Characterization of the amino acid transporter AAP1 in *Arabidopsis thaliana* – an exploration of AAP1's role in plant amino acid metabolism

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ABSTRACT

Amino acids are essential molecules in plant metabolism. Amino acids carry reduced nitrogen while serving as precursors for protein synthesis and secondary metabolites. Translocation of amino acids in the cell is mediated by amino acid transporters. While about 100 transporters have been identified, only a dozen have been fully characterized. The regulation of amino acid transporters is not fully understood and stands as the basis of this study. Previous toxicity-based screenings of *Arabidopsis thaliana* mutants led to the isolation of a loss-of-function line and the phenylalanine insensitive growth (*pig1*) mutant capable of growth on toxic concentrations of phenylalanine. The *pig1-1* mutants also displayed a deregulated metabolism (1). We followed this work with a similar forward genetic screening of *Arabidopsis thaliana* that led to the identification of 18 mutants capable of growth in the presence of amino acids at toxic concentrations. From this screen, seven mutations were confirmed to affect the amino acid transporter AAP1. Here I demonstrate that, when expressed in yeast deficient for endogenous amino acid transporters, three variant *aap1* proteins restored growth similar to yeast complemented by wild type AAP1. Transport of radiolabeled Pro was abolished by variant *aap1* proteins while deletion of an intracellular loop spanning the 8th and 9th transmembrane domains reduced Pro transport in yeast. Site directed mutagenesis of this loop conferred a variant *aap1* protein which augmented Pro transport in yeast. Amino acid transport in loss-of-function *aap1* plants display decreased uptake and increased efflux. In addition, *aap1* mutant plants accumulated between 2 and 8 times more free amino acids in the leaves than the wild type. These observations are not fully compatible with the accepted role of AAP1 in transport by the root. The present work describes how the amino acid transporter AAP1 could play a role in regulating amino acid metabolism. We hypothesize that the amino acid transporter AAP1 functions as a senor that is involved in amino acid homeostasis in addition to its established role as a transporter. Is true, this would make AAP1 the first identified amino acid sensor in plants. Knowledge of the mechanism of amino acid sensing would enable us to engineer crops for improved nutrition in a more efficient way than affecting metabolic enzymes.

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GENERAL ABSTRACT

Amino acids play essential role in crop metabolism. Amino acids are nitrogen containing molecules that are used to make protein and many other molecules. They are located through-out the plant and move from organ to organ by amino acid transporters. A dozen of approximately 100 known amino acid transporters have been studied in depth and are well understood. Interestingly, not much is known about these transporters and what controls their activity. A mutant weed, *Arabidopsis thaliana* mutant phenylalanine insensitive growth (*pig1*), was identified by its ability to survive in toxic environments with high amounts of the amino acid phenylalanine and also showed an irregular metabolism of amino acids (1). The Pilot Lab and I were able to identify 18 more mutants with similar abilities to survive in toxic amino acid conditions by performing similar experiments. Seven of the new mutants were found to have mutations that effected the amino acid transporters AAP1. Using yeast incapable of growing in nitrogen restricted conditions where amino acids are the only source of nitrogen, I found that three of the variants app1 proteins we identified were able to restore growth like wild type AAP1 yeast. These variant aap1 yeast did not show the ability to transport the acid proline, while other alter versions of the aap1 protein made to alter its structure and proposed significant parts were able to increase proline transport. Plants with no or mutant aap1 proteins showed a decreased ability to uptake amino acids in addition to increased efflux of amino acids. These plants also had a higher level of amino acids in their leaves than normal wild type plants. These results obtained in both plants and yeast with altered amino acid transporter aap1 do not agree with what we understand to be the accepted function of AAP1 transporting amino acids in plant roots. The work presented in this thesis discusses how AAP1 could be involved with controlling plant amino acid metabolism. It is my hypothesis that the transporter is serving two functions by both transporting and sensing amino acids. As a sensor, AAP1 serves to maintain a proper balance of amino acids for plant metabolism. If AAP1 does this, it would be the first of its kind to be identified in plants and help enhance crop engineering for better nutrition to better feed growing populations

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Table 1. Plant Mutant Lines in Plant Assay

Chapter One: Review

Nutrient Sensing, Transport, and Signaling

Amino acids are an essential organic molecule in plant metabolism. These molecules serve as the primary units to build proteins, are precursors to secondary metabolites, and carry organic nitrogen between plant organs. Amino acid transporters mediate the transport of amino acids across membranes and are thus involved in transport of amino acids in the cell and translocation between organs. There are over 100 identified amino acid transporters in Arabidopsis. The majority of these transporters are characterized with import function – transporting amino acids towards the cytosol. Transport can occur in an active process, requiring energy from a proton electrochemical gradient, or a passive process, through passive diffusion of the amino acids. Amino acid metabolism is regulated by both feed-back inhibition and transcriptional regulation. Most amino acid metabolic regulation is regulated though feed-back mechanisms where amino acid accumulation provides negative feedback to synthesis pathways. The enzymes synthesizing amino acids have been identified and their pathways are understood. Transcriptional regulation of the enzymes responsible for amino acid synthesis are dependent on amino acid sensing mechanisms. This process is not well understood. In stressful environments, plants accumulate amino acids at varying concentrations and the expression of enzymes responsible for synthesizing amino acids are deregulated. Amino acid sensing mechanisms have not been identified in plants. There are several hypotheses being explored pertaining to a mechanism for amino acid sensing. A recent review by Forde and Gent has summarized what is understood about amino acid sensing in plants. (2) The sensing mechanisms highlighted by Gent and Forde are dependent on several different proteins. These suggested potential mechanisms involve glutamate receptors in the root, PII proteins in the chloroplast, TOR signaling pathways in plants, GCN2 protein kinase pathways, and glutamate synthetase I like proteins. While many of these efforts have identified effects on amino acid metabolism, no direct sensor for amino acids has been identified. Studying the properties of nutrient sensors identified in yeast and mammals allows for potential amino acid sensing mechanisms to be identified in plants.

Cells gain information about extracellular nutrients from signals relayed by membrane receptor proteins. Receptor proteins sense metabolites and solutes after a solute interacts with the binding site of that receptor. Upon interaction, the receptor protein undergoes a conformational change which initiates a series of phosphorylation events or interactions with soluble cytosolic proteins leading to altered gene expression and regulation. Study of the sensor proteins such as the *Saccharomyces cerevisiae* amino acid transporter Sulfonylurea Sensitive on YPD 1 (Ssy1) and the human Sodium Glucose cotransporter hSGLT3 glucose sensor have elucidated the function of nutrient receptors. The Ssy1 Sensor acts as a unit of the SPS (Ssy1-Ptr3-Ssy5) amino acid sensing complex in yeast. Once an amino acid is bound to Ssy1, the receptor protein will change conformation which leads to phosphorylation of the Peptide Transport 3 (Ptr3) protein (3). This phosphorylation event continues with phosphorylation of Ssy5 and subsequent interactions with intracellular proteins. Altering this complex has led to changes in gene expression and cell growth phenotypes – demonstrating the importance of receptor proteins for cell growth and function (4).

Transporter proteins reside in the cell membrane, mediating transport of metabolites across membranes. Most transporters toggle between two functional conformations – an inward facing and an outward facing confirmation. In the case of importers, interactions between the binding site and metabolite initiate transport in the outward facing confirmation. After binding, the transporter shifts confirmations to the inward facing structural form which releases the metabolite on the opposite side of the membrane. Transporter and receptor proteins are very similar structurally, but fulfill two distinct roles for the cell. The hSGLT3 receptor protein is a member of a glucose transporter protein family, but

lacks the ability to transport glucose (5). Interestingly, structural analysis of hSGLT1 and hSGLT3 revealed the residue Q457 in hSGLT1, important for its transport function, is substituted with Glu at this position in hSGLT3 (6). Mutagenizing E457 to Gln in hSGLT3 rendered the protein able to transport glucose, similarly to hSGLT1, suggesting that while functioning solely as a sensor, hSGLT3 has not completely lost transport ability (7).

Structural similarities, like those in the hSGLT family, between many receptors and transporters suggest that receptors have emerged from transporters by losing transport function and gaining signal transduction function through intracellular interactions. This gain of function has given cells the ability to react to nutrient availability. Some proteins behave as a hybrid between these distinct classes of proteins, which have sensing/signaling functions and are still able to transport metabolites. This class of membrane proteins are called transceptors. A transceptor protein usually functions like a transporter protein – shifting between and outward and inward facing confirmation following the binding interaction between a metabolite and the protein binding site. In the process of a conformational shift, the transceptor initiates cytosolic signaling events within the cell similar to receptor protein (8). Two pathways which have been shown to be regulated by transceptor activity are the Protein Kinase A (PKA) pathway in yeast and the Target of Rapamycin (9) in yeast and mammals – two nutrient sensing pathways important for cell growth and metabolic regulation. Transceptors affecting the PKA pathway have been identified for macronutrients and micronutrients including amino acids (Gap1)(8, 10, 11), ammonium (12, 13), iron (Ftr1)(14), phosphate (15), sulfate (Sul1, Sul2)(16), and zinc (Zrt1)(14). The diversity of nutrients regulating this pathway allude to the complexity of regulating cell growth and metabolism.

Arabidopsis thaliana Amino Acid Permease 1

Before the availability of genome sequences, early plant amino acid transporters were identified by screening for growth complementation of yeast strains deficient for amino acid transport by plant genes. Two groups separately reported the isolation of an amino acid transporter by such an assay: a neutral transport system (called Neutral Amino Acid Transporter 2 - NAT2) using a His transport deficient yeast strain (17); and the Amino Acid Permease 1 (AAP1) using a Pro deficient transport yeast strain (18). Sequence analysis displayed that NAT2 and AAP1 are the same gene, and the AAP1 name was kept in subsequent literature. AAP1 is the first of 8 amino acid transporters in the AAP family in Arabidopsis. The cDNA of AAP1 is 1.7 kB long (17) with six exons and five introns (19) coding for a 486 amino acid, 52.9 kDa protein (17, 18). Biochemical analysis confirmed that AAP1 contains 11 transmembrane domains (17, 20, 21) with a cytosolic N-terminus and extracellular C-terminus (21). Transport analyses in presence of protonophores and different outside pH have revealed that AAP1 is a proton-amino acid symporter (18, 22), transporting both amino acids and protons simultaneously with more than one binding site for both protons and amino acids (23). AAP1 is specific to amino acids, and displays a broad specificity, since it is able to transport many amino acids (18). It favors neutral amino acids, especially those with short, non-aromatic side chains (20, 23, 24). Substrate recognition and transport by AAP1 is stereospecific and sensitive to the proximity of the amino and carboxyl groups (18, 25) in addition to the position of the alpha carbon of the amino acid (24). Further studies utilizing mutagenesis techniques have identified His47 and His337 as required for transport (26), and an extracellular loop between the fifth and sixth transmembrane domains required for substrate recognition (21).

