Regulation of plant amino acids metabolism and transport by a bacterial pathogen

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ABSTRACT

Plant immune responses to invading bacteria are initiated by the recognition of Pathogen-Associated Molecular-Patterns (PAMPs) such as bacterial flagellin in the leaf apoplast. Pathogenic bacteria have evolved virulence mechanisms that suppress plant immunity, a necessary condition to produce infections. *Pseudomonas syringae*, an important bacterial pathogen that infects leaves of several crops as well as the model plant *Arabidopsis* (*Arabidopsis thaliana*), suppresses plant immunity in part through the secretion of the small molecule coronatine (COR). It is well documented that bacterial COR mimics the plant-made intracellular signaling molecule jasmonic acid, whose activation suppresses plant defense signaling initiated by PAMPs and by the plant-made intracellular signaling molecule salicylic acid. In this study, I investigated the mechanisms by which COR promotes bacterial growth in *Arabidopsis* beyond its known function as a suppressor of salicylic acid- and PAMP-induced signaling. Since *P. syringae* colonizes the leaf apoplast, changes in extracellular concentrations of amino acids have a profound impact on bacterial infections (Zhang et al 2023). While the elicitation of PAMP-triggered immunity (PTI) induces an early increase and a late decrease in extracellular concentrations of several amino acids, including Gln and Ser, in *Arabidopsis* seedlings (Zhang et al 2022), I found that COR counters the effect of flagellin by promoting the late increase of extracellular concentrations of Glu and Asp, among other amino acids.

Furthermore, I unveiled that an intact jasmonic acid signaling pathway is required for COR to drive these changes, and that salicylic acid signaling suppression is not required. Gene expression analyses of *Arabidopsis* amino acid transporters (AATs) in seedlings revealed that COR induces the expression Usually Multiple Acids Move In and out Transporters (UMAMITs) family of AATs, suggesting that some of these transporters would contribute to

the late increase in extracellular amino acids concentrations observed in COR-treated *Arabidopsis* seedlings. In a reverse genetic screening of *Arabidopsis* loss-of-function mutants for COR-responsive AATs, I found that three COR-induced AATs, *UMAMIT18*, *UMAMIT21*, and *UMAMIT42*, are necessary for the late increase in extracellular concentrations of amino acids in response to COR in *Arabidopsis* seedlings. These data suggest that, while AATs that facilitate the late export of amino acids would contribute to producing robust bacterial infections, AATs that contribute to the late uptake of amino acids would suppress bacterial infections. Bacterial infection *in planta* showed that *umamit18*, *umamit21*, and *umamit42* single mutants were as susceptible to *P. syringae* infections as wild-type plants, suggesting that functional redundancy may compensate for the loss of function of any one of these genes individually. On the other hand, the loss-of-function for the COR-suppressed amino acid importers *AAP6* (*Amino Acid Permease 6*) and *LHT7* (*Lysine Histidine Transporter 7*), produced enhanced susceptibility against *P. syringae* infections suggesting that these genes play a role in suppressing bacterial infections by lowering the concentration of amino acids in the leaf apoplast. Moreover, *aap6* failed to load amino acids into the phloem and was compromised to transport amino acids away from the infected leaves to distal tissues, a response associated with the restriction of *P. syringae* growth in wild-type plants. Further analysis revealed that the expression of other AAP genes was downregulated in *aap6*, suggesting that the *aap6* phenotypes result from the concerted activity of several members of the AAP family of importers with partially overlapping functions. These studies demonstrate that COR plays a major role in extracting amino acids from the host, an important function at late stages of infections, when leaf apoplast colonizing bacteria have already suppressed plant immunity and require a readily usable source of carbon and nitrogen to support fast growth.

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LIST OF ABBREVIATIONS/ACRONYMS

AA	Amino Acid
AAP	Amino Acid Permease
AAT	Amino Acid Transporter
ABA	Abscisic Acid
ABC	ATP-binding cassette
ABRC	Arabidopsis Biological Resource Center
ACT2	Actin 2
AHA	Arabidopsis H+-ATPase
ANT	Aromatic and neutral amino acid transporter
APC	Amino acid, polyamine, and choline transporter superfamily
ASN	Glutamine-dependent asparagine synthase
ATP	Adenosine Triphosphate
AUX	Auxin resistant
AWF	Apoplasmic washing fluid
BSMT1	Benzoic/salicylic acid carboxyl methyltransferase
CAB1	Chlorophyll A/B binding protein 1
CAT	Cationic amino acid transporter
CFA	Coronafacic acid
CFL	Coronafacate ligase
CFU	Colony forming unit
CMA	Coronamic acid
COI1	Coronatine insensitive 1
Col	Columbia ecotype
COR	Coronatine
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Etheylene-diamine-tetra acetic acid
ET	Ethylene

ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
FLS2	Flagellin-sensitive 2
G6PD	Glucose-6-phosphate dehydrogenase
GABA	Gamma-aminobutyric acid
GAT	Gama-aminobutyric acid transporter
GFP	Green fluorescent protein
GLN1	Glutamine synthase 1
HCL	Hydrochloric acid
HAI	Hours after infection
HPE	Hours post elicitation
HR	Hypersensitive reaction
ICS1	Isochorismate synthase
JA	Jasmonic acid
JAT1	jasmonic acid transporter 1
JAZ	Jasmonate zim-domain repressor
KOH	Potassium hydroxide
LAP2	Luecyl aminopeptidase 2
LAT	L-type amino acid transporter
LB	Luria Broth
LHT	Lysine histidine transporter
LRR	Leucine rich repeat
MAMP	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinases
MED25	Mediator 25
MES	2-(N-morpholino) ethane sulfonic acid.
MS	Murashige and Skoog
MTI	MAMP-triggered immunity
PCR	Polymerase chain reaction
RT-qPCR	Reverse-Transcription coupled with quantitative PCR

PR1	Pathogenesis-related gene 1
PTI	PAMP triggered immunity
RBCS	Rubisco small subunit
RCA1	Rubisco activase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SWEET	Sugars Will Eventually Be Exported Transporter
T3E	Type-III effectors
T3SE	Type-III secreted effectors
T3SS	Type-III secretion system
TPL	Topless
UMAMIT	Usually multiple amino acids move in and out transporter
UPLC	Ultra Performance Liquid Chromatography
VSP2	Vegetative storage protein 2
WT	Wild type

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Chapter 1. Introduction

As the global human population continues to grow, so does the need for food. It is expected that by 2050, we will need an extra 200,000 billion calories per year to feed a projected population increase of 2.2 billion people. The problem of limited dietary supply in relation to increasing demand is a complex issue that is influenced by a variety of factors, including plant pathogens, farming practices, the use of chemical fertilizers, and climate change. Plant pathogens significantly threaten global food security as they can affect production, quality, and nutritional value. It is estimated that pathogens account for crop yield losses of up to 20% of the annual world harvest (Savary et al., 2019). Thus, a better understanding and management of plant pathogens is essential to improve food security. In this chapter, I will summarize the mechanisms through which pathogens infect plants and how plants defend themselves from pathogens, with special attention to molecular mechanisms that allow microbes to obtain nutrients from plants as well as those that restrict microbial nutrition during infection.

1.1 Overview of Plant immunity

In nature, plants live in diverse and complex environments where they constantly encounter insect herbivores and pathogenic microbes with a wide array of lifestyles and infection strategies. Plants have evolved complex mechanisms to defend themselves against microbes. Plant immunity is a multi-layered defense system that involves both physical and chemical defense mechanisms. Plant pathogens that feed on living host cells are known as biotrophs, while pathogens that feed on dead plant tissue are known as necrotrophs. The first line of protection against microbial infection is provided by physical barriers such as the cuticle and the cell wall (Gabler et al., 2003). Plant pathogens may invade the tissue within leaves via pre-existing openings such as stomata and hydathodes (Melotto et al., 2008). Once inside the leaf,

bacterial pathogens colonize the water and nutrient-rich liquid that exists between mesophyll cells (i.e., the leaf apoplast) and ultimately impact plant biomass production, decrease fertility, or even kill infected plants (Abramovitch et al., 2006). Plants have evolved two strategies to detect invading microbes. The first strategy relies on membrane receptors that recognize conserved molecules that only exist in microbes, such as bacterial flagellin or fungal chitin, collectively known as Pathogen-Associated Molecular Patterns (PAMPs) (Boller, 1995). PAMP perception typically induces an enhanced state of resistance to microbes known as PAMP-Triggered Immunity (PTI). Flagellin, for example, binds the extracellular domain of the Flagellin-Sensitive 2 (FLS2) receptor and activates downstream intracellular signaling cascades (Gómez-Gómez & Boller, 2000) that protect the plant from subsequent infections (Zipfel et al., 2004). FLS2 activation with flagellin leads to the synthesis of reactive oxygen species (ROS), the closure of stomata, callose deposition, lignin synthesis, the production of enzymes that hydrolyze the bacterial cell wall (Melotto et al., 2006), and the synthesis of secondary metabolites thought to be toxic to invading bacteria (Clay et al., 2009). Flagellin 22 (flg22), a 22-amino acid long synthetic peptide, contains the minimal epitope of the native flagellin protein. This peptide functions as a PAMP and can be used to study PTI in plants without bacterial infection (Gómez-Gómez et al., 1999).

PTI is such a sensitive and effective inducible immune program that only microbes that have evolved the ability to suppress it can infect plants. Plant pathogens promote disease by secreting virulence effectors in the extracellular matrix or into the plant cells to suppress PTI (Hann & Rathjen, 2010). Pathogenic bacteria use the Type-3 Secretion System (T3SS) protein complex to deliver small proteins called Type-3-Effectors (T3Es) into host cells (Tang et al., 2006). These bacterial effectors can dampen plant immune response by targeting apoplastic or

surface-exposed plant components needed for PTI signaling. The second layer of defense involves the recognition of T3Es by plant intracellular receptors encoded by 'disease-resistance' (R) genes. These receptors consist of a Nucleotide-Binding domain (NB), a **L**eucine-Rich Repeat domain (LRR), and a Coiled-Coiled (CC) or a Toll-Interleukin Receptor (TIR) domain and are generally known as NLRs (Couto & Zipfel, 2016).The recognition of bacterial effectors by NLRs induces an enhanced state of resistance known as the Effector-Triggered Immunity (ETI) (Jones & Dangl, 2006). Activation of the ETI typically leads to cell death-mediated defense, the so-called hypersensitive response (HR), which kills the cells in contact with the pathogen and the pathogen with them. Plants that cannot detect bacterial effectors that suppress PTI are susceptible to infections. While PTI does not produce visible symptoms and is effective against non-adapted pathogens, ETI produces visible areas of dead tissue and is active against pathogens that suppress PTI and can initiate infections.

1.2 *Pseudomonas syringae* virulence

The gram-negative phytopathogenic bacteria *Pseudomonas syringae* (*P. syringae*) causes a broad range of plant symptoms, including leaf spots, blights, cankers, and wilting. (Alfano & Collmer, 1996). More than 60 pathovars exist, and each pathovar infects a group of host plants, including tomatoes, beans, cereals, kiwi fruits, and beans (Bull et al., 2010). *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) and *Pseudomonas syringae* pv. maculicola strain ES4326 (*Psm* ES4326) are two strains commonly used as model pathogens in laboratory research. Both *Pst* DC3000 and *Psm* ES4326 can induce defense responses in *Arabidopsis* (Dong et al., 1991) and are widely used in research to study the interaction between plants and bacteria (Xin & He, 2013). The genome of *Pst* DC000 was completely sequenced in 2003

(Buell et al., 2003), making it an ideal strain for *P. syringae* genetics research. There are two growth phases observed *P. syringae*, with strain-specific predominant phases: the epiphytic phase, during which bacteria colonize the leaf surface, and the endophytic phase, where bacteria enter the leaf and colonize the leaf apoplast (Xin & He, 2013). Both strains, *Pst* DC3000 and *Psm* ES4326 are endophytic strains and do not produce epiphytic infections. *P. syringae* generally utilizes two major virulence strategies to disrupt and suppress the host immune system. The first strategy involves injecting T3Es, small bacterial proteins that enter the host cytosol through the T3SS (Lindeberg et al., 2012). *P. syringae*'s T3SS encodes the Hrp pilus that facilitates the delivery of T3Es into the plant apoplast (Arnold et al., 2001). A functional genomic study found that at least 68 T3Es exist in the genome of the *P. syringae* across different strains (http://www.*Pseudomonas-syringae*.org). These T3Es disrupt early plant immune signaling, inhibit vesicle trafficking, suppress the detection of other effectors, and increase the availability of nutrients (Melotto et al., 2006). For example, AvrPtoB and HopF2 can interfere with MAPK signaling downstream of *BAK1*, an FLS2 adaptor protein needed for the flg22 perception by plant cells (Cheng et al., 2011). HopM1 can block the burst of ROS and stomatal closure in response to flagellin and promote the development of a water-rich apoplast favorable to bacterial growth (Lozano-Durán et al., 2014; Xin et al., 2016). HopZ3 can disrupt multiple components of plant immune complexes, making the plants more vulnerable to infection (Jeleńska et al., 2021). Another virulence strategy used by *P. syringae* is the secretion of secondary metabolites toxic to plants, commonly known as phytotoxins, that disrupt the host metabolism, thereby causing damage to host cells and even mimicking plant hormones to suppress immune programs (Bender et al., 1999; Geng et al., 2014). Coronatine,

tabtoxins, syringomycins, and syringopeptins are some examples of toxins produced by *P. syringae* pathovars (Ichihara et al., 1977; Kinscherf et al., 1991).

1.3 Overview of the Jasmonic Acid signaling pathway

Jasmonic acid (3-oxo-2-2′-cis-pentenyl-cyclopentane-1-acetic acid, abbreviated as JA) is a plant hormone that plays a critical role in regulating various plant processes, including growth, development, and responses to biotic and abiotic stresses. Bioactive oxylipins such as Isoleucine-conjugated Jasmonic Acid (JA-Ile) and methyl jasmonate (MeJA) regulate plant growth, development, and immunity (Devoto & Turner, 2003). Under normal conditions, JASMONATE ZIM-DOMAIN REPRESSOR (JAZ) nuclear proteins inhibit jasmonic acid responses. JAZ repressors interact with the F-box protein *COI1 (CORONATINE SENSITIVE-1)* containing SKP1-CULLIN1-F-box-type (SCF) E3 ubiquitin ligase. JAZ proteins recruit co-repressors, such as TOPLESS (TPL) and NINJA (Novel Interactor of JAZ), to repress the transcriptional activity of downstream basic helix-loop-helix (bHLH) transcription factors *MYC2*, *MYC3*, and *MYC4* (Dombrecht et al., 2007; Pauwels et al., 2010). Wounding, herbivory, and necrotrophic plant pathogens lead to the rapid synthesis of jasmonic acid via the octadecanoid pathway. A member of the G-subfamily of ABC transporters, the jasmonate transporter (*AtJAT1/AtABCG16*) facilitates the nuclear transport of JA-Ile (Li et al., 2017) where it binds to *COI1* and promotes the interaction between the *COI1* complex and JAZ repressor proteins (Katsir et al., 2008; Sheard et al., 2010). As a result of this interaction, JAZ proteins are ubiquitinated and degraded via 26S proteasome, releasing the suppression of *MYC2*. (Thines et al., 2007). *MYC2* then recruits the Mediator complex subunit *MED25* to initiate the transcription of jasmonic acid-responsive genes (Cevik et al., 2012).

