Understanding the role of host amino acid transporters in nutrient acquisition by oomycete pathogens

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(ACADEMIC ABSTRACT)

Hyaloperonospora arabidopsidis (*Hpa*) is a naturally occurring oomycete pathogen on *Arabidopsis thaliana*. It is related to downy mildews of economically important crops such as cabbage, kale and broccoli, belonging to the *Brassicaceae* family. Downy mildew pathogens are obligate biotrophs that extract nutrients exclusively from living plant cells. As a part of its obligate biotrophy lifestyle, *Hpa* has lost the ability to assimilate inorganic nitrogen and sulfur. It thus has to acquire these nutrients from the host in an organic form; possibly amino acids. Using a reverse genetic approach, I was able to identify two host amino acid transporters that are up-regulated during Hpa infection: *AAP3* and *AAP6*. Both of these transporters are localized in the vasculature of the plant, AAP3 mostly in the root, and AAP6 in the roots and shoots. Using transgenic lines of Arabidopsis containing transcriptional and translational reporter fusion constructs for these genes, I found that *AAP3* displays increased mRNA accumulation which is attributable to an increased promoter activity in regions of shoot tissue colonized by Hpa. On the other hand, *AAP6* displays a mild increase in mRNA accumulation under *Hpa* infection, but the induction becomes more prominent at the protein level as seen by fluorescence from GFP fused to AAP6. Surprisingly, null mutants of *AAP3* did not impact *Hpa* growth whereas null mutants of *AAP6* made the plant more susceptible to *Hpa*. Furthermore, *aap6* mutants accumulate fewer free amino acids in the phloem compared to wild-type plants when infected with *Hpa*. Together, these results suggest that *AAP6* acts a nutritional starvation gene for the pathogen and hence aids the plant during infection. While we now know more about *AAP3*'s regulation during infection, its function remains to be elucidated. To successfully colonize a plant, a pathogen must be able to achieve both suppression of plant immunity and acquisition of nutrients from the plant host. While the former has been well studied, research on the latter is sparse. This work was a step in the direction to increase our understanding of potential players in nutrient acquisition by pathogens.

Understanding the role of host amino acid transporters in nutrient acquisition by oomycete pathogens

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(GENERAL AUDIENCE ABSTRACT)

A key aspect of achieving and maintaining food security is sustainable agricultural production. This is endangered by plant diseases that lead to large losses in crop production. All plant pathogens have to acquire food and nutrients from the plants they infect. Understanding how they acquire nutrients from the plant at a molecular level can give us insight into potential methods to prevent this and hence reduce the impact of plant diseases. One such nutrient is nitrogen. Nitrogen is essential to all of an organism's cellular and metabolic processes. Organisms utilize nitrogen by converting it from inorganic forms such as nitrates to organic forms such as amino acids. Some plant pathogens, such as *Hyaloperonospora arabidopsidis* (*Hpa*), which causes downy mildew disease on the model plant Arabidopsis, complete their entire life cycle on a living plant. They are also unable to convert the inorganic nitrogen to organic forms and hence depend on acquiring organic forms of nitrogen from the plant. Thus, it is important to understand how they acquire amino acids from the plant. Plants use amino acid transporters that serve as a siphon or a pump in moving amino acids from one region of the plant to another. It is possible that pathogens manipulate plant's amino acid transporters to move amino acids towards the infection site while, at the same time, plants might use another set of transporters to move amino acids away from the pathogen. This work was an attempt at understanding this potential role of plant amino acid transporters in plant-pathogen interactions using the model system of *Hpa* and Arabidopsis.

For Mom and Dad.
What is a tall tree without strong roots?

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Introduction

1.1 *Hyaloperonospora arabidopsidis*

Hyaloperonospora arabidopsidis (*Hpa*) is a biotrophic pathogen that causes downy mildew disease on Arabidopsis (Koch and Slusarenko, 1990). *Hpa* belongs to the group of fungus-like organisms called Oomycetes. Similar to fungi, they produce filamentous hyphae and employ modes of nutrition and reproduction that are like their fungal counterparts (Richards et al., 2006). However, DNA-based phylogenetic studies have shown that fungi and oomycetes fall into evolutionarily distinct lineages. While fungi belong to the same superkingdom as animals (Opisthokonta), oomycetes reside in the superkingdom Chromalveolata and are more closely related to brown algae and diatoms (Beakes and Sekimoto, 2009; Simpson and Roger, 2004).

Oomycetes consist of several crop pathogens with varied lifestyles (Fig. 1.1) ranging from the biotrophic downy mildews and white rust causing *Albugo* species to the necrotrophic *Pythium* species that cause damping off. The hemibiotrophic *Phytophthora* genus consists of the potato late blight pathogen *Phytophthora infestans*; infamous for causing the Irish potato famine. This pathogen alone leads to economic expenditures upwards of USD 3 billion annually at the global scale due to crop protection efforts and losses to damage (Fry, 2008). All the downy mildew species together are estimated to cause disease on about 15% of flowering plants, including several crops. Downy mildews are thus a threat to the $7.5 billion economy of vegetable, fruit and cereal crops that are susceptible to these diseases (Crandall et al., 2018; McDowell, 2014). *Hpa* is closely related to the downy mildew species of pathogens that cause disease on crucifer plants. However, *Hpa* is not known to cause disease on any other plant species apart from *Arabidopsis thaliana*, it is therefore considered a specialist pathogen (Holub and Beynon, 1997).

1.1.1 *Hpa's* life cycle

Owing to its obligate biotrophic lifestyle, *Hpa* has not yet been successfully cultured outside of its host. This means that it has to be continually propagated on Arabidopsis and also makes molecular manipulation of the pathogen difficult (Schlaich and Slusarenko, 2009). Similar to other downy mildews *Hpa* prefers high humidity and low temperatures for infection and asexual reproduction (Slusarenko and Schlaich, 2003).

The infection cycle (Fig. 1.2) starts with an *Hpa* spore landing and germinating on an Arabidopsis leaf. It forms an appressorium to attach to the host leaf surface. It then forms a penetration hypha that enters the leaf surface and grows though the epidermal layer of the leaf.

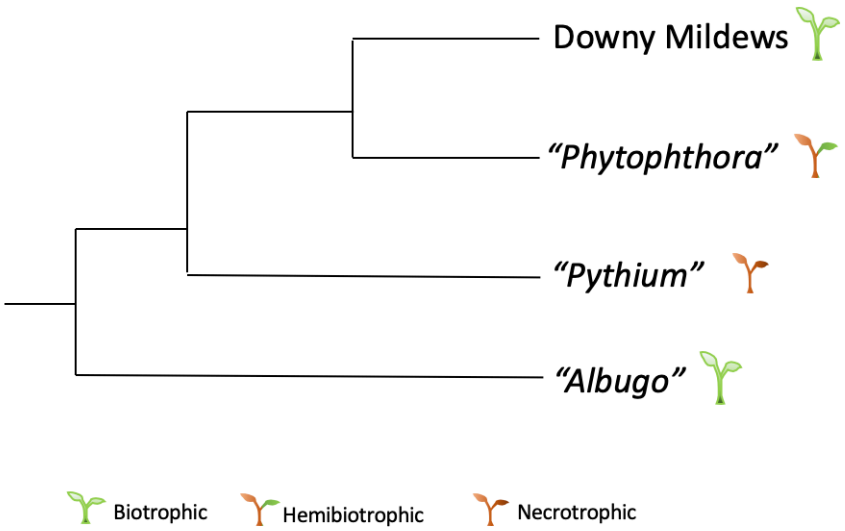

Figure 1.1: Schematic phylogenetic relationships of oomycete plant pathogens (adapted from Thines and Kamoun, 2010)

Upon reaching the mesophyll layer of the leaf, Hpa begins to branch and grow, and colonizes the intercellular spaces. The hyphae also differentiate to form a specialized structure called haustorium. The haustorium penetrates the mesophyll cell wall and invaginates the plasma membrane to form an intimate interface with the host cell (McDowell, 2014). Thus, the cell wall of the mesophyll cell is broken by the haustorium, but the plasma membrane remains intact. This host-derived membrane enveloping the haustorium is called the extra-haustorial membrane (EHM). EHM separates the haustorium from the plant cell's cytosol and serves as a point of interaction between the pathogen and the plant cell. It is a specialized membrane and has been shown to exclude several plasma membrane localized proteins (Bozkurt et al., 2014). The haustorium and the EHM are separated by the extra-haustorial matrix (EHMx) (Fig. 1.2). The haustorium has been traditionally believed to be a 'feeding structure' and the main site for the pathogen to acquire nutrients from the host cell. However, this is yet to be observed (McDowell, 2014). Haustoria are also suggested to be the site of effector delivery into the plant cells (Coates and Beynon, 2010). Effectors are pathogen proteins that suppress the plant's basal defense mechanisms and thus aid in pathogen virulence. Haustoria have been demonstrated to be the site for effector delivery in case of the flax rust fungi *Melamspora lini* (Dodds, 2004) and in *Phytophthora infestans* (Whisson et al., 2007; Wang et al., 2017). This is yet to be proven as a common function for the rest of the fungi and oomycetes (McDowell, 2014).

Hpa is self-compatible and reproduces sexually by forming oospores. They are formed by the fusion of hyphae that have differentiated to form antheridia and oogonia. Oospores serve as over-wintering structures until the conditions are favorable for infecting a compatible host (McDowell, 2014; Coates and Beynon, 2010). *Hpa* reproduces asexually by forming tree-like structures called sporagiophores which grow out of the stomata and carry spores that are dispersed aerially (Schlaich and Slusarenko, 2009).

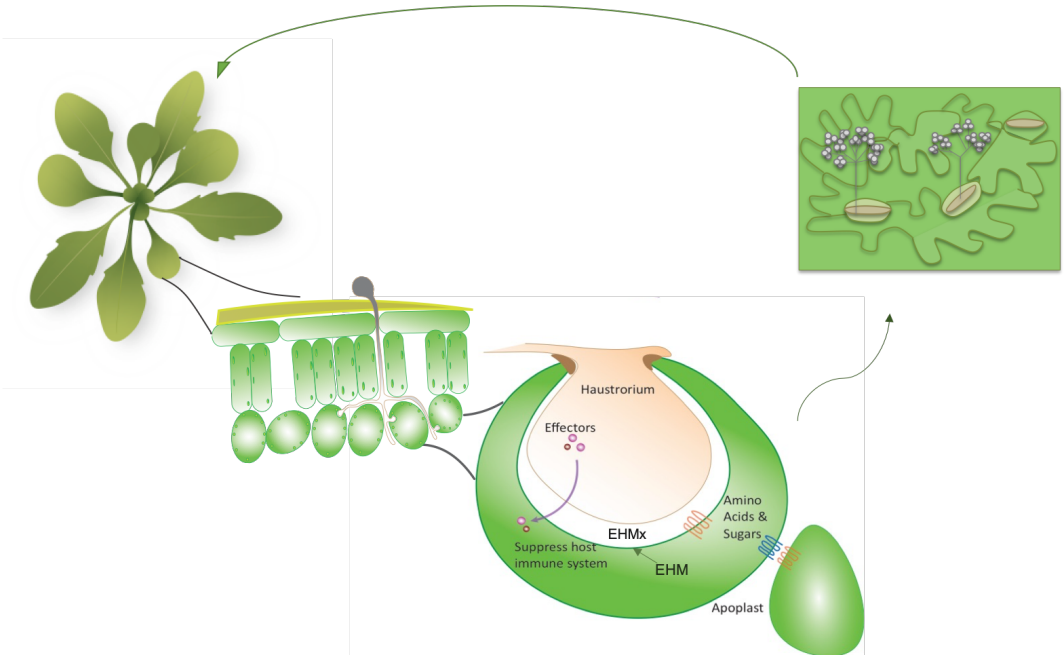

Figure 1.2: Schematic of *Hpa's* life cycle and its interaction with Arabidopsis in a haustoriated cell. EHMx = Extrahaustorial matrix, EHM = Extrahaustorial membrane

1.1.2 *Hpa's* role as a model pathogen in understanding nutrient acquisition

Even though *Hpa* is not a crop pathogen, it is considered an important oomycete phytopathogen since it is able to colonize Arabidopsis. *Hpa* colonizes naturally occurring accessions of Arabidopsis in the wild. This means that *Hpa* has co-evolved with Arabidopsis; evident from the genetic variability in disease resistance genes among Arabidopsis ecotypes against the corresponding effectors in *Hpa* (Holub, 2001, 2008). Using the *Hpa*-Arabidopsis pathosystem also allows us to take advantage of the molecular and genomic tools generated from Arabidopsis research (McDowell, 2014).

A more recent advantage of using the *Hpa*-Arabidopsis pathosystem comes from the sequencing of *Hpa's* genome. *Hpa's* genome sequencing revealed the loss of genes coding for nitrogen and sulfur assimilation enzymes. Compared to the hemibiotrophic *Phytophthora* species, *Hpa* lacks nitrate, nitrite, and sulphite reductases. It also lacks a nitrate transporter (Baxter et al., 2010). Sequencing of other fungal and oomycete pathogens such as poplar leaf rust, and barley and wheat stem rust fungi (*Melamspora larici-populina* and *Puccinia graminis* f.sp. *tritici*) (Duplessis et al., 2011; McDowell, 2011), barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) (Spanu et al., 2010) and white blister rust (*Albugo candida*) (Links et al., 2011) has revealed that these obligate biotrophic pathogens are deficient in inorganic nitrogen and sulfur assimilation pathways. These pathogens must thus depend on their host for their supply of organic forms of nitrogen and sulfur.

How pathogens acquire nutrients from their host is not well understood and how obligate biotrophs compensate for their metabolic deficiencies in the way they acquire nutrients compared to

their hemibiotrophic counterparts is unknown (Herlihy et al., under review). Exploiting the benefits that working with Arabidopsis offers, the *Hpa*-Arabidopsis interaction can be used as a model to probe the different host metabolic and transport genes that are important for nutrient acquisition by obligate biotrophs.

Since *Hpa* needs to acquire nitrogen exclusively in organic form, it is highly likely that amino acids form an important nitrogen source for *Hpa* and other biotrophic pathogens. The pathogen probably directly or indirectly manipulates host amino acid transporters to mobilize plant amino acids for its acquisition. Metabolomic and transcriptomic studies have revealed that some obligate fungi pathogens actively reprogram host metabolism to create a sink for nutrient acquisition (Spanu and Kämper, 2010). On the other hand, it is also possible that the plant uses its amino acid transporters to move amino acids away from infection site. Thus making the role and regulation of various plant amino acid transporters critical to disease outcome.

1.2 Amino Acid Transporters

1.2.1 Nitrogen uptake and assimilation in plants

Nitrogen is an essential macronutrient for plants and is important for almost all cellular and metabolic processes. In most agricultural land, nitrate and ammonium are the two main forms of nitrogen taken up by plant roots. However, in some cases organic forms of nitrogen such as amino acids could play an equally important role (Näsholm et al., 2009). Several low- and high-affinity nitrate and ammonium transporters are involved in taking up these compounds at epidermal and cortical cells of plant roots (Krapp, 2015).

Nitrate is first converted to nitrite, which is then converted to ammonium. Ammonium, either converted from nitrate or taken up directly from the soil, is assimilated into the amino acid pool via glutamine synthetase/glutamine-2-oxoglutarate aminotransferase (GOGAT) pathway. Both nitrate and ammonium can either be assimilated into amino acids in roots or loaded into the xylem and translocated to the shoot where they are assimilated in mature leaves (Xu et al., 2012; Giehl et al., 2017). These mature leaves and roots serve as source tissues for amino acids. These amino acids are then transported to sink tissues such as seeds, young leaves and flowers. Several amino acid transporters play a critical role in this process: translocation of amino acids from root-to-shoot via the xylem, loading of phloem with amino acids from the xylem, or mesophyll cells in source leaves, and finally during phloem unloading in sink tissues. Thus, amino acid transporters are required to maintain physiological functions such as plant growth and seed yield (Tegeder and Rentsch, 2010; Tegeder, 2014).

1.2.2 Amino acid transporters in plants

There are about 100 genes in Arabidopsis that code for amino acid tranporters. These transporters can be divided into three major groups: transporters belonging to amino acid-polyamine-choline (APC) superfamily, those belonging to the amino acid/auxin permease (AAAP) superfamily, and the newly found UmamiT transporters belonging to the drug/metabolite

transporter (DMT) superfamily (Pratelli and Pilot, 2014; Tegeder, 2012). The AAAPs and the APCs are further divided into subfamilies based on the sequence similarity of the transporters. AAAPs are divided into six subfamilies: i) AAPs (amino acid permeases), ii) LHTs (lysine-histidine transporters), iii) GAT (γ-aminobutyric acid transporter), iv) ProT (proline transporter), v) ANT1-like (aromatic and neutral amino acid transporter), and vi) AUX1 (auxin transporters). The APC superfamily can be divided into two subgroups: i) CAT (cationic amino acid transporters) and ii) LAT (L-type amino acid transporters).

Here, I summarize the known physiological roles of transporters belonging to the AAP and UMAMIT families since they are the main focus of this dissertation. For further reading on other families of transporters refer to Tegeder (2012); Rentsch et al. (2007); Pratelli and Pilot (2014).

AAPs consist of 8 members, AAP1 through 8. All of these AAPs (except for AAP7) have been functionally characterized via heterologous expression in yeast and *Xenopus* oocytes. Most of them were found to transport neutral and acidic amino acids with moderate affinity, while AAP6 was found to have the lowest K_m and is a high affinity transporter. (Fischer et al., 2002; Okumoto et al., 2002). AAP3 and AAP5 were found to also transport basic amino acids (Fischer et al., 2002). AAPs have been shown to be involved in a broad range of physiological processes: AAP1 and AAP5 have been implicated to function in root uptake of amino acids (Lee et al., 2007; Svennerstam et al., 2008), AAP2 and AAP3 are suggested to function in phloem loading (Okumoto et al., 2004; Zhang et al., 2010), AAP2 along with AAP6 is also indicated to function in xylem-to-phloem transfer (Zhang et al., 2010; Okumoto et al., 2002; Hunt et al., 2010), and AAP1 along with AAP8 are found to be important for seed loading via import of amino acids into the developing embryo (Sanders et al., 2009; Schmidt et al., 2007).

Arabidopsis contains 44 genes belonging to the UmamiT family. While several importers had been characterized from the AAAPs and APC families, identification of amino acid exporters remained elusive until the characterization of UmamiT18 (SIAR1, Siliques Are Red1) as a bidirectional amino acid facilitator (Ladwig et al., 2012). UmamiT05 (WAT1, WAlls are thin 1), another transporter from the same family, had been shown to facilitate auxin transport across the tonoplast membrane (Ranocha et al., 2010, 2013). Unlike importers, these transporters function in a proton-independent manner and mediate transport in both directions of the membrane, along the electrochemical gradient of the substrate (Müller et al., 2015). Since identification of UmamiT18 (SIAR1) and UmamiT05(WAT1), 6 other UmamiTs have been characterized. Many of these UmamiTs (UmamiT11, UmamiT14, UmamiT18 (SIAR1), UmamiT24, UmamiT25, UmamiT28 and UmamiT29) have been implicated to play a role in loading amino acids in seeds (Ladwig et al., 2012; Müller et al., 2015; Besnard et al., 2018). Some of them might also play additional roles in other plant organs. For example, UmamiT14 and Umamit18 have been shown to be important in phloem unloading in roots (Besnard et al., 2016). Furthermore, all the UmamiTs identified to-date have been found to be localized to the plasma membrane or the tonoplast, however proteomics datasets suggest that they might localize to all endomembranes (Karmann et al., 2018). Clearly, a lot remains to be understood about the breadth (the function of the rest of UmamiTs) and depth (different functions of each UmamiT) of these transporters in plants.

1.3 Goals and significance of this research

The main goal of this project was to examine if and what role Arabidopsis amino acid transporters play in its interaction with *Hpa*. Until now, most research on genetic mechanisms of crop protection against phytopathogens has been related to plant immunity, specifically *R* genes. Plants are able to recognize molecular patterns associated with microbes/pathogens (such as chitin and peptidoglycans) via pattern recognition receptors (PRRs). This initiates what is traditionally believed to be a basal defense mechanism in the plant, known as PTI (PAMP triggered immunity) (Jones and Dangl, 2006; Kourelis and van der Hoorn, 2018). Pathogens in turn deliver proteins known as effectors into plant cells to suppress this defense response. Resistant plants are able to recognize these effectors via several mechanisms that involve R proteins (Resistance proteins). This initiates a cascade of defense responses including hypersensitive response (HR) and is termed effector triggered immunity (ETI) (Jones and Dangl, 2006). Genomes of plant pathogenic oomycetes code for hundreds of effectors with similar functions. Hence, they can overcome resistance genes by losing these effectors with relative ease (Birch et al., 2008). Also, the co-evolutionary arms race between hosts and pathogens has led to extreme diversification of effector sequences, which have been found to undergo nonsynonymous substitutions due to positive selection (Schornack et al., 2010). This poses a major difficulty in developing durable resistance to oomycete pathogens by employing canonical resistance mechanisms alone. On the other hand, if the host is less susceptible to the pathogen due to reduction in the nutrients that the pathogen can acquire, it would conceivably be difficult and take longer for the pathogen to overcome such resistance. This would require identification of the host transporters and related pathways that are manipulated by the pathogen for nutrient acquisition. This dissertation is a step in the direction of understanding the role and regulation of amino acid transporters under pathogen infection.

The following chapter further describes the importance and potential of research in this field. It reviews previous research in published literature which taken together suggest that sugar and amino acid transporters play an important role in how plants respond to pathogen attack and in how pathogens acquire nutrients from their host.

Chapter 1. References

Baxter, L., Tripathy, S., Ishaque, N., Boot, N., Cabral, A., Kemen, E., Thines, M., Ah-Fong, A., Anderson, R., Badejoko, W. et al. (2010). Signatures of adaptation to obligate biotrophy in the *Hyaloperonospora arabidopsidis* genome. *Science* **330**, 1549–1551.

Beakes, G. W. and Sekimoto, S. (2009). The evolutionary phylogeny of oomycetes-insights gained from studies of holocarpic parasites of algae and invertebrates. In *Oomycete Genetics and Genomics: Diversity, Interactions, and Research Tools* (eds. K. Lamour and S. Kamoun), pp. 1–24. John Wiley & Sons, Inc. Hoboken, New Jersey.

Besnard, J., Pratelli, R., Zhao, C., Sonawala, U., Collakova, E., Pilot, G. and Okumoto, S. (2016). UMAMIT14 is an amino acid exporter involved in phloem unloading in Arabidopsis roots. *Journal of Experimental Botany* **67**, 6385–6397.

Besnard, J., Zhao, C., Avice, J.-C., Vitha, S., Hyodo, A., Pilot, G. and Okumoto, S. (2018). Arabidopsis UMAMIT24 and 25 are amino acid exporters involved in seed loading. *Journal of Experimental Botany* **69**, 5221–5232.

Birch, P. R., Boevink, P. C., Gilroy, E. M., Hein, I., Pritchard, L. and Whisson, S. C. (2008). Oomycete RXLR effectors: Delivery, functional redundancy and durable disease resistance. *Current Opinion in Plant Biology* **11**, 373–379.

Bozkurt, T. O., Richardson, A., Dagdas, Y. F., Mongrand, S., Kamoun, S. and Raffaele, S. (2014). The plant membrane-associated REMORIN1.3 accumulates in discrete perihaustorial domains and enhances susceptibility to *Phytophthora infestans*. *Plant Physiology* **165**, 1005–1018.

Coates, M. E. and Beynon, J. L. (2010). *Hyaloperonospora Arabidopsidis* as a pathogen model. *Annual Review of Phytopathology* **48**, 329–345.

Crandall, S. G., Rahman, A., Quesada-Ocampo, L. M., Martin, F. N., Bilodeau, G. J. and Miles, T. D. (2018). Advances in diagnostics of downy mildews: Lessons learned from other oomycetes and future challenges. *Plant Disease* **102**, 265–275.

Dodds, P. N. (2004). The *Melampsora lini* AvrL567 avirulence genes are expressed in haustoria and their products are recognized inside plant cells. *The Plant Cell* **16**, 755–768.

Duplessis, S., Cuomo, C. A., Lin, Y.-C., Aerts, A., Tisserant, E., Veneault-Fourrey, C., Joly, D. L., Hacquard, S., Amselem, J., Cantarel, B. L. et al. (2011). Obligate biotrophy features unraveled by the genomic analysis of rust fungi. *Proceedings of the National Academy of Sciences* **108**, 9166–9171.

Fischer, W. N., Loo, D. D., Koch, W., Ludewig, U., Boorer, K. J., Tegeder, M., Rentsch, D., Wright, E. M. and Frommer, W. B. (2002). Low and high affinity amino acid H+-cotransporters for cellular import of neutral and charged amino acids. *Plant Journal* **29**, 717–731.

Fry, W. (2008). *Phytophthora Infestans*: The plant (and R gene) destroyer. *Molecular Plant Pathology* **9**, 385–402.

Giehl, R. F., Laginha, A. M., Duan, F., Rentsch, D., Yuan, L. and von Wirén, N. (2017). A critical role of AMT2;1 in root-to-shoot translocation of ammonium in arabidopsis. *Molecular Plant* **10**, 1449–1460.

Herlihy, J., Ludwig, N. R., van den Ackerveken, G. and McDowell, J. M. (under review). Oomycetes used in Arabidopsis research. *The Arabidopsis Book* .

Holub, E. B. (2001). The arms race is ancient history in Arabidopsis, the wildflower. *Nature Reviews Genetics* **2**, 516–527.

Holub, E. B. (2008). Natural history of *Arabidopsis thaliana* and oomycete symbioses. *The Downy Mildews - Genetics, Molecular Biology and Control* **122**, 91–109.

Holub, E. B. and Beynon, J. L. (1997). Symbiology of mouse-ear cress (*Arabidopsis thaliana*) and oomycetes. In *Advances in Botanical Research*, volume 24, pp. 227–273. Academic Press.

Hunt, E., Gattolin, S., Newbury, H. J., Bale, J. S., Tseng, H. M., Barrett, D. A. and Pritchard, J. (2010). A mutation in amino acid permease *AAP6* reduces the amino acid content of the *Arabidopsis* sieve elements but leaves aphid herbivores unaffected. *Journal of Experimental Botany* **61**, 55–64.

Jones, J. D. G. and Dangl, J. L. (2006). The plant immune system. *Nature* **444**, 323–329.

Karmann, J., Müller, B. and Hammes, U. Z. (2018). The long and winding road: Transport pathways for amino acids in Arabidopsis seeds. *Plant Reproduction* **31**, 253–261.

Koch, E. and Slusarenko, A. (1990). Arabidopsis is susceptible to infection by a downy mildew fungus. *The Plant Cell* **2**, 437–437.

Kourelis, J. and van der Hoorn, R. A. L. (2018). Defended to the nines: 25 years of resistance gene cloning identifies nine mechanisms for R protein function. *The Plant Cell* **30**, 285–299.

Krapp, A. (2015). Plant nitrogen assimilation and its regulation: A complex puzzle with missing pieces. *Current Opinion in Plant Biology* **25**, 115–122.

Ladwig, F., Stahl, M., Ludewig, U., Hirner, A. A., Hammes, U. Z., Stadler, R., Harter, K. and Koch, W. (2012). Siliques Are Red1 from Arabidopsis acts as a bidirectional amino acid transporter that is crucial for the amino acid homeostasis of siliques. *Plant Physiology* **158**, 1643–1655.

Lee, Y.-H., Foster, J., Chen, J., Voll, L. M., Weber, A. P. M. and Tegeder, M. (2007). AAP1 transports uncharged amino acids into roots of Arabidopsis. *The Plant Journal* **50**, 305–319.

Links, M. G., Holub, E., Jiang, R. H., Sharpe, A. G., Hegedus, D., Beynon, E., Sillito, D., Clarke, W. E., Uzuhashi, S. and Borhan, M. H. (2011). De novo sequence assembly of *Albugo candida* reveals a small genome relative to other biotrophic oomycetes. *BMC Genomics* **12**, 503–503.

McDowell, J. M. (2011). Genomes of obligate plant pathogens reveal adaptations for obligate parasitism. *Proceedings of the National Academy of Sciences* **108**, 8921–8922.

McDowell, J. M. (2014). *Hyaloperonospora Arabidopsidis*: A model pathogen of Arabidopsis. In *Genomics of Plant-Associated Fungi and Oomycetes: Dicot Pathogens* (eds. R. A. Dean, A. Lichens-Park and C. Kole), pp. 209–234. Springer.

Müller, B., Fastner, A., Karmann, J., Mansch, V., Hoffmann, T., Schwab, W., Suter-Grotemeyer, M., Rentsch, D., Truernit, E., Ladwig, F. et al. (2015). Amino acid export in developing arabidopsis seeds depends on UmamiT facilitators. *Current Biology* **25**, 3126–3131.

Näsholm, T., Kielland, K. and Ganeteg, U. (2009). Uptake of organic nitrogen by plants: Tansley review. *New Phytologist* **182**, 31–48.

Okumoto, S., Koch, W., Tegeder, M., Fischer, W. N., Biehl, A., Leister, D., Stierhof, Y. D. and Frommer, W. B. (2004). Root phloem-specific expression of the plasma membrane amino acid proton co-transporter AAP3. *Journal of Experimental Botany* **55**, 2155–2168.

Okumoto, S., Schmidt, R., Tegeder, M., Fischer, W. N., Rentsch, D., Frommer, W. B. and Koch, W. (2002). High affinity amino acid transporters specifically expressed in xylem parenchyma and developing seeds of Arabidopsis. *Journal of Biological Chemistry* **277**, 45338–45346.

Pratelli, R. and Pilot, G. (2014). Regulation of amino acid metabolic enzymes and transporters in plants. *Journal of Experimental Botany* **65**, 5535–5556.

Ranocha, P., Denancé, N., Vanholme, R., Freydier, A., Martinez, Y., Hoffmann, L., Köhler, L., Pouzet, C., Renou, J. P., Sundberg, B. et al. (2010). Walls are thin 1 (WAT1), an Arabidopsis homolog of *Medicago truncatula* NODULIN21, is a tonoplast-localized protein required for secondary wall formation in fibers. *The Plant Journal* **63**, 469–483.

Ranocha, P., Dima, O., Nagy, R., Felten, J., Corratgé-Faillie, C., Novák, O., Morreel, K., Lacombe, B., Martinez, Y., Pfrunder, S. et al. (2013). *Arabidopsis* WAT1 is a vacuolar auxin transport facilitator required for auxin homoeostasis. *Nature Communications* **4**, 2625.

Rentsch, D., Schmidt, S. and Tegeder, M. (2007). Transporters for uptake and allocation of organic nitrogen compounds in plants. *FEBS Letters* **581**, 2281–2289.

Richards, T. A., Dacks, J. B., Jenkinson, J. M., Thornton, C. R. and Talbot, N. J. (2006). Evolution of filamentous plant pathogens: Gene exchange across eukaryotic kingdoms. *Current Biology* **16**, 1857–1864.

Sanders, A., Collier, R., Trethewy, A., Gould, G., Sieker, R. and Tegeder, M. (2009). AAP1 regulates import of amino acids into developing Arabidopsis embryos. *The Plant Journal* **59**, 540–552.

Schlaich, N. L. and Slusarenko, A. (2009). Downy Mildew of Arabidopsis Caused by *Hyaloperonospora arabidopsidis* (Formerly *Hyaloperonospora parasitica*). In *Oomycete Genetics and Genomics* (eds. K. Lamour and S. Kamoun), pp. 263–285. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Schmidt, R., Stransky, H. and Koch, W. (2007). The amino acid permease AAP8 is important for early seed development in Arabidopsis thaliana. *Planta* **226**, 805–813.

Schornack, S., van Damme, M., Bozkurt, T. O., Cano, L. M., Smoker, M., Thines, M., Gaulin, E., Kamoun, S. and Huitema, E. (2010). Ancient class of translocated oomycete effectors targets the host nucleus. *Proceedings of the National Academy of Sciences* **107**, 17421–17426.

Simpson, A. G. and Roger, A. J. (2004). The real 'kingdoms' of eukaryotes. *Current Biology* **14**, R693–R696.

Slusarenko, A. J. and Schlaich, N. L. (2003). Downy mildew of *Arabidopsis thaliana* caused by *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*). *Molecular Plant Pathology* **4**, 159–170.

Spanu, P. and Kämper, J. (2010). Genomics of biotrophy in fungi and oomycetes-emerging patterns. *Current Opinion in Plant Biology* **13**, 409–414.

Spanu, P. D., Abbott, J. C., Amselem, J., Burgis, T. A., Soanes, D. M., Stüber, K., van Themaat, E. V. L., Brown, J. K. M., Butcher, S. A., Gurr, S. J. et al. (2010). Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. *Science* **330**, 1543–1546.

Svennerstam, H., Ganeteg, U. and Näsholm, T. (2008). Root uptake of cationic amino acids by Arabidopsis depends on functional expression of amino acid permease 5. *New Phytologist* **180**, 620–630.

