et al., 2006; Walch-Liu and Forde, 2008). These data suggest that the *aap1-8* plants are altered in the sensing of Glu, by not properly responding to this amino acid. High NO_3^- application was shown to suppress lateral root activation, allowing the root to continually grow at the root tip, which is also modulating by internal N pools (Zhang and Forde, 1998). This model for regulating root architecture can actually be supported by the phenotype of the *aap1-8* mutants, which show no inhibition to Glu exposure and tolerance to other amino acids.

Chapter 4
Regulation of AAP1 Activity

Available data shows that the expression of *AAP1* responds to hormones, inorganic nitrogen, and amino acids: The stress hormone abscisic acid (ABA), applied at 40 µM for 24 h, increased the content of *AAP1* mRNA (Wang et al., 2017). Microarray and Northern blot data showed that *AAP1* expression increased in shoot tissue in response to 20 mM KNO_3, 10 mM Gln, and 10 mM Glu (Guo, 2004).

Beyond regulation at the transcript level, several transporters have been shown to be regulated at the protein level. For example, phosphorylation of the cytosolic loop in the iron transporter (IRT1) triggers to IDF1-mediated degradation and removal from the PM (Dubeaux et al., 2018). Similarly, phosphorylation of the cytosolic loop of NRT1.2 showed that the protein is degraded by ubiquitin proteins: UBC32, UBC33, and UBC34 (Zhang et al., 2021). Phosphorylation of the cytosolic facing domains, either as loops or the N- and C-termini, is common. Specifically, the phosphorylation of the T464 and T494 amino acids in the C-terminus of AMT1;3 (Wu et al., 2019) showed that the uptake activity of ammonium is altered. Therefore, it would not be surprising to find that AAP1 protein accumulation and activity is regulated by posttranslational phosphorylation.

This chapter explores how ABA, nitrogen, and the phosphorylation affect the expression and function of AAP1.

Results

Localization of AAP1 Expression is Modulated by ABA

Wang et al. (2017) showed that 40 µM ABA significantly increased *AAP1* mRNA accumulation after 24 h. To test the effect of ABA on the activity of the *AAP1* promoter, Arabidopsis plants expressing *AAP1p:GUS* were grown on ½ MS for 7-10 days, treated with 40 µM ABA for 24 h, and assayed for GUS activity. GUS activity was observed in the vasculature of the cotyledons of the ABA-treated plants after 3 h staining, while staining was barely visible after 12 h staining in plants not treated by ABA (Fig. 18). Only one line out of the 11 tested showed GUS-activity in the vasculature of the roots and in the lateral root tips. This line likely is not representative of the true expression pattern for *AAP1*, and this result was not taken into account, even if they matched previous studies (Lee, Wang). Without ABA treatment, staining was observed ubiquitously across the cotyledon (Fig. 18A), while ABA treatment enhanced the staining in the veins in shoots (not shown, see also enlargement on Fig. 18).

ABA Increases Efflux of Amino Acids

Since ABA was shown to increase *AAP1* transcript accumulation of and caused a re-localization of the expression to the vasculature, and expression of AAP1 in the phloem (*SUC2p:AAP1* construct tin Chapter 2) increase amino acid uptake, I suspected that ABA would also increase amino acid uptake. To test this hypothesis, wild-type (Col-0), *aap1-4,* and the *aap1* EMS-mutants were grown using ½ MS medium for 12 days and treated with ABA. The application of ABA did

not significantly decrease the uptake of 1 mM Gln, except in *aap1*-5 and *aap1*-7 mutants compared to the untreated plants. The efflux in all of the mutants was ~2-3 times greater in the ABA treated plants, compared to the untreated plants of the same line, all showing significant changes in efflux. These data showed that ABA treatment causes mutations to AAP1 to decrease uptake and efflux in wild-type plants. (Fig. 18B, C).

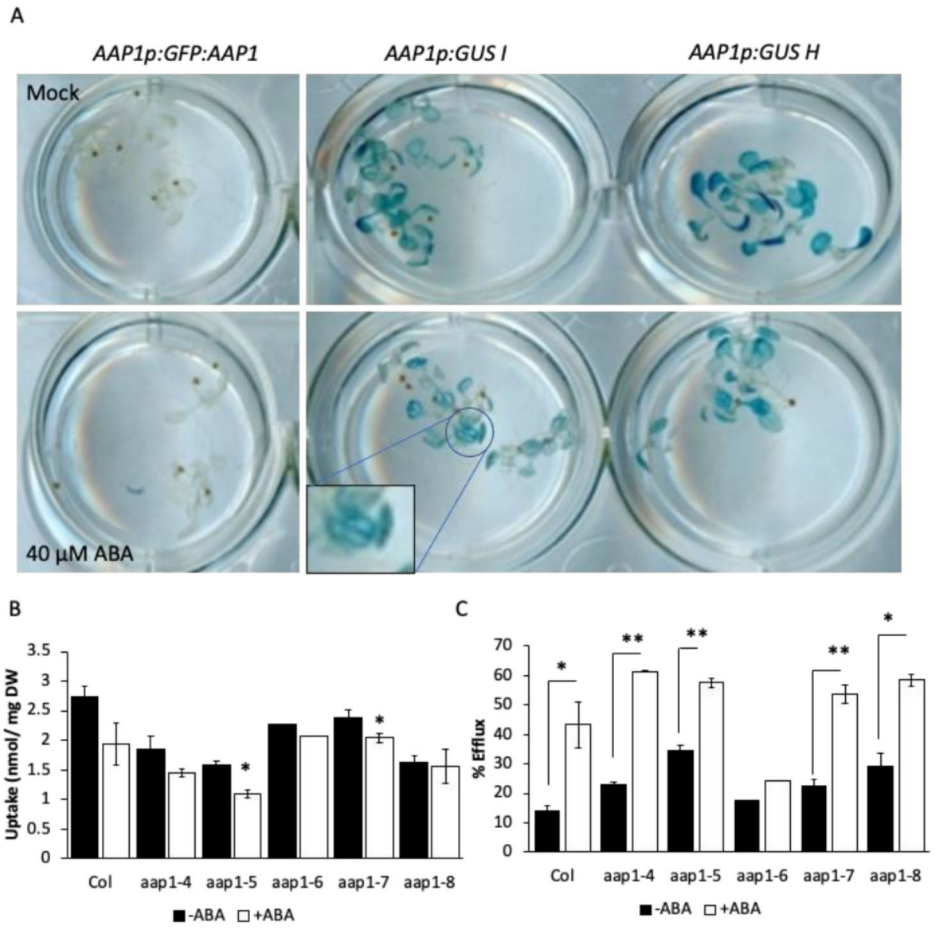


Figure 18. Effects of 40 µM ABA on *AAP1* localization and uptake. (A) Histochemical staining of *AAP1p:GUS* plants treated with 40 µM ABA for 24 h . B) Whole plant uptake of 1 mM Gln following 24 h treatment with 40 µM ABA. C) Percent efflux of total amino acid uptake following 24 h ABA treatment. Plants were incubated for 16 min uptake and 16 min efflux. Each bar represents the mean ± SD of three biological replicates. Significance was determined using a Two-tailed paired t-test (*p<0.05), (** p<0.001). All values in (C) were significantly different. Significance for *aap1-6* could not be calculated with too few arguments.

Growth Medium Influences Amino Acid Metabolism

To understand how inorganic nitrogen affects the expression of *AAP1*, plants expressing *AAP1p:GUS* were grown on 1 mM KNO_3 for 7 days, starved for nitrogen (100 µM KNO_3), subjected to different nitrogen treatments (100 µM KNO_3, 20 mM KNO_3, or 10 mM $(NH_4)_2SO_4$) for 4 hours, and subjected to GUS staining. The intensity of the staining increased in plants treated by 20 mM KNO_3 and 10 mM $(NH_4)_2SO_4$, with the most intense staining observed in the latter condition (Fig. 19A).The staining was homogenous across the whole leaf. To confirm these results at the mRNA level, plants were grown and treated similarly as above. Both 20 mM KNO_3 and 10 mM $(NH_4)_2SO_4$ significantly increased the accumulation of *AAP1* mRNA compared to the nitrogen-starved plants (100 µM KNO_3) in shoots only. The accumulation of mRNA increased in the roots, but not significantly (Fig. 19B). AAP1 mRNA content appears to respond to changes in nitrogen conditions rather than nitrogen content, since plants grown continuously on 1 mM KNO_3, 20 mM KNO_3, 10 mM NH_4NO_3, or 10 mM Gln did not show significant difference in *AAP1* mRNA accumulation in the roots ($p>0.05$) (data not shown).

At first, amino acid uptake assays in plants led inconsistent results. I later noticed that uptake results seemed to depend on the growth medium: *aap1-4* and Col-0 displayed the same transport activity when grown on 1 mM KNO_3, while *aap1-4* uptake activity was lower than Col-0 when grown on ½ MS. This suggested that nitrogen content and nature influenced the amino acid uptake capacity of plants, and that ammonium was the origin of the difference (the main difference between the two media was the additional presence of 10 mM NH_4NO_3 in ½ MS). To understand how nitrate, ammonium or the total nitrogen content affected the uptake of amino acids, plants were grown on 1 mM KNO_3 for 7 days, transferred to fresh medium containing the following nitrogen sources: 1 mM KNO_3, 30 mM KNO_3, or 15 mM $(NH_4)_2SO_4$ + 1 mM KNO_3. The ½ MS (20 mM KNO_3 and 20 mM NH_4NO_3) treatment corresponded to plants grown on ½ MS from the onset. Both the *aap1-4* and *aap1-8* plants took up Gln at levels similar to Col-0 in the 1 mM KNO_3 condition, while Gln uptake was ~50-65% lower than the wild-type when grown on the same concentration of nitrogen but supplied as ammonium (Fig. 19C). Uptake of 1 mM Gln was not different whether nitrate was supplied at 1 or 30 mM. This proved that ammonium was the reason for the difference in amino acid uptake.

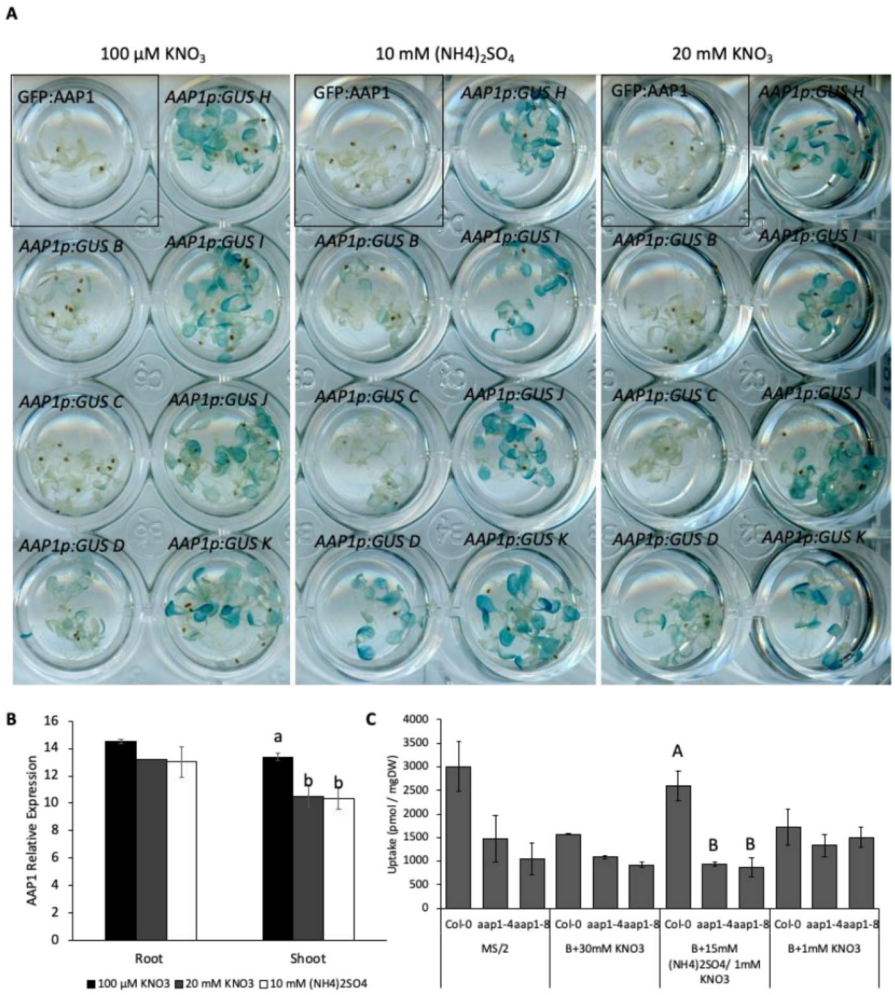


Figure 19. Response of *AAP1* expression and uptake to nitrogen form. (A) Histochemical staining of *AAP1p:GUS* plants treated with 100 µM KNO_3, 10 mM $(NH_4)_2SO_4$, or 20 mM KNO_3. (B) *AAP1* mRNA accumulation relative to *AtACTIN2* in 12-day old Col-0 plants treated with 100 µM KNO_3, 20 mM KNO_3 (p=0.046) or 10 mM $(NH_4)_2SO_4$ (p=0.006). Lower values = increased expression. Each bar represents the mean ± SE of three biological replicates; one-way ANOVA and Tukey HSD comparison. (C) Uptake of 1 mM [^{3}H]Gln by roots, excluding the hypocotyls placed on solid media with different forms and concentrations of inorganic nitrogen. Plants used in the ½ MS treatments were grown on ½ MS and uptake was on ½ MS. All other treatments were started on B medium + 1 mM KNO_3 for 7, d and then moved to medium for uptake. One-way ANOVA with Tukey HSD comparison for each treatment ($p<0.05$). Letters indicate significant differences.

N-terminal Phosphorylation Does Not Alter Function and Localization.

Mining phosphoproteomics databases identified Ser3 and Ser16 (located in the cytosol) as candidates for phosphorylation of AAP1. Based on the fact that the activity of several transporters are regulated by phosphorylation, I hypothesized that these sites could have a regulatory/stability role for AAP1. In an attempt to identify the role of these residues in the regulation of AAP1 activity, I mutated the Ser3 and Ser16 to amino acids that would mimic their phosphorylated or de-phosphorylated status: Asp is commonly used to mimic the phosphorylated status, while Ala is used for the dephosphorylated status. The wild-type, S3A S16A, and S3D S16D mutant AAP1 proteins were fused to the mCherry (AAP1:mCherry) and transiently expressed in *N. benthamiana* leaf epidermis. The C-terminal fusion of the fluorescent protein was chosen, because fusing the mCherry to the N-terminus might mask the phosphorylation signals born by Ser3 and Ser16. The accumulation of the fluorescent signal was not changed in the mutated versions of AAP1, compared to the wild-type protein (Fig. 20A). This showed that the stability of the protein in *N. benthamiana* is not affected by mutating these two Ser residues in the N-terminus of AAP1.

Since the localization of the variant proteins was not affected by these mutations, we thought maybe the mutations affected the function of AAP1. To test if the phosphorylation of the N-terminus on AAP1 affects the functional properties of the protein, the wild-type, S3A S16A, and S3D S16D variant proteins were expressed in yeast strain 22Δ10α. Yeast were grown on selective media containing only one amino acid as the sole nitrogen source. Cells expressing any of the AAP1 variants grew similarly as the cells expressing the wild-type AAP1, and much better than the vector control (Fig. 20B), suggesting that change in the phosphorylation status does not affect transport when measured in yeast.

