Role of Plant Amino Acid Transporters in Shaping Root—Beneficial Microbe Associations

Israel Deladem Kwashie Agorsor
Tsiame, Ghana

B.Sc., University of Cape Coast, 2007
M.Sc., Wageningen University, 2013

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ABSTRACT

Crop agriculture fueled by reliance on chemical fertilizers has environmentally-damaging consequences. Proposed alternative approaches to enhancing plant growth include the use of plant growth-promoting bacteria (PGPB). Large-scale field adoption of PGPB has been hindered by the low rhizosphere competence of PGPB, among others. Plant-derived amino acids in the rhizosphere promote bacterial growth, facilitate bacterial chemotaxis towards the roots, and enable microbial biosynthesis of plant growth-promoting compounds such as auxins. In this dissertation, I set out to identify and characterize Arabidopsis amino acid (AA) transporters that shape rhizosphere AA concentrations, and to determine whether these transporters modulate root—PGPB interactions and PGPB-mediated plant fitness. I found that the loss of Arabidopsis AA transporter LHT1 enhanced AA accumulation in the rhizosphere, indicating that LHT1 contributes to the retrieval of root-secreted amino acids. Consequently, both root exudates and root tissues of *lht1* plants promoted the growth of the PGPB *Pseudomonas simiae* WCS417r better than did wildtype root exudates and root tissues. Additionally, *lht1* root exudates enhanced biofilm formation by *Ps* WCS417r cells. Thus, engineering the rhizosphere to enhance AA levels by modifying amino acid transporter function would appear an important strategy to boost plant—PGPB interactions. Surprisingly, *Ps* WCS417r-mediated plant growth was attenuated in *lht1* plants, suggesting that rhizosphere AA homeostasis may be important for *Ps* WCS417r-mediated plant growth. In support of this hypothesis, I found plant growth was inhibited in *in vitro* experiments where wildtype roots were co-treated with glutamine and *Ps* WCS417r. Additional experiments are required to understand the mechanisms underlying how elevated concentrations of specific amino acids in the rhizosphere may impair rhizobacteria-mediated plant fitness. Furthermore, I found that Arabidopsis AA

transporter UMAMIT30 contributes to the availability of AA in the rhizosphere. Loss of UMAMIT30 function depleted *umamit30* root exudates of AAs. However, the low AA content of root exudates from *umamit30*, as well as from *umamit14* (previously characterized for its defective root AA secretion), did not impair *Ps* WCS417r growth. Importantly, *Ps* WCS417r-mediated plant growth remained intact in both *umamit30* and *umamit14* plants. These suggest that plant-derived AA metabolism by *Ps* WCS417r was not affected under these conditions of low AA concentrations so as to impair *Ps* WCS417r-mediated plant growth.

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LIST OF ABBREVIATIONS/ACRONYMS

µM: micromolar

AA: Amino Acid

AAs: Amino Acids

AM: Arbuscular Mycorrhiza

ANOVA: Analysis of Variance

ANT: Aromatic and Neutral Transporter

APC: Amino acid, Polyamine, Choline Transporter

AprA: Alkaline Protease

ATF: Amino acid Transporter Family

avrE: avirulence protein E

BGAL1: β-galactosidase 1

CAT: Cationic Amino acid Transporter

CFUs: Colony-Forming Units

DAMP: Damage-Associated Molecular Pattern

DNA: Deoxyribonucleic acid

DW: Dry Weight

EDTA: Ethylenediaminetetraacetic acid

EFR: EF-Tu Receptor (Elongation Factor Thermo-unstable Receptor)

ETI: Effector-Triggered Immunity

ETS: Effector-Triggered Susceptibility

Flg22: Flagellin 22

FLS2: Flagellin-Sensitive 2

Fv/Fm: Variable Fluorescence/Maximum Fluorescence

FW: Fresh Weight

GABA: gamma-aminobutyric acid

Gln: Glutamine

hrcC: hypersensitive response conserved C

IAA: Indole-3-Acetic Acid

ipdC: indole-3-pyruvate decarboxylase gene

LBP: Left Border Primer

LC-MS: Liquid Chromatography – Mass Spectrometry

LHT: Lysine Histidine Transporter

LP: Left Primer

LRRs: Leucine-Rich Repeats

MAMP: Microbe-Associated Molecular Pattern

MCPs: Methyl-accepting Chemotaxis Proteins

mM: millimolar

MS medium: Murashige and Skoog medium

MTI: MAMP-Triggered Immunity

NB-LRRs: Nucleotide-Binding Leucine-Rich Repeats

nM: nanomolar

OD: Optical Density

PCR: Polymerase Chain Reaction

Pf: *Pseudomonas fluorescens*

PGPB: Plant Growth-Promoting Bacteria

PNP: para-nitrophenol

ProT: Proline Transporter

PRRs: Pattern Recognition Receptors

Ps WCS417r: *Pseudomonas simiae* WCS417r

QS: Quorum sensing

RH: Relative Humidity

RNA: Ribonucleic acid

ropE: rhizosphere-expressed outer protein E

RP: Right Primer

rscC: rhizosphere-expressed secretion protein conserved C

RT-qPCR: quantitative reverse transcription PCR

SAR: Systemic Acquired Resistance

SE/CC: Sieve Elements/Companion Cells

SE: Standard Error

Ser: Serine

SiAR1: Siliques Are Red 1

T3SS: Type 3 Secretion System

T-DNA: Transfer DNA

UMAMIT: Usually Multiple Acids Move In and out Transporter

UPLC: Ultra-Performance Liquid Chromatography

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Chapter 1:

Introduction and Literature Review: Plant—Beneficial Microbe Interactions

1.1 OVERVIEW

In the last several decades, chemical fertilizers have greatly helped to raise crop yields, enabling the stability of food supplies and prices and raising agricultural productivity in general[1]. However, chemical fertilizers have also left damaging environmental footprints across the planet[2,3], emphasizing the unsustainability of agriculture fueled predominantly by chemical fertilizers. In the face of a rapidly warming climate and a rising global population, the search for more ecologically sustainable approaches to agriculture is imperative, and has explored the potential of large-scale deployment of plant growth-promoting bacteria (PGPB) to raise crop yields[4]. PGPB improve plant growth through various mechanisms, including enhancing nutrient uptake, biosynthesis of plant hormones such as cytokinin and gibberellins which boost plant growth through regulating germination, stem elongation, and cell division, as well as by conferring plant tolerance to biotic and abiotic stresses.

Meanwhile, our ability to take full advantage of beneficial microbes is hampered by our limited understanding of the plant host genetic factors that regulate plant—PGPB interactions. Many soil-resident microbes are drawn to the rhizosphere, the narrow, nutrient-rich 1-3 mm region around roots[5], by following chemical cues exuded by plant roots, in a process known as chemotaxis. Hence, these microbes are also known as rhizobacteria. Amino acids (AA) are abundant in plant root exudates, and serve important signaling functions, as well as providing C and N resources for soil microbes. For example, Oku et al[6] showed that wild type *Pseudomonas fluorescens* Pf0-1 significantly outcompetes a mutant deficient in three proteins that mediate chemotaxis towards amino acids, in colonizing tomato root tips, demonstrating that root-secreted AAs are important

signaling factors in recruiting PGPB. Still, the inconsistent performance of PGPB in field trials[7] is often traced to their low rhizosphere competence, which is the degree of root colonization, survival, and proliferation on root surfaces by PGPB in competition with other soil-resident microflora[8].

Importantly, till date, no study has identified and characterized the plant AA transporters that mediate the recruitment of PGPB to roots, and shape the plant—PGPB interactions. Our knowledge of which plant AA transporters mediate this important interaction will take us closer to the goal of enhancing the ability of plants to recruit and maintain their association with PGPB. Thus, the overall goal of this dissertation is to unravel the plant AA transporters that shape plant—beneficial microbe interactions. In Section 1.3 following the literature review section, I indicate the specific aims of this dissertation.

1.2 LITERATURE REVIEW

Plant-Beneficial Microbes: A Summary

Among the most important biotic factors that plants interact with are microbes. For a long time, microbes that cause plant diseases have been subjects of intense research due to the visible and measurable economic/social impacts of the diseases they cause[9]. While research efforts aimed at improving plant health have focused on elucidating plant disease resistance mechanisms, the realization that many plant-associated microbes confer benefits to the plant, has led to diversification of efforts aimed at improving plant health. To this end, understanding mechanisms that enhance association of plants with beneficial microbes has now taken a center stage. Among plant-beneficial microbes are

antagonistic Protobacteria and Actinobacteria that suppress the root-rotting fungus *Rhizoctonia* in soils[10]. Antagonistic microbes may produce effectors that interfere with virulence factors of pathogens[11]. The best-studied of the PGPB are the rhizobacteria. In the rhizosphere, rhizobacteria, including the free-living spp. (e.g., *Azotobacter*) and symbiotic spp. (e.g., root-nodulating *Rhizobium*), engage in nitrogen fixation, converting atmospheric nitrogen that is inaccessible to plants into ammonia that plants can use[5]. PGPB also help plant growth by outcompeting pathogens for niche. Beyond these, many rhizosphere-dwelling bacteria solubilize phosphates. Phosphorus is a growth-limiting factor for plants, and although often available in large amounts in the soil, are present in insoluble forms. Plants cannot utilize these unless they are solubilized into monobasic and dibasic ions. Phosphate-solubilizing bacteria include several species of the genus *Bacillus*[12].

Further, some species of *Bacillus* produce the plant hormone gibberellins[4,13], which can regulate various aspects of plant development such as germination and stem elongation. Additionally, the plant growth hormone cytokinin, which regulates cell division and promotes cell growth and differentiation, is also widespread in the genus *Bacillus*[4,13]. PGPB also produce molecules known as siderophores, which chelate ferric iron in the soil, making them available to plants as well[4]. Experiments have shown that when radiolabeled ferric-siderophore complex is made available to plants as the sole source of iron, plants are able to uptake these, and inoculating mung bean plants with siderophore-producing *Pseudomonas* strain GRP3 leads to higher chlorophyll content and reduced chlorosis, compared to control groups[4]. Under salt stress, plants may experience ion imbalance and hence osmotic stress, which may cause oxidative damage. Inoculating

maize rhizosphere with *Bacillus amyloliquefaciens* SQR9 promotes salt stress tolerance through enhanced peroxidase/catalase expression and glutathione content, enabling the scavenging of reactive oxygen species, and conferring reduced plant Na^+ levels to avoid Na^+ toxicity[14].

The plant hormone indole-3-acetic acid (IAA), a class of auxin, is commonly produced by plant-associated bacteria[15]. IAA, among others, may induce cell elongation and division, leading to plant growth. Additionally, the plant hormone ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) can be sequestered (and then hydrolyzed) by the bacterial enzyme ACC deaminase, which many PGPB possess, leading to disrupted ethylene signaling and hence tolerance to stress[16]. ACC deaminase production can protect from the damaging effects of the stress-response hormone ethylene, which is induced in response to a range of abiotic stressors such as temperature extremes, flooding, drought, salinity and metals and metalloids[4]. Thus, PGPB such as *Bacillus* confer enormous benefit to plants.

Mechanisms that Shape Beneficial Microbe Adaptation to the Plant Immune System

One of the most enduring questions in plant—microbe interactions research is how beneficial microbes are able to associate with plants without the microbial signature molecules triggering plant defense responses and leading to the termination of beneficial microbe colonization of plant tissues.

A snapshot of the plant immune response to pathogens is captured in a conceptual framework known as the zigzag model[17]. To fend off pathogen attack, plants have evolved elaborate defense mechanisms, including plasma membrane-resident receptors (known as Pattern Recognition Receptors, or PRRs) that recognize signature molecules in microbes (called Microbe-Associated Molecular Patterns, or MAMPs) that are conserved across related taxa, such as bacterial flagellin or fungal chitin, to initiate defense signaling. The defense response triggered following MAMP perception, which is basically the perception of non-self molecules, is known as MAMP-Triggered Immunity (MTI). This initial MTI response is sufficient to limit pathogen growth[18–20]. In an ongoing arms race, however, pathogens produce effectors, molecules that would normally lead to disease in the host, in a process known as Effector-Triggered Susceptibility (ETS). Strikingly, to get ahead of the pathogens, plants have evolved intracellular resistance proteins known as R proteins that recognize pathogen effectors, leading to Effector-Triggered Immunity (ETI)[17].

Compared to PRRs which are mainly composed of extracellular leucine-rich repeats (LRRs) and intracellular kinase domains (although they may also come in the form of receptor-like kinases without the extracellular LRR or receptor-like kinases without the intracellular kinase domains), the R proteins are typically nucleotide-binding leucine-rich repeats (NB-LRRs) that perceive the action of pathogen effectors[19]. The co-evolution between plants and pathogens as described indicates that both partners in the interaction are continuously undergoing innovations that enable them to get ahead of the other.

Beneficial microbes on the other hand are able to ‘coexist’ with the plant immune system, enabling efficient colonization of the plant organs. The signaling involved in ensuring the

endurance of this partnership is however not well-understood, and is a subject of continuing research. Below, I provide an overview of the main mechanisms through which microbes may suppress or evade the plant immune response.

Immune suppression:

As illustrated by the zigzag model discussed above, the deployment of effector molecules to trigger susceptibility (i.e., ETS) in plants by pathogens is a classic immune suppression mechanism. Interestingly, plant immune suppression has also emerged among beneficial microbes. For example, Arabidopsis and other non-leguminous plants are able to recognize the rhizobial Nod factor, the nodulation signal molecule responsible for initiating nodulation in leguminous plants in legume—*Rhizobium* symbioses, via a mechanism that comprehensively suppresses MTI[21]. Similarly, the ectomycorrhizal fungi *Laccaria bicolor*, a mutualistic fungus that closely resembles biotrophic pathogens in its lifestyle due to the invasive nature of the symbioses that it establishes with plants, is known to secrete a small effector molecule known as the Mycorrhiza-induced Small Secreted Protein 7 (MiSSP7) that blocks the action of the defense hormone jasmonic acid to facilitate host colonization[22,23].

A recent study has shown that the acidification of the rhizosphere is also a strategy that enables some bacteria to interfere with plant defense responses activated in roots. Perception of microbial signatures by plant cells typically triggers extracellular alkalinization as one of the early events in defense signaling in plants[18,24]. Through the production of gluconic acid, the plant growth-promoting bacteria *Pseudomonas capeferrum* WCS358 lowers the environmental pH, as a mechanism to suppress MTI in Arabidopsis. *P. capeferrum* WCS358 mutants that do not produce gluconic acid are

incapable of suppressing MTI and hence are unable to colonize Arabidopsis roots as efficiently as the wildtype[25]. Acidification of the rhizosphere is thought to be the same strategy utilized by the plant-beneficial bacteria *Pseudomonas fluorescens* WCS417r in suppressing defense responses activated in Arabidopsis roots and to enable plant root colonization[25,26].

Immune evasion:

Immune evasion mechanisms, compared to immune suppression mechanisms, are different in their conception in that they generally consist of alterations to microbial signatures that should normally set off the plant immune surveillance alarms when they come into contact with host plants. There are basically three of such mechanisms as currently recognized, and are summarized below.

a. MAMP Diversification

MAMPs are generally considered as evolutionarily-conserved molecules, but there are several lines of evidence suggesting that these molecules may accumulate mutations that enable the perturbation of the MTI response. For example, flg22, the 22-amino acid epitope within the flagellin of several bacterial species, is genetically diverse, and explains the variation in the strength of the MTI response triggered by these bacterial species[27–32]. Thus, the evolution of MAMP variants by microbes can allow for the evasion of the MTI, as these MAMP variants may no longer bind to their corresponding PRRs, or their binding might fail to initiate the corresponding immune signaling[33]. Intriguingly, the mutations that may accumulate in MAMP variants may also impose fitness costs; mutational analysis showed that loss of flg22 immunogenicity through amino acid

substitution also conditioned impaired or abolished swimming and swarming motilities of the bacteria, indicating that specific amino acid residues that are necessary for the innate flagellar function of motility can also betray the bacterium's presence in a host plant to trigger MTI responses[34].

However, several MAMP variants that can confer immune-evasion abilities are common among certain bacterial taxa. Genomic analysis of bacteria isolated from healthy Arabidopsis plants revealed the canonical MAMPs that can trigger MTI, in addition to several others that are sufficiently different from the canonical forms and hence may enable immune evasion. Specifically, of the flg22 peptide epitopes found in the bacterial isolates, about a quarter are at least 50% different from the canonical active sequence (i.e., when comparing sequences for similarity). In addition, of the elf18 variants uncovered, less than 1% are at least 50% different from the canonical elf18 sequence[33]. This might be explained by the flg22-triggered immunity being more costly for the bacteria than does elf18-triggered immunity, or the flg22 epitope being more responsive to evolution than does the elf18[33]. This proposition is in good agreement with the observation that EFR, the receptor for elf18, is not expressed in Arabidopsis roots (which are typically in contact with large numbers of bacterial species in natural environments), whereas FLS2, the receptor for flg22, is expressed[26,35].

The observations noted in the foregoing, about the diversity within microbial molecular signatures that may enable the evasion of plant immune response, point to the possibility of plant population level-imposed selection pressures. Evidence suggests the evolution of different plant hosts to respond differentially and specifically to different elicitors[36]. Additionally, microbial infection of different Arabidopsis and tomato lines reveals huge

variations in the response spectrum to different MAMPs, even to the same MAMP variant[37–39]. These studies suggest MAMP turnovers in microbial populations in response to host plant recognition.

b. MAMP Decay/Insulation

Though the evolution of MAMP variants, or MAMP diversification, affords microbes the leeway to adapt to plant defense responses, other strategies for coping with plant defenses exist, which might reflect the increasing selection pressure imposed upon microbes in the ongoing arms race in plant—microbe interactions. Similar to the opportunistic pathogen *Pseudomonas aeruginosa*, the plant pathogen *P. syringae* pv. *tomato* DC3000 secretes an alkaline protease AprA that degrades flagellin monomers, enabling the attenuation of MTI, with the result that DC3000 mutants deleted for the AprA protein effectively trigger MTI upon the recognition of their flagellin by the plant defense machinery, significantly decreasing their growth on both Arabidopsis and tomato plants in comparison to the wildtype[40].

Among fungi, a strategy to prevent the recognition of their chitin molecules—an elicitor of MTI, by the plant defense machinery, has been documented. Several fungal pathogens deploy effector proteins containing lysin motifs (LysM effectors) that bind chitin fragments to prevent plant cells from recognizing them[41–44]. Although MAMP degradation/insulation strategies among plant beneficial microbes is not widely known, a recent work uncovered the LysM effector RiSLM that is deployed by the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* DAOM197198 to subvert chitin-triggered immunity in *Medicago*

truncatula, enabling the establishment of AM symbiosis[45], suggesting that other plant beneficial microbes might possess this immune evasion strategy too.

c. MAMP alteration

Pathogens can modify their MAMP to evade recognition by PRRs. Flagellin, whose flg22 epitope serves as an MTI elicitor upon recognition by PRRs, is buried within a flagellin polymer, and recognition of the epitope requires hydrolytic release by plant-produced hydrolases[46]. It has been demonstrated that *Nicotiana benthamiana* produces β-galactosidase 1 (BGAL1) that degrades the flagellin polymer to present the epitope for recognition leading to plant defense responses. *N. benthamiana* mutants that do not produce BGAL1 are more susceptible to pathogen infection. Successful pathogens can suppress BGAL1 activity to counter plant defenses[46].

In some *Pseudomonas syringae* pathovars, the flagellin polymer is post-translationally modified through O-glycosylation, carrying glycans and several sugar molecules and a terminal modified viosamine. Pathovars carrying glycans without the modified viosamine effectively evade hydrolysis and host recognition[46]. Additionally, recognition of flagellin in the pathogen *Acidovorax avenae* K1 by rice cells is abolished due to the presence of 2150-Da glycan moieties, which are different from the 1600-Da glycan moieties in *Acidovorax avenae* N1141 flagellin which is recognized by rice cells to trigger immune responses[47]. At present, MAMP alteration has not been reported among beneficial microbes, but may be a strategy for immune evasion among these microbes considering that flagellin is an indispensable structure for plant-beneficial microbes too.

Root Exudates Shape Belowground Plant Microbiome

Plant-beneficial microbes occur aboveground and belowground, with the aboveground community located in the phyllosphere, the aerial surfaces of the plant. Belowground microbiome, on the other hand, are mainly located in the rhizosphere, which is a narrow (1-3 mm) region of soil around the root under the heavy influence of plant mucilage and root exudates[5]. Both aboveground and belowground microbes can be endophytic, inhabiting the internal tissues. Sugars and AAs are predominantly present in root exudates[48,49] and serve as signaling molecules and energy sources for the rhizosphere microbes.

Our knowledge of plant root exudate constituents comes from experiments that typically grow plants in sterile hydroponic systems, and exudates harvested from the culture and analyzed via techniques such as Ultra-Performance Liquid Chromatography (UPLC). Microbial biosensors have been used for real-time spatio-temporal mapping of root secretions, such as sugars, AAs, and secondary metabolites, revealing details of the formation of rhizobium—legume association[50], for example. Microbial biosensors have also helped to unravel nutrient cycling processes in the rhizosphere during interaction between root exudates and belowground microbes[49].

Many microbes are attracted to root exudates via chemotaxis. Attraction to root exudates, in some species of microbes, facilitates the establishment of host-microbe interactions. For example, *Bacillus subtilis* encodes several chemoreceptors whose ligands include many AAs. The perception of plant-secreted compounds, including AAs, thus facilitates root colonization by *B. subtilis*[51]. Upon root colonization, *B. subtilis* confers growth-enhancing effects to the plant. Generally, root exudates may serve feeding, antimicrobial,

and signaling functions[52]. And root exudation patterns may be developmental stage-dependent, correlating with the functions of soil-dwelling microbes. The importance of root exudates in shaping belowground microbial communities is further reflected in the fact that defects in root secretion of phytochemicals occasion an alteration of fungal and bacterial communities[53].

Plant AA Transport

AAs function as primary metabolites and signaling cues and are the prevalent forms of nitrogen transport in plants where they are transported by both the xylem and the phloem. In general, plants retrieve nitrogen from the soil in the form of inorganic salts, and these are converted into AAs in the roots or mature leaves. These AAs may be used in protein synthesis or serve as building blocks for other essential nitrogenous compounds in plants. Transportation of these AAs in the plant is primarily from the sites of assimilation to sites that require these for proper growth. That is, from the leaves after synthesis, to heterotrophic sinks that typically include developing roots, leaves, apical meristems, stem-localized cortical cells, as well as seeds and fruits[54].

Several AA importers facilitate the movement of AAs across multiple membranes throughout the plant. These fall into several gene families. The families of AA importers include the superfamilies APC (AA, polyamine, choline transporters) and ATF (AA transporter family)[54]. Within these superfamilies are the AA Permease (AAP), Aromatic and Neutral Transporter (ANT), Cationic AA Transporter (CAT), Lys His Transporter (LHT), Pro Transporter (ProT) and GABA transporter (GAT)[55]. Substrate binding assays

have revealed common substrates for many of these transporters, suggesting that functional specificity may reside in the tissue-expression patterns[54]. Indeed, AAP2, AAP4 and AAP5, for example, have floral stem-specific expression patterns, where they may supply AA to maturing embryos, and AAP3 is expressed in the roots, where it presumably participates in soil AA retrieval, or uptake from the phloem[56]. AA transporter expression is altered by environmental and temporal cues. AAP1 and AAP2 are highly expressed during the heart-stage of embryogenesis[57], and ProT1 and ProT2, both proline-specific AA transporters in Arabidopsis, are differentially regulated by salt and water stress[58].

Other transporters fall into AA exporter and facilitator categories. A previous study has described the functional characterization of UMAMIT14, an exporter belonging to the Usually Multiple Acids Move In and out Transporter (UMAMIT) family, which is expressed in root pericycles and phloem cells, and along with UMAMIT18, is involved in export of AA from the roots towards the soil, and loss of function of *UMAMIT14* and *UMAMIT18* significantly reduce secretion of glutamine and other amino acids[59]. The UMAMIT family share significant homology with the *Medicago truncatula* Nodulin 21, which presumably nourishes nitrogen-fixing bacteria[60], which live inside root nodules, which are protrusions on the surface of the root.

When AAs travel from the shoot towards the root, their secretion by the roots into the rhizosphere depends on their export from the sieve elements and companion cells of the phloem into the stele apoplasm. This critical export function is shown to be executed by UMAMIT14. Once in the stele apoplasm, the AAs may enter the pericycle or the endodermis cells via importers. From the endodermis, or from the cortex and/or epidermis (the outermost tissues of the root), the AAs will exit into the rhizosphere by freely leaking

through the apoplastic route, or via other exporters. UMAMITs expressed in the cortex-epidermis and in direct contact with the rhizosphere are excellent candidates for this final role. Thus, overall, the UMAMITs have a crucial role in sustaining root-to-soil AA secretion. This study, in part, focuses on uncovering additional root-expressed UMAMITs that may function as root-to-soil AA exporters, and their role in shaping root—PGPB associations.

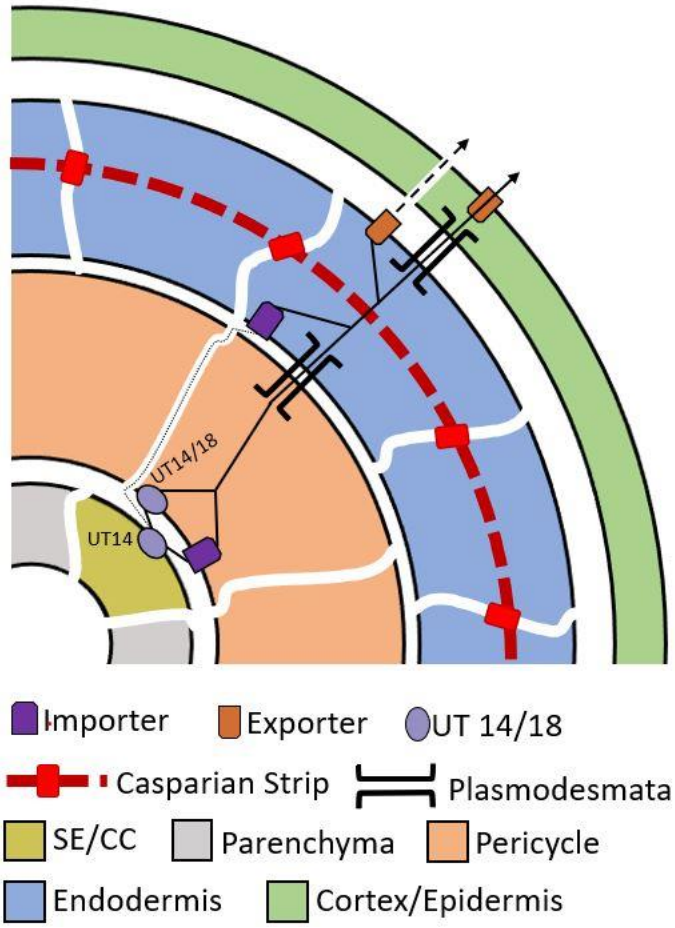


Fig. 1.1: Proposed model for radial AA transport in mature roots. AAs reaching the roots from the shoot will be exported from the sieve elements and companion cells (SE/CC) of the phloem into the stele apoplasm by UMAMIT14. Once in the stele apoplasm, AAs may enter the pericycle or enter the endodermis cells via importers (which may be UMAMIT14/18). From the endodermis, or from the cortex and/or epidermis (the outermost tissues of the root), AAs will exit into the rhizosphere by freely leaking through the apoplastic route, or via other exporters. UMAMITs expressed in the cortex-epidermis and the plasma membrane in the root are excellent candidates for this final role. Solid and dotted lines indicate symplasmic and apoplasmic routes, respectively. Figure is adapted from Besnard *et al*[59]. (Drawing credit: Philip J. Tubergen.)

How Plant-derived Amino Acids Shape Bacterial Phenotypes in the Rhizosphere

a. Chemotaxis

Chemotaxis, the process where bacteria move towards (i.e., positive chemotaxis) or away from (i.e., negative chemotaxis) chemical gradients, has been a long-studied phenomenon. Chemotaxis towards a chemical source may be driven by energy requirements, in which case the chemical source may be used in microbial metabolism, a process called energy taxis[61], as demonstrated in some *E. coli*[62], or the chemical attractant may simply have no role in nourishing the bacteria at all, as demonstrated in some *Bacillus subtilis*[62] and also in *Pseudomonas putida* G7 where attraction to the aromatic hydrocarbon biphenyl has no linkage with the compound's utility as an energy source[63].

Still, in the *P. fluorescens* WBC-3, attraction to the aromatic compounds such as methyl parathion or para-nitrophenol (PNP) has been shown[64]. While *P. fluorescens* WBC-3 could use these compounds as energy sources, this chemo-attraction was not abolished in the *pnpA* and *pnpC* disruption mutants that were incapable of growing on PNP as the sole carbon and nitrogen source, suggesting that the *P. fluorescens* WBC-3 chemotaxis towards these compounds was not metabolism-dependent per se[64]. In the *Pseudomonas* sp. B4, however, chemotaxis towards polychlorobiphenyls and chlorobenzoates were linked to the organism's ability to utilize these compounds as carbon and energy sources[65].

Colonization of plant roots by plant-beneficial bacteria, as well as by pathogens, is known to be driven by directed movement towards the rhizosphere/rhizoplane where nutrients

are highly abundant, followed by a signaling cascade that ensues when host-microbe contact is appropriately established, leading to the formation of root nodules, for example[66]. For chemotaxis to proceed, the microbe must possess and deploy chemosensory apparatus. In *B. subtilis* for example, an intact chemotaxis machinery is required for early root colonization[51]. Cell surface chemoreceptors known as methyl-accepting chemotaxis proteins (MCPs) bind chemotactic ligands and initiate chemotaxis signaling that is transmitted to flagellar motors, through an array of chemotaxis proteins (Che proteins)[51].

In the pathogenic microbe *Ralstonia pseudosolanacearum* Ps29, *mcpA* mutants showed a completely abolished response to 12 amino acids, and attenuated response to 4 other amino acids, compared to the wildtype which responded to all 16 amino acids. Further, in the beneficial *Pseudomonas* species, chemotaxis towards plant-derived metabolites has been demonstrated, whereby *P. fluorescens* WCS365 was shown to undergo chemotaxis towards tomato seeds and root exudates[67], as well as to amino acids[68]. In the beneficial microbe *Pseudomonas fluorescens* Pf0-1, mutants impaired in the chemotaxis machinery CheA, or deleted for proteins that mediate chemotaxis to amino acids are unable to efficiently colonize tomato roots compared to the wildtype[6]. These observations illustrate the importance of chemotaxis towards amino acids for the fitness of both beneficial and pathogenic microbes.

b. Biofilm formation and root colonization

An important trait that is crucial for plant root colonization by microbes is biofilm formation. How plant-derived amino acids regulate this aspect of plant—microbe interactions has not been extensively studied. Yet, it has been shown that although valine, for example,

may exert toxic effects on some bacteria, several Enterobacterial species are actually resistant to valine at high concentrations. This observation might be consistent with the expectation that bacterial colonization of ecological niches requires resistance/tolerance to the microenvironment such as the rhizosphere. It has been reported that some Gram-negative bacteria discharge huge amounts of valine when forming biofilm, resulting from metabolic changes occurring within the biofilm[69]. This observation experimentally demonstrates that some rhizosphere-resident bacteria may also possess the capacity to tolerate amino acid types/concentrations that may be toxic. In the rhizosphere-resident *Pseudomonas fluorescens* WCS365, biofilm formation is enhanced *in vitro* when the growth medium is supplemented with casamino acids[70], demonstrating a potential role for amino acids in shaping bacterial communities.

In general, the L-enantiomers of proteinogenic amino acids are the main forms of amino acids constituting cellular metabolites and participants in protein synthesis. The D-enantiomers, on the other hand, while featuring sparingly in biological systems[71], have shown up in numerous environments, including in plant tissues[72–74], and play important roles in biology. For example, D-Alanine is a constituent of the peptidoglycan cell wall in some bacteria[75], and D-serine may act as an agonist in plant signaling[76]. Interestingly, tyrosine, tryptophan, leucine and methionine can interfere with biofilm formation, as well as promote biofilm dispersal in *Bacillus subtilis*, when they occur as D-enantiomers, but not as L-enantiomers[77].

Finally, experiments have demonstrated that some microbes that dwell in the rhizosphere are impaired in root colonization when mutations are introduced into their genes for amino

acid assimilation and catabolism[78,79], further demonstrating the importance of rhizosphere amino acids in shaping root—bacterial interactions.

c. Metabolism and Growth

Inorganic nitrogen (specifically ammonium nitrogen) is the main source of N for soil-resident microbes[80]. And under conditions of ammonium N abundance, the uptake of N from other sources is downregulated in a process described as 'N regulation'[80]. However, nitrogen can be obtained through nitrate reduction, as well as from organic sources such as AAs. The uptake of organic N from the environment/growth medium may occur through uptake of intact organic N, such as AAs, which are then subjected to deamination/transamination. Alternatively, these can be directly utilized via peptide synthesis.

The importance of amino acids for microbial growth in the rhizosphere has recently been demonstrated. Analyzing the response of microbes to root exudate metabolites, it was shown that isolates with enhanced growth during root growth (i.e., positive responders) exhibited a far more impressive uptake of several metabolites, including amino acids, compared with those that do not show enhanced growth (i.e., negative responders)[81]. Additionally, the findings of enhanced amino acid and organic acid uptake by the positive responders are consistent with the observation that they encode significantly more transporters for amino acids and organic acids in their genomes than do the negative responders[81].

Other works have revealed the importance of amino acid uptake and metabolism in rhizosphere-dwelling bacteria. Utilizing technologies such as promoter-trapping and

promoter fusion[82–85], and microarray studies[86–88], genes deployed by some bacteria in amino acid uptake and metabolism were uncovered, indicated by their enhanced upregulation. Specifically, rhizosphere amino acids could enhance the expression of genes that regulate competence and sporulation in *Bacillus subtilis*, for example[89].

Injection studies involving the exogenous application of isotopically-labelled AA to the soil, followed by measurement of the extent to which plants versus soil-dwelling microbes compete for these exogenously supplied AA indicate that microbes consistently outcompete plant roots for AA. These studies indicate that rhizobacteria quickly respond to changes in the rhizosphere AA concentration (Reviewed in Ref[90]).

Bacteria, especially the rhizobacteria belonging to *Pseudomonas* and *Micromonospora* may prefer AAs to sugars[91–93], which are also abundant in the rhizosphere. In these groups, AAs may be selectively catabolized, since when both AAs and sugars are present, these bacteria reprogram their metabolism to repress the catabolism of glucose assimilation. Similarly, nutrient-limiting environments enable bacterial survival that is dependent on mutations that allow for enhanced AA catabolism[94]. Bioinformatics analysis suggests that between the rhizosphere-dwelling bacteria *P. putida* KT2440 and its close relative, the opportunistic human pathogen *P. aeruginosa*, the *P. putida* KT2440 encodes two-fold more genes with predicted function as AA uptake proteins than do *P. aeruginosa*. Overall, *P. putida* KT2440 encodes 15% more cytoplasmic membrane transport proteins than *P. aeruginosa*, and the genomic analysis suggests that *P. putida* KT2440 may be less capable of utilizing carbohydrates and dicarboxylates[95].

Both *P. putida* KT2440 and *P. fluorescens* efficiently catabolize AAs: in particular Asp, Glu, Asn, Gln. Growing these bacteria in the presence of the above-named AAs as sole

C and N sources lead to the induction of a unique group of proteins that mediate AA uptake and metabolism as revealed by proteomic analysis[96]. These studies, taken together, may suggest that rhizosphere-dwelling bacteria may have been selected for AA uptake and catabolism as energy sources.

d. Auxin Biosynthesis

The biosynthesis and regulation of the plant hormone auxin is dependent on amino acids[97,98], subsequently influencing a great variety of plant developmental process that occur throughout the plant life cycle, such as root growth and architecture[99]. Auxins are indole-derived compounds, and they also come in a number of different structural analogs. Of these, the indole-3-acetic acid (IAA) is the most extensively described[100]. The amino acid tryptophan is known to be the main precursor of IAA biosynthesis[97,100]. Auxin biosynthesis can occur directly via tryptophan or other tryptophan biosynthetic precursors in nearly all parts of the plant, as well as the roots[101]. The remarkable involvement of auxin in initiating lateral root formation is thought to be consistent with the observation that rhizosphere tryptophan concentrations are elevated along the root, and are substantially increased near the points of emergence of lateral roots[49].

While plants are the primary source of auxins in the rhizosphere[102], rhizosphere-resident bacteria have been suggested to be involved in the synthesis of auxins in the rhizosphere, with the possibility that these could be nearly 80% of the rhizosphere-resident bacteria[97].

In the PGPB *Enterobacter cloacae* UW5, the biosynthesis of IAA is regulated by both aromatic amino acids and branched chain amino acids. Whereas the indole-3-pyruvate decarboxylase gene (*ipdC*), coding for a key enzyme in the indole-3-pyruvic acid pathway

of IAA biosynthesis, is activated by the transcription factor TyrR, and its expression upregulated by the aromatic amino acids tryptophan, tyrosine, and phenylalanine[103,104], the branched chain amino acids valine, leucine, and isoleucine downregulate *ipdC* expression and IAA production[105]. Inoculation of cucumber roots with the plant growth-promoting bacteria *Bacillus amyloliquefaciens* SQR9 enhances the expression of the cucumber tryptophan transporter gene and hence tryptophan secretion from the roots, and thus IAA biosynthesis by *B. amyloliquefaciens* SQR9[106].

Significance of the Study

As global population rises to a projected 9 billion people by 2050, so is the need for adequate food supplies, as well as for fuel, feed and fodder, in order to continue to sustain life on Earth[107,108]. A longstanding practice for raising crop yields has been the application of chemical fertilizers. Yet, the environmental consequences of this approach are damaging, and include pollution of water resources[2] as well as the soil, which is the primary medium of plant growth[3]. Furthermore, the application of chemical pesticides has led to the evolution of pesticide-resistant strains of pathogens and insects[109]. Thus, the search for more sustainable approaches of increasing crop yields, has, among others, also focused on PGPB. PGPB are associated with many plant species[8], and benefit from root-secreted compounds for growth. Sugars and AAs are the most abundant of the compounds in root exudates, suggesting their importance for maintaining microbial life as they serve as signaling cues and C and N sources for soil-dwelling microbes[48,49]. While it is known that root-secreted AAs can attract beneficial microbes, the plant AA transporters involved in modulating root—beneficial microbe interactions are largely unknown. This

research focuses on identifying and characterizing candidate root-expressed AA importers and exporters that shape root-beneficial microbe associations.

1.3 AIMS OF THE STUDY

1. To establish a method to inoculate Arabidopsis growth medium with PGPB, and to assess root colonization and PGPB-mediated plant growth.

2. To identify and characterize Arabidopsis AA transporters that contribute to modifying AA concentration in the rhizosphere, and to determine whether these transporters modulate root—PGPB interactions and PGPB-mediated plant fitness.

Chapter 2:

Development and evaluation of a bioassay for studying plant—beneficial microbe interactions

2.1 BACKGROUND

Many different approaches for studying plant—bacterial interactions have been documented in the literature. These approaches vary, as expected, based on the exact goals of the study. For plant—beneficial microbe interactions, these methods range from exposure of whole plants to bacterial plates in enclosed containers with ports fitted at the top to allow air flow in and out, where volatiles emitted by the bacteria may be sufficient to induce plant growth[110], through to the direct inoculation of root tissues with the test bacterial species, or inoculation of the plant growth medium with the bacterial species[111], or merely by placing the bacterial inoculum beneath the root tips, in which case volatile compounds produced by the bacteria can trigger growth changes in the root and shoot of the plant[112]. In many cases, gnotobiotic systems are used, in which all other (micro)organisms, except the test bacterial species, are excluded, as they may impact the plant—bacterial species interaction of interest.[113]

Crucially, because plant growth medium components can interact with the test bacterial species, and hence altering the plant—bacterial interactions in ways that may confound the real effects of the bacteria on plant growth, plant growth media choice is paramount in unearthing the direct contributions of test bacterial species to plant growth. For example, growth media rich in carbon and nitrogen may not be suitable for uncovering whether plant root secretions can enhance bacterial association with roots. Thus, plant growth media rich in carbon and nitrogen may be replaced with one in which no sucrose is present, for example, just before bacterial inoculations[111]. Additionally, because different microbes may have different nutritional requirements which may impact their

plant growth-promoting activities, identifying the optimal assay is a *sine qua non* for making progress in plant—beneficial microbe interactions research. Thus, for the purposes of my dissertation research, I set out to determine the optimal bioassay, including the doses of the bacteria *Pseudomonas fluorescens* WCS417r, appropriate for inducing growth in Arabidopsis plants at the seedling and adult plant stages. Importantly, among others, I explored the utility of a system that enables "flood-inoculation" of root tissues, allowing the bacteria to swim freely to locate plant roots for colonization and bacterial-mediated plant growth.