AAP1 is characteristically expressed in the vascular system and cotyledons of developing seedlings (19, 20, 27, 28) and in the root tip, root hairs and root epidermal cells (27, 29). In vivo, AAP1 functions as an amino acid importer in roots to supply plants with organic nitrogen from the soil (29, 30) and is critical for supplying amino acids to developing embryo (31)**.**

Preliminary Work with AAP1

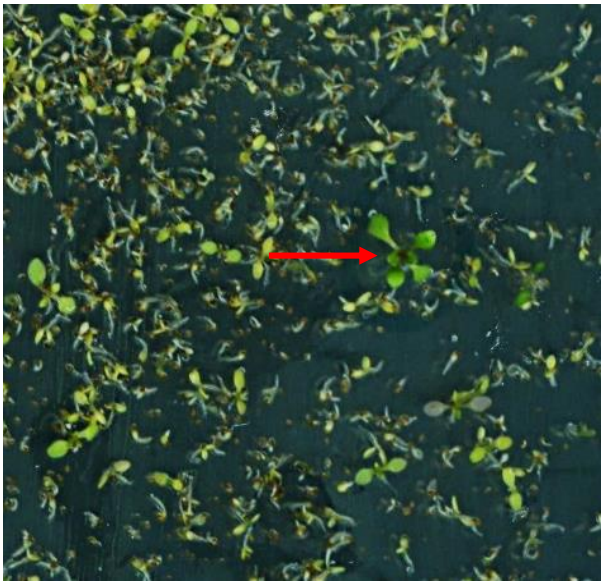
Work in the Pilot Lab has focused on identifying novel regulators of amino acid metabolism. To identify genes which function to regulate amino acid pathways, the Pilot Lab designed a tolerance assay screening using high concentrations of feed-back competent amino acids which alter feed-back regulated enzymes and thus amino acid homeostasis. *Arabidopsis thaliana* mutants were generated using ethyl-methane sulfonate (EMS) mutagenesis. Initial screening on arginine+valine and phenylalanine+valine lead to the isolation of 112 mutant plants capable of tolerating these toxic conditions (Fig. 1A and B). These mutants were confirmed using an extended set of amino acids and tested for their ability to tolerate amino acids more broadly. Those which grew best on the most diverse array of amino acids – 23 lines – were then selected for genetic mapping to identify the genes that were affected during mutagenesis that contribute to amino acid resistance.

The loci of genes affected in 12 of the mutants were predicted after genetic mapping – 4 mutants affected in Valine Tolerant 1 (VAT1), 1 mutant affected in Amino Acid Permease 5 (AAP5), and 7 lines affected in AAP1. The mapping approach could not resolve the affected loci of the remaining lines. To confirm the identity of the proposed mutations of these 12 mutant, the genomic DNA of each line was extracted. The proposed affected genes were amplified using PCR and sequenced for mutation in their respective proposed genes. The predicted mutations affecting VAT1, AAP5 and AAP1 were confirmed. Mutations for AAP1 corresponded to 4 missense mutations (S87F, G212D, G251E, A417T) and 3 nonsense mutations (Q241X, Q351X, W445X) (Figure 2). Mutants affecting AAP1 became the most interesting and significant set based on the number of mutants obtained from the screening. In addition, missense mutations are a unique opportunity to study the structure-function relationships of the protein. To test the function of each variant AAP1 protein, RNA was extracted, complementary DNA was synthesized by reverse transcription, and each of the coding sequences (CDS) were cloned into yeast expression vectors pDR196-WS and pDR-WY.

The work detailed in my Master's Thesis is the continuation of previous work in the Pilot Lab. Here I detail my findings with intent to (1) characterize the effect of mutations on AAP1 transport, (2) understand the effect these mutations had on AAP1's function in plants, and (3) gain insight to the activity and regulation of amino acid homeostasis.

Figures

A)



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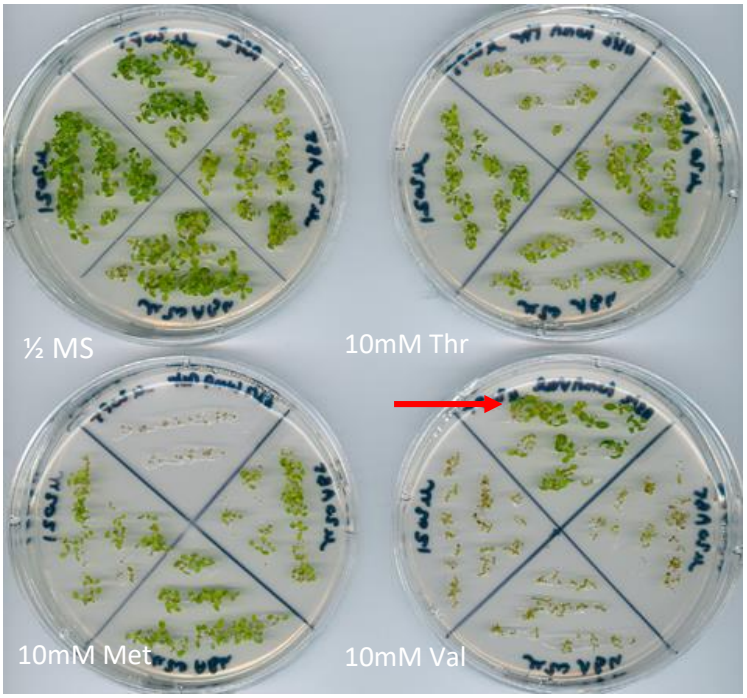


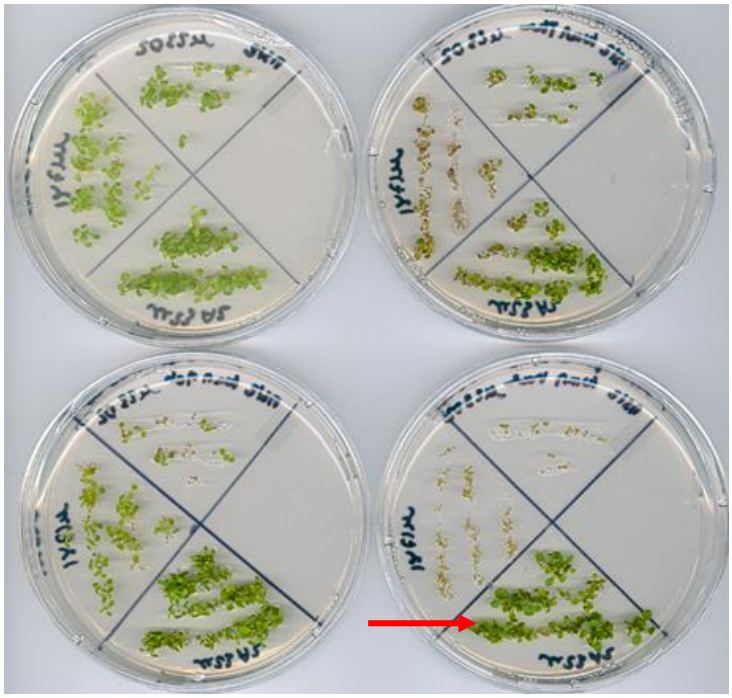


Figure 1. EMS Mutant Forward Genetic Screening. (A) EMS mutagenized seeds were plated on ½ MS media (0.5% Sucrose, 0.7% agar, pH 5.7) with 10mM Phe/Val or 10mM Arg/Val. Plants that displayed tolerance to amino acid conditions (indicated by a red arrow) were selected for additional confirmation resistance screenings. B) Selected mutants from preliminary screenings in (A) were further selected for their ability to tolerate amino acid conditions in conformational screenings using 10mM Thr, 10mM Met, and 10mM Val. Selected tolerant mutants were genetically mapped.