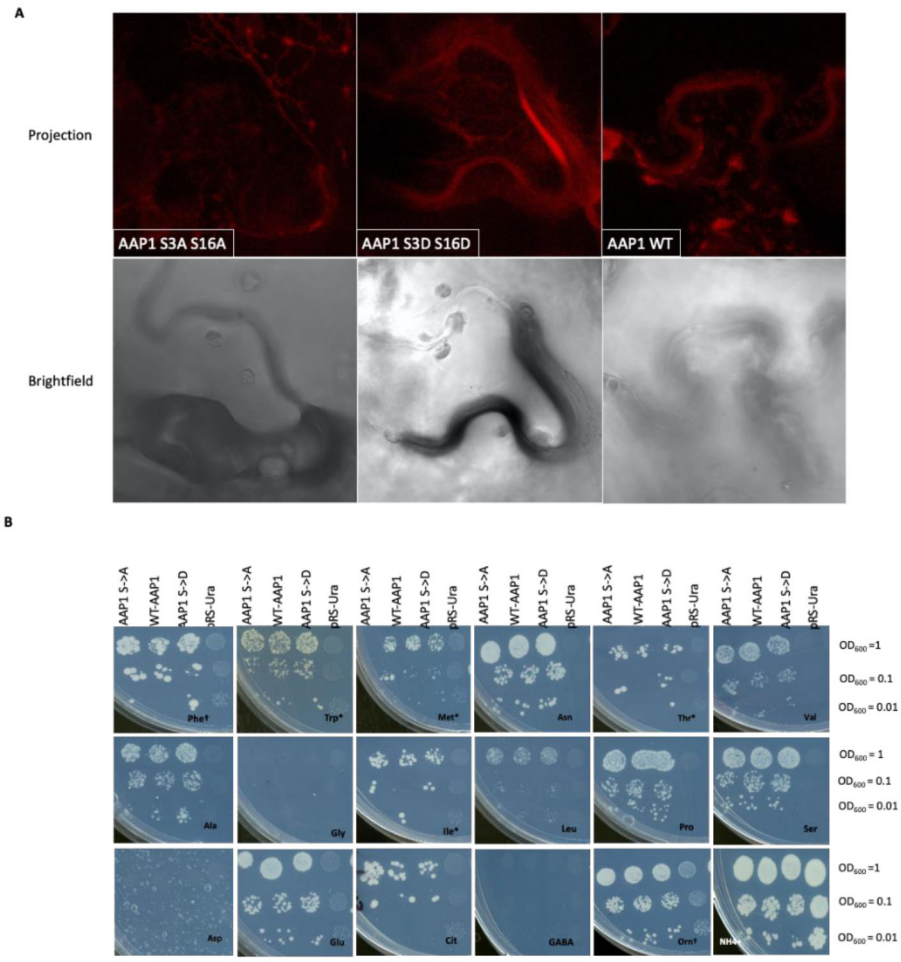


Figure 20. Functional characterization of N-terminal phosphorylation mimics of AAP1. (A) Expression of AAP1 phospho-mimic variant proteins in N. *benthamiana* leaves (Top: Projection of Z-stacks, Bottom: Brightfield images of cross sections from the compiled Z-stacks). (B) Yeast drop of 22Δ10α expressing WT-AAP1, AAP1 S3D S16D, or AAP1 S3A S16A grown on different amino acids as the sole nitrogen source. pRS-Ura is the vector control. Plates were scanned at 3, 6†, or 9* days.

Discussion

Nitrogen Source Affects Gln Uptake

Uptake of 1 mM Gln by *aap1-4* and Col-0 on different media showed that the uptake between these two lines was the least different in plants fed only KNO_3^- compared to plants grown on ½ MS or plants transferred to 15 mM $(NH_4)_2SO_4$ (Fig. X). Studies with Scots pine showed that the uptake of the amino acids studied increased in nitrogen-starved plants compared to fertilized plants (Persson and Näsholm, 2002). However, our data showed that increases in nitrogen content increased the uptake of amino acids, but the presence of NH_4^+ imparted more of an increase than NO_3^-. These data suggest that the presence of NH_4^+ is more important than the concentration of NO_3^- on modifying the uptake of amino acids. It is possible that *AAP1* is sensing external NH_4^+ levels in ½ MS (contains 20 mM NH_4NO_3) or B medium supplemented with 15 mM $(NH_4)_2SO_4$ and in order to decrease NH_4^+ toxicities, the uptake of amino acids, particularly Gln is increased. The work in Chapter 2 showed that loss of *AAP1* does not affect *AMT1;1* mRNA accumulation (Fig X). This work only looked at a small subset of other nitrogen-associated transporters with the untested ones potentially being responsible for changes nitrogen metabolism.

ABA Link to Uptake is Complicated

How nitrogen regulates the expression of AAP1 is still unknown; however, similar to Guo (2004), these data show that nitrogen increases the intensity of the *AAP1p:GUS* expression in the shoots (Fig. XA). ABA plays a role in stress responses and the specific role in the organization of AAP1 to the vascular tissues of the shoots is not understood. ABA alters the localization of *AAP1* in Arabidopsis after 24 h of treatment and increases the efflux of amino acids (Fig. X). The specific role for why plants decrease amino acid uptake and increase efflux in response to ABA is not known. The accumulation of *AAP1* mRNA appears to be correlated with stress responses (Wang et al., 2017) and increased export of amino acids is characteristic of stress responses (Pratelli et al., 2012). Yet, accumulation of the YFP signal for YFP:AAP1 expressed in *N. benthamiana* did not show changes in response to 50 µM ABA treated leaves, suggesting that responses to ABA are at the transcriptional level and not the protein level, as the ABRE were identified in the promoter (Hirner et al., 1998; Lee et al., 2007). The connection between ABA and uptake by AAP1 is not fully understood and work with ABA -synthesis and -insensitive mutants might better establish the role of ABA on AAP1 expression and amino acid uptake.

Future Directions

Since, ABA did not have an effect on changing protein localization at 6 h, we thought the regulation for protein activity was controlled via phosphorylation of two N-terminal residues Ser3 and Ser16. Both the expression in *N. benthamiana* and the yeast drop assays showed that both mutations allow yeast to import amino acids at the same level as the WT-AAP1 and are likely not involved in changing the activity of the protein. However, these studies only report on the expression of the mutated AAP1 protein in two heterologous systems. The iron transporter (IRT1) interacts with the kinase CIPK23 and an E3 ubiquitin ligase (IDF1), to relocate the transporter from the PM (Dubeaux et al., 2018). CIPK23 is shown to interact with NPF6.3 and AMT1;1 to regulate activity of these two proteins (Ragel et al., 2015; Straub et al., 2017) and may also

interact with AAP1 since NO_3^- and NH_4^+ appear to change the uptake and gene expression in WT plants. Yeast 2-Hybrid studies with CIPK23 and Ubiquitin E3 Ligases could identify missing links in the regulation of AAP1. Co-expression of the S3A S16A and S3D S16D with LOG2 (Pratelli et al., 2012) might be required for changing the localization of *AAP1* in *N. benthamiana*. When LOG2 was co-expressed with GDU1 (Pilot et al., 2004), the accumulation of AAP1 protein was not changed in *N. benthamiana* (Supplemental Fig. 3). In these studies, only the WT-AAP1 protein was tested, and the interaction with LOG2 and/ or GDU1 might depend on changes in the phosphorylation status of Ser3 and Ser16.

Chapter 5
Understanding the Role of the AAP-Specific Cytosolic Loop

Previous chapters challenged the model proposed by Lee et al. (2007) that AAP1 is expressed in the root tip and epidermis to mediate uptake of amino acids, and that reduction in uptake of amino acids renders plants resistant to toxic concentration of amino acid. Ectopic expression studies and *aap1* mutant analysis suggested that AAP1 is involved in the regulation of amino acid homeostasis. I the present Chapter, I to explore the possibility that some regions of the AAP1 protein are necessary for this regulatory role.

The Target of Rapamycin (TOR) and Sucrose non-fermenting 1 (SNF1)-related protein kinase 1 (SnRK1) are regulatory protein complexes that integrate multiple environmental and nutrient cues to promote different aspects of plant growth (McCready et al., 2020; O'Leary et al., 2020). Gln and other nitrogen sources induce TOR-mediated growth (Xiong and Sheen, 2014; Liu et al., 2021). In mammals, mTOR has been shown to be activated by several amino acid sensors, namely the amino acid transceptors PAT1 in the lysosome and SNAT2 at the plasma membrane (Goberdhan et al., 2016). Since AAP1 may impart some control over the metabolism, I hypothesized that AAP1 would signal the presence of amino acids and alter amino acid metabolism, using a similar signaling mechanism to PAT1 to repress activation under amino acid sufficient conditions.

This chapter focuses specifically on the characterization of a loop region linking transmembrane 8 and transmembrane 9 (T8/T9 loop) of AAP1, as a potential binding site for a signaling protein.

Results

The T8/T9 Cytosolic Loop is Unique to AAPs

A multiple sequence alignment of AAPs from Arabidopsis and rice orthologs compared to other amino acid transporters in the larger ATF-family (non-AAPs) showed an AAP-specific cytosolic loop that links transmembrane domains eight and nine. The alignment showed that the T8/T9 loop contains four conserved amino acid residues: P362, F366 , E370, and P376; and three semi-conserved residues: V/L/I367, V/L/I375, and V/L/I383 (Fig. 21).

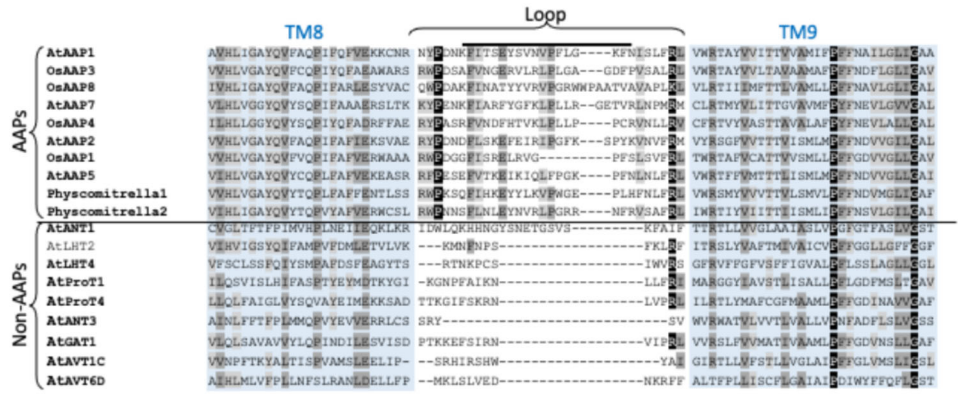


Figure 21. Multiple Sequence Alignment of Amino Acid Transporters. Subset of the protein alignment comparing the region linking transmembrane domains 8 and 9 between members of the AAP sub-family to other ATF transporters (Non-AAPs). Shading indicates conservation of amino acid residues, with darker shading representing more highly conserved residues.

AAP1Δloop can be Expressed in N. benthamiana.

To study the function of the AAP1 loop, a 16 amino acid deletion was made between amino acids F366 and F381 in the T8/T9 loop, leading to the AAP1Δloop variant. Co-expression of the YFP:*AAP1Δloop* with *AAP1:mCherry* in *N. benthamiana* epidermis cells showed that the YFP signal co-localized with the mCherry signal, in the ER (Fig. 22A), agreeing with the expression on the pattern for the wild-type protein. This showed that the loop is not important for the localization of AAP1 in *N. benthamiana*.

AAP1ΔLoop Increases Uptake in Arabidopsis plants

To study the functional properties of AAP1Δloop, Fiona Harris expressed this protein in 22Δ10α yeast and showed that protein was able to transport the same as the WT protein (data not shown). The uptake of 1 mM Gln by *aap1-4* roots expressing a genomic *AAP1p:AAP1Δloop* increased by 1.8 times compared to the uptake by wild-type plants, however the increase was not significantly different from the wild-type, but the uptake was significantly higher than the *aap1-4* mutant (Fig. 22B). The *aap1-4* mutants were also transformed with *AAP1p:AAP1Δloop* constructs causing the transgenic plants to lose some, but not all of the tolerance to 10 mM Phe (Fig. 23).

Similar to what was done in Chapter 3 with the four mutations aap1-5 to aaap1-8, the AAP1Δloop protein was expressed under the control of the dexamethasone promoter, to test for its transport activity in plants. Similar to the functional proteins aap1-5 and aap-8, induction of AAP1Δloop by DEX led to increased Gln import of whole plantlets (Fig. 22C), which would suggest that the protein is functional. Surprisingly, in these plants, Gln efflux was not significantly decreased from the un-induced plants, while it was in aap1-5 and aap1-8 expressing plants (Fig. 22D).

The effect of the deletion in the AAP1 T8/T9loop suggested that this loop does not have a major role in amino acid uptake but may have some regulatory function that affects amino acid efflux.

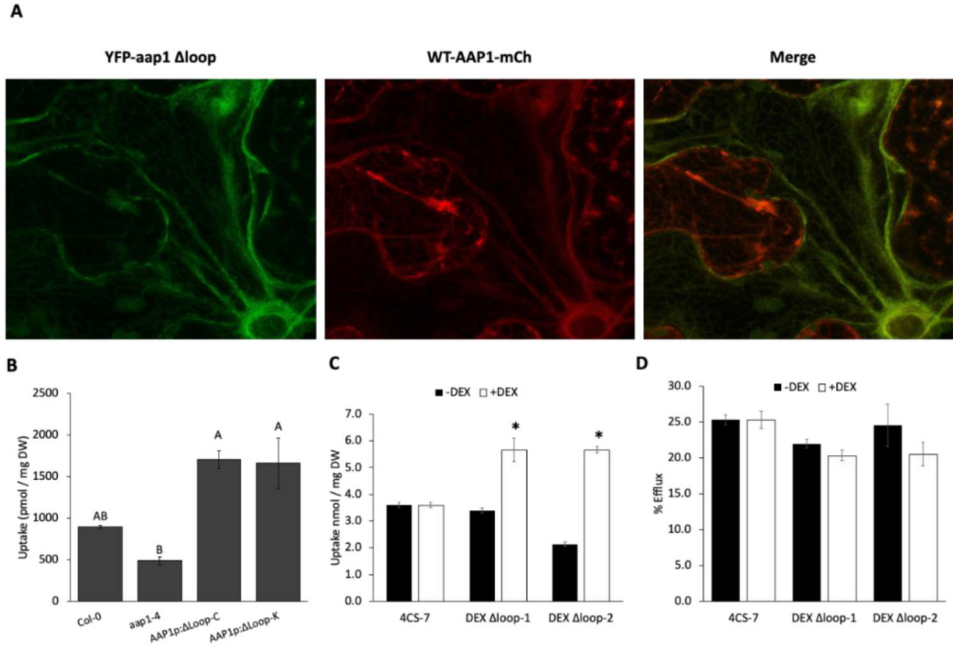


Figure 22. Functional characterization of aap1 Δloop proteins. (A) Projections of *N. benthamiana* cells co-expressing YFP:aap1Δloop variant protein with WT-AAP1:mCherry. (B) Root length uptake of 1 mM Gln with 7-day old *aap1-4* plants expressing aap1Δloop. Each bar represents the mean ± SE of two biological replicates. Different letters indicate significant differences between lines, tested by one-way ANOVA followed by Tukey's test ($p > 0.05$). (C) Whole plant (submerged) uptake in 1 mL of 1 mM Gln by 4C-S7 plants expressing aap1Δloop treated with 30 µM DEX for 24 h ($p<0.001$). (D) Percent export of amino acids taken up by plants in (C). Each bar represents the mean ± SE of three biological replicates. Asterisks indicated significant differences between lines treated by DEX, tested by paired t-Test ($p < 0.05$).