2.2 RESULTS

A system for inoculating Arabidopsis growth medium with PGPB and assessing root colonization and PGPB-mediated plant growth

- A single *P. fluorescens* WCS417r inoculation promotes Arabidopsis growth in peat pellets

To develop a system that allows me to evaluate the role of plant amino acid transporters in shaping plant—beneficial rhizobacteria interactions, I first tested the suitability of the plant-growth peat pellet (Jiffy-7® peat pellets; Jiffy products of America Inc, OH, USA) for this purpose, using the Arabidopsis—*P. fluorescens* WCS417r (hereafter 'Pf" or *P. fluorescens*) as a model system. Here, I hydrated peat pellets in tap water and allowed them to fully expand. I then placed twenty (20) pellets each in four plant-growth trays marked for four different experimental groups: Hoagland only (i.e., plant nutrient fertilizer as *positive control*), tap water (*negative control*), Hoagland + Pf ($1x10^5$ CFUs/g soil), and Hoagland + Pf ($4x10^7$ CFUs/g soil). The tap water-soaked peat pellets for the Hoagland

+ Pf ($1x10^5$ CFUs/g soil) and Hoagland + Pf ($4x10^7$ CFUs/g soil) groups were amended with a single dose of Pf [grown overnight in LB medium + Rifampicin (50 µg/mL) and washed with sterile water three times] to the indicated titers.

I then sowed wild type Arabidopsis seeds across all experimental groups and one week after germination, thinned out the excess seedlings, leaving two uniformly-growing plants per pellet. From one week onward, twice a week (Mondays and Wednesdays), I watered all pellets with 20 mL tap water each, and once a week (Fridays) with 20 mL modified half-strength Hoagland solution, except for the negative control group which was watered for the third time with tap water. At the end of week two, I completed a two-step thinning out process, leaving only one plant per pellet. The plants were allowed to grow through week seven and data were collected. Plants were grown in the walk-in Environmental Growth Chamber under the following conditions: 25 ºC, 65% RH, 9h Light/15h Dark, and a light intensity of 80 – 100 µmoles/m^2/s.

My results showed that one-time inoculation of the peat pellets with Pf is sufficient to boost plant growth, in a dose-dependent manner. Pellets amended with Pf to $1x10^5$ CFUs/g soil followed by weekly application of half-strength Hoagland recorded biomass that was marginally higher than (but not statistically significantly different from) Hoagland-only plants. At the dose of Pf $4x10^7$ CFUs/g soil, in conjunction with weekly application of half-strength Hoagland, however, plants were significantly larger than those under half-strength Hoagland-only application (Fig. 2.1).

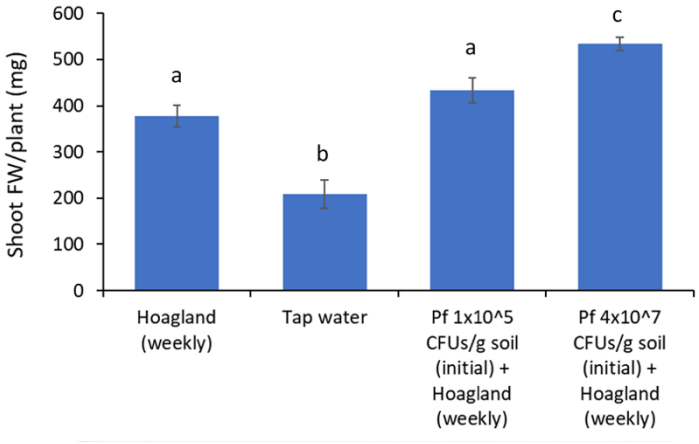


Fig. 2.1: One-time application of *P. fluorescens* WCS417r promotes Arabidopsis growth in peat pellets in a dose-dependent manner. Col-0 wild type seeds were sown and treated as described in the text. Shoot fresh weight (FW) was determined at the end of week seven. Data are average ± SE (*n*=19—20). Bars lettered differently are significantly different ($p<0.05$); One-Way ANOVA followed by Tukey's posthoc test. Hoagland = half strength.

At the heart of my experimental approach is the idea that the pellet-applied Pf will be recruited to the roots to intimately interact with the plants in order to promote plant growth. This would allow me to determine whether or not plant AA transporters expressed in the roots mediate the recruitment of Pf, impacting Pf growth/metabolism and hence the Pf-mediated plant growth. Thus, I needed to recover the roots from the peat pellets and to determine root biomass and the extent of root colonization by Pf (across the wild type and the different AA transporter mutants to be tested). Unfortunately, I found that I could not successfully recover intact roots from the pellets, as the roots were extensive and fragile, leading to substantial amounts of root tissue remaining unaccounted for following root biomass assessment. However, the data suggested that, consistent with previous reports[112,114], Pf could be used to study beneficial bacteria-mediated plant growth-promotion in Arabidopsis. I thus designed a new assay that enabled the assessment of root colonization by Pf (see below).

- *P. fluorescens* WCS417r promotes Arabidopsis growth in an inoculation titer-dependent manner in a plate assay

Most previous studies on the effect of PGPB on plants, focusing on seedling growth, utilized MS agar plates amended with sucrose as growth medium. However, I wanted a system that enables me to recover intact seedling roots easily and efficiently for root colonization assessment. To this end, I established a plate-based assay to grow Arabidopsis in the presence of chemical fertilizer-PGPB combinations in gnotobiotic conditions. Briefly, I employed 3MM Chromatography paper cut to the dimensions of 8cm x 7cm to fit a sterile 100mm x 100mm square plate with grids. The 3MM papers were sterilized by autoclaving at 121 ºC for 30 mins and then placed inside the square plates, followed by wetting with 6 mL of the appropriate inoculum. Surface-sterilized Arabidopsis Col-0 wild type seeds stratified for at least 48h at 4 ºC were then placed on the wetted paper, approximately 15 seeds per plate. The plates were sealed with parafilm and incubated vertically in a reach-in growth chamber (Conviron Adaptis 1000, Canada) (at 25 ± 2 ºC, 75% RH, 16h Light/8h Dark, and 100 µmoles/m^2/s light intensity) for 17 or 18 days. Shoot and root biomass (and other parameters) were then determined (see next section). For the seedling assays, all weight measurements were to the nearest 0.1 mg (Mettler Toledo, USA).

To utilize this plate-based assay to advance my objectives, I first tested the effects of different Pf inoculation titers on Arabidopsis growth, as previous studies have shown that growth promotion by PGPB may be inoculation titer-dependent[115,116]. Using sterile water and Hoagland as negative and positive controls, respectively, I showed that Pf promotes Arabidopsis growth in a dose-dependent manner, plant biomass recording a modest but

statistically significant growth with Pf titers of OD_{600}=0.2 and 0.4. At higher titers of OD_{600}=0.6 and 0.8, plant growth was severely inhibited, and not significantly different from plants under sterile water-only treatment (Fig. 2.2A). Root growth under the different treatments also followed a similar pattern as shoot growth (Fig. 2.2B). Shoot and root growth inhibition at higher OD_{600} titers is similar to results from a previous study showing that Arabidopsis seedlings exposed to *P. thivervalensis* MLG45 at titers beyond 10^6 CFU/mL were damaged irreversibly[116], and consistent with the expectation that in a nutrient-poor substrate such as my system, high inoculum titer would rapidly collapse, producing, among others, toxic products that may be inhibitory to plant growth.

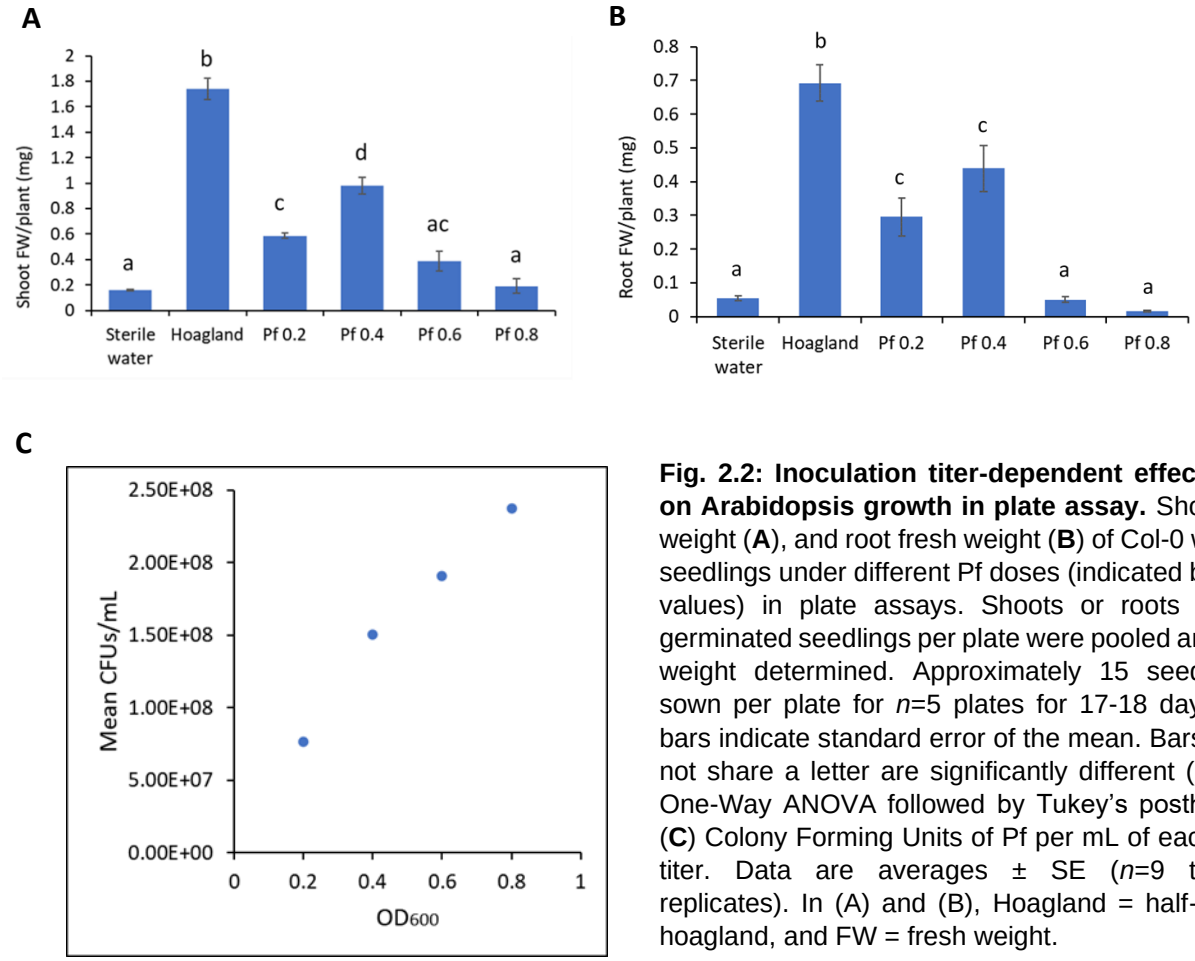


Fig. 2.2: Inoculation titer-dependent effects of Pf on Arabidopsis growth in plate assay. Shoot fresh weight (**A**), and root fresh weight (**B**) of Col-0 wild type seedlings under different Pf doses (indicated by OD_{600} values) in plate assays. Shoots or roots from all germinated seedlings per plate were pooled and mean weight determined. Approximately 15 seeds were sown per plate for *n*=5 plates for 17-18 days. Error bars indicate standard error of the mean. Bars that do not share a letter are significantly different ($p<0.05$); One-Way ANOVA followed by Tukey's posthoc test. (**C**) Colony Forming Units of Pf per mL of each OD_{600} titer. Data are averages ± SE (*n*=9 technical replicates). In (A) and (B), Hoagland = half-strength hoagland, and FW = fresh weight.

- *P. fluorescens* WCS417r inoculation titer, but not level of Arabidopsis root colonization, predicts shoot growth

To determine the suitability of my system for measuring plant root colonization, I measured root colonization by Pf across different doses of Pf inoculation. Here, I discontinued the application of higher bacterial titers, and instead, performed the analyses with Pf OD_{600}=0.002, 0.02, 0.2, and 0.4. Because Pf inoculation titers below 0.2 were unlikely to produce robust shoot and root biomass, all four Pf titers indicated were applied in combination with half-strength Hoagland solution, enabling robust root tissue growth to facilitate root colonization. To obtain root colonization data, I first excised shoots from all seedlings that germinated (approx. 15 seedlings per plate). Shoots and roots from each plate were collected separately into autoclaved 2.0 mL Eppendorf tubes using heat flame-sterilized razor blades and tweezers, and biomass was determined. Then, 400 µL of autoclaved water was added to each tube containing root biomass plus two tungsten beads (cleaned with ethanol) and the roots lysed (Qiagen Retsch TissueLyser II) at the freq. of 25/sec. for 5 minutes. One hundred (100) µL of each lysate sample was used to make 10-fold serial dilutions in sterile water. Five (5) µL of the dilutions were plated on LB agar (amended with Rifampicin 50 µg/mL) and incubated for 24h at 28 ^{o}C and the number of CFUs determined.

Consistent with my prediction, both OD_{600} titers below 0.2 (i.e., 0.002 and 0.02) were not sufficient to boost plant growth (Fig. 2.3A). However, in accord with my data in Fig. 2.2, both OD_{600}=0.2 and 0.4 in combination with half-strength Hoagland improved shoot growth, significantly higher than the Hoagland-only control group (Fig. 2.3A). Some PGPB are known to inhibit root elongation, in a species-dependent, or even strain-dependent

manner[116]. Pf is known to shorten primary root growth (although I did not find any remarkable root length reduction in my system, at titers equal to, or below OD_{600}=0.4), but promotes lateral root development[112], and this could modestly boost the root system and hence overall root biomass. Thus, I found that root biomass generally increased with Pf inoculation titer (Fig. 2.3B). Despite the root biomass enhancement associated with increasing Pf titer, both CFU/mg root FW and CFU/root system were indistinguishable for seedlings across the different treatments (Fig. 2.3C, 2.3D), and no strong correlation existed between shoot biomass and CFU/mg root FW or between shoot biomass and CFU/root system (Fig. 2.3F, 2.3G). Instead, Pf inoculation titer strongly predicted shoot growth (R^2=0.985, p=0.007; Fig. 2.3E). Thus, shoot growth under the different treatments is explained not by the level of root colonization by Pf, but by the titer of Pf to which the seedlings were exposed.

I note that the root colonization measurements likely reflected the number of bacteria on the root surface (i.e., the rhizoplane), plus the inside the roots (i.e., the root endosphere), whereas in natural settings, most plant growth-promoting activity by PGPB is attributed to PGPB occupancy of both the rhizoplane (plus root endosphere) and the rhizosphere (i.e., the 1-3 mm narrow region of soil around the roots under the influence of root exudates). Indeed, some PGPB, including Pf, could still boost Arabidopsis growth without direct contact with the plants, through volatile organic compounds that diffuse through the medium[110,112].

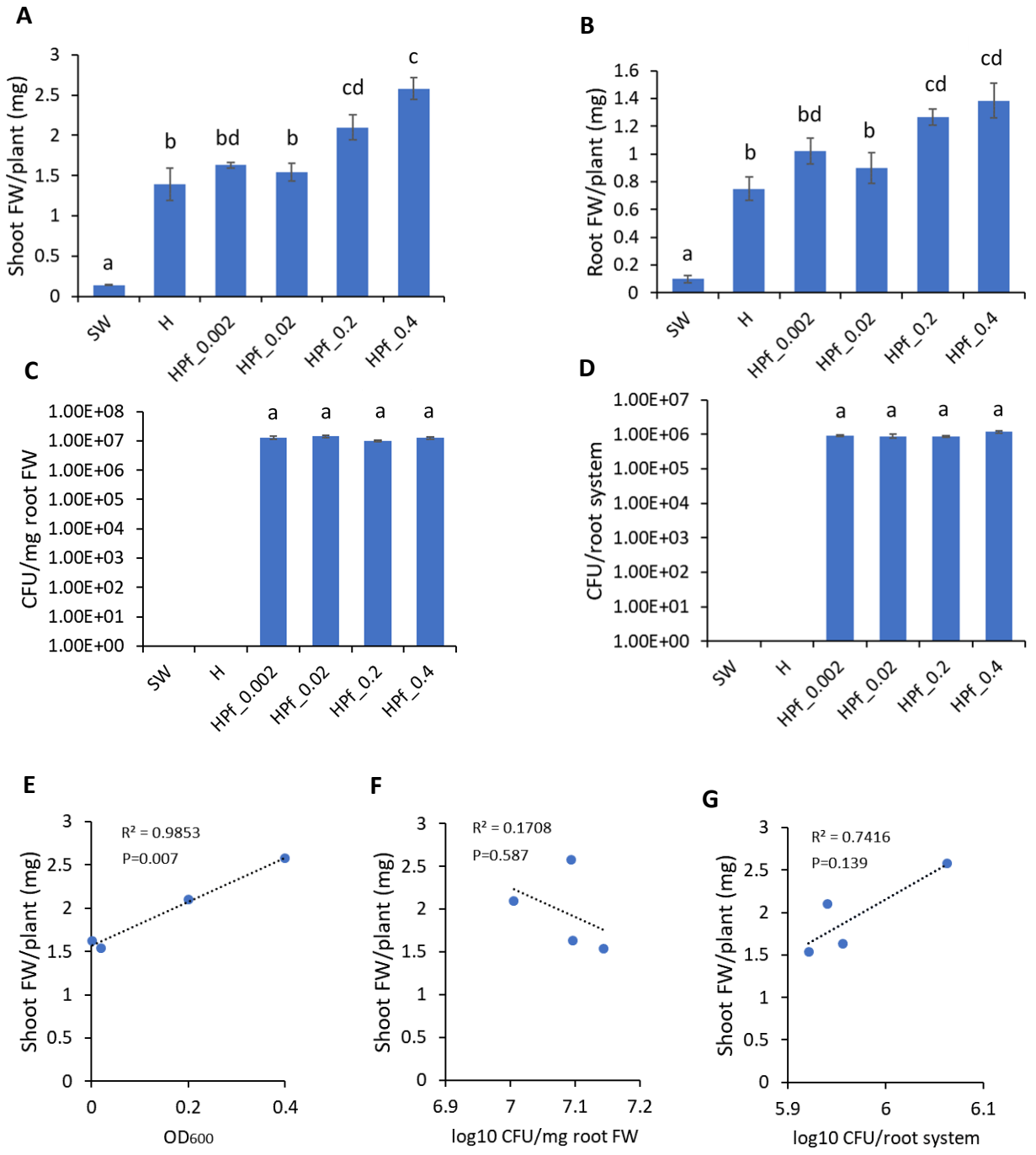


Fig. 2.3: Effect of Pf inoculation titer and level of root colonization on Arabidopsis growth. Shoot fresh weight (**A**), and root fresh weight (**B**) of Col-0 wild type seedlings treated with sterile water (SW), half-strength Hoagland (H), or H combined with different Pf doses (indicated by OD_{600} titers) in plate assays for 17-18 days. (**C**) CFU/mg root FW, (**D**) CFU/root system, and correlations between Pf dose vs shoot FW/plant; R^2=0.9853, p=0.007 (**E**), or between Log10 CFU/mg root FW vs shoot FW/plant; R^2=0.1708, p=0.587 (**F**), or between Log10 CFU/root system vs shoot FW/plant; R^2 =0.7416, p=0.139(**G**). Shoots or roots from all germinated seedlings per plate were pooled and mean weight determined. Approximately 15 seeds were sown per plate for *n*=5 plates. Error bars indicate standard error of the mean. CFUs were processed as described in the text. Bars that do not share a letter are significantly different (p<0.05); One-Way ANOVA followed by Tukey's posthoc test.

- Live *P. fluorescens* WCS417r are required for Arabidopsis growth promotion in plate assays

Because plants could utilize C and N from dead bacteria for growth, I wondered to what extent, under these experimental conditions, plant growth would be influenced by dead bacterial cells; that is, whether plant growth in my plate assay would largely require metabolically active (and not dead) bacteria. To test this, I applied OD_{600}=0.2 of live Pf or heat-killed Pf (autoclaved at 121 ^{o}C), along with my negative and positive controls (i.e., sterile water and half-strength Hoagland, respectively). Because Pf-only-mediated Arabidopsis growth is very modest, I also included Hoagland+Pf, and Hoagland+Heat-killed Pf combinations as additional experimental groups. I showed that metabolically inactive (heat-killed) Pf had no substantial effect on plant growth, being statistically indistinguishable from sterile-water-only effects ($p>0.05$; Fig. 2.4). Importantly, while Hoagland+Pf combination boosted plant growth above Hoagland-only levels ($p<0.05$), Hoagland+Heat-killed Pf combination returned plant growth to Hoagland-only level ($p>0.05$; Fig. 2.4). Thus, these data indicate that Pf-mediated plant growth in this system will largely require metabolically active Pf interacting with the plants.

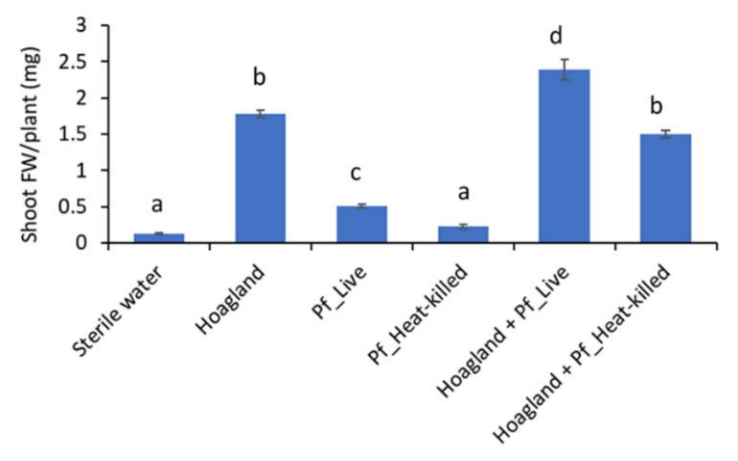


Fig. 2.4: Live *P. fluorescens* WCS417r (Pf) are required for Arabidopsis growth promotion in plate assays. Data are average ± SE of weights of Col-0 wild type shoots from all germinated seedlings per plate pooled together. Approximately 15 seeds were sown per plate for *n*=5 plates for 17-18 days. Bars that do not share a letter are significantly different ($p<0.05$); One-Way ANOVA followed by Tukey's posthoc test. Hoagland = half-strength hoagland, Pf (live or heat-killed) OD_{600} = 0.2

- Arabidopsis growth promotion in plate assay is specific to plant growth-promoting bacteria (PGPB)

In a final assay validation step, I tested whether the Pf-mediated plant growth-promotion observed in my system is specific to plant growth-promoting bacteria. For this, I utilized a commercially available consortium of PGPB consisting of four PGPB species of *Bacillus*, namely *B. licheniformis*, *B. subtilis*, *B. methylotrophicus*, and *B. amyloliquefaciens* (Pathway Biologic, Plant City, FL, USA), along with *E. coli* DH5α, as my test species. The negative and positive controls were sterile water and full-strength Hoagland (or full-strength Hoagland diluted with sterile water to 1:2 and 1:5), respectively. The results (Fig. 2.5) revealed that, indeed, plant growth promotion in this system is only specific to PGPB. In general, *E. coli* DH5α failed to boost plant growth, even in combination with Hoagland solution, as Hoagland+*E. coli* DH5α combinations generally produced plant biomass that did not substantially rise above those of Hoagland-only treatments. On the contrary, growth was significantly enhanced ($p<0.05$; Fig. 2.5) in the presence of the PGPB consortium, and when Hoagland+PGPB consortium was applied. These data suggest

that the plate-based assay described here is reliably sensitive enough to allow me to study Arabidopsis—PGPB interactions, and the role of plant amino acid transporters in shaping these interactions.

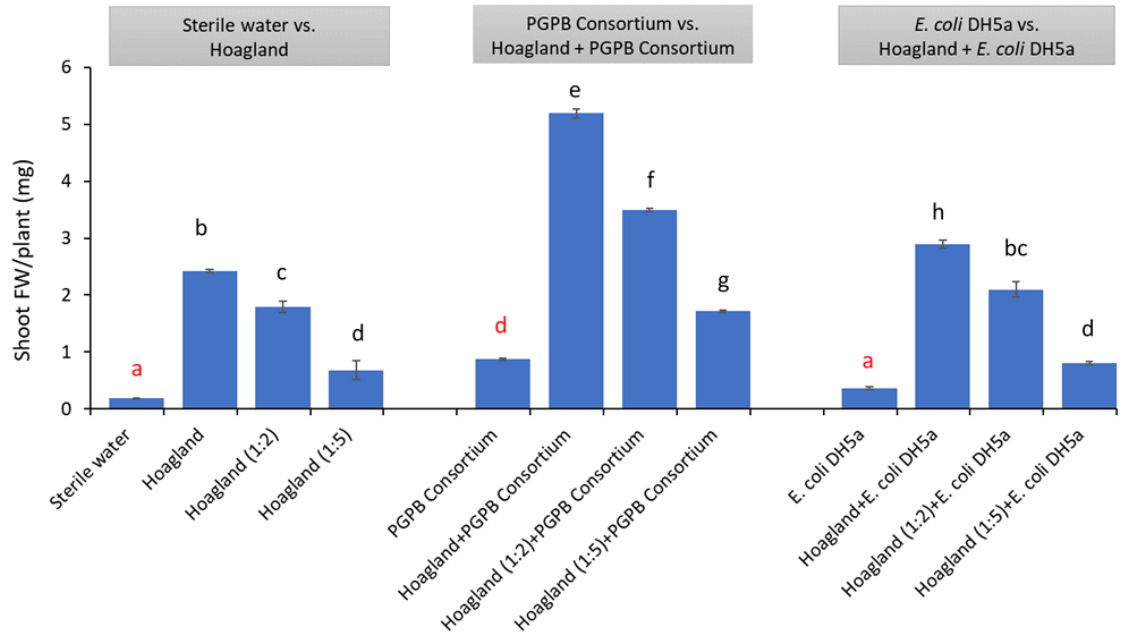


Fig. 2.5: Arabidopsis growth promotion in a plate assay is specific to plant growth-promoting bacteria (PGPB). Data are average ± SE of weights of Col-0 wild type shoots from all germinated seedlings per plate pooled together. Approximately 15 seeds were sown per plate for *n*=2 plates. Bars that do not share a letter are significantly different ($p<0.05$); One-Way ANOVA followed by Tukey's posthoc test. Hoagland = full-strength. PGPB consortium and *E. coli* DH5α OD_{600} = 0.4. Data were collected on 47-day old plants. Note that *E. coli* DH5α combined with full-strength Hoagland showed a slight but statistically significant growth-boosting effect compared to full-strength Hoagland-only treatment. This experiment has *not* been repeated.

2.3 DISCUSSION

Different bioassays are available for evaluating plant—beneficial microbe interactions, and depending on the objectives of the study, can be modified to achieve the intended goals. This has necessitated the continuing development and evaluation of different

bioassays from time to time. For example, two peat-based gnotobiotic plant growth systems called FlowPot system and GnotoPot system were recently developed[113]; the FlowPot enables the treatment of a substrate with inoculum such as microbiota suspensions, or with nutrients, via an irrigation port that enhances drainage to prevent root anoxia, for example, whereas the GnotoPot system uses the peat pellets used for the adult plant assay in this chapter, and affords the opportunity to inoculate peat pellets with microbiota of choice with minimal handling to reduce the chances of contamination.

Here, I tested a peat pellet-based system for growing Arabidopsis seedlings following substrate inoculation with the plant growth-promoting bacteria *P. fluorescens* WCS417r, and evaluating the plant response to the bacteria. Although axenic systems are most suitable for evaluating the effect of a microbial species or microbiota on plant growth, and different soil sterilization methods such as irradiation, application of gaseous chemical fumigants, and autoclaving have been applied, these may lead to phytotoxic effects[113]. Autoclaving may lower soil pH[117].

Indeed, in my hands, initial attempts at growing Arabidopsis in autoclaved peat pellets yielded poor and non-uniform plant growth. Thus, the peat pellet system was adopted without any sterilization, with the consideration that the pellets come relatively nutrient-poor, and flooding them with the test bacterial species in this study may allow the test bacteria to outcompete any microbes that may originate with the pellets. Using this system, I confirmed that a single inoculation of the peat pellet with WCS417r is sufficient to enhance Arabidopsis growth in the long-term (through seven weeks, at least), in an inoculation titer-dependent manner. This suggests that the inoculated bacteria survive in the peat pellet probably by feeding off plant-derived photosynthates, considering that the

peat pellet does not contain nutrients that can readily support microbial growth. A disadvantage of this system, however, was the difficulty in extracting intact root biomass to quantify bacteria associated with the root tissues.

While agar-based growth cultures have been employed for Arabidopsis seedling growth and examination of the effects of PGPB, root tissues may grow into the agar medium, sometimes rendering them non-extractable for the accurate assessment of root biomass and root tissue colonization by the inoculated bacteria.

To overcome these challenges, a plate-based system utilizing a 3MM paper which is a nutrient-poor substrate was used to assess the effect of WCS417r inoculation on Arabidopsis root and shoot growth. The 3MM paper is autoclavable without exerting any obvious negative effects on plant growth, and seedling roots remain on the surface of the paper, and can be easily extracted. This assay, using the 3MM paper, showed that Arabidopsis growth in response to WCS417r inoculation was dependent on the WCS417r titer (Fig. 2.3E), consistent with the PGPB effect on plant growth as reported previously for some other bacteria[116]. Yet, instructively, plant growth in response to the WCS417r inoculation was not dependent on the level of root colonization by WCS417r per se (Fig. 2.3F, 2.3G). These results suggest that occupancy of the root surface by WCS417r is not required for WCS417r-mediated Arabidopsis growth, and is consistent with previously-speculated WCS417r volatile-induced plant growth promotion[112]. While root tissue colonization is not required for WCS417r-mediated Arabidopsis growth, the results herein presented show that live WCS417r cells are required (Fig. 2.4), indicating that the bacteria may still need to feed off plant root exudate components to thrive in the rhizosphere in order to enhance the plant—beneficial microbe interactions. In this regard, to what extent

plant-derived amino acids secreted into the rhizosphere may shape these interactions is the subject of the remaining chapters of this dissertation.

MATERIALS AND METHODS

Plant growth and inoculation with bacteria

For all experiments presented in this chapter, wildtype plants were raised from Col-0 seeds, and seedlings and adult plants were raised as below:

Seedlings: For all seedling experiments, seeds were surface-sterilized using 10% bleach three times for two minutes each, followed by three washes with sterile water, and subsequently resuspended in 1% phytoagar and stratified in the dark at 4 ºC for at least two days. Seeds were then plated onto autoclaved 3MM paper cut to fit 100mm x 100 mm square plates and wetted with 6 mL of the appropriate inoculum. The 3MM papers were autoclaved at 121 ºC for 30 minutes. Where inoculum consisted of Hoagland's solution and Pf combinations, equal volumes of filter-sterilized full strength Hoagland's solution and washed Pf cells diluted to twice the required final titer (e.g., OD_{600}=0.4) were mixed together, bringing the Hoagland's solution to half-strength, and the Pf to the desired titer (i.e., OD_{600}=0.2 in this example). The plates were sealed with parafilm and incubated vertically in a reach-in growth chamber (Conviron Adaptis 1000, Canada) (at 25 ± 2 ºC, 75% RH, 16h Light/8h Dark, and 100 µmoles/m^2/s light intensity) for 17 or 18 days. Specific variations of this assay are described in the figure legends, as appropriate.

Adult plants: Peat pellets were hydrated in tap water and allowed to expand fully. Twenty (20) pellets were placed in each of four plant-growth trays marked for four different experimental groups: Hoagland only (i.e., plant nutrient fertilizer as *positive control*), tap water (*negative control*), Hoagland + Pf ($1x10^5$ CFUs/g soil), and Hoagland + Pf ($4x10^7$ CFUs/g soil). The tap water-soaked peat pellets for the Hoagland + Pf ($1x10^5$ CFUs/g soil) and Hoagland + Pf ($4x10^7$ CFUs/g soil) groups were amended with a single dose of Pf [grown overnight in LB medium + Rifampicin (50 µg/mL) and washed with sterile water three times] to the indicated titers.

Wildtype Arabidopsis seeds were then sown across all experimental groups and one week after germination, the excess seedlings thinned out, leaving two uniformly-growing plants per pellet. From one week onward, twice a week (Mondays and Wednesdays), pellets were watered with 20 mL tap water each, and once a week (Fridays) with 20 mL modified half-strength Hoagland solution, except for the negative control group which was watered for the third time with tap water. At the end of week two, a second thinning out was done, leaving only one plant per pellet. The plants were allowed to grow through week seven and data were collected. Plants were grown in the walk-in Environmental Growth Chamber under the following conditions: 25 ºC, 65% RH, 9h Light/15h Dark, and a light intensity of 80 – 100 µmoles/m^2/s.

Bacterial strains and growth conditions

Pseudomonas fluorescens WCS417r, also known as *Pseudomonas simiae* WCS417r which was initially isolated from lesions of wheat (*Triticum sativum*) roots[118] was maintained on LB plates supplemented with 50 µg ml^{-1} rifampicin. *E. coli* DH5α cells were maintained on LB plates. In preparation for root inoculation experiments, a single colony

was randomly picked from the appropriate plate and grown overnight in approximately 100 mL of LB at 28 ºC (for *Pseudomonas* strains) or 37 ºC (for *E. coli* DH5α cells) with shaking at 230 rpm till the cultures reached OD_{600} = 0.4 – 0.8. The cell culture was harvested and washed three times in sterile water, and then adjusted to the required inoculation titer with sterile water. For experiments where heat-killed bacteria were used, bacteria were autoclaved at 121 ºC for 30 minutes.

Quantifying bacterial growth on root tissues

To obtain root colonization data, roots from all seedlings that germinated (approx. 15 seedlings per plate) were collected into autoclaved 2.0 mL Eppendorf tubes using heat flame-sterilized razor blades and tweezers, and root biomass was determined. Then, 400 µL of autoclaved water was added to each Eppendorf tube containing root biomass plus two tungsten beads which were cleaned with 95% ethanol. The root tissues were lysed using Qiagen Retsch TissueLyser II at the freq. of 25/sec. for 5 minutes. One hundred (100) µL of each lysate sample was used to make 10-fold serial dilutions in sterile water. Five (5) µL of the dilutions were plated on LB agar (amended with Rifampicin 50 µg/mL) and incubated for 24h at 28 ºC and the number of CFUs determined. Number of CFUs were then normalized by the root fresh weight for CFU/mg root FW or by the number of seedlings from which the root biomass was obtained for CFU/ root system. For these seedling assays, all weight measurements were to the nearest 0.1 mg (Mettler Toledo, USA).

Statistical analysis

Data analyses were performed using the JASP open-source software v 0.14 and Excel, and graphs generated using Excel. Data were analyzed via a one-way ANOVA followed by Tukey's posthoc test.

Chapter 3:

Loss of rhizosphere amino acid homeostasis is associated with impaired rhizobacteria-mediated plant growth

3.1 BACKGROUND

Plant exudates broadly shape plant-microbe interactions belowground[52,119–124]. In the Arabidopsis root exudate, amino acids are among the most represented metabolites[48,49,125]. Some amino acids (e.g., tryptophan) released into the soil by plants are used by plant growth-promoting bacteria (PGPB) for the biosynthesis of plant growth-promoting substances such as indole-3-acetic acid (IAA), boosting plant growth[106]. Further, microbial products trigger the release of amino acids from plants roots[126], suggesting that plants and microbes could communicate with each other in a two-way traffic to enhance the beneficial association. In general, plant-derived molecules communicate with microbes to enhance plant fitness. For example, legume-derived flavonoids shape *Rhizobium meliloti*-mediated root nodulation in legumes by inducing the *R. meliloti* genes that regulate the nodulation process[127]. Arabidopsis root-expressed amino acid (AA) transporters are induced when Arabidopsis is grown on various amino acids (as well as on nitrate). This indicates that amino acid regulation of root-resident AA transporter expression may be a strategy for adaptation to local root AA homeostasis[128]. The importance of root-secreted AAs as signaling molecules and as C and N sources for microbes[48,49] is demonstrated by *Pseudomonas fluorescens* Pf0-1 effectively outcompeting a mutant that is deleted for three proteins that mediate chemotaxis towards AAs, in colonizing tomato root tips[6].

Different root exudate components could be released into the soil via different mechanisms, and a limited number of plant transport proteins have been shown or suggested to regulate the export (into the surrounding medium) and import (into the root tissue) of metabolites and other compounds. Yet, it remains largely unknown which AA

transporters are important for shaping plant—microbe interactions belowground[119,59]. Here, I show that the loss of LHT1, a single amino acid transporter in Arabidopsis, is sufficient to enhance the accumulation of amino acids in root exudates, promoting biofilm formation and growth of the PGPB *Pseudomonas simiae* WCS417r (*Ps* WCS417r) on root tissues. Despite this, rather remarkably, *lht1* plants are generally insensitive to *Ps* WCS417r-mediated plant growth. Further, *in vitro* experiments show that co-treatment of wildtype roots with glutamine and *Ps* WCS417r inhibit plant growth in a dose-dependent manner. This potentially suggests that altered amino acid homeostasis in *lht1* root exudates may underlie the impaired *lht1* plant response to *Ps* WCS417r treatment, although additional experiments are required to generate a robust support for this hypothesis. Altogether, my results suggest that Arabidopsis plants utilize LHT1 to re-uptake root-secreted amino acids to minimize the overaccumulation of amino acids in the rhizosphere, potentially to ensure proper amino acid metabolism of *Ps* WCS417r cells leading to enhanced plant fitness.

3.2 RESULTS

3.2.1 Mutant Screening for Alteration in Root Exudate Amino Acid Concentrations

To explore whether the rhizosphere can be rationally engineered to ensure the accumulation of plant-derived amino acids (AA) to support the growth of plant growth-promoting bacteria, I designed a screening strategy based on the hypothesis that root-expressed amino acid transporters shown or predicted to have a role in the uptake of exogenously supplied amino acids (from solid growth media[129,128], or from amino acid

solutions[130]) may also be involved in uptaking amino acids exported into the rhizosphere by the plant itself, a phenomenon that, to my knowledge, has not been previously demonstrated.

Earlier reports detailing the characterization of root-expressed AA transporters focused on identifying transporters that mediate uptake and acquisition of organic N from the soil, as in some environments, plants may acquire or benefit from N in organic forms[131–135]. Thus, whether or not any of the root-expressed AA transporters play a major role in plant-derived N cycling—by uptaking/importing back into the plant AA already exported into the rhizosphere—remains an obvious but open question.

Plant amino acid cycling involves transportation of AA from leaves to roots via the phloem, and back from the roots to the shoots via the xylem. Of the at least 53 putative AA transporters identified in the Arabidopsis genome[136,137], few have been characterized/examined for their role in (potential) AA uptake via the root, including members of the LHT family (lysine-histidine-like transporters), AAP family (amino acid permeases) and ProT family (proline transporters).

In Arabidopsis, there are 10 LHT isoforms (AtLHT1-10), 8 AAP isoforms (AtAAP1-8), and 3 ProT isoforms[138,139]. Of these, LHT1, LHT6, AAP1, AAP5 and ProT2 have been shown/suggested to be involved in root uptake of AAs[140,141]. ProT2 is expressed in the root cortex/epidermis, and is principally involved in root uptake of proline[138,142]. AAP1, on the other hand, while expressed in root tips and epidermis, was initially thought to uptake AAs in the concentrations greater than 50 μmol/L, more than typically found in the rhizosphere[143,144], but subsequently shown to uptake glutamate and neutral amino acids

at soil-level concentrations[141]. AAP5 is expressed in the root cortex of seedlings, and takes up basic amino acids in concentrations similar to those taken up by LHT1[145]. LHT6, expressed in the epidermis and cortex of roots, but also in the root hairs, functions in acquisition of acidic amino acids, glutamine, and alanine[141].