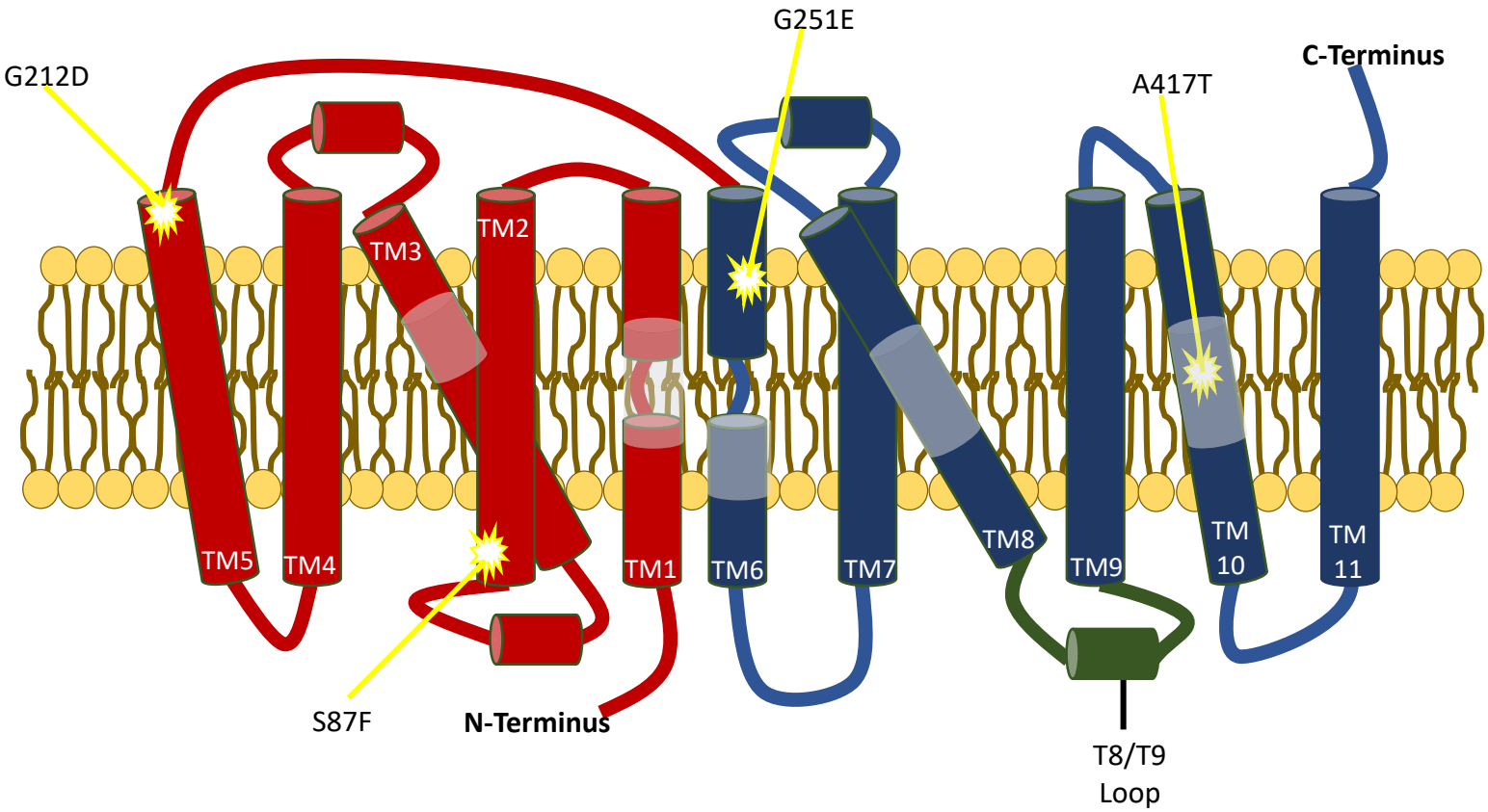


Figure 2. Confirmation of mutations in EMS mutant plants affecting AAP1. Genomic DNA of mutant plants was extracted and sequenced for mutations in proposed genes identified from the genetic mapping. Sequences were analyzed using NCBI Blast against WT sequences from The Arabidopsis Information Resource (TAIR). 3 nonsense and 4 missense mutations were confirmed by sequencing in proposed mutants for AAP1. The loop structure between the 8th and 9th transmembrane, unique to the AAP transporter subfamily, is highlighted in green. Transmembrane domains are numbered in white lettering. Potential amino acid binding sites in the 1st, 3rd, 6th, 8th, and 10th transmembranes are indicated by white highlighting.

Chapter Two: Characterization of AAP1 mutant plants

Results

AAP1 mutants resist toxic amino acids.

The growth of *aap1* mutant plants carrying the A417T, G251E, S87F, Q241X and W244X mutations, the *aap1-2* mutant (a knock out mutant which contains an insertion in the AAP1 gene) (29), *lht1-1* (a knockout mutant with an insertion in the root amino acid transporter) (32), *gdu1-1D* (an activation tagged insertion mutant in the GDU1 gene) (33), and the wild type Col-7 were characterized (Fig. 3). The *lht1-1* and *gdu1-1* mutants served here as controls for the effect of knocking out an amino acid transporter in roots and positive growth tolerance for high amino acid concentrations, respectively (33). Seeds were plated on ½ MS (0.5% sucrose, 0.7% agar) supplemented with toxic concentrations of various amino acids. All plants were capable of growing on ½ MS without amino acid supplementation (Fig. 3a). All but the loss of function mutant W244X were tolerant to 10 mM Glu and 10 mM Gln, used as a positive control, and 10mM Thr. Consistent with previous tests, *gdu1-1D* plants tolerated all tested amino acids (Fig. 3). *aap1-2* and the *aap1* A417T mutants were able to tolerate a broad range of the amino acids tested (Fig. 3b). The *aap1* G251E mutant displayed tolerance to all amino acid conditions, but not to the extent of the *aap1-2* and *aap1*A417T mutants. The *aap1* loss-of-function mutant W244X and *aap1* S87F mutant were weakly tolerant of 10 mM Gly (Fig. 3b). The *lht1-1* mutant did not display the same tolerance to the tested amino acid conditions as *app1* mutants. The *lht1-1* mutant displayed an intolerance to toxic amino acid conditions like the wild type. *aap1* mutants displayed varying tolerance to toxic amino acid conditions while the wild type and *lht1-1* mutants were intolerant of the same toxic amino acid conditions.

Mutations in AAP1 affect both amino acid uptake and efflux.

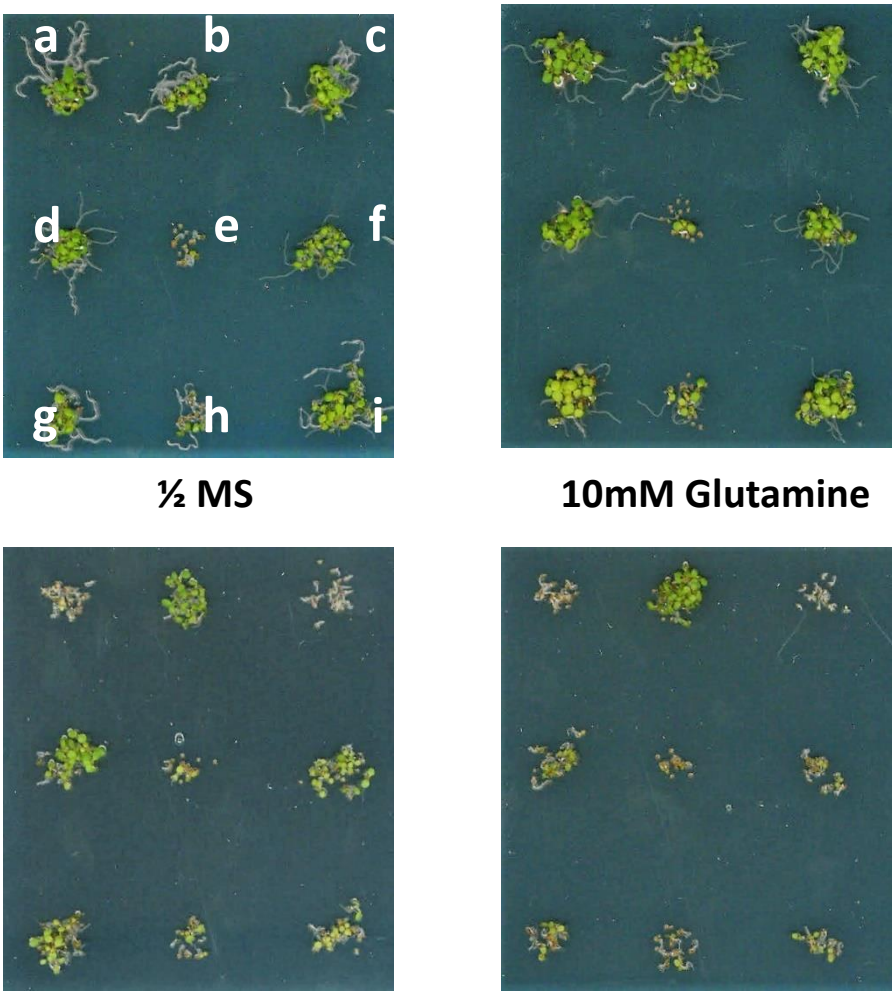
The uptake and efflux of 1mM L-^{3}H-Gln by Col-7, *lht1-1*, *gdu1-1D*, and *aap1* mutants G212D, A417T, G215E *aap1* mutants and *aap1-2* were analyzed (Fig. 4). The results were normalized to the uptake of Col-7. The *gdu1-1D* mutant has been identified by its reduction in amino acid uptake and increased amino acid efflux compared to wild type plants (34). *gdu1-1D* plants showed a reduced uptake (40%) of L-Gln, as expected. *lht1-1* plants were 82% as effective at taking up L-Gln as wild type plants. The *aap1* mutants exhibited a reduction in L-Gln uptake of between 55-78%, with *aap1* A417T and *aap1-2* plants displaying the greatest effect on uptake (55% the uptake of the wild type) (Fig. 4a). *gdu1-1D* plants showed 86% more efflux compared to the wild type, as expected (33). No significant difference was observed between the *lht1-1* and wild type plants with respect to L-Gln efflux. The *aap1* G212D and G251E mutants showed an increased efflux (122% and 110% of the wild type). Both the *aap1* A417T and *aap1-2* exhibited similar increased efflux with 131% and 133% efflux of the wild type (Fig. 4b). All mutants had an effect on amino acid transport over the entire plant. All mutants displayed a reduced uptake in comparison to wild-type plants. The *gdu1-1D* and *aap1* mutants displayed an additional increase in amino acid efflux while the mutant *lht1-1* displayed no effect on amino acid efflux.

Table 1. Plant Mutant Lines in Plant Assays

WT	– WT Control
gdu1-1D	– activation tag insertion in the GDU1 gene; over expression of GDU1, control
lht1-1	– T-DNA KO of the root bound AA transporter LHT1, negative control
aap1 KO	– T-DNA KO of AAP1
AAP1 A417T	– mutation affecting potential binding site
AAP1 G212D	– nonfunctioning fully translated protein
AAP1 G251E	– mutation near potential binding site
AAP1 S87F	– nonfunctioning fully translated protein
AAP1 Q241X	– truncated non-translated protein
AAP1 W244X	– truncated non-translated protein

Figures

A)



B)

	1/2 MS	Lys	Leu	Val	Met	Ile	Ser	Gly	Arg	Glu	Gln	Thr
Col-7												
GDU1 OE												
lht1-1												
aap1 KO Col-0												
AAP1 A417T												
AAP1 G251E												
AAP1 S87F												
AAP1 W244X												

Growth

No growth

Figure 3. AAP1 Amino Acid Tolerance Assays. **A)** AAP1 mutant plated on ½ MS (0.5% sucrose, 0.7% agar, pH 5.7) with high concentrations of amino acids to test tolerance [(a) Col-7, (b) GDU1 OE (c) lht1-1, (d) aap1 KO Col-0, (e) AAP1 Q241X, (f) AAP1 W244X, (g) AAP1 A417T, (h) AAP1 S87F, (i) AAP1 G251E] **B)** Table demonstrating relative growth of mutant plants in comparison to WT plants. Green indicates germination and growth while red represent no germination and no growth. White indicates some germination and some growth. The growth of each line was assessed by visual comparison of the wild type and gdu1-1D control lines. Lysine concentration is 3mM, Alanine concentration is 80mM, and all other amino acid concentrations are 10mM.