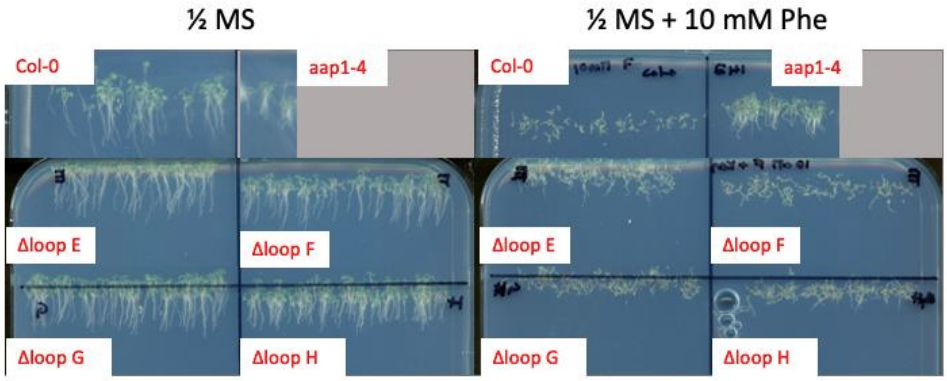


Figure 23. Complementation assay of the *aap1-4* mutant plants expressing *AAP1p:AAP1Δloop*. Complementation assay of 8-day old Arabidopsis plants grown on ½ MS ± 10 mM Phe.

Exploring Potential Interactions with Cytosolic Loop

To identify potential interactors of the T8/T9 loop, a yeast-two-hybrid screening was performed by former Pilot lab member Dr. Chengsong Zhao. This screening identified 4 clones presumably encoding proteins that interact with the loop. I retested these clones and showed they did not interact with the Δloop peptide (Fig. 21A). These four clones encoded the same protein, RabG3F (At3G18820), a GTPase involved in the fusion of endosome vesicles with the tonoplast (Rodriguez-Furlan et al., 2019). In mammals, Rag GTPase proteins interact with both TOR and PAT1 to regulate and signal amino acids (Goberdhan et al., 2016). Therefore, I hypothesized that RabG3F might be a link between TOR and AAP1, assuming that AAP1 could play the role of the SNAT2 amino acid transceptor.

Re-cloning of RabG3F using the GoldenGate technology led to a construct that could not mediate interaction with the AAP1 T8/T9 loop (Fig. 24A, column 4). Careful examination of the sequence of each of these clones showed a 15-amino acid region missing in the 5’ UTR of clone #3 that was not found in #1, #2, or #4, which was likely responsible for the interactions (Fig. 24B).

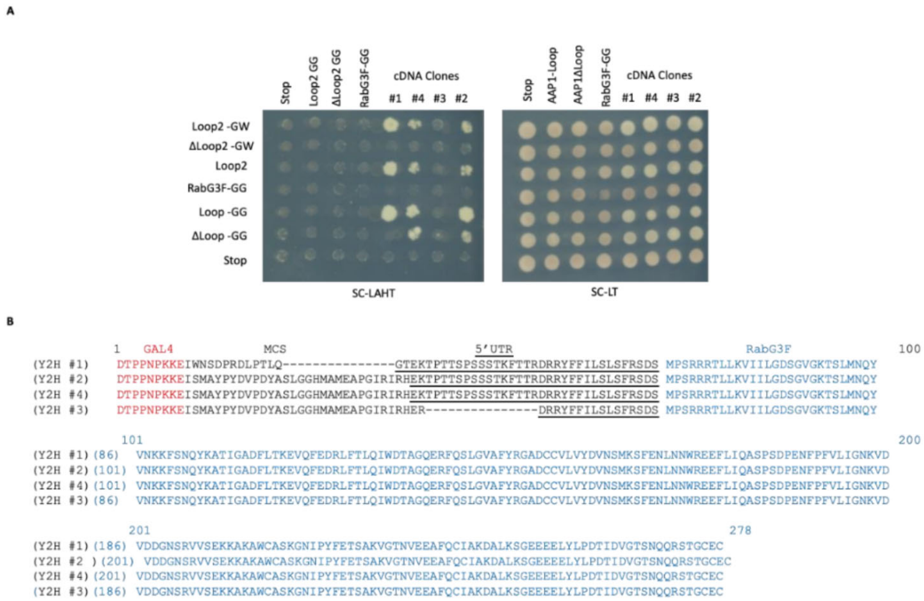

A
Stop
Loop2 GG
ΔLoop2 GG
RabG3F-GG
cDNA Clones
#1 #4 #3 #2
Stop
AAP1-Loop
AAP1ΔLoop
RabG3F-GG
cDNA Clones
#1 #4 #3 #2
Loop2 -GW
ΔLoop2 -GW
Loop2
RabG3F-GG
Loop -GG
ΔLoop -GG
Stop
SC-LAHT
SC-LT
B
1 GAL4 MCS 5'UTR RabG3F 100
(Y2H #1) DTPPNPKKEIWNSDPRDLPTLQ---------------GTEKTPTTSPSSSTKFTTRDRRYFFILSLSFRSDS MPSRRRTLLKVIILGDSGVGKTSLMNQY
(Y2H #2) DTPPNPKKEISMAYPYDVPDYASLGGHMAMEAPGIRIRHEKTPTTSPSSSTKFTTRDRRYFFILSLSFRSDS MPSRRRTLLKVIILGDSGVGKTSLMNQY
(Y2H #4) DTPPNPKKEISMAYPYDVPDYASLGGHMAMEAPGIRIRHEKTPTTSPSSSTKFTTRDRRYFFILSLSFRSDS MPSRRRTLLKVIILGDSGVGKTSLMNQY
(Y2H #3) DTPPNPKKEISMAYPYDVPDYASLGGHMAMEAPGIRIRHER---------------DRRYFFILSLSFRSDS MPSRRRTLLKVIILGDSGVGKTSLMNQY
101 200
(Y2H #1) (86) VNKKFSNQYKATIGADFLTKEVQFEDRLFTLQIWDTAGQERFQSLGVAFYRGADCCVLVYDVNSMKSFENLNNWREEFLIQASPSDPENFPFVLIGNKVD
(Y2H #2) (101) VNKKFSNQYKATIGADFLTKEVQFEDRLFTLQIWDTAGQERFQSLGVAFYRGADCCVLVYDVNSMKSFENLNNWREEFLIQASPSDPENFPFVLIGNKVD
(Y2H #4) (101) VNKKFSNQYKATIGADFLTKEVQFEDRLFTLQIWDTAGQERFQSLGVAFYRGADCCVLVYDVNSMKSFENLNNWREEFLIQASPSDPENFPFVLIGNKVD
(Y2H #3) (86) VNKKFSNQYKATIGADFLTKEVQFEDRLFTLQIWDTAGQERFQSLGVAFYRGADCCVLVYDVNSMKSFENLNNWREEFLIQASPSDPENFPFVLIGNKVD
201 278
(Y2H #1) (186) VDDGNSRVVSEKKAKAWCASKGNIPYFETSAKVGTNVEEAFQCIAKDALKSGEEEELYLPDTIDVGTSNQQRSTGCEC
(Y2H #2) (201) VDDGNSRVVSEKKAKAWCASKGNIPYFETSAKVGTNVEEAFQCIAKDALKSGEEEELYLPDTIDVGTSNQQRSTGCEC
(Y2H #4) (201) VDDGNSRVVSEKKAKAWCASKGNIPYFETSAKVGTNVEEAFQCIAKDALKSGEEEELYLPDTIDVGTSNQQRSTGCEC
(Y2H #3) (186) VDDGNSRVVSEKKAKAWCASKGNIPYFETSAKVGTNVEEAFQCIAKDALKSGEEEELYLPDTIDVGTSNQQRSTGCEC

Figure 24. Interactions of the T8/T9 loop in AAP1 with other proteins. (A) Yeast-two-hybrid Matrix testing the interaction of RabG3F with AAP1 T8/T9 **loop**. (B) Multiple Sequence Alignment of Clones #1, #2, #3 and #4.

Discussion

This work aimed to characterize the function of the cytosolic loop linking transmembrane domain 8 and transmembrane 9 (T8/T9) and trying to establish the signaling mechanism for AAP1. Deletion in the AAP1 T8/ T9 cytosolic loop did not affect the expression and localization in *N. benthamiana* but the protein did change the uptake of 1 mM Gln relative to wild-type plants and the tolerance of the plants to 10 mM Phe. It can be hypothesized that the AAP1 T8/T9 loop is a regulation site since it is unique to AAPs among the plant amino acid transporters (Fig. 18). I thus looked at the T8/T9 loop as a potential site for interaction with cytosolic proteins to signal the presence of amino acids. However, the AAP1 T8/T9 loop did not interact with the potential binding partners tested. This negative result does not exclude the hypothesis that the AAP1 T 8/9 loop could still participate in the signaling and/ or regulation of *AAP1*, only that the interacting partner (if one exists) is not yet determined. The loop does contain three potential sites for phosphorylation Thr368, Ser369, and Ser372, which, unfortunately, are not predicted as phosphorylation sites by the ATHENA (athena.proteomics.wzw.tum.de) or PhosPhAT tools (Heazlewood et al., 2008).

Chapter 6
General Discussion

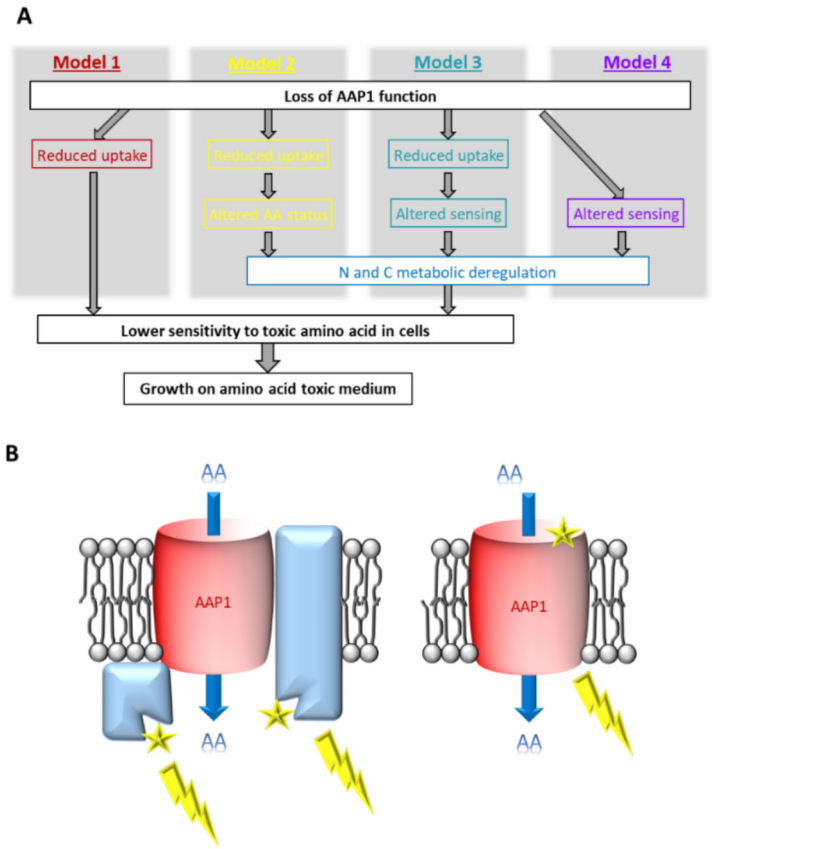


Figure 25: Models for tolerance of Arabidopsis plants to toxic amino acid medium mediated by AAP1. (A) Model proposed by (Lee et al., 2007) compared to three alternative models exposed from this dissertation. (B) Model for AAP1 as a transporter/ sensor complex or transceptor.

AAP1 from Arabidopsis was the first amino acid transporter isolated from plants, in 1993 (Frommer et al., 1993), and has been the subject of over a dozen publications since then. It is considered the most well-characterized amino acid transporter with thoroughly explored functional properties (Hsu et al., 1993; Kwart et al., 1993; Fischer et al., 1995; Boorer et al., 1996; Chang and Bush, 1997; Ortiz-Lopez et al., 2000; Fischer et al., 2002) and extensively studied role in the plant (Hirner et al., 1998; Lee et al., 2007; Sanders et al., 2009; Svennerstam et al., 2011; Elashry et al., 2013; Perchlik et al., 2014; Wang et al., 2016).

Loss-of-function mutations in AAP1 have been shown to promote tolerance to toxic concentrations of amino acids. Since these plants displayed lower amino acid uptake by the root, the following model was proposed by Lee et al. (2007): in normal conditions, AAP1 takes up amino acid in the roots from the soil solution, in the low millimolar range; when an amino acid is supplied at toxic concentrations, it accumulates in the root, and inhibit the synthesis of other amino acids and/or disturbs the metabolism, leading to growth arrest. When AAP1 is knocked out, the uptake of the amino acid is reduced, its intracellular concentration is lower, and the plant is able to grow better than the wild-type on the corresponding medium (Fig 25A, Model 1).

Current Data do NOT Fully Support Model 1

Available data and my results on AAP1, presented in this dissertation, argue against this model, and challenge the validity of some of the data and the conclusion presented by Lee et al. (2007):

(1) Transcriptomics results obtained from cell sorting (Cartwright et al., 2009), translatome (Mustroph et al., 2009), single cell sequencing (Cartwright et al., 2009; Denyer et al., 2019; Jean-Baptiste et al., 2019; Zhang et al., 2019) localized AAP1 mRNA in the root cortex. It has never been detected at significant levels in the root tip, phloem or epidermis, contrary to the results from (Kwart et al., 1993; Lee et al., 2007; Wang et al., 2017). My results are in good agreement with the transcriptomics data: the AAP1p-GFP-AAP1g-AAP1t construct expressed in the root cortex (Chapter 2).

(2) AAP1 has been claimed to localize in the plasma membrane by Lee et al. (2007), by expressing GFP-AAP1 in onion epidermis cells. Based on my experience with subcellular imaging of proteins, and to my opinion, the quality of the published picture makes this statement doubtful. I found that both in the *N. benthamiana* epidermis heterologous expression system and in Arabidopsis root cortex cells, GFP-AAP1 and AAP1-mCherry localize in the ER (Chapter 2). My result is supported from a similar expression localization of OsAAP1 and OsAAP1, close homologs of AtAAP1, in onion epidermis cells and Arabidopsis roots (Taylor et al., 2015). It should be noted that minute localization at the plasma membrane cannot be excluded, since it is not distinguishable from the ER by confocal microscopy.

(3) If AAP1 mediates uptake of amino acid in the root cortex, expressing it in the cortex of the aap1-4 knockout mutant using specific promoters should complement the phenotype of aap1-4, namely amino acid tolerance. This result was not observed (Chapter 2). In the contrary, I found that expressing AAP1 in other cells, i.e., root tip and phloem, complemented the phenotype of aap1-4. I further demonstrated that the amino acid uptake was recovered to at least the wild-type levels in the main root only when AAP1 was expressed in the phloem or root tip (Chapter 2). This result clearly shows a disconnect between localization of the expression of AAP1 and amino acid uptake, not supporting Model 1 (Fig. 22A).