However, LHT1 is the most well-characterized of these importers, and is known to have a broad substrate specificity, with the first report showing that LHT1 has a particularly high affinity for Lys and His (hence the name histidine-lysine-like transporter), and some affinity for Glu and Leu, as well as for Ala, Ser, and Gly[129]. Subsequent works showed that LHT1 has a high affinity for, or is necessary for, uptaking Gly, Ala, and Ser, but also Pro, Asp, His, and Gln. However, these studies could not confirm if LHT1 is necessary for Lys uptake, or if LHT1 is a high affinity transporter of Glu[130,128]. LHT1 is expressed in the root endodermis and cortex/epidermis (and hence in direct contact with the rhizosphere), and uptakes AAs in the concentrations typically found in the rhizosphere (2 – 50 µmol/L)[145].

Thus, I focused on LHT1 in my preliminary screening for root-expressed AA importers that may play a major role in recycling plant-derived N, and whose loss of function will lead to the accumulation in the rhizosphere AAs exported by the plant into the soil/growth medium, which may potentially benefit soil microbiota, including plant growth-promoting bacteria.

Loss of Arabidopsis amino acid transporter LHT1 alters amino acid homeostasis in root exudates

My root exudate screening approach for uncovering alteration in root-secreted AAs is a modification of a previously published method[111]. Briefly, this screening strategy involves growing Arabidopsis seedlings initially in full-strength MS medium with sucrose (0.5%) for 12 days, and changing the medium to half-strength MS without sucrose for 3 days, with the roots separated from the shoots by autoclaved mesh, to enable the collection of root exudates for further downstream processing (Fig. 3.1A). Using this approach, I evaluated the root exudates of wildtype and *lht1* mutant for alterations in root-secreted AA profile. The results indicate a consistent and reproducible accumulation of AAs in *lht1* root exudates, compared to wildtype root exudates (Fig. 3.1C), suggesting that the loss of a single root-expressed AA importer can significantly alter the root-secreted AA profile in Arabidopsis. This result highlights the previously undemonstrated role of root AA importers; that is, root AA importers are not only involved in uptaking AA already present in soil/growth medium, but may be actively involved in recycling AA that is exported into the rhizosphere back into the plant.

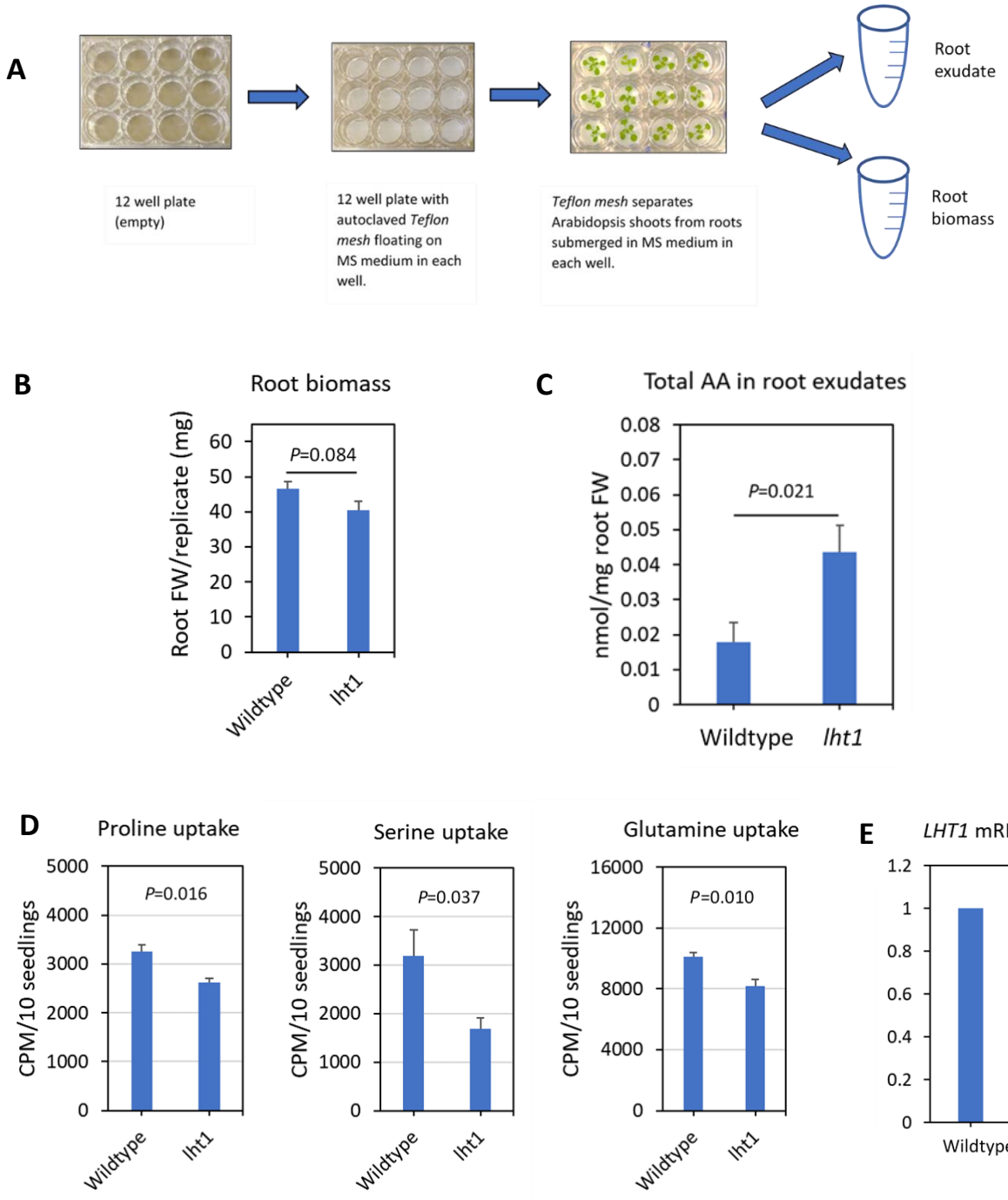


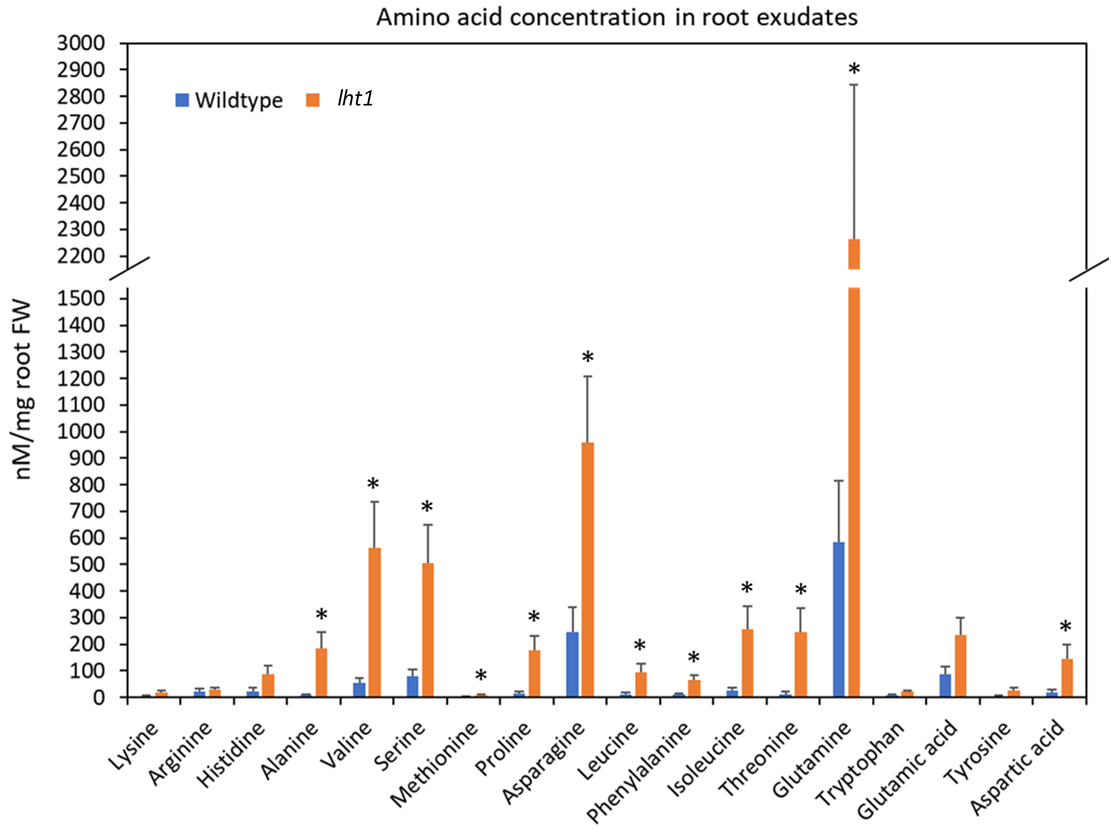
Fig. 3.1: Quantification of total amino acids in root exudates of wildtype and *lht1* seedlings and exogenous amino acid uptake from growth medium by wildtype and *lht1* seedlings. (**A**) Schematic for root exudate collection assay. (**B**) Root biomass of wildtype and *lht1* seedlings. (**C**) Total amino acids in root exudates of wildtype and *lht1* seedlings. Total amino acids were quantified using the L-Amino Acid Quantitation Colorimetric/Fluorometric Kit (BioVision, Catalog #K639-100) following manufacturer's instructions. Root exudates were filter-sterilized through a 0.22 µm-filter before use. Samples were composed of root biomass or root exudates collected from 5 seedlings per well pooled from 12 wells (approximately 60 seedlings) for n=1. Data are averages ± SE [n=6 (1n~60 seedlings)]. Two-sided Student's

t-test was used for pairwise comparison of means. Experiment described in (A), (B), and (C) was performed three times with similar results. (**D**) Uptake of ^{14}C-labelled Proline, Serine and Glutamine from growth medium by wildtype and *lht1* seedlings confirming the previously-reported amino acid import function of LHT1. Data were analyzed via Two-sided Student's *t*-test. *n*=4 for wildtype and *lht1* samples for Serine and Glutamine uptake assays; *n*=4 for wildtype samples and *n*=3 for *lht1* samples for Proline uptake assay. (**E**) *LHT1* mRNA accumulation in 2.5-week-old Arabidopsis wildtype and *lht1* mutant root tissues. *LHT1* mRNA levels were determined by RT-qPCR and normalized by *ACTIN2* mRNA levels. mRNA levels in *lht1* mutants are expressed relative to the levels in the wildtype, with the wildtype mean mRNA level being set to unity (i.e., 1). Significant difference between the means ($P<0.05$) was determined via a Welch's *t*-test. *** indicates $P<0.001$. There were 4 biological replicates for both groups, each replicate consisting of root samples pooled from approximately 12 seedlings. Primer information is located in Supplementary Table S3.1.

Concentration of several amino acids are increased in *lht1* root exudates

While the data on the root-secreted AAs from *lht1* plants clearly show enhanced accumulation of AAs in *lht1* root exudates, it was unclear what the exact signatures of this altered AA profile are. Thus, to examine the exact nature of the altered root-secreted AA profile of *lht1* plants, I subjected filter-sterilized (0.22 µm) and 10x concentrated root exudates from both wildtype and *lht1* to further analysis via LC-MS. The results indicate that several amino acids are over-represented in *lht1* root exudates, compared to wildtype root exudates ($p<0.05$; Fig. 3.2). In particular, glutamine, previously shown to be one of the AAs that LHT1 uptakes with high affinity, accumulates substantially in the *lht1* root exudates (88359.67 nM), along with alanine (7267.353 nM), valine (22295.19 nM), serine (19791.19 nM), methionine (333.7057 nM), proline (7030.316 nM), asparagine (37648.41 nM), leucine (3695.29 nM), phenylalanine (2598.73 nM), isoleucine (10194.54 nM), threonine (9861.754 nM), and aspartic acid (5753.898 nM).

A


Amino acid concentration in root exudates
Wildtype
lht1
nM/mg root FW
3000
2900
2800
2700
2600
2500
2400
2300
2200
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Lysine
Arginine
Histidine
Alanine
Valine
Serine
Methionine
Proline
Asparagine
Leucine
Phenylalanine
Isoleucine
Threonine
Glutamine
Tryptophan
Glutamic acid
Tyrosine
Aspartic acid

B

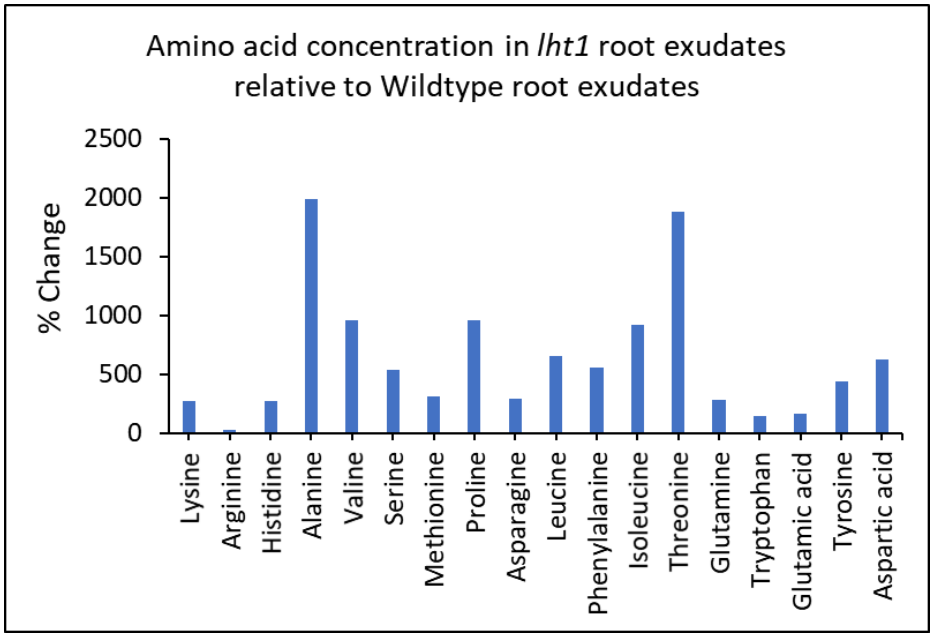

Amino acid concentration in lht1 root exudates
relative to Wildtype root exudates
% Change
2500
2000
1500
1000
500
0
Lysine
Arginine
Histidine
Alanine
Valine
Serine
Methionine
Proline
Asparagine
Leucine
Phenylalanine
Isoleucine
Threonine
Glutamine
Tryptophan
Glutamic acid
Tyrosine
Aspartic acid

Fig. 3.2: Separation of individual amino acids in wildtype and *lht1* root exudates via LC-MS. (A) Amino acid concentrations were determined using root exudates (as well as unplanted MS medium as vehicle for collecting root exudates) collected following 3 days of exudation, filter-sterilized through a 0.22 µm-filter, lyophilized and concentrated 10x before LC-MS analysis. Data are averages ± SE [*n*=6]. Two-sided Student's *t*-test was used for pairwise comparison of means. Samples were pooled from two independent experiments, consisting of root biomass or root exudates collected from approximately 5 seedlings per well pooled from 12 wells (approximately 60 seedlings) for 1*n*; *n*=3 for experiment 1, and root biomass or root exudates collected from approximately 5 seedlings per well pooled from 9 wells (approximately 45 seedlings) for 1*n*; *n*=3 for experiment 2. Bars with asterisk (*) are amino acid concentrations in *lht1* root exudates significantly different from wildtype concentrations ($p<0.05$). **(B)** Percent change in amino acid concentration in *lht1* root exudates relative to wildtype root exudates, computed from the data presented in (A). Samples were vacuum-dried and reconstituted in 100 µL of buffer containing 0.1% formic acid (FA), and subsequently analyzed on Thermo Exploris 480 Mass spectrometer using ZipChip Interface. Standard curves of 20 amino acids were generated to obtain absolute quantification of the concentration of amino acids. Glycine data was omitted from final analysis as MS medium (Cat#M519, *Phyto*Technology Laboratories, LLC) for collecting root exudates contained high background amounts of glycine (2 mg/L). For data analysis, raw data files for both the standard amino acids and root exudate and unplanted samples were uploaded into Thermo XCalibur software (https://www.thermofisher.com/order/catalog/product/OPTON-30965#/OPTON-30965), and targeted peak detection done using ICIS peak integration algorithm. Thermo Quantitative analysis software (Quan Browser) was then used to generate calibration curves, followed by the determination of the concentration of the amino acids in the root exudate and unplanted samples. The LC-MS analysis was performed at a third-party facility.

Loss of other AA importers does not lead to the accumulation of amino acids in root exudates

Because the accumulation of AAs in the *lht1* root exudates may be a general effect that may also be observed for the loss of function of other amino acid importer genes in the root, I sought to test whether similar levels of amino acids would accumulate in the root exudate of other root-expressed AA importer mutants. To this end, of the other root-expressed AA importers with a role in root uptake of AAs[140,141] (i.e., LHT6, AAP1, AAP5 and ProT2), I subjected only *AAP5* and *ProT2* mutants to additional root exudate screening rounds as I could not successfully retrieve *LHT6* and *AAP1* homozygous mutants. These analyses show that amino acids do not typically accumulate in root exudates of *aap5* and *ProT2* plants (Fig. 3.4; $P>0.05$). Thus, these data suggest that although other root-expressed AA importers execute the uptake of amino acids from the

external environment, root-expressed LHT1 in particular is principally responsible for the re-uptake of plant-derived amino acids exported into the rhizosphere. In other words, LHT1 executes the uptake of both plant-derived AAs and AAs from other sources from the rhizosphere.

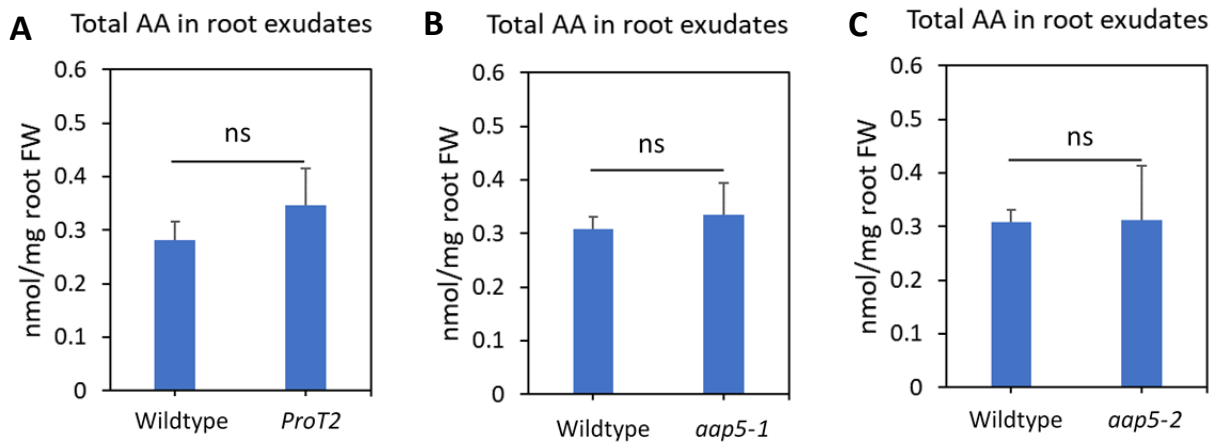


Fig. 3.4: Amino acid concentrations are unchanged in root exudates of (A) *ProT2,* (B) *aap5-1* and (C) *aap5-2* mutants, compared to the amino acid concentrations in wildtype root exudates. Total amino acids were quantified using the L-Amino Acid Quantitation Colorimetric/Fluorometric Kit (BioVision, Catalog #K639-100) following manufacturer's instructions. Root exudates were filter-sterilized through a 0.22 µm-filter before use. All samples were composed of root biomass or root exudates collected from 5 seedlings per well pooled from 6 wells (approximately 30 seedlings) for n = 1. For all experiments, data are averages ± SE (n=6), except for *ProT2* samples where n=5. Two-sided Student's t-test was used for pairwise comparison of means, and 'ns' on top of bars indicates no significant difference (P>0.05). Experiment with the *ProT2* line (along with the wildtype control) was performed twice, yielding similar results, whereas experiments with the two independent *aap5* lines (*aap5-1* and *aap5-2*) were performed once each, confirming the lack of enhanced AA accumulation in the mutant root exudates when compared to the wildtype root exudate.

3.2.2 *lht1* root exudates enhance *Ps* WCS417r growth

To determine whether the altered AA profile for root exudates from *lht1* plants can support the growth of the plant growth-promoting bacteria *Ps* WCS417r, I examined the growth of *Ps* WCS417r in wildtype versus *lht1* root exudates, as well as in the unplanted growth medium (half-strength MS without sucrose). Growth was measured over 24 h with intermittent shaking, in the microplate reader SpectraMax® i3x (Molecular Devices). The data show that *lht1* root exudates support bacteria growth better than do wildtype root exudates (Fig. 3.5). Importantly, the differential *Ps* WCS417r growth in these root exudates is not explained by differences in root biomass (Fig. 3.1B). To determine whether the differences in growth in the root exudates are statistically significant, I used the 'Compare Groups of Growth Curves' method as previously described[146] (See Figure legend for further description). These analyses reveal that the *Ps* WCS417r growth differentials between the two genotypes is statistically significant ($P<0.05$), and notably, that MS medium (without sucrose) does not support *Ps* WCS417r growth. Thus, the difference in *Ps* WCS417r growth in the wildtype and *lht1* root exudates is likely due to the difference in the amino acid content of the root exudates. However, the extent to which *lht1* root exudates support *Ps* WCS417r growth is still modest, when compared to rich medium support for *Ps* WCS417r growth, relative to *Ps* WCS417r growth in MS medium (Fig. 3.5B). Considering that the rich medium KB is composed mainly of peptone which is a nitrogen source, the modest support for *Ps* WCS417r growth in *lht1* root exudates reflects its rather modest AA levels (when compared to wildtype root exudates), as well as the presence of some specific AAs and other metabolites that may not efficiently support *Ps* WCS417r growth.

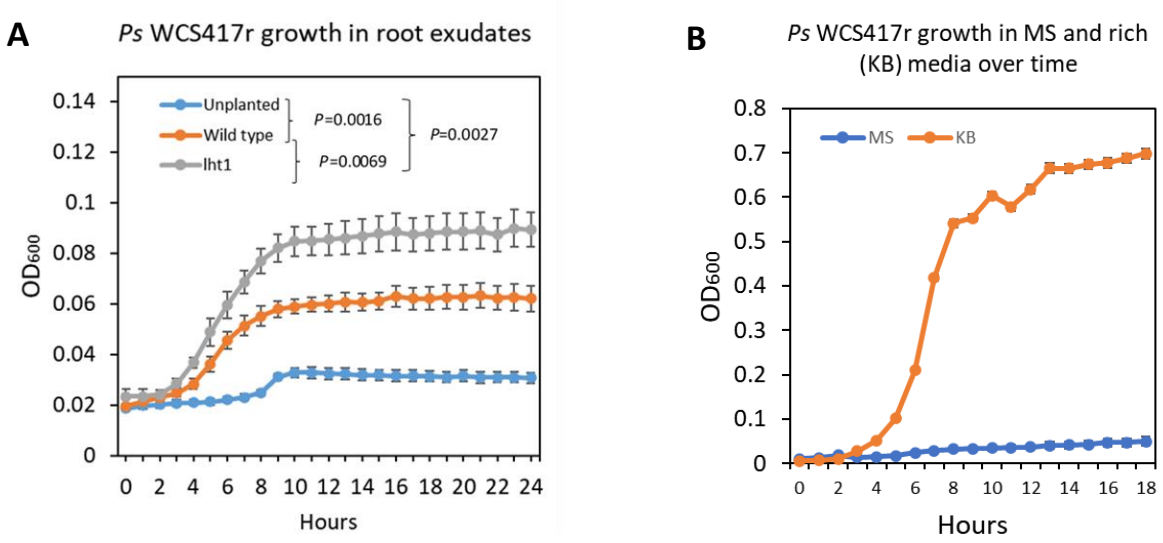


Fig. 3.5: (A) *Ps* WCS417r growth in root exudates. (B) *Ps* WCS417r growth in MS and rich (i.e., King's B or KB) media. For data in (A), *Ps* WCS417r cells from overnight culture were washed three times in sterile water and resuspended in sterile water to OD_{600}=0.2. Ten (10) µL was added to 90 µL of the unplanted growth medium (unplanted) or to 90 µL wildtype root exudates or 90 µL *lht1* root exudates, to a final OD_{600}=0.02. The test samples were filter-sterilized through a 0.22 µm-filter before use. Growth was measured over 24 h, with intermittent shaking, in the microplate reader SpectraMax® i3x (Molecular Devices). Data are average ± SE (*n*=6 biological replicates). Statistical analysis was performed using 'Compare Groups of Growth Curves' method as previously described[146]. In brief, a statistical permutation test was used to compare pairs of samples (i.e., unplanted vs. wildtype, OR unplanted vs. *lht1*, OR wildtype vs. *lht1*) over the course of growth (24 hours). The test statistic (mean *t*) is the two-sample *t*-statistic to compare the OD_{600} values between the two groups at each hour, averaged over the course of growth (24 hours). A P-value was obtained for the test statistic by simulation. Experiment was performed three times with similar results. For data in (B), *Ps* WCS417r cells from overnight culture were washed three times in sterile water and resuspended in sterile water to OD_{600}=0.2. Ten (10) µL was added to 90 µL of 0.5x MS medium (same as unplanted in (A)) or to 90 µL King's B (or KB) medium, to a final OD_{600}=0.02. Non-inoculated MS and KB were included as blank for background control. Data are average ± SE (*n*=36 technical replicates).

3.2.3 *Ps* WCS417r grows more robustly on *lht1* roots without activating local root immune responses

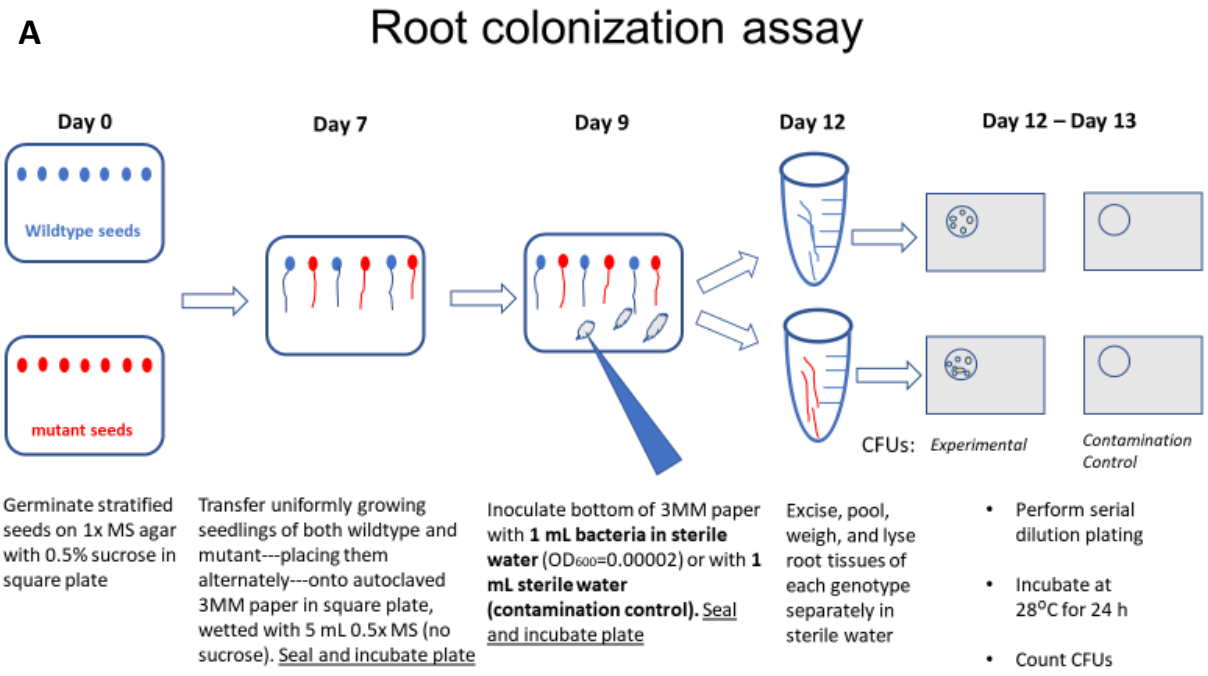
To extend the results from the growth-enhancing effects of the *lht1* root exudates on *Ps* WCS417r, I examined the extent to which *Ps* WCS417r directly grows on *lht1* roots compared to wildtype roots. To this end, I designed a root colonization assay in which both wildtype and *lht1* seedlings are exposed to a defined dose of *Ps* WCS417r in the same system, allowing the two genotypes to 'compete for' and support the growth of *Ps* WCS417r on their roots (Fig. 3.6A). These root colonization assays reveal that *Ps* WCS417r cells preferentially grow on *lht1* roots, consistent with the growth phenotypes obtained for root exudates (Fig. 3.6B).

Soil microbes that colonize plant roots can trigger the induction of plant immune responses, leading to the exclusion of some harmful microbes, but not PGPB, from colonizing roots. It has previously been shown that *Ps* WCS417r suppresses the activation of local immune responses[26], through gluconic-acid mediated lowering of environmental pH; the activation of Microbe-Associated Molecular Pattern (MAMP)-triggered defense signaling occurs at alkaline pH, and gluconic acid production by *Ps* WCS417r enables the bacteria to interfere with the induction of the root immune responses, facilitating *Ps* WCS417r colonization of the root[25]. Although it is unclear whether the growth of PGPB in large amounts on roots can perturb the host immune response, it has been shown that pathogen load may be a determinant of plant immune response activation. Immune activation in plants following exposure to MAMP/DAMP (i.e., Microbe-Associated Molecular Patterns/Danger-Associated Molecular Patterns) is dose-dependent[27,147], suggesting that plants may possess the capacity to monitor microbial

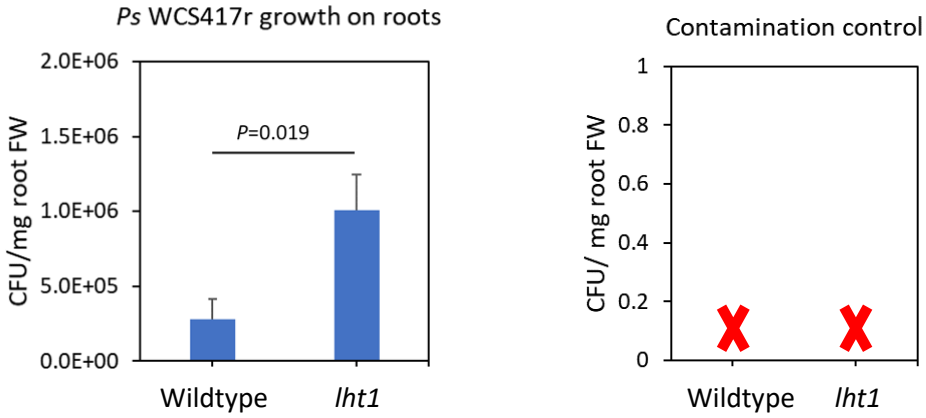
load and to limit growth to levels that ensure the required balance between plant growth and defense is maintained[148].

To address the question as to whether the enhanced accumulation of *Ps* WCS417r on *lht1* roots may activate MAMP-triggered immune responses in roots, I examined the induction of MAMP-Triggered Immunity (MTI) marker gene expression in wildtype and *lht1* roots exposed to *Ps* WCS417r for 24 h. I profiled the defense markers *PR1*, *MYB51*, and *WRKY29* in RT-qPCR assays. *PR1*, a defense response marker, is typically activated in response to pathogen exposure and is a marker for systemic acquired resistance (SAR). MYB51 is a transcription factor essential for the regulation of indole-glucosinolate biosynthesis[149]. WRKY29 is a transcription factor that regulates the expression of defense genes in innate immune response of plants and acts downstream of the flagellin receptor FLS2, a leucine-rich-repeat (LRR) receptor kinase[150]. These analyses show that *Ps* WCS417r cells, under conditions where they are expected to grow more robustly on *lht1* roots, are capable of suppressing local root immune responses to the same extent as they would while growing on wildtype roots (Fig. 3.6C). Under my experimental conditions, I did not detect *PR1* expression in roots, consistent with a previous report[111].

A


Root colonization assay
Day 0
Day 7
Day 9
Day 12
Day 12 – Day 13
Wildtype seeds
mutant seeds
CFUs:
Experimental
Contamination Control
Germinate stratified seeds on 1x MS agar with 0.5% sucrose in square plate
Transfer uniformly growing seedlings of both wildtype and mutant---placing them alternately---onto autoclaved 3MM paper in square plate, wetted with 5 mL 0.5x MS (no sucrose). Seal and incubate plate
Inoculate bottom of 3MM paper with 1 mL bacteria in sterile water (OD600=0.00002) or with 1 mL sterile water (contamination control). Seal and incubate plate
Excise, pool, weigh, and lyse root tissues of each genotype separately in sterile water
Perform serial dilution plating
Incubate at 28°C for 24 h
Count CFUs

B


Ps WCS417r growth on roots
CFU/mg root FW
2.0E+06
1.5E+06
1.0E+06
5.0E+05
0.0E+00
P=0.019
Wildtype
lht1
Contamination control
CFU/ mg root FW
1
0.8
0.6
0.4
0.2
0
Wildtype
lht1

C

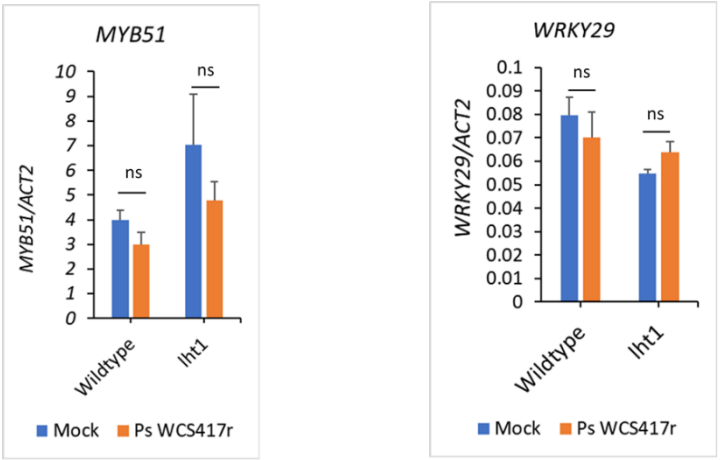

MYB51
MYB51/ACT2
ns
ns
Wildtype
lht1
Mock
Ps WCS417r
WRKY29
WRKY29/ACT2
ns
ns
Wildtype
lht1
Mock
Ps WCS417r

Fig. 3.6: *Ps* WCS417r root colonization profiles of wildtype and the *lht1* mutant, and immune response activation in roots. (**A**) Schematic for root colonization assay used to evaluate the extent of wildtype and *lht1* root colonization by *Ps* WCS417r (**B**) Extent of wildtype and *lht1* root colonization by *Ps* WCS417r cells. For each genotype, roots were removed from 3 seedlings and pooled into 1 for *n*=1. The experiment was composed of *n*=8 for the experimental group (Left), and *n*=3 for the contamination control group (Right). 'X' as indicated in the results for the contamination control experiment indicates no bacteria growth, suggesting that root colonization profiles as obtained for the experimental group are not due to assay contamination. (**C**) Gene expression analysis of immune response activation in wildtype and *lht1* roots under *Ps* WCS417r colonization. Seeds were grown on 1xMS agar with 0.5% sucrose for 10 days and seedlings transferred to 3MM paper with full-strength Hoagland, 12 seedlings per plate, for additional 7 days. Seedlings were then root-flood inoculated with 3 mL sterile water (Mock) or *Ps* WCS417r (OD_{600}=0.2) for 24 h. Root tissues were harvested and pooled (3 plates=*n*=1 biological replicate) for gene expression analysis. Data are average ± SE (*n*=3). Two-sided Student's *t*-test was used for pairwise comparison of means. Experiment was performed twice with similar results. For gene expression analysis, primer information is located in Supplementary Table S3.1.

3.2.4 Competitive chemotaxis assays reveal *lht1* root exudates do not significantly enhance *Ps* WCS417r chemotaxis to *lht1* roots

Root-secreted AAs shape root-beneficial microbe associations in a number of ways. AA perception by bacterial chemoreceptors promote chemotaxis towards these compounds, and hence towards plant roots that exude AAs, enabling root colonization. For example, *Bacillus subtilis* encodes several chemoreceptors whose ligands include many AAs. The perception of plant-secreted compounds, including AAs, thus facilitates root colonization by *B. subtilis*[51]. Upon root colonization, *B. subtilis* confers growth-enhancing effects to the plant.

Chemotaxis has been well-demonstrated in *Pseudomonas* species too. In one study, *Pseudomonas lachrymans* chemotaxis towards water droplets collected from the leaf surface of plants was directly correlated with the concentrations of amino acids and carbohydrates in these droplets[151]. In particular, the role of root-secreted amino acids in attracting microbes, and as energy sources for microbes[48,49] is demonstrated for *Pseudomonas fluorescens* Pf0-1, which outcompetes a mutant that lacks three proteins

that mediate chemotaxis towards amino acids, and hence colonizes tomato root tips more efficiently[6]. This suggests that chemotaxis is an important determinant of root colonization.

As shown in Fig. 3.6B, *Ps* WCS417r cells grow more robustly on *lht1* roots, when both wildtype and *lht1* plants are exposed to *Ps* WCS417r cells. To examine whether the enhanced *Ps* WCS417r proliferation on *lht1* roots is explained by more efficient chemotaxis towards *lht1* roots due to the elevated AA accumulation phenotype of the *lht1* roots (Fig. 3.1C), I designed a competitive chemotaxis assay (Fig. 3.7A), based on a chemotaxis assay originally developed for *E. coli* by Adler[152] and optimized by others for studying chemotaxis in *Pseudomonas*[153–155].

Briefly, *Ps* WCS417r culture grown at 28 ºC overnight with shaking at 230 rpm is washed three times with sterile water, and resuspended in chemotaxis buffer [10 mM potassium phosphate (pH 7.2), 1 mM $MgCl_2$, and 0.1 mM EDTA] to OD_{600}=0.002. Forty (40) mL is loaded into a sterile Petri dish, and left to stand on a horizontal laminar airflow hood. Then, 200 µL of the test samples (unplanted MS, wildtype root exudates, and *lht1* root exudates) are loaded into sterile 1 mL syringes and dipped in the chemotaxis buffer + *Ps* WCS417r inside the Petri dish. The rationale is that if there is a sufficient concentration gradient difference between the test samples, the one with highest AA concentration would attract the greatest number of *Ps* WCS417r cells over time.

The results from this analysis reveal no significant differences between the number of *Ps* WCS417r cells drawn to wildtype root exudates and *lht1* root exudates (Fig. 3.7B, 3.8C). This indicates that the enhanced proliferation of *Ps* WCS417r on *lht1* roots (Fig. 3.6B) is not necessarily due to more efficient chemotaxis towards *lht1* roots, compared to wildtype

roots. Instead, it seems likely that similar numbers of *Ps* WCS417r cells are drawn to the roots of both genotypes, with the result that the *lht1* roots are more efficient in enhancing the growth of the *Ps* WCS417r cells.