Tegeder, M. (2012). Transporters for amino acids in plant cells: Some functions and many unknowns. *Current Opinion in Plant Biology* **15**, 315–321.

Tegeder, M. (2014). Transporters involved in source to sink partitioning of amino acids and ureides: Opportunities for crop improvement. *Journal of Experimental Botany* **65**, 1865–1878.

Tegeder, M. and Rentsch, D. (2010). Uptake and partitioning of amino acids and peptides. *Molecular Plant* **3**, 997–1011.

Wang, S., Boevink, P. C., Welsh, L., Zhang, R., Whisson, S. C. and Birch, P. R. J. (2017). Delivery of cytoplasmic and apoplastic effectors from *Phytophthora infestans* haustoria by distinct secretion pathways. *New Phytologist* **216**, 205–215.

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., Armstrong, M. R., Grouffaud, S., Van West, P., Chapman, S. et al. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* **450**, 115–118.

Xu, G., Fan, X. and Miller, A. J. (2012). Plant nitrogen assimilation and use efficiency. *Annual Review of Plant Biology* **63**, 153–182.

Zhang, L., Tan, Q., Lee, R., Trethewy, A., Lee, Y.-H. and Tegeder, M. (2010). Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in arabidopsis. *The Plant Cell* **22**, 3603–3620.

Chapter 2

Review: Functional linkages between amino acid transporters and plant responses to pathogens

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2.1 Summary of key steps in nitrogen metabolism in plants

Nitrogen is arguably the most limiting factor of growth for all autotrophic organisms. Plants employ complex strategies to take up, reuse, recycle and transport nitrogen to different organs in order to optimize growth under changing conditions (Tegeder and Masclaux-Daubresse, 2018). Plants take up nitrogen from the soil as peptides, amino acids, ammonium or nitrate, the latter of which is reduced into ammonium, to be incorporated into glutamine. A large number of successive reactions enable the nitrogen from glutamine to be used for the synthesis of all amino acids (Pratelli and Pilot, 2014). Apart from their role in protein synthesis, amino acids are extensively used for synthesis of nitrogenous metabolites, and as storage and transport forms of nitrogen. Amino acids flow from source tissues where they are synthesized (mainly mature leaves) to the sink tissues where they are utilized (typically developing organs, like seeds, roots, and growing leaves). Amino acid fluxes between intracellular compartments and between various organs are controlled by amino acid transporters (Tegeder and Masclaux-Daubresse, 2018). These proteins are integral to cell membranes and transfer of amino acids from one side of the membrane to the other, typically between the cytosol and the lumen of the organelles or between the cytosol and the apoplasm. Numerous amino acid transporter genes are found in plant genomes (e.g., about 100 in Arabidopsis); the corresponding proteins serve diverse functions depending on the membrane to which they are targeted, and where (organ or tissue) and when they are expressed (Tegeder and Rentsch, 2010). Functions include transport to and from the vacuole, the chloroplast or the mitochondrion, transport across the plasma membrane that in turn controls the flux to and from the xylem and phloem, and consequently translocation between the roots, stem, leaves, and reproductive organs. Importantly, the role of each amino acid transporter encoded in plant genomes is far from being understood; only about 20 of the 100 Arabidopsis genes are currently fully characterized (Tegeder, 2012; Dinkeloo et al., 2018).

2.2 Plant pathogens and pests have evolved diverse nutrient acquisition strategies

Plants are subject to diseases caused by diverse pathogens and pests, including bacteria, fungi, oomycetes, and nematodes (Agrios, 2005). These organisms have evolved diverse strategies for acquiring nutrients from their plant hosts. Bacterial plant pathogens proliferate in the apoplasm and extract nutrients directly from apoplasmic fluid (Rico et al., 2011). Fungi and oomycetes produce filamentous hyphae that grow in the apoplasm, and many species produce feeding structures (e.g., haustoria from oomycetes and fungi, or stylets from nematodes, Fig. 2.1) that penetrate plant cell walls and form intimate associations with plant cell membranes (Szabo and Bushnell, 2001). Moreover, nematodes along with some bacteria and fungi can even alter root or leaf cell development to form giant cells or syncytia that serve as metabolic sinks (Agrios, 2005; Williamson and Gleason, 2003). Some plant pathogens are necrotrophic, meaning that they destroy plant cells and feed from the nutrients that are released (Mengiste, 2012). Others are biotrophic, meaning that they can obtain nutrients from living plant cells (Yi and Valent, 2013). Some biotrophic pathogens cannot be cultured apart from their hosts and are considered to be obligate (McDowell, 2011). This article focuses on plant interactions with bacteria, fungi, oomycetes, and nematodes that employ biotrophy as a major component of their colonization strategy.

2.3 Plant-derived amino acids are likely to be important nutrients for diverse pathogens

One of the major challenges faced by plant pathogens is to acquire and metabolize the nitrogen provided by the plant during infection. Within the plant, the most prevalent forms of metabolizable nitrogen include nitrate, ammonium, amino acids, and short peptides. While the majority of plant-pathogenic bacteria and fungi are capable of metabolizing most or all of these compounds, several lines of evidence underscore the importance of amino acids as a source of nitrogen and carbon (Solomon et al., 2003; Divon and Fluhr, 2007). First, amino acids are often abundant at pathogen infection sites. For example, 18 of 20 amino acids are present at detectable levels in the apoplasm of tomato infected by the biotrophic fungus *Cladiosporum fulvum*; The concentration of several amino acids reached millimolar amounts; the most prevalent of which was the non-proteogenic amino acid γ-amino-butyric acid (Solomon and Oliver, 2001). A subsequent study indicated that *Cladiosporum fulvum* metabolizes γ-amino-butyric acid and has thus adapted to the nutritional environment in the host (Solomon and Oliver, 2002). Importantly, a mutant of *Cladiosporum fulvum* that cannot utilize nitrate retains full pathogenicity (Solomon et al., 2003). The same was shown for nitrate non-utilizing mutants of the fungal phytopathogens *Magnaporthe oryzae* (Lau and Hamer, 1996) and *Stagonospora nodorum* (Cutler et al., 1998), while a *Magnaporthe oryzae* methionine auxotroph (Balhadère et al., 1999) and a *Fusarium oxysporium* arginine auxotroph (Namiki et al., 2001) are non-pathogenic. These results suggest that host-derived amino acids are much more important than nitrate for growth in planta. Moreover, comparative genomics has demonstrated that genes for reduction and/or transport of nitrate have

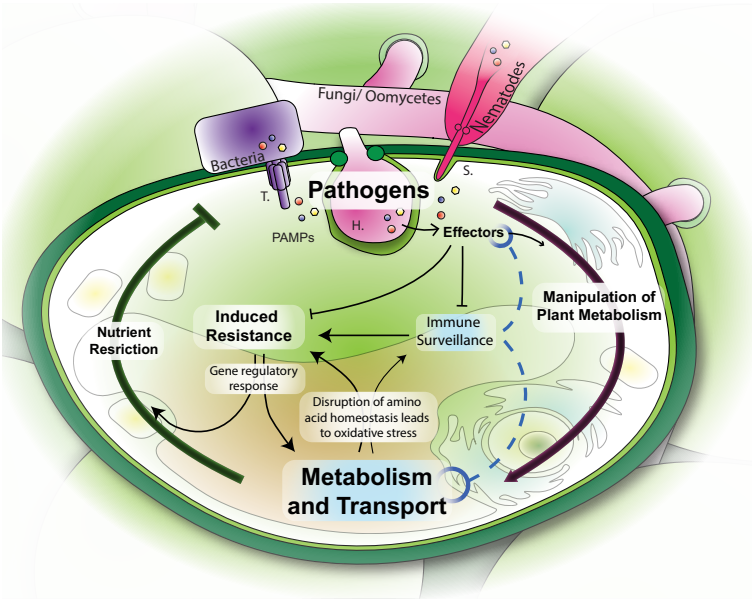


Figure 2.1: (A) **Proposed model for the cross-talk between metabolism and immune responses during pathogen infection.** Pathogen presence and/or secretion of effectors, and alteration of nutrient homeostasis are detected by the immune surveillance mechanism (dashed blue lines). Induced resistance is then obtained by activating several pathways, one of which is metabolism (for both production of defense compounds and restriction of nutrient availability for the pathogens; left, dark green arrow). At the same time, effectors suppress immune response and manipulate the host metabolism to extract nutrients (right, brown arrow). Effects of genetic alteration (e.g. by using knockout or over-expressor mutants) of host amino acid metabolism or transport genes is detected by the immune surveillance mechanism, and triggers the immune response, independent on the presence of pathogens. T: Type 3 Secretion system from bacteria; H: haustorium from oomycetes and fungi; S: stylet from nematodes.

been deleted independently in at least two lineages of obligate fungi (i.e., ascomycete powdery mildews (Spanu et al., 2010) and basidiomycete rusts (Duplessis et al., 2011)) and in two oomycete lineages (i.e., downy mildews (Baxter et al., 2010) and white rusts (Kemen et al., 2011)). It is likely that this energetically expensive pathway was lost as the pathogens became evolutionarily committed to a plant-dependent lifestyle, necessitating that the pathogens acquire nitrogen via plant-derived organic compounds (McDowell, 2011).

Additional examples imply that plant pathogenic oomycetes, bacteria, and fungi have evolved to exploit host-derived amino acids. For example, induction of genes for amino acid transport and/or metabolism has been demonstrated for bacteria (Rico et al., 2011; O'Leary et al., 2016; Rico and Preston, 2008), fungi (Hahn et al., 1997; Struck et al., 2002; Lanver et al., 2018; Garnica et al., 2013; Struck et al., 2004) and oomycetes (Ah-Fong et al., 2017; Attard et al., 2014; Abrahamian et al., 2016) growing inside their hosts. Comparative genomics and functional assays indicate plant pathogenic *Pseudomonas* bacterial species have evolved to utilize the amino acids that are most abundant in the host apoplasm during infection and have lost genes for assimilation of amino acids that are not abundant in the apoplasm (O'Leary et al., 2016; Mithani et al., 2011). Pea cotyledons infected with virulent strain *Rhodococcus fascians* were found to have elevated expression of several amino acid transporter and sugar transporter genes compared to cotyledons infected with an avirulent/epiphytic strain, where the genes were induced much later in the infection. The authors note that these transporters are probably used by the bacteria to maintain the cotyledons as sink for the pathogen rather than as a source of amino acids and sugar for the plant (Dhandapani et al., 2017). Finally, maize leaves infected with the biotrophic smut fungus *Ustilago maydis* were found to behave as strong nitrogen sinks, with increased amount of nitrogen-rich amino acids in pathogen-induced tumors of the colonized leaves (Horst et al., 2010b,a). Assimilation of nitrate and ammonium was reduced in tumors, but export of amino acids from systemic leaves in plants infected with the fungus was doubled.

Several lines of evidence suggest that amino acid availability at local infection sites could define the success or the failure of the biotrophic pathogen *Pseudomonas syringae* to stablish robust infections. One line of evidence comes from the study of plant inducible defense pathways. The exposure of plants to purified molecules that are produced by pathogens (i.e. bacterial flagellin or Elongation Factor-Tu), called Pathogen-Associated Molecular Patterns (PAMPs, see glossary), induces enhanced resistance to pathogens. Activation of PAMP-triggered immunity with the purified Elongation Factor-Tu peptide correlates with a decrease in the concentrations of several amino acids and other metabolites in the liquid exudates of Arabidopsis seedlings (Anderson et al., 2014). Several of these metabolites and amino acids have been shown to support *Pseudomonas syringae* growth as the only source of carbon and nitrogen in vitro (Chatnaparat et al., 2015), suggesting that limiting the availability of carbon and nitrogen to the invading microbe may contribute to plant defense. Indeed, the concentrations of the same metabolites and amino acids in exudates from seedlings of the constitutively activated defense mutant *mkp1* were significantly lower than those in wild type plants (Anderson et al., 2014). Furthermore, the enhanced resistance of *mpk1* to *Pseudomonas syringae* infections was abrogated when the metabolite concentrations were restored to wild type levels via exogenous supplementation, suggesting a functional link

between pathogens growth and the availability of readily usable sources of carbon and nitrogen (i.e. amino acid) (Anderson et al., 2014). The authors concluded that the decrease in concentrations of citric acid, 4-benzoic acid and aspartic acid prevented *Pseudomonas syringae* from inducing the Type-3 Secretion System (T3SS; a protein complex involved in delivering effectors into the plant cell) at early time points during the infection. As the T3SS is necessary for *Pseudomonas syringae* to produce infection, the authors proposed that the decrease in apoplasmic concentrations of plant metabolites that are necessary to induce the expression of the T3SS in bacteria is a likely mechanism by which plants suppress bacterial growth during the immune response. The authors however, did not rule out the possibility that the decrease in amino acid concentrations could directly affect bacterial growth independently of the failure to induce the T3SS (Anderson et al., 2014). It is clear than modification of the apoplasm composition by the plant, likely using transporters, affects *Pseudomonas syringae* success. It remains to be determined if this is due to the subsequent withdrawal of a nitrogen source for the pathogen or to an inability of the bacteria to deliver effectors in these conditions.

Transcriptomic analysis of plants interacting with biotrophic pathogens revealed that the mRNA content of genes encoding amino acid transporters is modified during infection (Fig. 2.2A). Notably, the transporters *LHT7, LHT1, CAT1* show remarkable increased mRNA accumulations when the plant is challenged with most pathogens (namely biotrophic or necrotrophic bacteria, fungi and oomycetes). In a more focused study, infection by the root-knot nematode *Meloidogyne incognita* has been shown to modify the expression of four amino acid importers (Hammes et al., 2005) (see below for discussion on the role of the *AAP* genes). Moreover, closer inspection of the transcriptome of plants infected by the biotrophic pathogens *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis* revealed that the expression of amino acid transporters is different during compatible or incompatible interactions (in which the pathogen can or cannot cause disease on the host, respectively) (Fig. 2.2B); this is particularly obvious for genes from groups c, e and f of Fig. 2.2B. This observation suggests that successful pathogen colonization leads to a re-programming of the expression of plant amino acid transporters.

Taken together, the observations presented in this section provide evidence that diverse pathogens use plant-derived amino acids for their nutrition and, in at least some cases, might actively manipulate host amino acid transport pathways to increase the pool of amino acids at infection sites. It is important to emphasize that the transcriptomic data described above provide only correlative evidence; definitive evidence for a role of any transporter requires integration of multiple lines of evidence from genetic, molecular, and physiological experiments. This aspect is discussed in detail in sections 6 and 7. In this context, it is useful to consider the pioneering research that has identified plant carbohydrate transporters that either promote or restrict pathogens' access to sugars. In the next section, we summarize several of such studies that provide a framework for hypotheses about how plant amino acid transporters could influence pathogen interactions.

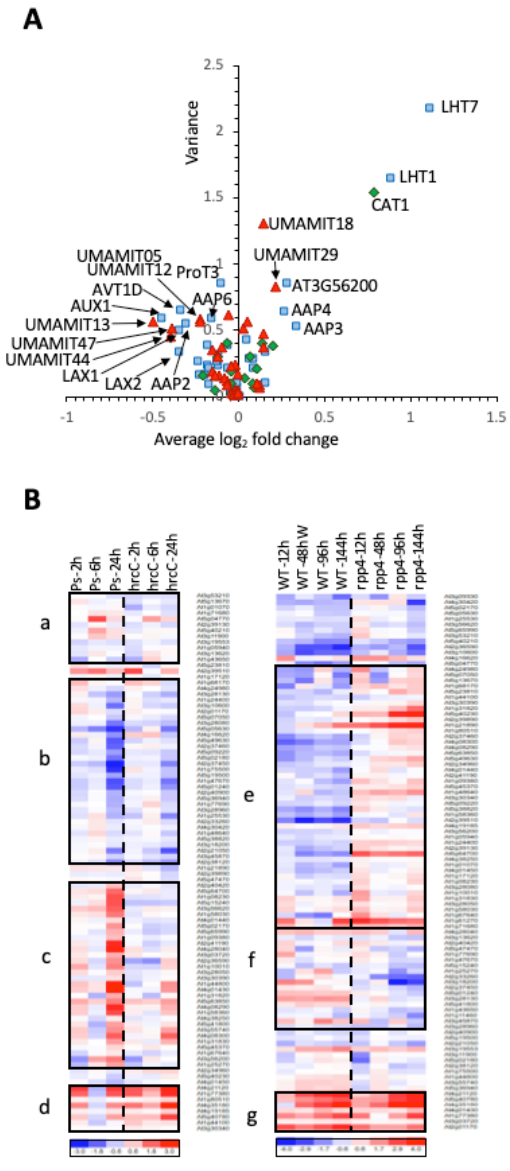


Figure 2.2: (A) Relationship between variance and average of expression change for the Arabidopsis amino acid transporter family genes in plants infected by various pathogens. Data were retrieved in May 2018 from Genevestigator (https://genevestigator.com/gv/). Labeled genes are among the ones responding to most to the infections. Blue squares: Amino Acid Auxin Permease (AAAP) family genes, Red triangles: Amino acid, Polyamine and organoCation (APC) family genes; Green diamonds: Usually Many Amino acids Move In and out Transporter (UMAMIT) family genes.(B) Clustering analysis of mRNA accumulation from the Arabidopsis APC, AAAP and UMAMIT family genes during the infection by the biotrophic pathogens *Pseudomonas syringae* (left panel) and *Hyaloperonospora arabidopsidis* (Hpa, right panel). Arabidopsis thaliana was infected by the virulent wild type *Pseudomonas syringae pv, Tomato* strain DC3000 (Ps) or the T3SS mutant (*hrcC*), which is non-pathogenic due to induction of pattern-triggered immunity. Arabidopsis Col-0 (resistant) or the defense response Arabidopsis mutant *rpp4* (susceptible) were infected by Hpa Emwa1 isolate for the indicated amounts of time. Log2 expression data for the APC, AAAP and UMAMIT family genes data were retrieved from the publicly available AtGenExpress dataset (`https://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp`; The *Pseudomonas syringae* data are from the Nürnberger laboratory (unpublished) and the *Hpa* data are from (Wang et al., 2011)) using e-Northern (Toufighi et al., 2005) and clustered using Dchip (`http://www.dchip.org/`). Major clusters (named a to g) are indicated for illustration purposes

2.4 Examples of plant nutrient transporters in pathogenicity: carbohydrate transporters can either promote or restrict pathogens' access to nutrients

2.4.1 Plant sucrose transporters are induced by pathogens to promote nutrient acquisition

Recent molecular and genetic studies have revealed that plant-encoded carbohydrate transporters can have major impacts on pathogens' capacity to proliferate in plant. This is exemplified by naturally occurring alleles of the *SWEET* genes in rice and other plants. Some SWEET proteins can export sucrose from the cytoplasm (Chen et al., 2010), and serve several functions in normal plant growth and development; for example Arabidopsis SWEET11 and SWEET12 are expressed in parenchymal cells to export sucrose to the apoplasm for phloem loading (Chen et al., 2012). However, some *SWEET* genes are also co-opted by pathogens to transport sugar to the apoplasm for uptake by the pathogen. The best studied case of co-option involves the rice *OsSWEET11* gene, which is ectopically activated by *Xanthomonas* species that secrete transcriptional activator-like (TAL) effector proteins that bind to the *OsSWEET11* promoter to activate *OsSWEET11* gene transcription in cells adjacent to the bacteria (Yang et al., 2006). The pathogen-induced OsSWEET11 protein enhances pathogen virulence, presumably by releasing sucrose to the apoplasm where the bacteria reside. Importantly, this exploitation has driven natural selection of *OsSWEET11* alleles that contain mutations in the promoter that abolish binding of the bacterial TAL effectors, such that the transporter gene can no longer be induced at feeding sites. These mutations render rice resistant; indeed, the *OsSWEET11* gene was initially discovered in breeding programs for *Xanthomonas*-resistant rice and named *xa13/Os8N3* (Yang et al., 2006). Additional examples of SWEET-based disease resistance have subsequently been documented, including a demonstration that Arabidopsis *SWEET2* plays a role in susceptibility to the root oomycete pathogen *Pythium irregulare* (Chen et al., 2015). Thus, this class of plant genes can promote disease by facilitating nutrient accumulation at pathogen feeding sites.

2.4.2 Plant monosaccharide transporters are activated during the immune response to starve pathogens

In contrast to the *SWEET* genes that are regulated by pathogens to enhance nutrient availability, some plant sugar transporters are regulated by the plant immune system to restrict nutrient availability. This is exemplified by the Arabidopsis monosaccharide transporter gene *STP13*, which is activated during immunity against bacteria to transport sugar from the apoplasm to the cytoplasm of mesophyll cells, thereby depleting the apoplasm of pathogen-accessible sugars (Yamada et al., 2016). *STP13* also plays a role in resistance to the necrotrophic fungus *Botrytis cinerea*, perhaps by depleting sugar from the apoplasm during the initial phase of colonization, prior to the onset of necrotrophy (Lemonnier et al., 2014). For both bacterial and fungal pathogens, mutation of *STP13* increases the plant's susceptibility to disease, while transgenic overexpression of *STP13* renders the plant more resistant (Yamada et al., 2016; Lemonnier et al., 2014). Moreover, the wheat

gene *Lr67*, which encodes the wheat ortholog of Arabidopsis *STP13*, is the source of resistance to multiple biotrophic rust and powdery mildew fungi (Moore et al., 2015), providing another example of naturally occurring resistance genes that correspond to nutrient transporters. In wheat however, resistance is provided by a non-functional allele of the transporter (Moore et al., 2015), contrasting with the situation described above for Arabidopsis *STP13*. This is likely explained by the different feeding strategies employed by the respective pathogens: rust and mildew fungi use haustoria to extract nutrients from the cytoplasm of living haustoriated cells, while bacteria and non-haustorial fungi extract nutrients from the apoplasm. It can be speculated that a functional wheat STP13, which transfers sugar from the apoplasm to the cytoplasm, may increase the amount of sugar available in the haustoriated plant cells, thereby promoting sugar availability to rust and mildew (Dodds and Lagudah, 2016). Thus, a loss-of-function mutation in this gene could restrict nutrient availability.

These examples illustrate how sugar transporter genes can promote or retard growth of bacterial and fungal pathogens in different ways, depending on the pathogen feeding strategy and the nature of the transport mediated by the protein. Moreover, such transporters can evolve to confer resistance. In the following sections, we critically evaluate evidence that plant amino acid transporters might operate in a similar fashion to either facilitate or restrict accumulation of an important source of nitrogen.

2.5 Amino acid transporters associated with pathogen/pest interactions

2.5.1 Plant amino acid permeases (AAPs) are linked to susceptibility to nematode infection

The expression of several amino acid transporters have been shown to be modified during infection by nematodes, namely *CAT6* (Hammes et al., 2006), *LHT1* (Elashry et al., 2013) and *AAPs* (Elashry et al., 2013; Pariyar et al., 2018), suggesting that they play a role in plant-nematode interactions. Genetic evidence of this role has been obtained for several *AAP* genes, but not for *CAT6* and *LHT1*. AAPs belong to the amino acid/auxin permease family (AAAP) of transporters, and eight members are present in Arabidopsis. They have been characterized as proton-coupled importers performing diverse roles such as taking up amino acids from soil, leaf export/phloem loading and transfer of amino acids from xylem to phloem (Tegeder and Ward, 2012). In addition to their physiological roles in the plant, AAPs are also involved in infection by parasitic nematodes. Reverse genetic experiments have demonstrated that Arabidopsis *AAP3* and *AAP6* are required for infestation by the root-knot nematode *Meloidogyne incognita* (Marella et al., 2013). Based on GUS reporter assays, expression of both transporters is increased in root tips infected by the nematode, and AAP6 expression is also upregulated in the giant cells that serve as feeding sites to the parasite. *aap3* and *aap6* knockout mutants display lower root-knot nematode infestation level compared to wild type plants (Marella et al., 2013). Double mutants of *aap3/aap6* however, do not display an additive effect in reduction of infestation level. This result, along with the GUS reporter assays,

indicates that AAP3 and AAP6 have synchronized functions wherein AAP3 acts distally to the site of infestation, potentially by transferring amino acids to the vascular tissue, and AAP6 acts proximally, by transferring amino acids into the giant cells (Marella et al., 2013).

The expression of all but two Arabidopsis *AAPs* (*AAP5* and *AAP7*) is also induced in the syncytia of the beet cyst nematode *Heterodera schachtii*, and loss-of-function mutations in *AAP1*, *AAP2* and *AAP8* lead to reduction in the number of female nematodes on these plants (Elashry et al., 2013). In another study, transcriptomic analysis of syncytia collected by micro-aspiration found *AAP6* and *AAP8* mRNA accumulation to be increased in the feeding structure (Szakasits et al., 2009). Furthermore, the wheat ortholog of AAP6 (*TaAAP6*) is also induced in infested roots of susceptible wheat accessions colonized by the cyst nematode *Heterodera filipjevi* (Pariyar et al., 2018). In contrast to previous research that showed no effect of the suppression of Arabidopsis *AAP6* expression on the reproductive success of the cyst nematode (Elashry et al., 2013), this study found that Arabidopsis *aap6* mutants displayed enhanced resistance to Heterodera schachtii (Pariyar et al., 2018). In addition to possible technical differences, this discrepancy could be attributed to the different Arabidopsis ecotypes used in the two studies (Col-0 vs. Ws-0).

The above examples indicate that AAPs serve as susceptibility genes during nematode infection and possibly play a role in the different steps involved in transferring amino acids from the plant to the nematode feeding structures. Not as much is known about the role of AAPs in case of other pathogens or pests. Public expression data sets indicate however that the expression of many of the AAPs change in response to infection by biotrophic pathogens. For example, expression of *AAP3* and *AAP4* is induced in response to various pathogens whereas expression of AAP2 and AAP6 is decreased (Fig. 2.2B). While expression of *AAP3* and *AAP4* is induced in response to virulent *Pseudomonas syringae* bacteria as well as PAMPs, *AAP1* expression is specifically increased only in response to infection by virulent *Pseudomonas syringae* bacteria, indicating that the AAPs are probably involved in different functions during the course of an infection (Yang et al., 2014). *AAP3* and *AAP4* are possibly involved in early nutrient restriction from a broad range of pathogens, while genes such as *AAP1* are likely more specific (Yang et al., 2014). Based on transcriptomic data and the research with nematodes, studying the role of AAPs in context of infection by other pathogens and pests is expected to be fruitful.

2.5.2 Amino acid transporters with genetic connections to plant immunity

In contrast to amino acid transporters that might be manipulated for pathogen feeding, other amino acid transporters appear to be linked to the plant's immune response. For example, some amino acid transport genes are induced upon exposure to PAMPs, suggesting that these genes are responsive to plant immune signaling pathways. An example of a PAMP-responsive transporter is the Arabidopsis Lysine-Histidine Transporter 1 (LHT1), a plasma-membrane localized transporter with a high affinity for glutamine (Chen and Bush, 1997; Hirner, 2006). The *lht1-1* knockout mutant confers broad resistance to both bacterial (*Pseudomonas syringae*) and fungal (*Colletotrichum higginsianum* and *Erysiphe cichoracearum*) pathogens (Liu et al., 2010). Aside from the presence of a constitutive stress response, the authors hypothesized that the resistance to pathogens could come from an increased speed of callose deposition, reactive oxygen species accumulation, and

cell death at the site of pathogen colonization (Liu et al., 2010). The authors attribute this enhanced resistance to LHT1's role in glutamine transport, such that, in its absence, leaf cells become glutamine deficient, which results in a disruption of the redox status of the cells (Liu et al., 2010). The authors also suggested that LHT1 is a negative regulator of the salicylic acid (SA) -dependent defense pathway, but the observation that *LHT1* expression is induced by SA and PAMPs is at odds with this conclusion (Liu et al., 2010). The metabolic disturbance caused by *LHT1* disruption could thus trigger of activation of the SA pathway, while, independently, LHT1 could be used by the plant to transfer amino acids or other compounds from the apoplasm to the cytosol as part of a defense mechanism. Interestingly, LHT1 can also take up 1-aminocyclopropane-1-carboxylic acid, a precursor of ethylene, which has a role in plant defense responses (Shin et al., 2015; Anver and Tsuda, 2015).

Another connection between plant immunity and amino acid export was provided by studies of the single transmembrane domain-protein Glutamine Dumper 1 (GDU1). Overexpression of *GDU1* stimulates amino acid export from cells, thereby profoundly altering the leaf amino acid profile (Pratelli and Pilot, 2007). Interestingly, GDU1-overexpressing plants exhibit constitutive defense responses (Liu et al., 2010; Pilot et al., 2004). It is thus conceivable that plant cells could perceive disturbances in amino acid balance (compartmentation, fluxes, or/and content) and/or alteration of the redox status (Liu et al., 2010) (or other effects yet to be determined) and trigger the onset of defense mechanisms in response to these changes.

The Arabidopsis Cationic Amino Acid Transporter 1 (*CAT1*) gene encodes a basic amino acid transporter with a high affinity for lysine. *CAT1* is responsive to PAMP treatments and is upregulated upon infection with virulent Pseudomonas syringae. Arabidopsis lines overexpressing *CAT1* showed increased Lys uptake, reduced biomass, and elevated resistance to pathogens (Yang et al., 2014). The authors noted that disruption in the Lys biosynthetic pathway is associated with altered SA levels, but were unable to draw a direct link between SA levels and the Lys-transporting CAT1. The closest potential link lies within the synthesis or transport of the Lys catabolite pipecolic acid, a non-proteogenic amino acid with roles in signaling/amplification or defense responses and systemic acquired resistance (Yang and Ludewig, 2014).

Similarly, a forward genetic screen revealed that a knockout mutant of an Arabidopsis amino acid transporter (AtUMAMIT36) confers resistance to the oomycete *Phytophthora parasitica*. Logically, the authors named the gene *Resistant to Phytophthora parasitica 1* (*AtRTP1*). The mutant showed early activation of defense responses to *Phytophthora parasitica*, *Pseudomonas syringae*, and *Golovinomyces cichoracearum* via increased activity of the SA pathway, localized cell death and rapid accumulation of reactive oxygen species (Pan et al., 2016). Importantly, RTP1 belongs to the Usually Multiple Acids Move In and out Transporters (UMAMIT) family, of which few members have been characterized. The majority of characterized UMAMITs are able to transport amino acids (Dinkeloo et al., 2018), but unlike most of the APC and AAAP transporters, they appear to be facilitators, mediating passive amino acid transport downwards the electrochemical gradient. The authors hypothesize that RTP1/AtUMAMIT36 plays a role as a negative regulator of plant immunity, but the link to its amino acid transport function remains to be established (Pan et al., 2016).

The above studies provide compelling evidence that plant amino acid transport pathways are connected to pathogen/pest resistance and susceptibility, but much work is still needed to establish clear mechanistic connections and to generalize the observations made on Arabidopsis to other plant-pathogen systems.

2.6 Bottlenecks and Opportunities

In this section, we provide a critical perspective on challenges and opportunities to establish a broader and deeper understanding of these connections. To begin with, it is notable that most of the studies described above are based on reverse genetics. We expect that this will continue to be a productive approach in Arabidopsis-based studies as well as in other species for which reverse genetics resources are established, including amenability to knockdowns via gene silencing (Senthil-Kumar and Mysore, 2014) or knockouts via gene editing (Belhaj et al., 2015). Considering the size and complexity of plant amino acid transporter gene families (Dinkeloo et al., 2018), future reverse genetic experiments must account for complications from genetic redundancy, in which the effect of a loss of function mutation in one gene could be masked by another gene that encodes a functionally equivalent transporter (i.e. another member of the gene family). A related pitfall, less straightforward to address, is physiological compensation (e.g. (Chen et al., 2012)), through which transporter knockout plants have adapted to loss of the transporter gene through compensatory pathways by the time they are subjected to infection. As with any reverse genetic approach, genetic redundancy can be addressed through judicious choices of candidate genes based on data from gene expression profiling, for example by constructing double mutants in genes that have similar pathogen-responsive expression profiles (e.g. (Elashry et al., 2013; Marella et al., 2013)). Another potentially productive approach is to make inducible knockdown lines (e.g. via a transgene encoding artificial micro RNAs designed to silence one or more transporters, driven by an inducible promoter). This provides capacity to knockdown multiple genes simultaneously (Li et al., 2013; Schwab et al., 2010) and to test the effects during the interval immediately following the knockdown, before the plant is able to compensate or otherwise respond to the reduction/loss of function of the targeted gene(s). Considering the highly buffered configuration of amino acid transport pathways in plants, such approaches might well provide insights that differ from those based on conventional analysis of knockout mutants.

Another complication lies in interpretation of expression patterns of amino acid transporter genes in response to pathogen invasion. In principle, pathogen-induced changes in expression of host amino acid transporters could occur because of pathogen intervention (e.g. reprogramming of plant gene expression via pathogen effectors), could be triggered by the host upon sensing the presence of the pathogen, or could result from a change in plant physiology due to the infection. It is difficult to ascribe an expression change to one or the other. A resolution can possibly be achieved in a case where these changes in expression could be recapitulated in response to a known elicitor that triggers an immune cascade in the plant, independent of the presence of a pathogen (e.g., a PAMP). The other approach would be to recapitulate the expression of the transporter upon transgenic expression of a pathogen effector (that is not recognized by the host immune surveillance system), suggesting that the pathogen is the trigger for the change in expression.