Plant defense responses are generally determined by the balance between jasmonic acid and salicylic acid. When facing herbivores and necrotrophic pathogens, plants initiate JA synthesis and signaling. However, plants trigger salicylic acid synthesis and signaling when responding to biotrophic and hemibiotrophic pathogens (Geng et al., 2014). Based on studies with tobacco and *Arabidopsis*, it has been demonstrated that mutual antagonism is the primary form of interaction between these two signaling pathways. For instance, *P. syringae*-infected *Arabidopsis* plants activate the salicylic acid-signaling pathway at the expense of suppressing jasmonic acid signaling-mediated defense against necrotrophic pathogens (Spoel et al., 2007). In support of this observation, salicylic acid-deficient *Arabidopsis NahG* plants accumulate 25-fold more jasmonic acid than wild-type plants in response to *P. syringae* infection (Spoel et al., 2003).

1.4 Coronatine

Coronatine (COR) is a non–host-specific phytotoxin produced by pathovars (pv.) of *P. syringae* including *P. syringae* pv. tomato, glycinea, maculicola, atropurpurea, and morspronum (Bender et al., 1999; Ullrich et al., 1993). COR chemical structure presents two distinct moieties, a polyketide called coronafacic acid (CFA) and an isoleucine-derived ethyl-cyclopropyl-amino acid called coronamic acid (CMA) (Bender et al., 1999; Ichihara et al., 1977; Parry et al., 1996). The polyketide pathway synthesizes CFA from one butyrate, one pyruvate, and three molecules of acetate residues (Parry et al., 1994). Genes of the *cfa* operon are responsible for the subsequent modification (Gross & Loper, 2009). L-alloisoleucine, a diastereomer of L-isoleucine, is the precursor of CMA, produced by the adenylating domain of the non-ribosomal peptide synthetase CmaA (Rohde et al., 1998). In the last step of the

synthesis, coronafacate ligase (cfl) links CFA and CMA by an amide linkage (Bender et al., 1993; Liyanage et al., 1995). *Pst* DC3000 COR-deficient mutants that cannot produce CFA or CMA reveal that a complete COR molecule is needed for maximum pathogenicity (Brooks et al., 2004). COR mimics JA-Ile structurally and functionally (Sheard et al., 2010). The cyclopentanone ring of COR is a stereoisomer of the JA-Ile (Wasternack & Xie, 2010) capable of binding to the jasmonic acid nuclear receptor plant protein COI1 and eliciting jasmonic acid-like transcriptional changes (Zhao et al., 2003).

1.5 The mode of action of Coronatine

The critical first step of endophytic leaf colonization is the passage through the stomata. Recognition of PAMPs can lead to the closure of stomata to prevent the entry of *P. syringae* (Melotto et al., 2006). The stomatal closure triggered by PAMP is dependent on at least hormonal two pathways, Salicylic acid and Abscisic acid (Cummins et al., 1971; Khokon et al., 2011). Coronatine can inhibit the flg22 and ABA-induced stomatal closure in *Arabidopsis* (Toum et al., 2016). COR triggers stomatal reopening by inhibiting the activity of *Arabidopsis* H+pump-ATPase 1 (*AHA1*) and *AHA2* and stimulating K+ inflow into guard cells (Melotto et al., 2017). Recent studies have shown that COR produced by *Pseudomonas cannabina* pv. alisalensis can inhibit stomatal closure in oat and cabbage (Sakata et al., 2021). Application of COR or COR-producing *P. syringae* causes severe chlorosis in the leaf tissue (Brooks et al., 2005). Several genes that are involved in chloroplast metabolism and photosynthesis are repressed by COR or MeJA. These genes include *CHLOROPHYLL A/B BINDING PROTEIN1(CAB1) and RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN*

(RUBISCO)(Attaran et al., 2014; Qi et al., 2015). COR alone can induce anthocyanin accumulation in *Arabidopsis* (Feys et al., 1994).

As COR is a mimic of JA-Ile, it activates the jasmonic acid signaling pathway and suppresses salicylic acid-mediated plant defense against *P. syringae* (Brooks et al., 2005). COR suppresses salicylic acid through MYC2-dependent activation of three NAC (petunia NAM and *Arabidopsis ATAF1*, *ATAF2*, and *CUC2*) transcription factors NAC019, NAC055, NAC072. These NAC transcription factors suppress salicylic acid signaling via the transcriptional down-regulation of *Isochorismate Synthase-1* (*ISC1*), the gene encoding the enzyme committed to the penultimate step in salicylic acid biosynthesis from Chorismate. In addition, these NAC proteins induce the expression of benzoic/salicylic acid carboxyl methyltransferase (*BSMT1*) gene encoding an enzyme that converts active salicylic acid into inactive methyl-salicylate (X. Y. Zheng et al., 2012). Thus, COR can increase the virulence of *P. syringae* by hijacking the jasmonic acid-signaling pathway and suppressing -mediated defense.

1.6 Amino acid transport in plants

Plants take up inorganic nitrogen from the soil and use that nitrogen to synthesize amino acids mainly in photosynthetic cells of leaves. Photosynthetic cells accumulate amino acids that passively leak out of the cell and into the apoplast from where they are taken up by active transporters. Some of these importers localize to the plasma membrane of mesophyll cells and allow for the reuptake of amino acids that have leaked into the apoplast. Others localize to the plasma membrane of phloem parenchyma cells and load amino acids into the phloem of the vascular system. Once in the phloem, amino acids can reach distant sink tissues such as apical

leaves, roots, flowers, and fruits, where they again leak out of the phloem parenchyma cells following a downward concentration gradient. They act as primary metabolites used for the synthesis of proteins, hormones, and secondary metabolites, and serve signaling functions as well (Dinkeloo et al., 2017).

Two superfamilies of amino acid importers have been identified: the amino acid, polyamine, and choline transporter superfamily (APC), and the amino acid transporter (ATF) superfamily (Ortiz-Lopez et al., 2000). The APC family consists of the CAT (cationic amino acid transporter) family and the LAT (L-type amino acid transporter) family. The ATF superfamily is further divided into six subfamilies: AAP (Amino acid permease), ProT (proline transporter), LHT (Lysine histidine transporter), ANT1-like (aromatic and neutral amino acid transporter), AUX (auxin resistant), and GAT (Gama-aminobutyric acid transporter) (Rentsch et al., 2007). Many of these transporters share a common substrate, suggesting their functional specificity may result from their tissue-specific expression patterns. *AAP1* and *AAP5,* along with *LHT1*, *LHT6* and *ProT2* are expressed in the root and have been shown to take up AAs from the soil (A. Hirner et al., 2006; Svennerstam et al., 2011). *AAP2* and *AAP6* are expressed in xylem parenchyma and phloem companion cells, respectively, and are involved in xylem to phloem transfer (Hunt et al., 2010; Okumoto et al., 2002; L. Zhang et al., 2010). The members of AAP family of transporters *AAP2*, and *AAP8* contribute to amino acids uptake by phloem (phloem loading) in mature leaves (Dinkeloo et al., 2017; Santiago & Tegeder, 2016). Based on localization and expression studies, other transporters such as *CAT1, CAT6,* and *CAT9* are also suspected to function in phloem loading (Hammes et al., 2006; Su et al., 2004). In addition to these transporters, *CAT2* and *CAT4* localize to the tonoplast and regulate the concentration of amino acids in the vacuole (Yang, Krebs, et al., 2014).

Only a few transporters of amino acids have been identified as exporters. UMAMITs (Usually Multiple amino acids Move In And Out Transporter) can export amino acids out of the cells following a concentration gradient (Denancé et al., 2014). AATs *UMAMIT11*, *UMAMIT14*, *UMAMIT28*, and *UMAMIT29* have secretory functions that are necessary for phloem unloading at the seed chalazal tissue and are necessary for embryo development (Muller et al., 2015). *UMAMIT18* is expressed in the root pericycle and phloem cells (Denancé et al., 2014). Along with *UMAMIT14*, it is involved in the export of amino acids from the roots toward the soil (Besnard et al., 2016).

1.7 Nutrient acquisition strategies during plant-pathogen interactions

Plant-pathogen interactions involve complex interplays between host plants and microbes. During these interactions, microbes can acquire nutrients from the host plant, which is necessary for their growth and survival. Thus, the availability of nutrients plays an important role in the progression of disease in infected plants. In plants, there are predominantly five nutrient-rich "niches" that are common targets of bacterial pathogens, namely the phloem, the xylem, the leaf apoplast, the root apoplast, and the cell organelles (Fatima & Senthil-Kumar, 2015). The phloem and the leaf apoplast mostly contain sugars and amino acids, while xylem and the root apoplast mostly contain minerals and water (Dinant et al., 2010; Rico & Preston, 2008). Most bacterial pathogens are restricted to the apoplast, but some can colonize in xylem or phloem cells in order to obtain nutrients (Fatima & Senthil-Kumar, 2015). Plants infected with pathogens exhibit altered physiology, including changes to nutrient uptake, assimilation, translocation, and utilization. High efflux of solute at the interface of plant-microorganism was observed in the plant membrane of lichens, and Rhizobium nodules (Patrick, 1989).

Based on the lifestyle, pathogens utilize different strategies to acquire nutrients from the host plant. Necrotrophic bacteria utilize a variety of mechanisms to cause cell death, including cell wall degradation enzymes, necrosis-inducing proteins, and toxins (Glazebrook, 2005). Nutrients are released from the cytosol and numerous organelles via activity of enzymes including pectate lyase, cellulase, and protease (Alfano & Collmer, 1996). The alkalinization of apoplasts by *P. syringae* may also affect nutritional content of the apoplast. There has been evidence that increasing the apoplastic pH decreases the activity of the symporters that load sucrose into the phloem, thereby increasing apoplastic sucrose levels (Atkinson & Jacyn Baker, 1989). Several microbial compounds such as phenazine, 2,4-diacetylphloroglunicol, and zearalenone increase the total net efflux of several amino acids in alfalfa roots (Phillips, 2004). By controlling the expression of the host genes that regulate the permeability of the cell membrane or the genes involved in transport, biotrophs and hemibiotrophs can obtain essential nutrients. For instance, some *P*. syringae pathovars can suppress the expression of stigmasterol biosynthesis-related gene *CYP710A1* to change the plant cell membrane permeability and favor the efflux of nutrients into the apoplast (K. Wang et al., 2012). Bacterial pathogen of the genus Xanthomonas can deliver Transcription Activator-Like Effectors that induce *SWEET (Sugars Will Eventually Be Exported Transporter)* transporters gene expression and enhance sugar efflux into the leaf apoplast where bacteria reside. Another rice pathogen*, X. oryzae pv. oryzae* PXO99 produces effector AvrXa7 which can regulate the expression of *SWEET14* transporter (L. Q. Chen et al., 2010). *X. oryzae pv. oryzae* PXO99 also utilizes ketoglutarate transport protein (*KgtP*), a T3E, to enhance the export of α -ketoglutaric acid from the host to the pathogen. Through *KgtP*, the pathogen indirectly affects the metabolism of infected plants by enhancing the expression of iso-citrate dehydrogenase enzyme in the host (Guo et al., 2012).

Another plant pathogen, *Ralstonia solanacearum* produces the effector protein Ripl to promote the activation of glutamate decarboxylase to enhance the production of GABA which can be used as a source of carbon and nitrogen during the infection (Xian et al., 2020). This evidence suggests that pathogens can increase nutrients available in infected tissues by manipulating host nutrient transporters. This may serve as an artificial sink upon which pathogens can feed (Tünnermann et al., 2022).

1.8 Research Objectives

As described earlier, the interaction between plants and pathogens is a complex and dynamic process that involves the exchange of signals, nutrients, and responses. Plant-made amino acids are key players in this interaction, as they can work as both carbon and nitrogen source that support bacterial growth and signaling molecules to modulate bacterial metabolism and virulence. For instance, the activation of PTI with purified flg22 leads to the accumulation of amino acids that suppress bacterial virulence at early infection time points, thus enhancing plant immunity (X. Zhang et al., 2023). At the same time, bacterial pathogens require to express virulence factors to rapidly suppress plant immunity and use plant-made apoplastic amino acids to support population growth and colonize the leaf apoplast. As described in a previous section, some *Xanthomonas sp* pathogenic bacteria have evolved TAL effectors to induce the expression of plant transporters to obtain sugars from the host (L. Q. Chen et al., 2010). *P. syringae* has not evolved TAL effectors and none of the known T3Es described so far seem to aid in the direct extraction of sugars or amino acids from the host. Therefore, understanding how *P. syringae* extracts nutrients from plant cells is crucial to understand how they produce infections. Since COR suppresses flg22-mediated defense responses (Mine et al., 2017; Young

Yi et al., 2014) and flg22 elicits changes in the extracellular concentrations of amino acids that are essential to suppress infections (X. Zhang et al., 2022, 2023), **I hypothesize that COR exploits the *Arabidopsis* jasmonic acid signaling pathway to make plant-made amino acids available at the leaf apoplast via the transcriptional regulation of plant membrane transporters.** By modulating amino acid concentration, *P. syringae* can potentially manipulate the plant's defense response and promote pathogenesis.

My specific research objectives are:

1. Determine if COR counters flg22-elicited changes in extracellular concentrations of amino acids.
2. Define if the jasmonic acid signaling pathway is needed for COR to alter the plant amino acid transport.
3. Identify the plant transporters that execute the changes in extracellular amino acid concentration.
4. Characterize the candidate genes that execute COR-mediated change in extracellular amino acid concentrations.

This study will advance our understanding of the mechanisms underlying bacterial pathogenesis and shed light on yet-unknown COR virulence functions.

Chapter 2. Coronatine induces the release of plant-made amino acids from *Arabidopsis* seedlings by activating the Jasmonic acid signaling pathway.

2.1 Abstract

Plant-made nutrients are essential for invading pathogens to produce infections. *P. syringae* produces the virulence factor COR, a functional mimic of jasmonic acid (JA)-isoleucine, to activate the host JA signaling pathway and suppress the salicylic acid-mediate plant immunity. This study investigated the role of COR in countering the flagellin-elicited responses that change the availability of extracellular amino acids and nullify flagellin-activated enhanced resistance to bacterial infections. Quantification of extracellular amino acids in the liquid culture of seedlings after COR treatment showed that COR induced an increase in extracellular amino acids that is dependent on senescence-induced proteolysis. Furthermore, COR regulated the expression of various plant AATs including *UMAMIT18*, *UMAMIT21*, and *UMAMIT42*, all of which are necessary for COR to increase the concentrations of extracellular amino acids. Gene expression analysis revealed that COR treatment induces senescence-related genes and proteolytic activity in the seedlings. These data support the hypothesis that COR hijacks the host jasmonic acid signaling pathway to upregulate senescence and proteolysis and allow amino acids to move out of the plant cells where they can be used to support bacteria growth.

2.2 Introduction

Plants are constantly exposed to a variety of biotic and abiotic stressors that can threaten their survival. To cope with these challenges, plants have evolved complex defense mechanisms that involve the activation of various signaling pathways, including the salicylic acid signaling pathway, and jasmonic acid signaling pathway. Jasmonic acid is a plant hormone that plays a

crucial role in regulating various physiological processes, including fertility, growth, development, and responses to biotic and abiotic stressors (Campos et al., 2014). Jasmonic acid signaling is initiated when the Isoleucine conjugate of jasmonic acid (JA-Ile) binds the nuclear receptor *CORONATINE INSENSITIVE1 (COI1)*, a component of the *SCF-COI1 E3* ubiquitin ligase complex. The binding of JA-Ile to *COI1* leads to the degradation of *JASMONATE ZIM-DOMAIN (JAZ)* transcriptional repressors. In turn, *MYC2* is released from JAZ-containing complexes by the jasmonic acid-dependent degradation of JAZ proteins, thus initiating transcriptional changes in jasmonic acid-responsive genes (Thines et al., 2007).