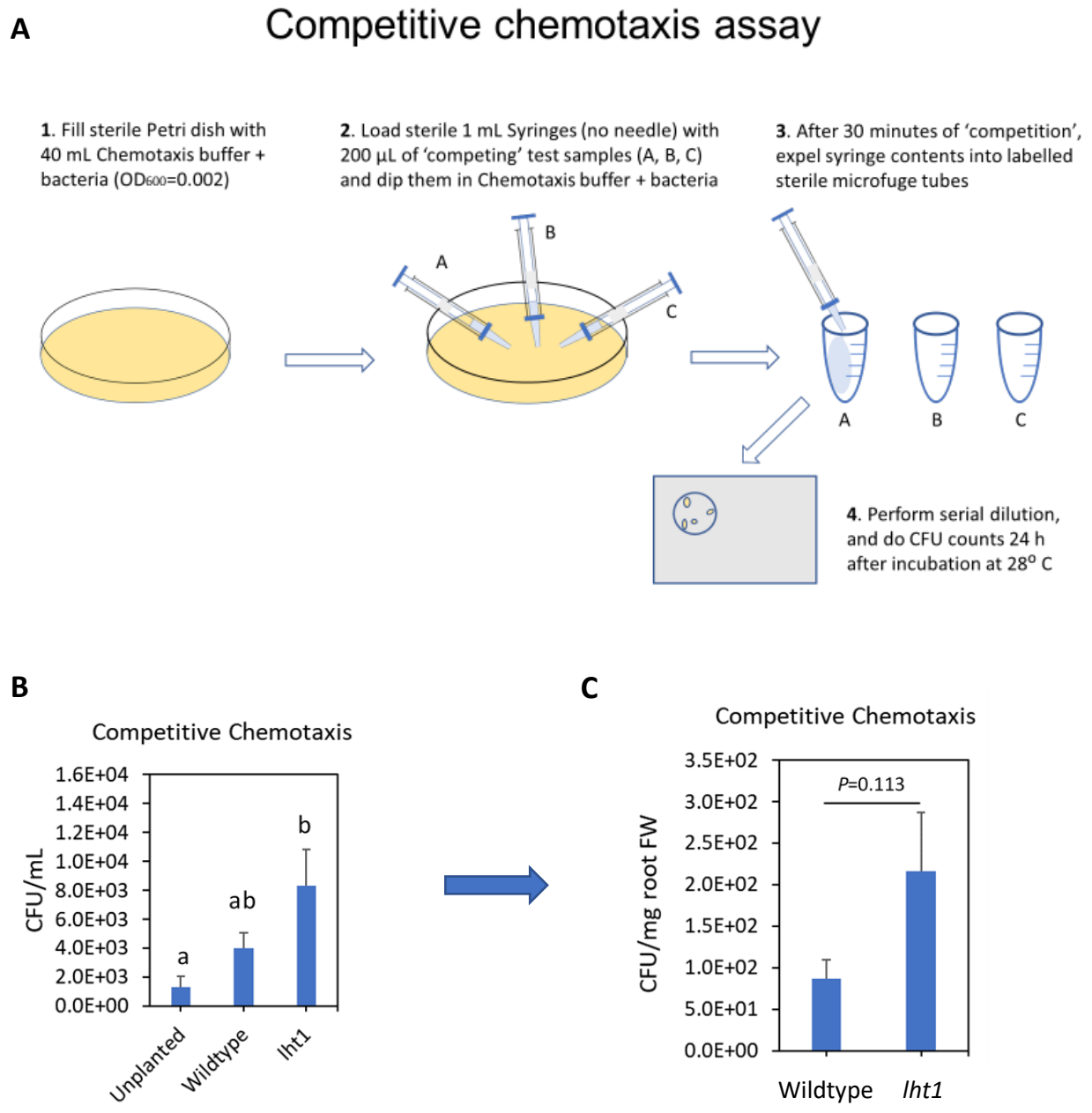


Fig. 3.7: (**A**) Schematic for competitive chemotaxis assay used to evaluate *Ps* WCS417r 'recruiting strength' of wildtype vs *lht1* root exudates. (**B**) Quantification of number of *Ps* WCS417r cells drawn to unplanted growth medium (1/2x MS without sucrose) and wildtype and *lht1* root exudates in a competitive chemotaxis assay 30 minutes after test samples (exudates and controls) were exposed to the *Ps* WCS417r cells resuspended in chemotaxis buffer to OD_{600} = 0.002. After 30 minutes of incubation, the test samples

were collected, serially diluted and plated on LB agar amended with rifampicin (50 µg/mL). CFUs were evaluated after overnight culture at 28ºC. (**C**) Data in (B) normalized by root fresh weight (FW), confirming that differences in CFU/mL numbers are not due to differences in root biomass. Data are average ± SE (*n*=6). Bars lettered differently are significantly different ($p<0.05$); One-Way ANOVA followed by Tukey's posthoc test (B). Two-sided Student's *t*-test was used for pairwise comparison of means for data presented in (C). Experiment was performed twice with similar results.

3.2.5 Biofilm formation by *Ps* WCS417r cells is modestly enhanced in *lht1* root exudates

Biofilm formation is an important trait exhibited by plant root-colonizing microbes. While a number of amino acids (tyrosine, tryptophan, leucine, and methionine) can interfere with biofilm formation in *B. subtilis* for example, even promoting biofilm disassembly, depending on the enantiomer[77], root colonization is impaired in rhizosphere-dwelling microbes carrying mutations in amino acid assimilation and catabolism genes[78,79].

To examine the mechanisms underlying the enhanced growth phenotype of the *Ps* WCS417r cells in *lht1* root exudates and root surfaces, I evaluated biofilm formation of the *Ps* WCS417r cells in wildtype and *lht1* root exudates, along with biofilm formation in unplanted controls, hypothesizing that the accumulation of amino acids in *lht1* exudates, while not sufficient to significantly enhance chemotaxis (Fig. 3.7), enables robust biofilm formation and hence the enhanced growth phenotypes of the *Ps* WCS417r cells in *lht1* root exudates relative to the growth phenotype in wildtype root exudates. I utilized the crystal violet staining assays for quantifying biofilm formation (See Materials & Methods) by *Ps* WCS417r cells in wildtype and *lht1* root exudates, along with the vehicle for root exudate collection as control.

Consistent with my hypothesis, biofilm formation by *Ps* WCS417r cells was modestly enhanced in *lht1* root exudates compared to wildtype root exudates (Fig. 3.8), suggesting that the accumulation of amino acids in *lht1* root exudates promotes biofilm formation. Considering that it is currently unknown whether wildtype and *lht1* root exudates are substantially similar in all other respect except their amino acid concentration levels, any biofilm formation-enhancing effects of *lht1* root exudates may not be solely due to its elevated amino acid concentration. Further experiments are required to evaluate this possibility.

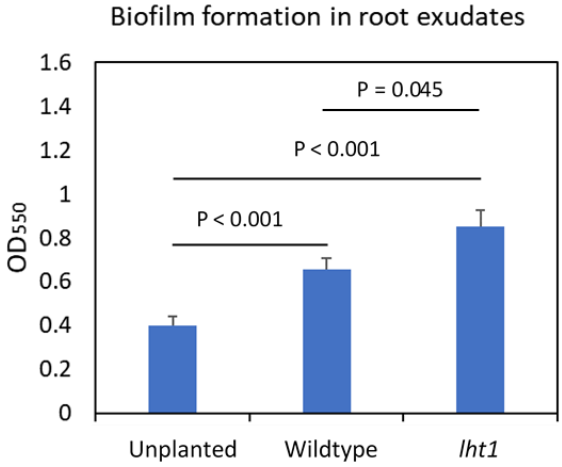


Fig. 3.8: Biofilm formation in wildtype and *lht1* root exudates, compared to the unplanted control. Data are average ± SE (*n*=30). Data were pooled from three independent experiments. Kruskal-Wallis Test was used to assess if the means are significantly different for these groups, followed by Dunn's post hoc test for separating the means (P<0.05).

3.2.6 Expression profiles of *Ps* WCS417r amino acid metabolism genes during WCS417r growth on root tissues

In some microbes such as *Staphylococcus aureus*, limitations to carbohydrate availability results in a metabolic shift towards the catabolism of peptides or amino acids to enhance growth[156]. Subsequently, this shift to amino acid catabolism generates key metabolites such as pyruvate, oxaloacetate, and 2-oxoglutarate that are central to their metabolism[157]. Considering that amino acid concentrations are altered in *lht1* root exudates, I reasoned that the overall amino acid metabolism of *Ps* WCS417r cells growing in association with *lht1* roots or in root exudates will be altered to reflect the abundance of these amino acids, relative to their concentration in wildtype root exudates. To test this, I examined the expression of selected *Ps* WCS417r AA metabolism genes during *Ps* WCS417r growth on wildtype and *lht1* roots.

ProA and *SerA* are core AA metabolism genes in *Ps* WCS417r regulating glutamate metabolism/proline biosynthesis and serine metabolism respectively in several bacterial species, and are present in the *Ps* WCS417r genome too[158]. Of note, both proline and serine concentrations are significantly higher in *lht1* root exudates than in wildtype root exudates (Fig. 3.2). To test the expression of the *ProA* and *SerA* genes in *Ps* WCS417r cells growing on *lht1* roots relative to their expression in cells growing on wildtype roots, 13-day old Arabidopsis seedlings grown on 1x MS agar plates with 0.5% sucrose were transferred to autoclaved 3MM paper wetted with 1/2x MS with no sucrose for 1 day, and then root-inoculated with 2 mL *Ps* WCS417r cells OD_{600}=0.2 for 72 hours. Root tissues were subsequently harvested and RNA extracted for *Ps* WCS417r gene expression analysis. Consistent with the expectation that *Ps* WCS417r cells will have altered AA

metabolism in response to their growth on *lht1* roots, the expression of *ProA* and *SerA* are mildly induced on *lht1* roots relative to their expression on wildtype roots (Fig. 3.9).

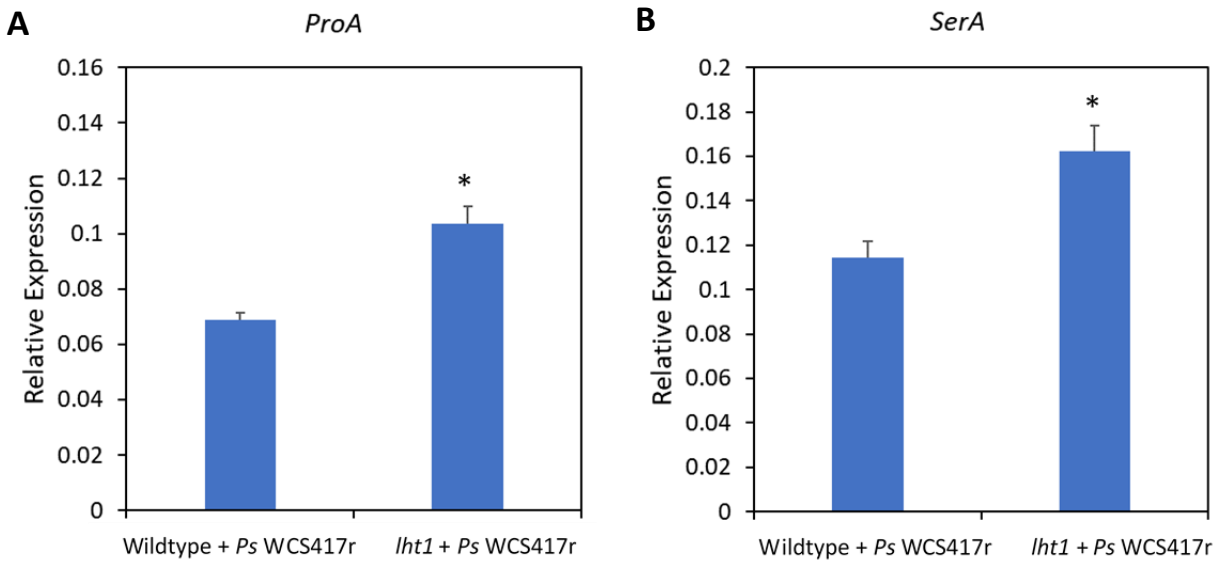


Fig. 3.9: **Induction of amino acid metabolism genes *ProA* (A) and *SerA* (B) in *Ps* WCS417r cells growing on wildtype and *lht1* roots.** Root tissues flood-inoculated with *Ps* WCS417r OD_{600}=0.2 for 72 hours were pooled from 15 seedlings into *n*=1 biological replicate. There were four (4) biological replicates with two technical replicates each for RT-qPCR run. Expression levels of *ProA* and *SerA* were relative to the expression level of the housekeeping gene *rpoB*. Data are averages ± SE. Two-sided Student's *t*-test was used for pairwise comparison of means. Asterisk (*) on bars indicate significant difference between the means ($P<0.05$). Experiment was performed twice with similar results. Primer information is located in Supplementary Table S3.1.

3.2.7 *Ps* WCS417r-mediated plant biomass accumulation is attenuated in *lht1* plants

Despite the fact that *lht1* plants accumulate more AA on their roots, which enhances *Ps* WCS417r growth, I observed that in a number of experiments, *lht1* plants typically do not respond to *Ps* WCS417r treatment. That is, *Ps* WCS417r-mediated plant growth is absent in *lht1* plants when compared to wildtype plants (Fig. 3.10A, 3.10B, 3.10D).

To characterize this phenotype, I considered the possibility that the impaired shoot growth might be due to the loss of LHT1 function. A previous report suggested that *LHT1* is upregulated in wildtype plants 1 h after treatment with *Ps* WCS417r[159] (Fig. 3.11A). Also, it is known that mycorrhizal fungi, for example, induce the expression of *LHT1* in Lotus japonicus[160], suggesting that microbes modulate AA transporter expression in plant roots to enable microbe-mediated plant growth. As shown, loss of LHT1 orchestrates the accumulation of amino acids in the root exudates, some of which (i.e., the branched chain amino acids valine, leucine, and isoleucine) may downregulate IAA production in some PGPB[105] and hence impairing the ability of PGPB to promote plant growth. Thus, I wondered whether LHT1 transporter regulation may be a strategy by *Ps* WCS417r to control the rhizosphere AA concentrations in order to retain the ability to promote plant growth.

To test this hypothesis, I extended the *LHT1* gene expression analysis in wildtype roots, but also in *lht1* roots, collecting root samples for analysis 24 h after inoculation with *Ps* WCS417r. The data reveal no upregulation of *LHT1* by *Ps* WCS417r in wildtype roots (and, as expected, not in *lht1* roots) (Fig. 3.11B). This result therefore ruled out *Ps*

WCS417r modulation of LHT1 expression as a strategy for enabling *Ps* WCS417r-mediated Arabidopsis growth.

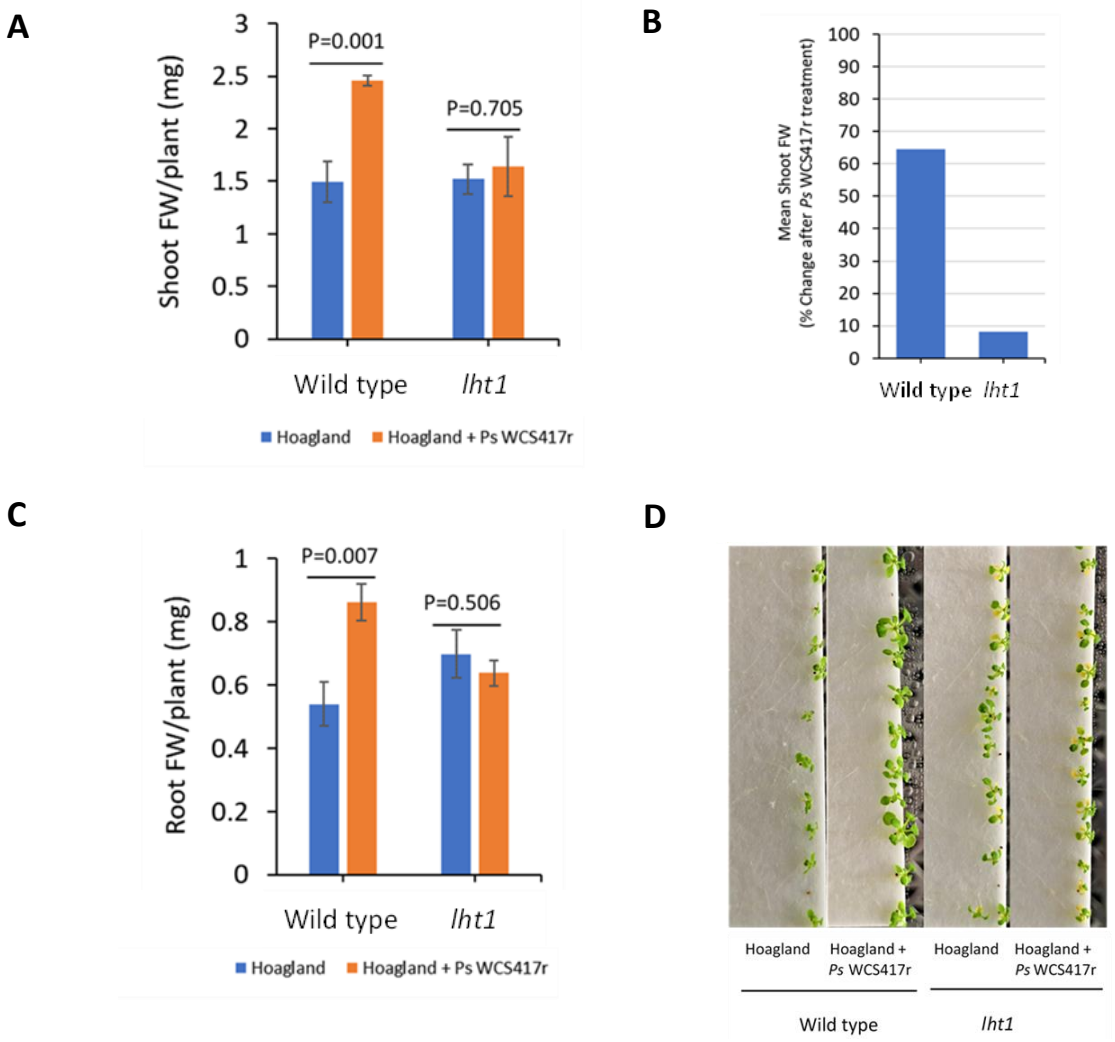


Fig. 3.10: **Arabidopsis *lht1* mutants are insensitive to plant growth-promotion signaling by *Ps* WCS417r**. (**A**) Shoot fresh weight of wildtype and *lht1* under Hoagland-only and Hoagland + *Ps* WCS417r combination treatments. (**B**) Percentage change in shoot fresh weight in response to Hoagland + *Ps* WCS417r combination for wildtype and *lht1* (**C**) Root fresh weight of wildtype and *lht1* under Hoagland-only and Hoagland + *Ps* WCS417r combination treatments. Data in (A) and (B) are averages ± SE of biomass from all germinated seedlings per plate pooled together. Approximately 15 seeds were sown per plate for *n*=5 plates. Two-sided Student's *t*-test was used for pairwise comparison of means. Hoagland = half-strength. *Ps* WCS417r OD_{600} = 0.2. (**D**) Wildtype and *lht1* seedlings under Hoagland and Hoagland + *Ps* WCS417r combination treatments. Images were collected from 16-day old seedlings in a representative experiment. Several *lht1* seedlings show chlorotic leaves characteristic of the mutant, associated with enhanced disease resistance[161]. Experiment was performed multiple times, and the impaired *lht1* response observed several times.

A

Gene Name	AGI Code	Down-/ Up-regulated by WCS417r in Arabidopsis roots? (Reference: Stringlis *et al.*, 2018; Plant J 93: 166-180)	Log2 Fold Change	p-value	FDR
EXPORTERS/ IMPORTERS/ FACILITATORS?					
UMAMIT2	AT4G19185	-			
UMAMIT4	AT3G18200	6h downregulated	-1.97542318	2.1281E-26	7.249E-24
UMAMIT5 (WAT1)	AT1G75500	6h downregulated	-1.084596269	4.9444E-14	2.523E-12
UMAMIT6	AT3G53210	-			
UMAMIT 11	AT2G40900	-			
UMAMIT 14	AT2G39510	6h upregulated	1.83913938	0.00388197	0.0158146
UMAMIT 17	AT4G08300	-			
UMAMIT 18	AT1G44800	-			
UMAMIT 20	AT4G08290	-			
UMAMIT 22	AT1G43650	6h downregulated	-1.149553753	4.746E-07	5.972E-06
UMAMIT 27	AT1G11450	3h downregulated	-1.110462405	2.14E-05	0.000466
UMAMIT 28	AT1G01070	-			
UMAMIT 29	AT4G01430	-			
UMAMIT 30	AT4G01450	-			
UMAMIT 31	AT4G01440	-			
UMAMIT 33	AT4G28040	-			
UMAMIT 34	AT4G30420	-			
UMAMIT 37	AT5G40230	-			
IMPORTERS					
LHT1	AT5G40780	1h upregulated	1.409114633	5.99998E-13	9.82E-11
AAP3	AT1G77380	3h upregulated, 6h upregulated	1.269418075 1.900018376	1.599E-06 7.1176E-11	5.5206E-05 1.993E-09
AAP6	AT5G49630	-			
CAT1	AT4G21120	6h downregulated	-1.209645853	9.5488E-06	8.639E-05
PROT2	AT3G55740	-			
PROT3	AT2G36590	-			

B

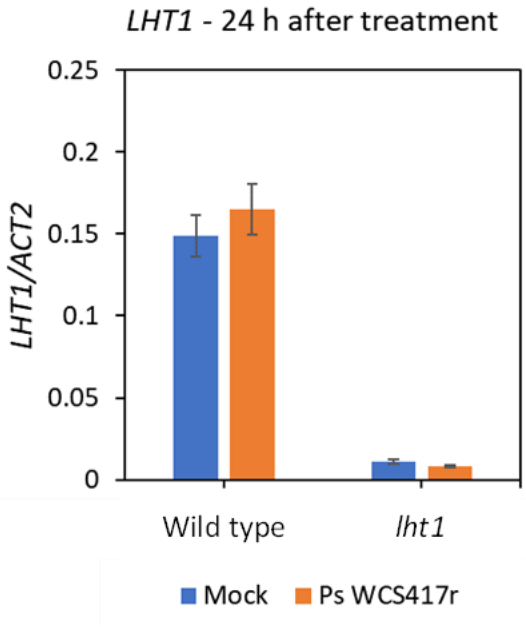


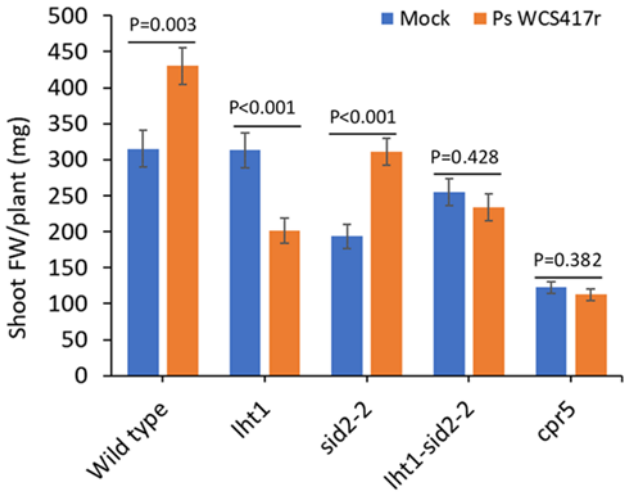
Fig. 3.11: **Transcriptional regulation of LHT1 transporter in wildtype and *lht1* mutants by *Ps* WCS417r.** (**A**) *Ps* WCS417r upregulates *LHT1* in wildtype plants 1 h after inoculation (See Red Rectangle). Table generated based on data extracted from Stringlis *et al.* 2018 (Ref[159]). (**B**) No induction of *LHT1* in response to *Ps* WCS417r treatment of wildtype and *lht1* roots 24 h after treatment. Data are averages ± SE (*n*=5). Two-sided Student's *t*-test was used for pairwise comparison of means. Primer information is located in Supplementary Table S3.1.

Next, I note that the *lht1* plants, as previously published, overaccumulate salicylic acid[161]. Salicylic acid (SA) accumulation in plants bolster plant defense against pathogens, but may also impair growth and development. According to the classic growth-defense tradeoff concept, plants with higher levels of SA, while resistant to pathogens, are also typically stunted, suggesting fitness costs are imposed by the SA-mediated constitutive defense responses that occur in the absence of stress[162]. Consequently, salicylic acid-deficient transgenic Arabidopsis *NahG* plants (carrying the salicylate hydroxylase gene *NahG*) and *sid2* mutants (mutated for the *ICS2* gene which encodes isochorismate

synthase which regulates the SA biosynthesis pathway) exhibit increased growth and have higher seed yield than wild type[163], but are highly susceptible to pathogens[164,165]. Also, a previous report has shown that SA shapes the root microbiome composition, with several bacterial families depleted in the root microbiome of Arabidopsis *cpr5* mutants which constitutively produce SA, suggesting that SA can negatively impact some bacterial families[166]. In that report, exogenous application of SA positively or negatively affected different bacteria[166]. However, a prior study that examined the response of *NahG* plants to different PGPB provided no clarity on this, as *in vitro* and *in vivo* data were inconsistent, and responses were also PGPB-dependent[167].

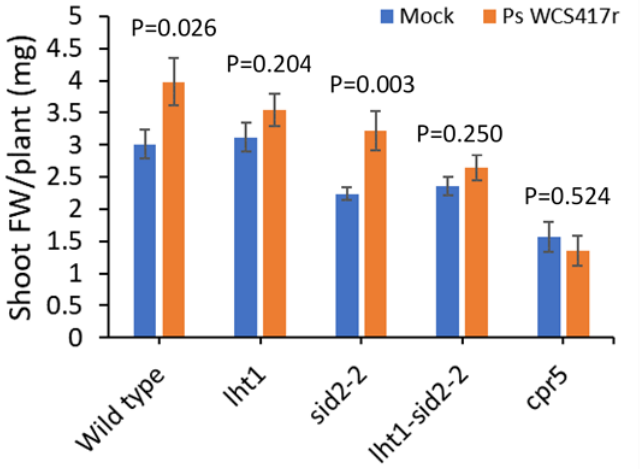
Because the *lht1* mutant overaccumulates SA, partly accounting for its higher-than-wild type-level of resistance to pathogens[161], I investigated the possibility that the impaired *lht1* response to WCS417r-mediated shoot growth is due to its relatively higher SA content. To this end, I evaluated the wild type and *lht1* responses to *Ps* WCS417r treatment, in comparison to *sid2-2*, *lht1-sid2-2*, and *cpr5* responses. Notably, *cpr5* plants did not respond to the *Ps* WCS417r treatment, confirming that SA may interfere with PGPB-mediated plant growth (Fig. 3.12). Interestingly, while the *sid2-2* plants accumulated more biomass in response to the *Ps* WCS417r treatment compared to wildtype plants, *lht1-sid2-2* plants did not respond (Fig. 3.12C). These results point to the fact that SA overaccumulation in the *lht1* plants does not explain its typically-impaired response to the *Ps* WCS417r-mediated shoot growth (Fig. 3.12).

A



Adult plants (*n* = 23—25)

B



Seedlings (*n* = 23—24)

C

% Change in biomass in response to *Ps* WCS417r treatment

	Adult plants	Seedlings
Genotype		
Wildtype	36.6	32.4
lht1	-35.7	13.9
sid2-2	60.4	44.0
lht1-sid2-2	-8.3	12.4
cpr5	-8.2	-13.5

Fig. 3.12: **Loss of *Ps* WCS417r-mediated plant growth in *lht1* mutants is independent of salicylic acid accumulation.** (**A**) **Adult plant assay:** Shoot fresh weight of wild type and mutants under mock treatment or *Ps* WCS417r treatment. (Mock treatment consists of initial inoculation of peat pellets with 5 mL tap water, and *Ps* WCS417r treatment consists of initial inoculation of peat pellets with 5 mL *Ps* WCS417r at OD_{600}=1.0. One week after sowing seeds, pellets in both Mock and *Ps* WCS417r groups were treated as follows: bottom irrigation with tap water twice weekly, and half-strength Hoagland once weekly for 30 seconds. Data are averages ± SE of fresh shoot biomass from *n*=23—25 plants. Two-sided Student's *t*-test was used for pairwise comparison of means. (**B**) **Seedling assay:** Shoot fresh weight of wild type and mutants under mock treatment or *Ps* WCS417r treatment. Seedlings were germinated on 1x MS agar plates (with 0.5% sucrose) for seven (7) days. Uniformly-growing seedlings were then transferred to

autoclaved 3MM paper wetted with filter-sterilized 5mL half-strength Hoagland (Mock) or 5mL half-strength Hoagland + *Ps* WCS417r (OD_{600} = 0.2). Plates were incubated vertically in a growth chamber for ten (10) days and processed for shoot biomass data. Data are averages ± SE of fresh shoot biomass from *n*=23—24 plants. (**C**) Percentage change in shoot fresh weight in response to *Ps* WCS417r treatment computed for the data in (A) and (B). Two-sided Student's *t*-test was used for pairwise comparison of means. Experiments were performed at least two times, with similar results.

3.2.8 Exogenous glutamine supplementation inhibits Arabidopsis growth in the presence of *Ps* WCS417r cells

Thus far, the data herein presented demonstrate that LHT1 may in fact also be involved in recycling plant-derived amino acids exported into the rhizosphere back into the plant. Hence, I hypothesized that one of the roles of LHT1 activity in root tissues is to maintain amino acid homeostasis in the rhizosphere to prevent microbial "overgrowth" and thus ensuring plant fitness. This hypothesis is in line with one of the hypotheses put forward previously to explain why high and low molecular weight compounds are actively cycled in the rhizosphere, namely that the active cycling of these compounds, among others, enables the sustenance of the plant's carbon budget and to "reduce rhizosphere microbial growth and pathogen attack."[168]

As shown in Chapter 2 of this dissertation and also reported by previous research, plant growth promotion by PGPB may be dose-dependent, with bacteria doses beyond an optimum bacteria titer damaging plant growth altogether[116] (See also Fig. 2.2). Thus, to test the hypothesis that the LHT1 function is to maintain rhizosphere AA homeostasis to prevent microbial overgrowth that may lead to damaging effects on the plant, I set up a suite of experiments. First, I designed a "rhizosphere inoculation assay" (See Materials and Methods), which enabled me to expose roots only of two-week-old Arabidopsis seedlings to *Ps* WCS417r only or *Ps* WCS417r supplemented with 5 μM, 50 μM or 500

μM of Gln, as well as to 5 μM, 50 μM or 500 μM of Gln only or mock only. All inocula were sufficiently buffered with 5 mM MES-KOH, and pH adjusted to 5.7. I chose Gln because I reasoned that since it is the most abundant AA in the phloem, and is the most depleted from the root exudate of previously published Arabidopsis root-to-medium AA transporter mutants[59], *Ps* WCS417r would have been adapted to utilize it for growth and metabolism, and thus additional Gln available in the rhizosphere resulting from the loss of LHT1 function (Fig. 3.2A) would boost *Ps* WCS417r growth. In these experiments, the seedlings under these treatments were inspected daily for any obvious phenotypic responses to *Ps* WCS417r + Gln combinations, through the next 10 days. There were no obvious treatment-induced phenotypic changes and hence no phenotypic data were collected and analyzed.

It is unclear whether amino acid concentrations in the *lht1* rhizosphere/rhizoplane could reach millimolar concentrations under the conditions where bacteria grow directly on root surfaces. However, to induce microbial overgrowth in the rhizosphere, I tested millimolar concentrations of Gln in combination with *Ps* WCS417r cells, for any potential debilitating effects on plant growth. While the 3-day root exudation data obtained for wildtype and *lht1* plants put the amino acid concentration for *lht1* root exudates conservatively at relatively high micromolar concentrations, I found evidence for continuing amino acid accumulation in *lht1* root exudates when root exudation experiments were performed for extended time [e.g., wildtype vs *lht1*: 5.2 vs 6.8; 3.3 vs 6.7; 5.9 vs 13.7 nmol/μl for 3, 6, and 9-day exudations, respectively (presented as percentage change in Fig. 3.13D)]. Surprisingly, I found that inoculating the rhizosphere with *Ps* WCS417r cells in combination with 10 mM Gln severely damaged plant roots and inhibited shoot biomass

growth, while the Gln alone or the *Ps* WCS417r at the dose tested did not cause any plant growth defects (Fig. 3.14).

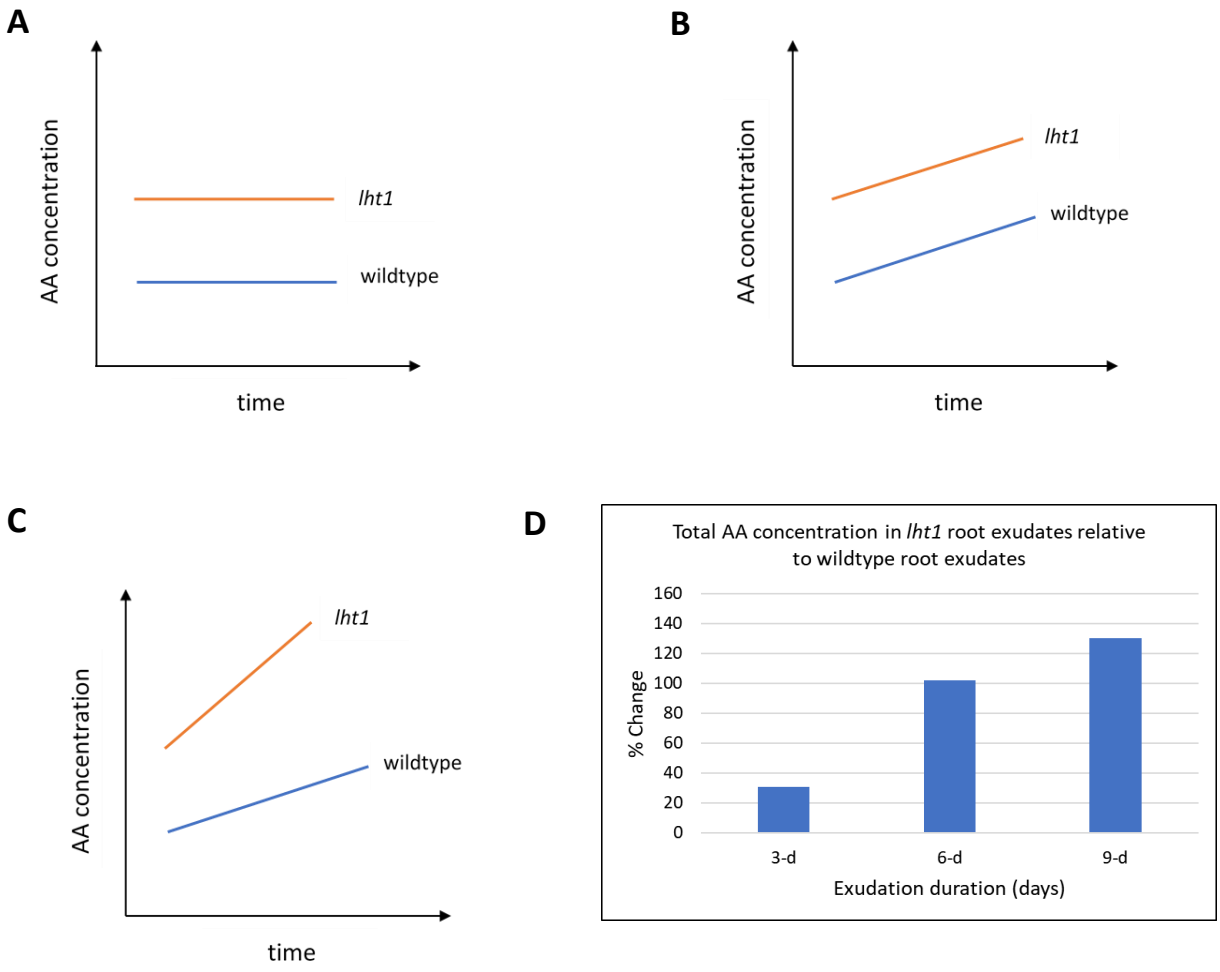


Fig. 3.13: **(A – C)**: **Predicted temporal patterns of root amino acid exudation in wildtype and *lht1* plants. (D): Amino acid accumulation in *lht1* root exudates, relative to wildtype root exudates, over an extended exudation period.** Total amino acids were quantified using the L-Amino Acid Quantitation Colorimetric/Fluorometric Kit (BioVision, Catalog #K639-100) following manufacturer's instructions. Root exudates were filter-sterilized through a 0.22 µm-filter before use. All samples were composed of root exudates collected from 5 seedlings per well pooled from 4 wells (approximately 20 seedlings) for *n*=1. Data are averages ± SE (*n*=5-6).

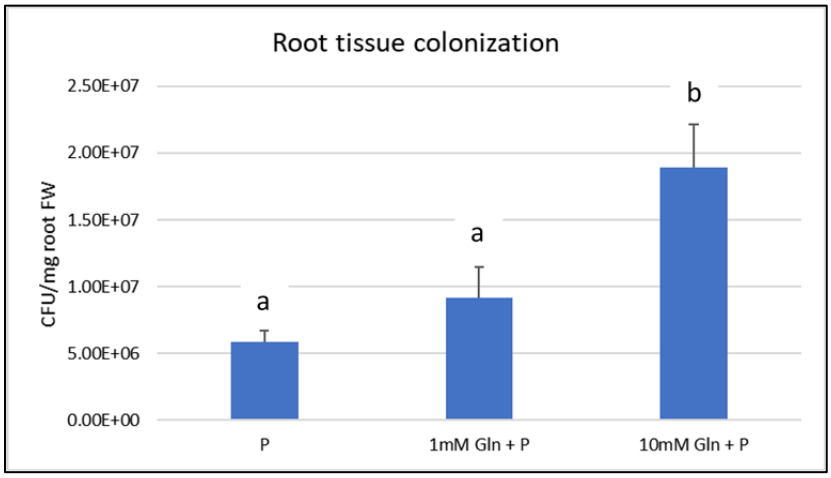
Because the above-described Arabidopsis growth defects were dependent on Gln concentration, with 1 mM Gln in combination with *Ps* WCS417r cells not causing any visible plant growth inhibition, I wondered whether the damaging effects were due to the increased bacterial numbers proliferating on the root tissues inoculated with the 10 mM Gln + *Ps* WCS417r (Fig. 3.14A).

To test this hypothesis, I tested the effect of 20 mM Ser + *Ps* WCS417r, to match the molarity of the 10 mM Gln + *Ps* WCS417r inoculation, and to compare the effect of the bacterial numbers under these two treatment conditions on Arabidopsis root and shoot growth. While 20 mM Ser alone seemed to affect root growth (but not shoot growth), unlike 10 mM Gln alone which caused no damage to roots and shoots, the 20 mM Ser + *Ps* WCS417r treatment orchestrated no further damage to root tissues or to shoot biomass, despite the 20 mM Ser + *Ps* WCS417r-treated roots carrying higher bacterial load than the 10 mM Gln + *Ps* WCS417r-inoculated roots (Fig. 3.15). This result suggests that the root and shoot growth inhibition triggered in Arabidopsis plants root-treated with 10 mM Gln + *Ps* WCS417r is unrelated to the enhanced proliferation of *Ps* WCS417r cells on the root tissues.

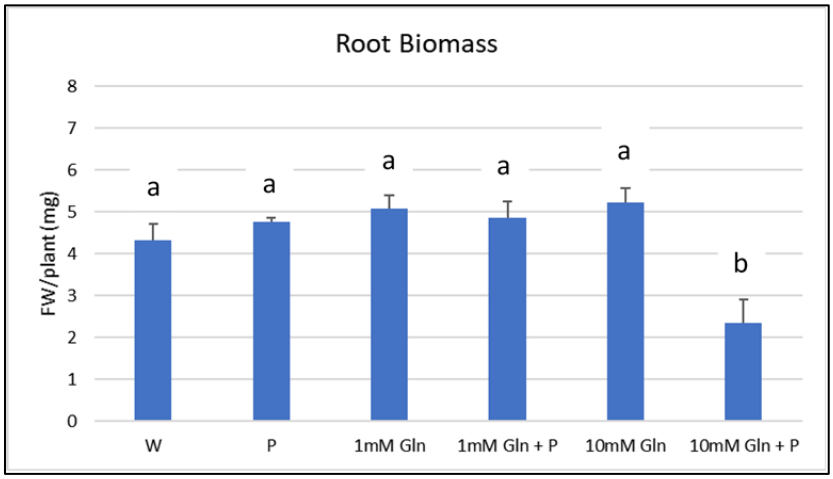
Intriguingly, the leaf pathogenic bacteria *Pseudomonas syringae* DC3000 (Pst) which is not a root pathogen or a root colonizer induced the same effect on Arabidopsis roots and shoots, despite not being able to grow on the root tissues in the presence of 10 mM Gln as compared to the *Ps* WCS417r cells under my experimental conditions. (Fig. 3.16). Although I have not tested other bacteria genus for a similar effect, these results, considered together, strongly suggest a dysregulated Gln utilization by *Pseudomonas*

species which results in damaging effects on plant growth, which effects only occur at relatively higher Gln concentrations.