Because amino acid metabolism and transport are closely linked to plant immunity, alteration of the expression of transporters via loss- or gain-of-function mutations could alter general defense responses and confound the role of specific transporters. For instance, LHT1 and CAT1 may play an essential positive or a negative role on *Pseudomonas syringae* infections via providing or restricting amino acids necessary to support bacterial growth. However, loss-of-function mutant of *LHT1* and the constitutive over-expression of *CAT1* leads to the accumulation of SA, which in turns enhances the resistance of these plants. An example of this sort of complication has been reported for loss-of-function mutants of Arabidopsis SWEET transporters. The Arabidopsis *sweet11/sweet12* double loss-of-function mutant exhibits an enhanced resistance phenotype against the fungus *Colletotrichum higginsianum* (Gebauer et al., 2017). The double mutants accumulated significantly more hexoses and sucrose compared to the wild type and single mutants in non-infected conditions and the increased accumulation became more pronounced after infection with *Colletotrichum higginsianum*. The increase in hexoses and sucrose was also accompanied by a higher increase in SA levels in the double mutant compared to the other genotypes (Gebauer et al., 2017). Furthermore, crossing the *sweet11/sweet12* double mutant to the *sid2* mutant, which cannot synthesize SA, produced plants that exhibited the enhanced susceptibility of *sid2*, instead of the enhanced resistance of *sweet11/sweet12*. This indicated that the enhanced resistance of the double mutant is dependent on an SA-mediated defense, which confounds the potential pathogen nutrition role of SWEET11 and SWEET12 (Gebauer et al., 2017). Amino acid transporter knockout mutants should hence be examined closely for changes in accumulation of amino acids before and after infection, and for temporal expression of key marker genes belonging to various defense pathways, such as SA, jasmonic acid and ethylene, to test whether the changes in the susceptibility to a pathogen is linked to changes in the immune response of the plant (for an example on the latter, see (Ried et al., 2018)). As described above (section 5.2) it is plausible that knocking out a host amino acid transporter leads to both, reduction in flux of amino acids to the pathogen and perturbation of immunity, and the effect of the former could be masked by the latter. This can again be resolved by creating knockout mutants for amino acid transporters of interest in a mutant background for defense response pathways (such as sid2 for SA response). Such a strategy could assist in deconvoluting the contribution of immune responses in an enhanced resistance phenotype resulting from loss-of-function mutants for amino acid transporter genes.

Extraction of nutrients from the plant by the pathogen is expected to result in changes in the flow of amino acids not only at the level of the plant cells (from the cytosol to the apoplasm or to the feeding structure), but also at the organ or whole plant level. The demand for amino acids to an organ heavily infected by a pathogen would likely exceed the capacity of the neighboring cells to provide amino acids. Therefore, it is reasonable to consider that nutrients would need to be diverted from distant cells in the same organ or from other organs/tissues to feed the pathogen: the infected organ becomes a sink, towards which amino acids are flowing. High concentrations of several amino acids have been reported in Arabidopsis leaves infected by Pseudomonas syringae (Návarová et al., 2012), which indicates that the physiology of infected mature leaves shifts from being a source to being a sink of nutrients. This also shows that during an infection with a Pseudomonas syringae virulent strain, which suppresses SA-mediated defense responses, the accumulation of amino acids positively correlates with bacterial growth. In this situation, the mature leaf, which is typically a

source organ, becomes a sink, and it is conceivable that the flow of phloem is inverted, flowing towards the leaf rather than outwards. Amino acid transporters could thus be involved in pathogen feeding by being active in not only cells in contact with the pathogen but also in more distant cells and tissues, like distant parenchyma cells, vascular parenchyma and phloem cells to promote amino acid flow towards the pathogen. The UMAMIT transporters, which have been shown to transport amino acids out of the cells following the concentration gradient, are the best candidates to serve pathogen feeding functions and supply the infected tissues with amino acids translocated from distal organs and tissues. These physiological changes can be studied by determining total or apoplasmic free amino acid content in the infected vs. uninfected organ, direction of phloem (for instance using radiolabeled sugars or amino acids) and change in phloem composition. Finding differences in content and flows of amino acids between a transporter mutant and the wild type plant during the course of an infection would provide the solid evidence that a given transporter is involved in the transfer of nutrient from the plant to the pathogen, provided that no significant changes are observed in non-infected mutant plants. Such physiological data provide a valuable component for interpreting the results from molecular and genetic experiments.

Activity of amino acid metabolism, and metabolism in general, is tightly adjusted to match changing growing condition, e.g. demand from growing organs (which depends heavily on abiotic factors), and availability of carbon, nitrogen and other nutrients. Relationship between immune response and nitrogen availability has been previously discussed (Fagard et al., 2014), and phosphate response has recently been linked to modulation of immune responses through the direct implication of Phosphate Starvation Response 1 (PHR1), a master regulator of phosphate starvation (Castrillo et al., 2017). In addition, mutations in several amino acid biosynthesis genes have been linked to pathogen resistance or susceptibility, further emphasizing the importance of the relationship between metabolic status and immune responses (van Damme et al., 2009; Stuttmann et al., 2011; Cecchini et al., 2011; Senthil-Kumar and Mysore, 2012; Liu et al., 2012; Alvarez et al., 2012; Zeier, 2013; Hiruma et al., 2013; Gao et al., 2014). This close relationship between amino acid metabolism/transport and immune response should be taken into account when assaying pathogen fitness on a transporter mutant: growth conditions need to be perfectly determined (light, temperature, nitrogen amount provided) and kept constant across many experiments to ensure consistent results. Unless constitutive defense responses are triggered by the mutation (see CAT1 over-expressor, *lht1-1* or *gdu1-1D* above), the disease resistance or susceptibility phenotype are expected to be subtle, and slight changes in growth conditions can result in different pathogen fitness/disease phenotypes.

2.7 Conclusion and outlook

The arms race between the pathogen effectors to suppress plant's immune response and the plant surveillance system to detect pathogen effectors or their activities is well-accepted (Jones and Dangl, 2006; Asai and Shirasu, 2015; Gust et al., 2017). In this model, recognition of effectors or their activities by plant resistance genes prompts the selection of pathogens expressing effectors that can avoid recognition or can target other presently un-monitored pathways. Based on the results concerning transporters and metabolism, we hypothesize the existence of a similar arms race

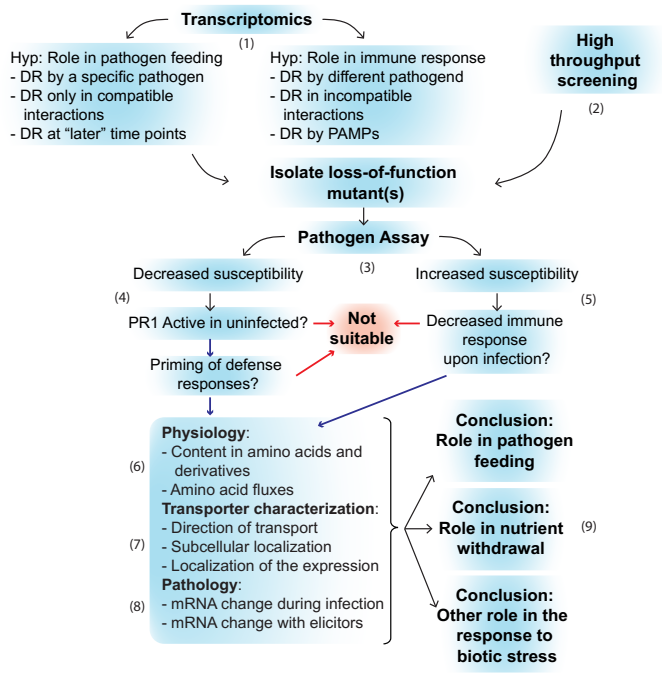


Figure 2.3: Proposed flowchart for the steps necessary for the elucidation of the role of an amino acid transporter as a susceptibility (involved in pathogen feeding) or resistance (involved in immune response) gene, combining pathogen assays, test for presence of immune response, functional properties of the amino acid transporter, and physiology of the plant with or without the presence of a pathogen. See main text, section 7 for more details. Blue and red arrows correspond respectively to "no" and "yes" to the questions from steps (4) and (5).

between the biotrophic pathogen modifying plant transporter expression to extract nutrients (sugars and amino acids) from the plant and the plant cell, which at the same time modifies transporter expression to restrict the access of the pathogen to nutrients. The best example supporting the existence of this arms race is the successive evolution of the TAL effector of *Xanthomonas* and the *OsSWEET11* gene to respectively hijack and restrict pathogen access to sugars (see 4.1 above). It also makes sense that the plant surveillance system also monitors metabolic activity, nutrient compartmentation and possibly nutrient fluxes across membranes, which could be indicators of the presence of a concealing pathogen (Fig. 2.1). This hypothesis would explain the constitutive enhanced resistance or priming of immune responses when the expression of amino acid or sugar transporters is genetically modified: an alteration in nutrient homeostasis may be interpreted by the plant as the signature of a lurking pathogen.

In this context, distinguishing between plant transporters involved in transfer of nutrients to the pathogen from the transporters used by the plant for defense purposes is challenging. We propose a succession of steps to this aim (Fig. 2.3). (1) Candidate transporters can be selected based on transcriptomics data, and mutants are isolated. It is important to emphasize that transcriptomics are only correlative, and that this approach could miss important genes that are regulated by other mechanisms. (2) Alternatively, candidate genes can be selected based on other approaches, like forward genetic screenings, high-throughput screening of proteins delocalized in cells in contact with the pathogen etc… (3) The mutations in the candidate genes can lead to increased or decreased susceptibility to the pathogen. (4) In case of decreased susceptibility (increased resistance), one must ensure that the mutation of the transporter does not affect immunity, either through constitutive

resistance or priming responses. (5) In case of increased susceptibility, one must ensure that the immune response is not decreased or delayed by the mutation. (6) When this has been ruled out, the role of the transporter in the modification of pathogen growth can be studied by measuring amino acid content, fluxes and compartmentation; (7) the functional characterization of the transporter, and its localization in the plant and in the cell in absence and upon infection will provide clues about the nature of the flux mediated by the protein; (8) determining the direction of the change in gene expression (mRNA / protein content or activity) upon infection and treatment with elicitor(s) will help establishing whether the change in expression is related to effects of pathogen effectors or plant defense mechanisms. Taken together, the results from (4) to (8) would determine if the transporter is required for transferring amino acids to the pathogen, if it is part of the plant defense response to withdraw amino acids from the pathogen or if it is involved in an indirect process, such as physiological and metabolic response to the biotic stress (9). While described here for the specific characterization of the role of amino acid transporters, this pipeline can be adapted to any other nutrient transporter that has a potential role in plant-pathogen interaction. It is important to emphasize that this pipeline is only a starting point to address the most common potential complications involved with understanding whether and how transporter genes affect plant-microbe interactions; however, this pipeline addresses many of the most common pitfalls.

This review has focused on foundational research. From a societal perspective, it is important to emphasize that this research could lay the groundwork for a new type of genetic resistance, which we term "nutritional resistance". This type of resistance encompasses any genetic modification to the plant host that reduces the supply of nutrients upon which the pathogen feeds. Such resistance could be achieved, in principle, by many types of genetic alterations, but we propose two mechanistic approaches. The first approach is based on creating loss-of-function mutations in genes encoding plant nutrient transporters that are co-opted during infection to transfer nutrients to pathogen feeding sites. A mutation in this type of gene would restrict the flow of nutrients to the pathogen and thereby retard pathogen growth. This type of mutation is exemplified by naturally occurring alleles of the SWEET sugar transporter genes described in 4.1 above. The second approach focuses on transporter genes that could be activated during immunity to sequester nitrogen or carbon away from the pathogen feeding site. This is exemplified by the monosaccharide transporters Arabidopsis STP13 and wheat Lr67 described above (Yamada et al., 2016; Moore et al., 2015). Genes of this type could be deployed as transgenes, driven by a promoter that is strongly induced at the site of infection. Such genes could deplete an important source of nitrogen and carbon from the apoplasm in situations where the pathogens' needs are most acute. Alternatively, loss-of-function mutants in such genes could be used to reduce nutrient availability inside haustoriated cells, mimicking the mechanism hypothesized for Lr67 (Moore et al., 2015).

Importantly, because all pathogens are dependent on nutrients supplied by the host, nutritional resistance genes could provide for broad-spectrum resistance. Thus, nutritional resistance genes could be more efficient than typical gene-for-gene resistance, in which a given R allele only recognizes one or a few strains of one pathogen species. Moreover, gene-for-gene resistance can often be defeated by a genetically simple loss-of-function of the corresponding effector gene, as described above. Contrastingly, pathogens would likely have to evolve a more complex

gain-of-function, perhaps in multiple genes, to increase fitness in a nutrient-limited environment and overcome a nutritional resistance gene. Thus, nutritional resistance could be more durable than gene-for-gene resistance. However, it is important to emphasize that no mechanism of host resistance is truly unbreakable; for example, Xanthomonas oryzae has evolved SWEET-targeting effectors multiple times to overcome SWEET-mediated resistance (Chen et al., 2010). Such gains-of-function are possible owing to the modularity of the TAL effectors as transcription factor mimics, the equivalent of which has not been found in other pathogen lineages. Contrastingly, *Lr67* has remained durable for decades. Thus, it is likely that the durability of nutritional resistance will likely vary from case to case, according to the details of the mechanisms of resistance. Accordingly, mechanistic understanding of nutrient exchange mechanisms will provide a sound basis for selecting candidate resistance genes that are most likely to be durable.

Despite these strong potential advantages, it is important to consider potential downsides of this strategy. The most obvious trade-off is potential pleiotropic effects from alteration of a transporter gene that results in insufficient nutrients to fuel normal development and growth of the plant. It is difficult to make predictions about the probability of such effects from mutation of any given transporter. Each candidate will likely need to be tested on a case-by-case basis. Another challenge is that different pathogens might have different nutritional requirements, and very little is known about which nutrients are potentially limiting for various classes of pathogens in the plant. Nevertheless, the doors are opening to a dramatic increase in understanding of how plant amino acid transporters mediate plant-pathogen interactions, and we anticipate that many potential candidate genes for nutritional resistance will emerge. Judicious development of this type of resistance could provide a significant step towards cost-effective mitigation of diseases that continuously limit agricultural productivity.

Overall, the study of carbon and nitrogen transfer from host plants to pathogens has a great potential to contribute with discoveries that will open new avenues to understand plant-microbe interactions. It also has the potential to contribute with transformative approaches to produce pathogen resistance crops. In the past few years, it has become evident that amino acids and sugar transport is intimately connected to general defense mechanisms, such that it makes difficult to elucidate whether amino acids or sugar transporters have functions related to supplying or depriving pathogens from gaining access to readily usable source carbon and nitrogen during the course of an infection. Hence, dissecting the physiological functions of individual transporters will require the use of molecular tools to fine-tune the tissue specific and transient down- or up-regulation of single genes or multiple genes simultaneously. With more 150 amino acid and sugar transporters in Arabidopsis still waiting to be characterized, this represents a major effort that requires a high level of investment in funds and time. The challenges ahead are big and require the immediate attention of funding agencies and the plant research community in general.

Chapter 2. References

Abrahamian, M., Ah-Fong, A. M., Davis, C., Andreeva, K. and Judelson, H. S. (2016). Gene expression and silencing studies in *Phytophthora infestans* reveal infection-specific nutrient transporters and a role for the nitrate reductase pathway in plant pathogenesis. *PLoS Pathogens* **12**.

Agrios, G. (2005). *Plant Pathology*. Academic Press, 5th edition.

Ah-Fong, A. M., Kim, K. S. and Judelson, H. S. (2017). RNA-seq of life stages of the oomycete *Phytophthora infestans* reveals dynamic changes in metabolic, signal transduction, and pathogenesis genes and a major role for calcium signaling in development. *BMC genomics* **18**, 198.

Alvarez, C., Bermudez, M. A., Romero, L. C., Gotor, C. and Garcia, I. (2012). Cysteine homeostasis plays an essential role in plant immunity. *New Phytologist* **193**, 165–77.

Anderson, J. C., Wan, Y., Kim, Y. M., Pasa-Tolic, L., Metz, T. O. and Peck, S. C. (2014). Decreased abundance of type III secretion system-inducing signals in Arabidopsis *mkp1* enhances resistance against Pseudomonas syringae. *Proceedings of the National Academy of Sciences* **111**, 6846–51.

Anver, S. and Tsuda, K. (2015). Ethylene and Plant Immunity. In *Ethylene in Plants* (ed. C.-K. Wen), pp. 205–221. Dordrecht: Springer Netherlands.

Asai, S. and Shirasu, K. (2015). Plant cells under siege: Plant immune system versus pathogen effectors. *Current Opinion in Plant Biology* **28**, 1–8.

Attard, A., Evangelisti, E., Kebdani-Minet, N., Panabieres, F., Deleury, E., Maggio, C., Ponchet, M. and Gourgues, M. (2014). Transcriptome dynamics of *Arabidopsis thaliana* root penetration by the oomycete pathogen *Phytophthora parasitica*. *BMC Genomics* **15**, 538.

Balhadère, P. V., Foster, A. J. and Talbot, N. J. (1999). Identification of pathogenicity mutants of the rice blast fungus *Magnaporthe grisea* by insertional mutagenesis. *Molecular Plant-Microbe Interactions* **12**, 129–142.

Baxter, L., Tripathy, S., Ishaque, N., Boot, N., Cabral, A., Kemen, E., Thines, M., Ah-Fong, A., Anderson, R., Badejoko, W. et al. (2010). Signatures of adaptation to obligate biotrophy in the *Hyaloperonospora arabidopsidis* genome. *Science* **330**, 1549–1551.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Patron, N. J. and Nekrasov, V. (2015). Editing plant genomes with CRISPR/Cas9. *Current Opinion in Biotechnology* **32**, 76–84.

Castrillo, G., Teixeira, P. J. P. L., Paredes, S. H., Law, T. F., de Lorenzo, L., Feltcher, M. E., Finkel, O. M., Breakfield, N. W., Mieczkowski, P., Jones, C. D. et al. (2017). Root microbiota drive direct integration of phosphate stress and immunity. *Nature* **543**, 513–518.

Cecchini, N. M., Monteoliva, M. I. and Alvarez, M. E. (2011). Proline Dehydrogenase Contributes to Pathogen Defense in Arabidopsis. *Plant Physiology* **155**, 1947–1959.

Chatnaparat, T., Li, Z., Korban, S. S. and Zhao, Y. (2015). The stringent response mediated by (p)ppGpp is required for virulence of *Pseudomonas syringae pv. Tomato* and its survival on Tomato. *Molecular Plant-Microbe Interactions* **28**, 776–789.

Chen, H. Y., Huh, J. H., Yu, Y. C., Ho, L. H., Chen, L. Q., Tholl, D., Frommer, W. B. and Guo, W. J. (2015). The Arabidopsis vacuolar sugar transporter SWEET2 limits carbon sequestration from roots and restricts Pythium infection. *The Plant Journal* **83**, 1046–1058.

Chen, L. and Bush, D. R. (1997). LHT1, a lysine- and histidine-specific amino acid transporter in Arabidopsis. *Plant Physiology* **115**, 1127–1134.

Chen, L. Q., Hou, B. H., Lalonde, S., Takanaga, H., Hartung, M. L., Qu, X. Q., Guo, W. J., Kim, J. G., Underwood, W., Chaudhuri, B. et al. (2010). Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature* **468**, 527–32.

Chen, L.-Q., Qu, X.-Q., Hou, B.-H., Sosso, D., Osorio, S., Fernie, A. R. and Frommer, W. B. (2012). Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. *Science* **335**, 207–211.

Cutler, S. B., Cooley, R. N. and Caten, C. E. (1998). Cloning of the nitrate reductase gene of *Stagonospora (Septoria) nodorum* and its use as a selectable marker for targeted transformation. *Current Genetics* **34**, 128–37.

Dhandapani, P., Song, J., Novak, O. and Jameson, P. E. (2017). Infection by *Rhodococcus fascians* maintains cotyledons as a sink tissue for the pathogen. *Annals of Botany* **119**, 841–852.

Dinkeloo, K., Boyd, S. and Pilot, G. (2018). Update on amino acid transporter functions and on possible amino acid sensing mechanisms in plants. *Seminars in Cell & Developmental Biology* **74**, 105–113.

Divon, H. H. and Fluhr, R. (2007). Nutrition acquisition strategies during fungal infection of plants. *FEMS Microbiology Letters* **266**, 65–74.

Dodds, P. N. and Lagudah, E. S. (2016). Starving the enemy. *Science* **354**, 1377–1378.

Duplessis, S., Cuomo, C. A., Lin, Y.-C., Aerts, A., Tisserant, E., Veneault-Fourrey, C., Joly, D. L., Hacquard, S., Amselem, J., Cantarel, B. L. et al. (2011). Obligate biotrophy features unraveled by the genomic analysis of rust fungi. *Proceedings of the National Academy of Sciences* **108**, 9166–9171.

Elashry, A., Okumoto, S., Siddique, S., Koch, W., Kreil, D. P. and Bohlmann, H. (2013). The *AAP* gene family for amino acid permeases contributes todevelopment of the cyst nematode *Heterodera schachtii* in roots of Arabidopsis. *Plant Physiology and Biochemistry* **70**, 379–386.

Fagard, M., Launay, A., Clément, G., Courtial, J., Dellagi, A., Farjad, M., Krapp, A., Soulié, M. C. and Masclaux-Daubresse, C. (2014). Nitrogen metabolism meets phytopathology. *Journal of Experimental Botany* **65**, 5643–5656.

Gao, D., Huibers, R. P., Loonen, A. E., Visser, R. G., Wolters, A. M. and Bai, Y. (2014). Down-regulation of acetolactate synthase compromises *Ol-1-* mediated resistance to powdery mildew in tomato. *BMC Plant Biology* **14**, 32.

Garnica, D. P., Upadhyaya, N. M., Dodds, P. N. and Rathjen, J. P. (2013). Strategies for wheat stripe rust pathogenicity identified by transcriptome sequencing. *PLoS One* **8**, e67150.

Gebauer, P., Korn, M., Engelsdorf, T., Sonnewald, U., Koch, C. and Voll, L. M. (2017). Sugar accumulation in leaves of Arabidopsis *sweet11/sweet12* double mutants enhances priming of the salicylic acid-mediated defense response. *Frontiers in Plant Science* **8**.

Gust, A. A., Pruitt, R. and Nürnberger, T. (2017). Sensing danger: Key to activating plant immunity. *Trends in Plant Science* **22**, 779–791.

Hahn, M., Neef, U., Struck, C., Gottfert, M. and Mendgen, K. (1997). A putative amino acid transporter is specifically expressed in haustoria of the rust fungus *Uromyces fabae*. *Molecular Plant-Microbe Interactions* **10**, 438–45.

Hammes, U. Z., Nielsen, E., Honaas, L. A., Taylor, C. G. and Schachtman, D. P. (2006). AtCAT6, a sink-tissue-localized transporter for essential amino acids in Arabidopsis. *The Plant Journal* **48**, 414–426.

Hammes, U. Z., Schachtman, D. P., Berg, R. H., Nielsen, E., Koch, W., McIntyre, L. M. and Taylor, C. G. (2005). Nematode-induced changes of transporter gene expression in Arabidopsis roots. *Molecular Plant-Microbe Interactions* **18**, 1247–1257.

Hirner, A. (2006). Arabidopsis LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. *The Plant Cell* **18**, 1931–1946.

Hiruma, K., Fukunaga, S., Bednarek, P., Piślewska-Bednarek, M., Watanabe, S., Narusaka, Y., Shirasu, K. and Takano, Y. (2013). Glutathione and tryptophan metabolism are required for Arabidopsis immunity during the hypersensitive response to hemibiotrophs. *Proceedings of the National Academy of Sciences* **110**, 9589–9594.

Horst, R. J., Doehlemann, G., Wahl, R., Hofmann, J., Schmiedl, A., Kahmann, R., Kamper, J., Sonnewald, U. and Voll, L. M. (2010a). *Ustilago Maydis* infection strongly alters organic nitrogen allocation in maize and stimulates productivity of systemic source leaves. *Plant Physiology* **152**, 293–308.

Horst, R. J., Doehlemann, G., Wahl, R., Hofmann, J., Schmiedl, A., Kahmann, R., Kamper, J. and Voll, L. M. (2010b). A model of *Ustilago maydis* leaf tumor metabolism. *Plant Signaling and Behavior* **5**, 1446–9.

Jones, J. D. G. and Dangl, J. L. (2006). The plant immune system. *Nature* **444**, 323–329.

Kemen, E., Gardiner, A., Schultz-Larsen, T., Kemen, A. C., Balmuth, A. L., Robert-Seilaniantz, A., Bailey, K., Holub, E., Studholme, D. J., Maclean, D. et al. (2011). Gene gain and loss during evolution of obligate parasitism in the white rust pathogen of *Arabidopsis thaliana*. *PLoS Biology* **9**, e1001094.

Lanver, D., Muller, A. N., Happel, P., Schweizer, G., Haas, F. B., Franitza, M., Pellegrin, C., Reissmann, S., Altmuller, J., Rensing, S. A. et al. (2018). The biotrophic development of *Ustilago maydis* studied by RNA-seq analysis. *The Plant Cell* **30**, 300–323.

Lau, G. and Hamer, J. E. (1996). Regulatory genes controlling *MPG1* expression and pathogenicity in the rice blast fungus *Magnaporthe grisea*. *The Plant Cell* **8**, 771–781.

Lemonnier, P., Gaillard, C., Veillet, F., Verbeke, J., Lemoine, R., Coutos-Thevenot, P. and La Camera, S. (2014). Expression of Arabidopsis sugar transport protein STP13 differentially affects glucose transport activity and basal resistance to *Botrytis cinerea*. *Plant Molecular Biology* **85**, 473–84.

Li, J.-F., Chung, H. S., Niu, Y., Bush, J., McCormack, M. and Sheen, J. (2013). Comprehensive protein-based artificial microRNA screens for effective gene silencing in plants. *The Plant Cell* **25**, 1507–1522.

Liu, G., Ji, Y., Bhuiyan, N. H., Pilot, G., Selvaraj, G., Zou, J. and Wei, Y. (2010). Amino acid homeostasis modulates salicylic acid-associated redox status and defense responses in Arabidopsis. *The Plant Cell* **22**, 3845–63.

Liu, S., Kandoth, P. K., Warren, S. D., Yeckel, G., Heinz, R., Alden, J., Yang, C., Jamai, A., El-Mellouki, T., Juvale, P. S. et al. (2012). A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens. *Nature* **492**, 256–260.

Marella, H. H., Nielsen, E., Schachtman, D. P. and Taylor, C. G. (2013). The amino acid permeases AAP3 and AAP6 are involved in root-knot nematode parasitism of Arabidopsis. *Molecular Plant-Microbe Interactions* **26**, 44–54.

McDowell, J. M. (2011). Genomes of obligate plant pathogens reveal adaptations for obligate parasitism. *Proceedings of the National Academy of Sciences* **108**, 8921–8922.

Mengiste, T. (2012). Plant immunity to necrotrophs. *Annual Review of Phytopathology* **50**, 267–294.

Mithani, A., Hein, J. and Preston, G. M. (2011). Comparative analysis of metabolic networks provides insight into the evolution of plant pathogenic and nonpathogenic lifestyles in *Pseudomonas*. *Molecular Biology and Evolution* **28**, 483–99.

Moore, J. W., Herrera-Foessel, S., Lan, C., Schnippenkoetter, W., Ayliffe, M., Huerta-Espino, J., Lillemo, M., Viccars, L., Milne, R., Periyannan, S. et al. (2015). A recently evolved hexose transporter variant confers resistance to multiple pathogens in wheat. *Nature Genetics* **47**, 1494–8.

Namiki, F., Matsunaga, M., Okuda, M., Inoue, I., Nishi, K., Fujita, Y. and Tsuge, T. (2001). Mutation of an arginine biosynthesis gene causes reduced pathogenicity in *Fusarium oxysporum* f. sp. *Melonis*. *Molecular Plant-Microbe Interactions* **14**, 580–4.

Návarová, H., Bernsdorff, F., Döring, A.-C. and Zeier, J. (2012). Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. *The Plant Cell* **24**, 5123–5141.

O'Leary, B. M., Neale, H. C., Geilfus, C. M., Jackson, R. W., Arnold, D. L. and Preston, G. M. (2016). Early changes in apoplast composition associated with defence and disease in interactions between *Phaseolus vulgaris* and the halo blight pathogen *Pseudomonas syringae* Pv. *phaseolicola*. *Plant Cell and Environment* .

Pan, Q., Cui, B., Deng, F., Quan, J., Loake, G. J. and Shan, W. (2016). *RTP1* encodes a novel endoplasmic reticulum (ER)-localized protein in Arabidopsis and negatively regulates resistance against biotrophic pathogens. *New Phytologist* **209**, 1641–1654.

Pariyar, S. R., Nakarmi, J., Anwer, M. A., Siddique, S., Ilyas, M., Elashry, A., Dababat, A. A., Leon, J. and Grundler, F. M. (2018). Amino acid permease 6 modulates host response to cyst nematodes in wheat and Arabidopsis. *Nematology* **20**, 737–750.

Pilot, G., Stransky, H., Bushey, D. F., Pratelli, R., Ludewig, U., Wingate, V. P. and Frommer, W. B. (2004). Overexpression of GLUTAMINE DUMPER1 leads to hypersecretion of glutamine from Hydathodes of Arabidopsis leaves. *The Plant Cell* **16**, 1827–40.

Pratelli, R. and Pilot, G. (2007). Altered amino acid metabolism in *glutamine dumper1* plants. *Plant Signaling and Behavior* **2**, 182–184.

Pratelli, R. and Pilot, G. (2014). Regulation of amino acid metabolic enzymes and transporters in plants. *Journal of Experimental Botany* **65**, 5535–5556.

Rico, A., McCraw, S. L. and Preston, G. M. (2011). The metabolic interface between *Pseudomonas syringae* and plant cells. *Current Opinion in Microbiology* **14**, 31–8.

Rico, A. and Preston, G. M. (2008). *Pseudomonas Syringae Pv. Tomato* DC3000 uses constitutive and apoplast-induced nutrient assimilation pathways to catabolize nutrients that are abundant in the tomato apoplast. *Molecular Plant-Microbe Interactions* **21**, 269–82.

Ried, M. K., Banhara, A., Binder, A., Hwu, F.-H., Gust, A. A., Höfle, C., Hückelhoven, R., Nürnberger, T. and Parniske, M. (2018). Symbiosis-related genes sustain the development of a downy mildew pathogen on Arabidopsis thaliana. *bioRxiv* p. 286872.

Schwab, R., Ossowski, S., Warthmann, N. and Weigel, D. (2010). Directed gene silencing with artificial microRNAs. In *Plant MicroRNAs: Methods and Protocols* (eds. B. C. Meyers and P. J. Green), Methods in Molecular Biology, pp. 71–88. Totowa, NJ: Humana Press.

Senthil-Kumar, M. and Mysore, K. S. (2012). Ornithine-delta-aminotransferase and proline dehydrogenase genes play a role in non-host disease resistance by regulating pyrroline-5-carboxylate metabolism-induced hypersensitive response. *Plant Cell and Environment* **35**, 1329–43.

Senthil-Kumar, M. and Mysore, K. S. (2014). Tobacco rattle virus-based virus-induced gene silencing in *Nicotiana benthamiana*. *Nature Protocols* **9**, 1549–62.

Shin, K., Lee, S., Song, W.-Y., Lee, R.-A., Lee, I., Ha, K., Koo, J.-C., Park, S.-K., Nam, H.-G., Lee, Y. et al. (2015). Genetic identification of *ACC-RESISTANT2* reveals involvement of *LYSINE HISTIDINE TRANSPORTER1* in the uptake of 1-aminocyclopropane-1-carboxylic acid in *Arabidopsis thaliana*. *Plant and Cell Physiology* **56**, 572–582.

Solomon, P. S. and Oliver, R. P. (2001). The nitrogen content of the tomato leaf apoplast increases during infection by *Cladosporium fulvum*. *Planta* **213**, 241–9.

Solomon, P. S. and Oliver, R. P. (2002). Evidence that gamma-aminobutyric acid is a major nitrogen source during *Cladosporium fulvum* infection of tomato. *Planta* **214**, 414–20.

Solomon, P. S., Tan, K.-C. and Oliver, R. P. (2003). The nutrient supply of pathogenic fungi; a fertile field for study. *Molecular Plant Pathology* **4**.

Spanu, P. D., Abbott, J. C., Amselem, J., Burgis, T. A., Soanes, D. M., Stuber, K., Ver Loren van Themaat, E., Brown, J. K., Butcher, S. A., Gurr, S. J. et al. (2010). Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. *Science* **330**, 1543–6.