(4) Importantly, the AAP1 mRNA levels of the ectopic over-expressors did not correlate with uptake activity, supporting the fact that localization rather than expression level was the reason for the recovery of uptake (Chapter 2) – see Supplemental Figure 4.

(5) According to Model 1, increased expression of AAP1 should lead to increased uptake. This was indeed observed by (Wang et al., 2017) and (Ren et al., 2019), who showed that expressing AAP1 under the CaMV35S promoter leads to higher Pro and an amino acid

derivative uptake. Yet, while ABA has been shown to induce the expression of AAP1 (Chapter 4, (Wang et al., 2017) and transcriptomics data), amino acid uptake is actually decreased by ABA (Chapter 4). This suggests that constitutive expression does not replicate endogenous expression, possibly by making AAP1 expressed in tissues other than root cortex. The effect would then be similar to the ectopic expression using root tip- and phloem-promoters (Chapter 2).

(6) Mutations in the AAP1 gene lead to modifications of metabolic activity, where amino acid turnover seemed to be accelerated (Chapter 3, (Voll et al., 2004)). While maybe not in complete disagreement with Model 1, this observation nevertheless questions the importance of the amino acid uptake activity mediated by AAP1 in the amino acid response (susceptibility/tolerance). It is possible that alterations in AAP1 function affect the metabolism, accelerating amino acid turn-over, thereby decreasing the cytosolic levels of the toxic amino acid, then rendering the plant more tolerant to amino acid.

(7) My analyses of *AAP1* expression at the transcriptional level in the aap1 mutant plants treated with inorganic nitrogen after starvation showed that altering the function of AAP1 leads to abnormal response of genes typically induced by organic nitrogen. I also found that the expression of the nitrate and ammonium transporters NPF6.3 and AMT1;1 is also affected by the mutations. In addition to (6), this approach suggests that the metabolic defects in the aap1 mutants are not confined to amino acid homeostasis but extending to the whole nitrogen metabolism.

Alternative Models

My work clearly conflicts with Model 1, and alternative models need to be formulated. I propose three other possibilities (Fig. 25A):

- Model 2: loss of AAP1 uptake activity triggers an imbalance in the C/N ratio, or a metabolic disturbance, increasing amino acid turn-over, which makes the plant tolerant to amino acids. The chain of events linking the loss of amino acid uptake at the ER membrane to the metabolic disturbance are yet to be elucidated.
- Model 3: loss of AAP1 uptake activity prevents an amino acid sensor to bind to the transported amino acid, leading to disturbed amino acid sensing, and metabolic disturbance.

Models 1, 2 and 3 require that AAP1 is only a transporter. Model 4 and 4' below posit that AAP1 is part of an amino acid sensing protein complex.

- Model 4: AAP1 interacts with a cytosolic or membrane amino acid sensor, that requires to be associated to AAP1 for effectively fulfil its sensing role. Any mutation that disrupts transport or binding to the sensor will affect sensing (Fig. 25B, left), leading to metabolic disturbance.
- Model 4': Alternatively, AAP1 is a transporter and a sensor, *i.e.,* a transceptor (Fig. 25B, right). Any mutation preventing binding to amino acids or sensing will lead to metabolic disturbance.

Data that Support an Alternative Model

AAP1-6 and AAP1-8 proteins are functional in yeast and localized similarly to the wild-type AAP1 in *N. benthamiana*. Overexpression of these two proteins in Arabidopsis increased Gln uptake proving that they are functional in Arabidopsis. Models 2 and 3 require that amino acid tolerance arise from loss of amino acid transport by AAP1. Yet, the corresponding aap1-6 and aap1-8 plants are amino acid tolerant, similarly to the *aap1-4* knockout, a fact incompatible with Models 2 and 3. These model are consequently invalid, leaving Model 4, in which AAP1 is part of a sensing complex or is a transceptor.

Most of my data can be explained by Model 4. One has to assume that aap1-6 and aap1-8 mutants have sensing defects (*i.e.,* these mutations uncouple transport and sensing, a key tool to prove that a transporter is also a sensor).Alteration of sensing by *AAP1* gene knockout or any of the four *AAP1* EMS mutations leads to disturbed nitrogen metabolism and triggers amino acid tolerance.

The differences in root length of plants grown on various combinations of nitrate and Glu of the aap1-8, aap1-4 and wild-type plants (Chapter 3), can be explained by the fact that these three lines have different sets of AAP1 functions: aap1-4 is lacking both transport and sensing, while aap1-8 is lacking only sensing. Similar differences observed upon growth on various combinations of phosphate and nitrate (Chapter 3) strengthen the conclusion above that alteration of the AAP1 sensing function has repercussions to the entire nitrogen metabolism, even when amino acids are not a variable in the experiment.

aap1 mutant plants display an increased amino acid efflux (Chapter 3), which could be the consequence of the metabolic disturbance, and not the loss of AAP1 import activity. It can be hypothesized that enhanced amino acid efflux leads to less accumulation of the toxic amino acid in the cytosol and is the cause to the amino acid tolerance. This hypothesis cannot be completely excluded, and the relative contribution of enhanced efflux and metabolic disturbance to the tolerance will need to be determined.

Postulated Molecular Role of AAP1 in Roots

The localization of AAP1 at the ER membrane could mean that AAP1 monitor amino acid levels in the cytosol endoplasmic reticulum, or that most of AAP1 accumulates in the ER in a non-functional state and is functional at the plasma membrane to sense external amino acids. It should be noted that the ER might have a role as amino acid reservoir, similar to the lysosome of animal cells, whose amino acid content is sensed by SNAT2 and transduced to the lysosome-bound TORc. In roots AAP1 could thus sense the levels of ER amino acids, possibly a proxy for nitrogen status. It is not clear at present what would be the role of AAP1 in shoots, because I have restricted by study to roots. The relocation of AAP1 expression in vasculature upon ABA treatment (Chapter 4) suggests that it can have a role in phloem loading upon stress.

As a sensor, AAP1 would require a mechanism to transduce a signal, likely through interaction with other membrane bound or soluble proteins. Similar to animals and yeast, amino acid signals are integrated by the TOR complex in Arabidopsis (O'Leary et al., 2020; Liu et al., 2021), but any connection with AAP1 has to be defined. Interestingly, ABA plays an important role in regulating growth and stress and has been shown to activate SnRK2 phosphorylation of RAPTOR to repress

TOR activity. TOR also represses ABA signaling in unstressed conditions by phosphorylating the ABA receptors (Wang et al., 2018).

As part of this work, I explored several avenues aimed at identifying any connection between ABA, AAP1 and signaling components/TOR. While an encouraging interactor of the T8/T9 loop was isolated (RabG3F, a protein from the same superfamily as the TORc-interactors RAG GTPase), I showed that this interaction is most likely artifactual (Chapter 5). Similarly, attempts at finding a role of the phosphorylated Ser in the N-terminus of AAP1 has so far been unsuccessful (Chapter 4). Yet, I believe that these are promising avenues to decipher AAP1 role in amino acid sensing.

Chapter 7
Material and Methods

Plant Maintenance

Transformant Arabidopsis seeds were selected on ½ MS supplemented with antibiotic for one weeks and transferred to soil - Sunshine Mix (Sungro Horticulture, MA, USA) under long-day conditions with 16 h of light @ 22°C and 8 h of dark @ 20°C in a Percival chambers. For Arabidopsis seed amplification and *N. benthamiana* plants, seeds were sown directly on soil.

Constructs

The tissue-specific promoters and other AAPs/ LHT1 and cDNAs were cloned by Gateway (Thermo Fisher, USA) with flanking attB sequences and placed into pDONRZeo vector before moving to pAAP1pWTkan or pWUTKan2 (*GUS*). pAAP1pWTkan was generated using the InFusion cloning system (Takara Bio, Japan) to generate a construct placing *AAP1* gDNA and the gateway cassette in pTkan2. The full-length AAP1 fusion construct used to express GFP:AAP1 genomic in Arabidopsis, was created by mutating a single nucleotide within Bsa I and Bpi I sites from in the 3.25 kbp promoter, genomic locus, and the 2 kbp *AAP1* terminator region downstream from the stop codon. The construct was assembled using the Golden Gate cloning system (*AAP1p:GFP:AAP1g:AAP1term*) and expressed in the Plant Level 2 Vector with a TBS26v insulator separating the KanR cassette from the GFP:AAP1 fusion cassette (Weber et al., 2011; Engler et al., 2014). For assembly of Level -1 to Level 1 vectors, the ligation was performed by loading 40 fmol of each plasmid and incubating with Bsa I or Bpi I (Thermo Fisher) and T4 Ligase (Promega) for 3 h at 37^{o}C in a thermo-mixing block. The ligation was stopped at 50^{o}C for 10 min. For Level 2 assembly, the reaction was alternated between 37^{o}C and 16^{o}C for 5 min each, with a final ligation step for 10 min at 16^{o}C.

Oligonucleotides used to generate all constructs are listed in Table 1 of the appendices section.

Amino Acid Tolerance and Complementation Screening Assays

Arabidopsis thaliana seeds were grown on ½ MS medium (1% sucrose, 0.75% agar, pH 5.7) supplemented with amino acids at known toxicities to the wild-type plants (Lee et al., 2007). Plants were grown under 16 hr/ 8hr light/ dark cycles at 22°C in a growth chamber for two weeks. For the Phe complementation assays, seeds were plated on ½ MS medium (1% sucrose, 1.2% agar, pH 5.7) supplemented with 10 mM Phe and 50 µg/ml kanamycin.

GUS Staining

Arabidopsis seedlings were sampled into 50 mM NaPi buffer (pH 7.2) 1.5% formaldehyde. Plates were placed in a vacuum for 1-2 min and allowed to prefix for 30 min at room temperature. Samples were washed two times in 1 mL 50 mM NaPi, 500 µM K-ferrocyanide, 500 µM K-ferricyanide. On the third wash, 1 mM X-Gluc was added, and vacuum was applied for 1-2 min. Samples were incubated in the dark at 37°C for 1-16 hours, until blue staining developed. Samples were de-stained twice with 70% EtOH for 15 min and cleared three times with 100% EtOH for more than 30 min each.

Sub-cellular localization in *N. benthamiana* and Arabidopsis

Leaves from four- to five-week-old *N. benthamiana* plants were used for transient expression via *Agrobacterium tumefaciens* infiltration. Agrobacteria transformed with constructs containing *AAP1* cDNA fused to YFP or mCherry under the control of the constitutive promoter C4H or CaMV35S infiltrated in the lower parenchyma. All constructs were co-expressed with p19 (Voinnet et al., 2003). Leaves were imaged using confocal microscopy 48-72 hours after infiltration. Arabidopsis plants were transformed with transgenic constructs using the floral dip method (Clough and Bent, 1998). In order to see GFP signals under the microscope Arabidopsis plants required 24 h treatment with 40-100 µM ABA.

RNA Extraction

RNA was isolated from 50-100 mg of tissue with a plastic pestles in 1.5 mL plastic tubes. Ground tissue was resuspended in 1 mL Tri-Reagent (Sigma, CAT#T9424), vortexed and placed on ice for 5-30 min. Samples were brought to room temperature for 5 min before 200 µL of chloroform was added. Samples were mixed vigorously for 15 s, left at room temperature for 10 min before centrifugation at 15,000 g for 10 min at 4°C. The aqueous phase was mixed with 100% isopropanol (2:1 v/v) and precipitated for 1-3 h at -20°C. The precipitated RNA was centrifuged at 15,000 g for 10 min at 4°C, washed with 75% Ethanol and re-pelleted for 5 min at 7,500 g at 4°C. The partially dried pellet was resuspended in 200 µL of DEPC-treated water, 100 µL 100% ethanol, and 10 µL Sodium Acetate. RNA was precipitated for 30 min at -20°C and centrifuged at 15,000 g for 10 min at 4°C. The pellet was washed with 70% ethanol, dried and solubilized in 50 µL of DEPC-treated water.

qRT-PCR Analysis

cDNA synthesis was completed using the RT III Enzyme Kit from Invitrogen. RNA was incubated for 5 minutes at 65°C with 10 µM of the dT18 oligonucleotide, 10 mM dNTPs. An ice-cold mixture of 5X RT buffer, RiboLock RNAse inhibitor, RT III, and 0.1 M DTT was added and incubated for 50 min at 50°C, followed by an incubation for 10 min at 80°C. qRT-PCR analysis was performed using 10 µL of the SYBR Power-Up Master Mix mixed with 1 µM forward and reverse oligonucleotides (Table 1) and 5 µL of the cDNA template (1:50 dilution). Gene expression changes were calculated by subtracting the Ct value of *AtACTIN2* to that of the gene of interest*;* a result, lower values correspond to higher accumulation of the mRNA.

Response to Nitrogen – Growth Conditions for qRT-PCR Analysis

Wild-type, *aap1-4*, and *aap1*-5, *-6, -7, -8* were germinated on B medium (3 mM $CaCl_2$, 1.5 mM $MgSO_4$, 1.25 mM KH_2PO_4, 0.1 mM NaFeEDTA, 1 mL/ L microelements (3.09 g H_3Bo_3/ 2.38 g $MnCl_2$/ 172 mg $CuCl_2$, 136 mg $ZnCl_2$, 10 mg $Mo_7O_{24}(NH_4)_6$ (w/v L)), 0.5% sucrose, 1% agar, pH 5.7) supplemented with 1 mM KNO3 (1% sucrose, 1% agar) on vertical plates. After 12 days, 10 plants were transferred to 1 mL B medium supplemented with 100 µM or 1 mM KNO3 for 24 h. Liquid was aspirated and replaced with the same medium for 30 min supplemented with 1 mM methionine sulfoximine (MSX). Medium was aspirated plants were treated for 3-4 h with 100 µM KNO3; 20 mM KNO3 + 20mM NH4NO3; 20 mM KNO3 + 20 mM NH4NO3 + 1 mM MSX or 20 mM

KNO3 + 20 mM NH4NO3 + 1 mM MSX + 10 mM Glu. Changes in lighting or the use of Gln, lack of starvation are all noted in the Figure legends.

Amino Acid Extraction and Analysis by UPLC (Free Amino Acid Content)
Plants were grown for 7 days on ½ MS (1% sucrose, 0.7% agar). Four plants were removed as a plug and transferred to rockwool plugs, saturated in J medium (1 mM KH2PO4, 0.5 mM MgSO4 7 H20, 0.1 mM NaFeEDTA, 1.3 mM $CaSO_4$, 1 mL microelements (3.09 g H_3Bo_3/ 2.38 g $MnCl_2$/ 172 mg $CuCl_2$, 136 mg $ZnCl_2$, 10 mg $Mo_7O_{24}(NH_4)_6$ (w/v - Liter) + 1 mM NH_4NO_3. After 14 days of growth, the rockwool plugs were removed and placed into blue 200 µL tip boxes and treated with J medium with 250 µM KNO_3, 1 mM NH_4NO_3, 20 mM KNO_3, or 10 mM $(NH_4)_2SO_4$; all containing 20 mM K supplemented as KCl when needed. Plants were grown until 21 days at 22°C under a 16 h/ 8 h light. Four plants (~50-100 mg tissue) were cut and collected in 1.5 mL tubes. Four replicates of each sample were collected in liquid nitrogen. After freezing, samples were lyophilized overnight and transferred to a fresh 1.5 mL tube with two 3-mm glass beads and pulverized with a bead-beater for 1 min. Approximately 1-3 mg of ground tissue was transferred to a fresh tube. Equal parts 10 mM HCl with 0.1 mM norvaline and 100% chloroform were added to the tissue sample and vortexed for 2 min. The vortexed sample was centrifuged at room temperature for 5 min at 20,000 g. The supernatant (120 µL) was transferred to a new tube. The extraction steps using HCl/ Norvaline, and Chloroform were completed again with centrifuged tissue sample from the first extraction. The supernatant (170 µL) was mixed with the previous extraction. The extracted samples were derivatized with the ACC-Tag reagent (Waters) according to the manufacturer's instruction and analyzed by Ultra High Performance Liquid Chromatography (Collakova et al., 2013).