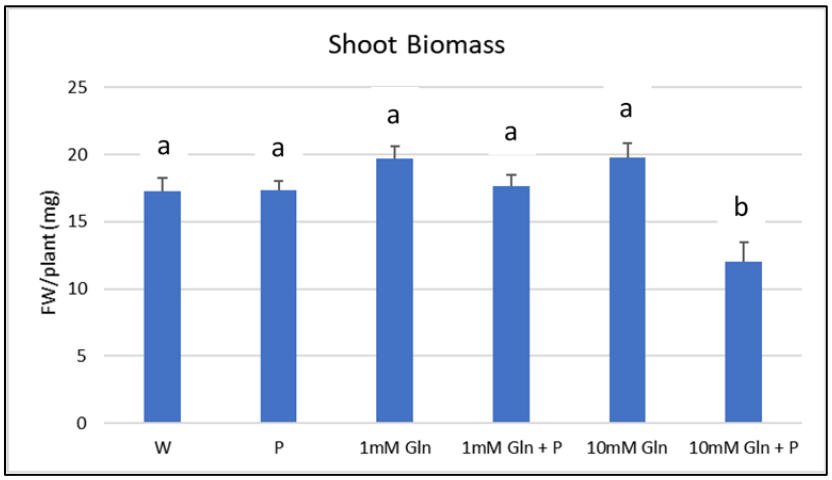
A



B



C



D

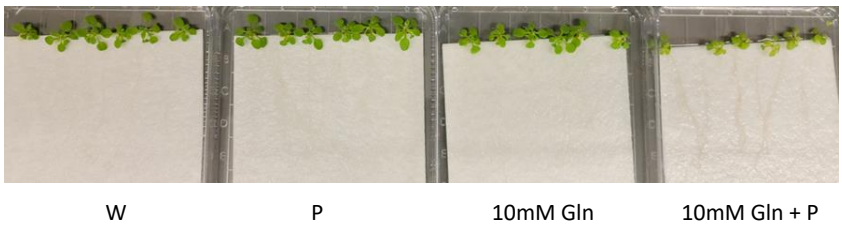


Fig. 3.14: **Glutamine-mediated *Ps* WCS417r inhibition/*Ps* WCS417r-mediated glutamine inhibition of Arabidopsis growth is glutamine dose-dependent** (**A**) Extent of Arabidopsis wildtype root colonization by *Ps* WCS417r cells only, or *Ps* WCS417r cells in combination with 1mM Gln (i.e., 1mM Gln + P) or 10mM Gln (i.e., 10mM Gln + P). Root biomass (**B**) and Shoot biomass (**C**) of Arabidopsis wildtype following root inoculation with sterile water (W), *Ps* WCS417r (P), 1mM Gln, 1mM Gln + P, 10mM Gln, or 10mM Gln + P. Seeds were grown on 1xMS agar with 0.5% sucrose for 12 days and seedlings transferred to 3MM paper wetted with 5 mL half-strength MS without sucrose, 6 uniformly-growing seedlings per plate, and plates horizontally incubated for one day to allow seedlings to stabilize. Seedlings were then root-flood inoculated with 2mL of the indicated inoculum for 72 h. *Ps* WCS417r inoculum titer was OD_{600}=0.2. All inocula were adjusted to pH 5.7 with 5mM MES-KOH. Root and shoot biomass were harvested and pooled (4 plates=*n*=1 biological replicate) for both biomass quantification and root-associated CFU counting. Data are average ± SE (*n*=4). Bars that do not share a letter are significantly different ($p<0.05$); One-Way ANOVA followed by Tukey's posthoc test. Experiment was subsequently repeated multiple times for the reproducibility of the effect seen with the 10 mM Gln + P, with identical results. A representative image is shown for these repeat experiments in panel **D**.

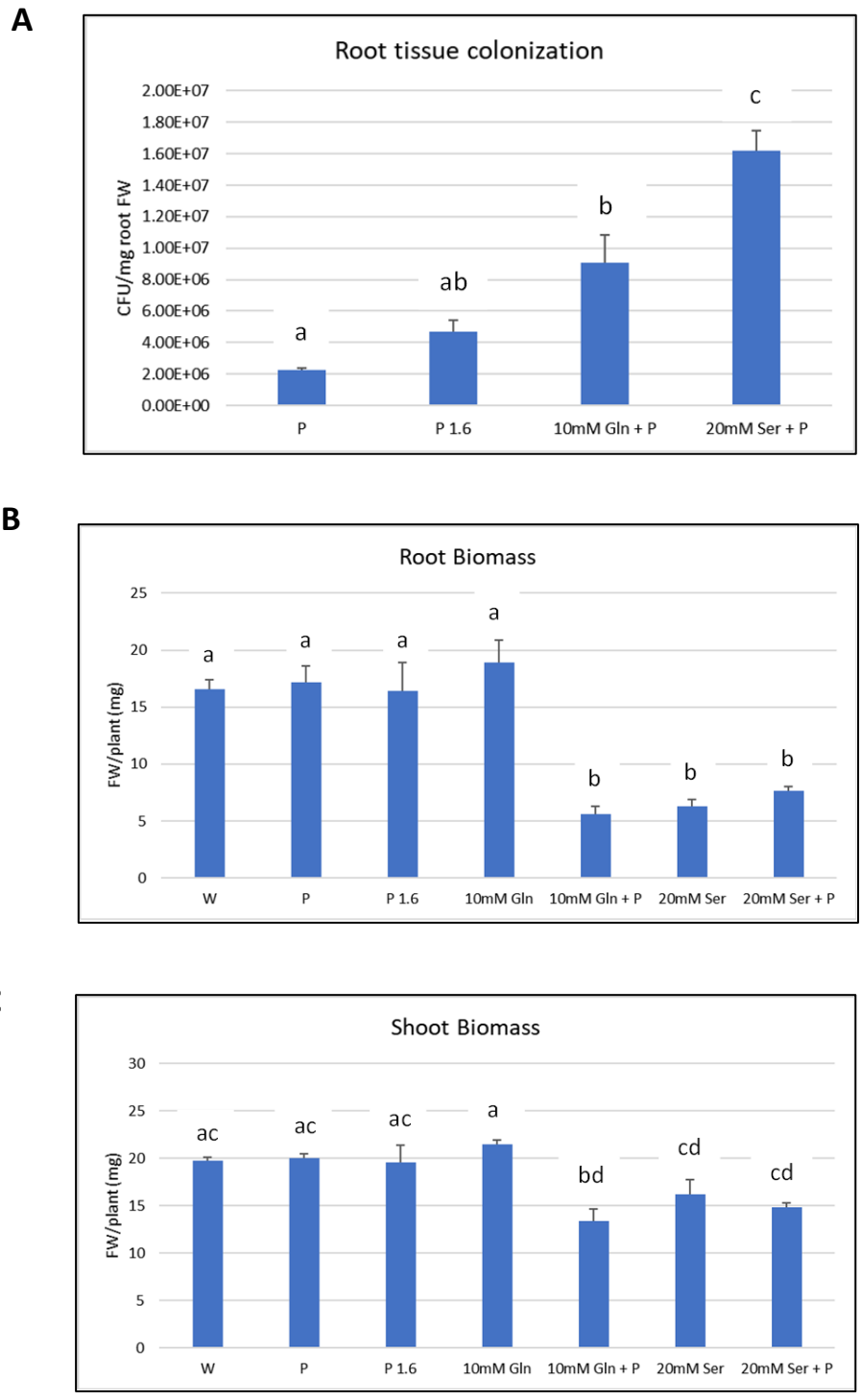


Fig. 3.15: **Glutamine-mediated *Ps* WCS417r inhibition/*Ps* WCS417r-mediated glutamine inhibition of Arabidopsis growth is unrelated to the extent of bacterial growth on root tissues** (**A**) Extent of Arabidopsis wildtype root colonization by *Ps* WCS417r cells only (P or P1.6), or *Ps* WCS417r cells in combination with 10mM Gln (i.e., 10mM G + P) or 20mM Ser (i.e., 20mM Ser + P). Root biomass (**B**) and Shoot biomass (**C**) of Arabidopsis wildtype following root inoculation with sterile water (W), *Ps* WCS417r (P), 10mM Gln, 10mM Gln + P, 20mM Ser, or 20mM Ser + P. Seeds were grown on 1xMS agar with 0.5%

sucrose for 12 days and seedlings transferred to 3MM paper wetted with 5 mL half-strength MS without sucrose, 6 uniformly-growing seedlings per plate, and plates horizontally incubated for one day to allow seedlings to stabilize. Seedlings were then root-flood inoculated with 2mL of the indicated inoculum for 72 h. For all inocula, *Ps* WCS417r inoculum titer was OD_{600}=0.2 except for P1.6 where titer was OD_{600}=1.6. All inocula were adjusted to pH 5.7 with 5mM MES-KOH. Root and shoot biomass were harvested and pooled (3 plates=*n*=1 biological replicate) for both biomass quantification and root-associated CFU counting. Data are average ± SE (*n*=3). Bars that do not share a letter are significantly different ($p<0.05$); One-Way ANOVA followed by Tukey's posthoc test for panels A and B, and Kruskal-Wallis Test, followed by Dunn's posthoc test for panel C.

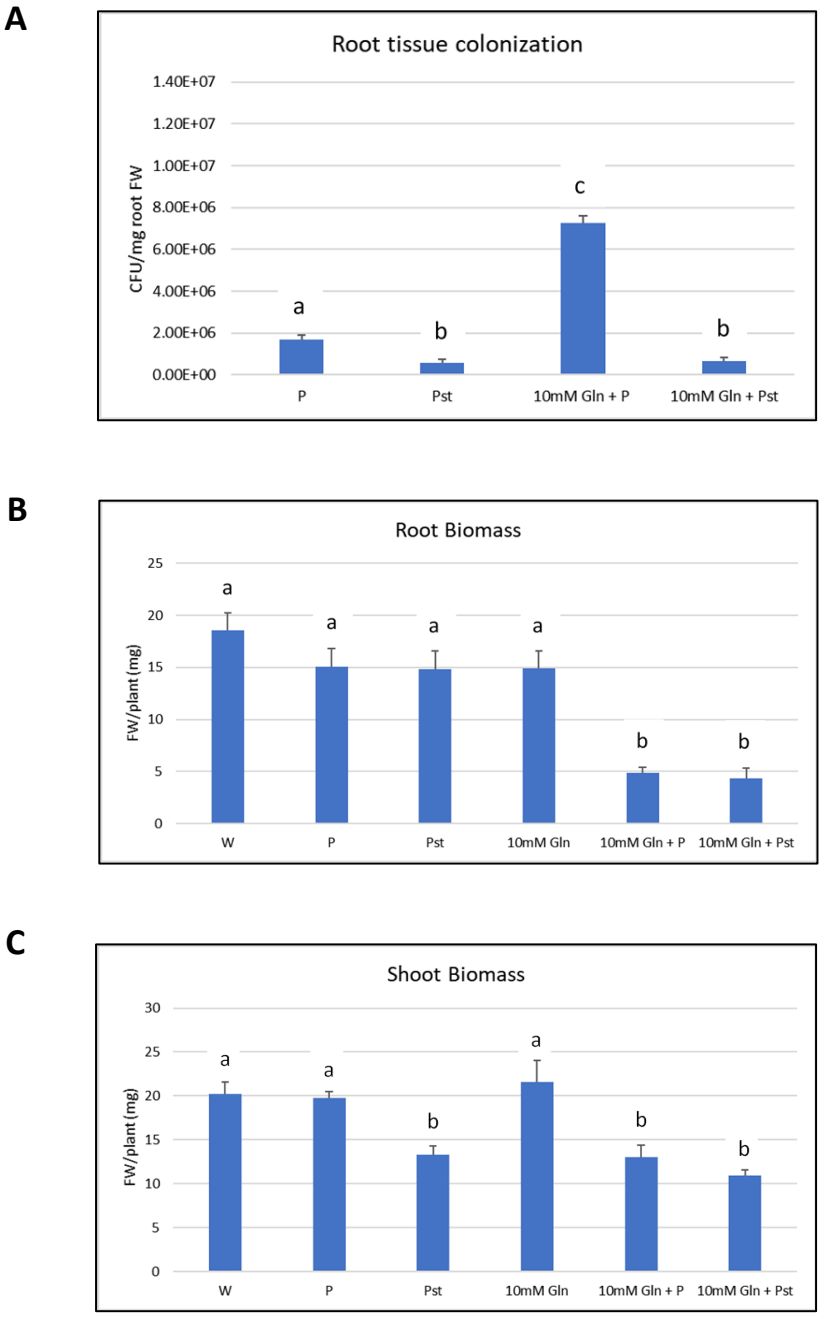


Fig. 3.16: **Glutamine-mediated bacterial inhibition/Bacterial-mediated glutamine inhibition of Arabidopsis growth occurs in the presence of both *Ps* WCS417r (P) and *Pseudomonas syringae* pv tomato DC3000 (Pst)** (**A**) Extent of Arabidopsis wildtype root colonization by *Ps* WCS417r cells only (P), or *Pseudomonas syringae* pv tomato DC3000 cells only (Pst) or P cells in combination with 10mM Gln (i.e., 10mM Gln + P) or *Pst* cells in combination with 10mM Gln (i.e., 10mM Gln + Pst). Root biomass (**B**) and Shoot biomass (**C**) of Arabidopsis wildtype following root inoculation with sterile water (W), P, 10mM Gln, 10mM Gln + P, or 10mM Gln + Pst. Seeds were grown on 1xMS agar with 0.5% sucrose for 12 days and seedlings transferred to 3MM paper wetted with 5 mL half-strength MS without sucrose, 6 uniformly-growing seedlings per plate, and plates horizontally incubated for one day to allow seedlings to stabilize. Seedlings

were then root-flood inoculated with 2mL of the indicated inoculum for 72 h. For all inocula, P or Pst inoculum titer was OD_{600}=0.2. All inocula were adjusted to pH 5.7 with 5mM MES-KOH. Root and shoot biomass were harvested and pooled (4 plates=*n*=1 biological replicate) for both biomass quantification and root-associated CFU counting. Data are average ± SE (*n*=4). Bars that do not share a letter are significantly different ($p<0.05$); One-Way ANOVA followed by Tukey's posthoc test.

As demonstrated thus far, Gln does not have to support bacteria growth to trigger bacteria-mediated root and shoot growth inhibition in Arabidopsis, as shown for Pst (Fig. 3.16). I thus tested whether ammonia, which is one of the most abundant nitrogen sources in the soil for microbial metabolism[80], can trigger the same effect in *Ps* WCS417r when supplied at the same concentration as Gln. Of note, ammonium sulfate did not boost *Ps* WCS417r growth on root tissues, compared to Gln, when both were supplied at the same concentration (i.e., 10 mM) (Supplementary Fig. S3.1A), and surprisingly, did not induce Arabidopsis root and shoot growth inhibition unlike Gln (Supplementary Fig. S3.1B & S3.1C). Because bacteria growth is not required for the Arabidopsis root and shoot growth inhibition, the observation that ammonium sulfate does not trigger a similar root and shoot growth inhibition effect in Arabidopsis in the presence of *Ps* WCS417r suggests that glutamine-specific perception by *Ps* WCS417r (and potentially other pseudomonads) at relatively high concentration of glutamine is required to trigger this plant growth inhibition effect.

3.2.9 Defense responses are transiently activated in Arabidopsis roots treated with 10 mM Gln + *Ps* WCS417r cells

To examine the potential mechanisms that may underlie the root and shoot growth inhibition following the exposure of Arabidopsis roots to 10 mM Gln + *Ps* WCS417r cells, I tested whether defense responses are activated in the root tissues following the exposure, hypothesizing that growth-defense tradeoff in which Arabidopsis would pull the

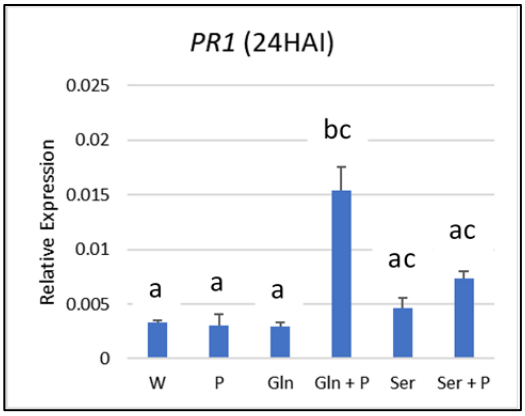
brakes on growth, so the plant diverts resources to defend itself against microbial attack, may contribute to, if not explain, the growth inhibition observed. As hypothesized, I found key immune response marker genes activated in the root tissues 24h after the exposure ($P<0.05$; Fig. 3.17A – C).

Because glutamine has been shown to induce virulence in some microbes[169], I tested the expression of Type 3 Secretion System (T3SS) genes of *Ps* WCS417r cells 24h following the treatment of Arabidopsis roots with the 10 mM Gln + *Ps* WCS417r inoculum. Among the T3SS genes present in the genome of *Ps* WCS417r are *ropE* and *rscC (i.e., rhizosphere-expressed outer protein E* and *rhizosphere-expressed secretion protein conserved C)*, which are homologs of the T3SS genes *avrE* and *hrcC* found in the pathogenic bacteria Pst DC3000[170]. This analysis shows that the expression of *ropE* and *rscC* are not significantly affected ($P>0.05$; Fig. 3.17 G & H), at least at the time-point examined (i.e., 24HAI), potentially suggesting that the possible induction of virulence in *Ps* WCS417r cells by glutamine would not explain the activation of defense responses in Arabidopsis roots as a counteracting force against *Ps* WCS417r virulence.

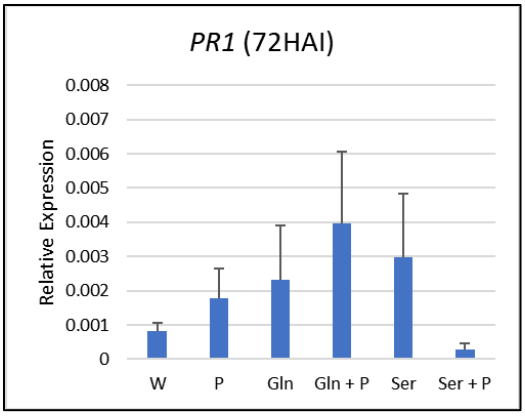
Of note, while T3SS effectors have been used by pathogenic bacteria to suppress host plant defense responses[171], these effectors may also betray the presence of the bacteria to activate host defenses, with these effectors thus acting as double agents.[172] In preliminary experiments, I tested whether the plant growth inhibition by the 10 mM Gln + *Ps* WCS417r inoculum also occurs in Arabidopsis *fls2* mutants. This is because the observed defense responses were reminiscent of MAMP-Triggered Immunity (MTI), and the receptor FLS2 which is expressed throughout the root of Arabidopsis[173] is a mediator of the canonical MTI defense responses. Interestingly, the root growth of the *fls2* mutants

is equally inhibited by the 10 mM Gln + *Ps* WCS417r inoculum ($P<0.05$; Fig. 3.17 I). This suggests that the activation of defense responses may not be the direct cause of the growth inhibition, but a mere by-product of the interaction, or that the glutamine-mediated activation of the defense response goes through another receptor other than FLS2. Importantly, considering that these defense responses are attenuated by 72HAI ($P>0.05$; Fig. Fig. 3.17 D – F), it is unlikely that the defense responses would fully explain the observed Arabidopsis growth arrest.

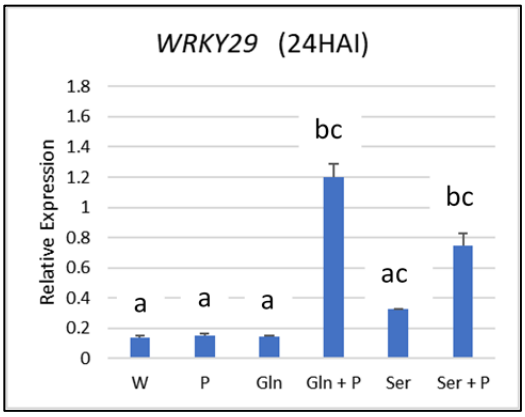
A


PR1 (24HAI)
Relative Expression
0.025
0.02
0.015
0.01
0.005
0
a
a
a
bc
ac
ac
W
P
Gln
Gln + P
Ser
Ser + P

D


PR1 (72HAI)
Relative Expression
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
W
P
Gln
Gln + P
Ser
Ser + P

B


WRKY29 (24HAI)
Relative Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
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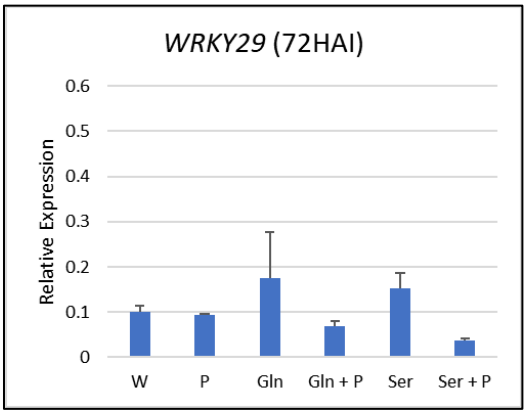
0
a
a
a
bc
ac
bc
W
P
Gln
Gln + P
Ser
Ser + P

E


WRKY29 (72HAI)
Relative Expression
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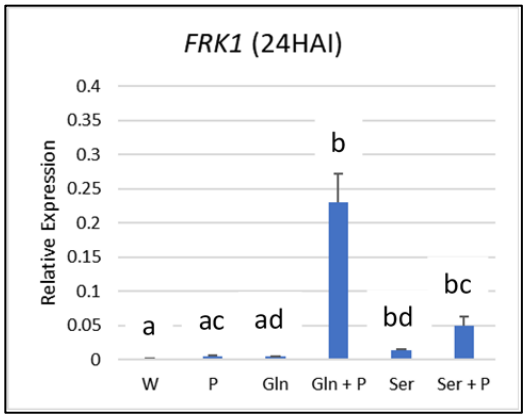
0.4
0.3
0.2
0.1
0
W
P
Gln
Gln + P
Ser
Ser + P

C


FRK1 (24HAI)
Relative Expression
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0.3
0.25
0.2
0.15
0.1
0.05
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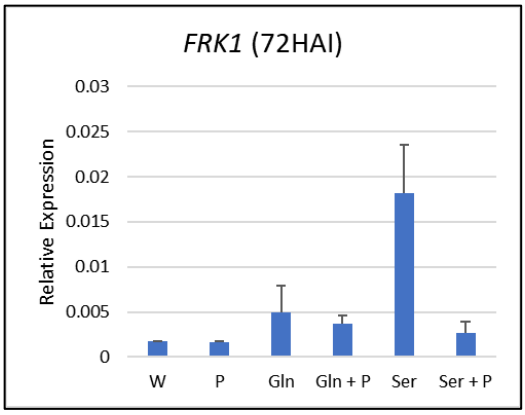
ac
ad
b
bd
bc
W
P
Gln
Gln + P
Ser
Ser + P

F


FRK1 (72HAI)
Relative Expression
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0.01
0.005
0
W
P
Gln
Gln + P
Ser
Ser + P

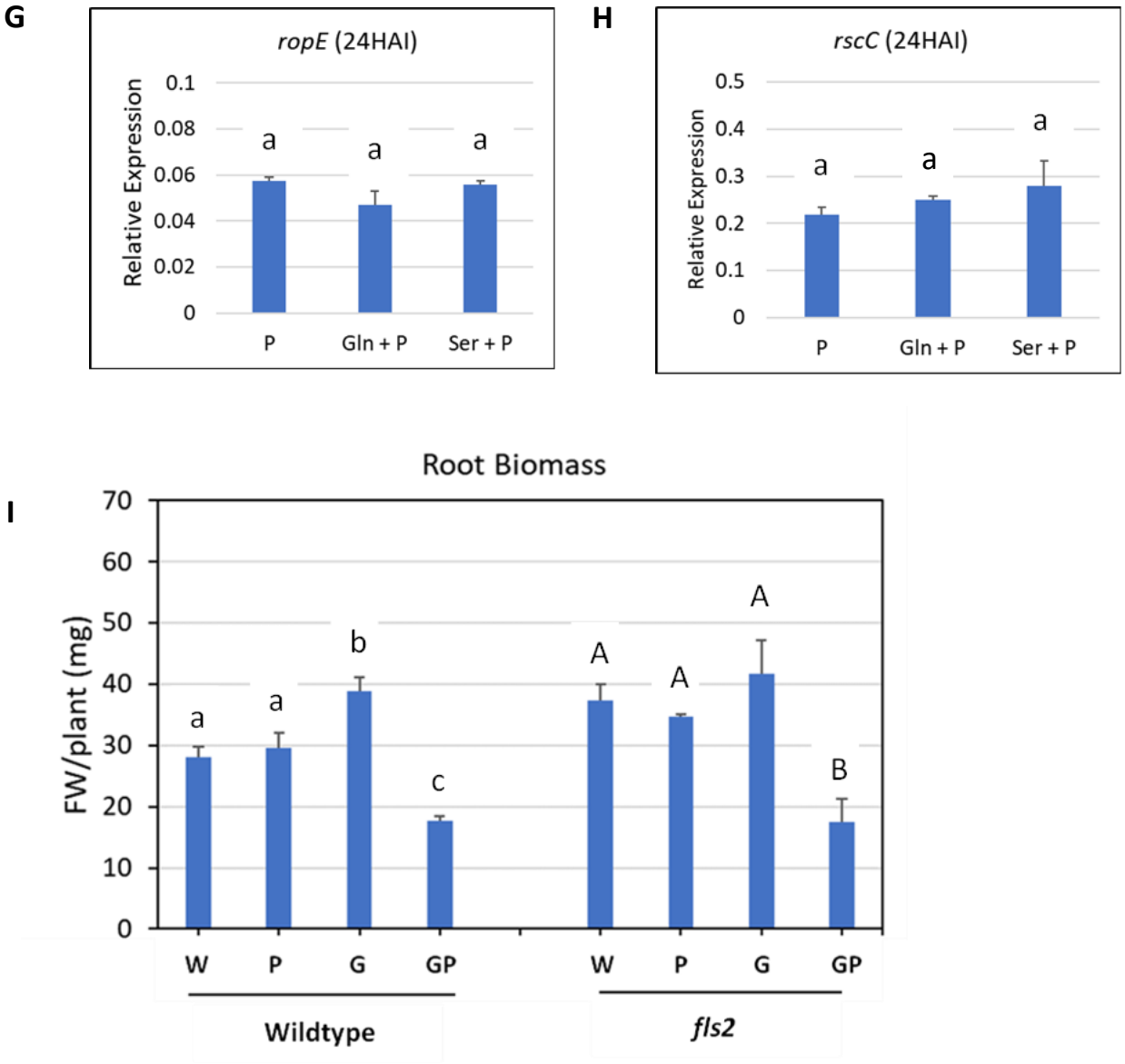


Fig. 3.17: Expression of immune response genes in Arabidopsis wildtype roots treated with sterile water (W), *Ps* WCS417r (P), 10mM Gln, 10mM Gln + P, 10mM Ser, or 10mM Ser + P, as well as T3SS expression in *Ps* WCS417r cells, and root biomass accumulation in FLS2 receptor mutant *fls2*. In Panel I, G indicates Gln. Seeds were grown on 1xMS agar with 0.5% sucrose for 12 days and seedlings transferred to 3MM paper wetted with 5 mL half-strength MS without sucrose, 6 uniformly-growing seedlings per plate, and plates horizontally incubated for one day to allow seedlings to stabilize. Seedlings were then root-flood inoculated with 2mL of the indicated inoculum for 24 h or 72 h for defense gene expression analysis (A-F) and 72 h for root biomass determination (I), and for 24 h for bacterial T3SS gene expression analysis (G, H). For all inocula, *Ps* WCS417r inoculum titer was OD_{600}=0.2. All inocula were adjusted to pH 5.7 with 5mM MES-KOH. Root biomass were harvested and pooled (2 plates=*n*=1 biological replicate). Data are average ± SE (*n*=3). Bars that do not share a letter are significantly different ($p<0.05$); Kruskal-Wallis Test, followed by Dunn's posthoc test for separation of means only when $p<0.05$ (panels A – H) and One-Way ANOVA, followed by Tukey's posthoc test for data in panel I. For gene expression analyses, primer information is located in Supplementary Table S3.1.

3.2.10 Bacterial metabolites in culture supernatants do not inhibit Arabidopsis root and shoot growth.

The effects of metabolites/drugs/chemicals on organisms can be mediated by the host-associated microbiota. These interactions may involve the microbes altering or accumulating the chemicals, which then impact host behavior, or the chemicals negatively impacting the microbes, which then modulate host biology[174]. The bidirectional nature of these interactions highlights the complexities of the biology of host-microbe interactions.

Because microbes have long been known to transform chemicals in their environment[175], I reasoned that the Arabidopsis growth inhibition occurring via exposure of roots to 10 mM Gln + *Ps* WCS417r cells could be due to the *Ps* WCS417r cells converting the glutamine into other metabolites that may directly target plant growth. This hypothesis was based, in part, on my observations that in *in vitro* experiments where *Ps* WCS417r cells were growing in the presence of 10 mM Gln (and appropriately buffered), the growth curves showed diauxic shifts (and in some cases multiauxic shifts), suggesting that the *Ps* WCS417r cells may be converting the glutamine into other usable carbon and nitrogen sources.

To test whether any metabolites that may result from the potential bioconversion of glutamine by *Ps* WCS417r cells could directly inhibit Arabidopsis growth, I collected and filtered culture supernatants from a culture of 10 mM Gln + *Ps* WCS417r cells (buffered), 30 mins, 24 h, and 48 h after incubation in a plant growth chamber, where Arabidopsis growth inhibition was observed following the root treatment with the 10 mM Gln + *Ps* WCS417r inoculum. As a control, supernatant was also collected from 10 mM Gln (buffered) incubated along with the 10 mM Gln + *Ps* WCS417r cells (buffered), 48 h after

incubation. Arabidopsis roots were then treated with the control supernatant, as well as the 30 mins, 24 h and 48 h supernatants from the 10 mM Gln + *Ps* WCS417r cells (buffered) (hereafter called experimental supernatant) in addition to the positive control, being freshly prepared 10 mM Gln + *Ps* WCS417r cells (buffered). These supernatants did not inhibit Arabidopsis root and shoot growth, as root and shoot biomass did not differ significantly from biomass of glutamine-treated only plants (P>0.05; Fig. 3.18), whereas the root and shoot from Arabidopsis root-treated with 10 mM Gln + *Ps* WCS417r were inhibited (P<0.05; Fig. 3.18). These results suggest that either the bacteria were required for the root and shoot growth inhibition effect, or that bacterially-produced metabolites that may orchestrate the effect are not soluble factors. Additional experiments will be required to test these hypotheses.

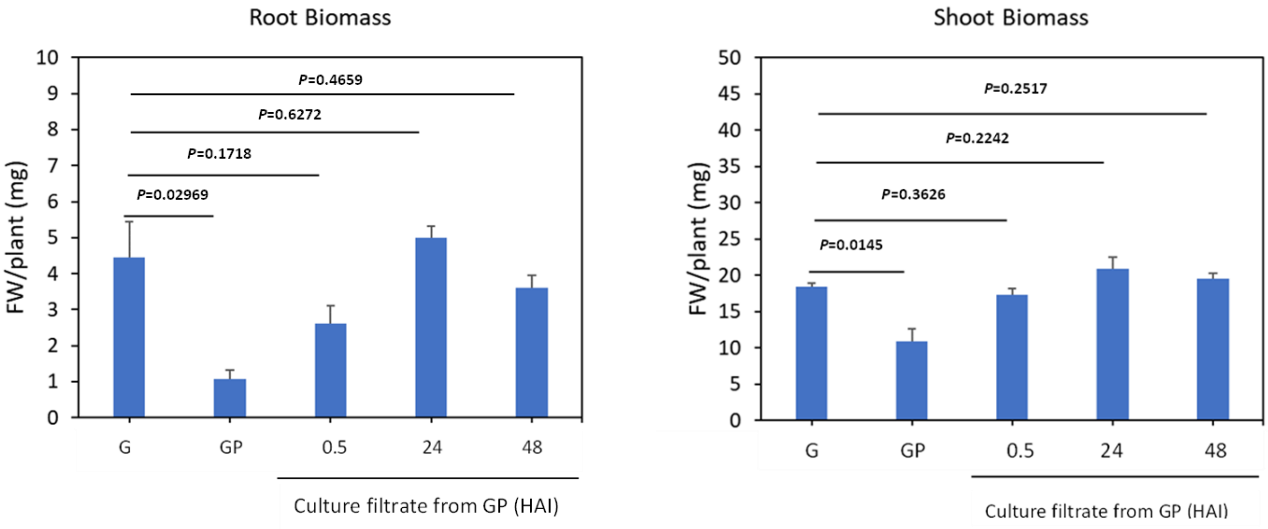


Fig. 3.18: 10mM Gln + *Ps* WCS417r culture supernatant filtrates do not inhibit Arabidopsis root and shoot growth. Root and shoot biomass of Arabidopsis root-treated with 10mM Gln (G), G + *Ps* WCS417r (GP), and filtrates from GP culture supernatants collected 0.5, 24, and 48h after incubation. Seeds were grown on 1xMS agar with 0.5% sucrose for 12 days and seedlings transferred to 3MM paper wetted with 5 mL half-strength MS without sucrose, 6 uniformly-growing seedlings per plate, and plates horizontally incubated for one day to allow seedlings to stabilize. Seedlings were then root-flood inoculated with 2mL of the indicated inoculum for 72 h. *Ps* WCS417r inoculum titer was OD_{600}=0.2 in the GP combination. Both G and GP inocula were adjusted to pH 5.7 with 5mM MES-KOH. Data are average ± SE (*n*=3 plates). Two-sided Student's *t*-test was used to determine statistical significance of means (P<0.05).

3.3 DISCUSSION

That plants deposit a large chunk of their metabolites into the rhizosphere is among the many intriguing observations in plant biology, leading to the proposition that soil-dwelling microorganisms would need to be regarded as important sinks of plant photosynthates[176], ultimately driving plant root exudation. Thus, export of plant metabolites such as amino acids into the rhizosphere has been hypothesized to play important roles in shaping the root microbiome[177]. Yet, the discovery of plant-root expressed transporters dedicated to the uptake of soil amino acids hints at the idea that plants may in fact exert control over the accumulation of plant-derived amino acids in the rhizosphere.

Here, I tested the hypothesis that the Arabidopsis amino acid transporter LHT1 previously described for its role in uptake of amino acids from growth medium and soil may in fact contribute to the recycling of plant-derived amino acids back into the plants. In support of this hypothesis, I found that the Arabidopsis LHT1 loss of function mutant *lht1* accumulates amino acids in root exudates. Extended root exudation experiments revealed that the accumulation of amino acids is amplified over time, suggesting that as amino acids collect around root tissues in the absence of LHT1, further uptake of amino acids may be inhibited. It has been speculated that active cycling of low molecular weight compounds in the rhizosphere is an important strategy that allows plants to, among others, control microbial growth and to dampen pathogen invasion of root tissues[168].

I initially reasoned that loss of LHT1 function would enable the accumulation of amino acids in the rhizosphere to promote colonization of the root tissues by artificially-inoculated rhizobacteria *Ps* WCS417r, which should consequently enhance plant growth. Beneficial rhizobacteria such as *Ps* WCS417r are known to induce the expression of

Arabidopsis root-specific transcription factor MYB72 which regulates the biosynthesis of scopoletin, a secondary metabolite with antimicrobial properties. Scopoletin specifically affects pathogens but not the beneficial rhizobacteria[178,179]. Thus, artificially inoculating the rhizosphere that is enriched with root-derived amino acids with beneficial rhizobacteria may be a strategy to boost plant performance. Therefore, the surprising results that inoculating the *lht1* rhizosphere with *Ps* WCS417r does not lead to improved plant growth indicated that, potentially, altered amino acid homeostasis in the rhizosphere could impair beneficial rhizobacteria-mediated plant growth. This could be due to either bacterial “overgrowth” in the rhizosphere or dysregulated amino acid metabolism by the rhizosphere bacteria, occasioned by the altered amino acid homeostasis, or due to some other mechanism.

Exogenous amino acid supplementation of the wildtype rhizosphere using millimolar concentrations of amino acids, while inhibiting plant growth in the presence of both *Ps* WCS417r and *Pst* DC3000 in the case of glutamine, clarified that bacterial “overgrowth” in the rhizosphere per se does not impair plant fitness. Yet, it is unclear if dysregulated metabolism of amino acids that may overaccumulate in the rhizosphere, by the root microbiota, would explain the observed plant growth inhibition. Soil-dwelling microbes play underestimated roles in affecting plant performance, through accumulation or transformation of chemicals or compounds available in the soil[175]. Transformation of chemicals by soil-dwelling microbes has been studied extensively with respect to chemicals of anthropogenic origin (such as the fate of pesticides applied to crop fields)[180]. In contrast, this work attempted to highlight the idea that plant rhizospheric control of exudation (to modulate the concentration of sugars, amino acids, among others in the

rhizosphere) may be an important strategy that enables plants to foster enduring beneficial interactions with their root microbiota. However, whether overaccumulation of amino acids in the rhizosphere can cause bacterial-mediated plant growth inhibition directly through dysregulated amino acid metabolism by the bacteria, or through some other mechanism, remains to be determined. For example, amino acids and amino-acid derived molecules can influence quorum sensing (QS) in microbes[181]. Virulence phenotypes such as biofilm formation is increased in some microbes such as *Vibrio cholerae* in the presence of increasing concentrations of indole[182]. Many plant-associated microbes can synthesize indole from tryptophan[97,100]. In this study, however, tryptophan levels in the wildtype and *lht1* root exudates were not different. But other amino acids could still regulate QS. Thus, whether plant-derived amino acid (and amino acid-derived molecule) regulation of quorum sensing in root-associated microbiota can impair plant growth will be important to study in the future.

Amino acids exported into the rhizosphere by plants mediate bacterial chemotaxis towards root tissues[6,51], and may thus drive biofilm formation on the root surfaces or in the rhizosphere. However, despite *lht1* root exudates accumulating more amino acids than the wildtype root exudates, I found that under my experimental conditions, *lht1* root exudates do not robustly promote bacterial chemotaxis, although they modestly enhance biofilm formation. Of note, my chemotaxis and biofilm formation experiments were performed using 3-day exudation samples, in which case the amino acid concentration difference between these samples was still modest, and not necessarily large enough to discriminate between the chemotaxis-promoting effects of the wildtype root exudates and the *lht1* root exudates. Still, considering that extended exudation experiments in which

root exudation collections ran for longer than three (3) days showed even larger amino acid concentration differences between the wildtype and *lht1* (Fig. 3.13D), future experiments could consider testing whether the enlarged differences under these experimental conditions could lead to an enhanced chemotaxis towards *lht1* root exudates.

While the collection of experiments reported here thus far demonstrate a strong correlation between altered rhizosphere amino acid homeostasis and impaired rhizobacteria-mediated plant growth, a previous work showed that *lht1* mutants typically grew smaller than wild type plants on fertilized soil. Because N supply was not expected to be limiting under this condition, it was speculated that the *lht1* phenotype on fertilized soil may be due to impaired AA transport activity at another location in the plant.[128] Based on the observation that the loss of LHT1 led to accumulation of amino acids in the leaf apoplasm, it was thought that generating a root-specific *lht1* knockout should lead to the rescue of the leaf phenotype associated with the *lht1* mutant.