The leaf apoplast is the battlefield where plants and bacterial pathogens define the outcome of the interaction. Recently published studies showed that PTI involves expression changes in amino acids and sugar transporters that modify the availability of amino acids and sugars in the leaf apoplast (Yamada et al., 2016; X. Zhang et al., 2022). Moreover, the increase concentration of Serine (Ser) and Glutamine (Gln) elicited by flg22 perception in the leaf apoplast delays the onset of bacterial virulence at early stages of the infection and compromises the ability of *P. syringae* to colonize the leaf apoplast (X. Zhang et al., 2023). Thus, to establish a successful infection, pathogens need to suppress plant immunity first and then alter the host metabolism to obtain nutrients. Plant pathogens use multiple strategies to manipulate the host to release nutrients. *Xanthomonas oryzae* pathovars use transcriptional activator-like (TAL) effector proteins that directly induce the expression of sugar transporters to increase the efflux of glucose from the plant cell into the leaf apoplast (L. Q. Chen et al., 2010). *Ralstonia solanacearum* effector protein Ripl hijacks plant metabolism by activating glutamate decarboxylase activity to increase the synthesis of GABA, which provides the pathogen with carbon and nitrogen during infections (Xian et al., 2020).

P. syringae produces a small molecule called coronatine (COR) that mimics the structure and function of JA-Ile. COR binds the *COI1* nuclear receptor (Xie et al., 1998) and activates *MYC2,* which induces the expression of the downstream transcription factors (Dombrecht et al., 2007) *NAC19*, *NAC55,* and *NAC72* (X. Y. Zheng et al., 2012), among others. COR regulates numerous aspects of *P. syringae* pathogenicity, including stomatal opening to enable access to the leaf apoplast (Melotto et al., 2006) and disease symptom development (Geng et al., 2014). Furthermore, COR suppresses the biosynthesis of salicylic acid and alters the expression of defense genes (X. Y. Zheng et al., 2012) that are necessary to restrict the growth of biotrophic pathogens like *P. syringae*. Additionally, COR interferes with plant defense signaling initiated in response to flg22 perception. Specifically, COR induces the expression of *HAI1*, an *Arabidopsis* gene encoding a protein phosphatases 2C that inactivates the MAPK3/6 signaling cascade, a key signaling nob required for downstream transcriptional responses to flg22 perception (Mine et al., 2017). At later time points in the apoplast colonization, COR progressively accumulates in infected leaves and produces disease symptoms such as chlorosis, wilting, and necrosis (Geng et al., 2014). Since flg22-elicited PTI induces changes in amino acid concentration and composition that restrict bacterial infections (X. Zhang et al., 2022, 2023) and COR is able to counter PTI (Mine et al., 2017), I sought to study if and how COR modifies the availability of amino acids in the leaf apoplast.

In this study, I present data supporting the hypothesis that COR increases the accumulation of the extracellular amino acids in the *Arabidopsis* seedlings. This enhanced accumulation of the extracellular amino acids required an intact jasmonic acid signaling pathway. After screening all the potential transporter genes whose expression changes in response to COR treatment, I found that three transporters, *UMAMIT18*, *UMAMIT21*, and *UMAMIT42* contribute to the

enhanced extracellular amino acids accumulation mediated by COR in the seedlings. In addition, I found that, in leaves of plants, COR also induces changes in amino acids that support bacterial infections.

2.3 Results

2.3.1 Coronatine suppresses flagellin-mediated enhanced resistance.

Flagellin-elicited PTI partially depends on an intact salicylic acid signaling pathway to induce enhanced resistance to bacterial infections. Since COR antagonizes salicylic acid signaling, it seems plausible that COR could antagonize flagellin enhanced resistance. To test this hypothesis, the growth of *P. syringae* in wild-type seedlings was assessed on COR, or flg22- and COR-treated seedlings (Figure 2-1A). In agreement with previously published data, *P. syringae* growth was compromised in seedlings pre-treated with flg22 (X. Zhang et al., 2022). Notwithstanding that the pre-treatment of seedlings with COR did not produce enhanced susceptibility, when flg22 and COR were combined, the growth of *P. syringae* was significantly higher than that observed in flg22-treatment seedlings. These data show that COR can suppress flagellin-mediated enhanced resistance. As the elicitation of PTI and downstream signaling is a time-dependent process, I hypothesized that the timing of COR treatment is important for the suppression of flg22-mediated responses. The flagellin receptor FLS2 is a cell surface-localized receptor that perceives flagellin outside the cell and initiates intracellular signaling (Gómez-Gómez & Boller, 2000). To understand how COR hinders flg22-induced resistance, I tested if COR interferes with flagellin-initiated and FLS2-mediated early signaling such as the production of reactive oxygen species (ROS) or the internalization of FLS2 that follows flg22 perception (Torres et al., 2006; Robatzek et al 2006). There was no effect of

COR treatment in the flg22-mediated internalization of FLS2 (Figure 2-1B). Treatment with flg22 produced significant ROS production while COR did not. When seedlings were treated simultaneously with COR with flg22, little to no effect on ROS accumulation was observed compared to flg22 treatment only (Figure 2-1C). As COR did not alter FLS2 internalization or ROS production, the data suggest that COR doesn't interfere with early flg22 signaling.

2.3.2 Coronatine elicits the accumulation of extracellular amino acids that support bacterial growth in seedlings.

Several studies, including a previous study from the Danna Lab, have suggested that invading pathogens utilize the amino acids available in the apoplast as sources of carbon and nitrogen (Figure 2-2). As COR is essential for *Psm* ES4326 to produce robust infection (Figure 2-3), I hypothesized that COR could promote pathogen growth by increasing the amino acids available to the pathogen in the leaf apoplast. Using liquid culture of seedlings, I can assess amino acid concentrations in the liquid medium where the seedlings grow. As they grow, seedlings exude metabolites normally found in the seedling apoplast and the phloem sap. Thus, a few days after germination, the liquid medium where seedlings grow contains sugars, amino acids, proteins, RNA, lipids, and other plant-made metabolites that were absent in the medium formulation (Weibull et al., 1990). Using this system, I monitored the concentration of free amino acids over a 48h period after COR treatment. I observed that the exudates collected 24h after COR treatment showed increased concentrations of amino acids compared to those obtained from both mock- and flg22-treated seedlings (Figure 2-4A). Also, the concentration of extracellular amino acids in flg22 and COR co-treated seedlings was significantly higher than that of flg22-only treated seedlings, suggesting that COR treatment functionally

suppressed the depletion of extracellular amino acids observed 24h post flg22 treatment (Zhang et al., 2022; Figure 2-4A). These data suggest that COR induces an increase in the concentration of amino acids in the seedling's apoplast and sap first, which is later exuded into the liquid medium. After profiling extracellular amino acids in the liquid exudates, I found that the concentration of Alanine (Ala), Asparagine (Asn), Aspartic acid (Asp), Glutamic acid (Glu), Glutamine (Gln), Proline (Pro), and Serine (Ser) has increased significantly in the exudates of COR-treated compared to mock-treated seedlings (Table 2-1). As the availability of nitrogen- and carbon-containing nutrients determines the growth of pathogens during infection (Rachel Jones, 2009; Rico & Preston, 2008), I tested if the liquid exudates collected 24h post COR treatment supported *P. syringae* growth to a higher extent than exudates obtained from mock- or flg22-treated seedlings. As shown by the shorter doubling time (Td), *Psm* ES4326 and *Pst* DC3000 grew faster in exudates of COR-treated seedlings than in mock-treated seedlings (Figure 2-4B, Figure 2-4C), suggesting that the COR-induced increase in extracellular amino acids concentration may allow bacteria to obtain nutrients to support full colonization of the leaf apoplast during the infection.

As flg22 modifies amino acid transport activity in seedlings over a period of 24h post-treatment (Zhang et al 2022), I hypothesized that COR could induce opposite changes in transport activity to counter those elicited by flg22. To test this hypothesis, I assessed the uptake of amino acids in mock vs COR-treated seedlings. Among several amino acids, Gln and Ser concentrations change in exudates of wild-type seedlings after COR (Table 2-1) or flg22 treatments (Zhang et al 2022). Thus, I used radiolabeled analogs of these amino acids to assess uptake activity in COR + flg22 treated seedlings. The data collected from several experiments

revealed that COR does not alter the basal uptake of ^{3}H-Gln, and ^{3}H-Ser but rather suppresses the enhanced uptake elicited 24HAE of seedlings with flg22 (Figure 2-5).

2.3.3 The COR-induced extracellular accumulation of amino acids requires jasmonic acid signaling induction but not salicylic acid signaling suppression.

The activation of the jasmonic acid signaling pathway in seedlings through COR treatment was confirmed by the induction of jasmonic acid markers *MYC2*, *VSP2*, and *BSMT1* 24 hours after treatment (Figure 2-6A). Having confirmed that COR induces jasmonic acid signaling markers, I tested if an intact jasmonic acid signaling pathway was required for COR to elevate the concentrations of extracellular amino acids in the exudates of liquid-grown seedlings. To this end, I tested the accumulation of extracellular amino acids in exudates of mutants of the JA-Ile nuclear receptor *COI1*, the *MYC2* transcription factor, and a triple loss of function mutants for *NAC019, NAC055*, and *NAC072*. While extracellular amino acids concentration increased 24h post COR treatment in wild-type seedlings, none of the mutants tested showed COR-induced increase concentrations of extra amino acids in the exudates (Figure 2-6C). Collectively these results suggest that the COR activates the *COI1–MYC2-NAC019/NAC055/NAC072* canonical axis of the jasmonic acid-signaling pathway and that the genes that lead to COR-induced increase in extracellular amino acid concentrations are likely downstream of the transcription factors tested.

As COR is known to suppress the salicylic acid signaling pathway, I hypothesized that the suppression of salicylic acid signaling could mediate the effect of COR. If this hypothesis was correct, salicylic acid mutants would display high concentrations of extracellular amino acid

without COR treatment, and they would not respond to COR by increasing the extracellular amino acid concentrations any further. To test this hypothesis, salicylic acid synthesis (*sid2*) and signaling (*cpr5*, *npr1*) mutants were treated with COR, and the concentration of extracellular amino acid was assessed in the exudates. The data showed the concentration of extracellular amino acids in *sid2*, *cpr5*, and *npr1* 24h post-mock or COR treatment was similar to that of wild-type seedlings (Figure 2-6B) suggesting that COR-induced increase in extracellular amino acids concentration does not depend on salicylic acid signaling suppression. In addition, COR did not increase the concentration of extracellular amino acids (Figure 2-6C) or lowered the doubling time of *Psm* ES4326 in the exudates obtained from jasmonic acid-signaling mutants (Figure 2-6D). Overall, these data suggest that an intact jasmonic acid signaling pathway is essential for COR to induce amino acid accumulation in the seedling exudates, and that salicylic acid signaling suppression is not required.

2.3.4 COR induces the secretion of amino acids via *UMAMIT18*, *UMAMIT21*, and *UMAMIT42*.

To identify amino acid transporters that contribute to increasing the concentrations of extracellular amino acids accumulation in exudates of COR-treated seedlings, I mined published global gene expression analysis datasets of *P. syringae* infected *Arabidopsis* plants (Table 2-2) (L. Wang et al., 2008) and designed a custom set of fluorescently labeled nanoString® probes to test the expression of COR-responsive *Arabidopsis* genes encoding AATs. For the analysis, I extracted RNA from seedlings 3h, 8h, and 24h post-mock or COR treatment. Several genes encoding influx and efflux AATs responded differentially to COR treatment at every timepoint tested (Table 2-3). Since previous results (Figure 2-4A) showed

that later time points were important for COR to increase the concentration of extracellular amino acids, I decided to use 8h and 24h post-treatment to prioritize candidate genes. Several *UMAMIT*s, and two influx AATs, *AAP4,* and *ProT3,* were highly induced by COR at both 8h and 24h (Table 2-3). To assess the potential contribution of these genes to COR-induced extracellular amino acid increase in seedling exudates, I quantified extracellular amino acids after COR treatment in the corresponding loss-of-function mutants (Figure 2-7). From several mutants tested, *umamit18*, *umamit21*, and *umamit42* did not show an increase in extracellular amino acids in the exudates of seedlings 24h post COR treatment (Figure 2-8A), suggesting that these three amino acid transporters play an important role in the secretion of amino acids elicited by COR treatment. To rule out the possibility that these UMAMIT mutants would be COR-insensitive, I tested the integrity of the COR signaling pathway by monitoring the expression of jasmonic acid marker genes. The expression of jasmonic acid markers *MYC2*, *BSMT1,* and *VSP2* was induced by COR in *umamit18*, *umamit21*, and *umamit42,* to the same extent as in wild-type seedlings (Figure 2-8B). Also, the expression of these 3 genes were not induced by COR in jasmonic acid signaling mutants as compared to wild type (Figure 2-9A, B, C). I also ran an *in-silico* analysis to identify binding sites of potential jasmonic acid-regulated transcription factors (TFs) in the promoter region (1500 nucleotides upstream of the start codon) of these three genes (http://www.athamap.de/). I identified several binding sites for jasmonic acid -regulated TFs such as *MYC*, *MYB*, and *NAC* in the promoter region of these genes (Figure 2-9D). These results show that *UMAMIT18*, *UMAMIT21*, and *UMAMIT42* are essential to allow intracellular amino acids to move out of the cells in response to COR treatment.

2.3.5 COR elicits a senescence program that increases the pool of free intracellular amino acids in seedlings.

A previously published gene expression analysis revealed that COR suppresses a large set of genes encoding proteins involved in photosynthesis (Attaran et al., 2014), suggesting that COR elicits the onset of senescence. One of the key processes that occur during senescence is the accumulation and remobilization of amino acids (Soudry et al., 2005). To test if COR induces senescence in seedlings, I monitored the expression of photosynthesis-associated genes (PAGs). The expression of *Rubisco Activase-1* (*RCA1*) and *Rubisco Small Subunit* (*RBCS*) was significantly suppressed by COR treatment at both 24h and 48h post-treatment (Figure 2-10A). As COR-mediated activity requires jasmonic acid signaling pathway, I tested if COR could suppress the expression of PAGs in the absence of jasmonic acid signaling transcription factors *MYC2* and *NAC19/55/72*. While COR significantly suppressed the expression of *RCA1* and *RBCS* in wild-type seedlings, it had no effect on the expression of senescence markers in the *myc2* and *nac19/55/72* triple mutant (Figure 2-10B). As senescence is associated with cell damage (Leshem, 1988; Thompson & Lake, 1987), I tested if COR would cause the leakage of cytosolic enzymes out of the cells. To this end, I tested the activity of the cytosolic enzyme Glucose-6-phosphate dehydrogenase (G6PD) in the liquid exudates of COR-treated seedlings (Figure 2-11). While high G6PD activity levels were detected in seedling lysates (positive control) exudates of COR-treated seedlings did not produce any significant enzymatic activity, suggesting there is minimal to no cell death in the conditions tested. During senescence, Gln, Glu, and Asp accumulate in leaves (Watanabe et al., 2013). The accumulation of free amino acids begins with the degradation and recycling of intracellular proteins. Then, different free amino acids are converted into Gln, Glu, and Asp, in preparation for phloem loading and long-

distance translocation (Nakano et al., 2017; Tilsner et al., 2004). This process demands high levels of proteases and Gln, Glu, and Asp biosynthesis enzymes. Gln synthetases use ammonium and Glu to produce Gln, while Asp synthetases are involved in nitrogen remobilization (Nakano et al., 2017). The LC_MS profiling of amino acids in exudates of COR-treated seedling showed higher concentration of Asn, Asp, Glu, and Gln, a signature profile found in senescing leaves (Couturier et al., 2010; Diaz-Mendoza et al., 2016; Watanabe et al., 2013). To test if COR induces a senescence program that elevate the concentration of intracellular amino acids, I checked the expression of *Glutamine Synthase* (*GLN1;1*), *Aspartyl Protease* (*AT5G48430*), *Glutamine-dependent Asparagine Synthase-1* (*ASN1*), and the *Cytosol Aminopeptidase family protein* in COR treated seedlings (Figure 2-10C). The expression of *GLN1;1* and *Aspartyl Protease* was highly induced 48h post treatment only, while *ASN1* was highly induced at both 24h and 48h post-treatment. Altogether these data suggest that COR triggers a senescence program that increases amino acid levels intracellularly.