Amino Acid Uptake and Partitioning
Plants were started on ½ MS (1% sucrose, 1.2% agar, pH 5.7) or B medium (3 mM $CaCl_2$, 1.5 mM $MgSO_4$, 1.25 mM KH_2PO_4, 0.5 mM NaFeEDTA, 1 mL/ L microelements 1 mL microelements (3.09 g H_3Bo_3/ 2.38 g $MnCl_2$/ 172 mg $CuCl_2$, 136 mg $ZnCl_2$, 10 mg $Mo_7O_{24}(NH_4)_6$ (w/v L), 0.5% sucrose, 1% agar, pH 5.7) and grown for 7-10 days. For whole plant amino acid uptake assays, 6-10 plants were transferred from solid medium to 1 mL of liquid ½ MS after 7 days. Uptakes with 12-day old plants varied between 10-20 minutes, with an equal time for efflux. For the root length uptakes, plants were germinated on 100-micron screens, and transferred to fresh medium containing radiolabeled amino acids for ~2-3 h. Hydrophobic parafilm was placed under the hypocotyl and leaves, leaving only roots exposed. A second piece of parafilm was placed under the root tip for the tissue-specific promoter uptake, to understand uptake by the cortex, when AAP1 in the root tip would not be exposed to the Gln. Plants were washed 3 times with 0.2 mM $CaSO_4$, dried and weighed. The dried samples were bleached with 500 µL of 50% NaClO solution for ≥ 4 h. Samples were allowed to ventilate for a minimum of 4 h to overnight. Samples were counted using a LSC6500 Scintillation Counter (Beckman Coulter, CA, USA) at 5 min/ sample in 5 mL Ultima Gold scintillation cocktail (Perkin Elmer, MA, USA).

For metabolic partitioning experiments, plants were started on solid ½ MS medium or B medium (3 mM $CaCl_2$, 1.5 mM $MgSO_4$, 1.25 mM KH_2PO_4, 0.5 mM NaFeEDTA, 1 mL/ L microelements 1 mL microelements (3.09 g H_3Bo_3/ 2.38 g $MnCl_2$/ 172 mg $CuCl_2$, 136 mg $ZnCl_2$, 10 mg $Mo_7O_{24}(NH_4)_6$

(w/v L), 0.5% sucrose, 1% agar, pH 5.7) supplemented with 1 mM KNO_3 + 10 mM Glu. Plants were either grown on ½ MS or ½ MS supplemented with 10 mM Glu. The submerged plants were fed 10 mM [^{14}C]Glu for 4 h, washed three times with $CaSO_4$ to remove the excess label, and placed in 100 µL $CaSO_4$ for 4 h, allowing plants to metabolize Glu. Fresh weights were measured after blotting, and flash frozen in liquid nitrogen. Amino acids were extracted by grinding ~50-100 mg fresh tissue in 750 µL 80% EtOH. Samples were incubated for 20 min at 80˚C, shaking at 1,400 rpm in a thermomixer (Eppendorf, Germany). Pellets and supernatant were obtained by centrifugation at 15,000 g for 3 min. The supernatant was placed on ice and the process was repeated with the resuspended pellet. The pooled supernatant was dried in a Vacufuge (Eppendorf, Germany) and resuspended in 150 µL water. Metabolites were separated by loading 145 µL of the concentrated supernatant onto a 1 mL bed volume of the cationic exchanger resin (Dowex AG 50W × 8), whose flow through was then loaded onto 1 mL bed volume anionic exchanger resin (Dowex AG 1 × 8) in the Acetic Acid form (Bio-Rad, Munich). The cationic exchange column was eluted with 1.5 mL 10N KOH, the anionic exchange column was eluted with 1.5 mL 2 N HCl. The radioactivity in each eluate and from the flow through of the second column was quantified by scintillation counting.

Yeast Two-Hybrid Assay

Yeast strains Y187 and AH109 were grown on solid rich medium. Yeast were sub-cultured, diluted to an OD_{600} of 0.3 in 50 mL of YPDA and grown at 30˚C for ~8 h, until OD_{600} = 1.5. Yeast was washed three times with sterile H_2O and resuspended in a final volume 800 µL. Yeast were transformed with DNA (~100-200 ng) using the LiAcetate-PEG method (Gietz, 2014). Vectors containing the GAL4-AD were introduced into AH109 and the GAL4-BD were introduced into Y187. Mating was performed after 2-3 days of growth by scraping yeast from the plates into 250 µL of water. An aliquot of 10 µL of each transformant was added to 50 µL of H_2O, mixed and plated on YPDA. The mated yeasts were transferred to 100 µL of water and 5 µL was plated on selective media for the interaction test.

Yeast Heterologous Complementation Assays

Yeast 22Δ10α was grown for 16 hours at 29˚C in selective medium. Using sterile water, yeast cultures were diluted in to OD_{600} 0.5. Samples were transformed using the LiAcetate-PEG method described above. Three ten-fold dilutions of each strain were made under the plating schematic. The medium solid consisted of a minimal medium (Jacobs et al., 1980) and 1.7% agar, and 3 mM amino acids as the sole source of nitrogen. Yeast were grown at 29˚C for 9 days, imaged at 3-day increments.

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Appendices:

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Col-0_Reference_3.5_kbp       CCTAAGTATTATAGTTGATTAGACTTAATTATCTTGCATGAACCTAATAGCTTTTGGTTA  60
Col-0_..._Lab_3.25_kbp_GG     ------------------------------------------------------------  1
Col-0_2.8_k...lot_Lab_GUS     ------------------------------------------------------------  1
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       CAATTTTGCGTTAAGTGAAGATTCGCCACCACAAGTTTTGATTTTTTAGGCAAGGAACCT  120
Col-0_..._Lab_3.25_kbp_GG     ------------------------------------------------------------  1
Col-0_2.8_k...lot_Lab_GUS     ------------------------------------------------------------  1
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       CAGATTCTCAGACGTTACACCTGTCTCCATATGCCATTTGTTGAAAGATTCATTAAGTGC  180
Col-0_..._Lab_3.25_kbp_GG     ------------------------------------------------------------  1
Col-0_2.8_k...lot_Lab_GUS     ------------------------------------------------------------  1
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       CATTATTGCTTATTAAAGCTTTATTAAAAAAAATTAGGTTTCGAATCTTTCTAGAAATTA  240
Col-0_..._Lab_3.25_kbp_GG     ------------------------------------------------------------  1
Col-0_2.8_k...lot_Lab_GUS     ------------------------------------------------------------  1
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       TCGATTATAAATCAATGAAAACCATGTGAATATGTGATTCACCTAATACGTAACTGTAGA  300
Col-0_..._Lab_3.25_kbp_GG     -----------TCAATGAAAACCATGTGAATATGTGATTCACCTAATACGTAACTGTAGA  49
Col-0_2.8_k...lot_Lab_GUS     ------------------------------------------------------------  1
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       CAAAGAAGTCAAACTTTCACATAAAAAGTTTTTTTGTTAATCATTATAAAATTAATCAGC  360
Col-0_..._Lab_3.25_kbp_GG     CAAAGAAGTCAAACTTTCACATAAAAAGTTTTTTTGTTAATCATTATAAAATTAATCAGC  109
Col-0_2.8_k...lot_Lab_GUS     ------------------------------------------------------------  1
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1
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Col-0_Reference_3.5_kbp       CTTTGACCAATCAAAAAACATTCAGCCATTTTCTATACGATTTCAATGATTTTTGTTCAT  420
Col-0_..._Lab_3.25_kbp_GG     CTTTGACCAATCAAAAAACATTCAGCCATTTTCTATACGATTTCAATGATTTTTGTTCAT  169
Col-0_2.8_k...lot_Lab_GUS     ------------------------------------------------------------  1
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       TCGTTGAAAAGAGTTTCACTGAACAATTGAAACAACTTGTCTCTTCAAGCGTATTAGAGC  480
Col-0_..._Lab_3.25_kbp_GG     TCGTTGAAAAGAGTTTCACTGAACAATTGAAACAACTTGTCTCTTCAAGCGTATTAGAGC  229
Col-0_2.8_k...lot_Lab_GUS     ------------------------------------------------------------  1
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       TATGAGAAAAAGGAGAAATATATCTTCTTTTTTTTTTTTTTTTTTTTCTTTTTTTTTGG  540
Col-0_..._Lab_3.25_kbp_GG     TATGAGAAAAAGGAGAAATATATCTTCTTTTTTTTTTTTTTTTTTTTCTTTTTTTTTGG  289
Col-0_2.8_k...lot_Lab_GUS     ------------------------------------------------------------  1
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       TAAACCAAGTAGAAATCTCTTCAGAAAACTATAAATGATGCTAATTATTGGTTTCCCGAT  600
Col-0_..._Lab_3.25_kbp_GG     TAAACCAAGTAGAAATCTCTTCAGAAAACTATAAATGATGCTAATTATTGGTTTCCCGAT  349
Col-0_2.8_k...lot_Lab_GUS     ----------------------------------------------GGTTTCCCGAT  11
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       TCGCTTACTCACAATTTGCTGCTTTATTAGAATTTTAGTACAATGTTTCTCTAAAAGTAA  660
Col-0_..._Lab_3.25_kbp_GG     TCGCTTACTCACAATTTGCTGCTTTATTAGAATTTTAGTACAATGTTTCTCTAAAAGTAA  409
Col-0_2.8_k...lot_Lab_GUS     TCGCTTACTCACAATTTGCTGCTTTATTAGAATTTTAGTACAATGTTTCTCTAAAAGTAA  71
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       GTTTTTTTCGTCCGGGATATATTTTCATAATTATAGTTTTTTTTTTGGGATAAAATGTAA  720
Col-0_..._Lab_3.25_kbp_GG     GTTTTTTTCGTCCGGGATATATTTTCATAATTATAGTTTTTTTTTTGGGATAAAATGTAA  469
Col-0_2.8_k...lot_Lab_GUS     GTTTTTTTCGTCCGGGATATATTTTCATAATTATAGTTTTTTTTTTGGGATAAAATGTAA  131
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       ATATTATTACCAAAAGGAACAATTTCTTTACAAGAGCTTGGCGTAGCTAGTGGTTACAAT  780
Col-0_..._Lab_3.25_kbp_GG     ATATTATTACCAAAAGGAACAATTTCTTTACAAGAGCTTGGCGTAGCTAGTGGTTACAAT  529
Col-0_2.8_k...lot_Lab_GUS     ATATTATTACCAAAAGGAACAATTTCTTTACAAGAGCTTGGCGTAGCTAGTGGTTACAAT  191
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Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       TAGACTAAAAAAAGAAGATTAAAAAGCCTAATTATGAAAAGAAGTTGTGAAAAAGCAGAG  840
Col-0_..._Lab_3.25_kbp_GG     TAGACTAAAAAAAGAAGATTAAAAAGCCTAATTATGAAAAGAAGTTGTGAAAAAGCAGAG  589
Col-0_2.8_k...lot_Lab_GUS     TAGACTAAAAAAAGAAGATTAAAAAGCCTAATTATGAAAAGAAGTTGTGAAAAAGCAGAG  251
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       ATTACTCATGCGCAAACCATTGTTGAAGCAGATTTGCAAAAGATGGATGTTTACGACGAG  900
Col-0_..._Lab_3.25_kbp_GG     ATTACTCATGCGCAAACCATTGTTGAAGCAGATTTGCAAAAGATGGATGTTTACGACGAG  649
Col-0_2.8_k...lot_Lab_GUS     ATTACTCATGCGCAAACCATTGTTGAAGCAGATTTGCAAAAGATGGATGTTTACGACGAG  311
Col-0_2.6_kbp_Wang_2017       ------------------------------------------------------------  1
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       CTAAAATTGTGTCTCTAATGTCTATCGACGTCTTTGAAAACCTTGAGGATCGTGGATGAG  960
Col-0_..._Lab_3.25_kbp_GG     CTAAAATTGTGTCTCTAATGTCTATCGACGTCTTTGAAAACCTTGAGGATCGTGGATGAG  709
Col-0_2.8_k...lot_Lab_GUS     CTAAAATTGTGTCTCTAATGTCTATCGACGTCTTTGAAAACCTTGAGGATCGTGGATGAG  371
Col-0_2.6_kbp_Wang_2017       ---------------TAATGTCTATCGACGTCTTTGAAAACCTTGAGGATCGTGGATGAG  45
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       ACATAGTTGTGCAAACGGTTGTTCCTCTCCTTCCAAGTCATGTTGAGGAACCGTTTATTT  1020
Col-0_..._Lab_3.25_kbp_GG     ACATAGTTGTGCAAACGGTTGTTCCTCTCCTTCCAAGTCATGTTGAGGAACCGTTTATTT  769
Col-0_2.8_k...lot_Lab_GUS     ACATAGTTGTGCAAACGGTTGTTCCTCTCCTTCCAAGTCATGTTGAGGAACCGTTTATTT  431
Col-0_2.6_kbp_Wang_2017       ACATAGTTGTGCAAACGGTTGTTCCTCTCCTTCCAAGTCATGTTGAGGAACCGTTTATTT  105
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       TTTATTTTATTGAAACAACAAGTTAAACCGAACTACTATTTATTGTTATTTGTTAATAGA  1080
Col-0_..._Lab_3.25_kbp_GG     TTTATTTTATTGAAACAACAAGTTAAACCGAACTACTATTTATTGTTATTTGTTAATAGA  829
Col-0_2.8_k...lot_Lab_GUS     TTTATTTTATTGAAACAACAAGTTAAACCGAACTACTATTTATTGTTATTTGTTAATAGA  491
Col-0_2.6_kbp_Wang_2017       TTTATTTTATTGAAACAACAAGTTAAACCGAACTACTATTTATTGTTATTTGTTAATAGA  165
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       GATGAAATTTCAAATCACATTTTTTTCTTCTTCTTATTTAACTAACAAGTAATAAATCAC  1140
Col-0_..._Lab_3.25_kbp_GG     GATGAAATTTCAAATCACATTTTTTTCTTCTTCTTATTTAACTAACAAGTAATAAATCAC  889
Col-0_2.8_k...lot_Lab_GUS     GATGAAATTTCAAATCACATTTTTTTCTTCTTCTTATTTAACTAACAAGTAATAAATCAC  551
Col-0_2.6_kbp_Wang_2017       GATGAAATTTCAAATCACATTTTTTTCTTCTTCTTATTTAACTAACAAGTAATAAATCAC  225
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1
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Col-0_Reference_3.5_kbp       GATTTGTTTATAAGAAGCATCATGAATATAAACGAGAGCAAAAAGACGATGATCTTCATA  1200
Col-0_..._Lab_3.25_kbp_GG     GATTTGTTTATAAGAAGCATCATGAATATAAACGAGAGCAAAAAGACGATGATCTTCATA  949
Col-0_2.8_k...lot_Lab_GUS     GATTTGTTTATAAGAAGCATCATGAATATAAACGAGAGCAAAAAGACGATGATCTTCATA  611
Col-0_2.6_kbp_Wang_2017       GATTTGTTTATAAGAAGCATCATGAATATAAACGAGAGCAAAAAGACGATGATCTTCATA  285
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       TATACAATTTCAATGATTTGCTGCTCATTCGTTGAAAGAGTTAAATTATATTAAACACTC  1260
Col-0_..._Lab_3.25_kbp_GG     TATACAATTTCAATGATTTGCTGCTCATTCGTTGAAAGAGTTAAATTATATTAAACACTC  1009
Col-0_2.8_k...lot_Lab_GUS     TATACAATTTCAATGATTTGCTGCTCATTCGTTGAAAGAGTTAAATTATATTAAACACTC  671
Col-0_2.6_kbp_Wang_2017       TATACAATTTCAATGATTTGCTGCTCATTCGTTGAAAGAGTTAAATTATATTAAACACTC  345
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       GACACAACTTGACTTTTCAAACGTGGTTAGAGTTAGATGAAAAATGAGAAGTCTTTTGAG  1320
Col-0_..._Lab_3.25_kbp_GG     GACACAACTTGACTTTTCAAACGTGGTTAGAGTTAGATGAAAAATGAGAAGTCTTTTGAG  1069
Col-0_2.8_k...lot_Lab_GUS     GACACAACTTGACTTTTCAAACGTGGTTAGAGTTAGATGAAAAATGAGAAGTCTTTTGAG  731
Col-0_2.6_kbp_Wang_2017       GACACAACTTGACTTTTCAAACGTGGTTAGAGTTAGATGAAAAATGAGAAGTCTTTTGAG  405
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       AAAACTATAAATGTTTTTAATTATTGGTTTCCAAATTCGCTTACTCACAATTTAATTCTT  1380
Col-0_..._Lab_3.25_kbp_GG     AAAACTATAAATGTTTTTAATTATTGGTTTCCAAATTCGCTTACTCACAATTTAATTCTT  1129
Col-0_2.8_k...lot_Lab_GUS     AAAACTATAAATGTTTTTAATTATTGGTTTCCAAATTCGCTTACTCACAATTTAATTCTT  791
Col-0_2.6_kbp_Wang_2017       AAAACTATAAATGTTTTTAATTATTGGTTTCCAAATTCGCTTACTCACAATTTAATTCTT  465
C24_2.1_kbp_Hirner_1998       ------------------------------------------------------------  1