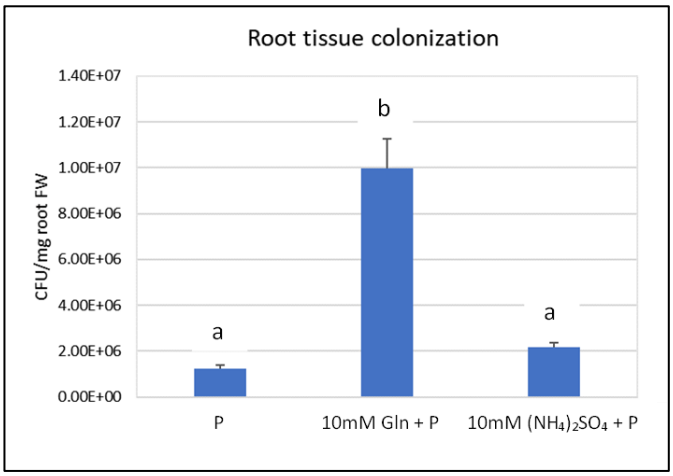
Indeed, the root-specific *lht1* knockout (Pst-LS1-LHT1) rescued the *lht1* phenotype on fertilized soil, but not the impaired *lht1* uptake of Asp, indicating that expression of *LHT1* in the root, and not in the leaf, is necessary for Asp uptake[128]. These results support the notion that normal cell-to-cell transport and cycling of amino acids is crucial for normal plant development[128]. These observations leave open the possibility that beyond the altered rhizosphere amino acid homeostasis in *lht1* plants which likely interfere with rhizobacteria-mediated plant growth, the loss of LHT1 function per se may also affect normal plant response to rhizobacteria. Of note, under my experimental conditions, for both seedlings and adult plants, wild type plants and *lht1* plants grew similarly, and

photosynthetic activity was similar when determined for adult plants via chlorophyll fluorescence measurements (Supplementary Fig. S3.2). Thus, whether and if LHT1 loss of function per se could contribute to the attenuation of rhizobacteria-mediated plant growth would remain a question for future determination.

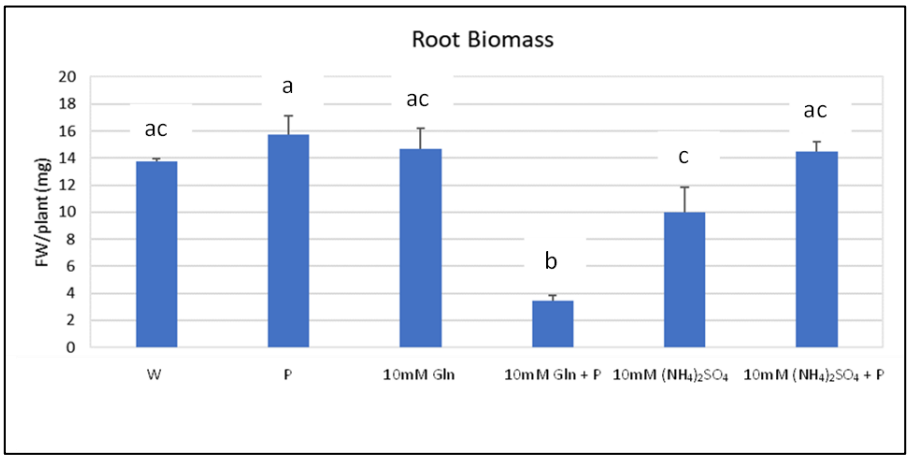
Finally, the results reported here hold important implications for current speculations that manipulating plant amino acid transporter activity in leaf tissues may confer resistance against leaf pathogens[183]. Indeed, *lht1* plants are resistant to some leaf pathogens as previously reported[161]. However, the altered belowground ecological interactions that may be attributable to the loss of rhizosphere amino acid homeostasis indicates that a fine balance must be struck between achieving disease resistance in aboveground plant tissues through amino acid transporter manipulation, and maintaining the integrity of rhizosphere ecological interactions. That is, the often-overlooked belowground interactions are just as important as aboveground interactions.

3.4 SUPPLEMENTARY FIGURES

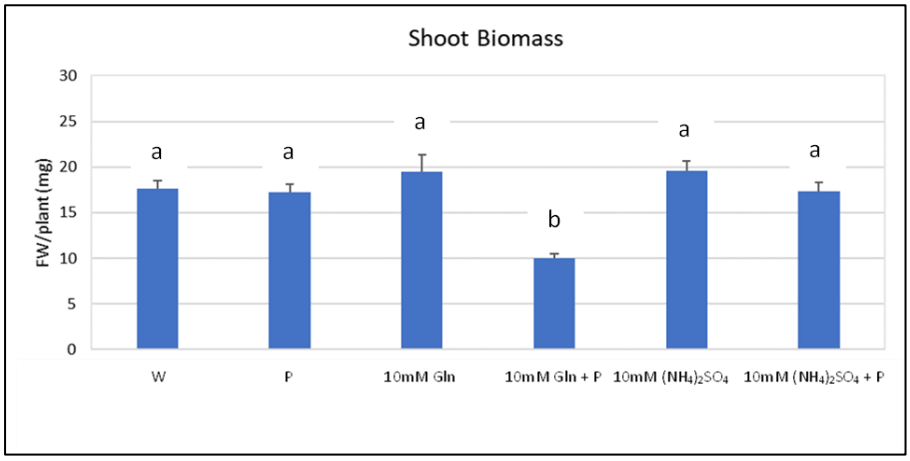
A



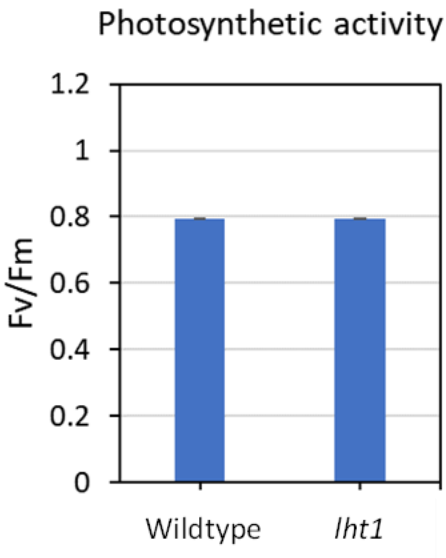
B



C



Supplementary Fig. S3.1: Glutamine (Gln), but not ammonium sulfate ($(NH_4)_2SO_4$), triggers root and shoot growth inhibition in Arabidopsis in the presence of *Ps* WCS417r cells (P). (**A**) Extent of Arabidopsis wildtype root colonization by *Ps* WCS417r cells only (P), or P cells in combination with 10mM Gln (i.e., 10mM Gln + P) or P cells in combination with 10mM $(NH_4)_2SO_4$ (i.e., 10mM $(NH_4)_2SO_4$ + P). Root biomass (**B**) and Shoot biomass (**C**) of Arabidopsis wildtype following root inoculation with sterile water (W), P, 10mM Gln, 10mM Gln + P, 10mM $(NH_4)_2SO_4$ or 10mM $(NH_4)_2SO_4$ + P. Seeds were grown on 1xMS agar with 0.5% sucrose for 12 days and seedlings transferred to 3MM paper wetted with 5 mL half-strength MS without sucrose, 6 uniformly-growing seedlings per plate, and plates horizontally incubated for one day to allow seedlings to stabilize. Seedlings were then root-flood inoculated with 2mL of the indicated inoculum for 72 h. For all inocula, P inoculum titer was OD_{600}=0.2. All inocula were adjusted to pH 5.7 with 5mM MES-KOH. Root and shoot biomass were harvested and pooled (4 plates=*n*=1 biological replicate) for both biomass quantification and root-associated CFU counting. Data are average ± SE (*n*=4). Bars that do not share a letter are significantly different (p<0.05); One-Way ANOVA followed by Tukey's posthoc test for panels A and C; Kruskal-Wallis Test, followed by Dunn's posthoc test for panel B.



Supplementary Fig. S3.2: Photosynthetic activity in leaves of 6-week-old wildtype and *lht1* plants. Leaves were dark-adapted for 30 minutes and the chlorophyll fluorescence measured using the OS-30p+ Chlorophyll Fluorometer (Opti-Sciences, Inc., USA). Data are averages (±SE) of *n*=8 leaves per genotype. No difference between the means (P>0.05); Two-sided Student's *t*-test.

MATERIALS AND METHODS

Plant Growth

For all experiments, wildtype plants were raised from Col-0 seeds, and all mutants utilized were derived from the Col-0 background. Seedlings and adult plants were raised as below:

Seedlings: For all seedling experiments, seeds were surface-sterilized using 10% bleach three times for two minutes each, followed by three washes with sterile water, and subsequently resuspended in 1% phytoagar and stratified in the dark at 4 ºC for at least two days. Seeds were then plated onto 1x MS agar plates (100mm x 100mm square plates; Fisher Scientific; Cat#FB0875711A), composed of 4.4 g/L Murashige and Skoog Basal Medium with Vitamins (PhytoTech; M519), 0.5% sucrose (Sigma; S7903), 0.5 g/L MES (Sigma; M8250), and 0.7% PhytoAgar (PlantMedia; Cat#40100072-2), pH 5.7 and the plates were sealed with parafilm and incubated vertically in a reach-in growth chamber (Conviron Adaptis 1000, Canada) at 25 ± 2 ºC, 75% RH, 16h Light/8h Dark, and 100 µmoles/m^2/s light intensity for two weeks. Uniformly growing seedlings were then selected and transferred to autoclaved 3MM paper cut to fit 100mm x 100 mm square plates and wetted with 5 mL 0.5x MS medium without sucrose, pH 5.7. These plates were incubated horizontally under the same conditions as above. One day was allowed for seedlings to stabilize and then the appropriate treatment applied, which for many experiments included inoculation of wildtype roots by wetting the entire paper with 2 mL sterile water (for mock), or 2 mL *Pseudomonas simiae* WCS417r (OD_{600}=0.2), or inoculation of

wildtype and mutant roots with 2 mL *Pseudomonas simiae* WCS417r (OD_{600}=0.2). Other variations of this assay are described in the figure legends, as appropriate.

Adult plants: For adult plant assays, stratified seeds were sown in peat pellets (Jiffy-7, Jiffy Products Ltd, Shippagan, Canada), 4-5 seeds per pellet, in trays, and covered with a translucent plastic dome to maintain high humidity. These were transferred to Growth Chambers with controlled conditions at 25 ± 2 ºC, 75% RH, 9h Light/15h Dark, and 100 µmoles/m^2/s light intensity) for the next 6 weeks. One week after sowing the seeds, the domes were taken off and seedlings thinned out leaving 1-2 seedlings per pellet. A second thinning out was performed at the end of week two, leaving 1 seedling per pellet through the end of the experiment. Plants were watered with Hoagland's solution three times weekly.

Bacterial strains and growth conditions

Pseudomonas fluorescens WCS417r, also known as *Pseudomonas simiae* WCS417r which was initially isolated from lesions of wheat (*Triticum sativum*) roots[118], along with *Pseudomonas syringae* pv tomato DC3000 (Pst DC3000) were maintained on LB plates supplemented with 50 µg ml^{-1} rifampicin. In preparation for root inoculation experiments, a single colony was randomly picked from the appropriate plate and grown overnight in approximately 100 mL of LB at 28 ºC with shaking at 230 rpm till the cultures reached OD_{600} = 0.4 – 0.8. The cell culture was harvested and washed three times in sterile water, and then adjusted to the required inoculation titer with sterile water.

Rhizosphere inoculation assays

Seeds were grown on 1xMS agar with 0.5% sucrose for approximately two weeks (under the conditions indicated above for seedling growth) and seedlings transferred to 3MM paper wetted with 5 mL half-strength MS without sucrose, six (6) uniformly-growing seedlings per plate, and plates horizontally incubated for one day to allow seedlings to stabilize. Seedlings were then root-flood inoculated with 2 mL of the indicated inoculum for the desired duration of exposure (See Figure Legends).

Root exudate collection assays

For root exudate screening, root exudates were collected using a modification of a previously published method[111]. Briefly, Arabidopsis seedlings were grown initially in 1x MS medium (i.e., full-strength) in 12-well plates (USA Scientific; Cat#CC7682-7512) containing 0.5% sucrose for 12 days, by placing ca. 5 seeds per well on an autoclaved mesh disc (McMaster-Carr; Cat#1100t41) sitting on top of the medium, and the medium changed to 0.5x MS medium (i.e., half-strength) containing no sucrose for 3 days, with the roots separated from the shoots by the autoclaved mesh. Root exudates were then collected and filter-sterilized through 0.22 µm filter for further downstream processing. Throughout the experiment, plates were sealed with parafilm and incubated in a reach-in growth chamber (Conviron Adaptis 1000, Canada) at 25 ± 2 ºC, 75% RH, 16h Light/8h Dark, and 100 µmoles/m^2/s light intensity.

Plant tissue gene expression analysis

Harvested root/leaf tissues were frozen in dry ice. RNA was isolated using TRIzol® Reagent (Fisher Scientific; Cat#15596018) and quantified in a NanoDrop-ND1000

spectrophotometer (Thermo Fisher Scientific Inc.). Two (2) µg of total RNA was used in 1st strand cDNA synthesis after DNaseI treatment. The 2 µg RNA was incubated together with 2 µl Random Decamers and appropriate volume of DEPC-treated water to a final volume of 15 µl at 70 ºC for 5 minutes, and removed to ice immediately. Subsequently, cDNA synthesis reaction mixture containing 10 mM dNTPs (2 µl), Promega RNase inhibitor (1 µl), Promega M-MLV-RT (1 µl), 5x Promega M-MLV Buffer (5 µl) and DEPC-treated water (1 µl) was added, to a final volume of 25 µl and incubated at room temperature for 1 minute and then at 37 ºC for 60 minutes. For qPCRs, 1 µl of cDNA was used in a total reaction volume of 20 µl containing 10 µl SyBr Green mix, 2 µl of 5 µM primers (forward and reverse), and 7 µl DEPC-treated water. There were two technical replicates per biological replicate. The reactions were performed using the ABI 7500 and 7500 Fast Real-Time PCR system v2.3

Biofilm formation assays

Two technical replicates of each sample (Unplanted, and Wildtype and *lht1* root exudates; *n* = 6 biological replicates x 2 technical replicates = *n* = 12) were set up by adding 200 µl of the sample into the appropriately labelled well of a polyvinyl chloride 96 well round-bottom plate (Catalog # CLS2797, Sigma). Each well was inoculated with 2 µl of a liquid culture of *Ps* WCS417r grown overnight in LB medium. Biofilms were grown statically for 48 h at 28 ºC. Subsequently, the medium and non-adherent bacterial cells were removed by carefully pipetting with a multi-pipette and then gently washing the plate with sterile water, without disturbing the biofilms attached to the bottom of the plate. The plate was air-dried for about 5 minutes. Next, the wells were stained with 125 µl of 0.1% (wt/vol) solution of crystal violet in water for 10-15 minutes. The stain was decanted and the plate

gently rinsed with sterile water, leaving the stained biofilm in place in each well. One hundred and fifty (150) µl of a solution containing 30% methanol and 10% acetic acid (i.e., 6:3:1 of sterile water: methanol: acetic acid) was added to each well to solubilize the crystal violet (10-15 minutes). To quantify biofilm biomass, 125 µl of the 150 µl solution was transferred into a well of a polyvinyl chloride 96 well flat-bottom plate (Catalog # CLS2595, Sigma), and the absorbance at 550 nm read in a microplate reader SpectraMax® i3x (Molecular Devices).

Amino acid uptake in seedlings

Arabidopsis seedlings were grown on a full-strength Murashige and Skoog (MS) agar plate supplemented with 0.5% sucrose, vertically incubated under 16h of light, 80 – 120 µmol $m^{-2}s^{-1}$ at 21 ºC for 3 days. Ten seedlings were then transferred from the MS plates into 1 mL of 1x MS medium with 0.5% sucrose (pH 5.7) in 12-well plates until day 8 when the medium is replaced with fresh MS medium. Two days after media change (i.e., day 10), the seedlings were washed with 1x MS medium with 0.5% sucrose and immersed in fresh medium containing between 3.7 and 7.4 kBq U ^{14}C radiolabeled substrate (Perkin-Elmer) and 100 µM unlabeled amino acid substrate. The seedlings were allowed to take up the radiolabeled substrate for 0.5 h and then rinsed twice with 0.2 mM $CaSO_4$. The seedlings were then lyophilized overnight, and then crushed into powder, and digested in 1 mL of 10% bleach for 1 h. The clear lysates were subsequently transferred to 96-well Isoplates (Perkin-Elmer) and scintillation cocktail added for measuring radioactivity. Radioactivity in the samples were then determined using a 1450 Microbeta Trilux Liquid Scintillation and Luminescence counter (Perkin-Elmer™ Life Sciences).

Genotyping of Arabidopsis mutants

To identify homozygous individuals for the Arabidopsis T-DNA insertion lines employed in this study, two PCRs were performed, the first using a left primer (LP) and a right primer (RP) that together amplify the wildtype CDS which would be expected in the wildtype plant (and also in a heterozygous plant) but absent in the homozygous plant, and a second primer pair, the RP and a left border primer (LBP) that together will amplify the T-DNA/genomic DNA junction sequence, which product will be absent in the wildtype plant but present in the homozygous plant (but also in the heterozygous plant). Primers were used at a final concentration of 10 µM. Total volume of 20 µL reaction was performed using Quick-Load® Taq 2x Master Mix (New England BioLabs) (10 µl), primer pairs (1 µl), genomic DNA (2 µl), and sterile water (7 µl). Primer sequences are shown in Supplementary Table S3.1. For genotyping each line, an additional control PCR was performed using wildtype genomic DNA, as well as a blank reaction in which DNA template was replaced with sterile water. PCR conditions are as indicated below:

Step 1: Initial denaturation at 94 ºC for 5 mins; Step 2: Denaturation at 94 ºC for 30 s; Step 3: Annealing at 54 - 56 ºC for 30 s; Step 4: Extension at 68 ºC for 1 min; Step 5: Final Extension at 68 ºC for 5 mins. Steps 2-4 were repeated for a total of 35 cycles; Step 6: Hold at 12 ºC. PCR products were resolved on 1% (w/v) ethidium bromide-stained agarose gel run at 100V. Gels were visualized using the Spectroline UV transilluminator Select™ Series, and images obtained via the AlphaImager 2200.

Statistical analysis

Data analyses were performed using the JASP open-source software v 0.14 and Excel, and graphs generated using Excel. A two-sided Student's *t*-test was performed for statistical comparison of two means, or a Welch's *t*-test for two means with unequal variances, when relevant. For comparison of more than two means, a one-way ANOVA followed by Tukey's posthoc test, or a Kruskal-Wallis test for unequal variances followed by Dunn's posthoc test was performed, as indicated in the relevant figure legends.

For statistical analysis of bacterial growth curves, the CGGC (Comparison of Groups of Growth Curves) permutation test[146] was used to compare pairs of samples (i.e., unplanted vs. wild type; unplanted vs. *lht1*; wild type vs. *lht1*) over the course of growth (24 hours). The test statistic (mean *t*) is the two-sample *t*-statistic to compare the OD_{600} values between the two groups at each hour, averaged over the course of growth (24 hours). A P-value was obtained for the test statistic by simulation. Samples were randomly allocated to each of the two groups and the mean *t* was recalculated for 10 000 data sets generated through this permutation. The P-value is the proportion of permutations where the mean *t* is greater in absolute value than the mean *t* for the original data set. (That is, the number of times the absolute mean *t* from the permutations is greater than the absolute mean *t* for the original data set, all divided by 10 000.) As always, this P-value was interpreted as the probability of obtaining the mean *t* obtained for the original data set if the null hypothesis was true.

Supplementary Table S3.1: List of primers used

Primer Name	**Sequence (5' → 3')**
GENOTYPING	
SALK Border Primer (LBb1.3)	ATTTTGCCGATTTCGGAAC
SALK_041999_LP (*aap5-1*)	AAAGATTCGAAAGAACGGCTC
SALK_041999_RP (*aap5-1*)	GCAATGTACATTTCCACTGGG
SALK_099586_LP (*aap5-2*)	TTATTCTGCCATTGGACTTGG
SALK_099586_RP (*aap5-2*)	AGCAGGAGTTTATTTTTCCCG
SALK_034566_LP (*lht1-1*)	CTGTACATCCCCAAAAATCATG
SALK_034566_RP (*lht1-1*)	ACCTGAGAGACATAACGGCAG
GENE EXPRESSION	
ACT2-U4	GTACGGTAACATTGTGCTCAGT
ACT2-L4	GAGATCCACATCTGCTGGAATGT
LHT1-F2	GTGGTGGTACTCTGCTTTTCAC
LHT1-R2	ACACTGCAATTCCCGGTCC
FRK1-F	CGGTCAGATTTCAACAGTTGTC
FRK1-R	AATAGCAGGTTGGCCTGTAATC
ropE-F1	TGTCCCTTGACGAAGTGCTC
ropE-R1	ATGGACCTGAAGCTGGTTGG
rpoB-F2	TCGCGTATGAACGTTGGTCA
rpoB-R2	GCAGGAACTTACGCAGGTCT
rscC-F1	TTCCAGCAGGCCTTCGATTT
rscC-R1	TATGAGGCCGACCACAAACC
ProA-F2	TCCACTTCGGTGAGGAAACG
ProA-R2	CATCAACACCTACGGCTCCA
PR1-U1	TTCTTCCCTCGAAAGCTCAA
PR1-L1	AAGGCCCACCAGAGTGTATG
SerA-F2	CAGAACATCCCTGGCGTGAT
SerA-R2	CTCGTTGGTCTGCAGGAACT
MYB51-F	ACAAATGGTCTGCTATAGCT
MYB51-R	CTTGTGTGTAACTGGATCAA
WRKY29-F	ATCCAACGGATCAAGAGCTG
WRKY29-R	GCGTCCGACAACAGATTCTC

Chapter 4:

Mutation in Arabidopsis *UMAMIT30* depletes root exudates of amino acids without impacting a root—beneficial microbe interaction

4.1 BACKGROUND

Despite the large presence of amino acids (AAs) in Arabidopsis root exudates, until very recently, it has been unknown how these AAs are released from the plant. Although physiological assays have facilitated the detection of AA export systems in plant cell membranes, the exact molecular identities of AA exporters have been elusive thus far[184]. However, a previous study uncovered UMAMIT14 (a member of the Usually Multiple Acids Move In and out Transporters), which is expressed in Arabidopsis root pericycle and phloem cells, as an exporter involved in exporting AAs from the root phloem into the stele apoplasm, a crucial step for sustaining root-to-soil AA secretion[59]. Loss of function of *UMAMIT14* or *UMAMIT18* leads to a significant reduction in the root exudate AA concentration of both *umamit14* and *umamit18* mutants. This reduction in root AA secretion in the single mutants is further amplified in the double mutant *umamit14-umamit18*[59].

In general, the UMAMIT family of transporters have emerged as candidates for root-to-soil AA export. Several other *UMAMIT* genes are expressed in roots, and UMAMIT transporters share significant homology with *Nodulin 21* (MtN21) from *Medicago truncatula*, which presumably has a role in nurturing nitrogen-fixing bacteria in root nodules[60]. For example, UMAMIT04 is expressed in root hairs and atrichoblasts, and UMAMIT06, UMAMIT37, and UMAMIT42 are expressed in trichoblasts[185], and hence are strategically positioned to execute the ultimate steps in the root-to-soil AA secretion.

In this chapter, I report the discovery of *UMAMIT30* as an additional AA exporter gene in Arabidopsis which contributes to the export of amino acids into root exudates. *UMAMIT30* is highly expressed in root tissues of Arabidopsis, but modestly in shoot tissues, and the

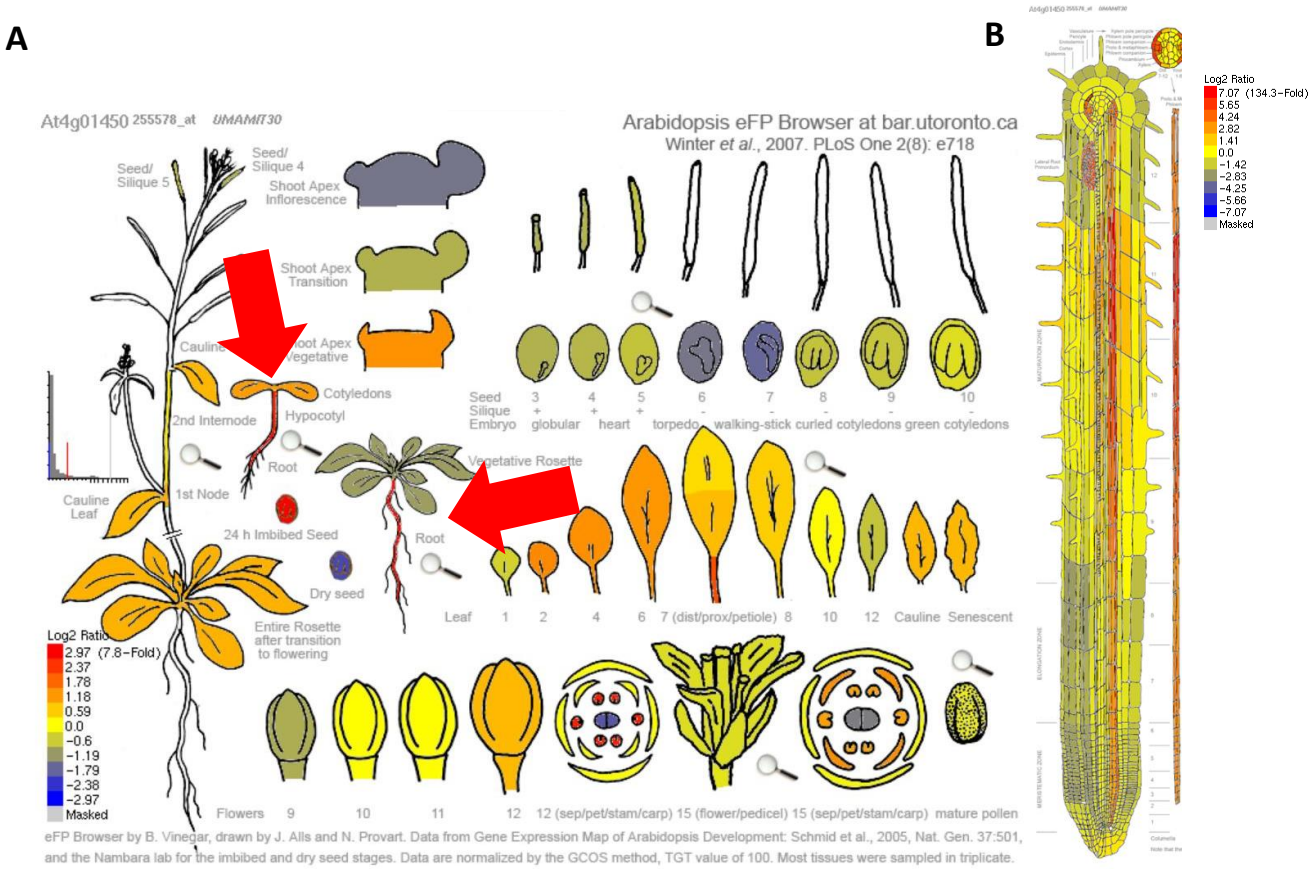
loss of *UMAMIT30* function depletes AA concentration in root exudates. I found that amino acid export out of *umamit30* shoots is diminished, as evidenced by impaired secretion of radiolabeled glutamine by *umamit30* shoots. This suggests that the depletion of amino acids from *umamit30* root exudates may be specifically due to impaired export of the amino acids from the shoots towards the roots. Yet, the amino acid depletion in *umamit30* root exudates was not sufficient to negatively affect the growth of the beneficial rhizobacteria *Pseudomonas simiae* WCS417r (*Ps* WCS417r). Similarly, the *UMAMIT30* mutation did not affect *Ps* WCS417r-mediated Arabidopsis growth.

4.2 RESULTS

4.2.1 *UMAMIT30* is highly expressed in root tissues in steady-state conditions

To uncover the identities of other AA exporters that might exist in Arabidopsis roots, and potentially contributing to the availability of plant-derived AAs in the rhizosphere, I scanned publicly-available microarray data sets to examine the expression profiles of several *UMAMIT* genes across different tissues and developmental stages. This examination revealed that, along with others, *UMAMIT30* is strongly expressed in Arabidopsis roots, relative to its expression in other tissues (particularly the shoots), during the seedling and vegetative stages (Fig. 4.1A; see inserted bold red arrows). To confirm these expression patterns of the *UMAMIT30* gene, I performed RT-qPCR using cDNA derived from roots and shoots/leaves of wildtype seedlings and adult plants growing in standby conditions (i.e., not subjected to any experimental treatment—biotic or abiotic), which showed a very strong expression of the *UMAMIT30* gene in the root

tissues when compared to the expression level in the leaf tissues (Fig. 4.1C and Fig. 4.1D). Remarkably, the publicly-available root tissue-specific expression data show that *UMAMIT30* expression is strongest within the vasculature (Fig. 4.1B). This expression pattern is consistent with a potential role for UMAMIT30 in unloading AAs from the phloem into the stele apoplasm, an important step contributing to amino acid transport from root tissues into the surrounding medium.



Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi)

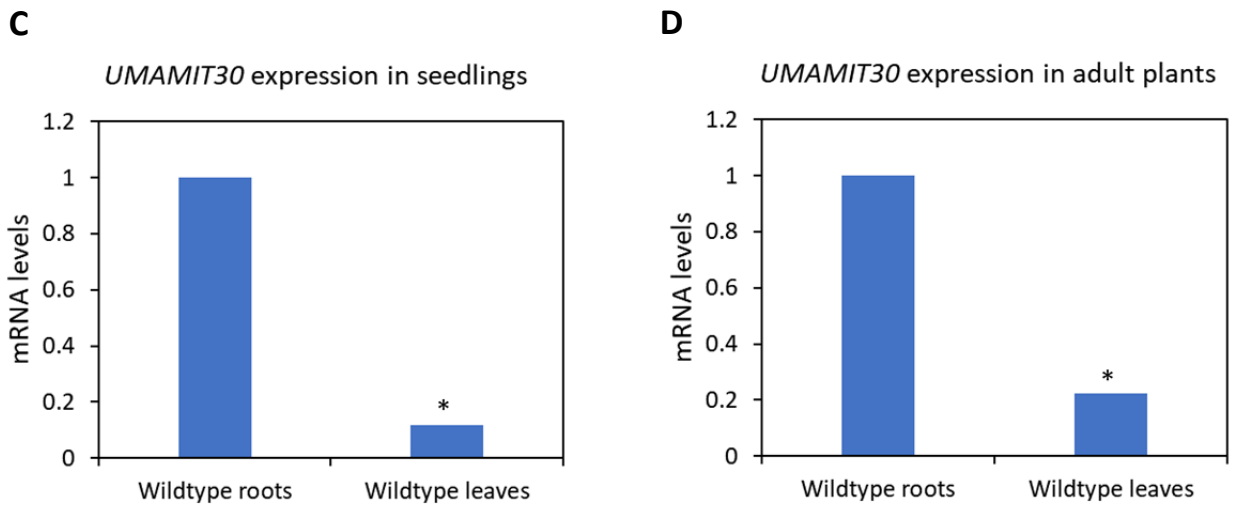


Fig. 4.1: Pictographs of Arabidopsis Developmental Map **(A)** and Arabidopsis root tissue-specific gene expression pattern **(B)** showing expression patterns of the *UMAMIT30* gene based on data from publicly-available microarray data sets retrieved via the Arabidopsis eFP browser[186] (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Signals less than or equal to 20 expression units (i.e., the background value of the AtGEN Express data sets[187,188]) were masked with a grey color. Signals greater than 20 expression units were color-coded in relation to their average levels in the respective data sets (color scales for the data sets used in generating pictographs in A and B above are shown). Inserted red arrows point to a two-leaf stage seedling and a vegetative stage adult plant showing enhanced expression of the *UMAMIT30* gene in the hypocotyl and root of the seedling, and in the roots of the adult plant. Confirmation of *UMAMIT30* gene expression in roots and leaves of 2-week-old wildtype seedlings **(C)** and 6-week-old wildtype plants (i.e., adult plants) **(D)** by RT-qPCR, using RNA from seedlings grown using root exudate collection assays, and RNA from peat pellet-grown adult plants. *UMAMIT30* gene expression levels were normalized against the expression levels of the housekeeping gene *ACTIN2*. The *UMAMIT30* expression level in wildtype leaves is set relative to the expression level in wildtype roots, which is set to unity i.e., 1 (or 100%). Asterisk (*) indicates significantly different (P<0.05) mRNA level via Two-sided Students *t*-test (C) and Welch's *t*-test (D). There were 3 biological replicates for both root and leaf samples. For seedling gene expression analysis, root tissues from ~ 5 seedlings per well were pooled from all 12 wells of a 12-well plate for 1 biological replicate, and leaves from ~ 5 seedlings per well were pooled from 3 wells of a 12-well plate for 1 biological replicate. For adult plants, root was excised from a single plant and washed gently under tap water for 1 biological replicate, and four leaves were collected from a single plant for 1 biological replicate. Samples were frozen in TRIzol® Reagent immediately until used for RNA isolation. Two (2) µg of DNase-treated RNA were used to synthesize first strand cDNA for all samples. (Note: Error bars are not shown, as normalized mean values, rather than mean values, were plotted). Primer information is located in Supplementary Table S4.1.

4.2.2 UMAMIT30 expression is knocked out in *umamit30* mutants

To analyze UMAMIT30 function *in planta*, I isolated a homozygous Arabidopsis *UMAMIT30* T-DNA insertion line (SALK_140547C; hereafter called *umamit30-1*) initially via genotyping with PCR assays on genomic DNA with primers on both sides of the insertion — a left border primer and a downstream primer (see Materials and Methods) — confirming the disruption of the gene loci by the insertion, with the T-DNA insertion putatively localized in the first intron. To be able to confirm any functional phenotype attributable to the loss of *UMAMIT30* function, I isolated a second *UMAMIT30* T-DNA insertion line (SALK_146977C; hereafter called *umamit30-2*), carrying the T-DNA insertion, putatively, in the first exon. I then analyzed the *UMAMIT30* transcript accumulation in two-week-old seedlings grown using the root exudate collection assays described in Chapter 3 (Fig. 3.1). For this analysis, both root and shoot tissues were harvested for RNA isolation and cDNA synthesis. RT-PCR assays confirmed that *UMAMIT30* expression is indeed knocked out in the *umamit30* mutants, as no *UMAMIT30*-specific bands were detected in the RT-PCR product of both root and shoot tissues of both *umamit30-1* and *umamit30-2*, whereas the *UMAMIT30*-specific band was present in the wildtype products (Fig. 4.2).

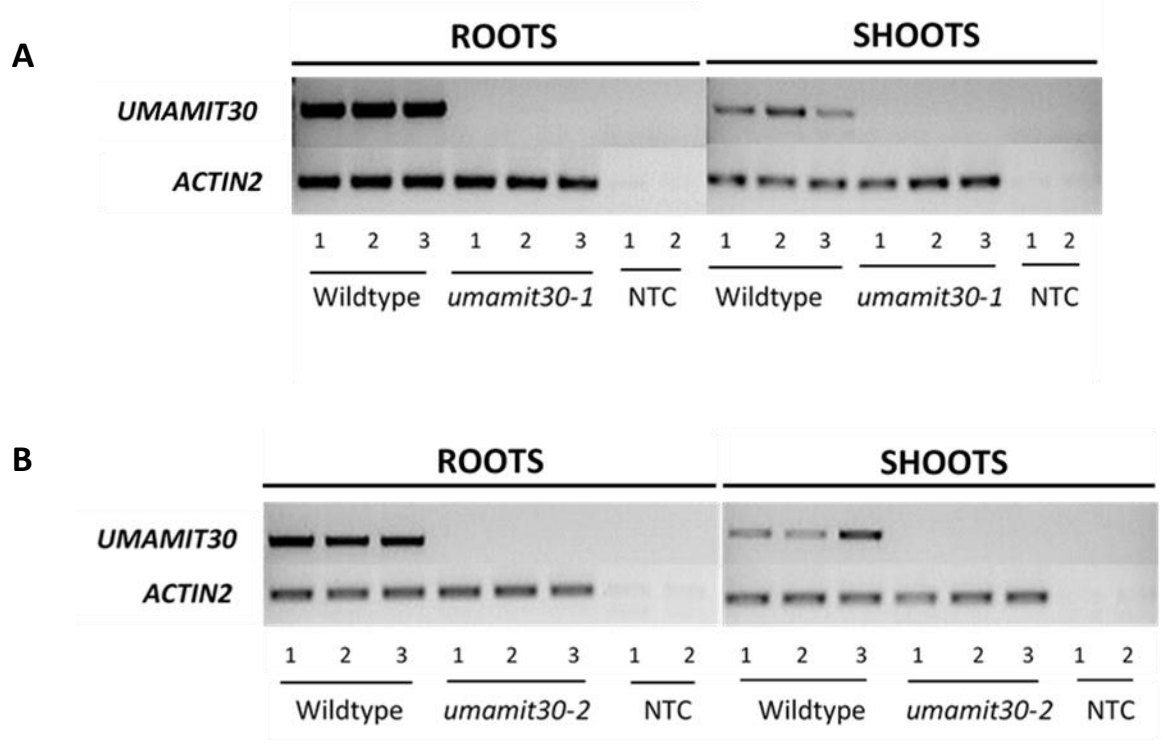


Figure 4.2: Analysis of *UMAMIT30* gene expression in wildtype and *umamit30* plants. Analysis of *UMAMIT30* gene expression in roots and shoots by RT-PCR using RNA from 2-week-old seedlings of **(A)** wildtype and *umamit30*-1 plants, and **(B)** wildtype and *umamit30*-2 plants grown using root exudate collection assays. *ACTIN2* expression was used as a control for equal concentrations of cDNA. NTC = No Template Control. For root tissue analyses, root tissues from ~ 5 seedlings per well were pooled from all 12 wells of a 12-well plate for 1 biological replicate. For shoot tissue analyses, shoots from ~ 5 seedlings per well were pooled from 3 wells of a 12-well plate for 1 biological replicate. There were three (3) biological replicates (i.e., 1, 2, and 3, as in the images above) for both root and shoot samples for both wildtype and *umamit30* genotypes. Samples were frozen in TriZol® immediately until used for RNA isolation. Two (2) µg of DNase-treated RNA were used to synthesize first strand cDNA for all samples. There were two (2) technical replicates for NTC samples (i.e., 1, 2 as in the images above). Two (2) µl of cDNA was used as template in RT-PCR analysis. For NTC samples, cDNA was replaced with two (2) µl of sterile water. Primer information is located in Supplementary Table S4.1.

4.2.3 Root exudate profiling of *umamit30-1* and *umamit30-2* mutants for alteration in AA levels reveals UMAMIT30 as a candidate AA exporter in the root

Because the *UMAMIT30* gene is strongly expressed in root tissues (in the vasculature in particular; Fig. 4.1B), I hypothesized that the UMAMIT30 protein functions in root-to-medium AA transport. To test this hypothesis, I employed the root exudate collection and analysis assay described in Chapter 3 (Fig. 3.1A), adapted from a previous paper[111] and

similar to one of the assays used to confirm UMAMIT14 and UMAMIT18 as root-expressed AA exporters in Arabidopsis[59]. Briefly, seedlings of wildtype and *umamit30-1* or *umamit30-2* were grown for 12 days in full-strength MS medium with 0.5% sucrose. The medium was then replaced with half-strength MS without sucrose and root exudates collected three (3) days later. Seedling roots were separated from the shoots using an autoclaved mesh through which roots could grow into the liquid medium. AA concentration was also quantified in unplanted MS (included with the set up for wildtype and mutants and incubated under the same growth conditions) for background control/subtraction. Analysis of the root exudates (following filter-sterilization through a 0.22 µm-filter) of the *umamit30-1* and *umamit30-2* mutants along with the root exudates of the wildtype, showed significant depletion of AA concentration in the root exudate of these mutants (Fig. 4.3). These data potentially indicated that UMAMIT30 may function in exporting AA out of Arabidopsis roots.