2.3.6 COR induces the accumulation of amino acids in *Arabidopsis* leaf and leaf apoplast in plants.

So far, I have shown that COR treatment increases the concentration of extracellular amino acids in the exudates of liquid-grown seedlings. The next question to address is whether a similar phenomenon occurs in the leaves of plants in response to COR, as such information could inform of the role of COR in bacterial leaf colonization. To address this question, I quantified the amino acids in leaf lysates and leaf apoplast of COR-infiltrated leaves. While the concentration of total amino acids did not change in leaf lysates after COR treatment at any time points (Figure 2-12A), UPLC-mediated profiling of amino acids showed that the overall

representation of Asp, Glu, and Val increased in leaves 3h post COR treatment (Table 2-4A). In the leaf apoplast, the concentration of amino acids only increased at 3h post COR infiltration (Figure 2-12B).

Previously, I showed that exudates collected from COR-treated seedlings supported robust bacteria growth *in-vitro* (Figure 2-4B, C). Thus, I hypothesized that COR could facilitate the acquisition of amino acids to support bacterial growth in the leaf apoplast. To test this hypothesis, I began by monitoring the growth of *Psm* ES4326 and *Psm* ES4326 *cfa6*, a mutant strain compromised for the synthesis of COR, in minimal M9 media supplemented with 1 mM or 10 mM Glu+Asp. The doubling time of *Psm* ES4326 (Figure 2-13A) and *Psm* ES432 *cfa6* (Figure 2-13B) supplemented with Glu and Asp decreased significantly compared to bacteria growing in M9 media only. During the infection of *Arabidopsis* leaves, *P. syringae* starts to produce detectable amounts of COR 20h after inoculation (de Torres Zabala et al., 2016). To mimic the impact that COR could have on *Psm* ES4326-infected leaf, I inoculated *Arabidopsis* leaves with *Psm* ES4326 *cfa6,* and 24h later I supplemented the infected leaves with 1 mM or 10 mM Glu+Asp. The infection titers obtained 24h after Glu+Asp supplementation show that both 1 mM and 10 mM Glu+Asp, significantly increased the growth of the COR synthesis mutant strain without having an effect on *Psm* ES4326 growth at lower concentrations (Figure 2-13C), suggesting that the wild type strain already expresses high levels of virulence to support maximum growth.

In addition to being a source of carbon and nitrogen to support growth, amino acids regulate the expression of bacterial T3SS genes relevant to producing infections (X. Zhang et al., 2023). To address the impact of Glu+Asp on bacterial virulence, the expression of the T3SS master regulator *hrpL* was tested via RT-qPCR analysis. The data showed that Glu+Asp significantly

induces *hrpL* expression in the *Psm* ES4326 *cfa6* (Figure 2-13D). Intriguingly, Glu+Asp supplementation did not induce T3SS in *Psm* ES4326, suggesting that the wild-type strain is able to extract enough Glu and Asp from the host to express optimal levels of virulence expression.

2.4 Discussion

Coronatine nullifies late plant immune responses that would otherwise restrict bacterial growth.

The data presented in Chapter 2 show that COR counters the changes in extracellular concentrations of amino acids elicited by flg22 without interfering with the early flg22-elicited signaling events (Figure 2-4A). From a biological perspective, these data align well with both the timing at which plant cells perceived flg22 at the onset of an infection, and the time that it takes for COR accumulates in the leaf tissue. While the perception of flg22 by the plant FLS2 receptor elicits signaling events within minutes (Gómez-Gómez & Boller, 2000), COR takes several hours to accumulate to detectable levels in the leaf tissue during infections (de Torres Zabala et al., 2016) and the plant responses initiated by COR take several hours to manifest. In this context, it seems likely that COR could begin to counter the responses elicited by flg22 perception only several hours after *P. syringae* enters the leaf apoplast. Indeed, only when pretreated 24h ahead of flg22 treatment in the seedlings, COR is able to suppress flg22 early signaling events (Young Yi et al., 2014). When applied together with flg22, COR suppresses the enhanced amino acid uptake activity and nullifies the drop in extracellular cellular concentrations of amino acids elicited by flg22 in seedlings 24h post-treatment (Figure 2-5). These data suggest that COR, rather than interfering with early flg22-elicited signaling, elicits

a parallel program in the plant cells that counters the late responses induced by flg22 (Figure 2-1, Figure 2-4A). By suppressing the influx but not the efflux of amino acids, COR could favor the accumulation of amino acids in the leaf apoplast. In previous studies, my colleagues and I (X. Zhang et al., 2023) have shown that the accumulation of amino acids in the leaf apoplast, specially Gln and Ser, delays the onset of *Pst* DC30000 virulence and compromises the bacterial colonization of the leaf apoplast. Those studies were made using flg22 infiltrations 24h prior to bacterial infiltration into the leaf apoplast. In such conditions, PTI runs free of interference from the bacterial T3SS that would otherwise suppress plant immunity within the first 3-6h after in a natural infection (Nobori et al., 2020; X. Zhang et al., 2023). In natural infection conditions, *Pst* DC3000 would first express T3SS and T3Es to suppress PTI, and only after would it begin to divide and colonize the leaf apoplast. At that point, typically 24h after inoculation, *Pst* DC3000 would express virulence to maintain the suppression of plant immunity and extract plant metabolites that can be used to sustain fast bacterial growth. My data suggests that COR serves such a function.

One important factor that needs to be considered is that the amino acid quantification and profiling were obtained from liquid exudates of seedlings instead of plants and may not fully inform the compositional changes that take place in the leaf apoplast of infected plants. The reason for using seedlings instead of plants is that adult plants quickly degrade COR (de Torres Zabala et al., 2016) after the infiltration of leaves. *Pst* DC3000 synthesizes and secretes COR as it colonizes the leaf apoplast, perhaps to compensate for plant-mediated degradation. In the settings used in this study, COR is added to the liquid surrounding the seedlings where it may remain stable for longer periods of time, thus allowing for the study of its long-term impact on plant immunity. Indeed, the amino acidic composition of the leaf apoplastic fluids recovered

from COR-infiltrated leaves is slightly different from that obtained from COR-treated seedling exudates (Table 2-1, Table 2-4). Importantly, while the concentration of most amino acids increases in the exudates of COR-treated seedlings, COR infiltration in leaves induces an increase in Glu, Asp, Phe, and Thr concentrations, and concomitantly decreases the concentration of Ser in the leaf apoplast (Table 2-4). This profile of amino acids is also different from the one elicited by flg22 in leaves, where the concentration of several amino acids, including Gln and Ser, increases significantly (X. Zhang et al., 2023). In the presence of fructose and other organic acids found in the leaf apoplast, Glu and Asp have been shown to induce *Pst* DC3000 virulence genes *in vitro* (Anderson et al., 2014; Yan et al., 2020). My data show that Glu and Asp supplementation 24h after the inoculation of leaves with a COR-deficient mutant strain also induces the expression of T3SS and COR synthesis genes, strongly suggesting that the mutant strain is unable to extract enough Glu and Asp to support high levels of virulence expression during the infection (Figure 2-13). Overall, the data presented in Chapter 2 supports a model where the accumulation of COR, which takes place 18h-24h after the inoculation of *Pst* DC3000 into the leaf apoplast, induces plant cells to increase the secretion of free amino acids, specially Glu and Asp, to the leaf apoplast where they can be used to support bacterial growth and maintain high levels of expression of virulence genes.

COR hijacks the host jasmonic acid-signaling pathway to induce a program that makes amino acids available in the leaf apoplast.

The jasmonic acid signaling pathway consists of a nuclear receptor and several interacting proteins that form a complex that suppresses the expression of a large number of genes (Attaran et al., 2014; Campos et al., 2014). The data presented in Chapter 2 show that all major proteins

needed for jasmonic acid to induce or suppress genes are also necessary for COR to induce the accumulation of amino acids in the leaf apoplast (Figure 2-6C). While many of the important functions the COR serves during the course of infections involve the suppression of the host salicylic acid-mediated immunity, the induced increase in apoplastic amino acid concentrations does not seem to require the suppression of salicylic acid signaling (Figure 2-6B). Rather than suppressing salicylic acid immunity, COR triggers the onset of a senescence program that likely increases the cytosolic concentrations of free amino acids (Figure 2-10A). The COR-induced senescence program involves the suppression of genes encoding proteins needed for photosynthesis and induces the expression of proteases and enzymes involved in the synthesis of Gln and Asp (Figure 2-10C), two amino acids that are normally used by plants to mobilize nitrogen out of senescing leaves (Masclaux-Daubresse et al., 2008; Watanabe et al., 2013).

Among a large number of genes that respond transcriptionally to *P. syringae* infections in leaves or COR treatment in *Arabidopsis* seedlings, several amino acid importers were suppressed at the same time that exporters were induced (Table 2-2, Table 2-3), suggesting a role for these *Arabidopsis* genes in the net increase in leaf apoplastic amino acid concentrations elicited by COR (Table 2-4). A large number of the COR-induced or suppressed AATs (Table 2-3) responded similarly to *Pst* DC3000 infection (Table 2-2), suggesting that COR modulates their expression during the course of an infection. Three of the *UMAMIT*s that are induced by COR and *Pst* DC3000 in infected leaves are necessary for COR to increase the concentration of extracellular amino acids in seedling exudates (Figure 2-8), strongly suggesting that they allow *Pst* DC3000 to obtain amino acids in the leaf apoplast during infections.

Overall, the data presented support a model (Figure 2-14) where COR gradually accumulates as *Pst* DC3000 begins to replicate and colonize the leaf apoplast. By inducing the plant jasmonic acid-signaling pathway, COR induces a senescence program that increases the concentration of intracellular amino acids. As the concentration increases in the cytoplasm, *UMAMIT18*, *21*, and 42, allow amino acids to move out into the leaf apoplast. Further studies will be necessary to define the tissue-specific and subcellular localization of these transporters and how they contribute to supporting bacterial infections.

2.5 Future directions

Future directions for this project should focus on understanding how COR the plant's metabolic pathways to increase amino acids in the plants. Metabolic profiling could be conducted on COR-treated plants to identify any changes in primary and secondary metabolites. This could provide additional information on the specific metabolic pathways affected by COR, as well as potential insights into the function of COR in the plant-microbe interaction.

Also, the effect of COR in the specific expression of the UMAMIT18, UMAMIT21, and UMAMIT42 genes needs to be studied. In silico promoter analysis was done to identify potential transcription factors. Further analysis of their promoter regions using chromatin immunoprecipitation (ChIP)-seq could be performed to identify potential COR-responsive cis-elements. Mutational analysis of these elements and their impact on gene expression could help to determine whether they are indeed involved in regulating COR-induced amino acids export. Also, subcellular localization of these transporters needs further exploration.

The virulence gene expression after amino acids supplementation was different in *P. syringae* depending on their ability to synthesize COR or not. To resolve this, investigation of the potential role of COR in nutrient acquisition by *P. syringae* needs to be done. Experiments could be designed to examine the impact of COR on the expression of Pseudomonas genes involved in amino acids utilization. RNA-seq analysis of Pseudomonas cells grown in the presence or absence of COR could help to identify specific genes that are up- or down-regulated by COR and may shed light on the potential mechanisms underlying the observed increase in amino acids concentration in the plant exudates.

I used radiolabeled amino acids to measure the effect of coronatine on the uptake and secretion of specific amino acids. Uptake experiments were somewhat conclusive, but secretion

experiments were not. It would be interesting to set up optimal secretion conditions to examine the role of COR in secretion in whole seedlings and plant protoplast and compare the secretion capacity of wildtype against *umamit18*, *umamit21*, and *umamit42* mutants.

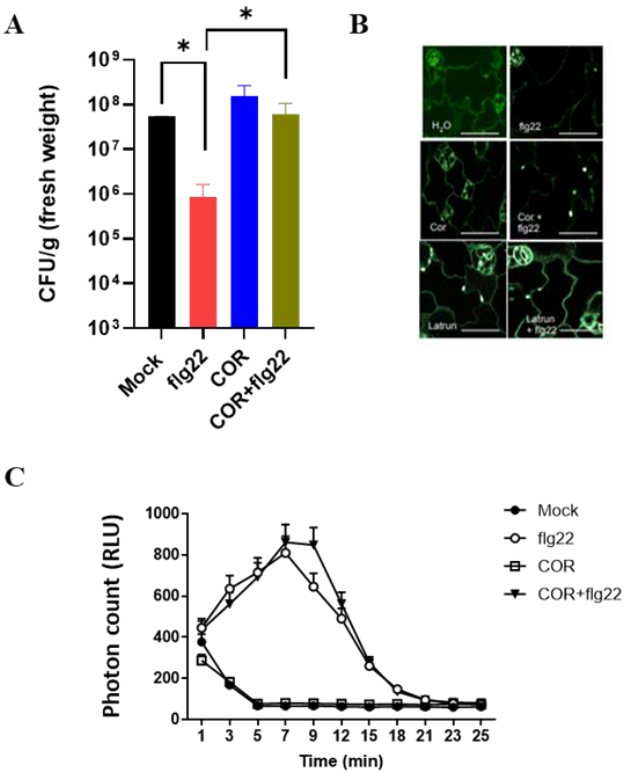

Figure 2- 1. COR suppresses flg22-mediated late defense response.

A) *P. syringae* growth in the *Arabidopsis* seedlings pretreated with water (Mock), flg22, COR, and COR+flg22. Bacterial inoculation was performed 24 hours post-treatment. Samples were collected 24 Hai (Hours after infection). Bacterial CFUs (Colony forming units) were normalized by fresh weight of seedlings. Mean ± SEM (n = 3). **B**) GFP signals in epidermal cells of Ws-0/FLS2-GFP-derived detached leaves 30 min post-treatment with flg22, COR, and COR and flg22 co-treatment. Bar: 10 µm. Background autofluorescence of chloroplasts appears in yellow. **C**) Reactive oxygen species (ROS) were measured by the peroxidase luminol enhanced chemiluminescence method and are expressed as relative light units (RLU). Values are averages of total photon counts from each treatment. Data analysis: Student's t-test. Statistically significant differences at P values < 0.05 (*) are shown. Similar results were obtained in three out of three independent experiments.