Col-0_Reference_3.5_kbp       TATTAGACTTAAGTACCATGTTTCTCTATAACTAAGCTTTTTCATATTTAGATAAATTTT  1440
Col-0_..._Lab_3.25_kbp_GG     TATTAGACTTAAGTACCATGTTTCTCTATAACTAAGCTTTTTCATATTTAGATAAATTTT  1189
Col-0_2.8_k...lot_Lab_GUS     TATTAGACTTAAGTACCATGTTTCTCTATAACTAAGCTTTTTCATATTTAGATAAATTTT  851
Col-0_2.6_kbp_Wang_2017       TATTAGACTTAAGTACCATGTTTCTCTATAACTAAGCTTTTTCATATTTAGATAAATTTT  525
C24_2.1_kbp_Hirner_1998       ----------------------------------GCTTTTTCATATTTAGATAAATTTT  25

Col-0_Reference_3.5_kbp       TTTTTTTTTTTTCGTAATTAAAACAACTAGTTAAACCGAACTATTTTTGATTGCTAATAAA  1500
Col-0_..._Lab_3.25_kbp_GG     TTTTTTTTTTTTCGTAATTAAAACAACTAGTTAAACCGAACTATTTTTGATTGCTAATAAA  1249
Col-0_2.8_k...lot_Lab_GUS     TTTTTTTTTTTTCGTAATTAAAACAACTAGTTAAACCGAACTATTTTTGATTGCTAATAAA  911
Col-0_2.6_kbp_Wang_2017       TTTTTTTTTTTTCGTAATTAAAACAACTAGTTAAACCGAACTATTTTTGATTGCTAATAAA  585
C24_2.1_kbp_Hirner_1998       TTTTTTTTTTTTCGTAATTAAAACAACTAGTTAAACCGAACTATTTTTGATTGCTAATAAA  85

Col-0_Reference_3.5_kbp       GATGATACTTTGTTTTTTGAGAAAGAATGAACATGATATTTCAACTCGATTTTTCTTCTT  1560
Col-0_..._Lab_3.25_kbp_GG     GATGATACTTTGTTTTTTGAGAAAGAATGAACATGATATTTCAACTCGATTTTTCTTCTT  1309
Col-0_2.8_k...lot_Lab_GUS     GATGATACTTTGTTTTTTGAGAAAGAATGAACATGATATTTCAACTCGATTTTTCTTCTT  971
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Col-0_2.6_kbp_Wang_2017        GATGATACTTTGTTTTTTGAGAAAGAATGAACATGATATTTCAACTCGATTTTTCTTCTT  645
C24_2.1_kbp_Hirner_1998        GATGATACTTTGTTTTTTGAGAAAGAATGAACATGATATTTCAACTCGATTTTTCTTCTT  145

Col-0_Reference_3.5_kbp        CTTATATTAAACAAGTAAATAAATCACCATTTGTTTCTAAAGAAGCAACATGAATATATA  1620
Col-0_..._Lab_3.25_kbp_GG      CTTATATTAAACAAGTAAATAAATCACCATTTGTTTCTAAAGAAGCAACATGAATATATA  1369
Col-0_2.8_k...lot_Lab_GUS      CTTATATTAAACAAGTAAATAAATCACCATTTGTTTCTAAAGAAGCAACATGAATATATA  1031
Col-0_2.6_kbp_Wang_2017        CTTATATTAAACAAGTAAATAAATCACCATTTGTTTCTAAAGAAGCAACATGAATATATA  705
C24_2.1_kbp_Hirner_1998        CTTATATTAAACAAGTAAATAAATCACCATTTGTTTCTAAAGAAGCAACATGAATATATA  205

Col-0_Reference_3.5_kbp        TATAAATTAAAGAACGATGGACATCTATGTCACGGTCTAACATTTTTACTGGCAAAGGGC  1680
Col-0_..._Lab_3.25_kbp_GG      TATAAATTAAAGAACGATGGACATCTATGTCACGGTCTAACATTTTTACTGGCAAAGGGC  1429
Col-0_2.8_k...lot_Lab_GUS      TATAAATTAAAGAACGATGGACATCTATGTCACGGTCTAACATTTTTACTGGCAAAGGGC  1091
Col-0_2.6_kbp_Wang_2017        TATAAATTAAAGAACGATGGACATCTATGTCACGGTCTAACATTTTTACTGGCAAAGGGC  765
C24_2.1_kbp_Hirner_1998        TATAAATTAAAGAACGATGGACATCTATGTCACGGTCTAACATTTTTACTGGCAAAGGGC  265

Col-0_Reference_3.5_kbp        ATTTGCATTATTTGACCTGATTGAGCATGATTAACCTTTATAAGATAATGTAATCTCTTA  1740
Col-0_..._Lab_3.25_kbp_GG      ATTTGCATTATTTGACCTGATTGAGCATGATTAACCTTTATAAGATAATGTAATCTCTTA  1489
Col-0_2.8_k...lot_Lab_GUS      ATTTGCATTATTTGACCTGATTGAGCATGATTAACCTTTATAAGATAATGTAATCTCTTA  1151
Col-0_2.6_kbp_Wang_2017        ATTTGCATTATTTGACCTGATTGAGCATGATTAACCTTTATAAGATAATGTAATCTCTTA  825
C24_2.1_kbp_Hirner_1998        ATTTGCATTATTTGACCTGATTGAGCATGATTAACCTTTATAAGATAATGTAATCTCTTA  325

Col-0_Reference_3.5_kbp        ATAGCCAAAACCTACGTTTACGCTTTTACCCTTCATCCGTTTTTTTGTTCAACTTTTTAC  1800
Col-0_..._Lab_3.25_kbp_GG      ATAGCCAAAACCTACGTTTACGCTTTTACCCTTCATCCGTTTTTTTGTTCAACTTTTTAC  1549
Col-0_2.8_k...lot_Lab_GUS      ATAGCCAAAACCTACGTTTACGCTTTTACCCTTCATCCGTTTTTTTGTTCAACTTTTTAC  1211
Col-0_2.6_kbp_Wang_2017        ATAGCCAAAACCTACGTTTACGCTTTTACCCTTCATCCGTTTTTTTGTTCAACTTTTTAC  885
C24_2.1_kbp_Hirner_1998        ATAGCCAAAACCTACGTTTACGCTTTTACGCTTCATCCGTTTTTTTGTTCAACTTTTTAC  385

Col-0_Reference_3.5_kbp        TTTTTGTTTCAGTCAACACACGTTTACTCTTCATCAAACTCAAACCTTAAATTTGTTTAT  1860
Col-0_..._Lab_3.25_kbp_GG      TTTTTGTTTCAGTCAACACACGTTTACTCTTCATCAAACTCAAACCTTAAATTTGTTTAT  1609
Col-0_2.8_k...lot_Lab_GUS      TTTTTGTTTCAGTCAACACACGTTTACTCTTCATCAAACTCAAACCTTAAATTTGTTTAT  1271
Col-0_2.6_kbp_Wang_2017        TTTTTGTTTCAGTCAACACACGTTTACTCTTCATCAAACTCAAACCTTAAATTTGTTTAT  945
C24_2.1_kbp_Hirner_1998        TTTTTGTTTCAGTCAACACACGTTTACTCTTCATCAAACTCAAACCTTAAATTTGTTTAT  445

Col-0_Reference_3.5_kbp        TTTTTAGTTTTATTTTGGTAGAACATTGAAAACCAAAATTTGAAGATCAAATAAATCAAA  1920
Col-0_..._Lab_3.25_kbp_GG      TTTTTAGTTTTATTTTGGTAGAACATTGAAAACCAAAATTTGAAGATCAAATAAATCAAA  1669
Col-0_2.8_k...lot_Lab_GUS      TTTTTAGTTTTATTTTGGTAGAACATTGAAAACCAAAATTTGAAGATCAAATAAATCAAA  1331
Col-0_2.6_kbp_Wang_2017        TTTTTAGTTTTATTTTGGTAGAACATTGAAAACCAAAATTTGAAGATCAAATAAATCAAA  1005
C24_2.1_kbp_Hirner_1998        TTTTTAGTTTTATTTTGGTAGAACATTGAAAACCAAAATTTGAAGATCAAATAAATCAAA  505
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Col-0_Reference_3.5_kbp       GTTATTTATGGTAGATAAGTAGCTTCTCTCGTCGTTTCAAGATGCTTAAATAAGTAGTAT  1980
Col-0_..._Lab_3.25_kbp_GG     GTTATTTATGGTAGATAAGTAGCTTCTCTCGTCGTTTCAAGATGCTTAAATAAGTAGTAT  1729
Col-0_2.8_k...lot_Lab_GUS     GTTATTTATGGTAGATAAGTAGCTTCTCTCGTCGTTTCAAGATGCTTAAATAAGTAGTAT  1391
Col-0_2.6_kbp_Wang_2017       GTTATTTATGGTAGATAAGTAGCTTCTCTCGTCGTTTCAAGATGCTTAAATAAGTAGTAT  1065
C24_2.1_kbp_Hirner_1998       GTTATTTATGGTAGATAAGTAGCTTCTCTCGTCGTTTCAAGATGCTTAAATAAGTAGTAT  565

Col-0_Reference_3.5_kbp       CTTCTGACTTTCTGATTCCCTCAATTAACTGAAAGAATGGTTCAAGGATTTGTTAGCTCT  2040
Col-0_..._Lab_3.25_kbp_GG     CTTCTGACTTTCTGATTCCCTCAATTAACTGAAAGAATGGTTCAAGGATTTGTTAGCTCT  1789
Col-0_2.8_k...lot_Lab_GUS     CTTCTGACTTTCTGATTCCCTCAATTAACTGAAAGAATGGTTCAAGGATTTGTTAGCTCT  1451
Col-0_2.6_kbp_Wang_2017       CTTCTGACTTTCTGATTCCCTCAATTAACTGAAAGAATGGTTCAAGGATTTGTTAGCTCT  1125
C24_2.1_kbp_Hirner_1998       CTTCTGACTTTCTGATTCCCTCAATTAACTGAAAGAATGGTTCAAGGATTTGTTAGCTCT  625

Col-0_Reference_3.5_kbp       TAGGATACATTATATATAAGCCGTTTGAAAAACAACTAGGTATGTTCGACCAAGTAGTAC  2100
Col-0_..._Lab_3.25_kbp_GG     TAGGATACATTATATATAAGCCGTTTGAAAAACAACTAGGTATGTTCGACCAAGTAGTAC  1849
Col-0_2.8_k...lot_Lab_GUS     TAGGATACATTATATATAAGCCGTTTGAAAAACAACTAGGTATGTTCGACCAAGTAGTAC  1511
Col-0_2.6_kbp_Wang_2017       TAGGATACATTATATATAAGCCGTTTGAAAAACAACTAGGTATGTTCGACCAAGTAGTAC  1185
C24_2.1_kbp_Hirner_1998       TAGGATACATTATATATAAGCCGTTTGAAAAACAACTAGGTATGTTCGACCAAGTAGTAC  685

Col-0_Reference_3.5_kbp       GAAAAACTAACTCCTAACTAAACGAGAAAAAAACATTAAGAGAATGAGAGTTCCAACATG  2160
Col-0_..._Lab_3.25_kbp_GG     GAAAAACTAACTCCTAACTAAACGAGAAAAAAACATTAAGAGAATGAGAGTTCCAACATG  1909
Col-0_2.8_k...lot_Lab_GUS     GAAAAACTAACTCCTAACTAAACGAGAAAAAAACATTAAGAGAATGAGAGTTCCAACATG  1571
Col-0_2.6_kbp_Wang_2017       GAAAAACTAACTCCTAACTAAACGAGAAAAAAACATTAAGAGAATGAGAGTTCCAACATG  1245
C24_2.1_kbp_Hirner_1998       GAAAAACTAACTCCTAACTAAACGAGAAAAAAACATTAAGAGAATGAGAGTTCCAACATG  745

Col-0_Reference_3.5_kbp       ATTTCTATTATTCTTGTATGAATGAGTTTATGAGAATTTGGAATAATTCATGAATCTACC  2220
Col-0_..._Lab_3.25_kbp_GG     ATTTCTATTATTCTTGTATGAATGAGTTTATGAGAATTTGGAATAATTCATGAATCTACC  1969
Col-0_2.8_k...lot_Lab_GUS     ATTTCTATTATTCTTGTATGAATGAGTTTATGAGAATTTGGAATAATTCATGAATCTACC  1631
Col-0_2.6_kbp_Wang_2017       ATTTCTATTATTCTTGTATGAATGAGTTTATGAGAATTTGGAATAATTCATGAATCTACC  1305
C24_2.1_kbp_Hirner_1998       ATTTCTATTATTCTTGTATGAATGAGTTTATGAGAATTTGGAATAATTCATGAATCTACC  805