Struck, C., Ernst, M. and Hahn, M. (2002). Characterization of a developmentally regulated amino acid transporter (AAT1p) of the rust fungus *Uromyces fabae*. *Molecular Plant Pathology* **3**, 23–30.

Struck, C., Mueller, E., Martin, H. and Lohaus, G. (2004). The *Uromyces fabae UfAAT3* gene encodes a general amino acid permease that prefers uptake of in planta scarce amino acids. *Molecular Plant Pathology* **5**, 183–9.

Stuttmann, J., Hubberten, H.-M., Rietz, S., Kaur, J., Muskett, P., Guerois, R., Bednarek, P., Hoefgen, R. and Parker, J. E. (2011). Perturbation of Arabidopsis amino acid metabolism causes incompatibility with the adapted biotrophic pathogen *Hyaloperonospora arabidopsidis*. *The Plant Cell* **23**, 2788–2803.

Szabo, L. J. and Bushnell, W. R. (2001). Hidden robbers: The role of fungal haustoria in parasitism of plants. *Proceedings of the National Academy of Sciences* **98**, 7654–5.

Szakasits, D., Heinen, P., Wieczorek, K., Hofmann, J., Wagner, F., Kreil, D. P., Sykacek, P., Grundler, F. M. W. and Bohlmann, H. (2009). The transcriptome of syncytia induced by the cyst nematode *Heterodera schachtii* in Arabidopsis roots. *The Plant Journal* **57**, 771–784.

Tegeder, M. (2012). Transporters for amino acids in plant cells: Some functions and many unknowns. *Current Opinion in Plant Biology* **15**, 315–321.

Tegeder, M. and Masclaux-Daubresse, C. (2018). Source and sink mechanisms of nitrogen transport and use. *New Phytologist* **217**, 35–53.

Tegeder, M. and Rentsch, D. (2010). Uptake and partitioning of amino acids and peptides. *Molecular Plant* **3**, 997–1011.

Tegeder, M. and Ward, J. M. (2012). Molecular evolution of plant AAP and LHT amino acid transporters. *Frontiers in Plant Science* **3**, 21–21.

Toufighi, K., Brady, S. M., Austin, R., Ly, E. and Provart, N. J. (2005). The botany array resource: E-Northerns, expression angling, and promoter analyses. *The Plant Journal* **43**, 153–63.

van Damme, M., Zeilmaker, T., Elberse, J., Andel, A., der Velden, M. d. S.-v. and van den Ackerveken, G. (2009). Downy mildew resistance in Arabidopsis by mutation of *HOMOSERINE KINASE*. *The Plant Cell* **21**, 2179–2189.

Wang, W., Barnaby, J. Y., Tada, Y., Li, H., Tör, M., Caldelari, D., Lee, D. U., Fu, X. D. and Dong, X. (2011). Timing of plant immune responses by a central circadian regulator. *Nature* **470**, 110–115.

Williamson, V. M. and Gleason, C. A. (2003). Plant-nematode interactions. *Current Opinion in Plant Biology* **6**, 327–33.

Yamada, K., Saijo, Y., Nakagami, H. and Takano, Y. (2016). Regulation of sugar transporter activity for antibacterial defense in Arabidopsis. *Science* **354**, 1427–1430.

Yang, B., Sugio, A. and White, F. F. (2006). *Os8N3* is a host disease-susceptibility gene for bacterial blight of rice. *Proceedings of the National Academy of Sciences* **103**, 10503–8.

Yang, H. and Ludewig, U. (2014). Lysine catabolism, amino acid transport, and systemic acquired resistance. *Plant Signaling and Behavior* **9**, e28933.

Yang, H., Postel, S., Kemmerling, B. and Ludewig, U. (2014). Altered growth and improved resistance of *Arabidopsis* against *Pseudomonas syringae* by overexpression of the basic amino acid transporter *AtCAT1*. *Plant, Cell and Environment* **37**, 1404–1414.

Yi, M. and Valent, B. (2013). Communication between filamentous pathogens and plants at the biotrophic interface. *Annual Review of Phytopathology* **51**, 587–611.

Zeier, J. (2013). New insights into the regulation of plant immunity by amino acid metabolic pathways. *Plant, Cell and Environment* **36**, 2085–2103.

Chapter 3

Role of amino acid permease 3 and 6 in the interaction of Arabidopsis with its downy mildew pathogen *Hyaloperonospora arabidopsidis*

(Manuscript in preparation)

3.1 Introduction

Amino Acid Permeases (AAPs) play important roles in transporting amino acids at different stages of the plant's life such as seed filling, taking up amino acids at root tip and long-distance transport (see section 1.2 for the various physiological roles AAPs are known to be important for in the plant) and have also been linked to susceptibility to nematode infection (see subsection 2.5.1 for detail).

Briefly, *AAP3* and *AAP6* have been found to be important during root-knot nematode infection (Marella et al., 2013). *AAP3* and *AAP6* mRNA accumulation along with other *AAPs* is also increased in the nematode-induced synctia during beet cyst nematode infection (Elashry et al., 2013). Furthermore, the wheat ortholog of *AAP6* (*TaAAP6*) also appears to be important for cereal cyst nematode infection in wheat (Pariyar et al., 2018). Based on publicly available microarray and RNA-seq datasets, on average *AAP3* and *AAP6* appear to be transcriptionally regulated in opposite directions in response to various pathogen infection (See part A in Fig. 2.2). While *AAP3* expression is induced on average, *AAP6* expression appears to be repressed. These genes thus aligned well with our objective of identifying Arabidopsis amino acid transporters that could be important during its interaction with its downy mildew pathogen *Hyaloperonospora arabidopsidis*. In this work, we show that *AAP3* and *AAP6* are involved and important in the *Hpa*-Arabidopsis interaction, respectively.

3.2 Results

AAP3 and *AAP6* are induced in response to *Hpa* infection

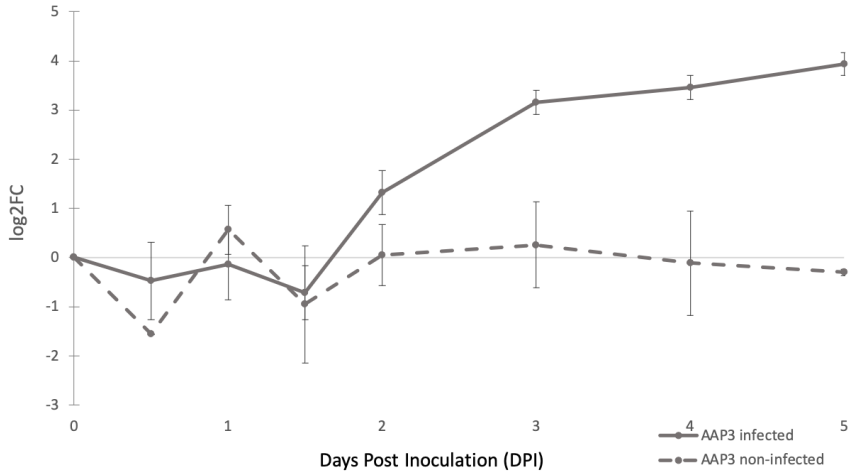
Since *AAP3* and *AAP6*, on average, were transcriptionally induced and repressed respectively in response to pathogen attack (Fig. 2.2), we started out by testing if this was true in the case of *Hpa*-Arabidopsis interaction. We used wild-type Col-0 plants infected with *Hpa*'s virulent isolate Noco2 and monitored the mRNA accumulation of these transporters over the course of the infection. Another set of plants grown and maintained in the same environment were mock-treated with sterile water for comparison. *AAP3* expression was robustly induced as measured by its increase in mRNA accumulation under *Hpa* infected conditions compared to mock-treated control plants (Fig. 3.1(a)). The increase in *AAP3* mRNA amount did not start until 2 days after infection, indicating that AAP3 possibly becomes important later in the infection process. On the contrary, no significant increase in mRNA accumulation of *AAP6* was observed over the course of infection (Fig. 3.1(b)).

To explore the importance of expression pattern of these transporters in the context of *Hpa* infection, we used transgenic plants carrying the constructs of *GUS* gene driven by either *AAP3* or *AAP6* promoters to visualize changes in promoter activity as proxy for changes in spatial expression. Since previous work by Okumoto et al. (2004) had shown that elements upstream of up to 3.8 kb region of *AAP3* start site were required for achieving GUS activity that matched RNA gel blot analysis, we used constructs containing 3.8 kb region upstream of *AAP3* driving the *GUS* gene as well. In case of *AAP6*, a more traditional 2 kb fragment upstream of *AAP6* start site was used.

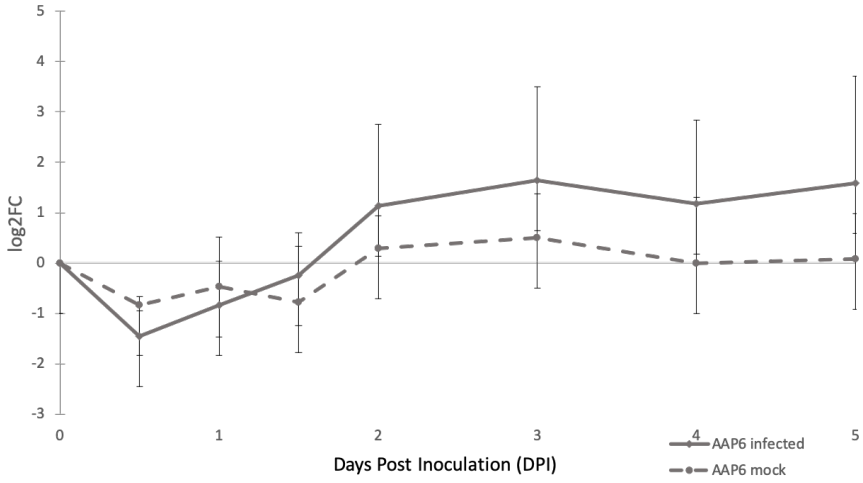
Similar to Okumoto et al. (2004) , GUS staining was observed mostly in the vasculature of roots and cotyledons of mock-treated *AAP3p*:GUS plants. Upon infection with virulent isolate (Noco2) of *Hpa*, promoter activity increased in the shoot vasculature. GUS staining was not only observed in the major veins of infected leaves, but in secondary and tertiary veins as well (Fig. 3.2).

In order to test if this increase and change in spatial expression pattern of *AAP3p*:GUS was dependent or independent of host susceptibility to the pathogen, we also challenged these plants with an avirulent isolate (Emwa1) of *Hpa*. We observed increased staining for GUS activity in secondary and tertiary veins of infected leaves compared to those of mock-treated plants, similar to what we had seen in experiments with Noco2 . This gave us an initial suggestion that the transcriptional induction of *AAP3* is probably controlled by the plant since the changes in promoter activity occur regardless of pathogen recognition in case of the incompatible interaction of Col-0 with Emwa1 (wherein the resistance to Emwa1 is mediated via the *R*-gene *RPP4* (Biezen et al., 2002)).

To further investigate if *AAP3* was being induced systemically or was more localized to the regions of colonization by *Hpa*, we drop inoculated *Hpa* spores on only 2-3 leaves of *AAP3p*:GUS transgenic plants. The entire plant was then dual-stained for β-glucuronidase activity (Magenta-Gluc) and pathogen structures (Trypan Blue). We found that the vasculature of *Hpa* colonized leaves displayed stronger magenta-gluc (seen as purple) staining compared to the



(a)



(b)

Figure 3.1: mRNA accumulation of (a) *AAP3* and (b) *AAP6* under Hpa infection
10-12 days old Col-0 seedlings sprayed with Noco2 (solid line) or mock-treated with sterile water (dashed line) were used to obtain cDNA over a time-course of 5 days and primers specific to *AAP3* and *AAP6* were used to measure the mRNA accumulation via qRT-PCR. Values were normalized to Actin2 abundance, which were then normalized to 0 dpi(days post inoculation) time-point. Each data point represents a mean of 2 biological replicates (except AAP3 infected which represents 3), error bars represent standard error of the mean.

Figure 3.2: promoter-GUS analysis of plants carrying *AAP3p*:GUS construct under infection by Noco2

(a) Transgenic plants expressing GUS under the control of 3.8 kb region upstream of *AAP3* start site were infected with virulent isolate Noco2 (left) or mock-treated with sterile water (right) and stained for β-glucuronidase activity at 5 dpi. Images from two representative independent lines are shown.(b) Magnified images of leaves from line 1

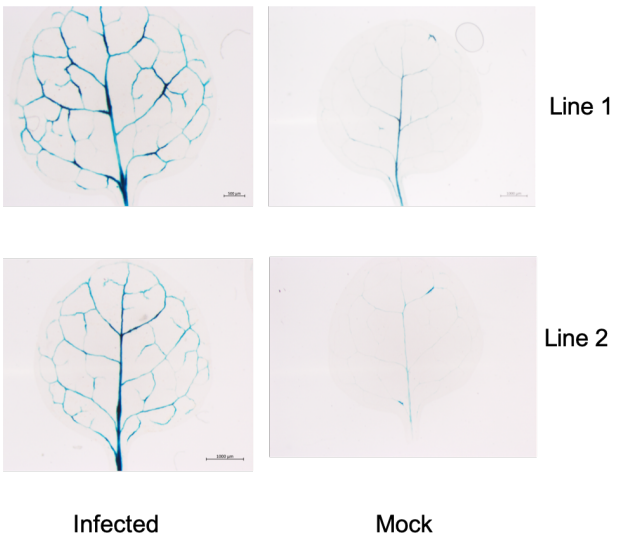


Figure 3.3: promoter-GUS analysis of plants carrying *AAP3p*:GUS construct under infection by Emwa1

Transgenic plants expressing GUS under the control of 3.8 kb region upstream of *AAP3* start site were infected with avirulent isolate Emwa1 (left) or mock-treated with sterile water (right) and stained for β-glucuronidase activity at 5 dpi. Images from two representative independent lines are shown.

non-infected leaves from the same plant (Fig. 3.4). This suggests that *AAP3* is not induced systemically, but is preferentially up-regulated in shoot tissue colonized by the pathogen.

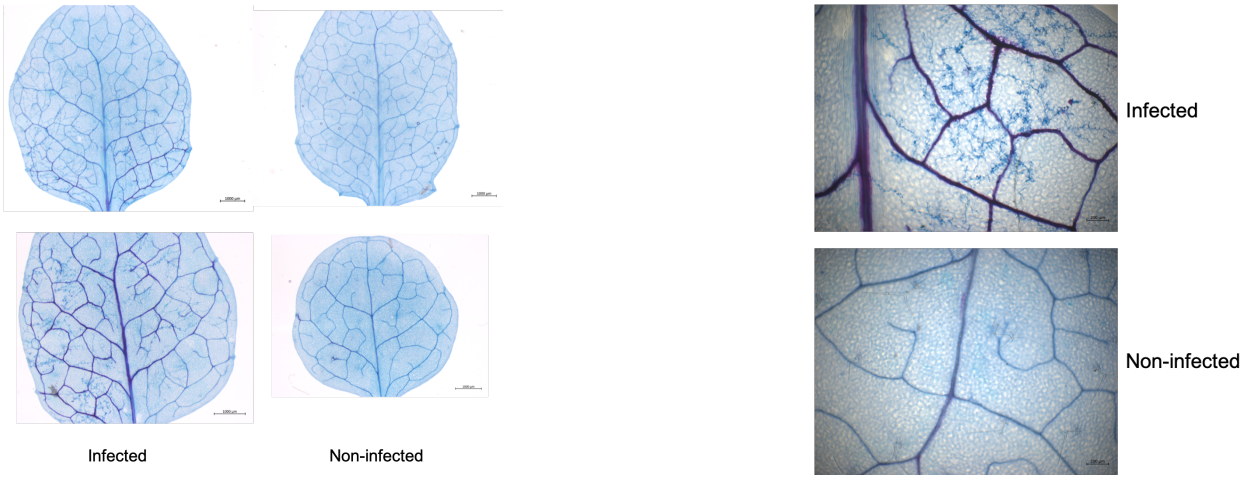


Figure 3.4: Dual-staining of plants carrying *AAP3p*:GUS construct under infection by Noco2
2-3 leaves of transgenic plants expressing GUS under the control of 3.8 kb region upstream of *AAP3* start site were drop inoculated with virulent isolate Noco2. The whole plant was dual-stained for β-glucuronidase activity (using magenta-gluc) followed by trypan blue staining at 5 dpi. All 4 leaves shown are from the same plant. (b) Magnified images of infected and non-infected leaves

We also tested whether there were any other levels of post-transcriptional or post-translational regulation of *AAP3* by challenging transgenic plants carrying GFP fused to the N-terminus of full-length AAP3 under the control of the *AAP3* promoter. We were able to detect little to no signal in the leaves of these plants under mock-treated conditions (Fig. 3.5), which is in line with what we saw using promoter:GUS analysis (Fig. 3.2). Upon infection with Noco2, we saw a small increase in GFP fluorescence in the vasculature. The weaker GFP signal is most likely related to *AAP3*'s basal expression level being low, we consider this further in the discussion section below (section 3.3).

In case of *AAP6p*:GUS mock-treated plants, we observed staining in the root vasculature, all veins of cotyledons and leaves, and in the hydathodes of the transgenic plants as reported previously by Okumoto et al. (2002) (Fig. 3.6, right). This spatial pattern did not change when the plants were infected with Noco2 (Fig. 3.6). We also tested for changes in promoter activity within the same plant and did not find any significant difference in staining between infected and non-infected leaves from the same plant (Fig. 3.S1). This data along with expression data in Fig. 3.1(b) indicates that the level of *AAP6* expression in Noco2 interaction with Col-0 is not regulated transcriptionally in a major way.

We also challenged transgenic plants carrying full-length *AAP6* gene construct (*AAP6p*:GFP-AAP6). Corroborating the localization from the promoter-GUS analysis (Fig. 3.6), AAP6 fused to GFP localized in the vasculature of the leaves in both mock and Noco2 infected

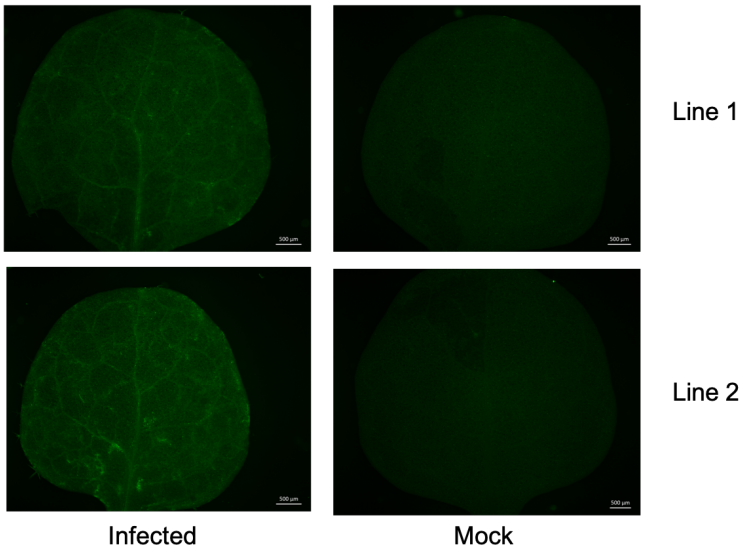


Figure 3.5: GFP localization in plants carrying *AAP3p*:GFP-AAP3 constructs under infection by Noco2 Transgenic plants expressing GFP fused to the N-terminus of AAP3 protein under the control of the native *AAP3* promoter were infected with Noco2 (left) or mock-treated with sterile water (right). Leaf samples were imaged at 6 dpi. Representative images from two independent lines are shown.

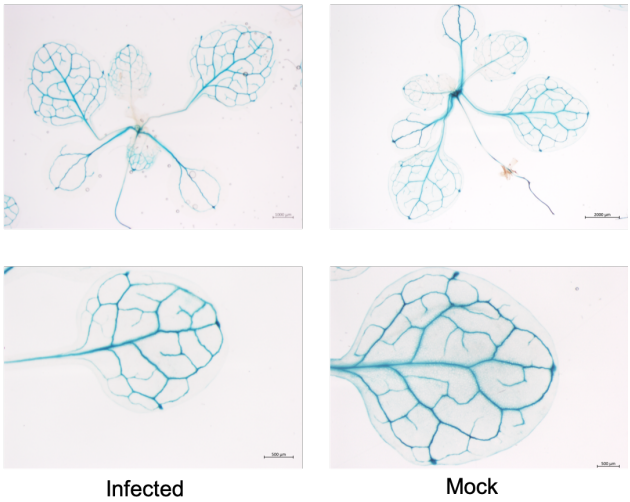


Figure 3.6: promoter-GUS analysis of plants carrying *AAP6p*:GUS construct under infection by Noco2
Transgenic plants expressing GUS under the control of 2 kb region upstream of *AAP6* start site were infected with virulent *Hpa isolate* Noco2 (left) or mock-treated with sterile water (right) and stained for β-glucuronidase activity at 5 dpi. Images from two representative independent lines are shown.

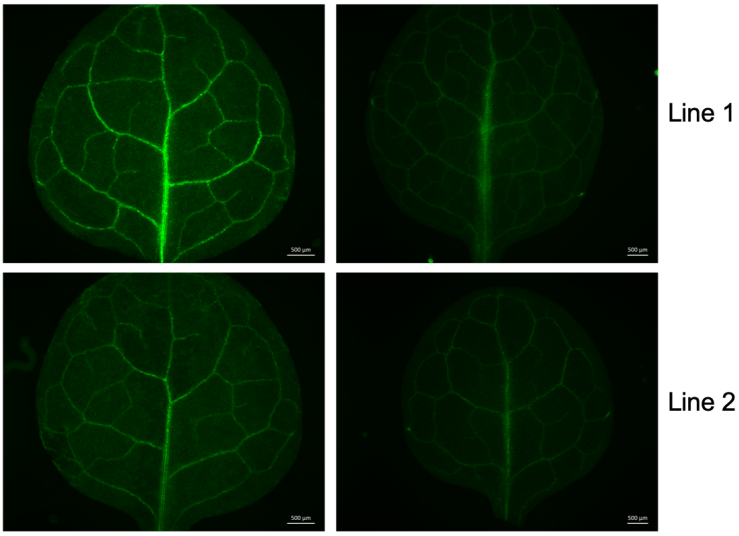


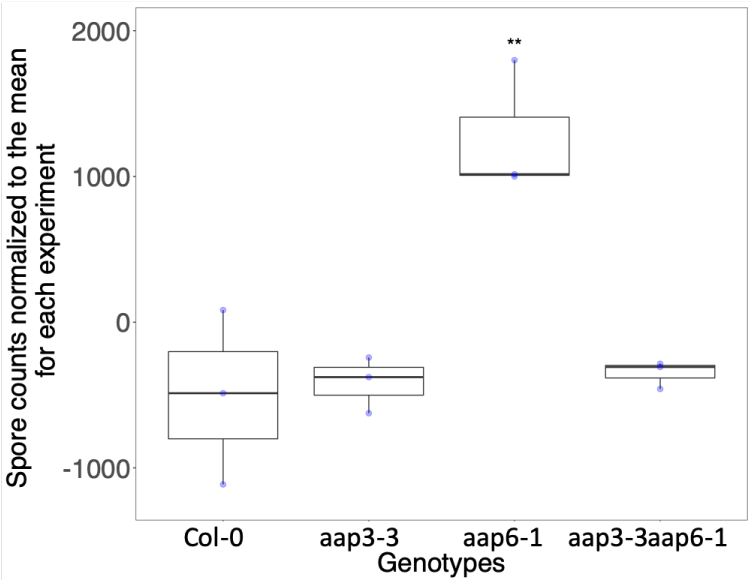
Figure 3.7: GFP localization in plants carrying *AAP6p*:GFP-AAP6 constructs under infection by Noco2 Transgenic plants expressing GFP fused to the N-terminus of AAP6 protein under the control of the native *AAP6* promoter were infected with Noco2 (left) or mock-treated with sterile water (right). Leaf samples were imaged at 6 dpi. Representative images from two independent lines are shown.

plants. However, the leaves infected with Noco2 displayed an increase in GFP fluorescence which was much more pronounced than that seen with mRNA accumulation or GUS staining (Fig. 3.7).

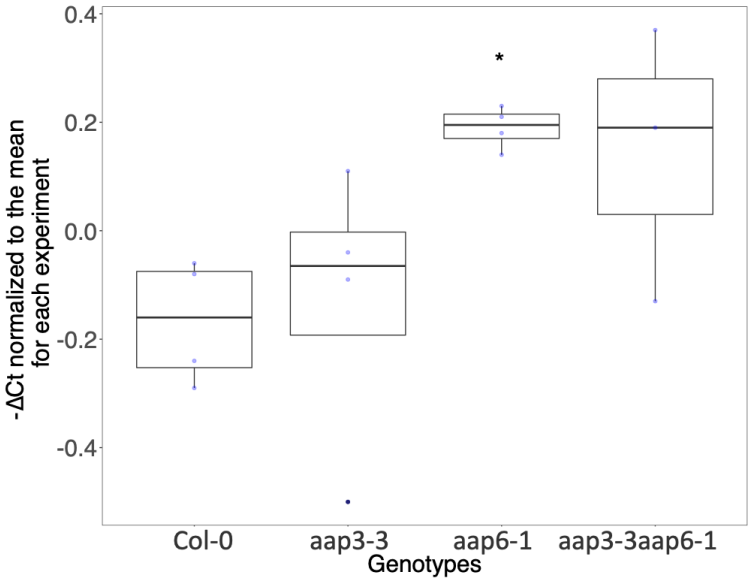
Loss of *AAP6* makes the plant more susceptible to *Hpa* infection, and is not due to perturbed immunity

We hypothesized that if these transporters are important for Arabidopsis-*Hpa* interaction we would see a change in pathogen growth in their loss-of-function mutants. We challenged loss-of-function T-DNA insertion mutants *aap3-3*, *aap6-1* and the double mutant *aap3-3aap6-1* with the virulent isolate Noco2. We found that *aap6-1* was more susceptible to *Hpa* compared to Col-0 (Fig. 3.8). Surprisingly, even though *AAP3* was induced in response to Noco2 infection (Fig. 3.1(a)), *aap3-2* did not show a consistent difference in pathogen growth compared to Col-0. Epistasis between the *aap3* and *aap6* mutations is inconclusive since the double mutant *aap3-2aap6-1* supports similar pathogen reproduction as *aap3-2* (Fig. 3.8(a)), but is more variable in vegetative pathogen growth (Fig. 3.8(b)).

We also challenged these mutants with the avirulent isolate Emwa1 and found both *aap3* and *aap6* mutants similar or slightly more susceptible to the avirulent isolate compared to the wild-type (Fig. 3.S2(a)). However, qualitative analysis of the hypersensitive response using trypan blue staining displayed similar cell death in the mutants as in wild-type plants (Fig. 3.S2(b)). This suggests that the increased susceptibility in these mutants, especially in case of *aap6*, is not a result



(a) Counts from Noco2 spores, 7 dpi



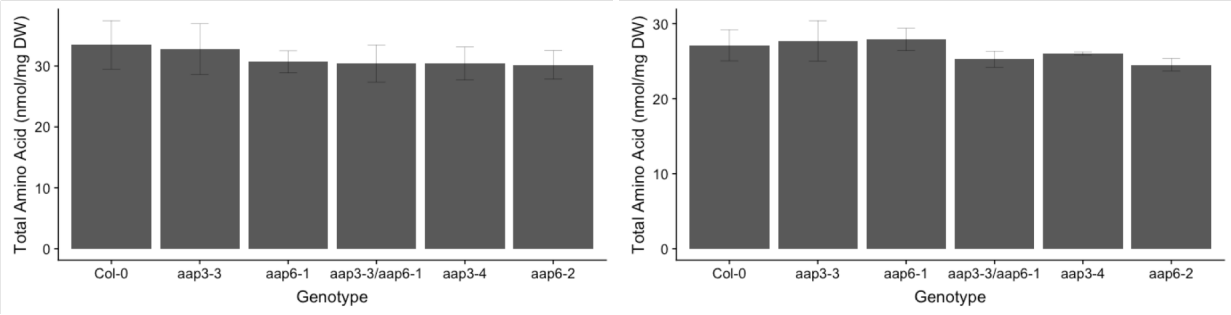
(b) qPCR assay for Noco2 growth, 5 dpi

Figure 3.8: Pathogen growth on Noco2 infected plants (a) Spores re-suspended in water were counted at 7 dpi. The number of spores was normalized to fresh weight of leaves. The value for every genotype was normalized to the mean of each experiment. Each data point per genotype is from an independent replicate (n=3). (b) Ct values from *Hpa* actin2 were normalized to Arabidopsis actin2. The -ΔCt value for every genotype was normalized to the mean for that experiment, (n=4). *, ** indicate p-value<=0.05 and <=0.01 when compared to Col-0 (reference group) by Dunnett's Test

of perturbed immunity.

aap6 loss-of-function mutants accumulate less amino acids in the phloem on infection with Noco2

To better understand the physiology behind the the increased susceptibility of *aap6* mutants to *Hpa* we looked at how these mutations affect the amino acid content and distribution in the plant, with or without *Hpa* infection. We started out by measuring the total free amino acid content in leaves of plants infected with Noco2. We did not observe any change in the total free amino acid content in leaves of infected plants compared to those treated with sterile water (Fig. 3.9). We did observe a general increase of about 5nmol/mg in total free amino acids in infected plants compared to the mock-treated plants, irrespective of the genotype.
Since the two transporters are active in the vasculature, we looked at the amino acid content in the phloem sap of infected and non-infected plants. We found that the total amino acid content in the phloem sap increased on infection with *Hpa*. However, the *aap6* mutants accumulated less amino acids in the phloem compared to Col-0 when infected with Noco2 (Fig. 3.10). This was a result of an overall decrease in the accumulation of most amino acids in the *aap6* mutants instead of a single amino acid (Fig. 3.S3). This can also be seen in the stacked graph with the total amino acid content scaled to hundred percent, wherein the proportion of amino acids is mostly similar compared to wild-type (Fig. 3.11).



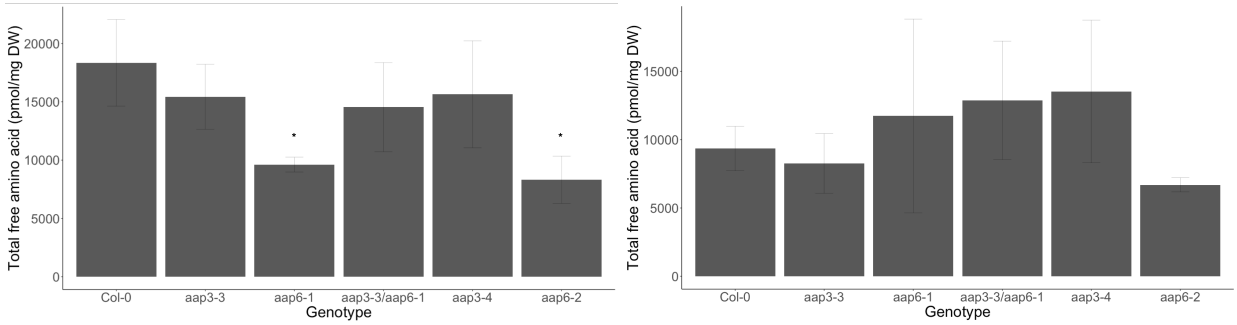
(a) Total free amino acid content in leaves infected with Noco2

(b) Total free amino acid content in mock-treated leaves

Figure 3.9: Total free amino acid content in *aap3* and *aap6* mutants under Noco2 infection
3-week old plants were infected with Noco2 or mock-treated with sterile water. Free amino acid content whole leaves was measured from samples collected at 5 dpi. The amino acid content was normalized to the dry weight of the sample. Error bars represent standard deviation among three replicates. This experiment was repeated three times with similar results.

Sub-cellular localization of AAP3 and AAP6

When transiently expressed in *Nicotiana benthamiana* epidermal cells, GFP-AAP3 was found to localize to punctate vescicle-like structures in the cytosol. This was corroborated by the localization via transient expression in Arabidopsis epidermal cells as well (Fig. 3.12(a) and Fig. 3.12(b)). This is also consistent with the observation of Okumoto et al. (2004)

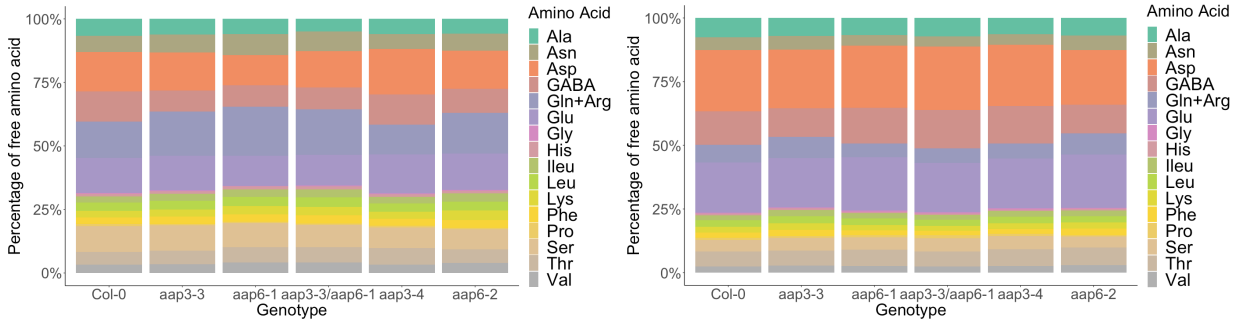


(a) Total free amino acid content in phloem sap of Noco2 infected plants

(b) Total free amino acid content in phloem sap of mock-treated plants

Figure 3.10: Total free amino acid content in phloem sap of *aap3* and *aap6* mutants under Noco2 infection

3-week old plants were infected with Noco2 or mock-treated with sterile water. Free amino acid content in phloem sap was measured from samples collected at 5 dpi. The amino acid content was normalized to the dry weight of the sample. Error bars represent standard deviation among three replicates.