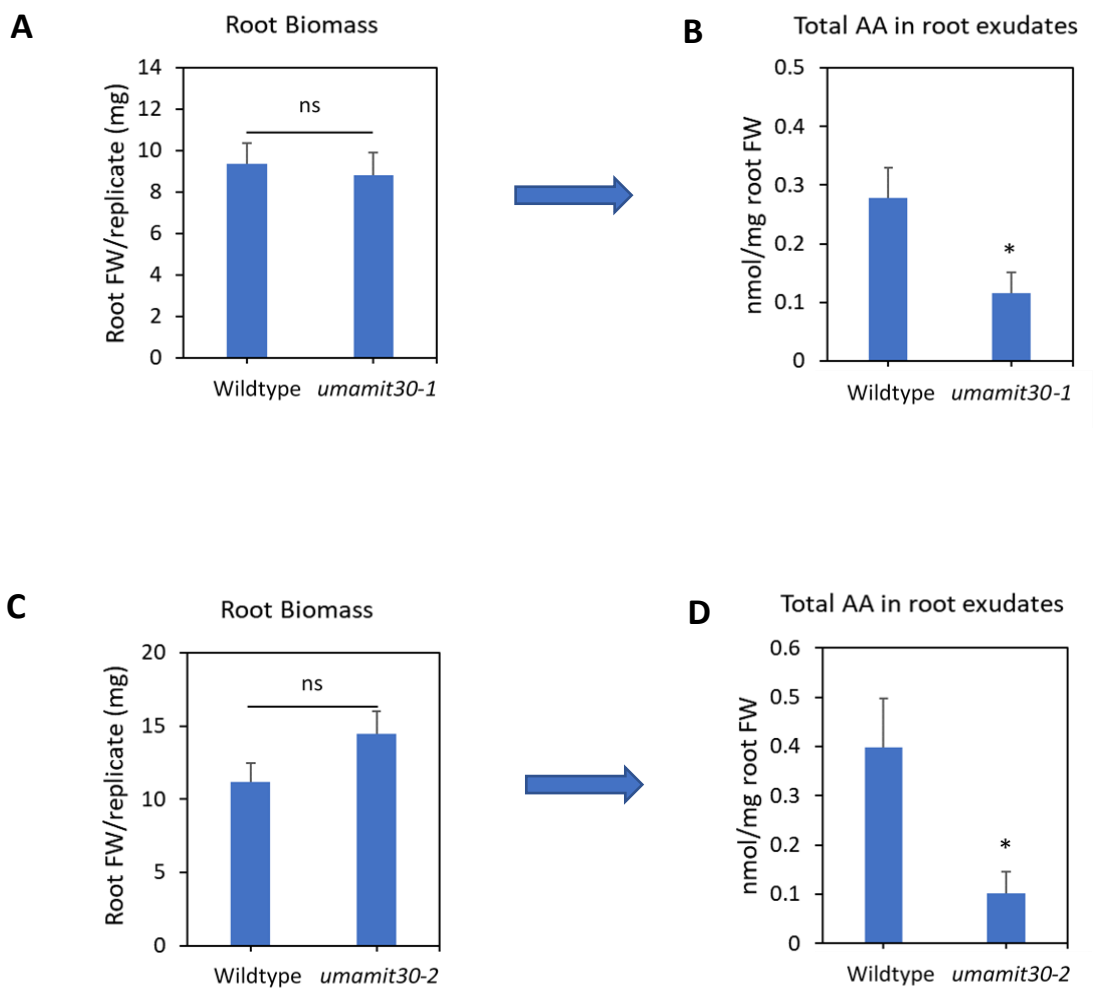


Fig. 4.3: Amino acid concentrations are depleted in root exudates of *umamit30-1* and *umamit30-2* mutants, compared to the amino acid concentrations in wildtype root exudates. (A, C): Root biomass of wildtype vs *umamit30-1* plants and wildtype vs *umamit30-2* plants, respectively, from which root exudates were collected for amino acid quantification. **(B, D):** Concentration of total amino acids (AA) in root exudates of wildtype vs *umamit30-1* plants and wildtype vs *umamit30-2* plants, respectively. Total amino acids were quantified using the L-Amino Acid Quantitation Colorimetric/Fluorometric Kit (BioVision, Catalog #K639-100) following manufacturer's instructions. Root exudates were filter-sterilized through a 0.22 µm-filter before use. For wildtype vs *umamit30-1*, samples were composed of root biomass or root exudates collected from 5 seedlings per well pooled from 4 wells (approximately 20 seedlings) for n = 1. For wildtype vs *umamit30-2*, samples were composed of root biomass or root exudates collected from 5 seedlings per well pooled from 6 wells (approximately 30 seedlings) for n = 1. Data are averages ± SE (*n*=6). Two-sided Student's *t*-test was used for comparison of means, and asterisk (*) indicates a statistically significant difference (P<0.05). 'ns' indicates no statistically significant difference between the means. Experiments with both *umamit30* lines (along with the wildtype control) were performed at least 3 times, yielding similar results.

Concentration of several amino acids are decreased in *umamit30* root exudates

Despite the fact that the data on root exudate AA concentrations for both wildtype and *umamit30* plants (Fig. 4.3) clearly show depleted accumulation of AAs in *umamit30* root exudates, it was not immediately clear if all amino acids or only particular amino acids were depleted. Previously characterized AA transporters in Arabidopsis tend to have a higher affinity for particular amino acids. Thus, to verify the amino acid accumulation signatures of the *umamit30* root exudates, I subjected filter-sterilized (0.22 µm) and 10x concentrated root exudates from both wildtype and *umamit30* to further analysis via LC-MS.

Consistent with the amino acid accumulation profile of the *umamit30* root exudates (Fig. 4.3), the LC-MS analysis showed a noticeable reduction in the concentration of almost all amino acids in the *umamit30* root exudates relative to the wildtype root exudates, except arginine and methionine which were increased (Fig. 4.4). While in most species of plants, glutamine is one of two amino acids (the other being asparagine) found in the xylem sap, all other amino acids are present in the phloem sap[189–193], indicating that abolishing UMAMIT30 function could affect the concentration of several amino acids in the phloem.

A recent report focusing on the characterization of UMAMITs in Arabidopsis confirms the previously reported publicly-available data on the expression pattern of the *UMAMIT30*, utilizing a promoter-GUS analysis to show that in root tissues, the *UMAMIT30* gene is expressed in the phloem, but also in the stele of leaf tissues and in the phloem of floral tissues[194]. In the report, the amino acid export activity of UMAMIT30 was demonstrated in yeast cells expressing UMAMIT30, which were capable of exporting at least seven amino acids including GABA, Met, Glu, Ser, Pro, Thr, and Asp (which were the significant

changes compared to the export activity of the yeast cells carrying the empty vector), under their experimental conditions[194]. This strongly suggests that in Arabidopsis root tissues, the *UMAMIT30* gene may encode a transport protein capable of executing root-to-medium transport of amino acids.

Although the amino acid export profile of UMAMIT30 revealed via the screening approach described in the aforementioned report is different (in terms of the significant changes) from the profile obtained via my screening approach, in general, both approaches show that UMAMIT30 is capable of transporting a broad array of amino acids.

Considering that there are other root-to-medium AA exporters in Arabidopsis previously characterized (e.g., UMAMIT14) and potentially others yet to be characterized, it is not surprising that the loss of a single, presumptive root-to-medium AA exporter, in this case UMAMIT30, does not significantly reduce the concentration of every individual amino acid in root exudates collected from such a transporter mutant.

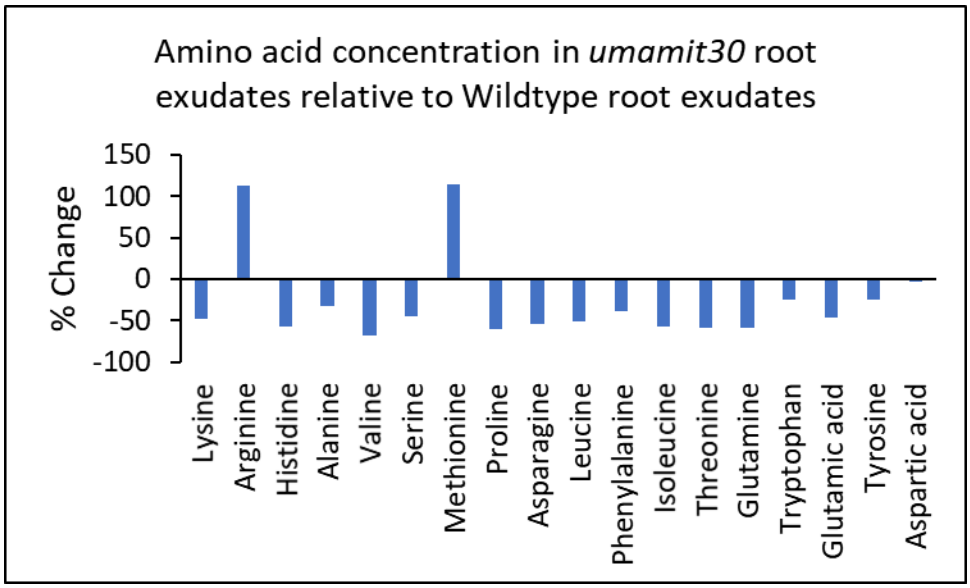


Fig. 4.4: Amino acid concentration in *umamit30* root exudates relative to wildtype root exudates. The individual amino acids in both wildtype and *umamit30* root exudates, along with the unplanted control, were separated via LC-MS. Root exudates (as well as the unplanted control) were filter-sterilized through a 0.22 µm-filter, lyophilized and concentrated 10x before LC-MS analysis. Samples were pooled from two independent experiments, with root exudates and root biomass collected from approximately 5 seedlings per well pooled from 4 wells (approximately 20 seedlings) for 1 biological replicate (n); *n*=3 for each experiment. Amino acid concentrations were normalized by root biomass, and the amino acid concentrations in the *umamit30* root exudates expressed as percentage change relative to the concentrations in the wildtype root exudates. For LC-MS analysis, samples were vacuum-dried and reconstituted in 100 µL of buffer containing 0.1% formic acid (FA), and subsequently analyzed on Thermo Exploris 480 Mass spectrometer using ZipChip Interface. Standard curves of 20 amino acids were generated to obtain absolute quantification of the concentration of amino acids. Glycine data was omitted from final analysis as MS medium (#M519, *Phyto*Technology Laboratories, LLC) for collecting root exudates contained high background amounts of glycine (2 mg/L). For LC-MS data analysis, raw data files for both the standard amino acids and root exudates and unplanted samples were uploaded into Thermo XCalibur software (https://www.thermofisher.com/order/catalog/product/OPTON-30965#/OPTON-30965), and targeted peak detection done using ICIS peak integration algorithm. Thermo Quantitative analysis software (Quan Browser) was then used to generate calibration curves, followed by the determination of the concentration of the amino acids in the root exudate and unplanted samples. The LC-MS analysis was performed at a third-party facility.

4.2.4 Assay validation confirms previously described root-to-medium AA export function for UMAMIT14, and lack of robust AA transport by UMAMIT05

To further confirm the reliability of my assay in isolating Arabidopsis AA transporter mutants with impaired root AA secretion phenotype, I aimed to confirm the impaired root AA secretion phenotype for *umamit14* plants as previously described[59]. To this end, root exudates were collected from both wildtype and *umamit14* seedlings and AA quantification performed essentially as described in Section 4.2.3. In addition, I quantified AA concentrations in root exudates of wildtype and *umamit05* seedlings following the same procedure to compare the results with a recent report suggesting that UMAMIT05 is capable of exporting only a single AA when expressed in yeast cells[194].

Consistent with the previous results, my screening strategy showed a significant depletion of AA in the root exudates of *umamit14* compared with wildtype (Fig. 4.5B), whereas no significant difference was found between the root exudate AA concentrations of *umamit05* and the wildtype (Fig. 4.5D). Taken together, these results confirm the root AA export function for UMAMIT14 and the limited (if any) role of UMAMIT05 in AA export in Arabidopsis as previously described, and indicate the reliability of my assay in detecting root exudate AA concentration differences between wildtype and AA transporter mutants.

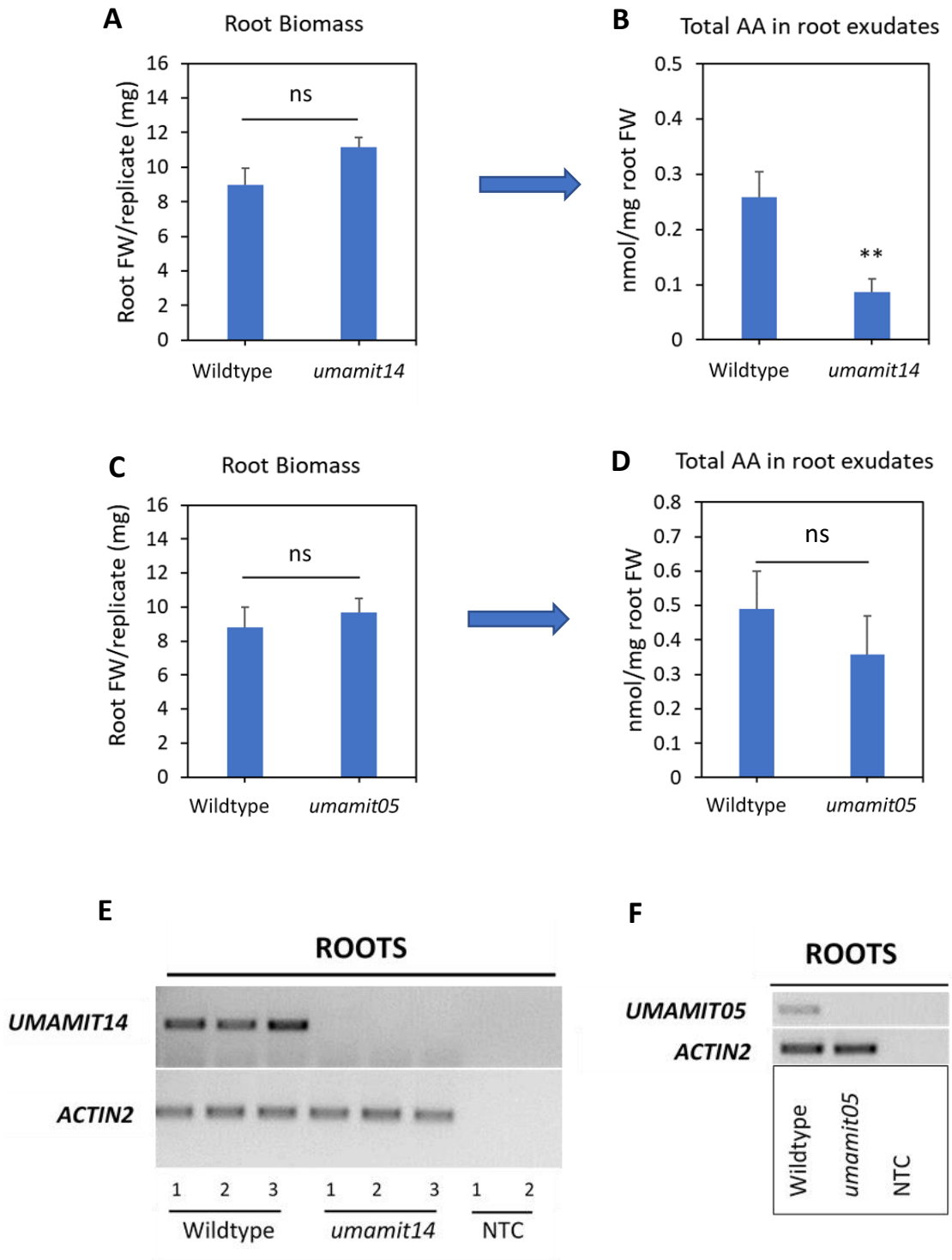

A
Root Biomass
Root FW/replicate (mg)
16
14
12
10
8
6
4
2
0
ns
Wildtype
umamit14
B
Total AA in root exudates
nmol/mg root FW
0.5
0.4
0.3
0.2
0.1
0
**
Wildtype
umamit14
C
Root Biomass
Root FW/replicate (mg)
16
14
12
10
8
6
4
2
0
ns
Wildtype
umamit05
D
Total AA in root exudates
nmol/mg root FW
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ns
Wildtype
umamit05
E
ROOTS
UMAMIT14
ACTIN2
1 2 3 1 2 3 1 2
Wildtype
umamit14
NTC
F
ROOTS
UMAMIT05
ACTIN2
Wildtype
umamit05
NTC

Fig. 4.5: Amino acid concentrations are depleted in root exudates of *umamit14* mutants, but not in *umamit05* mutants, compared to the amino acid concentrations in wildtype root exudates. (A, C): Root biomass of wildtype vs *umamit14* plants and wildtype vs *umamit05* plants, respectively, from which root exudates were collected for amino acid quantification. **(B, D):** Concentration of total amino acids (AA) in root exudates of wildtype vs *umamit14* plants and wildtype vs *umamit05* plants, respectively. Total amino acids were quantified using the L-Amino Acid Quantitation Colorimetric/Fluorometric Kit (BioVision, Catalog #K639-100) following manufacturer's instructions. Root exudates were filter-sterilized through a 0.22 µm-filter before use. Samples were composed of root biomass or root exudates collected from approximately 5 seedlings per well pooled from 4 wells (approximately 20 seedlings) for n = 1. Data are averages ± SE (n=6). Two-sided Student's t-test was used for comparison of means, and asterisks (**) indicate a statistically significant difference (P<0.01). 'ns' indicates no statistically significant difference between the means. Experiments were performed twice, yielding similar results. **(E):** Analysis of *UMAMIT14* gene expression in wildtype and *umamit14* root tissues confirming the loss of function of *UMAMIT14* in *umamit14* plants. RT-PCR was performed using RNA from 2-week-old seedlings grown using root exudate collection assays. *ACTIN2* expression was used as a control for equal concentrations of cDNA. NTC = No Template Control. Root tissues from ~ 5 seedlings per well were pooled from all 12 wells of a 12-well plate for 1 biological replicate. There were three (3) biological replicates (i.e., 1, 2, and 3, as in the image above) for the root samples, and two (2) technical replicates for NTC samples (i.e., 1, 2 as in the image above). **(F):** Analysis of *UMAMIT05* gene expression in wildtype and *umamit05* root tissues confirming the loss of function of *UMAMIT05* in *umamit05* plants. RT-PCR was performed using RNA from 2-week-old seedlings grown using root exudate collection assays. *ACTIN2* expression was used as a control for equal concentrations of cDNA. NTC = No Template Control. Root tissues from ~ 20 seedlings per well were bulked together. For the analyses in both panels E & F, biological samples were frozen in TriZol® immediately until used for RNA isolation. Two (2) µg of DNase-treated RNA were used to synthesize first strand cDNA for all samples. Two (2) µl of cDNA was used as template in RT-PCR analysis. For NTC samples, cDNA was replaced with two (2) µl of sterile water. For gene expression analyses, primer information is located in Supplementary Table S4.1.

4.2.5 Analyses of growth phenotypes reveal no phenotypic differences between wildtype and *umamit30* plants

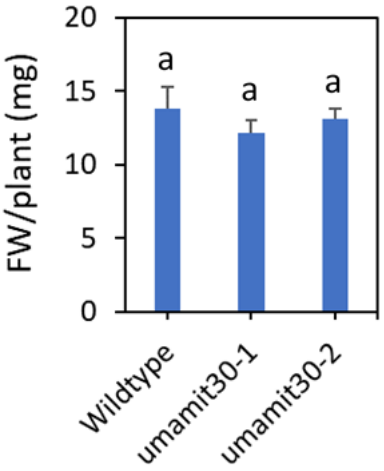
Amino acid transporter mutations may alter amino acid homeostasis in plants and affect growth phenotypes (e.g., *LHT1* mutation leads to a smaller rosette size for adult plants under some growth conditions, and to amino acids accumulating in the leaf apoplasm[128], and *AAP6* mutation leads to a larger mean rosette width at flowering time, and a larger number of cauline leaves as well as a relatively larger seed size compared to the wildtype seeds[195]). To examine if the *UMAMIT30* mutation alters the overall amino acid homeostasis in the mutants which may affect root or shoot growth and potentially AA

transport, I analyzed the fresh and dry shoot weights of 14-day-old wildtype, *umamit30-1* and *umamit30-2* plants grown on 1x MS agar with 0.5% sucrose, in addition to their fresh root weight (Fig. 4.6), as well as the fresh and dry shoot weights of 6.5-week-old plants grown in peat pellets (Fig. 4.7). These analyses reveal no growth defects in the root (determined for seedlings only) and shoot growth (determined for both seedlings and adult plants) of the *umamit30-1* and *umamit30-2* plants when compared to the wildtype plants.

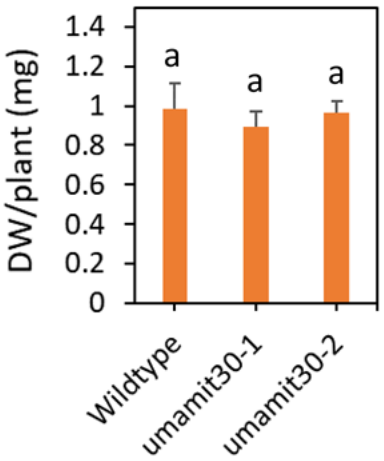
While for adult plants the shoots of *umamit30-1* plants appear slightly larger than those of the wildtype, these differences were not significantly different (at least not at 6.5 weeks; Fig. 4.7A & B). Similarly, the shoots of *umamit30-2* plants are also not phenotypically different from those of the wildtype plants (Fig. 4.7C & D). I extended the phenotypic characterization to 9-week-old plants grown in long day conditions in a climate-controlled growth chamber, and found no differences between the mutants and the wildtype (Table 4.1). Thus, the depletion of the root exudate AA concentration in the mutants is likely not explained by any significant loss of AA homeostasis in the mutants, which would have led to altered growth phenotypes.

A

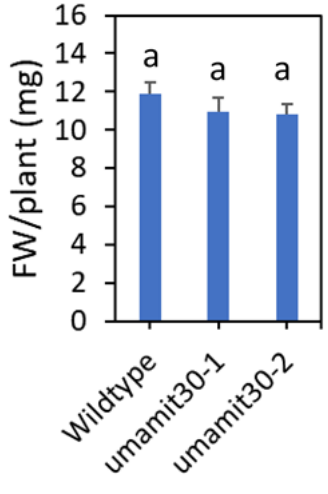
B Whole plant biomass



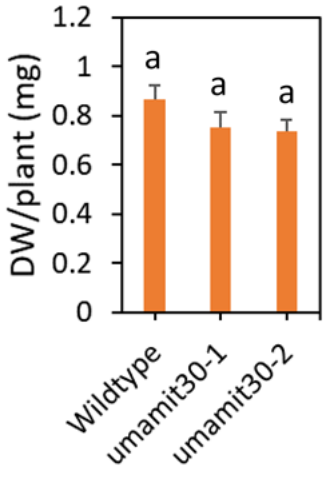
C Whole plant biomass



D Shoot biomass



E Shoot biomass



F Root biomass

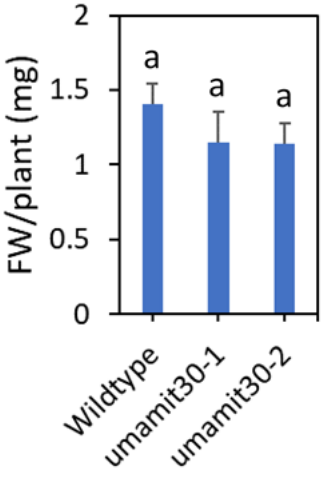


Fig. 4.6: Analyses of growth phenotype of wildtype and *umamit30* seedlings. (A) Two-week-old seedlings of the wildtype, *umamit30-1*, and *umamit30-2* plants growing on 1x MS agar with 0.5% sucrose. Fresh and dry weights (blue and orange graphs, respectively) of whole plants **(B, C)** and shoots **(D, E)** of wildtype, *umamit30-1*, and *umamit30-2,* and fresh weight of roots **(F)** of wildtype, *umamit30-1*, and *umamit30-2* plants. No dry weight data is provided for roots which were too small to weigh accurately for individual plants after drying. Data are averages ± SE (*n*=15). For whole plant analysis, a separate set of plants was used, and a separate and second set was used for shoot and root biomass analysis. That is, the whole plant data are not derived from the total of shoot and root data points as presented. For dry weight analysis, samples were freeze-dried in a lyophilizer (Labconco) overnight. Bars carrying same letters indicate no statistically significant difference between the means (P>0.05; One-way ANOVA. Kruskal-Wallis test for unequal variances). Experiment was performed twice with similar results.

Table 4.1: Characterization of 9-week-old Arabidopsis wildtype and *umamit30* mutants grown in peat pellet in long day conditions in a climate-controlled growth chamber.

	Plant height (cm)	Biomass, FW (g)	Weight of 100 seeds (mg)	Number of seeds per silique
Wildtype	53.5 ± 0.65 (a)	3.7 ± 0.15 (a)	2.1 ± 0.17 (a)	44.0 ± 2.68 (a)
umamit30-1	52.0 ± 1.22 (a)	3.3 ± 0.30 (a)	2.4 ± 0.03 (a)	43.0 ± 2.16 (a)
umamit30-2	50.3 ± 2.06 (a)	3.5 ± 0.21 (a)	2.4 ± 0.05 (a)	42.3 ± 1.25 (a)

NOTES: All traits were analyzed using one-way ANOVA in conjunction with Tukey's posthoc test, except 'Weight of 100 seeds' which were analyzed via the Kruskal-Wallis Test followed by Dunn's post hoc test. Data represent mean ± S.E. (*n*=4 biological replicates). For all traits analyzed, no statistically significant differences were found across the genotypes (p>0.05), represented by same letters in bracket. Biomass indicates fresh weight (FW) of all aerial parts, including fresh siliques still attached to the plants after collecting dry siliques for weight of 100 seeds (mg).

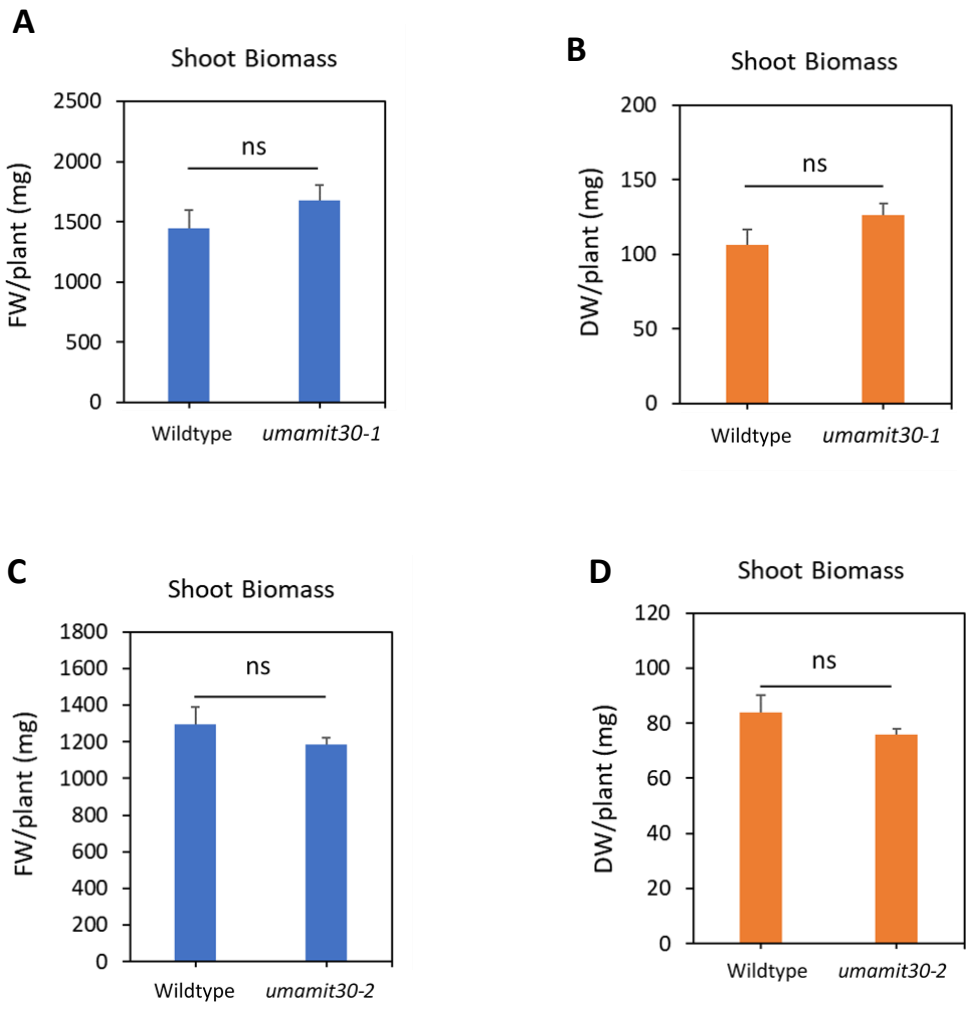


Fig. 4.7: Analyses of growth phenotype of wildtype and *umamit30* adult plants. Fresh and dry weights (blue and orange graphs, respectively) of shoots of 6.5-week-old wildtype and *umamit30-1* plants **(A, B)** and shoots of 6-week-old wildtype and *umamit30-2* plants **(C, D)**. For dry weight analysis, samples were dried in an oven at 55 ºC overnight. Data are averages ± SE (*n*=10). 'ns' indicates no statistically significant difference between the means (P>0.05; Two-sided Students *t*-test). Experiment was performed twice with similar results.

4.2.6 *umamit30* seedlings modestly accumulate AAs in their shoots

As originally hypothesized, and consistent with the model where UMAMIT30 is strongly expressed in the root tissues and functions in export of AAs out of the roots, I expected that AAs may accumulate inside the root tissues, in the absence of *UMAMIT30* function in the root. To test this hypothesis, I collected root and shoot tissues from both wildtype and *umamit30-1* seedlings for AA extraction and quantification via LC-MS. I found the surprising result that there was no significant difference between the AA content of the roots of wildtype and *umamit30-1* seedlings ($P>0.05$). On the other hand, the AA content of the shoots of the *umamit30-1* seedlings was modestly elevated ($P<0.05$), compared to the AA content of the shoots of wildtype seedlings (Fig. 4.8).

At first glance, these results seem to contradict the model where a defective root-to-medium export of AAs would explain the relatively low concentration of AAs in *umamit30* root exudates. However, *umamit14*, the previously characterized Arabidopsis mutant with decreased root exudate AA content was found to not accumulate AAs in root tissues. In this case, it was observed that the decrease in the root exudate AA content, measured over the duration of root exudation, was occurring at the estimated rate of 2.9 nmol mg^{-1} DW d^{-1}; this is negligible when compared to the total AA content of the root tissues (i.e., 393.8 nmol mg^{-1} DW)[59].

Additionally, it was speculated that if AAs were being retained within the root phloem, homeostatic mechanisms regulating the partitioning of AAs in the plant would ensure that AAs arriving from the leaf phloem towards the root are redirected into the xylem[59]. While the proposed redirection of AAs from the roots into the xylem (and hence towards the aerial parts of the plant) may also explain the modest accumulation of AAs in the shoots

of the *umamit30* seedlings, further experiments indicated that export of amino acids from *umamit30* shoots is likely impaired by the loss of *UMAMIT30* function (Fig. 4.9).

The foregoing suggests that phloem loading of AAs in shoots of *umamit30* seedlings may be defective due to the loss of *UMAMIT30* function, limiting how much AAs eventually reach the root tissues and hence accumulate in root exudates. Although I examined this possibility, collecting leaf phloem sap from fully expanded adult leaves of wildtype and *umamit30* plants (because collecting leaf phloem sap from seedlings would be technically challenging), I found no difference in the phloem sap AA concentrations (Supplementary Fig. S4.1). However, an important caveat to observe in interpreting this result is that the expression pattern of *UMAMIT30* is not necessarily identical for seedlings and adult plants (Fig. 4.1), and thus in seedlings, a loss of *UMAMIT30* function may still contribute to impaired phloem loading in the shoots, accounting for the observed root exudate AA concentration decline.

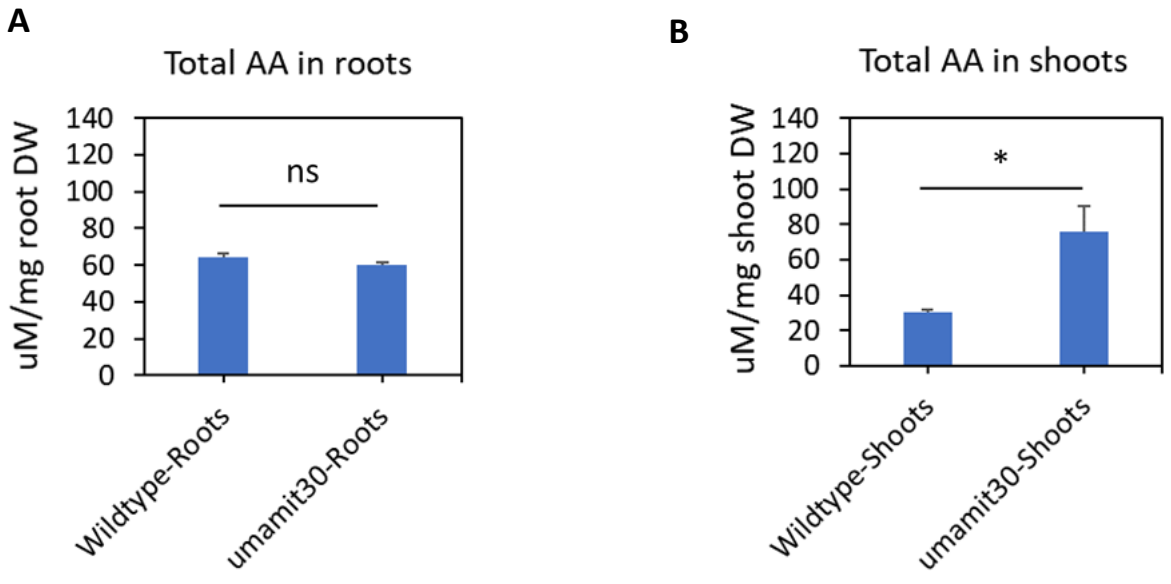


Fig. 4.8: Accumulation of amino acids in roots and shoots of wildtype and *umamit30* seedlings. Amino acids were extracted from tissues using chloroform extraction (see Materials and Methods), quantified via LC-MS, and normalized by dry weight of extracted plant sample. Data are averages ± SE (*n*=3-4). Asterisk (*) indicates statistically significant difference between means (P<0.05; Two-sided Students *t*-test). NOTE: A close examination of the LC-MS data suggested the presence of a single potential outlier for both the WT and *umamit30-1* shoot AA concentrations (out of the four samples analyzed for each group). A box and whisker plot was therefore used to detect outliers. This analysis confirmed that a single data point (i.e., the mean shoot AA concentration for sample WT-3S) in the WT-shoot group was indeed an outlier. Further, Grubb's test confirmed this data point as an outlier (P<0.05). Thus, this data point was excluded from further analysis.

4.2.7 Efflux of amino acids from shoots is impaired in *umamit30* seedlings

Following the observation that *umamit30-1* seedlings accumulate modest amounts of AAs in their shoots, compared to the shoots of wildtype seedlings (Fig. 4.8), I tested whether loss of function of *UMAMIT30* affects the secretion of AAs by both roots and shoots, by measuring the efflux of a radiolabeled glutamine, i.e., [^{3}H]Gln, from roots and shoots independently in the wildtype and *umamit30* mutant. While the root efflux data were inconclusive, it was found that [^{3}H]Gln efflux from shoots decreased significantly for

umamit30-1 seedlings, compared to the shoots of the wildtype seedlings. These data, considered together with the observation that *umamit30-1* seedlings modestly accumulate AA in their shoots, suggest that the depletion of AAs in the root exudates of the *umamit30* seedlings may be related to impaired export of the AAs out of leaf tissues (i.e., a potentially defective phloem loading), in addition to a potentially defective root-to-medium AA export, which could not be determined conclusively in this study. Among others, root tissues generally had a considerably lower AA uptake compared to the shoot tissues, making secretion measurement for root tissues unreliable under my assay conditions.

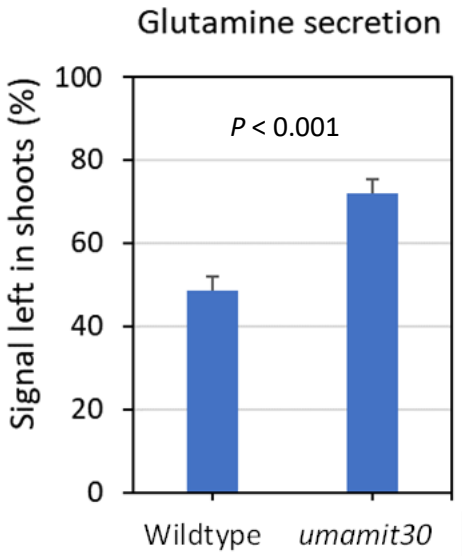


Fig. 4.9: Secretion of [^{3}H]Gln by shoots of wildtype and *umamit30* seedlings. Uptake and secretion of [^{3}H]Gln lasted for 20 minutes each. Secretion was computed as a percentage of total uptake and informed as radioactive signal left in samples. Higher signal left in sample indicates lower secretion. Data are averages ± SE (*n*=5 - 6). Statistical significance of the difference between the means was determined via a Two-sided Students *t*-test; P<0.05. Experiment was performed twice with similar results.

4.2.8 Depletion of AAs in *umamit30* root exudates does not affect root—beneficial microbe interactions

A few examples of the functional significance of plant amino acid transporters in mediating plant—biotic interactions are known. For example, the amino acid transporter CAT6 as well as some transporters belonging to the AAP family are known to be modified during nematode invasion of plant tissues[196–198]. In the Arabidopsis *aap3* and *aap6* mutants, root-knot nematode infestation is reduced when compared to infestation levels of the wildtype plant[198]. Additionally, the loss of the transporter AAP6 function in Arabidopsis leading to the reduction in AA concentrations in sieve elements affects the reproductive performance of aphid herbivores feeding on the *aap6* plants only slightly, as well as causing a substantial delay in their ingestion of sieve element saps[195]. Several other aspects of the biology of the aphids, including honeydew amino acid concentrations and honeydew production, were unaffected by the alteration in the sieve element AA composition.

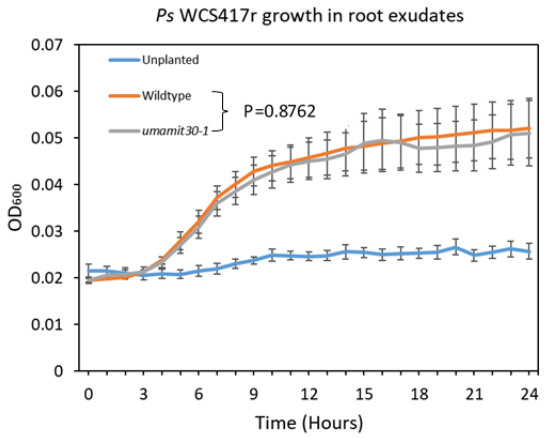
All the above examples focus on the effect of plant AA transporter gene mutation on parasites. In the context of the role of plant AAs in mediating plant—beneficial microbe interactions, no direct evidence exists, to the best of my knowledge, that the loss of a plant AA transporter impacts this interaction, except that beneficial microbes with a defect in their chemotaxis machinery governing chemotaxis towards plant-derived AAs could lose their plant tissue colonization capacity[6].

To determine whether decreased AA content of root exudates could abolish a beneficial microbe interaction with the mutant plants, I first examined the growth of *Ps* WCS417r in

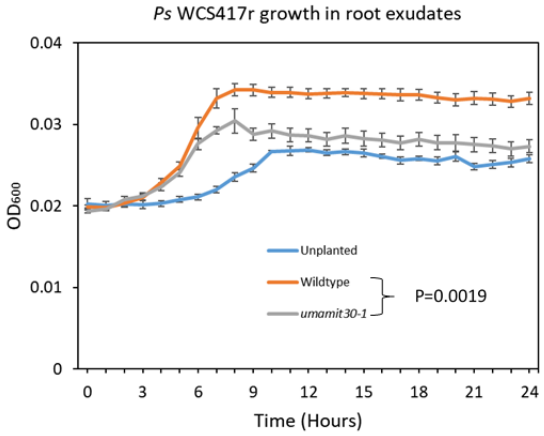
wildtype and *umamit30* root exudates. The results show that in general, *umamit30* root exudates are just as efficient as wildtype root exudates in supporting *Ps* WCS417r growth (Fig. 4.10). Similarly, *umamit14* root exudates are just as efficient as wildtype root exudates in supporting *Ps* WCS417r growth (Fig. 4.11). Because root exudates are complex and contain a variety of nutrients capable of supporting microbial nutrition, mere depletion of root exudates of amino acids may not be sufficient to significantly impair the growth of *Ps* WCS417r cells in the root exudates. Considering that *Ps* WCS417r growth in both *umamit30* and *umamit14* root exudates is not impacted, these data suggest that the loss of a single AA transporter in Arabidopsis is not sufficient to abrogate Arabidopsis root—*Ps* WCS417r interactions, even if it is assumed that plant-derived amino acid metabolism by *Ps* WCS417r is an important feeding strategy for these cells.