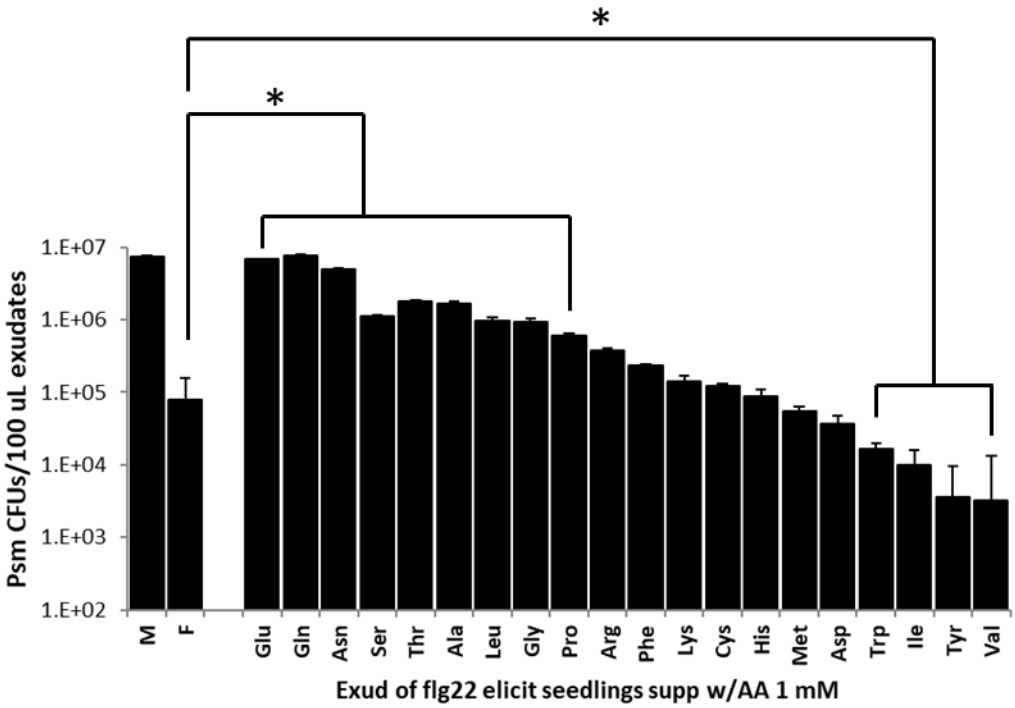

Figure 2- 2. Amino acids supplementation rescues bacterial growth in liquid exudates of flg22-treated seedlings.

Psm ES4326 growth in liquid exudates of flg22-treated seedlings supplemented with 1 mM of each amino acid as indicated. Bacterial CFUs (Colony forming units) were normalized by volume of exudates. Mean ± SEM (n = 3). Data analysis: Student's t-test. Statistically significant differences at P values < 0.05 (*) are shown. Similar results were obtained in three out of three independent experiments.

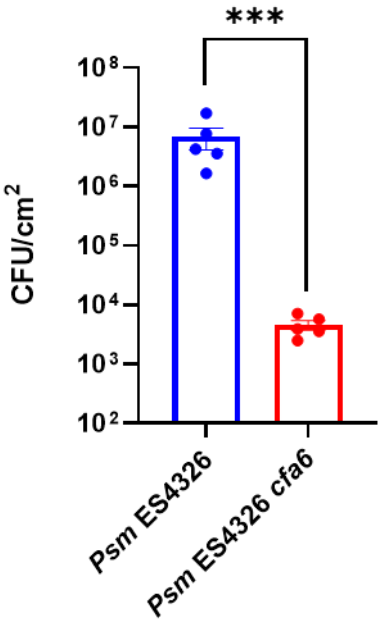

Figure 2- 3. COR is required to produce robust infection.

Bacterial growth in the leaves of wild-type Col-0 (WT) infiltrated with *Psm* ES4326 or *Psm* ES4326 *cfa6* (OD_{600nm} = 0.0002). Mean ± SEM (n = 6). Data analysis: Student's t-test. Statistically significant differences at P values < 0.001 (***) are shown. Similar results were obtained in three out of three independent experiments.

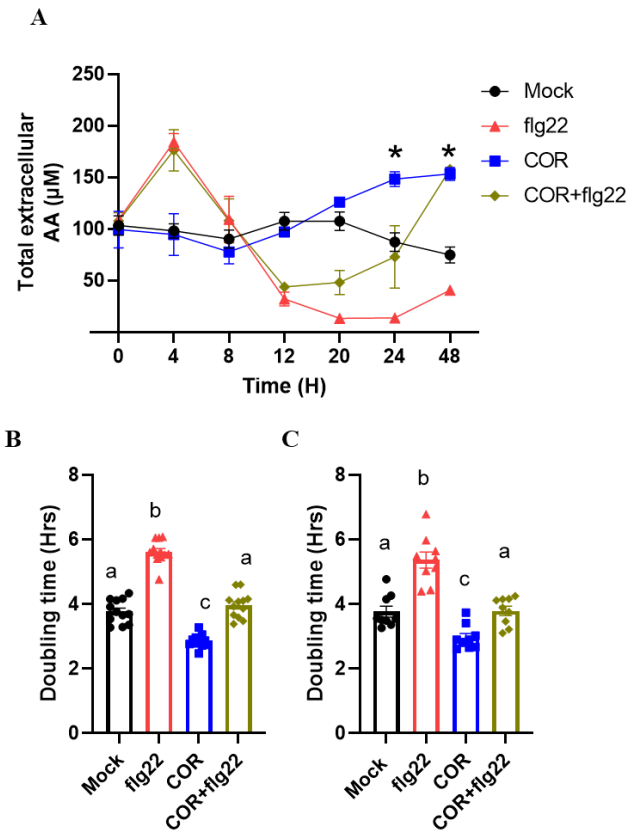

Figure 2- 4. COR elicits the extracellular accumulation of plant-made amino acids.

A) Concentration of total L-amino acid in liquid exudates of COR-treated seedlings 24 HPE. Mean ± SEM (n = 3). B) Doubling time of *Psm* ES4326 in the liquid exudates of seedling collected 24 HPE. Mean ± SEM (n = 12). C) *Pst* DC3000 doubling time in the exudates of seedlings collected 24 HPE. Mean ± SEM (n = 10). Data analysis: (A) Student's T-test; (B) and (C) One-Way ANOVA followed by Tukey's post hoc test. (A) Statistically significant differences at P values < 0.05 (*), or non-significant (ns) differences are shown. Different letters in (B) and (C) indicate statistically significant differences at $P < 0.05$. (A) Similar results were obtained in three out of three independent experiments. (B) (C) Data is combined from two independent experiments with similar results.

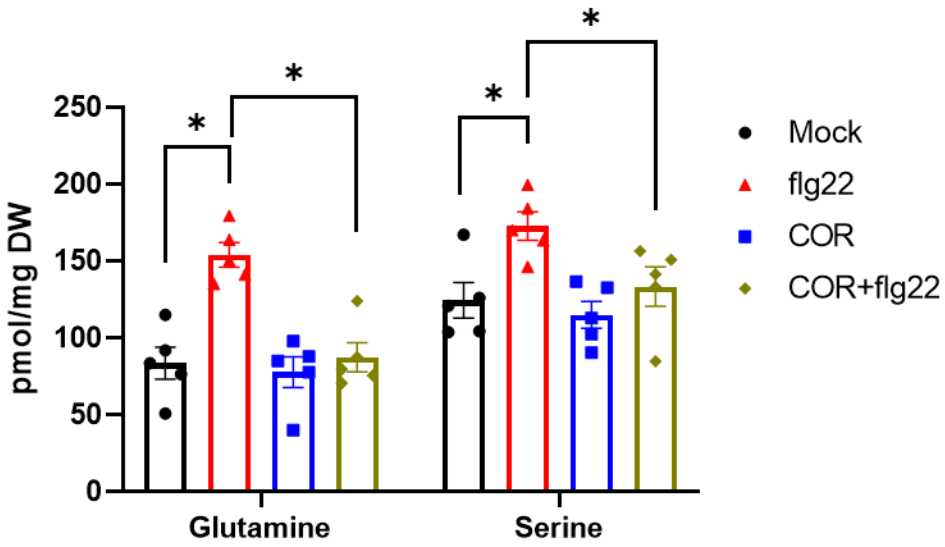

Figure 2- 5. COR suppresses the induced uptake of amino acids elicited 24 HPE with flg22 in seedlings.

Accumulation of ^{3H}Ser, and ^{3H}Gln in wild-type seedlings 24 HPE with water (mock), flg22, COR, and COR+flg22. Mean ± SEM (n = 4). Data analysis: Student's t-test. Statistically significant differences at P values < 0.05 (*), or non-significant (ns) differences are shown. Similar results were obtained in two out of two independent experiments.

	Mock		COR		
	Mean (µM)	SEM (µM)	Mean (µM)	SEM (µM)	P value of M vs C
Alanine	3.2426	0.5617	17.3938	3.3446	0.0003
Arginine	0.2118	0.0238	0.3473	0.1180	0.2265
Asparagine	36.7944	3.9568	54.9898	5.4242	0.0087
Aspartic acid	2.4154	0.3972	6.0785	1.5538	0.0428
Glutamic acid	6.0581	0.7791	20.4731	4.7458	0.0046
Glutamine	62.2783	11.8380	103.4364	13.2333	0.0207
Glycine	4.3506	1.0041	4.8057	0.8692	0.7057
Histidine	3.0035	0.4864	3.8491	0.6193	0.2470
Isoleucine	1.1331	0.2455	1.5767	0.4026	0.3078
Leucine	1.9328	0.5091	1.6051	0.2928	0.5402
Lysine	0.4420	0.0511	0.4426	0.0600	0.9933
Methionine	0.0580	0.0189	0.1626	0.0788	0.1680
Phenylalanine	0.5398	0.1206	0.8629	0.2445	0.2038
Proline	2.3592	0.4051	4.4921	0.5431	0.0033
Serine	7.4836	0.9039	16.9759	2.0598	0.0003
Threonine	2.3557	0.3854	3.1513	0.6691	0.2658
Tryptophan	0.2309	0.0565	0.2824	0.0711	0.5342
Tyrosine	0.3429	0.0670	0.5576	0.1922	0.2551
Valine	5.4711	1.4193	7.6145	1.9331	0.3317
Total	134.6591	20.3986	240.6428	29.7563	0.0052

Table 2- 1. LC-MS profiling of amino acids in exudates of wild-type seedlings 24 HPE with COR

Concentrations of free amino acids in exudate of water (mock) or COR-treated wild type seedlings 24HAE. Mean ± SEM, (n=8). Data analysis: Student's t-test. Statistically significant differences between mock and treatment for each amino acid concentration are highlighted in red, ($p < 0.05$). Data is combined from two independent experiments with similar results.

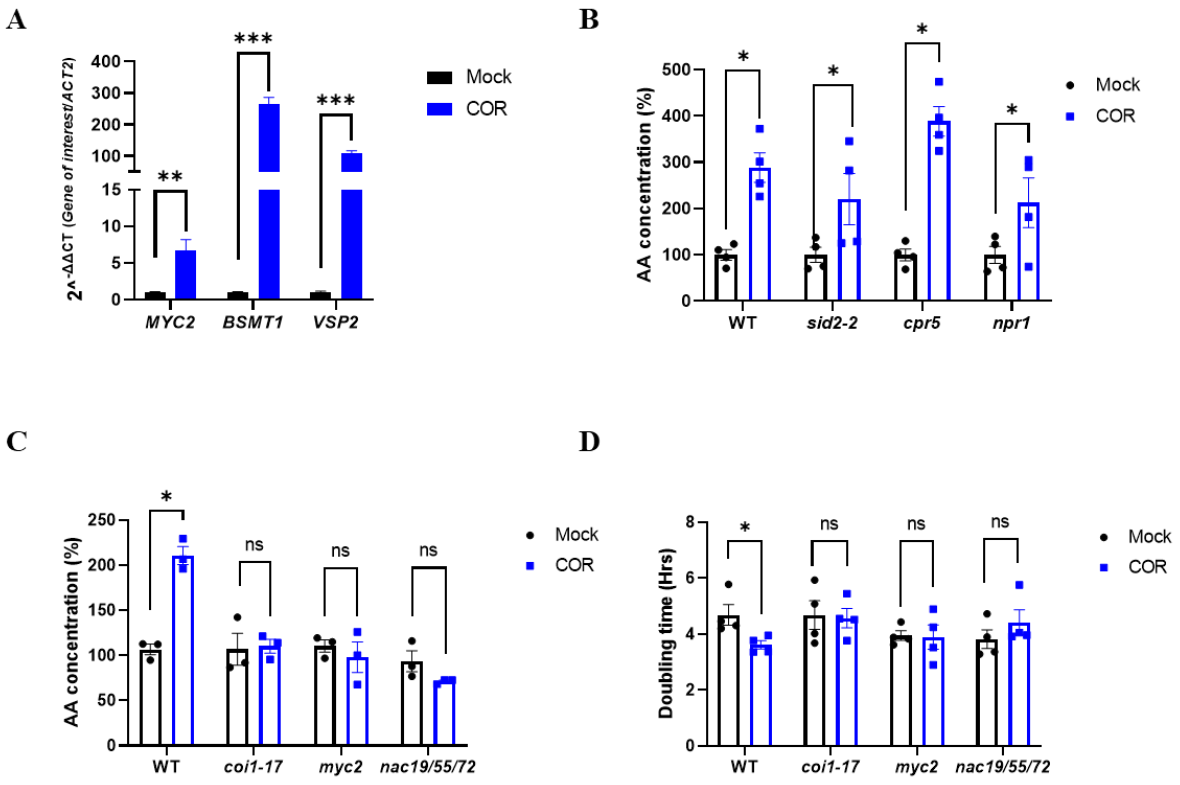

Figure 2- 6. COR-induced accumulation of extracellular amino acids requires jasmonic acid signaling but is independent of salicylic acid signaling suppression.

A) Expression of jasmonic acid signaling marker genes in wild-type seedlings 24 HPE with COR. Mean ± SEM (n = 3). B) Accumulation of total L-amino acids in salicylic acid signaling mutants; Mean ± SEM (n = 3). C) Accumulation of total L-amino acids in jasmonic acid signaling mutants; Mean ± SEM (n = 3). D) *Psm* ES4326 doubling time in liquid exudates of seedling collected 24 HPE; mean ± SEM (n = 4). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), < 0.01 (**), and < 0.001 (***), or non-significant (ns) differences are shown. Similar results were obtained in three out of three independent experiments.