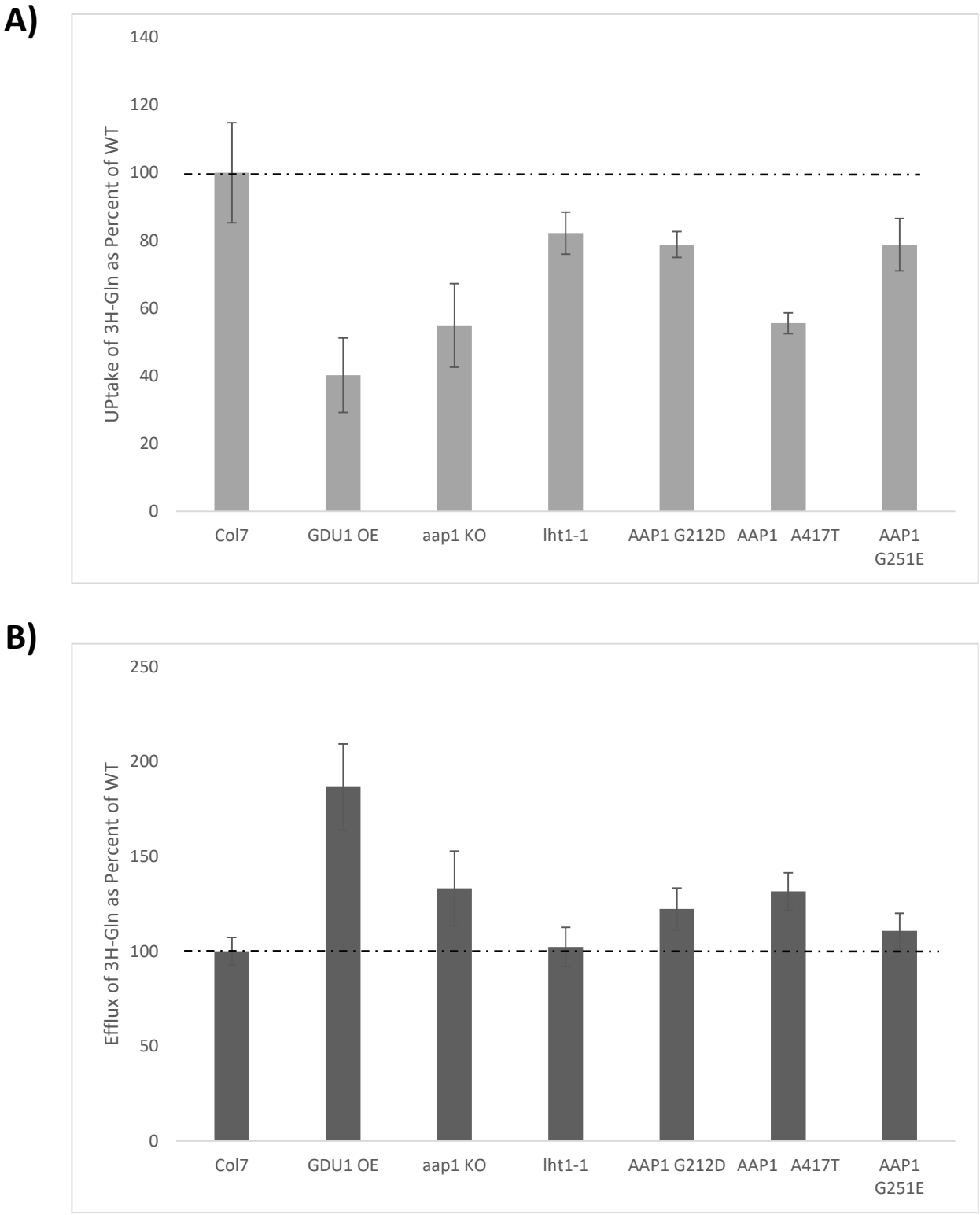


Figure 4. Transport assays of 1mM L-3H-Glutamine. Whole plants are submerged in liquid ½ MS inoculated with ^{3}H-glutamine. Whole plants were washed from external radiolabeled media and submerged in fresh ½ MS media (efflux media). Uptake is measured by the accumulation of radiolabel inside the plant. Efflux is a measure of the ratio between the total amount of radiolabel in efflux media and the total radiolabel accumulation. Total radiolabel accumulation is defined as the accumulation of radiolabel in the plant and the efflux media. **(A)** Uptake of 1mM glutamine was measured over 20 minutes. Data is an average of three replicates represented as a percentage of WT uptake, 100% uptake is equal to 1.132 nmols glutamine. **(B)** Efflux of 1mM glutamine. Efflux was determined as a percent of total glutamine transported during the assay. Data represents the average of three replicates as a percentage of WT efflux. Standard Error is represented by black bars.

Chapter Three: Characterization of APP1 variants in Yeast

Results

Heterologous Expression of AAP1 requires specific expression conditions.

AAP1 variants were expressed in mutant strain 22Δ10α. 22Δ10α is a mutant yeast strain deficient for endogenous amino acid transporters that is capable of growing on plates supplemented with amino acid as the sole source of nitrogen. Each *aap1* variant protein was expressed using several gateway compatible vectors – pDR196-WS, pDRWY, pRS-P-W-Ura, and pFL61-W, to observe growth patterns and determine the functional properties of each variant. Transformations with each vector resulted in different phenotypes with inconsistent colony size. Transformations in pDR196-WS and pDRWY resulted in varying colony sizes (i.e transformations of variants were different than those of wild type or the empty vector). The same phenotypes were noticed when using the pDRWY vector. Using a low copy vector, pRS-P-W-Ura, transformed yeast displayed variation in colony phenotypes on each plate across constructs, however when using these strains in uptake assays yeast cells demonstrated little or no uptake on a 4-minute time scale. Using the low expression vector, pFL61-W, transformed yeast displayed consistent colonies sizes on each plate and between constructs. Expression in this vector also resulted in measurable uptake assays for each variant. 12-minute time scales for uptake assays in pFL61-W yielded even greater measurable results (see below). The expression of AAP1 and *aap1* variants leads to varying phenotypes that are dependent on the type of expression vector being used. The yeast strain 22Δ10α displayed sensitivity to the expression of AAP1 and requires a specific set of expression conditions to express AAP1 in a way that enables the functional analysis of AAP1 proteins and the measurement of transport rates. The functional properties and rate of transport were able to be assessed after identifying expression conditions that resulted in consistent transformation phenotypes.

Functional complementation reveals functionality of the AAP1 variants.

A loop structure located between the 8th and 9th transmembrane of AAP1 was identified through sequence alignment of several know amino acid transporters. This loop was unique to the AAP subfamily of transporters. To identify its function, two constructs were generated effecting the loop – one which removed the entire loop from the AAP1 protein (*aap1* ΔLoop), and another which was created by site directed mutagenesis which substituted a Gly residue at 6 potential phosphorylation sites (*aap1* mutLoop). The functional properties of AAP1, *aap1* variants G212D and A417T, and both *aap1* variants affecting the loop spanning the 8th and 9th transmembrane domains were assessed. Each variant protein expressed using the pFL61-W expression vector in the mutant yeast strain 22Δ10α and grown on BA medium supplemented with amino acids as the sole source of nitrogen. Each *aap1* strain was capable of transporting ammonium (Fig 5a). The strain containing the empty vector pFL61-W was not able to restore grow on any amino acid media while the expression of the wild type AAP1 restored growth on all tested amino acids (Fig. 5b). *aap1* variant G212D displayed no to growth on any amino acid medium (Fig 5a). Variant proteins *aap1* A417T, *aap1* ΔLoop, and *aap1* mutLoop were all able to restore yeast cell growth on all tested amino acids. *aap1* A417T and *aap1* ΔLoop variants restored less growth of yeast cells than the wild type AAP1. Interestingly, variant *aap1* mutLoop restored growth as well as yeast expressing the wild type AAP1 protein on all amino acids and in some cases more growth (Fig 5a). Variant *aap1* proteins that restored growth on amino acid supplemented media demonstrated functional transport. Having identified functional variant *aap1* proteins which restore growth in mutant yeast and enable amino acid tolerance in plants, the transport kinetics for each of the *aap1* variants was assessed.

Transport of 1mM L-^{3}H-Proline is altered by variant AAP1 transporters.

The transport efficiency of AAP1, *aap1* G212D, *aap1* A417T, *aap1* ΔLoop, and *aap1* mutLoop was assessed by measurement of ^{3}H-proline accumulation in yeast cells over a 12-minute time course (Fig. 6). The transport rate displayed by each variant *aap1* protein was normalized to the wild type AAP1. Both *aap1* G212D and *aap1* A417T variants were unable to transport proline (Fig 6a). Lack of transport by *aap1* G212D confirmed results of the functional complementation in which *aap1* G212D was unable to promote growth of yeast cells. Complementation assays with *aap1* variant A417T led to the hypothesis that transport of proline would be reduced. Here, *aap1* variant A417T displayed no transport of proline (Fig. 6). This finding was inconsistent with the observations made during complementation assays. Consistent with complementation assays, the *aap1* ΔLoop variant displayed a 62% reduction in proline transport compared to wild type AAP1 (Fig. 6b). AAP1 mutLoop displayed a 40% increase in proline transport compared to wild type AAP1 (Fig. 6b). Variant aap1 proteins displayed altered transport kinetics than the wild type protein revealing functional significance for these structures in AAP1.