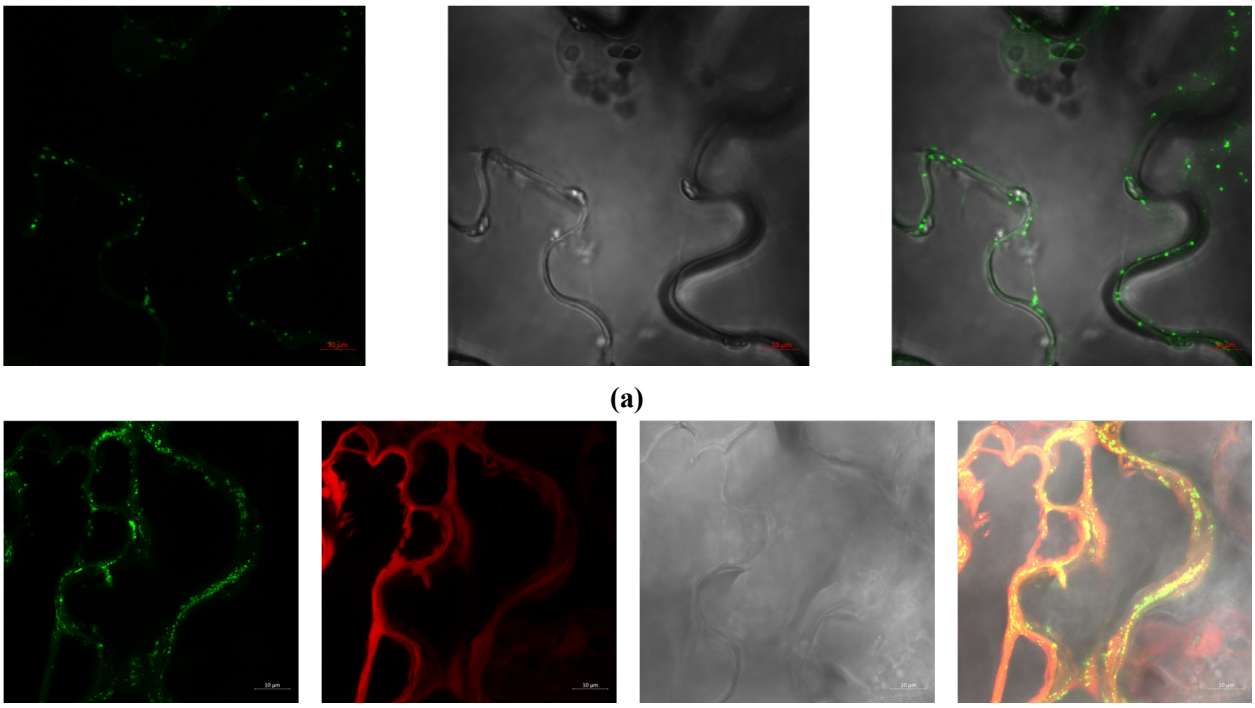
(a) Percentage of free amino acids in phloem sap of Noco2 infected plants

(b) Percentage of free amino acids in phloem sap of mock-treated plants

Figure 3.11: Percentage of free amino acids in phloem sap of *aap3* and *aap6* mutants under Noco2 infection

3-week old plants were infected with Noco2 or mock-treated with sterile water. Free amino acid content in phloem sap was measured from samples collected at 5 dpi. The amino acid content was normalized to the dry weight of the sample. Total amino acid content in these samples has been scaled to hundred percent and the content of each amino acid has been represented as a percentage of the total.

in *N. benthamiana* protoplast cells as well. When transiently expressed in *N. benthamiana*, AAP6-mCherry was found to localize in the endoplasmic reticulum (ER) (Fig. 3.13(a)). This result was also corroborated by GFP-AAP6 in stable Arabidopsis transgenic plants carrying *AAP6p*:GFP-AAP6 constructs (Fig. 3.13(b)).



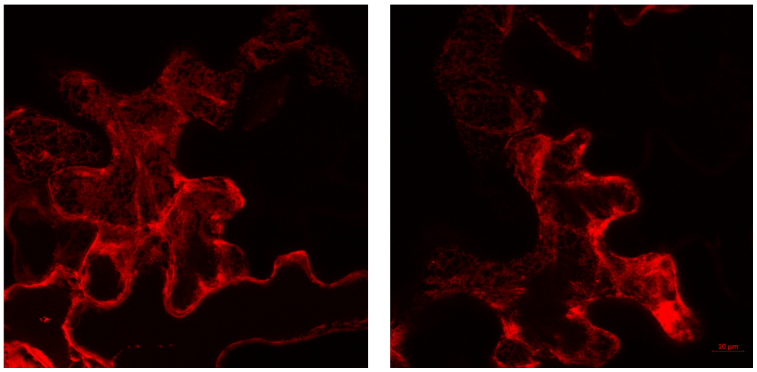
(a)

(b)

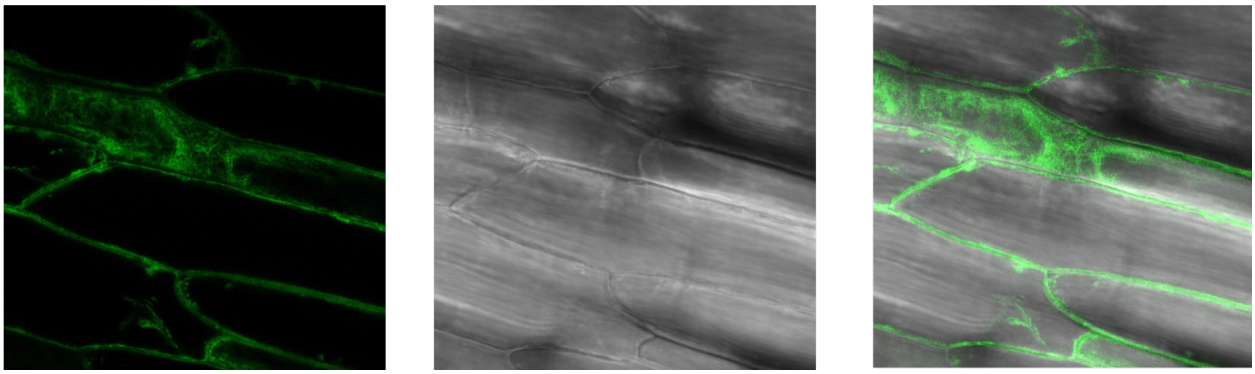
Figure 3.12: Sub-cellular localization of AAP3 in *N. benthamiana* and Arabidopsis epidermal cells GFP fused to AAP3 at the N-terminus was transiently expressed and visualized in (a) *N. benthamiana* and (b) Arabidopsis cells. In (b) AAP3 was co-expressed with free mCherry to aid in visualization of the cell.

3.3 Discussion

Obligate biotrophic pathogens such as *Hpa* that have lost the ability to assimilate inorganic nitrogen (Baxter et al., 2010) are entirely dependent on the living host for acquiring nitrogen in an organic form. In this work we report how the host could be utilizing amino acid transporters such as AAP6 to mobilize amino acids away from the pathogen. We also describe our findings on the regulation of *AAP3* under *Hpa* infection and discuss further in the following sections the possible reasons for why a gene that appears to be important for the plant during biotic interactions does not have any effect on the pathogen when knocked out.



(a)



(b)

Figure 3.13: Sub-cellular localization of AAP6 in *N. benthamiana* and Arabidopsis mCherry fused to C-terminus of AAP6 was transiently expressed in (a)*N. benthamiana* epidermal cells and (b) cells in pertiole of stable Arabidopsis transgenic plants carrying *AAP6p*:GFP-AAP6 constructs were visualized using confocal microscopy

AAP3 and *AAP6* are involved in the plant's interaction with *Hpa*

The expression of both *AAP3* and *AAP6* is induced during *Hpa* infection. Most of the induction of *AAP3* expression seems to be a result of increase in promoter activity (as seen from promoter:GUS analysis and mRNA accumulation assays). Based on promoter:GUS analysis, in an uninfected plant, AAP3 is mostly present in the roots. In the shoots it is only found to be active in the vasculature of cotyledons and main vein of some leaves, similar to what has been previously reported by Okumoto et al. (2004) as well. However, under infected conditions, it also appears to be expressed in the secondary and tertiary veins of leaves. The increase in promoter activity appears to be limited to the those leaves that were colonized by the pathogen (Fig. 3.4). This indicates that the induction of *AAP3* expression is not systemic. Even though *AAP3* expression is significantly induced during infection, *AAP3* itself is not a highly expressed gene in non-infected conditions, especially in the shoot tissue. This appears to be true with regards to promoter activity and mRNA accumulation (in uninfected conditions *AAP3* accumulates 2-fold less mRNA than *AAP6*). It is also corroborated at the protein level by the images of GFP localization in *AAP3p*:GFP-AAP3 plants (see Fig. 3.5, wherein there is little to no GFP fluorescence in mock treated leaves of the plants). Hence, even though the increase in *AAP3*'s promoter activity upon infection by *Hpa* appears to be consistent and specific, the increase itself is not very large.

Under mock-treated conditions, AAP6 already localizes to the vasculature of both roots and shoots. Under infected conditions, the induction of *AAP6* at the transcriptional level is more subtle. Promoter:GUS analysis revealed no evidence of increase in promoter activity and the increase in mRNA content was small. On the other hand, GFP intensity in infected transgenic plants carrying *AAP6p:*GFP-AAP6 constructs displayed a substantial increase compared to that of mock-treated plants. This indicates that post-transcriptional regulation may play a role in the expression of AAP6. Again, as described above, even though mRNA accumulation of *AAP3* is induced under infected conditions, the basal expression of AAP3 is lower than that of AAP6. This might explain the differences in these transporters' regulation during infected conditions.

Role of *AAP6* as nutritional starvation gene

Based on previous promoter:GUS analysis by Okumoto et al. (2002), AAP6 is probably present in the xylem parenchyma cells. When *AAP6* is knocked out, the plant becomes more susceptible to *Hpa*. While the amino acid content in the phloem sap appears to increase under infected conditions in wild-type plants, *aap6* mutants accumulate fewer amino acids in the phloem. Since AAP6 is thought to play a role in uptake of amino acids into the xylem parenchyma cells from the tracheids and hence aid in xylem-phloem transfer (Okumoto et al., 2002; Hunt et al., 2010), the lack of AAP6 in *aap6* mutants could become more apparent during infected conditions; hence explaining the reduction of amino acids in the phloem sap.
Further, the increase in expression of GFP-AAP6 in the vasculature and the increased susceptibility of aap6 mutants to Noco2 suggests that AAP6 is used by the plant to move amino acids away from the pathogen (and loaded into the phloem), hence AAP6 could serve as a nutritional starvation gene. However, exactly how AAP6 could help the plant in moving amino acids away from the mesophyll cells (where the pathogen grows), remains to be elucidated.

Alternatively, *aap6* mutants could have altered C:N ratios, which could in turn affect the pathogen's growth. This has been seen before in case of *aap2* mutants, wherein loss of AAP2, which is also responsible for xylem-phloem transfer of amino acids, leads to a decrease in free amino acids in source leaves along with increase in protein content, increase in leaf area, and also increase in photosynthesis (measured as μmol of CO2 per m2/s) and sugar content (Zhang et al., 2010). Hunt et al. (2010) found that while *aap6* mutants were similar to wild-type in terms of developmental stages, at flowering stages the mutant displayed small increase in rosette leaf width, number of cauline leaves and seed volume. Since the increased leaf area is only seen later in plant growth (post bolting), increase in leaf area playing a role in C:N ratio of *aap6* mutants used in this study is not very likely.

Role of AAP3 in Arabidopsis-*Hpa* interaction

AAP3 expression is induced under *Hpa* infection. It has also been found to be up-regulated in response to several other biotic interactions (Yang et al., 2014; Sonawala et al., 2018) and hence it appears to be a common response to pathogen perception by the plant. AAP3 localizes in the phloem (Okumoto et al., 2002) and is suggested to be involved in phloem loading. It could thus be used in moving amino acids away from the pathogen. Surprisingly, *aap3* mutants do not display significant differences in pathogen growth or reproduction compared to wild-type plants.

Why do we not see larger effects on pathogen fitness?

The most likely explanation for not seeing any difference in pathogen fitness in *aap3* mutants is compensation; either physiological or during the infection process or some combination of both. Plants have a highly buffered network of amino acid transporters. The compensation could occur at several levels: At the phloem loading during xylem-phloem transfer; involving a transporter such as AAP2 (Zhang et al., 2010) or during phloem unloading by transporters such as UMAMITs.

Even in the case of the *aap6* mutants, we don't see any difference in amino acid content in whole leaves under mock or infected conditions, and no significant difference in phloem sap under mock conditions alone. This indicates that the plant physiologically compensates for the lack of these transporters. While Hunt et al. (2010) found that the amino acid content was reduced in the sieve element sap of *aap6* plants, we did not see significant changes in the mock treated phloem sap of *aap6* plants: a couple of differences could explain this variation; the plants used in their experiments were physiologically older since they were grown in long day conditions and the sieve element sap was collected from stems using aphids whereas we collected phloem sap from rosette leaves.

Differences in effect of *aap6* mutation on *Hpa* compared to nematodes

Knocking out *AAP6* makes the plant more susceptible to *Hpa*, whereas it makes the plant more resistant to root knot and cyst nematodes (Marella et al., 2013; Pariyar et al., 2018). The most likely reason for this difference is the contrast in organs and tissue infected by these pathogens/parasites. While *Hpa* colonizes the mesophyll cells in the shoot tissue, both root knot and cyst nematodes

infect the roots. The second difference is their mechanism of nutrient acquisition. *Hpa* could acquire nutrients from the apoplast using hyphae or from haustoriated mesophyll cells or both; the comparative importance of hyphae and haustoria in nutrient acquisition remains to be understood (McDowell, 2014). Root knot and cyst nematodes induce the formation of giant cells and synctia, respectively. Both giant cells and early stage synctia are suggested to acquire nutrients apoplastically from the phloem whereas the synctia mostly acquire nutrients via the symplast at later stages (Hoth et al., 2008; Pariyar et al., 2018). Furthermore, *AAP6* expression is found to be up-regulated in the giant cells and synctia themselves (Marella et al., 2013; Pariyar et al., 2018). Under *Hpa* infection increase in GFP intensity in *AAP6p*-GFP-AAP6 plants was only observed in the vasculature. We did not observe any visible induction in cells more local to the pathogen. This difference in disease physiology and local vs. distal localization of AAP6 in case of nematodes and *Hpa* probably leads to different outcomes for pathogen/parasite fitness in *aap6* mutant plants.

Potential regulators of *AAP3* and *AAP6* during infection

The regulation of amino acid transporters for the most part is not very well understood. Most of our understanding of transcriptional regulation of these transporters comes from biotic and abiotic perturbations of particular transporters (Pratelli and Pilot, 2014). Since plant hormones such as SA (salicylic acid), JA (jasmonic acid) and ABA (abscisic acid) play an integral role in signalling during biotic and abiotic stresses, it is possible that hormonal regulation of these transporters especially during perturbations could be important. Based on promoter analysis, we found that both *AAP3* and *AAP6* could contain ABA responsive elements (ABREs) upstream of the start site (data not shown, analyzed via Promomer at `bar.utoronto.ca`). *PDF1.2 (plant defensin 1.2)*, which is activated by JA and other fungal pathogens (Manners et al., 1998) is also induced by *Hpa*. Additionally, publicly available expression data and unpublished data in the Pilot and McDowell laboratories suggest that *AAP3* could be differentially regulated in response to SA and JA. It is possible that on pathogen recognition these hormones along with other players are used by the plant to induce amino acid transporter genes that mobilize amino acids away from the pathogen. Further experiments should help us get a better glimpse on how this is orchestrated.

3.4 Future directions and suggested follow-up experiments

Future directions for this project should focus on understanding how AAP6 is able to move amino acids from the pathogen and what the changes in regulation of *AAP6* mean for the pathogen. Since it is suspected that there are other players involved in the function of AAP3, identifying these compensatory mechanisms could help understand the function of *AAP3* better.

Dexamethasone inducible overexpressors of *AAP3* and *AAP6* have been developed and while these still retain the caveat of any form of non-targeted (not being tissue specific) overexpression, they should at least reduce the pleitropic effects that come with constitutively overexpressing a gene. Future experiments with these transgenic plants might help us further support the hypothesis of *AAP6* functioning as nutritional starvation gene and may even give us more information towards the function of *AAP3*.

Another layer of regulation that could be important and needs further exploration is the role of subcellular localization of these transporters under infection. When transiently expressed in *N. benthamiana* and Arabidopsis epidermal cells, AAP3 fused to GFP was found to localize to punctate vescicle-like structures in the cytosol (Fig. 3.12). When transiently expressed in *N. benthamiana* and in stable Arabidopsis transgenic plants, AAP6 (fused to mCherry or GFP, respectively) was found to localize in the endoplasmic reticulum (ER) (Fig. 3.13). These localizations indicate constant cycling of the transporter between the plasma membrane and the endomembrane trafficking system. The localization of AAP3 and AAP6 in the vasculature and not being able to tag *Hpa* with a fluorescent marker might pose some hurdles in identifying the changes in subcellular localization of these proteins. One solution would be to use other related hemibiotrophic oomycetes such as *Phytophthora capsici*, that can be transformed and do infect *N. benthamiana*, to test for these changes.

Furthermore, the loss-of-function mutants should be tested with other plant pathogens in order to discern the similarities and differences in their function when infected with a pathogen that is not *Hpa*. *Phytophthora capsici* is a good candidate since it is a related oomycete, but is a root pathogen and will thus complement some of the information from *Hpa* as well.

Finally, some targeted experiments looking at the regulation of these transporters when treated with plant hormones such as SA, JA and ABA would hopefully add another piece of information towards solving this puzzle.

3.5 Materials and Methods

Plant lines and growth conditions

Plants were transformed with respective constructs using the floral dip method (Clough and Bent, 1998). Plants used for *Hpa* assays were grown in short-day conditions with 8 h of light at 22 °C and 16 h of dark at 20 °C in Percival chamber, using Sunshine Mix (Sungro Horticulture, MA, USA). Information about T-DNA mutant lines *aap3-3* (SALK_148822), *aap3-4* (SAIL_98_B12), *aap6-1* (SALK_013231), and *aap6-2* (SALK_14038) was obtained using the T-DNA express tool for SIGnAL database (Alonso et al., 2003)

Pathogen assays

Hpa was propagated and maintained on Col-0 or Ws-0 ecotype for Noco2 and Emwa1, respectively. A suspension of 50,000 spores/ml in sterile water was used to spray either 11 day old seedlings or 3 weeks old plants, as specified. For stable GFP transgenics, seedlings from T2 generation were grown on 1/2MS medium with Kanamycin for 8-10 days and then transplanted to soil and infected with *Hpa* at 2 weeks. Propagation, sporangiaphore assays, trypan staining, DAB staining were carried out as described in (McDowell et al., 2011)

Constructs and transformation

AAP3 and *AAP6* promoters and cDNAs were cloned using Gateway Cloning technology (Thermo Fisher) They were PCR cloned with attB sites flanking the product and moved to pDONRZeo vector. They were then moved to destination vectors; pWUTKan for promoters and pC4H-YFP-W/35S-W-mCherry for cDNAs. For transient expression in Arabidopsis, the cDNA of AAP3 was moved to 35S:GFP-W vector. For the full-length *AAP3p*:GFP-AAP3 and *AAP6*:GFP-AAP6 constructs, InFusion HD cloning kit (Invitrogen) was used. The primers were designed as per manufacturer's protocol, and the amplified fragments were cloned into the linearized pTKan vector by following the InFusion kit protocol.

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

4-5 week old *N. benthamiana* leaves were used for transient expression via *Agrobacterium tumifaciens* infiltration. Agrobacterium transformed with constructs containing amino acid transporter cDNA fused to GFP or mCherry and driven by constitutive promoter C4H or CaMV35S was used to infiltrate *N. benthamiana* leaves. All constructs were coexpressed with p19 (Voinnet et al., 2003), The samples were imaged using confocal microscopy 2-3 days after infiltration. For transient expression in Arabidopsis AGROBEST protocol was followed as described in Wu et al. (2014); Wang et al. (2018)

GUS staining

Around 12 independent lines were used to determine initial localization. 2-3 independent lines representative of the localization, and segregating for a single insertion were used for further studies

with pathogens and localization within organs. Fixing and staining for β-glucuronidase activity was performed as described by Lagarde et al. (1996). Samples were incubated in the dark at 37˚C until staining became visible. In case of infected and mock-treated plants, both treatments were treated to same amount of incubation period. For dual staining with trypan blue, X-gluc was replaced with Magenta-gluc (Gold Biotechnology). Post-staining the samples were rinsed in sodium phosphate buffer and followed with trypan staining as described in (McDowell et al., 2011).

DNA extraction and qPCR

DNA extraction for pathogen quantification was performed using BioSprint DNA Plant kit (Qiagen) according to manufacturer's protocol. qPCR assay for pathogen quantification was carried out as described in (Anderson and Mcdowell, 2015), with modifications (using TaqMan mastermix, probes and primers instead of SYBR Green).

RNA extraction and qRT-PCR

Total RNA was extracted using RNeasy plus mini kit (Qiagen) and the RNA was DNAse treated for removal of any genomic DNA contamination using DNA-free kit (Ambion) according to manufacturer's protocol. RNA integrity was verified by running 1-2µg of denatured RNA on an agarose gel. cDNA was synthesized using Super Script IV reverse transcriptase first-strand synthesis kit (Invitrogen) according to manufacturer's instructions, scaled down to a total of 10µL reaction. For the real-time PCR 20µL reactions consisting of 10µL of 2X PowerUp SYBR green master mix (Applied Biosystems), 5µL of appropriate primer mix (each at 1µM) for respective genes, and 5µL of diluted template.

Analytical techniques

For total amino acid content, 2-3 lyophilized whole leaves per plant were used to extract free amino acids in 10mM HCl and 0.1mM Norvaline, and chloroform. This formed one replicate. Three such replicates were collected per genotype and treatment. Phloem sap was collected as described in Corbesier et al. (2001). Briefly, Arabidopsis rosettes were cut at the base with a razor blade and placed in 10mM EDTA solution. 2-3 leaves were cut at the base of the petiole per plant in the solution, rinsed in 5mM EDTA solution and then placed in a microcentrifuge tube containing 5mM EDTA. This formed one replicate. Three such replicates were collected per genotype and treatment. The amino acids extracted from whole leaves and those from the phloem sap were were derivatized using the AccQ-Tag Ultra derivatization kit (Waters). The derivatized amino acids were used to determine amino acid content via UPLC. The amount of amino acids for each sample was normalized to the dry weight of the leaves.

Microscopy

For confocal microscopy, Zeiss LSM 880 with Airyscan was used for optical sectioning of images. GFP was excited using 488nm argon and mCherry/RFP was excited using 543nm HeNe lasers, and imaged using Airyscan detectors. The optical sections were processed using maximum intensity projections. The widefield images were taken using Zeiss Zoom.V16 fitted with a HBO

100 illuminating system. For all imaging mock and infected samples sets were subjected to identical imaging and post-imaging processing.

3.6 Supplementary Material

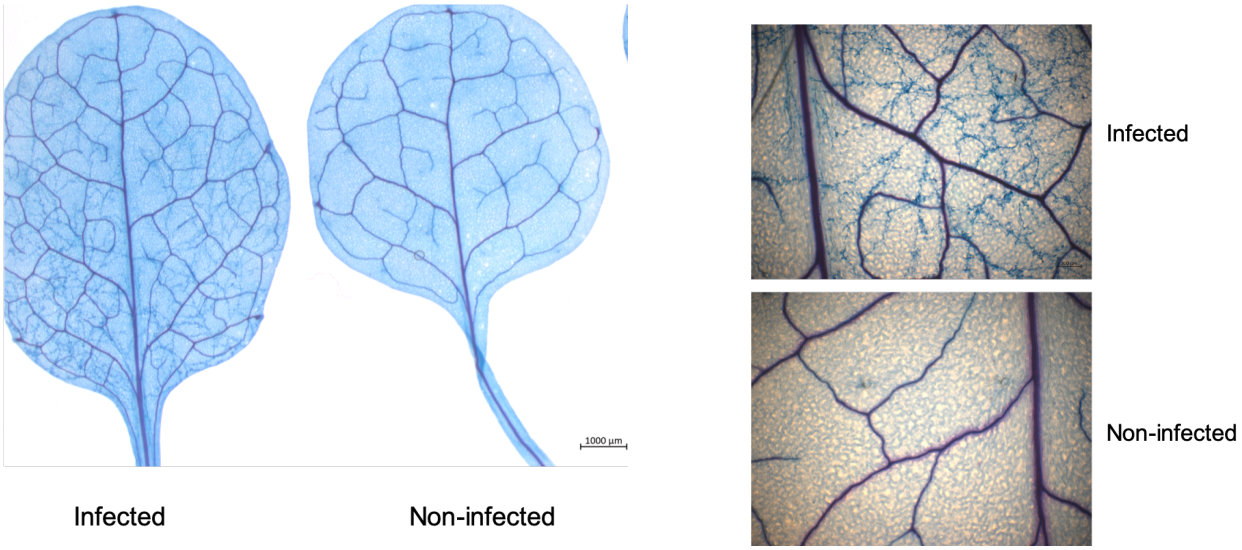


Figure 3.S1: Dual-staining of plants carrying *AAP6p*:GUS construct under infection by Noco2
2-3 leaves of transgenic plants expressing GUS under the control of 2kb region upstream of *AAP6* start site were drop inoculated with virulent isolate Noco2. The whole plant was dual-stained for β-glucuronidase activity (using magenta-gluc) followed by trypan blue staining at 5 dpi. Both leaves shown are from the same plant. (b) Magnified images of infected and non-infected leaves

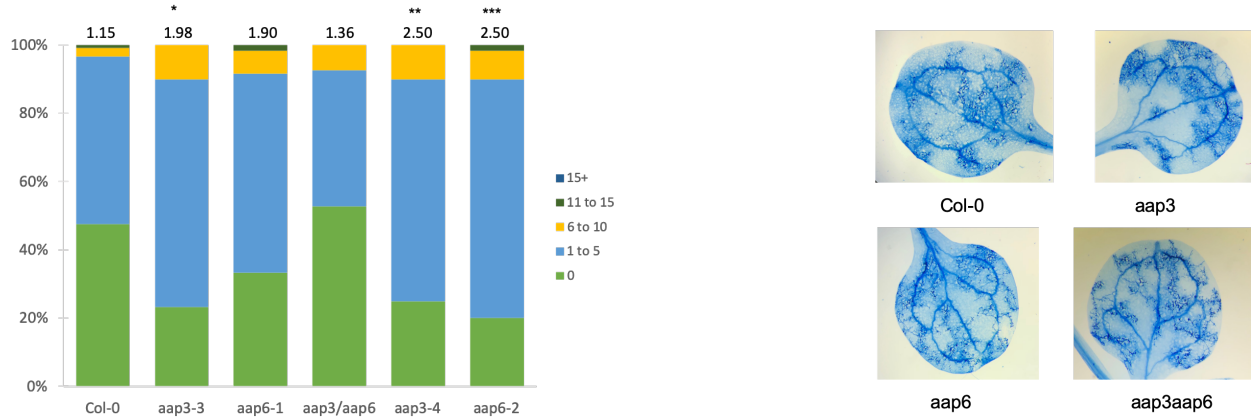


Figure 3.S2: Pathogen reproduction and trypan stains of Emwa1 infected plants 11 days old seedlings were inoculated with the avirulent isolate Emwa1 (a) Sporangiophores were counted on >40 cotyledons per genotype at 7 dpi. Counts were divided into bins and the frequency percentage of counts within those bins was plotted as a stacked bar. The numbers above the bars represent average sporangiophore counts per cotyledon. *,**,*** indicate p-value <=0.05,0.01,0.001 using Kruskal-Wallis test (b) Emwa1 infected seedlings were trypan stained at 5 dpi to enable visualization of hypersensitive response and pathogen strucutres. This experiment was conducted 2 more times with similar results.

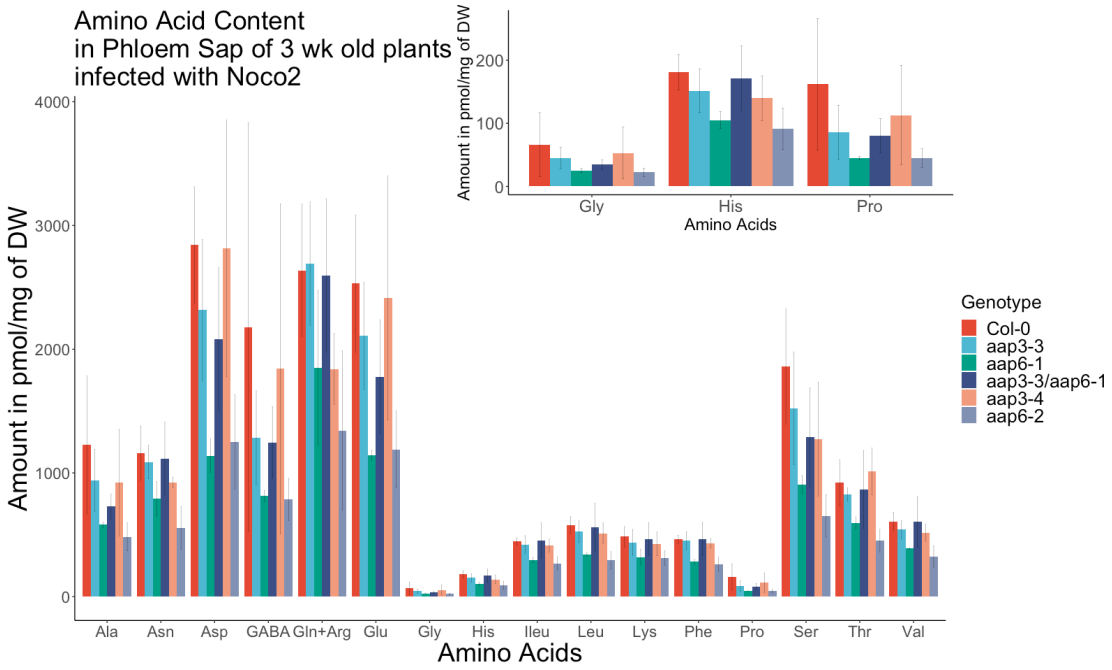


Figure 3.S3: Amino acid content in phloem sap of Noco2 infected plants

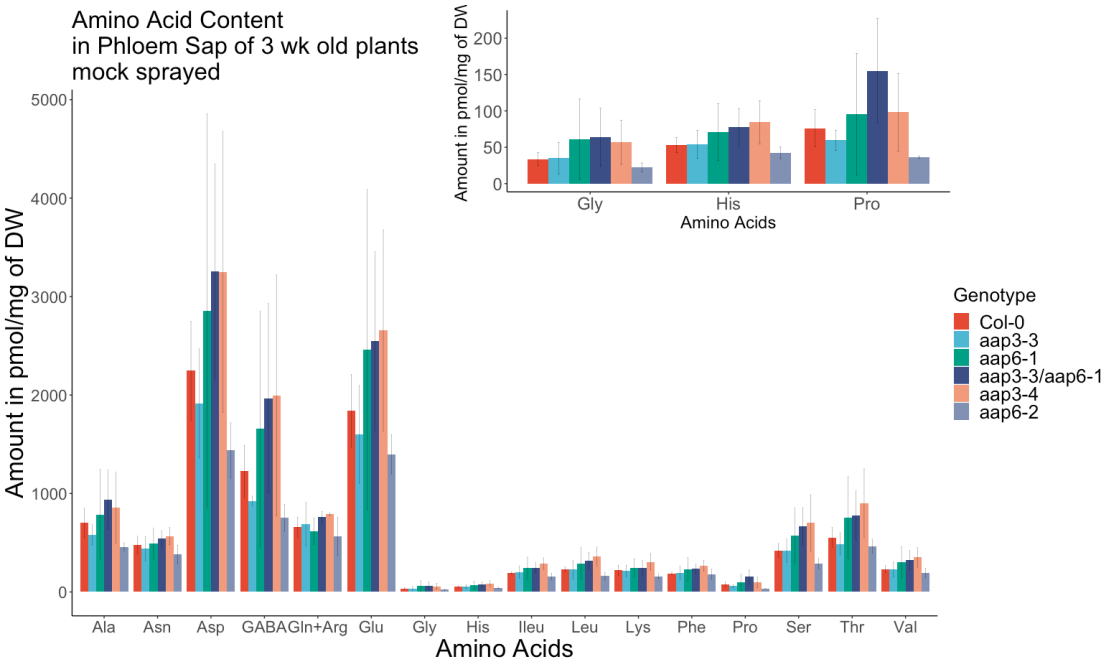


Figure 3.S4: Amino acid content in phloem sap of mock-treated plants

Chapter 3. References

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R. et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**, 653–657.