Gene	DC3000-Mock	q DC3000 vs Mock	Psm-Mock 24h	q Psm vs Mock
AAP1	2.1625	0.0000	1.3893	0.0012
AAP2	-1.1523	0.0053	-2.6179	0.0014
AAP3	1.4602	0.0009	1.3722	0.0055
AAP4	1.1433	0.0003	1.4442	0.0003
AAP6	-2.0189	0.0001	-1.8052	0.0017
CAT1	1.8763	0.0003	3.3433	0.0009
CAT2	1.6382	0.0001	0.7852	0.0007
CAT3	-0.7492	0.0021	-0.7740	0.0005
CAT4	0.9098	0.0004	1.1495	0.0006
CAT7	-0.3424	0.0058	-0.4106	0.0003
CAT9	-0.1048	0.3346	0.5824	0.0008
LHT1	1.1832	0.0151	1.9525	0.0033
LHT2	-0.7165	0.0002	-0.2728	0.1021
LHT3	0.0062	0.9078	0.0360	
LHT4	-0.9631	0.0005	-1.0099	0.0003
LHT7	0.2901	0.5594	4.3154	0.0013
LHT10	-1.4350	0.0000	-1.3629	0.0066
PROT2	0.7669	0.0078	0.5289	0.0329
PROT3	1.3434	0.0080	-0.9579	0.0551
PROT4	1.2353	0.0013	0.3215	0.1893
PUT1	1.2794	0.0030	2.1450	0.0018
PUT2	1.1209	0.0002	1.3876	0.0013
PUT3	-0.5267	0.0015	-0.2924	0.0998

Gene	DC3000-Mock	q DC3000 vs Mock	Psm-Mock 24h	q Psm vs Mock
UMAMIT01	0.5597	0.0009	1.0490	0.0047
UMAMIT02	0.1987	0.0799	1.2969	0.0003
UMAMIT05	-2.4701	0.0003	-3.3607	0.0002
UMAMIT06	-0.2879	0.0485	-0.3628	0.0442
UMAMIT07	0.0270	0.8340	0.2038	0.0431
UMAMIT10	2.0443	0.0016	1.4145	0.0027
UMAMIT11	-0.6470	0.0078	-0.8429	0.0189
UMAMIT12	-1.8710	0.0025	-1.0944	0.0012
UMAMIT13	-1.9650	0.0000	-3.2339	0.0004
UMAMIT17	1.0535	0.0120	0.9196	0.0048
UMAMIT18	3.3125	0.0000	2.8754	0.0004
UMAMIT20	2.2274	0.0000	1.8712	0.0002
UMAMIT21	1.1538	0.0004	1.8954	0.0022
UMAMIT22	-0.4428	0.0293	-1.0849	0.0017
UMAMIT28	0.5246	0.0048	0.2742	0.5009
UMAMIT29	3.2879	0.0001	3.3083	0.0003
UMAMIT30	-0.6643	0.0106	-0.0320	0.8788
UMAMIT31	0.5181	0.0079	0.2288	0.2798
UMAMIT33	2.3321	0.0001	2.7742	0.0006
UMAMIT41	0.7321	0.0005	0.6072	0.0219
UMAMIT42	0.3811	0.2328	1.1796	0.0420
UMAMIT44	-1.2440	0.0033	-2.1946	0.0035
UMAMIT47	-1.8873	0.0006	-2.8714	0.0003
VAAT1	-0.5227	0.0035	-1.1180	0.0086
VAAT2	2.2414	0.0000	1.7514	0.0016
VAAT3	2.0926	0.0003	2.0888	0.0044
VAAT4	-0.0207	0.9321	-1.0007	0.0050
VAAT6	0.0075	0.9671	0.2327	0.0203
VAAT8	1.1378	0.0072	0.0689	0.3873
VAAT9	-1.4937	0.0007	-1.6011	0.0003

Table 2- 2. Expression of *Arabidopsis* AATs in *P. syringae*-infected leaves.

Log2 fold changes of the transcriptional levels of amino acid transporter (AAT) genes that respond in adult leaves at 24 hours after *Psm* ES4326 or *Pst* DC3000 infection. Data analysis: Student's t-test (two tails) was used to obtain the P values for comparisons. The P values were corrected for multiple testing using the Benjamini- Hochberg FDR, and the corrected values were designated as the q values. ($q < 0.01$) Statistically significant differences between mock and infection for each AATs are highlighted in red. Data source: L. Wang et al., 2008.

Gene	8h wt-C/M	P value	24h wt-C/M	P value
AAP1	1.76	0.00123989	3.02	0.00001189
AAP2	-2.65	0.00111614	1.56	0.00025501
AAP3	2.6	0.001169	5.59	0.00043746
AAP4	9.61	0.0000021	7.31	0.00000444
AAP6	2.95	0.00283198	2.92	0.00177096
CAT1	-1.35	0.2135029	-1.1	0.47964522
CAT2	-1.17	0.01401982	1.05	0.17645875
CAT3	1.2	0.0045146	1.29	0.00154331
CAT6	-1.01	0.87661225	-1.73	0.01509874
CAT7	1.91	0.04528542	1.21	0.38002026
LHT1	-1.28	0.43475291	1.74	0.00054575
LHT2	1.44	0.0066749	2.39	0.00075974
LHT4	-1.2	0.09653461	1.48	0.01044935
LHT7	3.25	0.01050687	1.27	0.08137884
ProT2	1.15	0.09794402	1.6	0.00037874
ProT3	8.91	0.0000003	15.39	0.00003794
ProT4	-1.07	0.1622934	-1.09	0.24272467
PUT2	1.51	0.00024259	1.9	0.00000914
PUT3	-1.01	0.85186017	1.15	0.03479137
PUT4	4.06	0.00012409	4.52	0.00020588

Gene	8h wt-C/M	P value	24h wt-C/M	P value
UMAMIT04	-4.22	0.00905697	-6.77	0.00001761
UMAMIT05	-1.26	0.0083567	1.44	0.00534424
UMAMIT08	-3.38	0.00008125	-2.48	0.00061204
UMAMIT10	3.89	0.00032712	5.53	0.0000575
UMAMIT11	-1.19	0.04852065	1.57	0.00604462
UMAMIT12	1.55	0.0406082	3.43	0.00000432
UMAMIT13	1.07	0.30954781	-1.21	0.08161879
UMAMIT14	-2.07	0.02626557	1.22	0.01690872
UMAMIT17	1.28	0.13951391	2.96	0.00270446
UMAMIT18	2.85	0.00010178	6.44	0.00008258
UMAMIT19	1		1	
UMAMIT20	1.07	0.1156536	1.83	0.00001913
UMAMIT21	1.55	0.02010418	2.18	0.00015926
UMAMIT24	1		1	
UMAMIT25	-1.39	0.28199717	2.98	0.00077746
UMAMIT27	1.56	0.03415238	2.42	0.00114342
UMAMIT28	-1.24	0.03756888	1.48	0.00533314
UMAMIT29	1.12	0.12323471	2.11	0.00016259
UMAMIT30	3.08	0.00032879	4.29	0.00040405
UMAMIT33	-3.64	0.03602516	-2.51	0.00274634
UMAMIT42	32.6	0.00048651	15.28	0.0007308
UMAMIT44	-1.16	0.12321029	1.02	0.54418713
UMAMIT47	-1.25	0.06450728	-1.03	0.49177155
VAAT1	1.4	0.03098663	1.79	0.01877681
VAAT2	3.29	0.0006341	4.57	0.00011241
VAAT3	-1.92	0.00682289	-1.08	0.24080661
VAAT8	36.01	0.0005027	21.42	0.00001863
VAAT9	-1.61	0.00659215	-2.83	0.00179831

Table 2- 3. Expression of *Arabidopsis* AATs in COR treated seedlings.

Log2 fold changes of the transcriptional levels of amino acid transporter (AAT) genes that respond in seedlings 8 or 24 hours after COR treatment. Gene expression was assessed via nanoString hybridization®. Data analysis: Student's t-test (two tails) was used to obtain the P values for comparisons. Statistically significant differences between mock and treatment for each AATs are highlighted in red ($P < 0.05$).

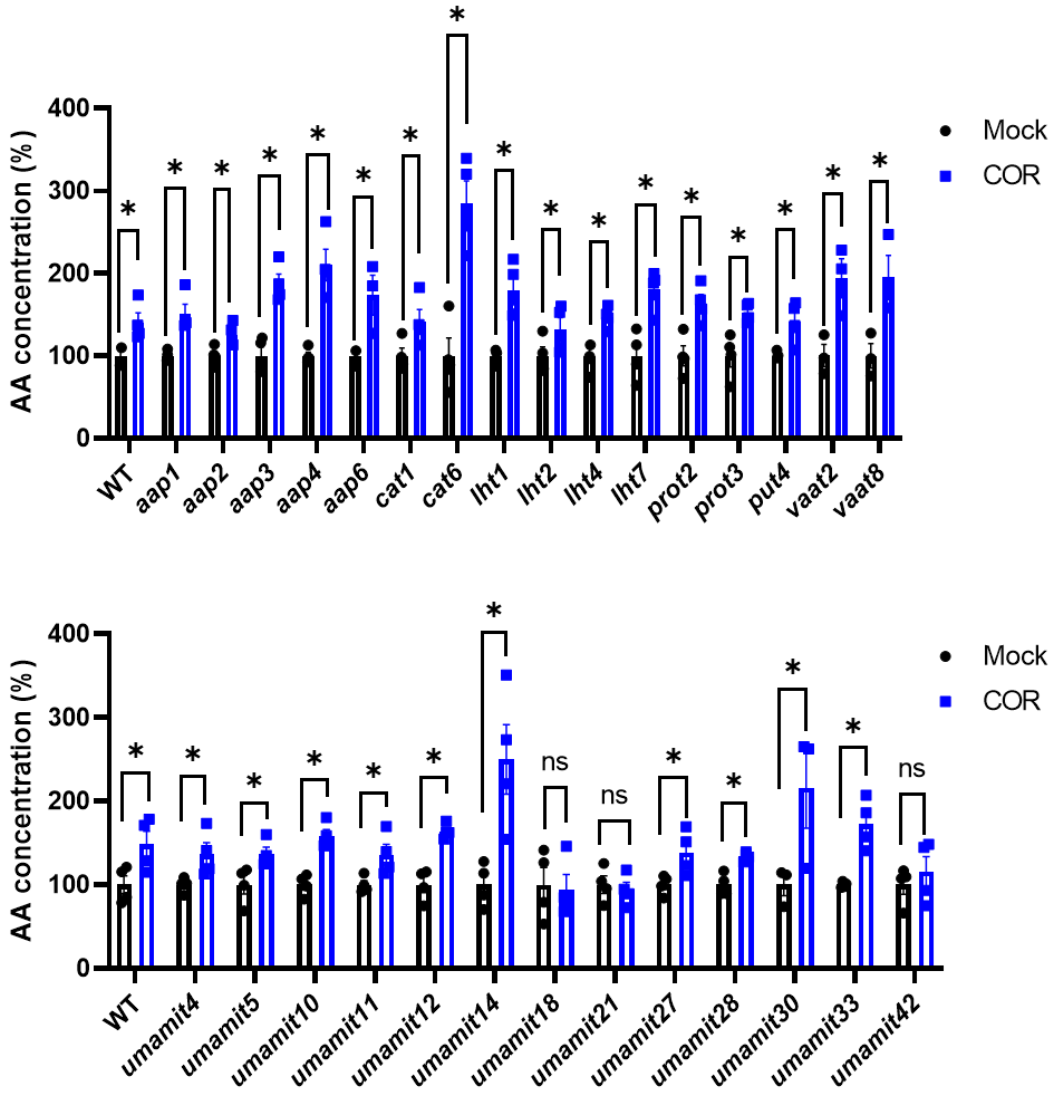

Figure 2- 7. Genetic screening of loss-of-function mutants for COR-responsive genes.

Accumulation of total L-amino acids in the liquid exudates of AATs mutant seedlings 24 HPE with COR. Mean ± SEM (n = 4). Data analysis: Student's t-test. Statistically significant differences at P values < 0.05 (*), or non-significant (ns) differences are shown. Similar results were obtained in three out of three independent experiments.

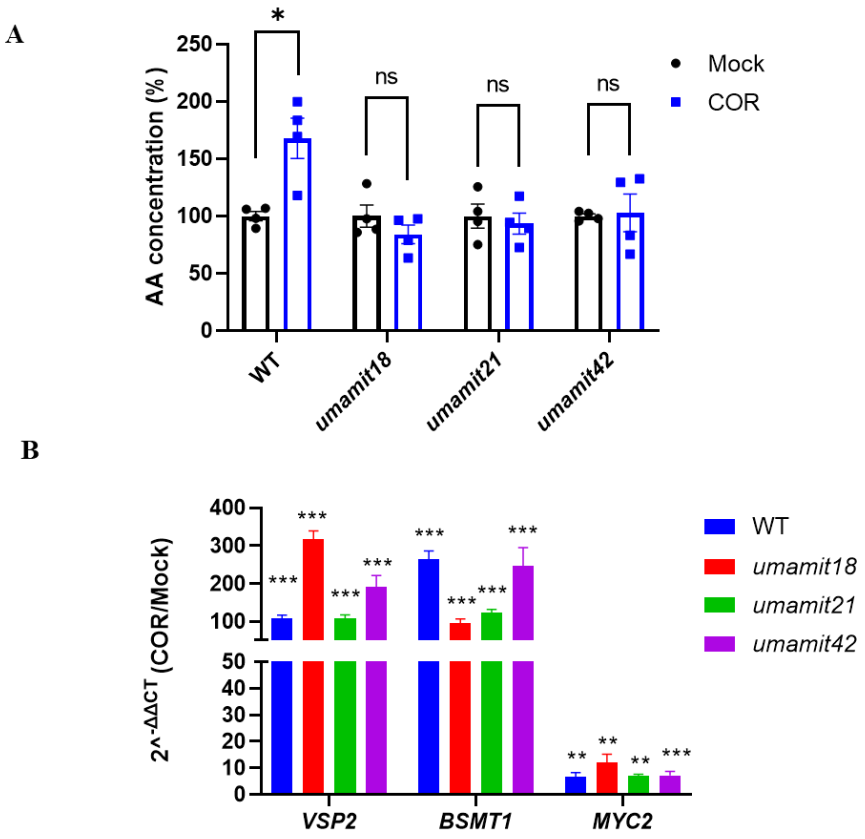

Figure 2- 8. *UMAMIT18*, *UMAMIT21*, and *UMAMIT42* are required for the accumulation of amino acids in exudates of COR-treated seedlings.

A) Accumulation of total L-amino acids in *umamit18*, *umamit21*, and *umamit42* mutant seedlings 24 HPE with COR. Mean ± SEM (n = 4). B) Expression of jasmonic acid signaling marker genes in *umamit18*, *umamit21*, and *umamit42* mutant seedlings treated with COR for 24 hours. Mean±SEM, (n = 3). Data analysis: (A) Student's t-test, (B) One tailed t-test; Statistically significant differences at P-values of < 0.05 (*), < 0.01 (**), and < 0.001 (***), or non-significant (ns) differences are shown. Similar results were obtained in three out of three independent experiments.