Figures

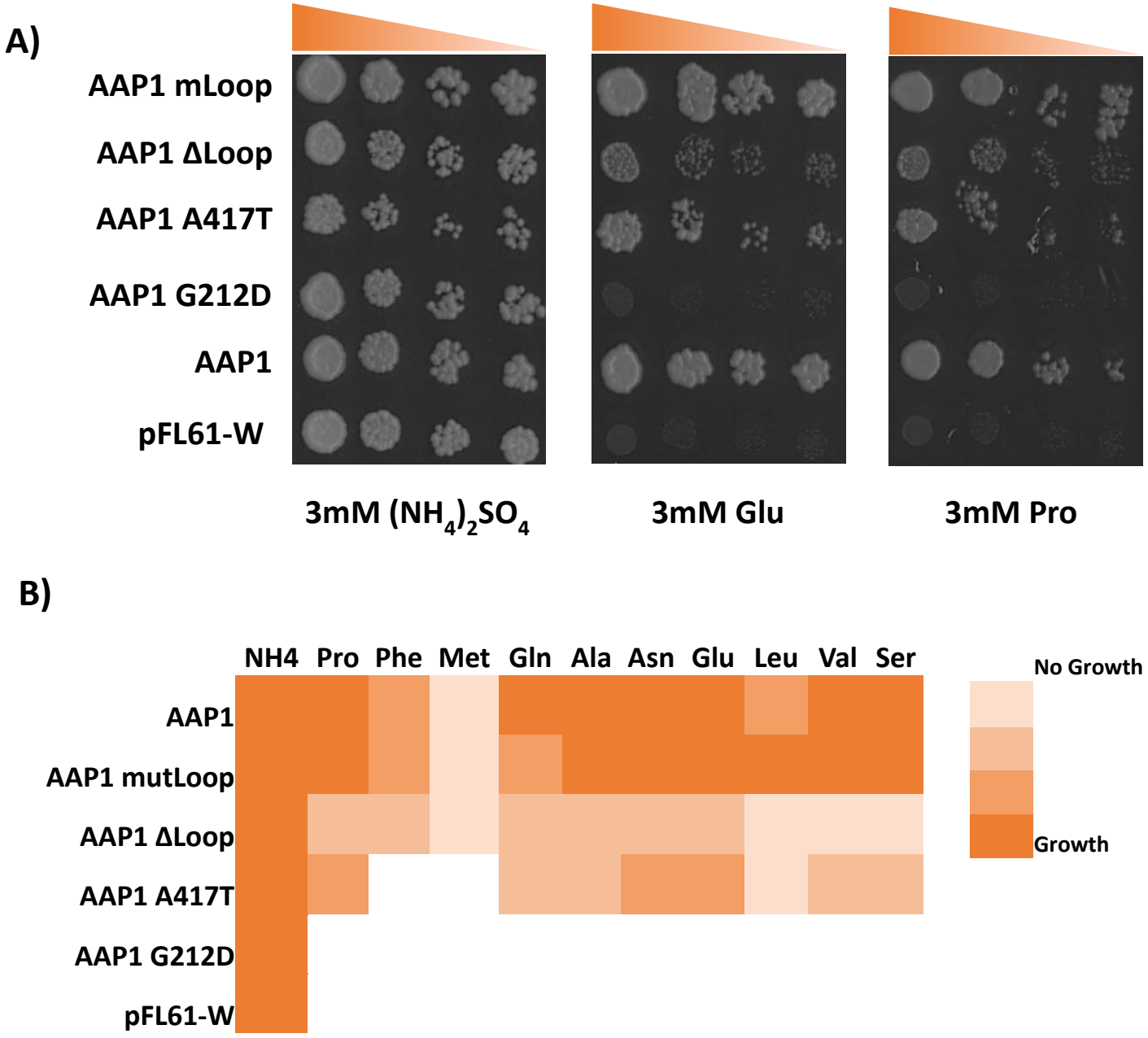


Figure 5. Functional heterologous complementation of yeast deficient for endogenous amino acid transporters – 22Δ10α. (A) *aap1* variant proteins complementing mutant yeast strain 22Δ10α were plated on BA medium containing 3 mM Pro and Glu as the sole source of nitrogen. Yeast were dropped on amino acid plates and grown at 30°C for 4 days **(B)** The growth of each variant yeast on an expanded array of amino acids was assessed. Plates were made, dropped, and grown under the same conditions as A. Growth of each variant yeast was visually assessed in comparison to the yeast complemented with wild-type AAP1 and the empty vector pFL61-W. Orange indicates growth of the yeast strain while white indicates no growth.

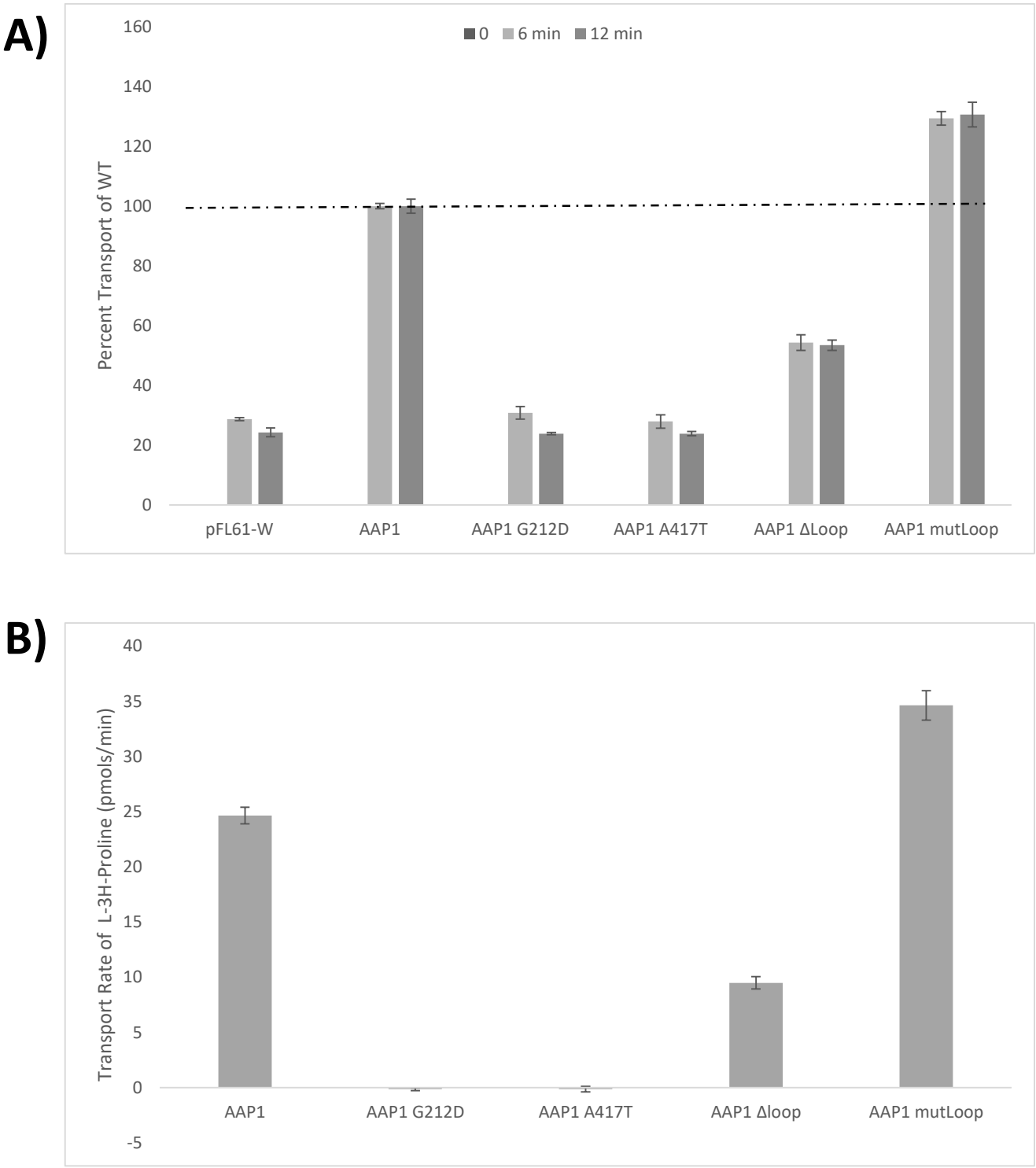


Figure 6. Transport of 1mM ^{3}H-Proline by AAP1 variant in 22Δ10α. (A) Transport of ^{3}H-Proline by AAP1 variants in 22Δ10α over a time course of 0, 6, and 12 minutes. Data is an average of three replicates for each variant, and expressed as a percent of WT transport. 100% transport is equal to 213 pmol at t=6 and 390 pmol at t=12. Standard Error is represented by black bars. **(B)** Transport rate of ^{3}H-Proline over a 12-minute time course. Rate is expressed as pmol/min and corrected for background import. Standard Error is represented by black bars.

Chapter Four: Exploring another potential role for AAP1 in *Arabidopsis thaliana*

Results

AAP1 effects the uptake of amino acids in over the entire root in seedlings.

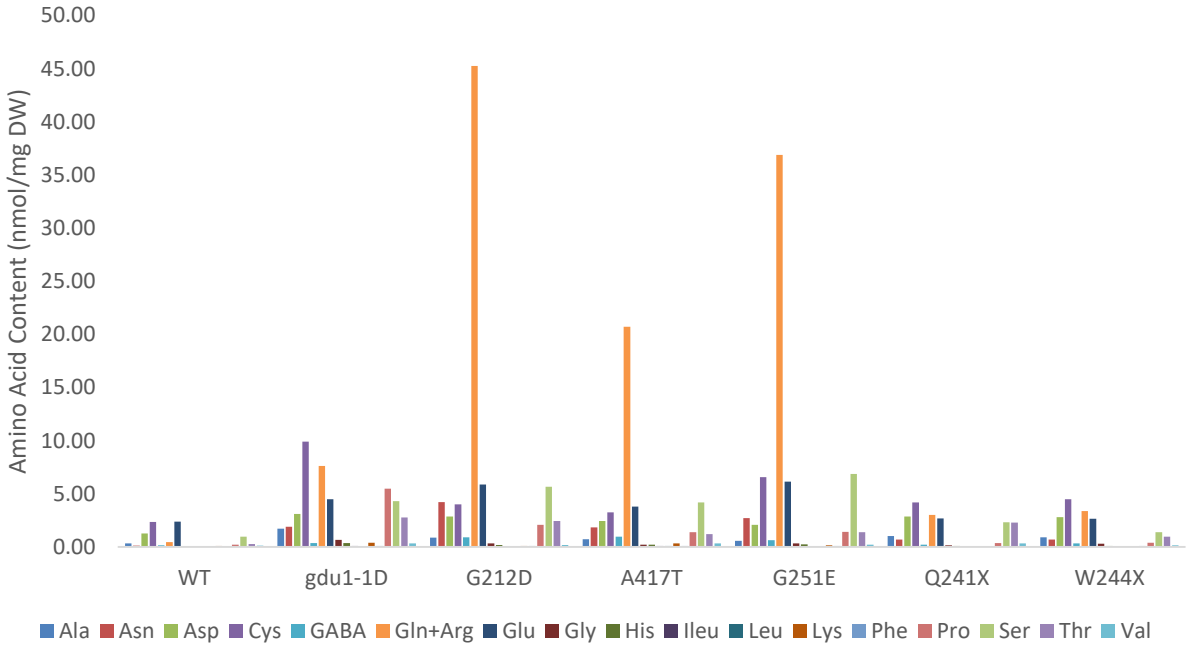
Previous transport assays evaluated the uptake and efflux of glutamine over the entire plant with both roots and shoots. To gain a better understanding of where uptake, assays were developed to determine the location of uptake glutamine uptake along the root. Seedlings were exposed to ^{3}H –glutamine at either the tip of the root; where AAP1 is typically expressed; or over the entire root. Uptake was measured by counting the accumulation of ^{3}H –glutamine in the shoots of seedlings. Here the effect of AAP1 on root uptake was measured using *aap1-2* and wild type Col-0 seedlings. Wild-type seedlings whose root tips were exposed to glutamine displayed little uptake of glutamine compared to wild-type seedlings whose entire root was exposed to ^{3}H –glutamine. Glutamine uptake by *aap1-2* seedlings whose root tip was exposed to ^{3}H –glutamine was similar to wild-type seedlings. Interestingly, *aap1-2* seedlings did not display the same level of glutamine uptake when the entire root was exposed to ^{3}H –glutamine. Glutamine uptake by *aap1-2* was similar to root tip uptake of the *aap1-2* and wild-type seedlings (Fig. 7). Knocking out AAP1 reduced glutamine uptake along the entire root and not just at the root tip where AAP1 is found to be expressed. Finding an effect on uptake over the whole root prompted the evaluation of other potential effects of AAP1 outside of the root tip.