Anderson, R. G. and Mcdowell, J. M. (2015). A PCR assay for the quantification of growth of the oomycete pathogen *Hyaloperonospora arabidopsidis* in *Arabidopsis thaliana*. *Molecular Plant Pathology* **16**, 893–898.

Baxter, L., Tripathy, S., Ishaque, N., Boot, N., Cabral, A., Kemen, E., Thines, M., Ah-Fong, A., Anderson, R., Badejoko, W. et al. (2010). Signatures of adaptation to obligate biotrophy in the *Hyaloperonospora arabidopsidis* genome. *Science* **330**, 1549–1551.

Biezen, E. A. V. D., Freddie, C. T., Kahn, K., Parker, J. E. and Jones, J. D. G. (2002). Arabidopsis *RPP4* is a member of the *RPP5* multigene family of TIR-NB-LRR genes and confers downy mildew resistance through multiple signalling components. *The Plant Journal* **29**, 439–451.

Clough, S. J. and Bent, A. F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735–743.

Corbesier, L., Havelange, A., Lejeune, P., Bernier, G. and Périlleux, C. (2001). N content of phloem and xylem exudates during the transition to flowering in Sinapis alba and Arabidopsis thaliana. *Plant, Cell & Environment* **24**, 367–375.

Elashry, A., Okumoto, S., Siddique, S., Koch, W., Kreil, D. P. and Bohlmann, H. (2013). The *AAP* gene family for amino acid permeases contributes todevelopment of the cyst nematode *Heterodera schachtii* in roots of Arabidopsis. *Plant Physiology and Biochemistry* **70**, 379–386.

Hoth, S., Stadler, R., Sauer, N. and Hammes, U. Z. (2008). Differential vascularization of nematode-induced feeding sites. *Proceedings of the National Academy of Sciences* **105**, 12617–12622.

Hunt, E., Gattolin, S., Newbury, H. J., Bale, J. S., Tseng, H. M., Barrett, D. A. and Pritchard, J. (2010). A mutation in amino acid permease *AAP6* reduces the amino acid content of the *Arabidopsis* sieve elements but leaves aphid herbivores unaffected. *Journal of Experimental Botany* **61**, 55–64.

Lagarde, D., Basset, M., Lepetit, M., Conejero, G., Gaymard, F., Astruc, S. and Grignon, C. (1996). Tissue-specific expression of *Arabidopsis AKT1* gene is consistent with a role in K+ nutrition. *The Plant Journal* **9**, 195–203.

Manners, J. M., Penninckx, I. A., Vermaere, K., Kazan, K., Brown, R. L., Morgan, A., Maclean, D. J., Curtis, M. D., Cammue, B. P. and Broekaert, W. F. (1998). The promoter of the plant defensin gene PDF1.2 from Arabidopsis is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid. *Plant Mol Biol* **38**, 1071–80.

Marella, H. H., Nielsen, E., Schachtman, D. P. and Taylor, C. G. (2013). The amino acid permeases AAP3 and AAP6 are involved in root-knot nematode parasitism of Arabidopsis. *Molecular Plant-Microbe Interactions* **26**, 44–54.

McDowell, J. M. (2014). *Hyaloperonospora Arabidopsidis*: A model pathogen of Arabidopsis. In *Genomics of Plant-Associated Fungi and Oomycetes: Dicot Pathogens* (eds. R. A. Dean, A. Lichens-Park and C. Kole), pp. 209–234. Springer.

McDowell, J. M., Hoff, T., Anderson, R. G. and Deegan, D. (2011). Propagation, storage, and assays with *Hyaloperonospora arabidopsidis*: A model oomycete pathogen of Arabidopsis. In *Methods in Molecular Biology (Clifton, N.J.)*, volume 712, pp. 137–151. Humana Press.

Okumoto, S., Koch, W., Tegeder, M., Fischer, W. N., Biehl, A., Leister, D., Stierhof, Y. D. and Frommer, W. B. (2004). Root phloem-specific expression of the plasma membrane amino acid proton co-transporter AAP3. *Journal of Experimental Botany* **55**, 2155–2168.

Okumoto, S., Schmidt, R., Tegeder, M., Fischer, W. N., Rentsch, D., Frommer, W. B. and Koch, W. (2002). High affinity amino acid transporters specifically expressed in xylem parenchyma and developing seeds of Arabidopsis. *Journal of Biological Chemistry* **277**, 45338–45346.

Pariyar, S. R., Nakarmi, J., Anwer, M. A., Siddique, S., Ilyas, M., Elashry, A., Dababat, A. A., Leon, J. and Grundler, F. M. (2018). Amino acid permease 6 modulates host response to cyst nematodes in wheat and Arabidopsis. *Nematology* **20**, 737–750.

Pratelli, R. and Pilot, G. (2014). Regulation of amino acid metabolic enzymes and transporters in plants. *Journal of Experimental Botany* **65**, 5535–5556.

Sonawala, U., Dinkeloo, K., Danna, C. H., McDowell, J. M. and Pilot, G. (2018). Review: Functional linkages between amino acid transporters and plant responses to pathogens. *Plant Science* **277**, 79–88.

Voinnet, O., Rivas, S., Mestre, P. and Baulcombe, D. (2003). Retracted: An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *The Plant Journal* **33**, 949–956.

Wang, Y.-C., Yu, M., Shih, P.-Y., Wu, H.-Y. and Lai, E.-M. (2018). Stable pH Suppresses Defense Signaling and is the Key to Enhance Agrobacterium -Mediated Transient Expression in Arabidopsis Seedlings. *Scientific Reports* **8**, 1–9.

Wu, H.-Y., Liu, K.-H., Wang, Y.-C., Wu, J.-F., Chiu, W.-L., Chen, C.-Y., Wu, S.-H., Sheen, J. and Lai, E.-M. (2014). AGROBEST: An efficient Agrobacterium-mediated transient expression method for versatile gene function analyses in Arabidopsis seedlings. *Plant Methods* **10**, 19.

Yang, H., Postel, S., Kemmerling, B. and Ludewig, U. (2014). Altered growth and improved resistance of *Arabidopsis* against *Pseudomonas syringae* by overexpression of the basic amino acid transporter *AtCAT1*. *Plant, Cell and Environment* **37**, 1404–1414.

Zhang, L., Tan, Q., Lee, R., Trethewy, A., Lee, Y.-H. and Tegeder, M. (2010). Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in arabidopsis. *The Plant Cell* **22**, 3603–3620.

Chapter 4

Engineering a yeast strain used to characterize plant amino acid transporters and findings from its genome's sequencing

(Manuscript in preparation)

4.1 Introduction

We can use various genetic tools to study the function of a gene in an entire organism, such as a whole plant. This can lead us to an understanding of the major role of the gene and the mechanism of its function in the plant, but it might still be difficult to deduce the biochemical function and properties of the protein encoded by this gene of interest. Basic cell biology and metabolism is conserved among all eukaryotic organisms; hence simpler eukaryotic organisms such as *Saccharomyces cerevisiae* can be used to study the function of proteins from more complex organisms such as plants. This approach of expressing a gene in a simpler system to further understand its function is known as heterologous expression (d'Enfert et al., 1995).

Many plant genes have been studied by expressing them in baker's yeast (*Saccharomyces cerevisiae*) (Frommer and Ninnemann, 1995). The function of a plant gene can be studied by expressing it in a yeast strain where the yeast gene encoding the same function has been deleted. This yeast exhibits a phenotypic defect (such as auxotrophy), which is restored by expression of the plant gene of interest. This method is called functional complementation of the yeast mutant (Frommer and Ninnemann, 1995).

Functional complementation for studying plant amino acid transporters can be achieved in two ways: a transport complementation or complementation of a biosynthesis auxotrophy. In the first case, a mutant yeast lacking amino acid transporters for the transport of one or more amino acids is grown in a medium with the amino acid of interest as the sole nitrogen source. This yeast thus relies on the function of the heterologously expressed transporter for its nitrogen needs. In the second case, a mutant yeast unable to transport a specific amino acid nor synthesize it is used to study the foreign transporter via uptake of the amino acid of interest from the medium and complementation of the auxotrophy. Both of these methods have inherent advantages and disadvantages based on how they work. In case of a pure transport complementation, the same mutant yeast strain that is unable to transport several amino acids can be used to study the transport capabilities of a plant transporter for all of those amino acids. However, since the yeast relies on this amino acid as its sole nitrogen source, it is difficult to use lower concentrations of amino acids. On the other hand,

in case of biosynthesis complementation the yeast does not depend on the particular amino acid as its sole nitrogen source; hence can be used to study transport of that amino acid at much lower concentrations. However, several mutant strains lacking the ability to synthesize each amino acid of interest need to be generated. Furthermore, since certain amino acids can be synthesized via several biosynthetic pathways, this approach would not work for all amino acids.

Functional complementation of yeast was first used to study plant amino acid transporters in the early 1990s. The yeast strain JT16 lacking both the histidine permease (HIP1) and an enzyme required in the synthesis of histidine (HIS4) was used to screen for a plant amino acid transporter that could uptake histidine (Hsu et al., 1993). Around the same time, yeast strain 22574d (Fig. 4.1) lacking a broad specificity permease (GAP1), a γ-aminobutyric acid transporter (UGA4) and a high-affinity proline permease (PUT4) was used to identify a plant amino acid transporter that was able to transport proline (Frommer et al., 1993). Both groups had simultaneously cloned the first secondary-active amino acid transporter, AAP1 (Amino Acid Permease 1) (Chang and Bush, 1997). Several other plant amino acid transporters were identified by using complementation of these yeast mutants lacking their endogenous amino acid transporters in the later half of the 90s (Fischer, 1998) (See Kwart et al. (1993); Fischer et al. (1995); Rentsch et al. (1996) for identification of other AAPs and related proline transporters, Frommer et al. (1995) for identification of cationic amino acid transporter (CAT1) and Chen and Bush (1997) for identification of lysine-histidine transporter (LHT1)).

Baker's yeast contains several endogenous amino acid transporters, 22 of them are localized to the plasma membrane (Popov-Čeleketić et al., 2016). They all belong to the APC (Aminoacid-Polyamine-organoCation) superfamily and are further divided into the YAT (Yeast Amino acid Transport), LAT (L-type Amino acid Transport), and ACT (Amino acid Choline Transporter) families (Gournas et al., 2016). Some of these transporters display broad specificity for amino acids, whereas others are more specific and transport a few amino acids (Van Belle and André, 2001).

The mutant yeast strain 22574d is still able to transport other amino acids apart from γ-aminobutyric acid (GABA) and proline by using the remaining endogenous amino acid transporters. To enable study of lysine transport via heterologous expression, Fischer et al. (2002) deleted three other yeast amino acid transporters in this background responsible for transporting arginine (CAN1) and lysine (LYP1 and APL1) along with an enzyme required for biosynthesis of lysine (LYS2). This mutant yeast strain was named 22Δ6AAL (after the 6 deleted amino acid transporters in this strain and the biosynthesis enzyme LYS2). This is another example of a yeast mutant useful for studying transport of an amino acid via complementation of biosynthesis auxotrophy. Since the strain cannot synthesize lysine and lacks major endogenous lysine transporters, it is mostly only useful for studying lysine transport. To study transport of other amino acids, two other transporters were deleted in the 22Δ6AA background (HIP1 and DIP5 to disrupt histidine, and glutamate and aspartate transport respectively) leading to the strain 22Δ8AA (Fischer et al., 2002) (see Fig. 4.1). At 3mM concentration, 22Δ8AA is unable to grow on 5 amino acids as its sole nitrogen source, including the non-proteinogenic amino acids: GABA and citrulline. This strain is still able to grow on 13 other amino acids, including threonine. Based on the identification of the yeast amino acid transporters AGP1 and GNP1 as the major transporters of threonine (along with GAP1, which

is already deleted in 22Δ8AA) (Velasco et al., 2004), we previously reported deleting these two transporters in the 22Δ8AA background to increase the number of amino acids whose transport can be studied. This led to the strain 22Δ10α (Besnard et al., 2016). On characterization of this strain, we found that in addition to threonine 22Δ10α is not able to grow on 9 additional amino acids as its sole nitrogen source at 3mM concentration (Fig. 4.2 Fig. 4.2) (Besnard et al., 2016). This is probably because AGP1 is also a broad specificity amino acid transporter in yeast (Iraqui et al., 1999).
In order to reduce the background transport for the remaining aromatic amino acids, we deleted 5 other endogenous amino acid transporters in the 22Δ10α background in 3 different combinations. Apart from reducing the background transport of aromatic amino acids, we also wanted to engineer a yeast strain that could be used to study transport at higher concentrations than 3mM, which would be useful in characterizing low-affinity plant amino acid transporters with high K_m values.
Here, we describe our work on these further deletions of endogenous amino acid transporters in the 22Δ10α background, characterization of these higher deletion yeast mutant strains, and our findings from the genome sequencing of 22Δ10α.

4.2 Results

Deletion of amino acid transporters in 22Δ10α

Based on the work by Regenberg et al. (1999), the endogenous transporters BAP2, BAP3, TAT1 and TAT2 are important for transport of aromatic amino acids (Phenylalanine, Tryptophan and Tyrosine), BAP2, BAP3 and TAT2 were also found to be important for transport of the branched-chain amino acids (Leucine, Isoleucine and Valine) and to a lesser degree alanine and glycine (Regenberg et al., 1999).
Another endogenous yeast transporter AGP3 has been shown to transport leucine, and becomes more important for yeast growth in low nutrient conditions or in yeast mutants lacking the broad-specificity transporters (GAP1 and AGP1) (Schreve and Garrett, 2004). Hence, we deleted these 5 endogenous amino acid transporters in the following combination: deletion of BAP3 leading to 22Δ11, deletion of BAP2 and TAT1 in 22Δ11 background leading to 22Δ13, and deletion of AGP3 or TAT2 in the 22Δ11 background each leading to 22Δ12 or 22Δ12' respectively (Fig. 4.1). Several attempts were made to make further deletions in the 22Δ12 and 22Δ13 background with no success.

Characterization of 22Δ11 and 22Δ13

To test if these deletions would reduce the background growth of these mutants on higher concentrations of amino acids as sole nitrogen source, we grew the yeast mutant strains 22Δ10α, 22Δ10 and 22Δ13 transformed with empty vector pRS on minimal medium with varying concentrations of each amino acid: 0.5mM, 3mM. 9mM and 12mM. We also complemented these strains with the broad-specificity yeast amino acid transporter GAP1 (Jauniaux and Grenson, 1990) as a positive control.

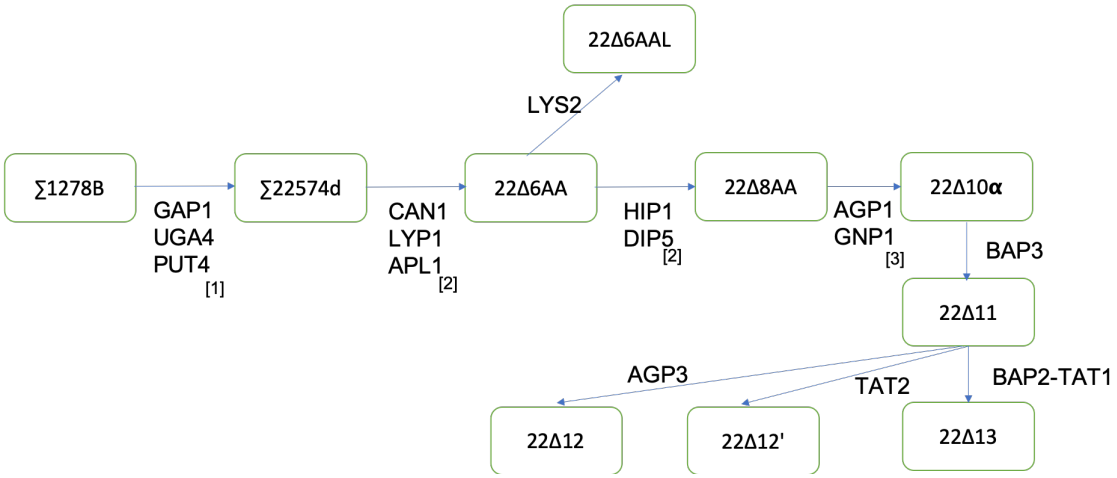


Figure 4.1: Short schematic displaying the genealogy leading to 22Δ10α and its progenies The major strain names are indicated in green boxes and the knock-out of genes encoding given amino acid transporters are noted beneath the arrow. 1. Jauniaux and Grenson (1990), 2. Fischer et al., (2002) and 3. Besnard et al., (2016)

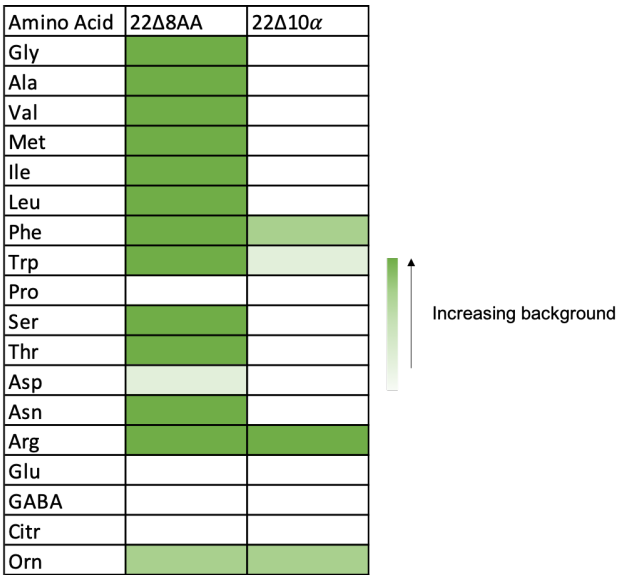


Figure 4.2: Background growth of 22Δ8AA and 22Δ10α (3mM amino acid concentration) 22Δ8AA and 22Δ10α were grown on minimal medium with 3mM concentration of the given amino acid as sole nitrogen source (except for Gln at 1.5mM concetraion). The background growth of the mutant strain transformed with empty vector (see Fig. 4.S1) is represented here by the increasing intensity of green color, with white meaning little to no growth.

We found that when grown on higher concentrations of 9 and 12mM of amino acids, the comparisons in background growth between 22Δ10α, and 22Δ11 and 22Δ13 can be divided into four sections (Fig. 4.3, Fig. 4.S2): i) In case of methionine, leucine and ornithine we saw an increase in background growth in 22Δ10α (compared to the lower 3mM amino acid concentration) and a corresponding reduction or lack of background growth in 22Δ11 and 22Δ13 ii) For isoleucine, phenylalanine, alanine, valine and asparagine we saw a small increase in background growth in 22Δ10α and a lack of background in 22Δ10 and 22Δ13 iii) In case of glycine and glutamine we saw an increase in background growth of all three mutant yeast strains iv) In case of proline, glutamate, aspartate, threonine, serine, GABA and citrulline we did not see an increase in background growth in any of the three mutant yeast strains.
Overall, we only saw a substantial reduction in background growth of 22Δ11 and 22Δ13 when grown on higher concentrations of methionine, leucine and ornithine. For other amino acids, 22Δ10α had similar or no background growth either.

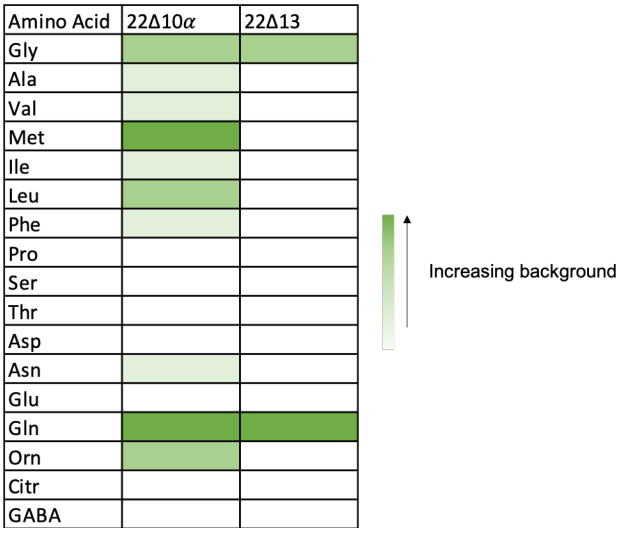
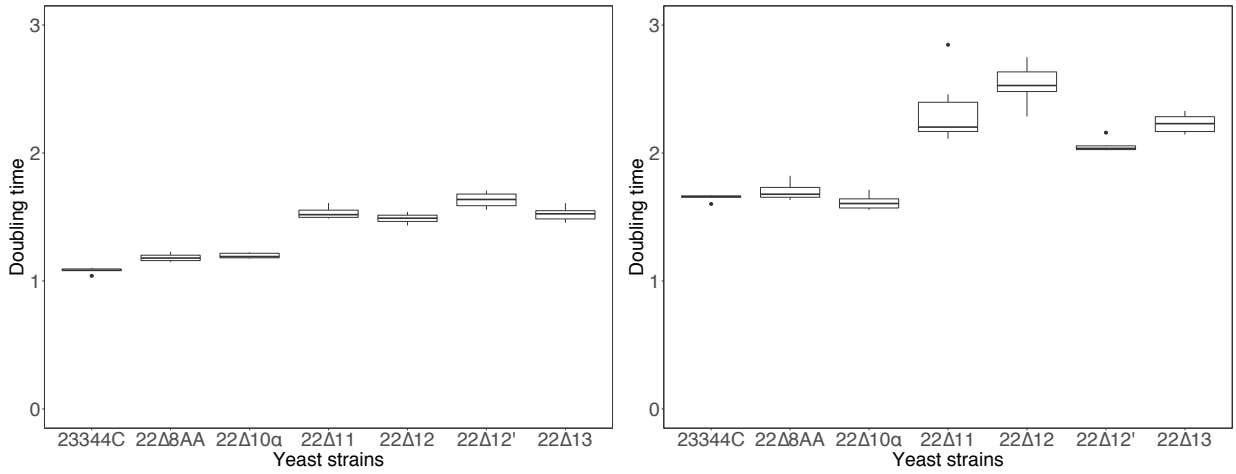


Figure 4.3: Background growth of 22Δ10α and 22Δ13 (for 9mM and 12mM amino acid concentration) 22Δ10α and 22Δ13 were grown on 9mM or 12mM concentration of given amino acid as sole nitrogen source (see Fig. 4.S2). The background growth of the mutant yeast strain transformed with empty vector is represented here by the increasing intensity of green color, with white indicating little to no growth.

22Δ11 and 22Δ13 display slower growth rate

While working with 22Δ11 and 22Δ13, we noticed that these two strains grew appreciably slower compared to 22Δ10α and sometimes led to poor transformation efficiency. We calculated the growth rate of these strains and the corresponding doubling time in nutrient rich YPDA medium and the minimal SD medium (Fig. 4.4) . We found that in the nutrient rich YPDA medium 22Δ11, 22Δ12, 22Δ12' and 22Δ13 took 25-30 minutes more to double compared to the wild-type yeast strain. The impeded growth was magnified when these mutants were grown in minimal SD medium with 22Δ11, 22Δ12 and 22Δ13 doubling up to 40-50 minutes slower than 23344C.



(a) Doubling time of yeast strains in YPDA **(b)** Doubling time of yeast strains in SD

Figure 4.4: Doubling time of yeast strains in (a) YPDA and (b)SD medium. Yeast strains were grown in the respective medium and their ODs measured using a plate reader, every 5 min for 15 hours. The data were fitted to a standard form of logistic equations (see Fig. 4.S3 for the fit) to get the growth characteristics including doubling time. Each boxplot represents six data points per strain.

Genome sequencing of 22Δ10α

The mutant yeast strains starting from 22574d have been used to study plant amino acid transporters for several years. However, none of these yeast strains apart from the parent Σ1278b have been sequenced. Previous deletion studies, such as those for yeast deletion collection, have found that deleting yeast genes can cause off target deletion or gene duplications, and sometimes chromosomal rearrangements (Giaever et al., 2002; Giaever and Nislow, 2014). Although rare, such collateral changes and off-target effects to the genome are possible. In order to aid future research with these mutant strains, we sequenced 22Δ10α's genome. Owing to the slower growth and reduced transformation efficiency of 22Δ11 and 22Δ13 we chose to sequence 22Δ10α, which would be used more frequently than those with higher deletions.
Using high-coverage of long-read PacBio sequencing, we were able to assemble 22Δ10α's genome *de novo* in 21 contigs (yeast contains 16 nuclear chromosomes and 1 mitochondrial chromosome) with a 12.7Mbp assembly size (Koren et al., 2017). When aligned to the reference genome (S288C) (Engel et al., 2014), the dotplot showed coverage over all chromosomes further supporting the assembly (Fig. 4.S4, Fig. 4.S5).

Confirmation of expected indels in 22Δ10α's genome

PacBio reads from 22Δ10α were aligned to the reference genome and expected disruptions in the ten amino acid transporter genes were visualized using Integrated Genome Viewer (IGV) (Fig. 4.5). We were able to confirm all ten expected disruptions in amino acid transporter genes. In case of insertions, reads spanning the insertions were used to identify the insert using BLAST

(Fig. 4.5(a)). These insertions are a result of the scar left behind by the deletion cassette used to disrupt the genes, except *PUT4* and *UGA4* which were disrupted as a result of a transposon insertion (Jauniaux and Grenson, 1990). 22547d and all of its progenies also have a disrupted *URA3* gene which codes for a pyrimidine synthesis enzyme. This auxotrophy has been used as a selection marker during yeast transformation. With high-read coverage, we were able to identify a potential SNP disrupting this gene, which aligns with the ethyl methansulfonate (EMS) based mutagenesis that lead to this mutation (Grenson, 1969).

Gene	Indel information
GAP1	4kb deletion
PUT4	6kb insertion (Transposon)
UGA4	6kb insertion (Transposon)
CAN1	250bp insertion (HisG scar)
ALP1—LYP1	2.5kb deletion
HIP1	800bp insertion (HisG scar)
DIP5	1200bp insertion (HisG-URA3-HisG scar)
AGP1	2kb deletion
GNP1	2kb deletion

(a) Expected indels in 22Δ10α

(b) GAP1 deletion viewed in IGV

Figure 4.5: Confirmation of expected indels in 22Δ10α's genome (a)Ten amino acid transporter genes expected to be disrupted in 22Δ10α and further information gained from the genome's alignment to the reference (b)Deletion in *GAP1* as viewed in Integrated Genome Viewer (IGV). The 4kbp deletion is visualized by the drop in read coverage of the reference genome.

4.3 Discussion

In this work, we report deleting three more endogenous amino acid transoprters (BAP3, BAP2 and TAT1) in the 22Δ10α background and characterizing the resulting two yeast strains: 22Δ11 and 22Δ13.

Since BAP2, BAP3, TAT1 along with GAP1 are the most important amino acid transporters for the three aliphatic amino acids Leucine, Isoleucine, Valine (Regenberg et al., 1999), the reduction in background growth of 22Δ13 when grown on higher concentration of these amino acids seems to be a result of the deletion of these transporters. Regenberg et al. (1999) also found that apart from methionine specific high-affinity transporters, this amino acid is also transported by some combinatorial contribution from AGP1, BAP2 and BAP3. BAP2 and BAP3 appear to be more important at the 9mM and 12mM concentrations of amino acids since 22Δ10α lacking AGP1 is not able to grow on 3mM methionine as sole nitrogen source, but does grow at 9 and 12mM. We also found that 22Δ11 and all of its progenies (22Δ12, 22Δ12' and 22Δ13) display a growth penalty, especially in minimal medium. This is probably because many of these endogenous transporters become more important for the yeast when its other broad-specificity transporters are lost (Schreve and Garrett, 2004). Furthermore, the growth penalty means that these strains sometimes transform poorly and probably explains why we were not able to make any further deletions in the 22Δ13 background. We thus recommend using this strain only when researchers need to study transport of amino acids such as Methionine, Leucine and Ornithine at higher concentrations. For most other purposes, 222Δ10α should suffice.

As described in the results section above, the genomes of none of the mutant yeast strains used to study plant amino acid transporters had been sequenced to date. We report genome sequencing of 22Δ10α strain and generating a high coverage assembly of the genome using long-read PacBio sequencing data. We were able to confirm the ten expected indels in the amino acid transporter genes and acquire a list of other structural variants. Since the reads were initially mapped to the reference genome S288C (to take advantage of its genome quality and annotation), this list of variants also includes any variation between the reference and 22Δ10α's parent strain (Σ1278b). We are working on using the sequence of the parent strain (albeit of lower quality than S288C) to get more information on the origin of variation.

4.4 Materials and Methods

Cloning and transformations

cDNA/DNA for amino acid transporters were PCR-amplified from Col-0 cDNA or yeast genomic DNA respectively. They were cloned into pDONRZeo entry vector and were then moved to the binary destination vectors pDR196-Ws or pRS-Ura-Ws using Gateway technology (ThermoFisher Scientific). Deletions of yeast genes were performed as described by Güldener et al. (1996). Briefly, forward and reverse primers flanking the KanMX casette were designed such that 40bp would anneal to the 5' and 3' of the gene to be deleted, respectively. The PCR product from using plasmid pUG6 as template was used to transform the yeast. The recombinants were selected for by growing the yeast on YPDA medium with 200 µg/ ml of antibiotic G418. The insertion of KanMX and loss of gene to be deleted was verified by PCR. The KanMX casette was removed by transforming the yeast with the plasmid pSH47, which contains the Cre recombinase construct under the control of GAL4 promoter. Yeast were grown on synthetic complete medium (SC) lacking uracil to select for pSH47 following which Cre expression was induced by growing the yeast in YPG medium (using galactose instead of dextrose). Yeast induced by galactose were streaked on SC (lacking uracil) plates and were tested for loss of KanMX by lack of growth on mediium containing and G418 and via PCR. The plasmid pSH47 was removed by using minimum medium plates supplemented with 1mg/ml 5-Fluoroorotic Acid (5-FOA) for counter-selection.

DNA extraction and sequencing

High molecular weight DNA from yeast was extracted using MagAttract HMW DNA kit (Qiagen) by following the manufacturer's protocol, with following modifications for use with yeast: Yeast cells were grown until they reached log phase (two doublings), washed and resuspended in 1M Sorbitol. They were then converted to spheroplasts using Zymolyase 100T solution (United States Biological). The spheroplasts were washed with 1M Sorbitol solution, centrifuged and then used for DNA extraction. DNA was tested for purity and molecular weight with NanoDrop (OneC, Thermo Scientific) and by running it on a gel with Lambda DNA/HindIII marker (NEB), respectively. DNA was sequenced using Sequel platform from PacBio.

Genome Assembly and Analysis

The PacBio sequences yeast genome was assembled *de novo* using canu (Koren et al., 2017) and was then aligned to the S288C reference genome (Engel et al., 2014) using the whole genome aligner (Marçais et al., 2018). Corrected reads from canu were also aligned to the S288C reference genome using minimap2 (Li, 2018) and freebayes (Garrison and Marth, 2012) was used to detect variants compared to the reference genome. The aligned reads and coverage of particular regions of the genome were visualized using Integrative Genome Viewer (IGV) (Robinson et al., 2011)

Yeast growth rate and doubling time measurements

For measuring the growth rate and doubling of yeast strains, a single colony was used to start a culture in YPDA overnight at 30˚C, shaking at 200rpm (Three such cultures were started and maintained for the rest of the experiment per strain, serving as biological replicates). This liquid culture was then used the next morning to start a subculture in either YPDA or SD(+Uracil) medium at OD of 0.1. The subculture was allowed to grow for 5-6 hours (in order to achieve 2-3 doublings). This was then used to start a third subculture in 96-well plates at an OD of about 0.05-0.1 in 200µL of either YPDA or SD(+Uracil) as specified. biological replicate per strain was also technically replicated twice on the 96-well plate, leading to 6 total wells per strain. Synergy H1 Hybrid Multi-Mode plate reader (Biotek) was used for measuring the OD of the cultures with the following settings: temperature set at 30˚C, measurements taken every 5min at 600nm, continuous orbital shaking at 559nm (slow speed) for 15 hours. The growth curve from the data was fit to logistic equation and growth characteristcs were measured using Growthcurver package in R (Sprouffske and Wagner, 2016).