However, these results contrast with the finding that overaccumulation of AAs in *lht1* root exudates boosts *Ps* WCS417r growth (Chapter 3). This may mean that when AAs accumulate substantially beyond wildtype root exudate levels, *Ps* WCS417r cells may reprogram their metabolism to utilize the AAs for enhanced growth. On the other hand, when AA concentrations are low, relative to wildtype levels (such as in *umamit30* and *umamit14* root exudates), *Ps* WCS417r cells may adjust their metabolism to also utilize other carbon sources (such as sucrose) which are present in the root exudates.

EXPERIMENT 1:



EXPERIMENT 2:



EXPERIMENT 3:

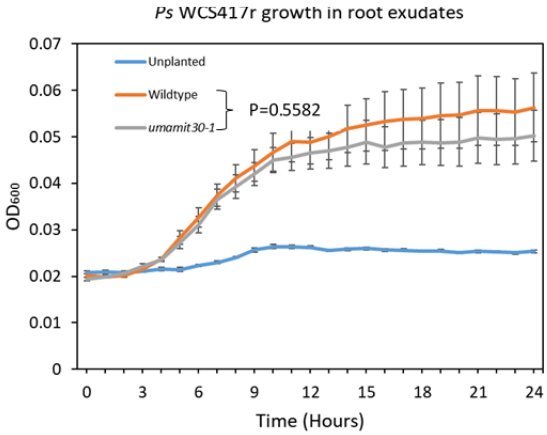


Fig. 4.10: *Ps* WCS417r growth in wildtype and *umamit30* root exudates, and in unplanted control. *Ps* WCS417r cells from overnight culture were washed three times in sterile water and resuspended in sterile water to OD_{600}=0.2. Ten (10) µL was added to 90 µL of the unplanted growth medium (unplanted) or to 90 µL wildtype root exudates or 90 µL *umamit30-1* root exudates, to a final OD_{600}=0.02. The test samples were filter-sterilized through a 0.22 µm-filter before use. Growth was measured over 24 h with intermittent shaking, in the microplate reader SpectraMax® i3x (Molecular Devices). Data are average ± SE (*n*=6 biological replicates). Statistical analyses were performed using 'Compare Groups of Growth Curves'

method as previously described[146]. In brief, a statistical permutation test was used to compare pairs of samples (i.e., unplanted vs. wildtype, OR unplanted vs. *umamit30-1*, OR wildtype vs. *umamit30-1*) over the course of growth (24 hours). The test statistic (mean *t*) is the two-sample *t*-statistic to compare the OD_{600} values between the two groups at each hour, averaged over the course of growth (24 hours). A P-value was obtained for the test statistic by simulation. Results from three independent experiments are presented.

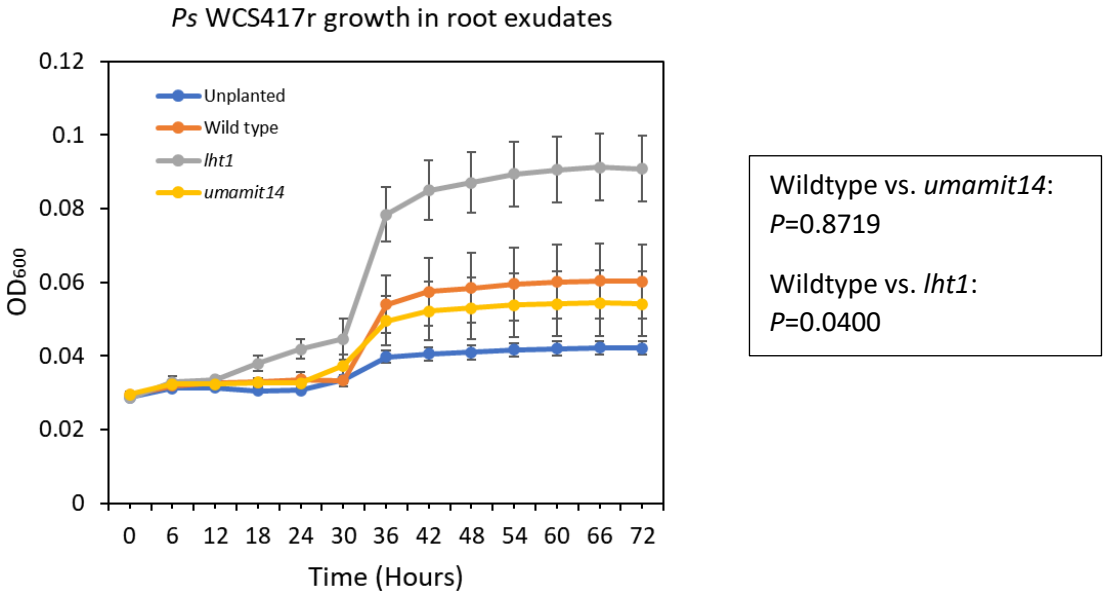


Fig. 4.11: *Ps* WCS417r growth in wildtype, *lht1*, and *umamit14* root exudates, as well as in unplanted control. *Ps* WCS417r cells from overnight culture were washed three times in sterile water and resuspended in sterile water to OD_{600}=0.2. Ten (10) µL was added to 90 µL of the unplanted growth medium (unplanted) or to 90 µL wildtype root exudates or 90 µL *lht1* root exudates or 90 µL *umamit14* root exudates, to a final OD_{600}=0.02. The test samples were filter-sterilized through a 0.22 µm-filter before use. Growth was measured over 72 h with intermittent shaking, in the microplate reader SpectraMax® i3x (Molecular Devices). Data are average ± SE (*n*=6 biological replicates x 2 = 12 replicates). Statistical analyses were performed using 'Compare Groups of Growth Curves' method as previously described[146]. In brief, a statistical permutation test was used to compare pairs of samples (e.g., wildtype vs. *umamit14*) over the course of growth (72 hours). The test statistic (mean *t*) is the two-sample *t*-statistic to compare the OD_{600} values between the two groups at the sampled time-points, averaged over the course of growth (72 hours). A P-value was obtained for the test statistic by simulation.

To further examine whether decreased root AA exudation could impact beneficial microbe-mediated plant growth, I treated the roots of both *umamit14* and *umamit30* mutants, along with the wildtype, with *Ps* WCS417r cells. I found that *Ps* WCS417r-mediated plant growth was not impaired in either the *umamit14* or *umamit30* mutants (Fig. 4.12). These results indicate that impaired root AA exudation, at least under the conditions tested, is not sufficient to abrogate *Ps* WCS417r-mediated plant growth.

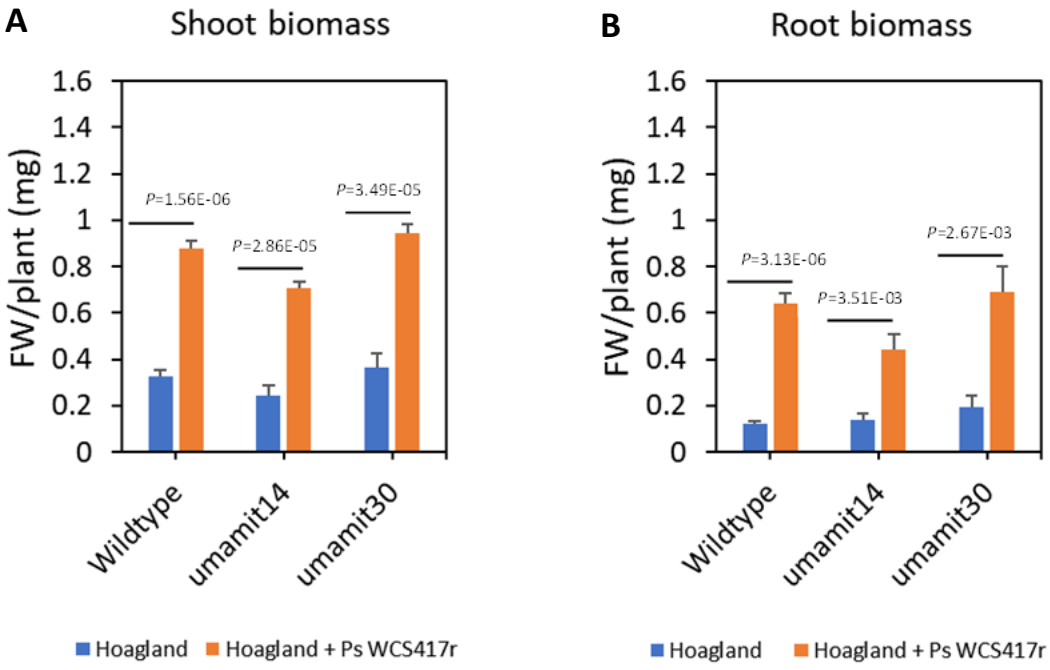


Fig. 4.12: ***Ps* WCS417r-mediated Arabidopsis growth is intact in *umamit14* and *umamit30* mutants** (**A**) Shoot fresh weight of 12-day old wildtype, *umamit14,* and *umamit30* seedlings under Hoagland-only and Hoagland + *Ps* WCS417r combination treatments. (**B**) Root fresh weight of 12-day old wildtype, *umamit14,* and *umamit30* seedlings under Hoagland-only and Hoagland + *Ps* WCS417r combination treatments. Data are averages ± SE of biomass from all seedlings per plate pooled together. Twelve (12) seeds were sown per plate for *n*=5 plates. Two-sided Student's *t*-test was used for pairwise comparison of means. Hoagland = half-strength. *Ps* WCS417r OD_{600} = 0.2. Experiment was performed twice with similar results.

4.3 DISCUSSION

The identities of the long-sought amino acid transporters that contribute to amino acid presence in root exudates of Arabidopsis are only now beginning to emerge. Following the isolation and characterization of SiAR1/UMAMIT18 as the first transport protein with export properties in Arabidopsis[117], UMAMIT14, expressed in the root pericycle and phloem cells of Arabidopsis, was recently shown to contribute to phloem unloading of amino acids, and thus may facilitate root-to-soil amino acid transport[59]. However, amino acid composition of *umamit14* mutants is only modestly depleted, compared with the wildtype exudates, suggesting that several other amino acid export proteins might be expressed in the root tissues.

Based on publicly available data showing the strong expression of *UMAMIT30* in Arabidopsis root tissues, I hypothesized that *UMAMIT30* is an additional root-to-medium amino acid exporter. Using a basic root exudate screening strategy, I found significant and reproducible decrease in the amino acid concentration of *umamit30* mutant root exudates (Fig. 4.3). Assuming that this decline is specifically due to loss of UMAMIT30 function in root tissues, amino acids would be expected to be trapped in the *umamit30* mutant root tissues. However, I found, through LC-MS analysis of amino acid content of root and shoot extracts, that amino acids instead appear to modestly accumulate in the shoot tissues of *umamit30* mutants (Fig. 4.8). In *Ricinus communis*, for example, a substantial amount of the amino acids delivered to the roots are redirected to the ascending xylem sap[199]. This leaves open the possibility that even if amino acids were to accumulate in *umamit30* root tissues, homeostatic mechanisms might ensure the

redistribution of the accumulating amino acids, as previously speculated for amino acid distribution in *umamit14* mutants[59].

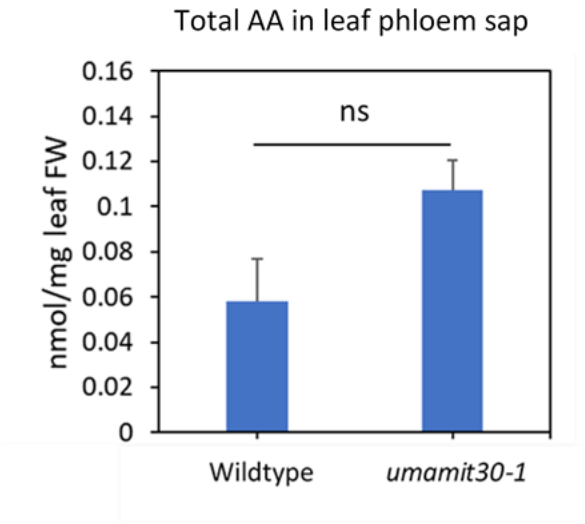
However, a complementary assay to study the uptake and secretion of radiolabeled glutamine by wildtype and *umamit30* root and shoot tissues showed that *umamit30* shoots are compromised for glutamine secretion (Fig. 4.9). Because the radiolabeled glutamine secretion capacity of the roots could not be conclusively determined from these experiments, these results indicate, at least in the interim, that the root exudate amino acid composition depletion phenotype of the *umamit30* seedlings may be due to a defect in shoot-to-root transport of amino acids, as well as to a potential defect in root-to-medium export of amino acids. Future experiments would help clarify the extent to which a defective root-to-medium export contributes to this phenotype.

Because soil-resident microbes are attracted by root exudate metabolites including sugars and amino acids, I tested the hypothesis that the depletion of amino acids in *umamit30* mutant root exudates (as well as in *umamit14* root exudates) may impair the growth of the rhizobacteria *Ps* WCS417r cells in these root exudates, compared to their growth in the wildtype root exudate. *Ps* WCS417r growth was generally not affected, with only one in three experiments showing that *umamit30* root exudates could impair bacteria growth (Fig. 4.10), similar to the observation that *umamit14* root exudates do not impair *Ps* WCS417r growth (Fig. 4.11).

This result is not necessarily surprising, given that, first, there is no known evidence that *Ps* WCS417r has an amino acid auxotrophy, and is therefore expected to be a general feeder. Second (and directly related to the first), both *UMAMIT30* and *UMAMIT14* mutations (considered individually) do not completely deplete root exudates of amino

acids, and affect nearly all amino acids similarly (suggesting that these transporters have a broad affinity for several amino acids) (e.g., Fig. 4.4). Thus, *Ps* WCS417r growth may not be significantly impaired under the growth conditions tested. Indeed, if maintenance of beneficial interactions with soil-resident microbes is a function of root exudate components such as amino acids, it would not be expected that loss of a single amino transporter would completely deplete root exudates of amino acids. Similarly, *Ps* WCS417r-mediated Arabidopsis growth was unaffected in both *umamit14* and *umamit30* mutants under my experimental conditions (Fig. 4.12). Thus, future experiments could consider testing the effect of root exudates from double and triple *UMAMIT* mutants (e.g., *umamit14-umamit18* and *umamit14-umamit18-umamit30*) on the growth of *Ps* WCS417r cells. These future experiments should also evaluate the *Ps* WCS417r-mediated plant growth in these double and triple *UMAMIT* mutants. Nevertheless, the results herein presented, in this Chapter, contribute to our knowledge of the identities of the amino acid export proteins that affect the amino acid composition of Arabidopsis root exudates.

4.4 SUPPLEMENTARY FIGURE(S)



Supplementary Fig. S4.1: Analysis of leaf phloem sap of 6-week-old wildtype and *umamit30-1* plants reveals no significant difference between AA concentrations in these samples (P>0.05; Two-sided Students *t*-test). Data are mean ± SE; *n*=5. Leaf phloem saps were collected from three source leaves per plant for *n*=1 into 5 mM EDTA solution (pH 7.0) and analyzed for AA content. AA content was normalized by fresh leaf weight. Experiment was performed three times with similar results. (See Materials and Methods for details.)

MATERIALS AND METHODS

Plant Growth

For all experiments, wildtype plants were raised from Col-0 seeds, and all mutants utilized were derived from the Col-0 background. Seedlings and adult plants were raised as below:

Seedlings: For all seedling experiments, seeds were surface-sterilized using 10% bleach three times for two minutes each, followed by three washes with sterile water, and subsequently resuspended in 1% phytoagar and stratified in the dark at 4 ºC for at least two days. Seeds were then plated onto 1x MS agar plates (100mm x 100mm square plates; Fisher Scientific; Cat#FB0875711A), composed of 4.4 g/L Murashige and Skoog Basal Medium with Vitamins (PhytoTech; M519), 0.5% sucrose (Sigma; S7903), 0.5 g/L MES (Sigma; M8250), and 0.7% PhytoAgar (PlantMedia; Cat#40100072-2), pH 5.7 and the plates were sealed with parafilm and incubated vertically in a reach-in growth chamber (Conviron Adaptis 1000, Canada) at 25 ± 2 ºC, 75% RH, 16h Light/8h Dark, and 100 µmoles/m^2/s light intensity for two weeks. Uniformly growing seedlings were then selected and transferred to autoclaved 3MM paper cut to fit 100mm x 100 mm square plates and wetted with 5 mL 0.5x MS medium without sucrose, pH 5.7. These plates were incubated horizontally under the same conditions as above. One day was allowed for seedlings to stabilize and then the appropriate treatment applied, which for many experiments included inoculation of wildtype roots by wetting the entire paper with 2 mL sterile water (for mock), or 2 mL *Pseudomonas simiae* WCS417r (OD_{600}=0.2), or inoculation of

wildtype and mutant roots with 2 mL *Pseudomonas simiae* WCS417r (OD_{600}=0.2). Other variations of this assay are described in the figure legends, as appropriate.

Adult plants: For adult plant assays, stratified seeds were sown in peat pellets (Jiffy-7, Jiffy Products Ltd, Shippagan, Canada), 4-5 seeds per pellet, in trays, and covered with a translucent plastic dome to maintain high humidity. These were transferred to Growth Chambers with controlled conditions at 25 ± 2 ºC, 75% RH, 9h Light/15h Dark, and 100 µmoles/m^2/s light intensity) for the next 6 weeks. One week after sowing the seeds, the domes were taken off and seedlings thinned out leaving 1-2 seedlings per pellet. A second thinning out was performed at the end of week two, leaving 1 seedling per pellet through the end of the experiment. Plants were watered with Hoagland's solution three times weekly.

Bacterial strain and growth conditions

Pseudomonas fluorescens WCS417r, also known as *Pseudomonas simiae* WCS417r which was initially isolated from lesions of wheat (*Triticum sativum*) roots[118] were maintained on LB plates supplemented with 50 µg ml^{-1} rifampicin. In preparation for root inoculation experiments, a single colony was randomly picked from the appropriate plate and grown overnight in approximately 100 mL of LB at 28 ºC with shaking at 230 rpm till the cultures reached OD_{600} = 0.4 – 0.8. The cell culture was harvested and washed three times in sterile water, and then adjusted to the required inoculation titer with sterile water.

Root exudate collection assays

For root exudate screening, root exudates were collected using a modification of a previously published method[111]. Briefly, Arabidopsis seedlings were grown initially in 1x

MS medium (i.e., full-strength) in 12-well plates (USA Scientific; Cat#CC7682-7512) containing 0.5% sucrose for 12 days, by placing ca. 5 seeds per well on an autoclaved mesh disc (McMaster-Carr; Cat#1100t41) sitting on top of the medium, and the medium changed to 0.5x MS medium (i.e., half-strength) containing no sucrose for 3 days, with the roots separated from the shoots by the autoclaved mesh. Root exudates were then collected and filter-sterilized through 0.22 µm filter for further downstream processing. Throughout the experiment, plates were sealed with parafilm and incubated in a reach-in growth chamber (Conviron Adaptis 1000, Canada) at 25 ± 2ºC, 75% RH, 16h Light/8h Dark, and 100 µmoles/m^2/s light intensity.

Plant tissue gene expression analysis (RT-qPCR)

Harvested tissues (roots/shoots/leaves) were frozen in dry ice. RNA was isolated using TRIzol® Reagent (Fisher Scientific; Cat#15596018.) and quantified in a NanoDrop-ND1000 spectrophotometer (Thermo Fisher Scientific Inc.). Two (2) µg of total RNA was used in 1st strand cDNA synthesis after DNaseI treatment. The 2 µg RNA was incubated together with 2 µl Random Decamers and appropriate volume of DEPC-treated water to a final volume of 15 µl at 70 ºC for 5 minutes, and removed to ice immediately. Subsequently, cDNA synthesis reaction mixture containing 10 mM dNTPs (2 µl), Promega RNase inhibitor (1 µl), Promega M-MLV-RT (1 µl), 5x Promega M-MLV Buffer (5 µl) and DEPC-treated water (1 µl) was added, to a final volume of 25 µl and incubated at room temperature for 1 minute and then at 37 ºC for 60 minutes. For qPCRs, 1 µl of cDNA was used in a total reaction volume of 20 µl containing 10 µl SyBr Green mix, 2 µl of 5 µM primers (forward and reverse), and 7 µl DEPC-treated water. There were two technical

replicates per biological replicate. The reactions were performed using the ABI 7500 and 7500 Fast Real-Time PCR system v2.3

Analysis of leaf phloem sap

Three uniformly growing source leaves per plant were sampled (for n=1) by cutting the leaves at the base of the petiole in a solution of 10 mM EDTA solution (pH 7.0). The leaves were placed on top of each other in the same orientation, and the petioles, pooled together, were rinsed in 5 mM EDTA solution (pH 7.0). Then, the petioles were immersed in 400 µl of 5 mM EDTA solution (pH 7.0) in a microtube. The set-ups were incubated for 6 h in the dark at 90-100% RH. The leaves were subsequently removed and weighed, and the solution analyzed for total amino acids using the L-Amino Acid Quantitation Colorimetric/Fluorometric Kit (BioVision, Catalog #K639-100) following manufacturer's instructions. Leaf petiole exudation as a proxy for leaf phloem loading was then informed as nmol/mg leaf FW. Assay was a modification of the protocol reported by Araya et al. (2015)[200].

Genetic analysis of *umamit* mutants

Genotyping:

Insertion alleles for the *UMAMIT30* gene were identified in the Salk Arabidopsis T-DNA Insertion Mutant Collection[201]. The *umamit30-1* mutant corresponds to Salk_140547C, and genotyping was performed using LBb1.3 and the following gene specific primers: Forward, 5'-GCATTGAAGCGTACCAAAGAC-3', and Reverse 5'-TTCTTGATGGAGGCATCAATC-3' (LBb1.3+Reverse yields the *umamit30-1* insertion-specific product). The *umamit30-2* mutant corresponds to Salk_146977C, and genotyping

was performed using LBb1.3 and the following gene-specific primers: Forward, 5'-GAAATATTGCATCAAGCTCGC-3', and Reverse, 5'-CCTTCCACCGAGAAAAACAG-3' (LBb1.3+Reverse produces the *umamit30-2* insertion-specific product). Primers were used at a final concentration of 10 µM. Total volume of 20 µL reaction was performed using Quick-Load® Taq 2x Master Mix (New England BioLabs) (10 µl), primer pairs (1 µl), genomic DNA (2 µl), and DEPC-treated water (7 µl). For genotyping each line, an additional control PCR was performed using wildtype genomic DNA, as well as a blank reaction in which DNA template was replaced with DEPC-treated water. PCR conditions are as indicated below:

Step 1: Initial denaturation at 94 ºC for 5 mins; Step 2: Denaturation at 94 ºC for 30 s; Step 3: Annealing at 55 - 56 ºC for 30 s; Step 4: Extension at 68 ºC for 1 min; Step 5: Final Extension at 68 ºC for 5 mins. Steps 2-4 were repeated for a total of 35 cycles; Step 6: Hold at 12 ºC. PCR products were resolved on 1% (w/v) ethidium bromide-stained agarose gel run at 100V. Gels were visualized using the Spectroline UV transilluminator Select™ Series, and images obtained via the AlphaImager 2200.

RT-PCR:

Roots/shoots/leaves harvested from wildtype and *umamit30* mutants as well as *umamit14* and *umamit05* mutants were frozen in dry ice. RNA was isolated using TRIzol® Reagent (Fisher Scientific; Cat#15596018.) and quantified in a NanoDrop-ND1000 spectrophotometer (Thermo Fisher Scientific Inc.). Two (2) µg of total RNA was used in 1st strand cDNA synthesis after DNaseI treatment. The 2 µg RNA was incubated together with 2 µl Random Decamers and appropriate volume of DEPC-treated water to a final volume of 15 µl at 70 ºC for 5 minutes, and removed to ice immediately. Subsequently,

cDNA synthesis reaction mixture containing 10 mM dNTPs (2 µl), Promega RNase inhibitor (1 µl), Promega M-MLV-RT (1 µl), 5x Promega M-MLV Buffer (5 µl) and DEPC-treated water (1 µl) was added, to a final volume of 25 µl and incubated at room temperature for 1 minute and then at 37 ºC for 60 minutes. For PCR analysis, 2 µl of cDNA was used in a total reaction volume of 20 µl containing 10 µl Quick-Load® Taq 2x Master mix, 1 µl of 5 µM primers (forward and reverse), and 7 µl DEPC-treated water. Primer sequences for the RT-PCR analyses are shown in Supplementary Table S4.1. A "no template control" (NTC) PCR was performed in which cDNA template was replaced with DEPC-treated water. PCR conditions are as indicated below:

Step 1: Initial denaturation at 94 ºC for 5 mins; Step 2: Denaturation at 94 ºC for 30 s; Step 3: Annealing at 56 ºC for 30 s; Step 4: Extension at 68 ºC for 1 min; Step 5: Final Extension at 68 ºC for 5 mins. Steps 2-4 were repeated for a total of 35 cycles; Step 6: Hold at 12 ºC. PCR products were resolved on 1% (w/v) ethidium bromide-stained agarose gel run at 100V. Gels were visualized using the Spectroline UV transilluminator Select^{TM} Series, and images obtained via the AlphaImager 2200. Additional details for the RT-PCR analyses are indicated in the respective figure legends, as appropriate.

Amino acid uptake and secretion in planta

Arabidopsis seedlings were grown for two weeks using the root exudate collection assay described above, in a full-strength Murashige and Skoog (MS) medium supplemented with 0.5% sucrose. Roots and shoots were separated and each sample was transferred into 1-ml full-strength MS medium supplemented with 0.5% sucrose in 12-well plates, for 5 h under agitation on a rocking surface (8 rpm). Amino acid uptake was performed for

20 min in a solution containing 1 ml of full-strength MS medium supplemented with 0.5% sucrose and 100 µM glutamine + [^{3}H]Gln with a final specific activity of 3.7 kBq µmol–1. Efflux was performed for 20 min. Radioactivity in the root and shoot samples were then determined using a 1450 Microbeta Trilux Liquid Scintillation and Luminescence counter (Perkin-Elmer™ Life Sciences).

Amino acid extraction for LC-MS analysis

Plant material was collected in 2 mL Eppendorf tube (round-bottom), and lyophilized overnight in the lyophilizer (LABCONCO). A defined biomass was then weighed into a new Eppendorf tube, frozen in liquid nitrogen and homogenized with the tissue lyser (TissueLyser II, Qiagen) for 3 minutes at 15 1/s. Next, 700 µl of 100% methanol was added to each sample, and vortexed briefly. The samples were subsequently shaken for 15 minutes at 4 ºC with the tubes opened approximately 2 minutes after start of incubation to release the pressure built up in the tubes. Centrifugation was carried out at approximately 14000 rpm for 10 minutes, and the supernatant transferred to a new tube. Next, 375 µl of chloroform was added to each supernatant, plus 750 µl of water (Nanopure). The samples were vortexed for 15 s each, and then centrifuged for 15 minutes at 4000 rpm. The entire upper phase (polar phase) was collected into new Eppendorf tubes and then dried using the Savant DNA120 SpeedVac Concentrator (Thermo Electron Corporation). These extracts were stored at -80 ºC until LC-MS analysis.

Statistical analysis

Data analyses were performed using the JASP open-source software v 0.14 and Excel, and graphs generated using Excel. A two-sided Student's *t*-test was performed for statistical comparison of two means, or a Welch's *t*-test for two means with unequal variances, when relevant. For comparison of more than two means, a one-way ANOVA followed by Tukey's posthoc test, or a Kruskal-Wallis test for unequal variances followed by Dunn's posthoc test was performed, as indicated in the relevant figure legends.

For statistical analysis of bacterial growth curves, the CGGC (Comparison of Groups of Growth Curves) permutation test[146] was used to compare pairs of samples (e.g., wild type vs. *umamit30*) over the course of growth (e.g., 24 hours). The test statistic (mean *t*) is the two-sample *t*-statistic to compare the OD_{600} values between the two groups at each hour, averaged over the course of growth (e.g., 24 hours). A P-value was obtained for the test statistic by simulation. Samples were randomly allocated to each of the two groups and the mean *t* was recalculated for 10 000 data sets generated through this permutation. The P-value is the proportion of permutations where the mean *t* is greater in absolute value than the mean *t* for the original data set. (That is, the number of times the absolute mean *t* from the permutations is greater than the absolute mean *t* for the original data set, all divided by 10 000.) As always, this P-value was interpreted as the probability of obtaining the mean *t* obtained for the original data set if the null hypothesis was true.

Supplementary Table S4.1: List of primers used

Primer Name	**Sequence (5' → 3')**
GENOTYPING	
SALK Border Primer (LBb1.3)	ATTTTGCCGATTTCGGAAC
SALK_140547C-F	GCATTGAAGCGTACCAAAGAC
SALK_140547C-R	TTCTTGATGGAGGCATCAATC
SALK_146977C-F	GAAATATTGCATCAAGCTCGC
SALK_146977C-R	CCTTCCACCGAGAAAAACAG
RT-PCR	
UT30-RT-PCR-F1 (SALK_140547C)	CGATGGAAAGTGGGCACCAATG
UT30-RT-PCR-R1 (SALK_140547C)	AACGACGGTGATGATGGTAAGC
UT14-RT-PCR-F1 (SALK_037123)	GGCCTCGAGATCACAACACT
UT14-RT-PCR-R1 (SALK_037123)	TCAGGGATTGGTTACTACGTTCA
UT05-RT-PCR-F1 (GK-799H03)	GCGTAGAGGATGGTGAAAAGC
UT05-RT-PCR-R1 (GK-799H03)	ACAGAAGAGACGGTATCTCCAA
GENE EXPRESSION (RT-qPCR)	
ACT2-U4	GTACGGTAACATTGTGCTCAGT
ACT2-L4	GAGATCCACATCTGCTGGAATGT
UT30-P3-F (SALK_140547C)	AGGGCGAGCTTGATGCAATA
UT30-P3-R (SALK_140547C)	ATGGCCCAGAAAGCAGATCC

Chapter 5:

General Discussion, Conclusions, and Outlook

Past and ongoing work on plant—beneficial microbe interactions have significantly enhanced our understanding of the ways in which plants benefit from the soil microbiota, evidenced by the preservation of rhizodeposition of metabolites into the soil by nearly all plant species[202] despite this representing a clear cost for the plants. As has been thoroughly discussed elsewhere in this dissertation, plant-associated microbes contribute to plant adaptation to biotic and abiotic stresses. However, a more complete understanding of the plant host genetic factors facilitating plant—beneficial microbe interactions is warranted in the face of continuing challenge to plant growth and health, challenges that are likely going to be amplified by effects of climate change and human population growth.

Previous reports have documented the effects of specific plant secondary metabolites such as flavonoids and strigolactones in mediating the interactions between plants and the soil microbiota[203,204]. Additionally, the effects of plant-produced primary metabolites such as sugars and amino acids in sustaining the beneficial interactions whereby these metabolites may serve as nutrients, or signaling cues, have been documented across many plant species. A long-standing question as to whether the presence of amino acids in root exudates occurs as a simple, unregulated passive process, has been challenged by several observations including that, plants express and regulate amino acid transporters that modify amino acid concentrations in the different compartments of the plant itself. Yet, whether specific amino acid transporters have an outsized effect on root exudation in the rhizosphere, either by contributing to the export of amino acids from root tissues or by being involved in the retrieval of root-secreted amino acids and thereby

affecting the overall accumulation of amino acids in the rhizosphere have been speculated but generally not well-documented.

In this dissertation, I showed that the Arabidopsis amino acid transporter LHT1 contributes to the retrieval of root-secreted amino acids back into the plant, suggesting that amino acids deposited into the soil by plants are not irretrievably lost. While soil microbiota and other organisms in the rhizosphere may benefit from root-secreted amino acids, the observation that plants could actually take back some of the amino acids already secreted suggests that plants may be competing with the soil microbiota for amino acids, or may be monitoring amino acid utilization by the soil microbiota to adjust the concentrations in response to the need to avoid the accumulation of amino acid metabolic by-products that may negatively affect plant health.

While it is known that plant roots compete with the soil microbiota for exogenously supplied amino acids[90], the hypothesis that the root-secreted amino acid re-uptake mechanism mediated by transporters such as LHT1 may enable plants to avoid the accumulation of potentially toxic by-products of amino acid metabolism in the rhizosphere remains to be tested. In a preliminary experiment, I exposed Arabidopsis wildtype roots to increasing doses of the beneficial rhizobacteria *Ps* WCS417r, to examine whether the expression of the LHT1 transporter gene may increase with increasing bacterial dose, with the idea that root-reuptake of root-secreted amino acids from the rhizosphere via LHT1, for example, may function in limiting bacterial overgrowth in the rhizosphere. However, *LHT1* expression did not significantly change with increasing *Ps* WCS417r dose. Possibly, because plant roots are already exposed to large numbers of bacteria in

the rhizosphere, using LHT1 transporter expression to control bacterial numbers may be a rather high-energy costing activity for the plant.

Instead, I propose that plants may monitor the accumulation of by-products of plant-derived amino acid metabolism from rhizosphere-dwelling bacteria, as a way to regulate amino acid availability in the rhizosphere. Consistent with this hypothesis, treating Arabidopsis wildtype roots with a combination of a relatively high glutamine concentration (i.e., 10 mM Gln) and *Ps* WCS417r or the pathogenic bacteria *Pst* DC3000 ultimately inhibited Arabidopsis root and shoot growth, even though under my experimental conditions, only the *Ps* WCS417r numbers were boosted by the glutamine supplementation. This suggests that bacteria-mediated inhibition of plant root and shoot growth which may occur in response to amino acid accumulation in the rhizosphere may occur independently of bacteria growth. Thus, plants may ensure their own fitness not necessarily by monitoring bacterial overgrowth in the rhizosphere, but perhaps by detecting changes in the expression levels of bacteria virulence machinery which may increase in response to high concentrations of specific amino acids, or by monitoring the accumulation of amino acid metabolism by-products from the microbiota, which may negatively affect plant growth and health (See overall proposed model: Fig. 5.1).

Indeed, it is known that microbial associations with wildtype plant tissues may be under genetic regulation, and that alterations leading to an imbalance to the numbers and relative abundances could dramatically affect plant health[205,206]. In a recent report, it was shown that the receptor kinase FERONIA (FER) in Arabidopsis played a role in limiting the number of *P. fluorescens* WCS365 cells associated with Arabidopsis roots via regulation of reactive oxygen species. Loss of the FER function increased the number of

WCS365 cells colonizing root tissues. Interestingly, while *fer-8*, a *FER* loss of function mutant, looked stunted, compared to the wildtype, this stunting phenotype was specifically due to the loss of FER function, as microbiome transfer experiments in which *fer-8*-grown soils (i.e., natural soil in which *fer-8* was grown) were subsequently used to grow wildtype plants conferred growth benefits to these wildtype plants, due to the enrichment of the *fer-8*-soil for beneficial pseudomonads.[207] These suggest that enhanced colonization of root tissues, per se, by beneficial Pseudomonads may not be problematic.

Nevertheless, the dose-dependent effects of *Ps* WCS417r-mediated plant growth as documented in this dissertation indicates that there is not just a single optimum dose, but several optimum doses of *Ps* WCS417r which promote plant health, beyond which plant health will be negatively impacted (Fig. 2.2). Of note, FER restriction of Pseudomonad growth is not only mediated by FER-8, but by other FER proteins, as FER-4 loss of function mutant *fer-4* is also enriched for pseudomonads to levels similar to those found in *fer-8*, while fer-5 is enriched only modestly because it is a partial loss-of-function allele[208]. It is thus conceivable that simultaneously deleting these FER proteins might push WCS365 numbers to levels beyond the enrichment magnitude associated with *fer-8* roots, and possibly damaging plant health. Regardless, it would remain a goal for the future to determine whether plant root-expressed amino acid importers such as LHT1 may be upregulated in response to increasing bacterial numbers in the rhizosphere under some specific growth conditions in order to regulate amino acids accumulation in the rhizosphere to prevent microbial overgrowth.

As clearly shown by the results of this study, LHT1 contributes to the capacity of Arabidopsis to re-uptake amino acids secreted by the roots. I have provided evidence here that Arabidopsis UMAMIT30 which has recently been shown to contribute to amino acid export when expressed in yeast cells is involved in contributing to root exudation of amino acids, as loss-of-function *umamit30* mutants contain less amino acids in their root exudates. As would be expected, the loss of this single amino acid transporter is not sufficient to considerably alter root exudate amino acid concentrations such that the root—beneficial bacteria *Ps* WCS417r interactions is negatively affected; growing *Ps* WCS417r in wildtype and *umamit30* root exudates reveals no significant impact of the *UMAMIT30* mutation on *Ps* WCS417r growth (Fig. 4.10).

Amino acid secretion from the roots may not occur uniformly along the root length, as Trp secretion from *Avena barbata* roots, for example, peaks at regions close to the root tip[49]. Thus, it remains to be determined whether bacterial growth on *umamit30* root tissues is spatially distinct from their growth on wildtype root tissues, and how or if the spatially-distinct *Ps* WCS417r distribution along the roots might alter how the plant copes with pathogen attack of the root tissues. It would be noted that an important ecological function of beneficial rhizobacteria is to outcompete pathogens for niche, and that ultimately, differential amino acid exudation along the root tissues may modify these interactions, and hence impacting plant adaptation and fitness.

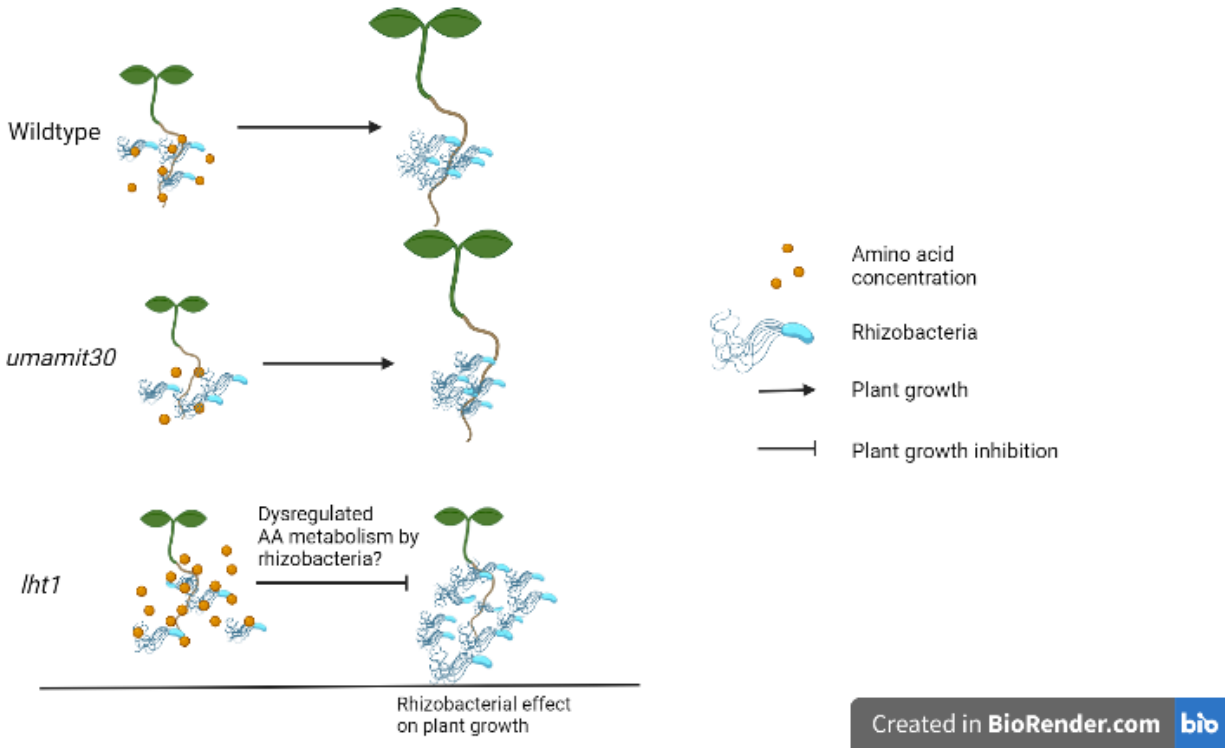


Fig. 5.1: Proposed model showing how plant-derived amino acid (AA) concentration in the rhizosphere as modulated by amino acid transporter mutations (*umamit30* and *lht1*) may impact rhizobacterial-mediated plant growth. Elevated amino acid concentration may impair rhizobacterial-mediated plant growth via yet-to-be-determined mechanisms.

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