High amino acid content in the leaves of mutant *aap1* plants.

Having already explored the effects of mutations in AAP1 on whole plant uptake and identifying an effect on whole root uptake, it was hypothesized that AAP1 could display an effect in other areas of plants outside of the root tip. Previous uptake assays illuminated the effects of AAP1 at the roots in seedlings and over the whole plant, but have not specifically evaluated potential effects in the leaves. To address the potential effect of AAP1 in leaves, the free amino acid content of wild type Col-7, *gdu1-1D*, *aap1* mutants G212D, A417T, G251E, Q241X and W244X were profiled using UPLC analysis (Fig. 8). *gdu1*-1D plant leaves have been shown to accumulate twice as many free amino acids as the wild type (33). The accumulation of amino acids in the leaves of *gdu1-1D* plants was 4.8 fold higher than wild type plants, as expected (Fig. 8B). Additionally, increased accumulations of Thr, Pro, Gln+Arg, Cys, and Asp were observed in *gdu1-1D* lines. *aap1* mutants G212D, A417T, and G251E contained 8.3, 4.6, and 7.3, times higher free amino acids than the wild type, respectfully (Fig. 8B). Each of these mutants also contained higher free Gln+Arg compositions (Fig. 8A). Two *aap1* loss–of-function mutants Q241X and W244X accumulated twice the amount of amino acids than wild type plants. Notably, Gln+Arg and total amino acid accumulation were much higher in functional *aap1* mutants than the *gdu1-1D* mutants (Fig. 8). All *aap1* variants displayed higher free amino acid concentrations in their leaves than the wild-type and in the case of *aap1* G212D, G251E, and A417T, higher than *gdu1-1D* mutants. Increasing concentrations of free amino acids in the leaves by mutating a root-tip bound amino acid importer was intriguing and prompted the evaluation of other potential roles for AAP1.

Figures

A)



B)

Fold Change in Total AA content	
WT	1.0
gdu1-1D	4.8
G212D	8.3
A417T	4.6
G251E	7.3
Q241X	2.3
W244X	2.1

Figure 8. Free `Amino Acid Content in Leaves of AAP1 mutants. Free amino acids were extracted from the leaves of wild-type, *gdu1-1* mutant plants, and *aap1* mutant plants. The concentration of free amino acids was determined using UPLC analysis. (A) UPLC analysis of free amino acid extractions from leaf tips of AAP1 mutant plants. (B) Fold Change of total amino acid content in AAP1 mutants. Data represent the average of 5 replicates of 4 leaf samples each.

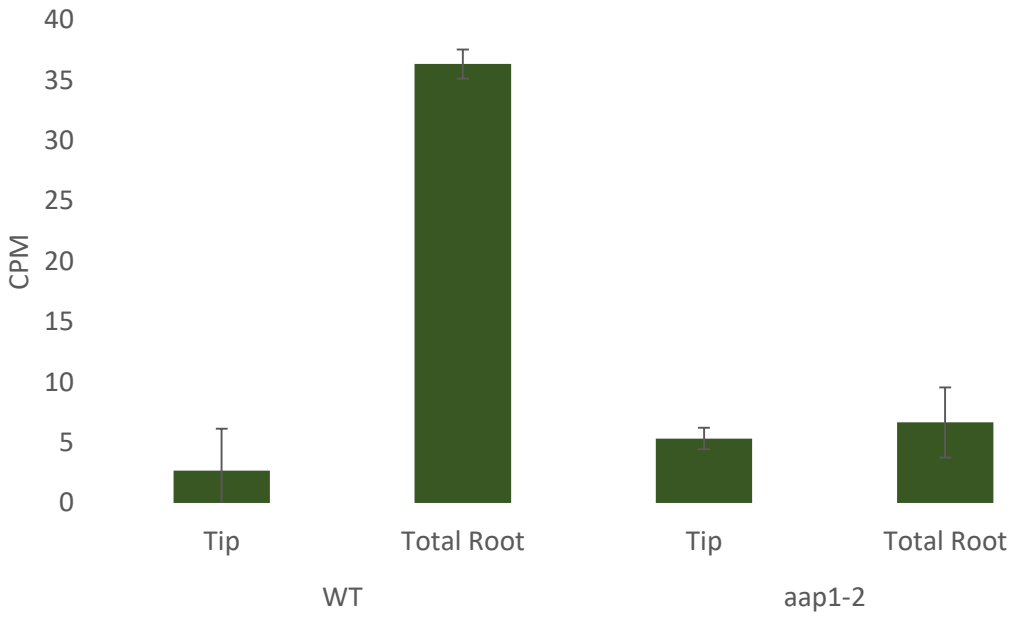


Figure 7. Effects of AAP1 on amino acid metabolism. The effect of AAP1 on root uptake was assessed over the entire root. The roots of Col-0 and *aap1-2* mutant seedling were exposed to radiolabeled glutamine at the root tip or over the entire root. The accumulation of radiolabel counted in the shoot of exposed seedlings was counted. The accumulation of radiolabel in the shoots of seedlings correlates to the uptake of glutamine by the root of the seedling. Data represents the average of three replicates. Standard error is indicated by black bars.

Chapter Five: Discussion

Structural mutations in AAP1 effect transport function

Previous studies using site directed mutagenesis have identified residues critical for transport and substrate specificity such as His47 and His337 (26) and amino acid in the extracellular loop located between the 5th and 6th transmembrane domain (21). A homology model of the AAP1 protein based on the crystallized AdiC transporter from E. coli enabled us to identify V52, G134, V135, Y139, T142, and G416 as residues likely involved in substrate binding and residues Y257 and F345 important for transport. The mutations identified from the Pilot lab screening (A417T and G251E) are located in close proximity to these proposed important residues, suggesting that they also are involved, maybe indirectly, in substrate binding or transport. Sequence alignment of Arabidopsis amino acid transporters from the AAAP family (to which AAP1 belong) revealed that the intracellular loop between the 8th and 9th transmembrane domain is unique to the genes from the AAP subfamily and display high sequence conservation among these members.

In this work, the effect of missense mutations and alterations to a loop between the 8th and 9th transmembrane domains on functional properties of AAP1 were studied in a heterologous system in the mutant yeast strain 22Δ10α. *aap1* G212D was not able restore growth in mutant yeast 22Δ10α when amino acids were supplied as the sole nitrogen source (Fig. 5) and completely abolished transport of Pro. These two findings reveal that residue G212 is critical for amino acid transport by AAP1. Residue G212 is located extracellularly and away from any proposed binding sites for AAP1. Although not directly directing effecting amino acid binding, the displayed effects on AAP1 transport function by G212D reveals that this mutation is capable of abolishing amino acid transport. It is hypothesized that this mutation inhibits AAP1 from shifting from its outward facing conformation to its inward facing conformation. By inhibiting AAP1 conformation transitions, amino acids are not fully transported and released into the cytoplasm. *aap1* A417T restored yeast growth of 22Δ10α on most amino acids as a sole nitrogen source but displayed less growth than yeast complemented by wild type AAP1 protein. Unexpectedly, aap1 A417T displayed no transport ability in transport assays with Pro (Fig. 5b); an amino acid on which A417T has displayed sufficient transport for growth restoration in 22Δ10α. These results provide discrepancies which make discerning the functionality of *aap1* A417T inconclusive. It is not clear why or how this discrepancy occurs, but could result from the difference in the time scale of each experiment. Transport assays were tested over 12 minutes while functional complementation assays spanned the duration of 4 to 7 days. It is possible that 12-minute transport assays were not discerning enough to detect such minimal transport activity that over a longer period of time would be capable of supplying sufficient amino acids to restore growth. The residue A417 is positioned inside the proposed pore in AAP1 and in a proposed amino acid binding region. Evidence provided here does not conclude enough to suggest how A417T affects transport, however, functional complementation suggests that *aap1* A417T may reduce amino acid transport. Mutant yeast complemented with *aap1* A417T display growth and thus display some degree of transport. Because aap1 A417T affects potential binding sitre for AAP1 and complemented yeast do not grow as well, it is hypothesized that aap1 A417T effects the protein structure in a way that partially blocks the binding site. In turn, this larger Thr residue substitution alters the interaction between amino acids and the AAP1 binding site.

Yeast complemented with proteins effecting the intracellular loop between the 8th and 9th transmembrane domain also displayed altered transport function. Deleting this loop, thereby making the junction between the 8th and the 9th transmembrane domains the same length as other amino acid transport proteins not in the AAP family, lead to a functional AAP1 protein that enabled lesser growth rates than AAP1 complemented yeast. Transport assays revealed that by removing this loop, uptake of Pro was reduced by 47%. Mutagenizing large conserved residues in this loop with smaller residues such

as Ala, Gly, and Ser made the protein more active; displaying higher growth restoration than AAP1 complemented yeast and increasing Pro transport by 40% (Fig 5). It is probable that by deleting this loop in the protein and reducing the space between the 8th and 9th transmembrane domains that the protein does not shift between inward and outward facing conformations at the same rate as the wild-type which translates to alter transport rates. Conversely, by mutating conserved residues the protein is able to shift conformations at a higher rate and thus is able to transport amino acids at a higher rate. Affecting this loop confers proteins that are functional transporters and does not abolish transport; suggesting the hypothesis that this loop is not critical for amino acid transport by AAP1. While this loop structure between the 8th and 9th transmembrane domains is not critical for transport but conserved specifically among the AAP subfamily, it is hypothesized that this loop may serve some other function for AAP1 and other members of the AAP subfamily.