4.5 Supplementary Material

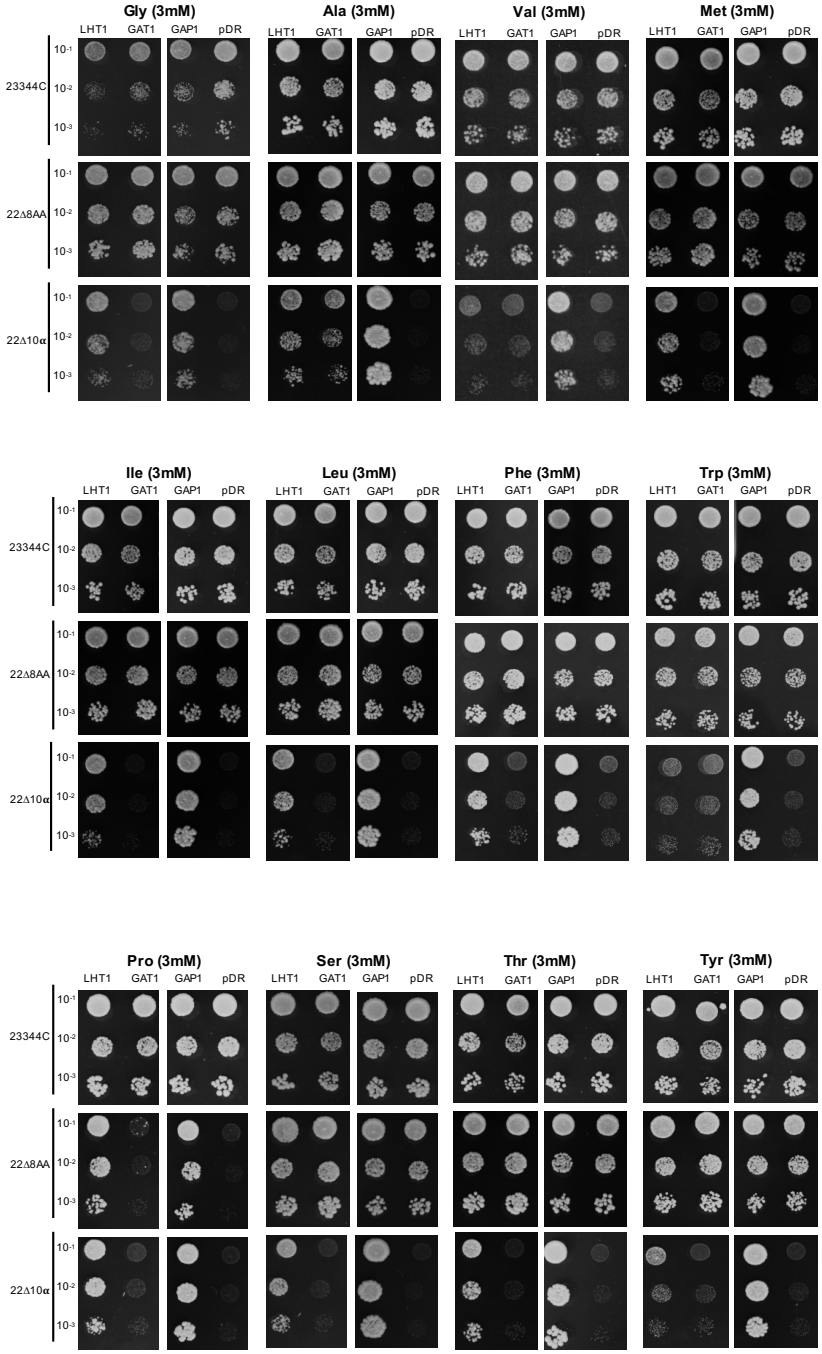


Figure 4.S1

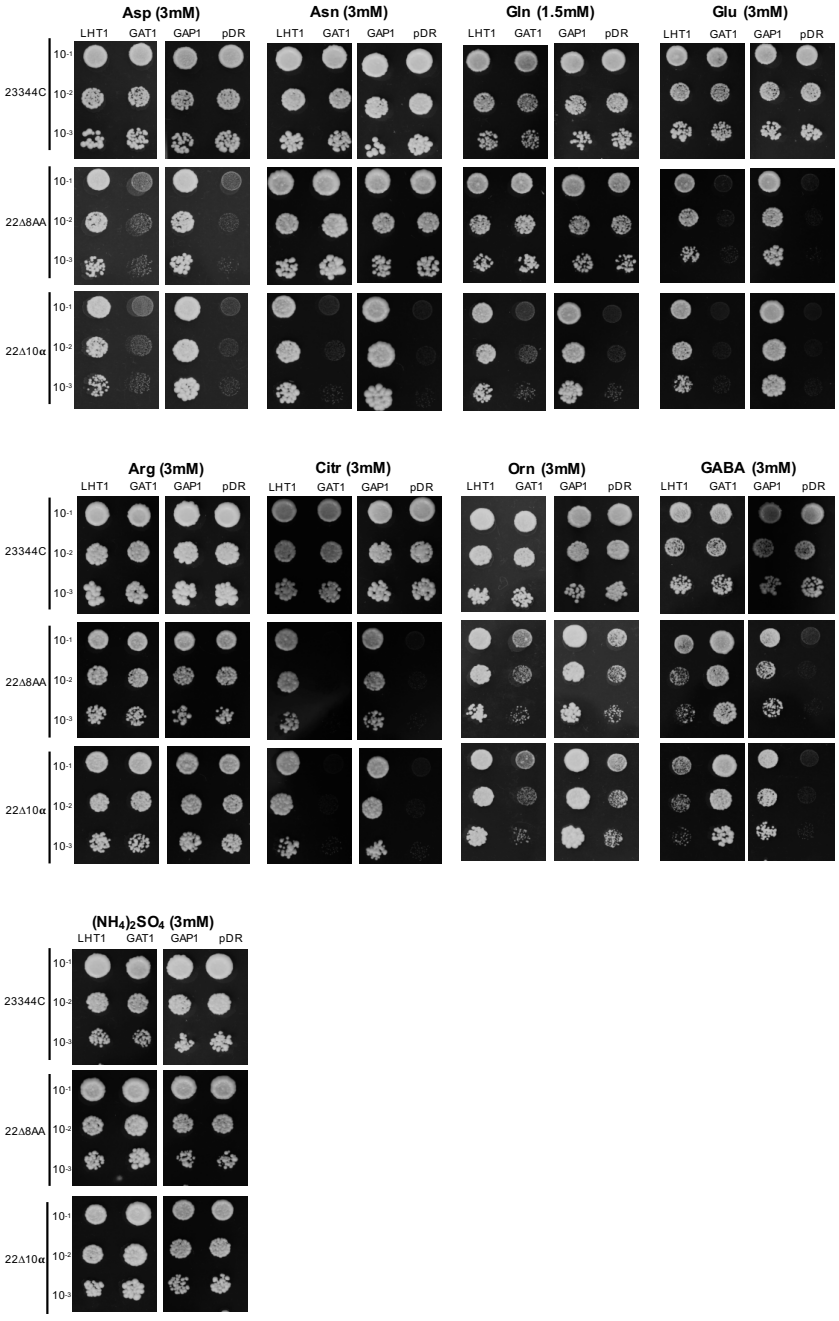


Figure 4.S1: Functional complementation of 22Δ8AA and 2210α. Wild-type yeast strain 23344C and mutant yeast strains 22Δ8AA and 22Δ10α were complemented with plant amino acid transporters LHT1 and GAT1, yeast broad-specificity amino acid transporter GAP1 and grown on given amino acids at 3mM concentrations (except Gln at 1.5mM). Published in Besnard et al. (2016).

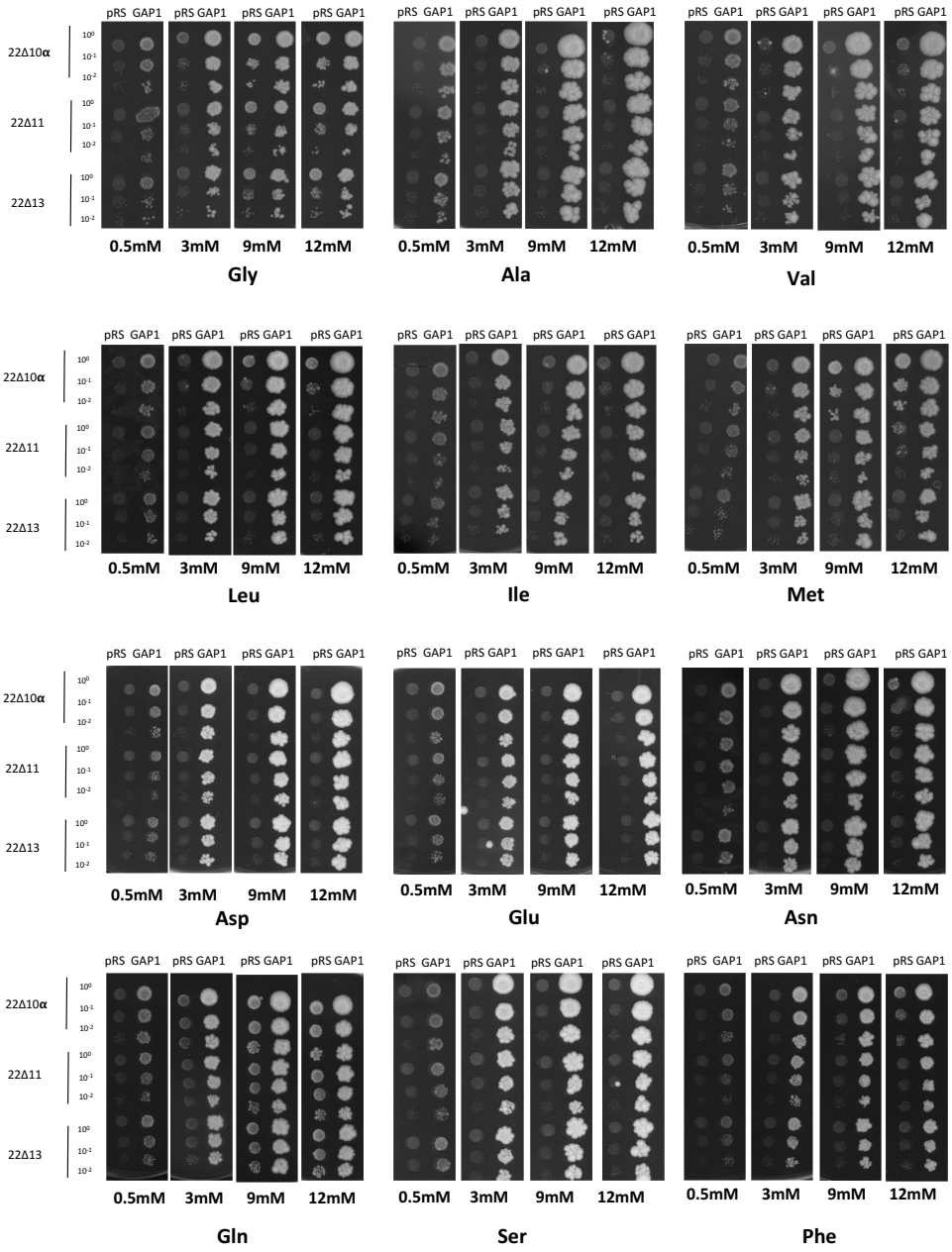

pRS GAP1
22Δ10α
22Δ11
22Δ13
0.5mM 3mM 9mM 12mM
Gly
Ala
Val
Leu
Ile
Met
Asp
Glu
Asn
Gln
Ser
Phe

Figure 4.S2

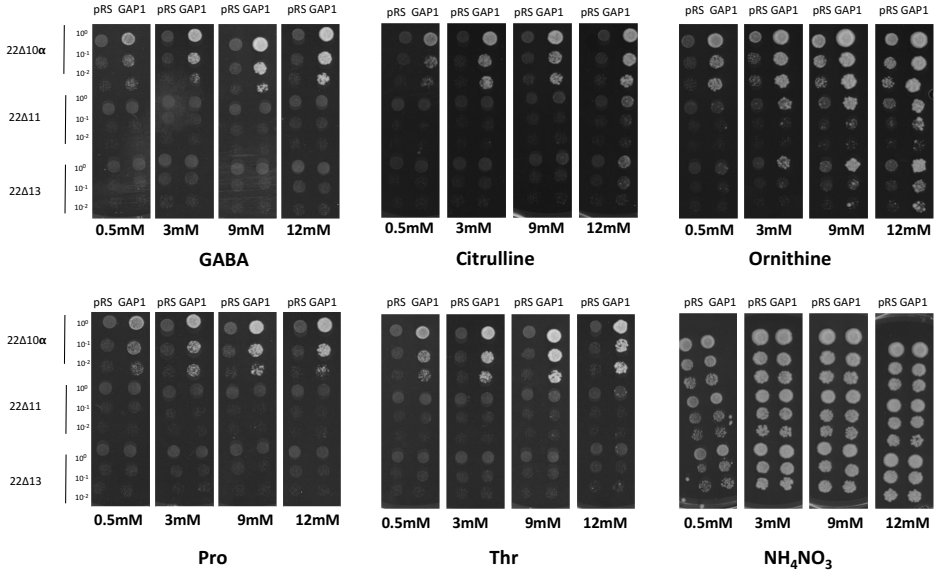
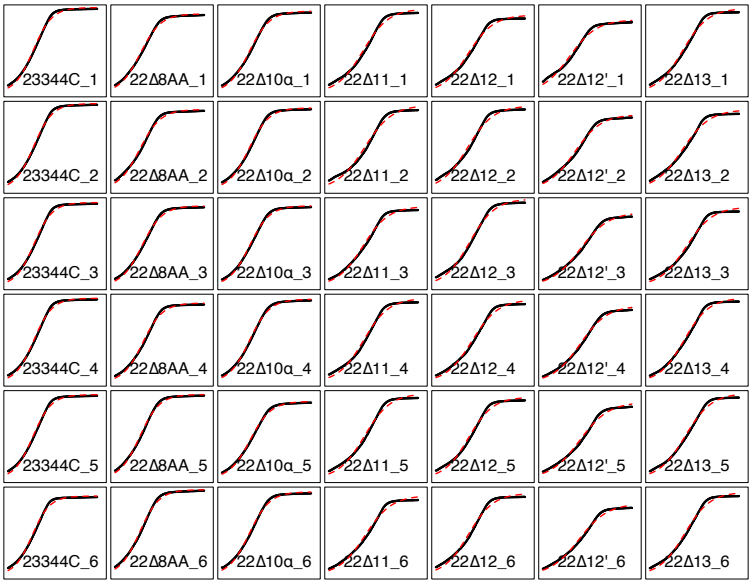
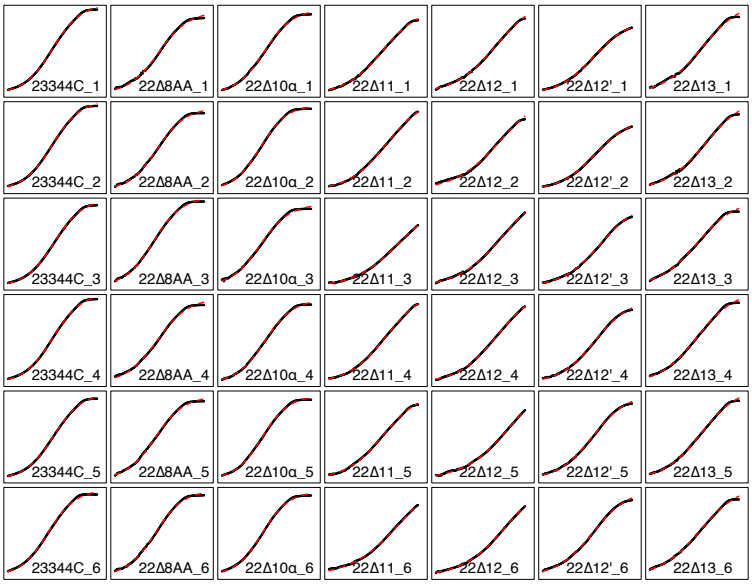


Figure 4.S2: Growth of 2210α, 22Δ11 and 22Δ13 on different concentrations of amino acids as sole nitrogen source. Mutant yeast strains 22Δ10α, 22Δ11 and 22Δ13 were transformed with empty vector pRS or complemented with yeast broad-specificity amino acid transporter GAP1 and grown on 0.5mM, 3mM, 9mM and 12mM concentrations of amino acids.



(a) Growth curves for yeast grown in YPDA



(b) Growth curve for yeast grown in SD

Figure 4.S3: Growth curve for yeast strains grown in (a) YPDA and (b)SD medium. Yeast strains were grown in the respective medium and their ODs measured using a plate reader, every 5 min for 15 hours. The data were fitted to a standard form of logistic equation. The black line indicates the actual data points for the given well and the dotted red line indicates the best fit curve for the given data.

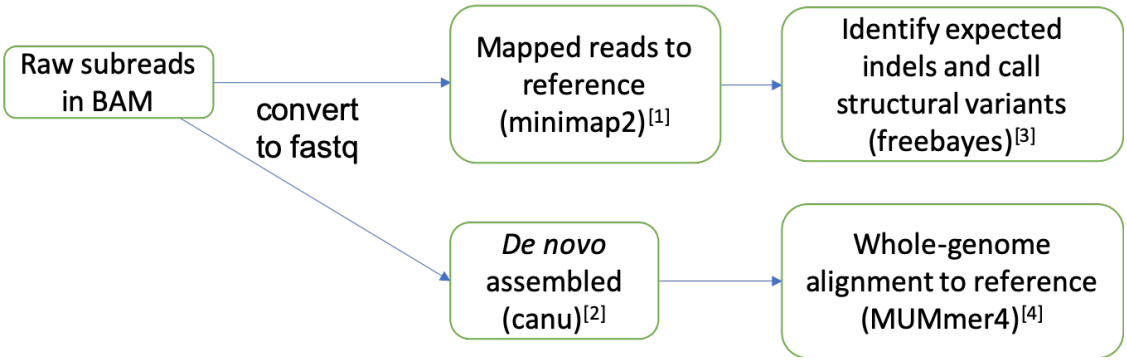


Figure 4.S4: Short schematic displaying the pipeline used for genome assembly and variant calling S288C was used as reference for mapping.
1.Li (2018), 2.Koren et al. (2017) and 3.Garrison and Marth (2012), 4.Marçais et al. (2018)

Assembly	Contigs
# contigs	21
Largest contig	2966298
Total length	12754172
GC (%)	38.37
N50	1035883
N75	699390
L50	4
L75	8

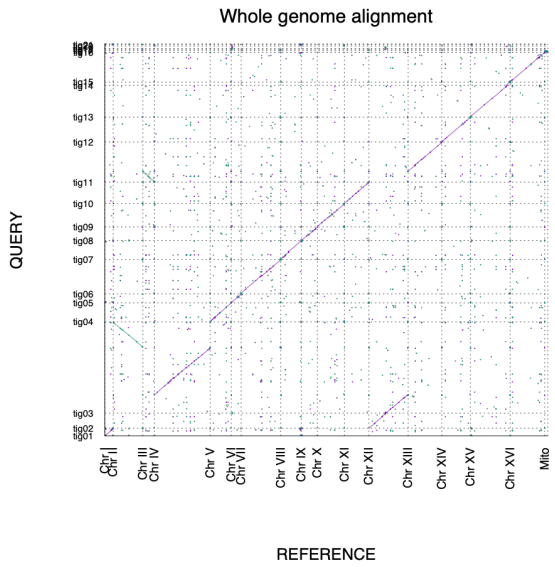


Figure 4.S5: Assembly statistics (left) and whole-genome alignment of 22Δ10α to the reference (right) MUMmer4 was used to align the two genomes and the alignment visualized using a dotplot (right).


NC_001147.6
1,450 bp
967,800 bp
968,000 bp
968,200 bp
968,400 bp
968,600 bp
968,800 bp
969,000 bp
Coverage
0 - 679
Aligned reads
6826
5,970
5,962
Sequence
XV
Annotations
PUT4
NM_001183768.1

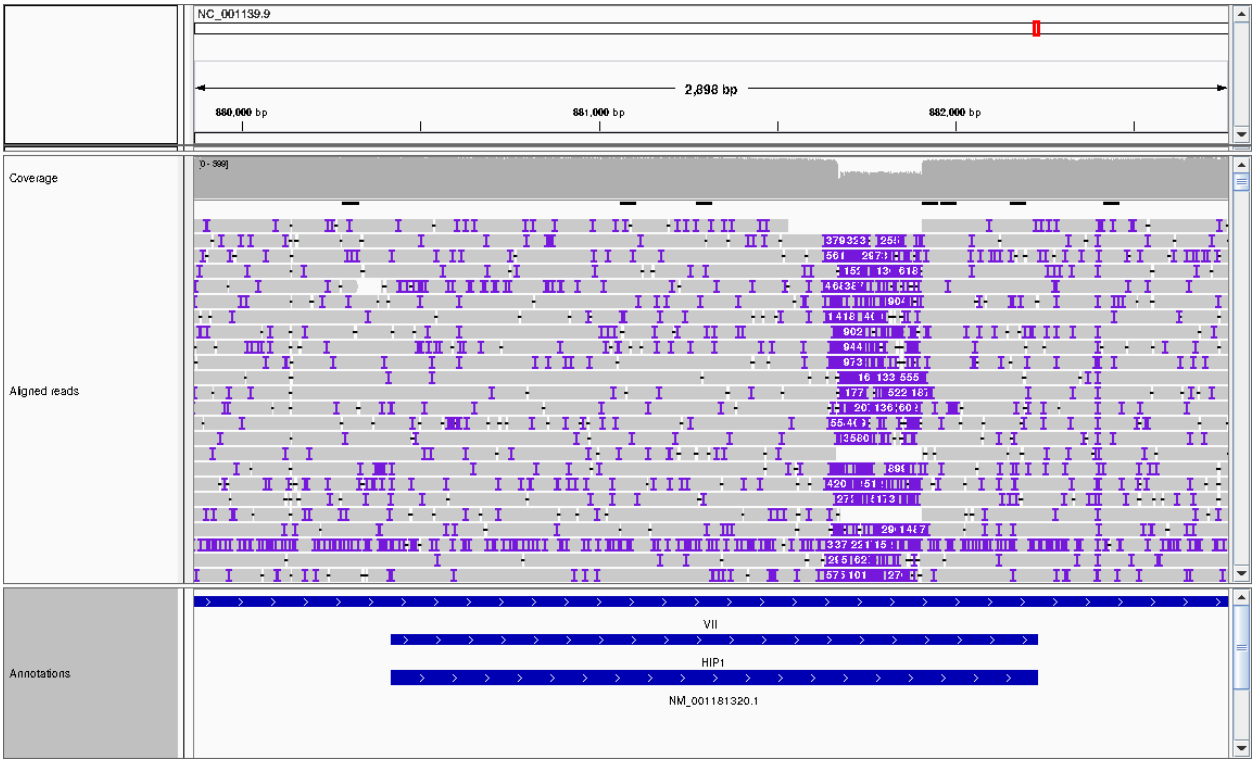
(a) PUT4


NC_001136.10
1,018 bp
84,800 bp
85,000 bp
85,200 bp
85,400 bp
85,600 bp
Coverage
0 - 843
Aligned reads
6,055
Sequence
IV
Annotations
NM_001180270.1
UGA4

(b) UGA4

(c) CAN1

(d) ALP1-LYP1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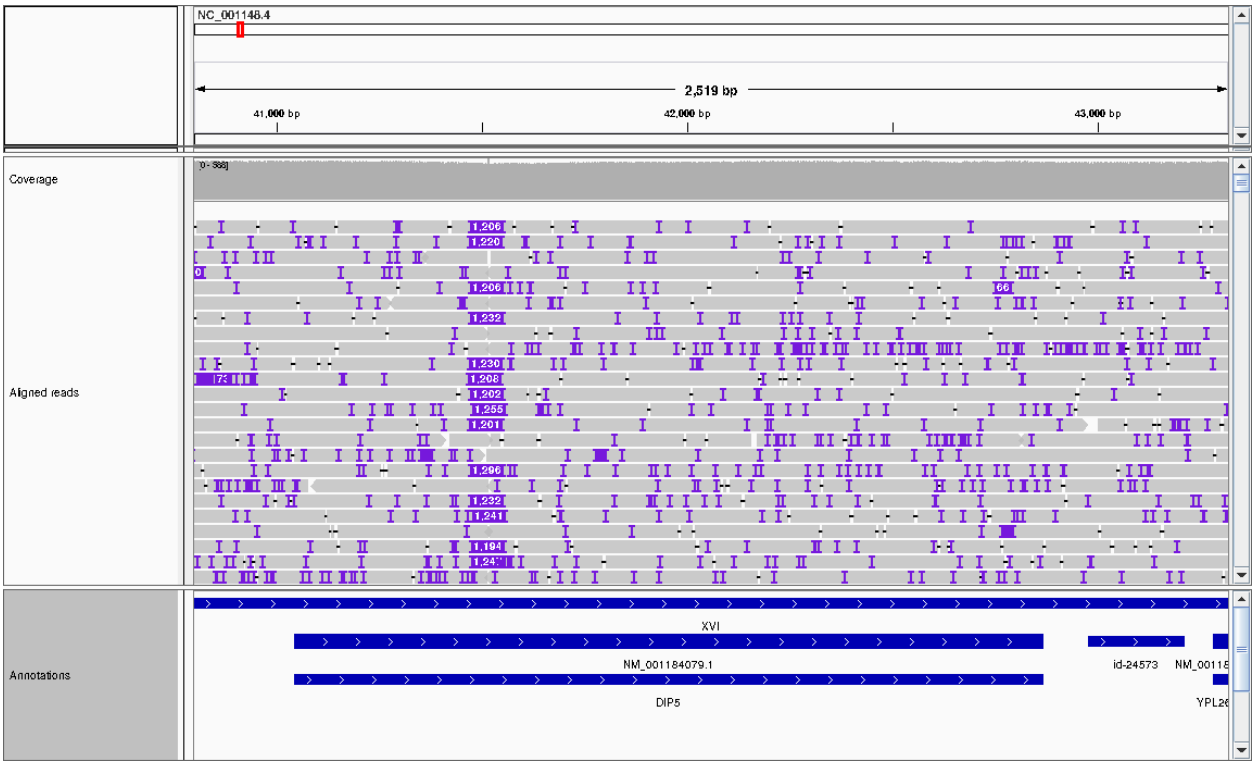
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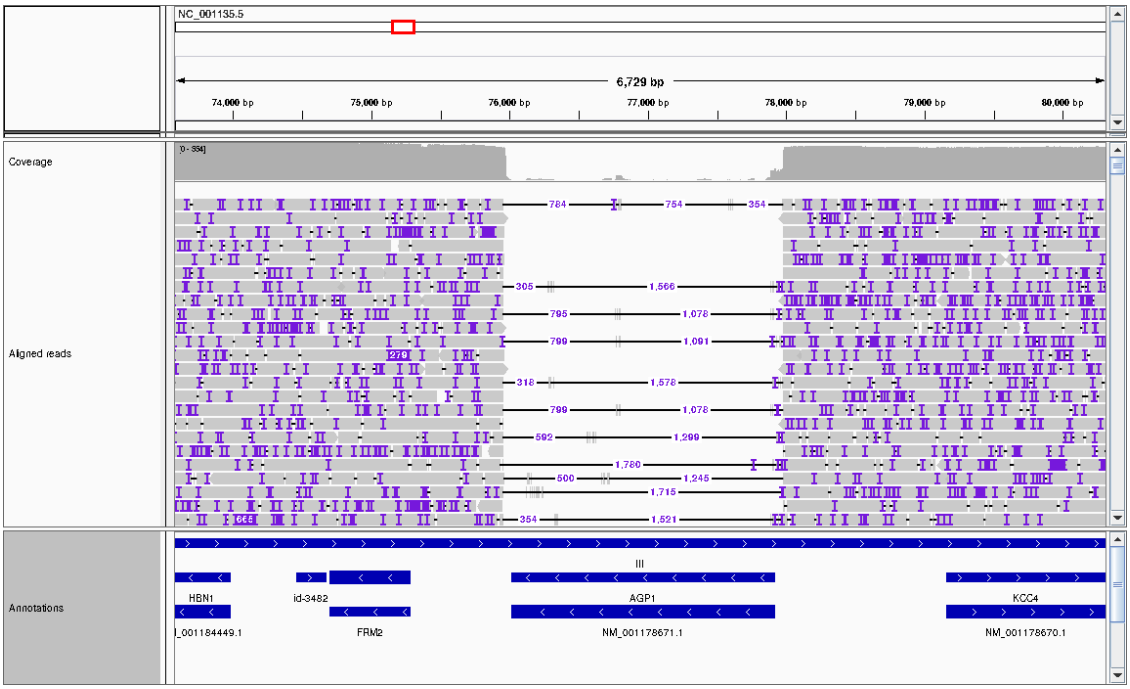
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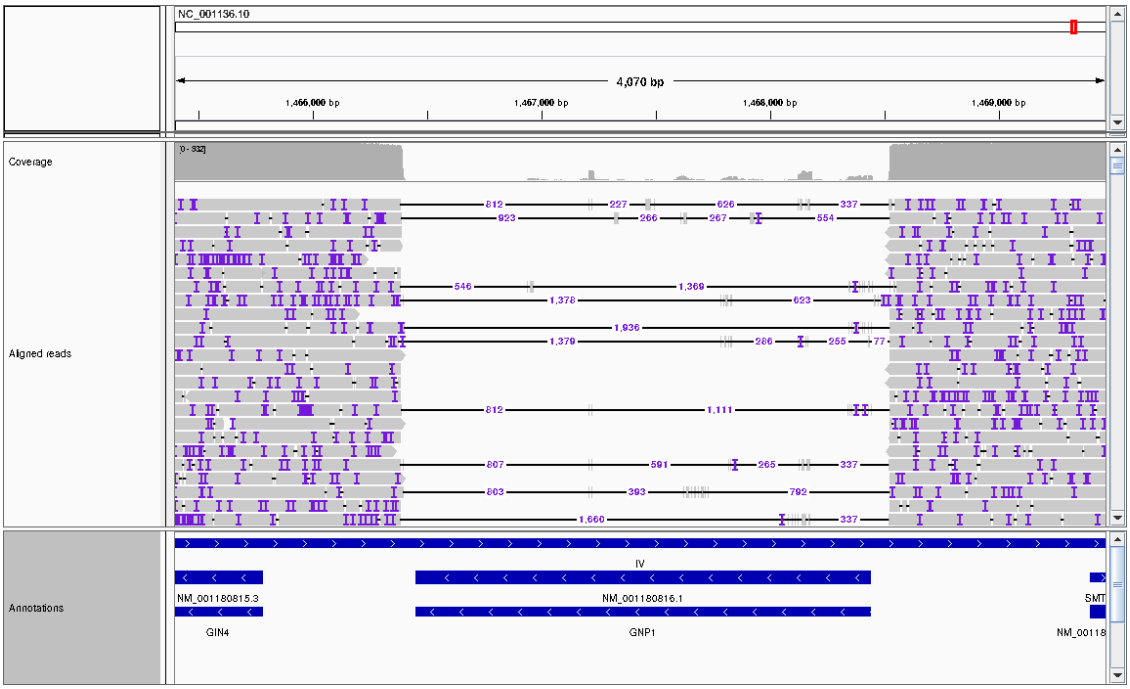
(g) AGP1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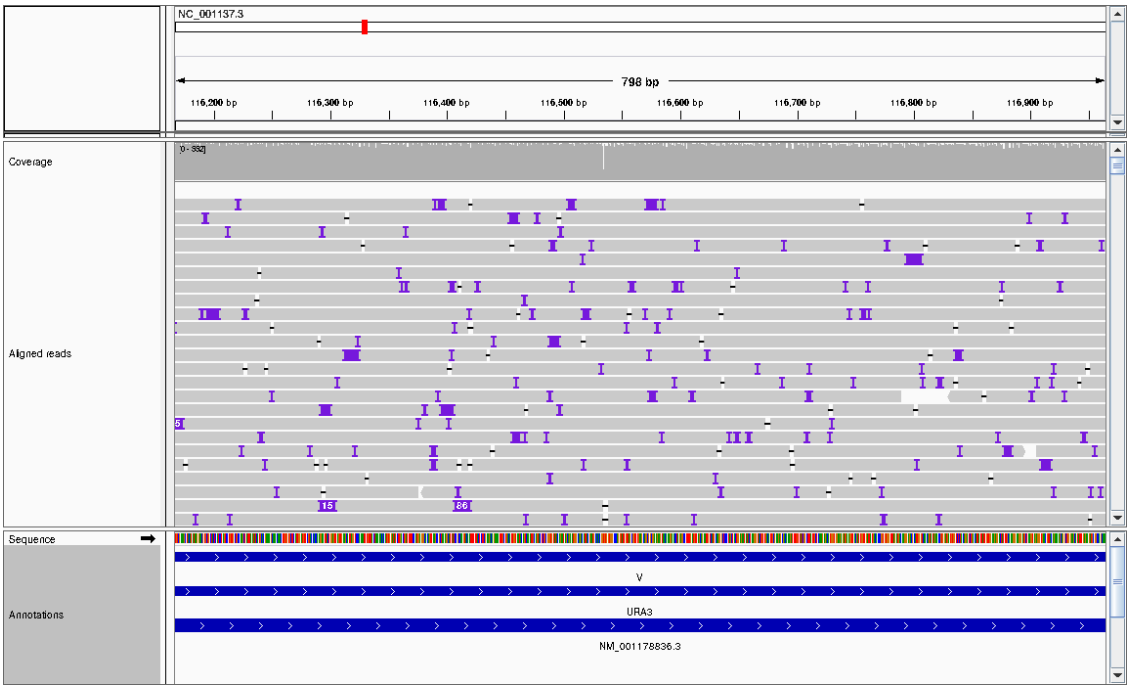
(h) GNP1



(i) URA3

Figure 4.S6: Integrated Genome Viewer images of indels in 22Δ10α(a-h)22Δ10α alined to the S288C reference genome were loaded in IGV (Robinson et al. (2011)) along with the reference genome and its annotation. Deletions result in drop in read coverage seen as white spaces, whereas insertions result in reads that do not map to the reference seen in purple (number indicates the length of insertion for that read)

Chapter 4. References

Besnard, J., Pratelli, R., Zhao, C., Sonawala, U., Collakova, E., Pilot, G. and Okumoto, S. (2016). UMAMIT14 is an amino acid exporter involved in phloem unloading in Arabidopsis roots. *Journal of Experimental Botany* **67**, 6385–6397.