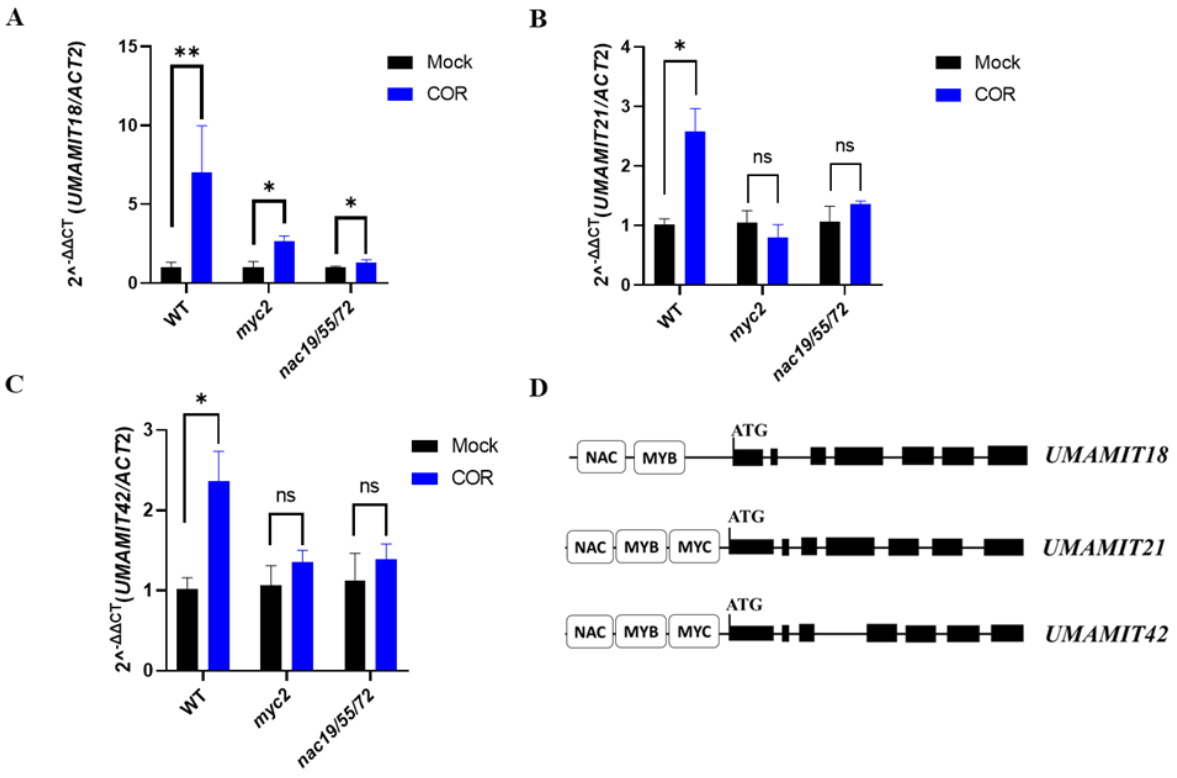

Figure 2- 9. *UMAMIT*s expression is regulated by COR via jasmonic acid signaling pathway.

Expression of A) *UMAMIT18*, B) *UMAMIT21*, and C) *UMAMIT42* genes in wildtype and jasmonic acid signaling mutants (*myc2* and *nac19/55/72*) seedlings treated with COR for 24 hours. Mean ±SEM, (n = 3). D) Schematic representation of the distribution of the jasmonic acid responsive transcription factors, NAC, MYB, and MYC2 in -1500 base pairs on the UMAMIT18, UMAMIT21, and UMAMIT42 genes. The black box denotes the exon regions, and ATG denotes transcription start site (http://www.athamap.de/). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), and < 0.01 (**), or non-significant (ns) differences are shown. (A), (B) and (C) Similar results were obtained in three out of three independent experiments.

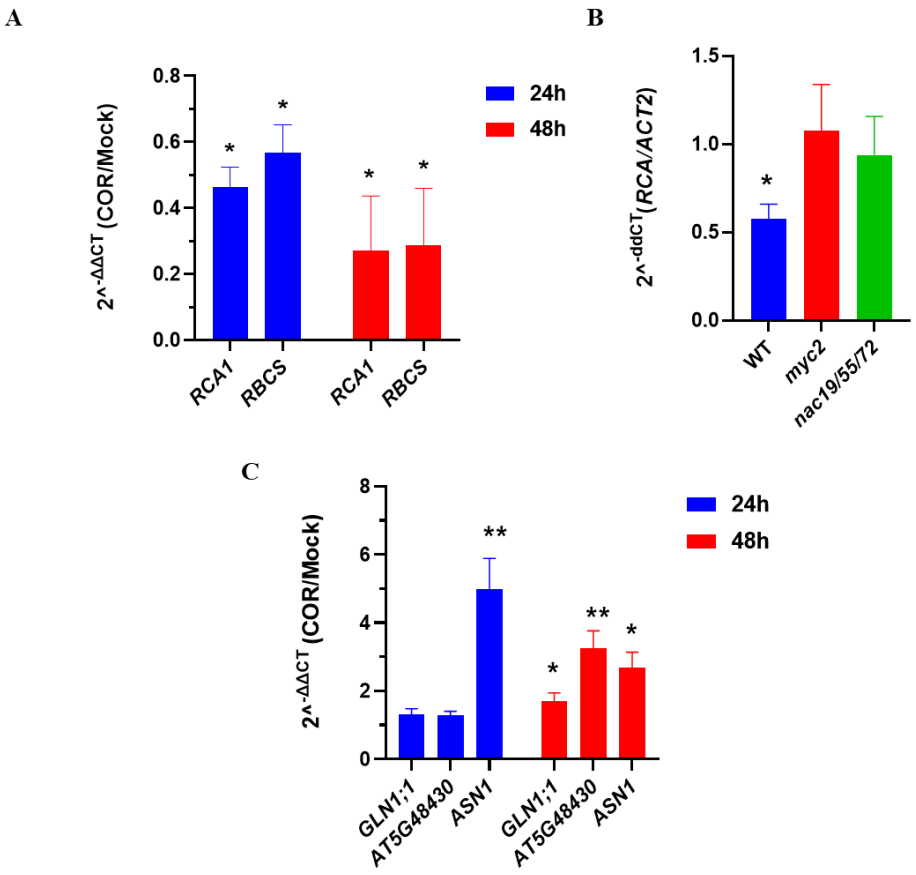

Figure 2- 10. COR induces the expression of plant metabolic genes and suppresses photosynthesis-related genes in seedlings.

A) Expression of photosynthesis-related genes *Rubisco Activase-1* (*RCA1*) and *Rubisco Small Subunit* (*RBCS*) in COR-treated wild-type seedlings. Mean ± SEM (n = 3). B) *RCA* expression in jasmonic acid signaling mutant seedlings 24 HPE with COR. Mean ± SEM (n = 3). C) Expression of plant metabolic genes *Glutamine* synthase (*GLN1*), Aspartyl-protease *AT5G48430*, *Glutamine-dependent Asparagine Synthase-1* (*ASN1*) in COR-treated seedlings. Mean ± SEM (n = 3). Data analysis: one tailed t-test Statistically significant differences at P-values of < 0.05 (*), and < 0.01 (**), or non-significant (ns) differences are shown. Similar results were obtained in three out of three independent experiments.

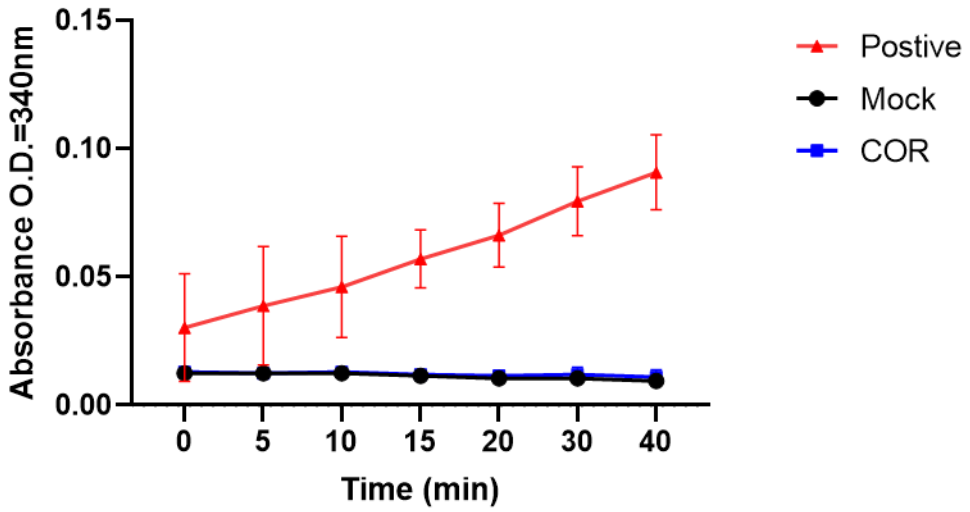

Figure 2- 11. COR-induced increase in extracellular amino acid does not involve damaging plant cells.

Glucose-6-phosphate dehydrogenase (G6PDH) activity in exudates of wild-type seedlings collected 24 HPE with COR. Seedling lysates were used as a positive control. Mean ± SEM (n=2). Similar results were obtained in three out of three independent experiments.

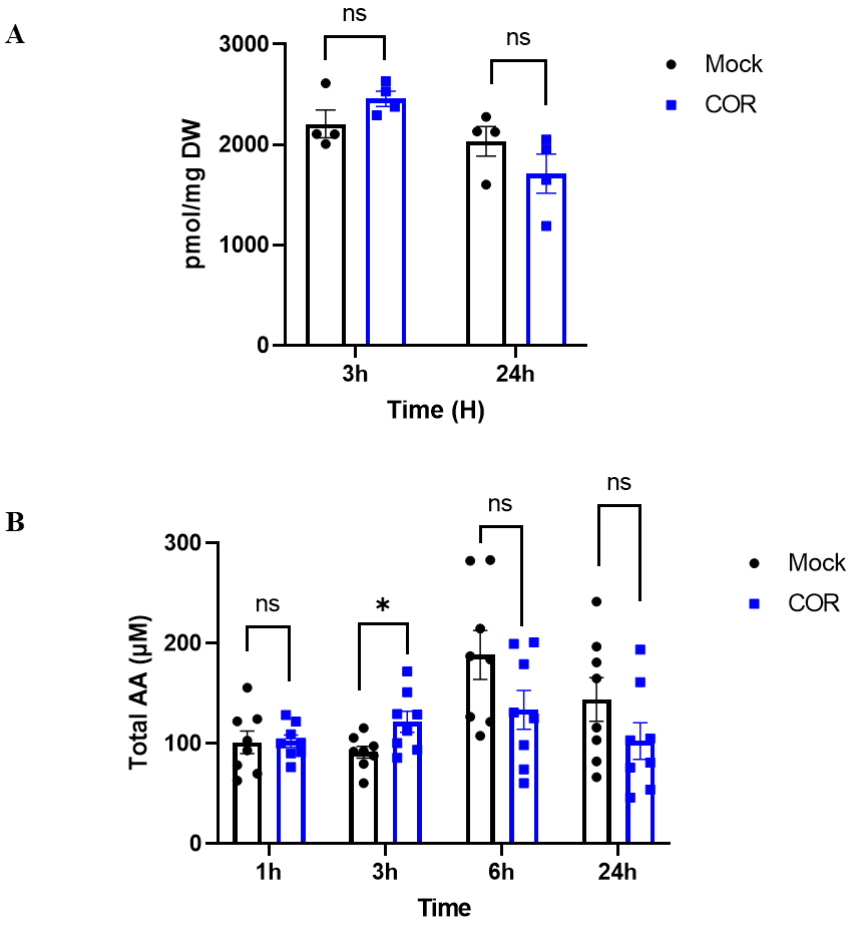

Figure 2- 12. COR induced amino acids accumulation in adult leaves.

A) Total free L-amino acids (pmol/mg DW) in lysates of water- (mock) or COR-infiltrated leaves of wild-type plants. Mean ± SEM, (n = 12). B) Concentration of total L-amino acids in apoplastic washing fluids (AWF) of leaves infiltrated with water (mock) or COR. Mean ± SEM (n = 8). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), or non-significant (ns) differences are shown. (A) Similar results were obtained in three out of three independent experiments. (B) Data combined from two independent experiments with similar results.

A

	Mock		COR		
	Mean (pmole/mg)	SEM	Mean (pmole/mg)	SEM	P value M vs C
Alanine	117.0	7.867	130.1	6.180	0.2029
Arginine	7.4	0.546	5.0	0.347	0.0014
Asparagine	75.3	5.233	75.6	4.409	0.9670
Aspartic acid	454.3	21.968	519.2	21.032	0.0441
Glutamine	345.0	16.548	295.5	36.189	0.2275
Glutamic acid	462.8	32.147	639.3	26.379	0.0003
Glycine	56.0	7.171	64.1	2.376	0.2977
Histidine	7.6	0.539	10.7	1.331	0.0467
Isoleucine	5.1	0.191	5.5	0.432	0.4916
Leucine	5.0	0.263	5.3	0.636	0.6866
Lysine	11.6	0.605	10.9	1.551	0.6791
Phenylalanine	8.2	0.175	16.4	0.961	0.0000
Proline	38.3	5.140	34.8	3.427	0.5763
Serine	371.0	22.664	292.3	23.097	0.0236
Threonine	114.6	11.955	137.3	10.278	0.1629
Valine	39.7	2.500	57.2	2.033	0.0000
Total	2118.9	62.858	2299.3	86.533	0.1058

B

	Mock		COR		
	Mean (µM)	SEM (µM)	Mean (µM)	SEM (µM)	P value of M vs C
Alanine	211.822	60.221	244.037	36.054	0.0773
Arginine	1.204	0.306	1.340	0.420	0.3072
Asparagine	24.382	4.908	21.732	8.488	0.2939
Aspartic acid	296.048	36.098	393.352	42.488	0.0000
Glutamine	97.216	23.294	108.673	14.926	0.1093
Glutamic acid	89.095	20.257	136.644	55.173	0.0037
Glycine	20.480	15.044	18.192	20.861	0.7261
Histidine	2.496	1.976	2.021	0.818	0.3800
Isoleucine	26.889	21.186	26.161	9.296	0.9038
Leucine	22.182	16.126	28.258	12.320	0.2563
Lysine	1.488	0.747	1.639	0.566	0.5299
Methionine	3.632	1.441	4.722	1.959	0.1360
Phenylalanine	18.444	13.285	29.173	14.613	0.0432
Proline	40.511	32.974	62.065	40.911	0.1120
Serine	166.219	47.389	109.375	27.416	0.0003
Threonine	51.401	14.559	68.968	16.132	0.0040
Tryptophan	4.023	2.862	5.254	2.082	0.1894
Tyrosine	9.359	11.436	12.838	8.026	0.3289
Valine	40.341	17.360	31.423	18.199	0.1770
Total	1127.232	274.557	1305.869	127.781	0.0280

Table 2- 4. Glutamic acid and Aspartic acid concentrations increase in leaf tissue and leaf apoplast after the infiltration with COR.

A) Total free amino acids (pmol/mg DW) in lysates of water- (mock) or COR-infiltrated leaves 3HPE. Mean ± SEM (n = 12). **B)** Concentrations of amino acids in apoplastic washing fluid (AWF) 3h post-infiltration of wild-type leaves with COR. Mean ± SEM (n = 15). Data analysis: Student's t-test (two tails) was used to obtain the P values for comparisons. Statistically significant differences between mock and treatment for each amino acid are highlighted in red ($P < 0.05$). (A) single experiment (B) Data combined from four independent experiments with similar results. independent experiments with similar results.