The effect of AAP1 on plant amino acid metabolism

The *aap1* mutants A417T, G251E, and *aap1-2* demonstrated an ability to resist to amino acids better than the wild type. The *aap1* mutant S87F and two loss of function mutants *aap1* Q241X and W244X were not able to resist to high concentrations of amino acids as well as the other AAP1 mutants (Fig3). *aap1* G212D (78%), *aap1* G251E (79%), *aap1* A417T (56%), *aap1-2* (56%), and *lht1-1* (82%) lines all revealed a reduced uptake of Gln (Fig. 4a). Altering or knocking out a plant amino acid importer is expected to decrease uptake of amino acids by roots. Interestingly, there was an increased efflux of Gln in the *aap1* mutants G212D (122%), G251E (110%), and A417T (132%) (Fig. 4b). By reducing uptake and increasing efflux, the accumulation of amino acids is could no longer toxic for the plant and could explain the resistance of *aap1* mutant plants to high concentrations of amino acids. However, *lht1-1* did not display any additional efflux (102%) nor enhanced tolerance (Fig. 4b). These features are thus specific to the *aap1* mutant plants.

The Weber group previously identified a mutant from an amino acid resistance screening (using 10 mM Phe as the toxic conditions) of EMS mutagenized *Arabidopsis thaliana* seedlings. **(1)** The isolated Phenylalanine Insensitive Growth 1 (*pig1-1*) mutant was characterized by its ability to tolerate abroad range of biosynthetically relevant and non-relevant amino acids supplied at toxic concentrations. The authors suggested that the *PIG1* gene encodes a regulator of amino acid metabolism, but could not confirm this hypothesis since the mutation had not been identified. Based on collaboration with Dr. Voll (University of Erlangen, Germany) we speculated that the *pig1-1* mutation could affect *AAP1*. I isolated the AAP1 locus of *pig1-1* and confirmed its identity as an AAP1 mutant with an exact match to our own isolated AAP1 G251E mutant. These mutants were thus independently isolated, since my mutant is from the Col7 ecotype and *pig1-1* from the WS ecotype. In addition to enhanced amino acid tolerance, *pig1-1* displayed increased leaf amino acid content, increased catabolism of phenylalanine in plant shoots, and was unaffected in root to shoot translocation with phenylalanine uptake by roots. (1) Loss of function EMS AAP1 mutants Q241X and W244X accumulated more amino acids in the leaves than the wild type, similar to the *gdu1-1D* mutant. (33) The *aap1*mutants G212D, G251E, and A417T all accumulated amino acids at much higher levels than the wild type, about 4.6 to 8.3 times (Fig. 8).

AAP1 is expressed at the root tip (27, 29) and in developing seedlings (19, 20, 27, 28)**.** Other amino acid transporters like LHT1 are also expressed at this location in the plant. (32) It is debated whether there is meaningful ecological relevance for AAP1 in the root tip when the natural relevant concentrations of amino acid found in soil is below the specificity for AAP1 transport. (35) Additionally, these concentrations are well within the range of transport of the LHT1 transporter. [36][35][34][15]

Our own observation show that by knocking out aap1, uptake over the entire root is affected, while AAP1 has been shown to be expressed mainly in the root tip, which demonstrates a distal effect away from its own expression area in the plant.

Our observations of mutations in AAP1 that alter uptake and efflux, translocation, and accumulation of amino acids in leaves cannot be fully explained by an alteration in transport function alone. What explanation could account for the complex phenotype in AAP1 mutants, seemingly incompatible with a simple role in amino acid uptake from the soil? It is plausible that perturbing the expression of AAP1 alters general amino acid metabolism as suggested by Voll et al. (1), correlating amino acid metabolism to transport. However, mutations affecting AAP1 have demonstrated an effect in functions other than transport which contribute to the complexity of the AAP1 mutant phenotype. Following the work by the Forde group, there is sensing occurring at the root tip where AAP1 is expressed. It is possible that AAP1 is involved in plant amino acid homeostasis as a part of an amino acid sensing mechanism in the root tip. AAP1 could contribute to amino acid sensing indirectly; transporting amino acids from the soil into the root where accumulation of amino acids is recognized by some intracellular sensor; or directly; acting as a sensor of extracellular amino acids itself. Accommodating both sensing and transport function would then classify AAP1 a plant amino acid transceptor rather than a transporter. Unpublished reports from the Bush group have noted the AAP1 expression is regulated by the status of carbon in the plant and light, and highly induced by nitrate following starvation (36). These observations demonstrate how AAP1 is receptive to multiple inputs for energy status much like other transceptors identified to be regulated by complex metabolic regulation pathways such as the TOR pathway. (37)

The identification and confirmation of other nutrient transceptors in yeast and mammals has established a method to explore AAP1's potential role as an amino acid transceptor. To justify AAP1 as a transceptor, there must be evidence to support amino acid transport, a direct sensing output from AAP1, increased AAP1 expression during amino starvation, and AAP1 must be rapidly degraded upon amino acid replenishment. AAP1 has previously been established as an amino acid transporter. The Pilot Lab will continue this work and explore the other transceptor characteristics for AAP1. To confidently establish a sensing function, there must be direct sensing output from AAP1 associated with amino acid binding and transport. The PKA pathway and mTOR pathway have been used to study sensing outputs in yeast and mammals. The plant TOR pathway could provide such a pathway to study sensing outputs by AAP1. The use of site directed mutagenesis has been employed in the past to establish sensing capabilities for transceptors. Point mutations affecting the plant nitrate transceptor NRT1.1 (38, 39), yeast phosphate transceptor; Pho84 (40), and yeast amino acid transceptor; Gap1 (8) (11) have been shown to dissociate these functions by abolishing transport yet retain sensing outputs . The Pilot Lab has confirmed mutants for AAP1 which transport but do not appear to sense and mutants which seem to sense and do not transport. With these tools and guidelines from previous transceptor identification studies, the Pilot Lab will be able to explore the role of AAP1 as an amino acid transceptor in plants.

Chapter Six: Materials and Methods

Genomic DNA Extraction for PCR

One selected Arabidopsis leaf was ground in a 1.5mL Eppendorf tube using a micropestle and CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM pH 8). Extraction Buffer (CTAB buffer with 1% v/v β-mercaptoethanol) was added and mixed with ground leaf and CTAB buffer mix. The complete mix was heated for 10 minutes at 65°C in an Eppendorf heat block. After heating, chloroform was added, mixed, and centrifuged for 10 minutes at 15,000 g at 12°C. The supernatant was added to an equal volume of 100% isopropanol, mixed, and centrifuged for 10 minutes at 15,000 g at 12°C. The resulting pellet was washed with 70% ethanol and solubilized in TE (10 mM Tris pH 8, 1 mM EDTA).

Sequencing Fragments and Plasmids

All PCR fragments and plasmids were sequenced using the services of the Biodiversity Institute at Virginia Tech or Eton Bio Sciences (Raleigh, NC).

RNA Extraction

Leaves samples were cut using RNAse Zap-treated forceps and scissors. Sample were collected in sterile 1.5 mL tubes and transferred to liquid nitrogen. Samples were ground using sterile plastic pestles in liquid nitrogen. TRIREAGENT (Sigma, CAT#T9424) was added to ground tissues and mixed. Processed samples were kept on ice for no longer than 30 minutes after mixing with TRIREAGENT. Samples were heated to room temperature and mixed for 5 minutes. Chloroform was added (1:5; Chloroform:TRIREAGENT), mixed, and incubated at room temperature for 15 minutes. Samples were then centrifuged for 15 minutes at 15,000 g at 4°C. The resulting upper aqueous phase was mixed with 100% isopropanol (1:2; Isopropanol:TRIREAGENT). RNA was precipitated overnight at -20°C. The next day, the mixture was centrifuged for 10 minutes at 15,000 g at 4°C. The resulting pellet was vortexed in 75% ethanol, centrifuged for 5 minutes at 7,500 g at 4°C. The pellet was dried and solubilized in diethylpyrocarbonate-treated water.

RT-PCR Reaction

RNA (10 µg) was treated with DNAse using Invitrogen's DNAse I and RNAse inhibitor RiboLock from Fermentas at 40U/µL for 30 minutes at room temperature. The reaction was stopped by addition of 25 mM EDTA (pH 8.0) and treatment for 10 minutes at 65°C. RNA was precipitated by addition of 3M NaAc and 100% ethanol overnight and solubilized in DEPC-treated water. cDNA synthesis was completed using using the RT III Enzyme Kit from Invitrogen. DNAse treated RNA was incubated for 5 minutes at 65°C with 10 µM of the dT18 oligonucleotide, 10 mM dNTPs. Synthesis mix was transferred to ice where RT buffer, RNAse inhibitor, RT III, and 0.1 M DTT. Once mixed, the complete synthesis reaction was incubated for 50 minutes at 50°C followed by and incubation for 10 minutes at 80°C.

Gateway Cloning Reactions (BP/LR)

Genes amplified using oligonucleotides designed with Gateway compatible sequences using synthesized complementary DNA. An attB1 sequence was added to the beginning of all forward primers and attB2 sequence to the end of all reverse primers. Genes were amplified according to the KOD Hot-Start Polymerase enzyme Kits and gel-purified according to the Gel Purification kit by Thermo-Scientific Kit. Purified amplicons were cloned in pDONR Zeo f1 donor plasmids using the BP clonase enzyme mix from ThermoScientific. The resulting reaction was used to transform Top10 heat shock competent *E. coli* cells, which were selected on Zeocin LB medium plates. Colonies of transformed cells were grown over night in a 5 mL liquid LB medium supplemented with Zeocin. Plasmids from selected cultures were extracted using the Thermo Mini Prep Kit and confirmed using restriction reactions (Thermo-Fischer) at 37°C. Confirmed plasmids were sequenced and analyzed using NCBI BLAST tool. Inserts with correct sequence were mobilized to the yeast expression vectors pRS-P-WS-Ura, pFL61-W, pDR196WS, and pDR-WY using LR clonase enzyme mix from ThermoScientific. The reaction product were used to transform Top10 competent E. coli cells which were selected on LB solid media plates supplemented with Ampicillin. Plasmid purification and confirmation was performed as for BP clones (see above).