Chang, H.-C. and Bush, D. R. (1997). Topology of NAT2, a prototypical example of a new family of amino acid transporters. *Journal of Biological Chemistry* **272**, 30552–30557.

Chen, L. and Bush, D. R. (1997). LHT1, a lysine- and histidine-specific amino acid transporter in Arabidopsis. *Plant Physiology* **115**, 1127–1134.

d'Enfert, C., Minet, M. and Lacroute, F. (1995). Cloning plant genes by complementation of yeast mutants. *Methods in Cell Biology* **49**, 417–430.

Engel, S. R., Dietrich, F. S., Fisk, D. G., Binkley, G., Balakrishnan, R., Costanzo, M. C., Dwight, S. S., Hitz, B. C., Karra, K., Nash, R. S. et al. (2014). The reference genome sequence of *Saccharomyces cerevisiae*: Then and now. *G3: Genes, Genomes, Genetics* **4**, 389–398.

Fischer, W. (1998). Amino acid transport in plants. *Trends in Plant Science* **3**, 188–195.

Fischer, W.-N., Kwart, M., Hummel, S. and Frommer, W. B. (1995). Substrate specificity and expression profile of amino acid transporters (AAPs) in Arabidopsis. *Journal of Biological Chemistry* **270**, 16315–16320.

Fischer, W. N., Loo, D. D., Koch, W., Ludewig, U., Boorer, K. J., Tegeder, M., Rentsch, D., Wright, E. M. and Frommer, W. B. (2002). Low and high affinity amino acid H+-cotransporters for cellular import of neutral and charged amino acids. *Plant Journal* **29**, 717–731.

Frommer, W. B., Hummel, S. and Riesmeier, J. W. (1993). Expression cloning in yeast of a cDNA encoding a broad specificity amino acid permease from *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* **90**, 5944–5948.

Frommer, W. B., Hummel, S., Unseld, M. and Ninnemann, O. (1995). Seed and vascular expression of a high-affinity transporter for cationic amino acids in Arabidopsis. *Proceedings of the National Academy of Sciences* **92**, 12036–12040.

Frommer, W. B. and Ninnemann, O. (1995). Heterologous expression of genes in bacterial, fungal, animal, and plant cells. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**, 419–444.

Garrison, E. and Marth, G. (2012). Haplotype-based variant detection from short-read sequencing. *arXiv:1207.3907 [q-bio]* .

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Véronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., André, B. et al. (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**, 387–391.

Giaever, G. and Nislow, C. (2014). The yeast deletion collection: A decade of functional genomics. *Genetics* **197**, 451–465.

Gournas, C., Prévost, M., Krammer, E.-M. and André, B. (2016). Function and regulation of fungal amino acid transporters: Insights from predicted structure. In *Yeast Membrane Transport* (eds. J. Ramos, H. Sychrová and M. Kschischo), Advances in Experimental Medicine and Biology, pp. 69–106. Cham: Springer International Publishing.

Grenson, M. (1969). The utilization of exogenous pyrimidines and the recycling of uridine-5′-phosphate derivatives in saccharomyces cerevisiae, as studied by means of mutants affected in pyrimidine uptake and metabolism. *European Journal of Biochemistry* **11**, 249–260.

Güldener, U., Heck, S., Fiedler, T., Beinhauer, J. and Hegemann, J. H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Research* **24**, 2519–2524.

Hsu, L. C., Chiou, T. J., Chen, L. and Bush, D. R. (1993). Cloning a plant amino acid transporter by functional complementation of a yeast amino acid transport mutant. *Proceedings of the National Academy of Sciences* **90**, 7441–7445.

Iraqui, I., Vissers, S., Bernard, F., de Craene, J.-O., Boles, E., Urrestarazu, A. and André, B. (1999). Amino acid signaling in *Saccharomyces cerevisiae*: A permease-like sensor of external amino acids and F-box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. *Molecular and Cellular Biology* **19**, 989–1001.

Jauniaux, J.-C. C. and Grenson, M. (1990). *GAP1*, the general amino acid permease gene of *Saccharomyces cerevisiae*: Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. *European Journal of Biochemistry* .

Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H. and Phillippy, A. M. (2017). Canu: Scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Research* **27**, 722–736.

Kwart, M., Hirner, B., Hummel, S. and Frommer, W. B. (1993). Differential expression of two related amino acid transporters with differing substrate specificity in Arabidopsis thaliana. *The Plant Journal* **4**, 993–1002.

Li, H. (2018). Minimap2: Pairwise alignment for nucleotide sequences. *Bioinformatics* **34**, 3094–3100.

Marçais, G., Delcher, A. L., Phillippy, A. M., Coston, R., Salzberg, S. L. and Zimin, A. (2018). MUMmer4: A fast and versatile genome alignment system. *PLOS Computational Biology* **14**, e1005944.

Popov-Čeleketić, D., Bianchi, F., Ruiz, S. J., Meutiawati, F. and Poolman, B. (2016). A plasma membrane association module in yeast amino acid transporters. *Journal of Biological Chemistry* **291**, 16024–16037.

Regenberg, B., Düring-Olsen, L., Kielland-Brandt, M. C. and Holmberg, S. (1999). Substrate specificity and gene expression of the amino-acid permeases in *Saccharomyces cerevisiae*. *Current Genetics* **36**, 317–328.

Rentsch, D., Hirner, B., Schmelzer, E. and Frommer, W. B. (1996). Salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases identified by suppression of a yeast amino acid permease-targeting mutant. *The Plant Cell* **8**, 1437–1446.

Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G. and Mesirov, J. P. (2011). Integrative genomics viewer. *Nature Biotechnology* **29**, 24–26.

Schreve, J. L. and Garrett, J. M. (2004). Yeast Agp2p and Agp3p function as amino acid permeases in poor nutrient conditions. *Biochemical and Biophysical Research Communications* **313**, 745–751.

Sprouffske, K. and Wagner, A. (2016). Growthcurver: An R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinformatics* **17**, 172.

Van Belle, D. and André, B. (2001). A genomic view of yeast membrane transporters. *Current Opinion in Cell Biology* **13**, 389–398.

Velasco, I., Tenreiro, S., Calderon, I. L. and André, B. (2004). *Saccharomyces Cerevisiae* Aqr1 is an internal-membrane transporter involved in excretion of amino acids. *Eukaryotic Cell* **3**, 1492–1503.

Appendices

Appendix A

Role of host amino acid transporters in Arabidopsis-*Hyaloperonospora arabidopsidis* interaction

A.1 Introduction

As described in chapter 1, chapter 2 and chapter 3, owing to its metabolic deficiency *Hpa* is entirely dependent on its host for acquiring an organic form of nitrogen. It is highly likely that it acquires this in the form of amino acids and might directly or indirectly manipulate the host's amino acid transporters to mobilize amino acids to the infection site.

In order to find Arabidopsis amino acid transporters that could be manipulated by *Hpa* we identified amino acid transporter genes that displayed increased mRNA accumulation in response to *Hpa* infection in a susceptible genotype, but was suppressed in a resistant genotype. This was achieved by using publicly available data from Wang et al. (2011) wherein the Col-0 genotype is resistant to the *Hpa* isolate Emwa1 and the genotype lacking the resistance gene (*rpp4*) is susceptible. This was based on the observation that the transcriptome of *rpp4* infected by *Hpa* was different than that of infected Col-0 (see Fig. 2.2B), especially in the case of amino acid transporter genes. The null mutants of amino acid transporter genes identified in this manner were then challenged with *Hpa* and genes whose null mutants displayed reduction in *Hpa* growth in these preliminary studies were used for further examination (McDowell lab, unpublished). Four of these amino acid transporter genes used for further analysis were *AAP2*, *CAT8*, *UMAMIT12* and *UMAMIT28*.

AAP2 is expressed in the phloem throughout the plant and plays a role in xylem to phloem transfer of amino acids (Zhang et al., 2010). Based on promoter:GUS analysis, *CAT8* has been found to be expressed in root tips and in actively dividing tissue in shoots such as young leaves and is suggested to play a role in proton-independent uptake of glutamine at the root tip (Su et al., 2004; Yang et al., 2010). UMAMIT28 is found to be associated with the vasculature of the plant and is important for export of amino acids in seeds (Müller et al., 2015). While no information on *UMAMIT12* exists in the published literature, based on unpublished promoter:GUS analysis data in the Pilot lab, it was known to be active in the vasculature of the plants.

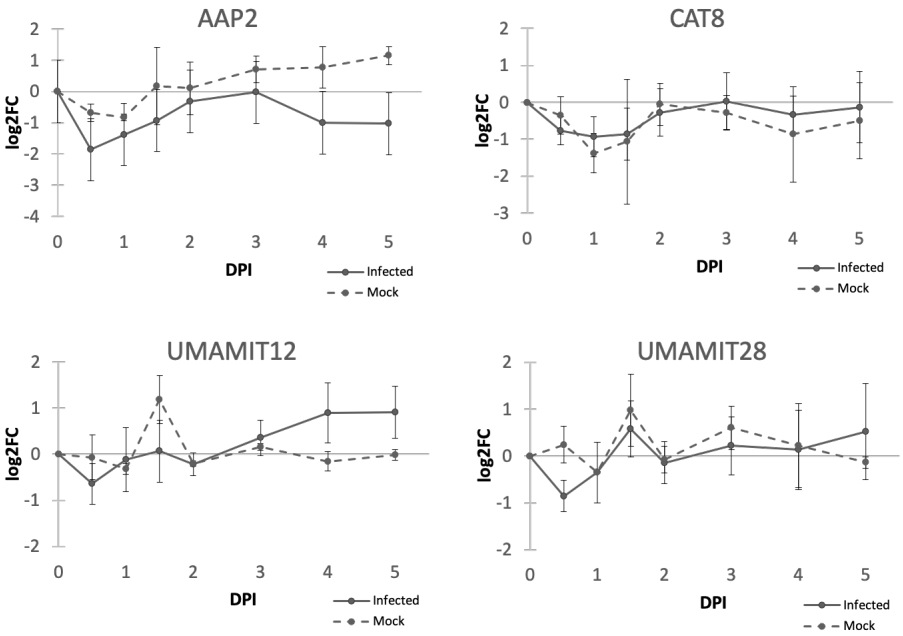
A.2 Results

To verify the increase in mRNA accumulation of these amino acid transporter genes in a compatible interaction, we challenged Col-0 plants with the virulent *Hpa* isolate Noco2 and measured the mRNA accumulation of these genes over the course of infection. We found that the changes in mRNA accumulation of these genes was small if any over the course of Noco2 infection. Accumulation of *AAP2* mRNA appeared to slightly reduce in infected samples and that of *CAT8* had no change compared to non-infected samples. *UMAMIT12* and *UMAMIT28* displayed a small increase in mRNA accumulation in two out of the three experiments (see Fig. A.1(a) and Fig. A.1(b)).

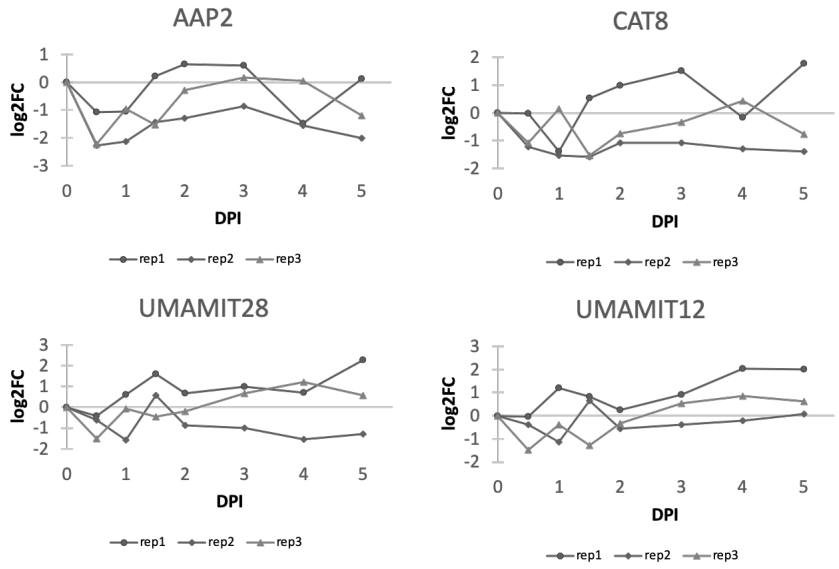
To test if there were any spatial changes in the expression pattern of the genes, we used transgenic plants carrying promoter:GUS constructs for the four amino acid transporter genes of interest. In mock-treated conditions (Fig. A.2), GUS staining was observed throughout the vasculature of *AAP2p*:GUS plants as described by Zhang et al. (2010). For *UMAMIT12p*:GUS plants, GUS staining was observed in the vasculature of the hypocotyl and roots, and little to no GUS staining was observed in the shoots. In case of *UMAMIT28p*:GUS, GUS staining was observed in the vasculature, mostly in the cotyledons of the shoots and not much in the leaves in these younger plants. GUS staining was observed throughout the plant in *CAT8p*:GUS plants. This is in contrast to the results from Su et al. (2004) where they found the promoter to be active mostly in actively dividing tissues such as young leaves, and in root tips. This was observed for four different independent lines (data not shown).

When challenged with Noco2, we found a strong increase in GUS staining in *UMAMITp*:GUS plants infected with *Hpa* compared to those that were mock-treated (Fig. A.2(d), Fig. A.S1). Some of the increased staining was observed in non-vascular tissue, the cellular location of which remains to be verified. In case of *AAP2p*:GUS a small decrease in the intensity of staining in the vasculature was observed in infected samples, mostly in line 2 (Fig. A.2(a)). Little to no change in GUS staining was observed in the infected and *CAT8p*:GUS and *UMAMIT12p*:GUS samples compared to the mock-treated samples (Fig. A.2(c), Fig. A.2(b)). This results were further corroborated by fluorometric quantification of GUS activity (Fig. A.S2), except in case of *CAT8p*:GUS which also displayed an increase in GUS activity in infected samples at 3 dpi.

The null mutants of all four of these transporters and two double mutants (*aap2cat8* and *umamit12umamit28*) were challenged with Noco2. After several repeats of this experiment, the phenotype of these mutants remained inconclusive. While single mutants of *cat8*, *umamit12* and *umamit28* did not appear to differ in pathogen biomass compared to wild-type, *aap2-1* and the double mutant *umamit12-1umamit28-1* appeared to be more resistant to Noco2 compared to wild-type Col-0 in initial experiments. However, in future experiments these mutants supported similar amount of Noco2 biomass as Col-0.

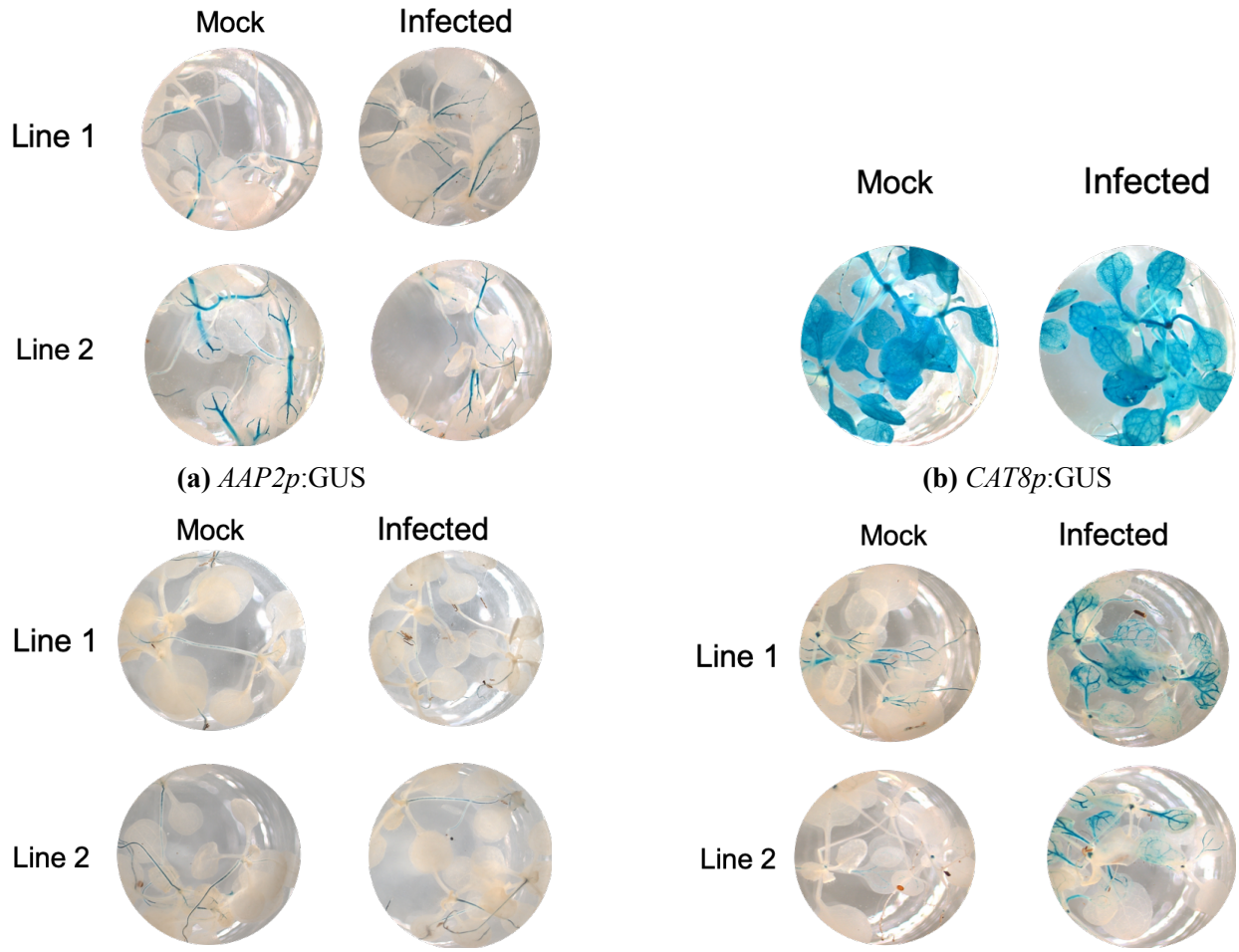


(a)



(b)

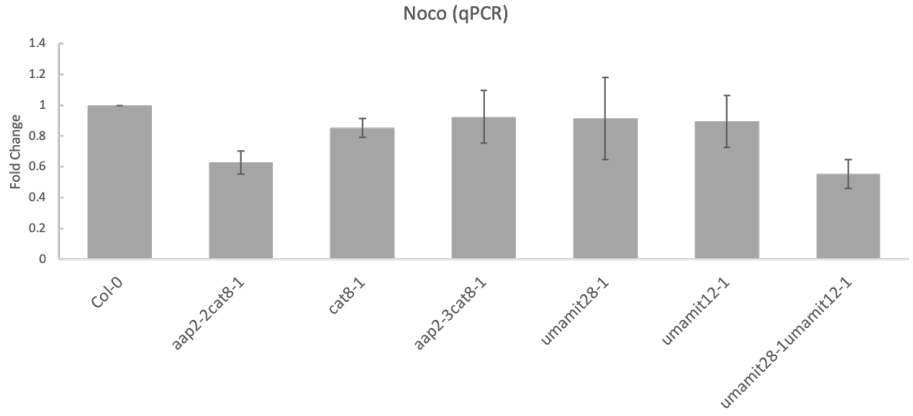
Figure A.1: mRNA accumulation of amino acid transporter genes under Noco2 infection10-12 day old seedlings of Col-0 were infected with virulent *Hpa* isolate Noco2 and mRNA content for given genes was measured over the course of infection via qRT-PCR. Values for each gene were normalized to Actin2 abundance, which were then normalized to 0 dpi time-point. (a) represents average of three independent biological replicates (b) represents each biological replicate from infected samples. Error bars represent standard error of the mean.



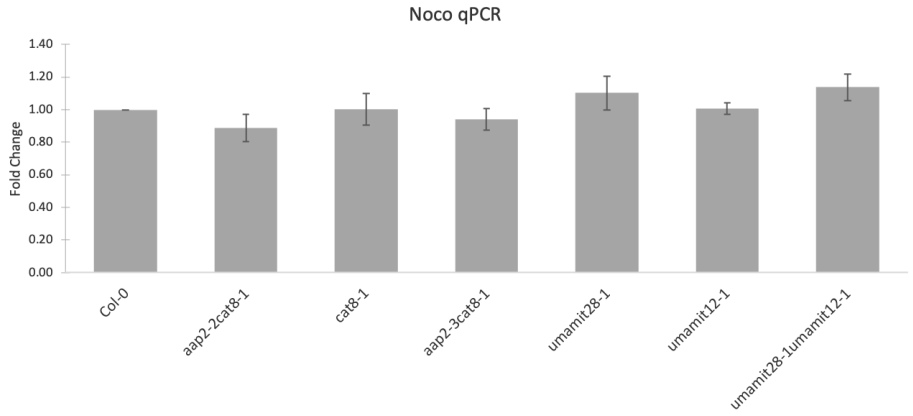
(a) *AAP2p*:GUS **(b)** *CAT8p*:GUS

(c) *UMAMIT12p*:GUS **(d)** *UMAMIT28p*:GUS

Figure A.2: promoter:GUS analysis of amino acid transporter genes under Noco2 infection Transgenic plants expressing GUS under the control of 2 kb region upstream of (a) *AAP2*, (b)*CAT8*, (c)*UMAMIT12* and (d)*UMAMIT28* start site were infected with virulent *Hpa isolate* Noco2 (right) or mock-treated with sterile water (left) and stained for β-glucuronidase activity at 5 dpi. Images from one/two representative independent lines are shown.



(a)



(b)

Figure A.3: Pathogen growth on amino acid transporter mutants 11 day old seedlings of Col-0 and amino acid transporter mutants were infected with virulent *Hpa* isolate Noco2. Pathogen growth was measured using quantitative PCR and is plotted as relative abundance of *HpaActin2* to *AtActin2*, and normalized to Col-0 using $2^{-}\Delta\Delta Ct$. For both (a) and (b) n=4 and error bars represent standard error of the mean

A.3 Discussion

We found a consistent increase in GUS activity in *UMAMIT28p*:GUS infected plants (Fig. A.S1, Fig. A.S2(d)), but a much weaker increase in accumulation of *UMAMIT28* mRNA in Noco2 infected samples. This is most likely due to higher stability of GUS mRNA. Currently, all evidence support an increase in activity of the *UMAMIT28* promoter. Whether *UMAMIT28* mRNA stability is important in how its expression is regulated during *Hpa* infection will require further investigation. Additionally, a small decrease was observed in *AAP2* promoter activity and mRNA accumulation upon infection. How this affects *Hpa*-Arabidopsis interaction remains to be answered.

The variability in pathogen biomass on *aap2* and *umamit12umamit28* mutants is probably a result of some change in environmental factors. Efforts were made to identify these via controlled nutrition and using hydroponically grown plants, however the reasons for the variability remained unresolved.

A.4 Material and Methods

For most material and methods refer to Chapter 3 (section 3.5)

Plant lines and crosses

T-DNA mutant lines for *aap2-2* (SALK_143597), *aap2-3* (SALK_020688), *cat8-1* (SALK_052450), *umamit28-1* (SALK_147481), *umamit12-1* (SALK_101986) were obtained from ABRC stock center (Ohio State University, OH). Information for these mutants was gathered using the T-DNA express tool for SIGnAL database (Alonso et al., 2003). Double mutant for *aap2-2cat8-1* was obtained by crossing pollens of *aap2-2* with ovules of *cat8-1* and that for *umamit28-1umamit12-1* was obtained by crossing pollens from *umamit28-1* and ovules from *umamit12-1*. In both cases, homozygotes were identified in the F3 generation via PCR.

GUS fluorometric assay

Transgenic plants carrying promoter:GUS GUS constructs infected with Noco2 or mock-treated with sterile water were collected at given time points and flash frozen. Total protein was extracted by grinding on ice in protein extraction buffer (50mM NaPi (pH 7.2), 10mM EDTA, 0.1%Triton, 0.1% Sarkosyl, 10mM β-mercaptoethanol, 0.5X cOmplete^{TM} protease inhibitor), centrifuging at 10,000g at 4˚C for 10min and then collecting the supernatant in a fresh tube. The total protein amount was measured via Bradford's assay (Fermentas) by following manufacturer's protocol, and quantified against a BSA standard curve. The fluorometric assay was performed as described in Gallagher (1992), the reaction with 4-MUG (4-methylumbelliferyl b-D-glucuronide) was incubated at 37˚C in dark for 30min, and fluorescence was measured using Synergy 4 (BioTek) with excitation at 365nm and emission at 455nm, and sensitivity set to 55.

A.5 Supplementary Material

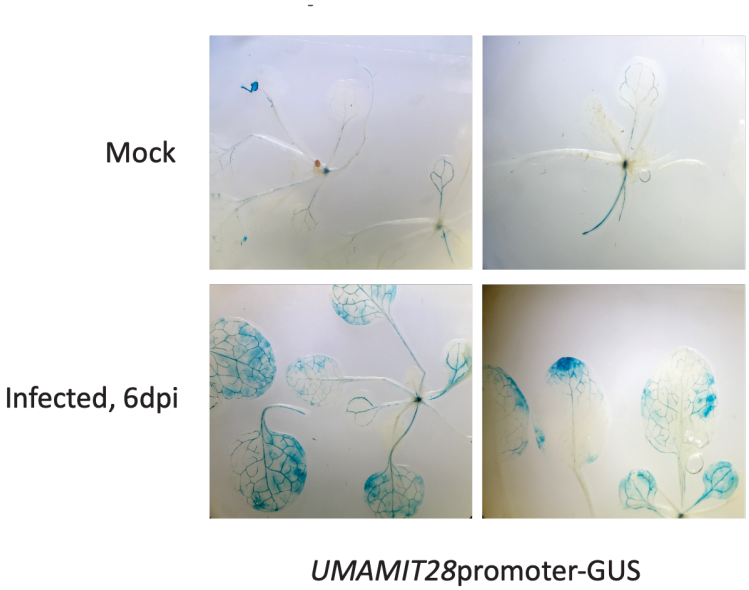
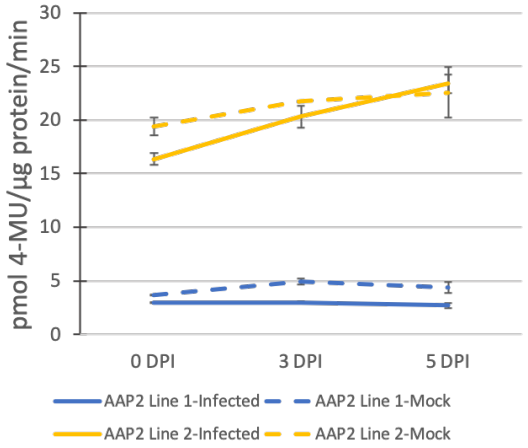
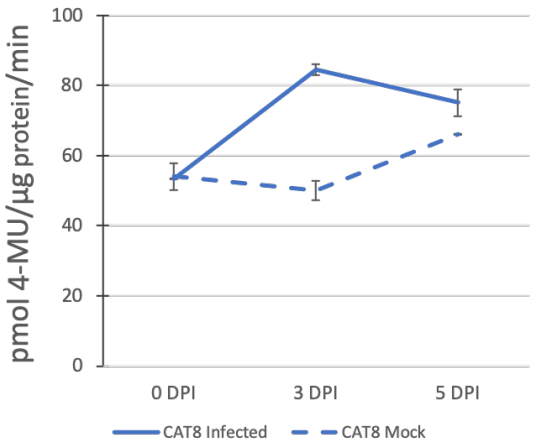


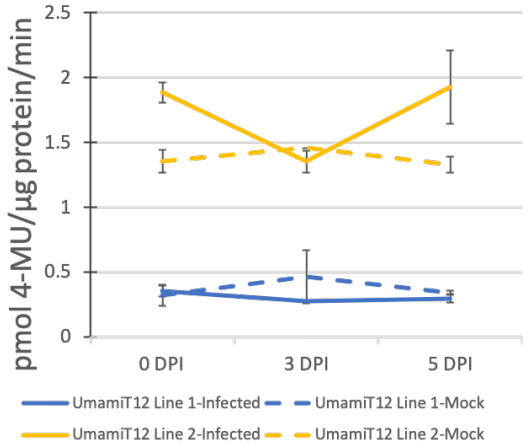
Figure A.S1: promoter:GUS analysis *UMAMITp*:GUS plants under Noco2 infection Transgenic plants expressing GUS under the control of 2 kb region upstream of *UMAMIT28* start site were infected with virulent *Hpa isolate* Noco2 (right) or mock-treated with sterile water (left) and stained for β-glucuronidase activity at 6 dpi. Images from two representative independent lines are shown.



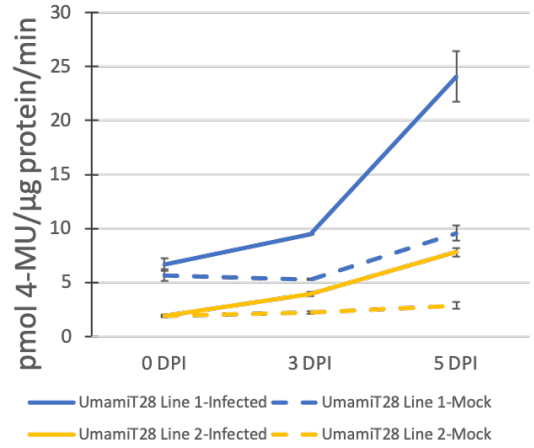
(a) *AAP2p*:GUS



(b) *CAT8p*:GUS



(c) *UMAMIT12p*:GUS



(d) *UMAMIT28p*:GUS

Figure A.S2: Fluorometric assay for GUS activity under Noco2 infection Transgenic plants expressing GUS under the control of 2 kb region upstream of (a) *AAP2*, (b)*CAT8*, (c)*UMAMIT12* and (d)*UMAMIT28* start site were infected with virulent *Hpa isolate* Noco2 (right) or mock-treated with sterile water (left). Samples for the assay were collected at 0, 3 and 5 dpi. GUS activity was measured as pmol of 4MU(4-Methylumbelliferone) produced per min per μg of protein.

Appendix A References

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R. et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**, 653–657.

Gallagher, S. R. (1992). Quantitation of GUS Activity by Fluorometry. In *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression* (ed. S. R. Gallagher), pp. 47–59. San Diego: Academic Press.

Müller, B., Fastner, A., Karmann, J., Mansch, V., Hoffmann, T., Schwab, W., Suter-Grotemeyer, M., Rentsch, D., Truernit, E., Ladwig, F. et al. (2015). Amino acid export in developing arabidopsis seeds depends on UmamiT facilitators. *Current Biology* **25**, 3126–3131.

Su, Y.-H., Frommer, W. B. and Ludewig, U. (2004). Molecular and functional characterization of a family of amino acid transporters from arabidopsis. *Plant Physiology* **136**, 3104–3113.

Wang, W., Barnaby, J. Y., Tada, Y., Li, H., Tör, M., Caldelari, D., Lee, D. U., Fu, X. D. and Dong, X. (2011). Timing of plant immune responses by a central circadian regulator. *Nature* **470**, 110–115.

Yang, H., Bogner, M., Stierhof, Y. D. and Ludewig, U. (2010). H+-independent glutamine transport in plant root tips. *PLoS ONE* **5**, e8917–e8917.

Zhang, L., Tan, Q., Lee, R., Trethewy, A., Lee, Y.-H. and Tegeder, M. (2010). Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in arabidopsis. *The Plant Cell* **22**, 3603–3620.