Col-0_Reference_3.5_kbp       CAAATTTTCATTATATATGGATTTATGAAACTTGGTAATTGATACGAAAGTATTTTGTTA  2280
Col-0_..._Lab_3.25_kbp_GG     CAAATTTTCATTATATATGGATTTATGAAACTTGGTAATTGATACGAAAGTATTTTGTTA  2029
Col-0_2.8_k...lot_Lab_GUS     CAAATTTTCATTATATATGGATTTATGAAACTTGGTAATTGATACGAAAGTATTTTGTTA  1691
Col-0_2.6_kbp_Wang_2017       CAAATTTTCATTATATATGGATTTATGAAACTTGGTAATTGATACGAAAGTATTTTGTTA  1365
C24_2.1_kbp_Hirner_1998       CAAATTTTCATTATATATGGATTTATGAAACTTGGTAATTGATACGAAAGTATTTTGTTA  865

Col-0_Reference_3.5_kbp       TTTTATTCTCGATATATTAACATTTTTTTTTTTTTTTTAAAAATAGTGTTTTTTTTTAAC  2340
Col-0_..._Lab_3.25_kbp_GG     TTTTATTCTCGATATATTAACATTTTTTTTTTTTTTTTAAAAATAGTGTTTTTTTTTAAC  2089
Col-0_2.8_k...lot_Lab_GUS     TTTTATTCTCGATATATTAACATTTTTTTTTTTTTTTTAAAAATAGTGTTTTTTTTTAAC  1751
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Col-0_2.6_kbp_Wang_2017        TTTTATTCTCGATATATTAACATTTTTTTTTTTTTTTTAAAAATAGTGTTTTTTTTTAAC  1425
C24_2.1_kbp_Hirner_1998        TTTTATTCTCGATATATTAACATTTTTTTTTTTTTTTTAAAAATAGTGTTTTTTTTTAAC  925

Col-0_Reference_3.5_kbp        ATGGGAGAGCACTTAACAGGACGGGTTCTCGCAGAAACGATATATATTTAGGTCAAATCT  2400
Col-0_..._Lab_3.25_kbp_GG      ATGGGAGAGCACTTAACAGGACGGGTTCTCGCAGAAACGATATATATTTAGGTCAAATCT  2149
Col-0_2.8_k...lot_Lab_GUS      ATGGGAGAGCACTTAACAGGACGGGTTCTCGCAGAAACGATATATATTTAGGTCAAATCT  1811
Col-0_2.6_kbp_Wang_2017        ATGGGAGAGCACTTAACAGGACGGGTTCTCGCAGAAACGATATATATTTAGGTCAAATCT  1485
C24_2.1_kbp_Hirner_1998        ATGGGAGAGCACTTAACAGGACGGGTTCTCGCAGAAACGATATATATTTAGGTCAAATCT  985

Col-0_Reference_3.5_kbp        GTTTTATCGGGGTTATTAGATTCTCTTGGCCCAACTGTAGTGTCAAATATCAAACCGATG  2460
Col-0_..._Lab_3.25_kbp_GG      GTTTTATCGGGGTTATTAGATTCTCTTGGCCCAACTGTAGTGTCAAATATCAAACCGATG  2209
Col-0_2.8_k...lot_Lab_GUS      GTTTTATCGGGGTTATTAGATTCTCTTGGCCCAACTGTAGTGTCAAATATCAAACCGATG  1871
Col-0_2.6_kbp_Wang_2017        GTTTTATCGGGGTTATTAGATTCTCTTGGCCCAACTGTAGTGTCAAATATCAAACCGATG  1545
C24_2.1_kbp_Hirner_1998        GTTTTATCGGGGTTATTAGATTCTCTTGGCCCAACTGTAGTGTCAAATATCAAACCGATG  1045

Col-0_Reference_3.5_kbp        TTATCTTATTAAAAAATTATATTAACATCAACTTAAATTGTTAAATGATCTACATACATG  2520
Col-0_..._Lab_3.25_kbp_GG      TTATCTTATTAAAAAATTATATTAACATCAACTTAAATTGTTAAATGATCTACATACATG  2269
Col-0_2.8_k...lot_Lab_GUS      TTATCTTATTAAAAAATTATATTAACATCAACTTAAATTGTTAAATGATCTACATACATG  1931
Col-0_2.6_kbp_Wang_2017        TTATCTTATTAAAAAATTATATTAACATCAACTTAAATTGTTAAATGATCTACATACATG  1605
C24_2.1_kbp_Hirner_1998        TTATCTTATTAAAAAATTATATTAACATCAACTTAAATTGTTAAATGATCTACATACATG  1105

Col-0_Reference_3.5_kbp        AATTTTACCCCATTATTTATTTTTATTTGTCGTCTTCGATTGTCCATAATTTCTAATCTG  2580
Col-0_..._Lab_3.25_kbp_GG      AATTTTACCCCATTATTTATTTTTATTTGTCGTCTTTGATTGTCCATAATTTCTAATCTG  2329
Col-0_2.8_k...lot_Lab_GUS      AATTTTACCCCATTATTTATTTTTATTTGTCGTCTTCGATTGTCCATAATTTCTAATCTG  1991
Col-0_2.6_kbp_Wang_2017        AATTTTACCCCATTATTTATTTTTATTTGTCGTCTTCGATTGTCCATAATTTCTAATCTG  1665
C24_2.1_kbp_Hirner_1998        AATTTTACCCCATTATTTATTTTTATTTGTCGTCTTCGATTGTCCATAATTTCTAATCTG  1165

Col-0_Reference_3.5_kbp        TAATGCCAAATTTGATGATCTTCAACTCGTTAAAAAAACCAAACCATAGAGATTTTGTAT  2640
Col-0_..._Lab_3.25_kbp_GG      TAATGCCAAATTTGATGATCTTCAACTCGTTAAAAAAACCAAACCATAGAGATTTTGTAT  2389
Col-0_2.8_k...lot_Lab_GUS      TAATGCCAAATTTGATGATCTTCAACTCGTTAAAAAAACCAAACCATAGAGATTTTGTAT  2051
Col-0_2.6_kbp_Wang_2017        TAATGCCAAATTTGATGATCTTCAACTCGTTAAAAAAACCAAACCATAGAGATTTTGTAT  1725
C24_2.1_kbp_Hirner_1998        TAATACCAAATTTGATGATCTTCAACTCGTTAAAAAAACCAAACCATAGAGATTTTGTAT  1225

Col-0_Reference_3.5_kbp        ATCCAATTTAGGGAATTCATTAGAGTTCAAGAAGTCTTCATAATTAGGTTGCATCTTTGA  2700
Col-0_..._Lab_3.25_kbp_GG      ATCCAATTTAGGGAATTCATTAGAGTTCAAGAAGTCTTGATAATTAGGTTGCATCTTTGA  2449
Col-0_2.8_k...lot_Lab_GUS      ATCCAATTTAGGGAATTCATTAGAGTTCAAGAAGTCTTCATAATTAGGTTGCATCTTTGA  2111
Col-0_2.6_kbp_Wang_2017        ATCCAATTTAGGGAATTCATTAGAGTTCAAGAAGTCTTCATAATTAGGTTGCATCTTTGA  1785
C24_2.1_kbp_Hirner_1998        ATCCAATTTAGGGAATTCATTAGAGTTCAAGAAGTCTTCATAATTAGGTTGCATCTTTGA  1285
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Col-0_Reference_3.5_kbp       ATACCTTTTTCTCATTTAGGCATAACAATATAATAATTTGTTTTTTGTTTTCATTTTCTT  2760
Col-0_..._Lab_3.25_kbp_GG     ATACCTTTTTCTCATTTAGGCATAACAATATAATAATTTGTTTTTTGTTTTCATTTTCTT  2509
Col-0_2.8_k...lot_Lab_GUS     ATACCTTTTTCTCATTTAGGCATAACAATATAATAATTTGTTTTTTGTTTTCATTTTCTT  2171
Col-0_2.6_kbp_Wang_2017       ATACCTTTTTCTCATTTAGGCATAACAATATAATAATTTGTTTTTTGTTTTCATTTTCTT  1845
C24_2.1_kbp_Hirner_1998       ATACCTTTTTCTCATTTAGGCATAACAATATAATAATTTGTTTTTTGTTTTCATTTTCTT  1345

Col-0_Reference_3.5_kbp       TTGGTGTCATCTTCAAAAATCTGTAAACCCAAAAGTTTGTATAACTTGTTTATTAAGATA  2820
Col-0_..._Lab_3.25_kbp_GG     TTGGTGTCATCTTCAAAAATCTGTAAACCCAAAAGTTTGTATAACTTGTTTATTAAGATA  2569
Col-0_2.8_k...lot_Lab_GUS     TTGGTGTCATCTTCAAAAATCTGTAAACCCAAAAGTTTGTATAACTTGTTTATTAAGATA  2231
Col-0_2.6_kbp_Wang_2017       TTGGTGTCATCTTCAAAAATCTGTAAACCCAAAAGTTTGTATAACTTGTTTATTAAGATA  1905
C24_2.1_kbp_Hirner_1998       TTGGTGTCATCTTCAAAAATCTGTAAACCCAAAAGTTTGTATAACTTGTTTATTAAGATA  1405

Col-0_Reference_3.5_kbp       TTTTTAATTAAATTTTTTTTTTTTGACATTTTTAAAAAATTATAAAGTGTTTTATGAATTT  2880
Col-0_..._Lab_3.25_kbp_GG     TTTTTAATTAAATTTTTTTTTTTTGACATTTTTAAAAAATTATAAAGTGTTTTATGAATTT  2629
Col-0_2.8_k...lot_Lab_GUS     TTTTTAATTAAATTTTTTTTTTTTGACATTTTTAAAAAATTATAAAGTGTTTTATGAATTT  2291
Col-0_2.6_kbp_Wang_2017       TTTTTAATTAAATTTTTTTTTTTTGACATTTTTAAAAAATTATAAAGTGTTTTATGAATTT  1965
C24_2.1_kbp_Hirner_1998       TTTTTAATTAAATTTTTTATTTTGACATTTTAAAAAATTATAAAGTGTTTTATGAATTT  1465

Col-0_Reference_3.5_kbp       AAGGAGTAAATAATATTTATTTAGAACACTATAAATTAGTTTTACAAGTTCTTAGAAATG  2940
Col-0_..._Lab_3.25_kbp_GG     AAGGAGTAAATAATATTTATTTAGAACACTATAAATTAGTTTTACAAGTTCTTAGAAATG  2689
Col-0_2.8_k...lot_Lab_GUS     AAGGAGTAAATAATATTTATTTAGAACACTATAAATTAGTTTTACAAGTTCTTAGAAATG  2351
Col-0_2.6_kbp_Wang_2017       AAGGAGTAAATAATATTTATTTAGAACACTATAAATTAGTTTTACAAGTTCTTAGAAATG  2025
C24_2.1_kbp_Hirner_1998       AGGGAGTAAATAATATTTATTTAGAACACTATAAATTAGTTTTACAAGTTCTTAGAAATG  1525

Col-0_Reference_3.5_kbp       TATCTGTAAATTTCAAAAAGGAAAAATATAGCATTTAATTTTGAAGATTTTTTTCTACAT  3000
Col-0_..._Lab_3.25_kbp_GG     TATCTGTAAATTTCAAAAAGGAAAAATATAGCATTTAATTTTGAAGATTTTTTTCTACAT  2749
Col-0_2.8_k...lot_Lab_GUS     TATCTGTAAATTTCAAAAAGGAAAAATATAGCATTTAATTTTGAAGATTTTTTTCTACAT  2411
Col-0_2.6_kbp_Wang_2017       TATCTGTAAATTTCAAAAAGGAAAAATATAGCATTTAATTTTGAAGATTTTTTTCTACAT  2085
C24_2.1_kbp_Hirner_1998       TATCTGTAAATTTCAAAAAGGAAAAATATAGCATTTAATTTTGAAGATTTTTTTCTACAT  1585

Col-0_Reference_3.5_kbp       TATATATATGATAAAAATATTGTATTTTGTACTTTGTAGTTACAAAAAGTCATTATATCA  3060
Col-0_..._Lab_3.25_kbp_GG     TATATATATGATAAAAATATTGTATTTTGTACTTTGTAGTTACAAAAAGTCATTATATCA  2809
Col-0_2.8_k...lot_Lab_GUS     TATATATATGATAAAAATATTGTATTTTGTACTTTGTAGTTACAAAAAGTCATTATATCA  2471
Col-0_2.6_kbp_Wang_2017       TATATATATGATAAAAATATTGTATTTTGTACTTTGTAGTTACAAAAAGTCATTATATCA  2145
C24_2.1_kbp_Hirner_1998       TATATATATGATAAAAATATTGTATTTTGTACTTTGTAGTTACAAAAAGTCATTATATCA  1645

Col-0_Reference_3.5_kbp       ACAAATCTAAATATAAAATATTTTTCTATATATTACTCCAAATTAACTGTCAGAATAAAA  3120
Col-0_..._Lab_3.25_kbp_GG     ACAAATCTAAATATAAAATATTTTTCTATATATTACTCCAAATTAACTGTCAGAATAAAA  2869
Col-0_2.8_k...lot_Lab_GUS     ACAAATCTAAATATAAAATATTTTTCTATATATTACTCCAAATTAACTGTCAGAATAAAA  2531
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Col-0_2.6_kbp_Wang_2017        ACAAATCTAAATATAAAATATTTTTCTATATATTACTCCAAATTAACTGTCAGAATAAAA  2205
C24_2.1_kbp_Hirner_1998        ACAAATCTAAATATAAAATATTTTTCTATATATTACTCCAAATTAACTGTCAGAATAAAA  1705

Col-0_Reference_3.5_kbp        AAGAAGAATAATTATTACAGAATCTGAACATTAAAATCGTCCCTCCATATGTGGTCTCTG  3180
Col-0_..._Lab_3.25_kbp_GG      AAGAAGAATAATTATTACAGAATCTGAACATTAAAATCGTCCCTCCATATGTTGTCTCTG  2929
Col-0_2.8_k...lot_Lab_GUS      AAGAAGAATAATTATTACAGAATCTGAACATTAAAATCGTCCCTCCATATGTGGTCTCTG  2591
Col-0_2.6_kbp_Wang_2017        AAGAAGAATAATTATTACAGAATCTGAACATTAAAATCGTCCCTCCATATGTGGTCTCTG  2265
C24_2.1_kbp_Hirner_1998        AAGAAGAATAATTATTACAGAATCTGAACATTAAAATCGTCCCTCCATATGTGGTCTCTG  1765

Col-0_Reference_3.5_kbp        TCTAGTCCAAAAGCAATTTACACATCCCAAGCCGAAACTATATTAAATAAACATTTTTTT  3240
Col-0_..._Lab_3.25_kbp_GG      TCTAGTCCAAAAGCAATTTACACATCCCAAGCCGAAACTATATTAAATAAACATTTTTTT  2989
Col-0_2.8_k...lot_Lab_GUS      TCTAGTCCAAAAGCAATTTACACATCCCAAGCCGAAACTATATTAAATAAACATTTTTTT  2651
Col-0_2.6_kbp_Wang_2017        TCTAGTCCAAAAGCAATTTACACATCCCAAGCCGAAACTATATTAAATAAACATTTTTTT  2325
C24_2.1_kbp_Hirner_1998        TCTAGTCCAAAAGCAATTTACACATCCCAAGCCGAAACTATATTAAATAAACATTTTTTT  1825