Yeast Transformation

Yeast strain 22D10α used for heterologous complementation assays and radiolabeled amino acid uptake assays were streaked on YPDA medium from glycerol stocks stored at -80°C and grown on for 3 days at 29°C. A 5 mL liquid culture of yeast was inoculated and grown for 16 hours. A 50 mL culture in YPDA was inoculated at an OD_{600} of 0.4 to a concentration and grown at 29°C until the OD reached 2. The cells were spun down for 5 minutes at room temperature and at 2,500 g. The yeast cell were resuspended in 25 mL of water and centrifuged for the same time and under the same conditions. This process was repeated twice more, and the final pellet was resuspended in 800 µl of water. The suspended pellet was left on the bench for 15 minutes. 15 µl of prepared cells were added to the prepared DNA solution (30 ng/uL plasmid DNA in TE mixed with 7.5 µL of 4 mg/mL salmon sperm, 0.2 M lithium acetate) and mixed with a solution of 1X TE, 100mM lithium acetate, and 40% PEG. The final cell solution was incubated for 30 minutes at 30°C followed by an additional 30-minute incubation at 42°C and plated on SC-LHATMU selective medium (0.17% w/v YNB without AA or ammonium sulfate, 38mM ammonium sulfate, 2% glucose, 0.1% w/v Drop out mix [7%w/w Arginine, 7% w/w Cysteine, 7% w/w Isoleucine, 7% w/w Lysine, 14% w/w Phenyalanine, 7% Serine, 7% Threonine, 10% Tyronsine, and 34% Valine]) using sterile 3-mm glass beads. Cells were grown for 3 days at 29°C.

Yeast Uptake Assays

A liquid culture of yeast was grown for 16 hours at 29°C in SC-uracil. A flask with a 15 mL culture was started in YPDA media at OD_{600} 0.1 at 29°C. Cultures were grown until cells were concentrated to OD_{600} 0.4. Cells were centrifuged for 5 minutes at room temperature at 2,500 g, and resuspended in 10 mL of water. Cells were centrifuged a second time under the same conditions and suspended in 1 mL of

water. A volume of cells was centrifuged so that when resuspended in 1 mL of uptake buffer (50mM potassium phosphate monobasic, 600 mL Sorbitol, pH 4.5), the OD600 of the solution is 5. Cells were then chilled on ice. 100 µL uptake aliquots of cells were prepared on ice and uptake solution (Uptake Buffer, 2X amino acid, 1% radiolabeled amino acid) was prepared. 10 µL of 1 M glucose was added to an aliquot of cells and incubated for 5 minutes at 30°C while shaking in an Eppendorf mixing block. 110 µL of uptake solution was added to incubating cells and shaken 7 minutes. After shaking, a 50 µL aliquot of cells was washed in a prepared filtration vacuum manifold (DHI Lab Products, CAT #EQU-FM-10X20-SET) with 5 mL cold uptake buffer and collected on 24 mm Whatman glass filters (CAT# 1822-024). Cold uptake buffer solution was drained and an additional 5 mL uptake buffer was added and drained in the vacuum manifold. An additional time point was collected and washed at 15 minutes. Filters with collected yeast cells were transferred to scintillation vials and mixed with 5 mL Ultima Gold XR scintillation cocktail and the radioactivity was counted using a liquid scintillation counter.

Yeast Heterologous Complementation Assays

A liquid culture of yeast was grown for 16 hours at 29°C in SC-U. Using sterile water, yeast cultures were diluted in to OD_{600} 0.5 and arranged according the yeast plating schematic in a sterile 96 well plate. Three ten-fold dilutions of each strain were made under the plating schematic. The medium solid consisted of BA medium and 1.7% agar, and 3 mM amino acids as the sole source of nitrogen. Yeast were grown at 29°C for two weeks – monitoring growth over time.

Amino Acid Resistance Screening Assays

Arabidopsis thaliana seeds were plated on ½ MS medium (33 Pilot 2004) supplemented with desired concentrations of amino acid or no amino acids. Plants were grown in 16hr/8hr light/dark conditions growth chamber for two weeks. Plant growth was closely monitored for two weeks following plating.

Amino Acid Extraction and analysis by UPLC

Plants grown soil for 4 weeks at 24°C using a 16hr/8hr light/dark scheme. The first third of four leaves (~50-150mg tissue) were cut and collected in 1.5 mL tubes. Four replicates of each sample were collected for extraction and analysis. After freezing, samples were lyophilized overnight. Lyophilized samples were transferred to a fresh 1.5 mL tube with two 3-mm glass beads and shaken in a bead beater for 1 min. Samples were removed from the bead beater, unpacked from the bottom of the tube, and shook a second time for 1 minute. 1.5 mg of dry 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 minutes. The vortexed sample was centrifuged at room temperature for 5 minutes at 20,000 g. 120 µL of the resulting supernatant was transferred to a new tube. The extraction steps using HCl/Norvaline and Chloroform were completed again using the same centrifuged tissue sample from the first extraction. Following the centrifugation, 170 µL of the supernatant was added to the previous supernatant. Extracted Samples were frozen at -80°C. Samples were derivatized with the ACC-Tag

reagent (Waters) according the manufacturer's instruction, and analyzed by Ultra High Performance Liquid Chromatography as described in (41).

Radio-labeled Transport Assays

Seeds were plated on ½ MS solid medium (1% sucrose, 0.7% agar, pH 5.7) and grown for one week at 24°C 16hrs/8hrs light/dark. After 7 days, seedlings were transferred to a 24 well plate – 4 seedlings per well -- in 3 mL of ½ MS liquid medium. Seedlings were grown in liquid media for 4 days using the same growth conditions. Plants were transferred to a 24 well plate with 1mL of ½ MS medium and acclimated to the uptake conditions for 2 hours, shaken at 300 rpm on an Eppendorf thermomixer. Uptake solution (11X select amino acid, 1% radiolabeled compound) was added to each well. After 20 minutes, the plants were transferred to a vacuum filtration manifold (DHI Lab Products, 10x20 mL, CAT#EQU-FM-10X20-SE) and washed three times with 5 mL of 0.2 mM calcium sulfate. After washing, plants were transferred to a 24 well plate with 1 mL ½ MS for 20 minutes. Efflux medium was collected in a scintillation vial and mixed with five mL Ultima Gold XR (Perkin Elmer). Plant samples were collected in scintillation vials and dried at 70°C for 3 hours. Following drying, plant samples were weighed and mixed with 500 µL 8% NaClO solution, bleached overnight and mixed with 5 mL Ultima Gold XR. Radioactivity was counted using a liquid scintillation counter.

Split Plate Radio-Labeled Assays

Seeds were plated on ½ MS solid medium (1% sucrose, 0.9% agar, pH 5.7) and grown vertically for one week in 16hrs/8hrs light/dark growth. After 6 days, seedlings were transferred to a prepared radiolabeled ½ MS plate. Plates were made by cutting a 3 mm gap between the top and bottom halves of the plate. An uptake solution was spread and diffused across the bottom half of the plate. The shoots of the seedlings were placed on the top (unlabeled) half of the plates while root were spread across the gap, and tips were in contact with the labeled half of the plate. After 4 hours, shoots and roots of seedlings were separated by cutting at the base of the shoot. Shoots were collected in scintillation vial, treated and the radioactivity was counted as described above for the transport assays.

Mutagenesis by Kunkel Method

This form of mutagenesis was used to create the AAP1 –Loop and AAP1 mutLoop constructs.

Single Strand DNA (ssDNA) Preparation

CJ236 competent cells were transformed by electroporation with plasmid containing the AAP1 CDS. Transformed cells were plated on LB medium containing zeocin and chloramphenicol and grown at 37°C. A 1 mL culture of selected transformed cells was grown in liquid LB with zeocin and chloramphenicol for 16 hours at 37°C. The next evening, a new 1mL culture was inoculated using 100 µL of the culture grown the evening before until turbid (approximately 1 hour and 30 minutes at 37°C, to OD_{600} 0.05). 1 uL of uridine at concentration 0.25µg/mL final and 1 µL of M13K07 phage stock (from NEB, 10^8 pfu/mL final) were added to the slightly turbid culture and grown for 2 additional hours. 500 µL

of the culture grown with phage was used to inoculate a 50 mL liquid culture with chloramphenicol, zeocin, kanamycin, and uridine and grown for 16 hours.

The cell culture was centrifuged for 10 minutes at room temperature at 5,500 g. 90% of the supernatant were transferred to a new tube and centrifuged an under the same conditions. This process was repeated four times total. 40mL of the final supernatant was transferred to a new tube and mixed thoroughly with 8 mL of a 20%PEG, 2.5M NaCl solution. The mixture was chilled on ice for 1 hour.

The phage was precipitated by centrifugation for 10 minutes at 4°C at 10,000 g. The resulting pellet was completely rid of the supernatant, vigorously re-suspended in 1.5 mL of TE, and transferred to a fresh 1.5 mL tube. The phage suspension was centrifuged for 5 minutes at room temperature at 15,000 g. 90% of the supernatant was transferred to a fresh 1.5 mL tube, mixed with 300µL of PEG/NaCl solution, and chilled on ice for 10 minutes. The chilled suspension was centrifuged for 10 minutes at 4oC at 15,000 g. The supernatant was completely discarded, and the resulting pellet was suspended in 1 mL TE. The re-suspended pellet was centrifuged for 5 minutes at room temperature at 15,000 g. The resulting supernatant was transferred to a new 1.5 mL tube. ssDNA was extracted using the Qiagen M13 spin kit.

Phosphorylating and Annealing Primers

The primers used for annealing and second strand synthesis were phosphorylated before use. A final primer solution (2.5 µM un-phosphorylated primer, 1X T4 PNK Buffer, 1 mM ATP, and 5U T4 Polynucleotide kinase) was incubated for 1 hour at 37°C. Following incubation, the T4 PNK kinase was deactivated by incubation for 10 minutes at 70°C. Phosphorylated primers were annealed to the extracted ssDNA template using a mix composed of 125 ng ssDNA, 0.3125 pmols phosphorylated primers, and 1.25X (final) NEB3 reaction buffer. Annealing the Primer/ssDNA mix was accomplished by heating to 96°C for 3 minutes, then cycling the mix at 96oC for 10 sec, followed by 95.3°C for 11 seconds; decreasing the temperature 0.7°C and increasing the time by 1 second for a total of 96 cycles.

Second Strand Synthesis and Transformation

The full mixture of ssDNA with annealed primers was mixed with a synthesis reaction mixture (1mM dTP, 0.4 mM ATP, 1X BSA, 2% v/v T7 DNA Polymerase, 0.4% v/v T4 ligase) and incubated for 16 hours. A dilution (1/100) of the resulting double strand plasmid was transformed by heat shock into TOP10 cells and grown on LB media and zeocin for selection.

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