Col-0_Reference_3.5_kbp        TTCTTTAACTAAAACATTTATAACATTTAACAATAAAAGTTAAAAATCGAACACGTATAA  3300
Col-0_..._Lab_3.25_kbp_GG      TTCTTTAACTAAAACATTTATAACATTTAACAATAAAAGTTAAAAATCGAACACGTATAA  3049
Col-0_2.8_k...lot_Lab_GUS      TTCTTTAACTAAAACATTTATAACATTTAACAATAAAAGTTAAAAATCGAACACGTATAA  2711
Col-0_2.6_kbp_Wang_2017        TTCTTTAACTAAAACATTTATAACATTTAACAATAAAAGTTAAAAATCGAACACGTATAA  2385
C24_2.1_kbp_Hirner_1998        TTCTTTAACTAAAACATTTATAACATTTAACAATAAAAGTTAAAAATCGAACACGTATAA  1885

Col-0_Reference_3.5_kbp        CGTATTTTTTTACGTATACGTCTTGTTGGCATATATGCTTAAAAACTTCATTACATACAT  3360
Col-0_..._Lab_3.25_kbp_GG      CGTATTTTTTTACGTATACGTCTTGTTGGCATATATGCTTAAAAACTTCATTACATACAT  3109
Col-0_2.8_k...lot_Lab_GUS      CGTATTTTTTTACGTATACGTCTTGTTGGCATATATGCTTAAAAACTTCATTACATACAT  2771
Col-0_2.6_kbp_Wang_2017        CGTATTTTTTTACGTATACGTCTTGTTGGCATATATGCTTAAAAACTTCATTACATACAT  2445
C24_2.1_kbp_Hirner_1998        CGTATTTTTTTACGTATACGTCTTGTTGGCATATATGCTTAAAAACTTCATTACATACAT  1945

Col-0_Reference_3.5_kbp        ATACAAGTATGTCTATATATATGATATTATGCAAACACAAATCTGTTGACTATAATTAGA  3420
Col-0_..._Lab_3.25_kbp_GG      ATACAAGTATGTCTATATATATGATATTATGCAAACACAAATCTGTTGACTATAATTAGA  3169
Col-0_2.8_k...lot_Lab_GUS      ATACAAGTATGTCTATATATATGATATTATGCAAACACAAATCTGTTGACTATAATTAGA  2831
Col-0_2.6_kbp_Wang_2017        ATACAAGTATGTCTATATATATGATATTATGCAAACACAAATCTGTTGACTATAATTAGA  2505
C24_2.1_kbp_Hirner_1998        ATACAAGTATGTCTATATATATGATATTATGCAAACACAAATCTGTTGACTATAATTAGA  2005

Col-0_Reference_3.5_kbp        CTTCTTCATTTACTCTCTCTCTGACTTAAAACATTTATTTTATCTTCTTCTTGTTCTCTC  3480
Col-0_..._Lab_3.25_kbp_GG      CTTCTTCATTTACTCTCTCTCTGACTTAAAACATTTATTTTATCTTCTTCTTGTTCTCTC  3229
Col-0_2.8_k...lot_Lab_GUS      CTTCTTCATTTACTCTCTCTCTGACTTAAAACATTTATTTTATCTTCTTCTTGTTCTCTC  2891
Col-0_2.6_kbp_Wang_2017        CTTCTTCATTTACTCTCTCTCTGACTTAAAACATTTATTTTATCTTCTTCTTGTTCTCTC  2565
C24_2.1_kbp_Hirner_1998        CTTCTTCATTTACTCTCTCTCTGACTTAAAACATTTATTTTATCTTCTTCTTGTTCTCTC  2065
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Col-0_Reference_3.5_kbp          TTTCTCTTTCTCTCATCACT  3500
Col-0_..._Lab_3.25_kbp_GG        TTTCTCTTTCTCTCATCACT  3249
Col-0_2.8_k...lot_Lab_GUS        TTTCTCTTTCTCTCATCACT  2911
Col-0_2.6_kbp_Wang_2017          TTTCTCTTTCTCTCATCACT  2585
C24_2.1_kbp_Hirner_1998          TTTCTCTTTCTCTCATCACT  2085
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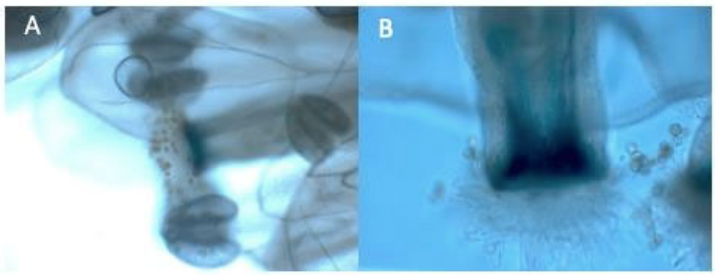
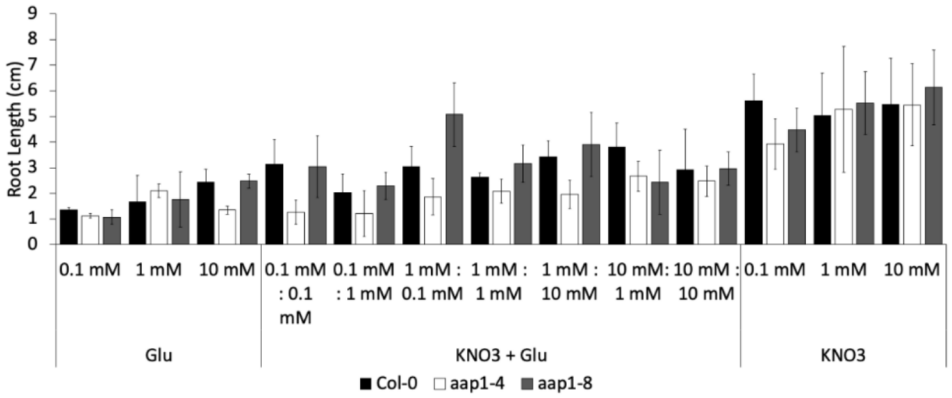
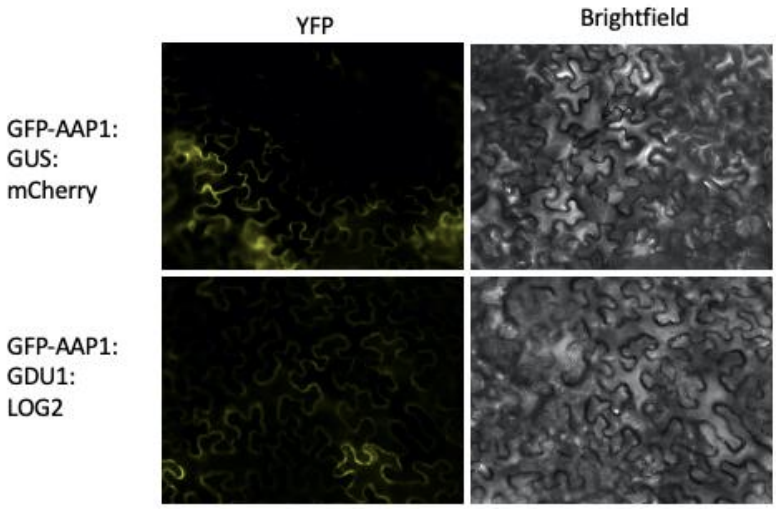
Supplemental Figure 1. Alignment of AAP1 Promoters from Different Labs used to Characterize AAP1 Expression. Highlighted residues represent deviation from the Col-0 reference sequence (3.5 kbp) upstream from the ATG. Yellow indicates naturally occurring mutations in the C24 sequence. Pink indicates introduced mutations as part of the GoldenGate domestication to remove Bsa I and Bpi I sites. Sequences were aligned with ClustalW with a 15-point opening and 6.66-point extension penalty.



Supplemental Figure 2. Histochemical staining of 6-wk-old Col-0 plants expressing *AAP1p:GUS*.
A) Open Flower, B) Pistil, early silique



Supplemental Figure 3. Root length (cm) of 10-day old wild-type, *aap1-4* and *aap1-8* plants grown on various concentrations of KNO_3 and Glu.



Supplemental Figure 4. Co-expression of AAP1 and LOG2/ GDU1 in *N. benthamiana*. YFP:AAP1 was co-expressed with mCherry fused to both GDU1 and LOG2. GUS:mCherry was used as a control to show that GFP:AAP1 is expressed (not shown).

Table 1. Oligonucleotides used for Cloning, qRT-PCR

AAP1 m S16D	TTCAACACAGAAGGACACAACCACTCCACGGCGGAAGATGGCGATGCCTAC
AAP1 m S16A	TTCAACACAGAAGGACACAACCACTCCACGGCGGAAGCCGGCGATGCCTAC
AAP1 m S3D GG Bpi f	TTGGTCTCAACATAATGAAGGATTTCAACACAGAAGGACACACCA
AAP1 m S3A GG Bpi f	TTGGTCTCAACATAATGAAGGCTTTCAACACAGAAGGACACACCA
AAP1 Q r new	ATCCGCAGTGTGCCCTT
NRT2.1 Q r	TTGTAACGGCATACCACAGAAT
NRT2.1 Q f	ATGGGAATCTTGGTGCTCAA
NRT1.1 Q f	TGCTATATTCGCCGCAATC
NRT1.1 Q r	GTGAGACGACGTTGTTGGATT
AAP5 Q f	ACTGGAGTCACTGTTGGGACA
AAP5 Q r	GCGTTATCCCCAAATGCT
AAP4 Q f	GACCAGATTTGGTGGCTCTC
AAP4 Q r	AGTGCTTGAAAGGTTCTCCAT
AAP8 Q f	TCCATACATGGCGGCATT
AAP8 Q r	TTCAGACGCAGTTACGTCCA
pGG0 lacZ GW attB2	GACCACTTTGTACAAGAAAGCTGGGTACTGGTCTCCCGAATTGT
pGG0 lacZ GW attB1	GACAAGTTTGTACAAAAAAGCAGGCTCAGGGTCTCAAATGTTGT
Xho GG term Ar	AGCGTTCCGATCTCGAGCTATTG
Xho GG term Af	GCTTCAATAGCTCGAGATCGGAA
Kpn GG P5UN Ar	CATTGTTAGGGTACCTTCCAGT
Kpn GG P5UN Af	GGAGACTGGAAGGTACCCTAAC
Mut AAP1t +3940 Bpi r new	TTGAAGACATCTTGTCTCAAAATAGTTATAATATATAGAAGTG
NRT2.2 Q r	ACATCATCATGGGAATCTTGGT
NRT2.2 Q f	ATTTGTAACGGCGTACCATAGA

TBSv26 BpiI r	TTGAAGACAAAGCGAGTTGTAATGAGTTGCT
Mut TBSv26 +690 f	TTGAAGACTGGCCTCCCCTTGGCT
Mut TBSv26 +690 r	TTGAAGACAGAGGCCAAAGTGTGCAGGCTGTTGA
TBSv26 BpiI f	TTGAAGACAAGGAGTTCCTAACACCTGGAGA
Mut AAP1t +3940 Bpi r	TTGAAGACATCTTGTCTCAAAATAGTTATAAT
Mut AAP1t +3940 Bpi f	TTGAAGACAACAAGATTTAACTGAAAACAAGCC
AAP1t GG Bpi r	TTGAAGACAAAGCGCGGGAAAATCACGGGTTTAC
AAP1t GG Bpi f	TTGAAGACAAGCTTGTTTGAGATCCTCAAGAGAGTCA
Xho GG term f	GGAGACTGGAAGGTACC
Xho GG term r	ATTGGTACCTTCCAGT
Kpn GG P5UN f	GCTTCTCGAGATCGGAA
Kpn GG P5UN r	AGCGTTCCGATCTCGAG
AKT2p attB2	GACCACTTTGTACAAGAAAGCTGGGTAGTCTGAGGATAAGTTGCA
AKT2p attB1	GACAAGTTTGTACAAAAAAGCAGGCTCAGGCTTCTAACCGTCTTTTGTG
CoYMVp attB2	GACCACTTTGTACAAGAAAGCTGGGTACTTGTTGTGTTGGTTTTCTAAGC
CoYMVp attB1	GACAAGTTTGTACAAAAAAGCAGGCTCACGATTTCTTAGGGGCTTCTCTC
SUC2p attB2	GACCACTTTGTACAAGAAAGCTGGGTAATTTGACAAACCAAGAAAGTAAGA
SUC2p attB1	GACAAGTTTGTACAAAAAAGCAGGCTCAGTTACTTTCTATTATTAACTGTTATAAT
Dummy C r	AAGCCTACTCAGTTA
Dummy C f	TTCGTAACTGAGTAG
Dummy N r	CATTTTTGTTT
Dummy N f	CCATAAACAAA
AAP1g GG Hind Bsa r	TTGGTCTCAACAACGAAGCTKACTCATGCATAGTCCGGAAGG
Mut AAP1g +3600 Bsa f	TTGGTCTCTCGTGTCGCTCTTAGCTGC
Mut AAP1g +3600 Bsa r	TTGGTCTCACACGATCAAGCAAACATAG

Mut AAP1g +2850 Bsa f	TTGGTCTCAACATTGATCTCTGACCCAAGGAA
Mut AAP1g +2850 Bsa r	TTGGTCTCAACAAATCAGAACTAAGTTAGTCCCCTC
AAP1g +1550 Bsa f	TTGGTCTCAACATACAATATGGGAATCTGATTGG
AAP1g +1550 Bsa r	TTGGTCTCAACAATTGTGCCACTCCACAGAGCT
AAP1g GG Bpi f	TTGGTCTCAACATAATGAAGAGTTTCAACACAGAA
AAP1p GG Bpi r	TTGAAGACAAATGGAGTGATGAGAGAAAGAGAAAGAG
Mut AAP1p -325 Bpi f	TTGAAGACGTTGTCTCTGTCTAGTCCAAAAG
Mut AAP1p -325 Bpi r	TTGAAGACGAGACAACATATGGAGGGAC
Mut AAP1p -825 Bpi f	TTGAAGACGTCTTGATAATTAGGTTGCATCT
Mut AAP1p -825 Bpi r	TTGAAGACATCAAGACTTCTTGAACTCTAATG
Mut AAP1p -945 Bpi f	TTGAAGACCTTTGATTGTCCATAATTTCTAA
Mut AAP1p -945 Bpi r	TTGAAGACAATCAAAGACGACAAATAAAAA
AAP1p GG Bpi f	TTGAAGACAAGGAGTCAATGAAAACCATGTGAATATG
PMEIp attB2	GACCACTTTGTACAAGAAAGCTGGGTAGCTGTTTTTCTTGAGGTTGAGAG
PMEIp attB1	GACAAGTTTGTACAAAAAAGCAGGCTCAGTCCCTTTTCACTTATCTCGAAT
AGP30p attB2	GACCACTTTGTACAAGAAAGCTGGGTAGTGTGACTTTGGGTTGATGTGA
AGP30p attB1	GACAAGTTTGTACAAAAAAGCAGGCTCACGTATTGTGGATGCCGTCG
TMO7p attB2	GACCACTTTGTACAAGAAAGCTGGGTAACAGAGATAGAAGCAAAGGCACT
TMO7p attB1	GACAAGTTTGTACAAAAAAGCAGGCTCATCGCTACTTTTCAACATACAAGGA