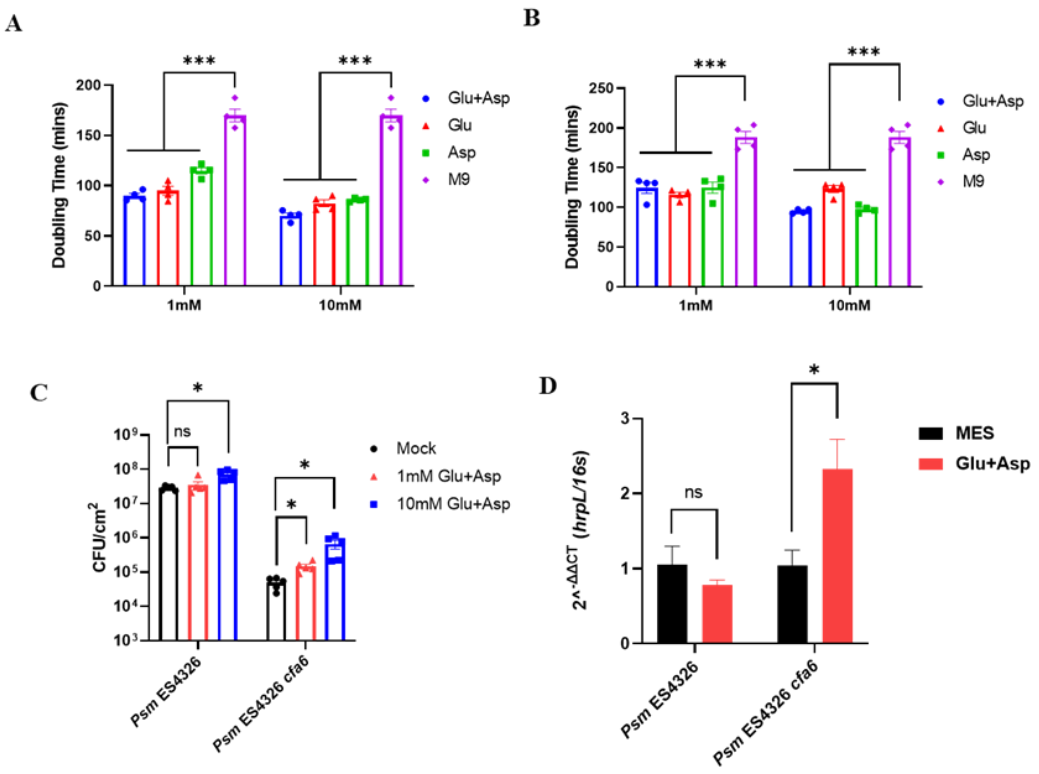

Figure 2- 13. Glutamic acid and Aspartic acid support bacterial growth *in vitro* and *in planta*.

A) Doubling time of *Psm* ES4326 in M9 minimal media supplemented with Glu, Asp, or both combined. Mean ± SEM (n = 4). B) Doubling time of the *cfa* mutant strain of *Psm* ES4326 in M9 minimal media supplemented with Glu, Asp, or both combined. Mean ± SEM (n = 4). C) Bacterial infection in leaves supplemented with 1 mM or 10 mM Glu+Asp 24 HAI with *Psm* ES4326 or *Psm* ES4326 *cfa6.* Infection titer (CFUs) was assessed 24 hours after amino acids supplementation. Mean ± SEM (n = 6). D) Bacterial gene expression in *Psm* ES4326 and *Psm* ES4326 *cfa6* 24 hours after amino acids supplementation and 48 HAI. Mean ± SEM (n = 4). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), < 0.01 (**), < 0.001 (***), or non-significant (ns) differences are shown. Similar results were obtained in three out of three independent experiments.

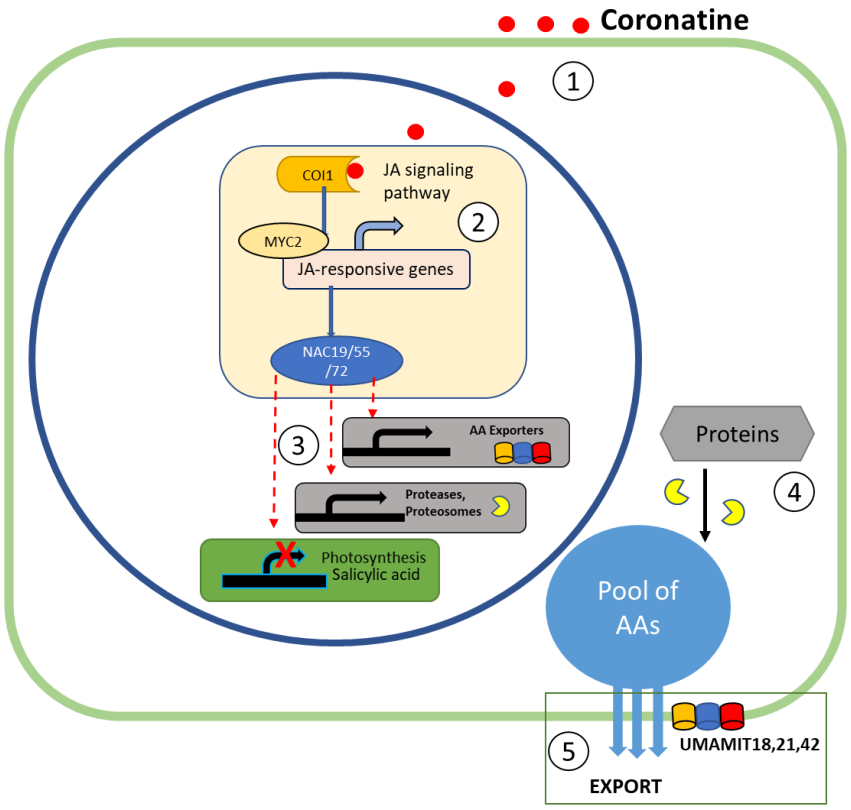

Figure 2- 14. A working model: COR makes Glu and Asp available in the leaf apoplast via the activation of a senescence program and the induction of *UMAMITs* that secrete amino acids out of the cells.

The model summarizes the potential role of COR in increasing the concentration of amino acids in the lead apoplast of infected leaves. **1.** *P. syringae* synthesizes COR at later time points during infection. **2.** Binding of COR to *COI1* activates the jasmonic acid signaling pathway. **3.** The expression of jasmonic acid-responsive genes will cause induction of *UMAMIT* genes, activation of proteases and proteasomes, and down-regulation of photosynthesis via suppression of *RCA1* and *RBCS*. **4.** In the cytoplasm, the proteases and proteasomes break down proteins into free amino acids. **5.** These amino acids are then exported out of the cell and into the leaf apoplast via *UMAMI18*, *UMAMIT21*, and *UMAMIT42*.

2.6 Materials and methods

Plant material

Arabidopsis thaliana ecotype wild type (Col-0) and T-DNA insertional lines were obtained from ABRC, Ohio State University. Whenever available, two independent alleles of previously uncharacterized insertional lines were tested. The lines used this study are, coi1-17, *myc2* (SALK_040500), *nac19/55/72*, *sid2-2*, *cpr5*, *npr1*, *aap1* (SAIL_871_C03), *aap2* (SALK_047237), *aap3* (SALK_148822c), *aap4* (Wiscseq_DsLox351C05.0) *aap6* (SALK140384), *cat1* (SALK_087921c), *cat6* (SALK_082408), *lht1* (SALK_026389), *lht2* (SALK_088896), *lht4* (SALK_204245c), *lht7* (SALK_043012), *prot2* (SALK_067508), *prot3* (SALK_116060), *prot4* (SALK_024507), *put2* (SALK_129045), *put4* (SALK_204857), *umamit4* (SALK_152243), *umamit5* (GK_799H03), *umamit10* (SALK_206142), *umamit11* (WiscDsLox_495C08), *umamit12* (SALK_057246), *umamit14* (SALK_037123), *umamit18* (SALK_123331c), *umamit21*(SALK_064834), *umamit27* (SAIL_359_G10), *umamit28* (SALK_099741), *umamit30* (SALK_140547c), *umamit33* (GK_702_E11), *umamit42* (SALK_011509c).

Seedlings exudate accumulation assay

Arabidopsis seeds were surface sterilized in 10% bleach and kept in dark at 4C for 24 hours. At least 15-18 seeds were planted in 1mL of 12-well culture plates in liquid Murashige and Skoog (MS) basal medium with vitamins (PhytoTech; M519), 0.5% sucrose (Sigma; S7903), 0.5 g/L MES (Sigma; M8250), pH 5.7 corrected with KOH. Plates were sealed with parafilm and incubated inside Conviron adatis A1000 growth chambers (Conviron, Inc.) under 16 hours of light photoperiod, 23^0C constant temperature, 100µmol light intensity, and 75% relative humidity. Liquid MS medium was changed on the 8th day after sowing. On the 10th day,

required treatment was added to the seedlings, and the same volume of sterile MS was added to the mock group. Liquid exudates were collected after 24 hours.

Uptake of Glutamine, and Serine

The seedlings were maintained for 10 days as described above. Then the seedlings were treated with flg22, COR and COR+flg22 on day 10. After 24 hours, Seedlings were washed in MS liquid media and then incubated in fresh MS media with 100 µM cold (unlabeled) Glutamine, or cold Serine, and 3.7 kBq of the corresponding U-^{3}H radiolabeled AA (Perkin Elmer). Plates with seedlings were then transferred to a growth chamber and incubated under slow shaking at 23C, 80% relative humidity, and 30 E.m^{-2} s^{-1} light energy for the duration of the uptake period. The uptake was stopped by washing seedlings with 0.2mM CaSO4 twice. Samples were then lyophilized, ground into powder using glass beads and a Tissue Lyser (QIAGEN) and digested in 1mL of 10% bleach overnight. Chlorine was removed by drying the samples at 55C overnight before resuspending the samples in 200µL of scintillation cocktail (Ultima Gold; Perkin Elmer) and transferring them to a 96-well Isoplate-96 microplates (Perkin Elmer). Radioactivity CPM (counts per minute) was measured with a MicroBeta® Trilux Scintillation Counter (Perkin Elmer). Unless otherwise indicated in the figure legends, seedlings were allowed to uptake ^{3}H-Gln, ^{3}H-Ser for 1H, and before processing the samples.

Bacterial strains and growth conditions

Psm ES4326 and *Pst* DC3000 were maintained on LB plates supplemented with 50µg/ml streptomycin and 50µg/ml Rafampicin respectively. In preparation of bacterial growth assay experiment, a single colony was randomly picked from appropriate plate and grown overnight in approximately 5 mL of King's B at 28C with shaking at 230 rpm till the cultures reached

OD_{600nm} = 0.4-0.8 (approx. 3-4 hours). The bacterial culture was harvested and washed three times in sterile water, and then adjusted to required inoculation titer with sterile water.

Quantification of free amino acids in seedling exudates

10µM COR was added to the seedlings on 10th day. Seedling exudates (MS medium containing seedling exudates) were collected by pipetting the medium out of 12-well plates wells containing 1mL of liquid MS medium on 11th day. Total L-AA concentration in the exudates was quantified with the commercial L-AA Colorimetric/Fluorometic Kit (BioVision/abcam).

Bacterial strains and growth conditions

Pseudomonas syringae pv. maculicola ES4326 and *Psm*Δcma6 were maintained on LB plates supplemented with 50µg/ml streptomycin and kanamycin respectively. In preparation of bacterial growth assay experiment, a single colony was randomly picked from appropriate plate and grown overnight in approximately 5 mL of King's B at 28C with shaking at 230 rpm till the cultures reached OD600 = 0.4-0.8. The bacterial culture was harvested and washed three times in sterile water, and then adjusted to required inoculation titer with sterile water.

Bacterial growth in seedling exudates assay

100uL of collected seedling exudates (Mock and COR treated) were transferred in the well of sterile 96 well flatbottom transparent plate (Corning, CLS3997). Appropriate volume of washed bacterial culture was added to the individual wells to make starting OD600 of 0.01 and briefly centrifuged. The plate was sealed with plastic film, and bacterial growth was recorded over the time at OD600 using SpectraMax i3x Multi-Mode Microplate Reader (Cat#). After extracting the data doubling time was calculated.

Total amino acid extraction

For amino acid extraction in leaves, 4th and 5th pairs of leaves were infiltrated with 10 µM COR. Samples were collected in 2 mL EP tubes and flash frozen after indicated time in the text. The dry weight of each sample is adjusted to the same amount (Usually 5 mg). Leaves were then crushed using Qiagen tissue lyser into powder. 200µl of HCL/Norvaline solution (10mM HCL and 0.1mM Norvaline which is the internal control) and 200µl chloroform are added to each sample and vortex for 2 minutes. The supernatant is transferred to a new tube for UPLC profiling. The profiling is done by our collaborators in Virginia tech.

Apoplastic washing fluid extraction.

Apoplastic washing fluid was extracted with infiltrating and centrifugation methods described by Lohaus et al (2001). 4th and 5th pairs of leaves were treated with COR and Mock. The leaves were cut, and 2 plants were pooled together to make 1 biological replicate. Leaves were vacuum infiltrated with sorbitol (0.24M) with luciferase yellow dye (1mg/100mL) and the pat dried. Leaves of 1 sample were stacked together and centrifuged in parafilm sealed 15ml tube with 5mL syringe (plunger removed) for 5 minutes at 500g (Beckman). Apoplastic washing fluid were then collected in a new tube and stored in -80C. Total L-AA concentration in the AWF was quantified with the commercial L-AA Colorimetric/Fluorometic Kit (BioVision/abcam) or in the University of Virginia-Biomolecular Analysis Facility Core using an UHPLC and a Q-Exactive HF-X mass spectrometer (Thermo Scientific).

Bacteria inoculation assays

6-week-old plants grown in pellets were used. Fully expanded 4th and 5th pairs fo the leaves are labelled before inoculation. For AA supplementation analysis, a single colony of *Psm*ES4326 and *Psm* Δcma6 from a fresh overnight plate was picked and transferred to fresh King's B media (5 mL) and shaken in 28C at 230rpm for 3-4 hours until the OD_{600nm} reached 0.4 – 0.8. The bacterial culture was harvested and washed three times in sterile water, and then adjusted to required inoculation titer with sterile water. The leaves were infiltrated with OD_{600nm} = 0.002 of each bacterial strain. After 24 hours the same leaf was infiltrated with 1 mM of Glutamic acid + Aspartic acid and 1mM of Serine only. Samples were collected in indicated time points in text. Leaf samples were grinded in 400ul sterile water and steel beads in a tissue lyser. The lysates are then diluted in a series of 10 times and transferred to LB plates containing respective antibiotics for CFU counting.

For mutant infection phenotype assay, OD_{600nm} = 0.0002 was used for infiltration and samples were collected after 72 hours.

RNA work and gene expression analysis

Harvested leaves were frozen in dry ice. RNA was isolated using TRIzol® Reagent (Fisher Scientific; Cat#15596018.) and quantified in a NanoDropND1000 spectrophotometer (Thermo Fisher Scientific Inc.). RNA samples (5 µg) were treated with DNAse-I Promega for genomic DN decontamination. For Nanostring® 200ng of RNA samples (3 biological replicates per condition) were used; hybridization of samples with florescent probe sets were done following standard protocol (Geiss et al., 2008). nSolver® software (NanoString Technologies Inc) was used to normalize the raw fluorescent counts. For qPCR, cDNA was synthesized with M-MLV

(Promega) retro transcriptase and random oligo-decamers (Invitrogen) using 2 µg of DNAse treated RNA. 1µL of cDNA was used in a total reaction volume of 20 µL containing 10 µL SyBr Green mix (Cowin Biosciences), 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.

Enzymatic activity of Glucose-6-phosphate dehydrogenase

The exudates collected after COR treatment were collected and stored on ice. 0,5 ml reaction solution (G6P/NADPsol) was prepared by adding µl G6P and 5 µl NADP to 495 µl of TrisHCl 0,1M pH=8. 0.5 ml of exudates were added to 0.5 ml of prepared reagent. The measurement of O.D was checked at 340nm, 10 mins for 1 hour. Crushed seedligns were used as positive control.

Statistical analysis

All data show one of three independent experiments and the comparisons between groups are two-tailed t-tests unless specified in the legends otherwise.

Chapter 3. COR induces the accumulation of amino acids in the leaf apoplast by suppressing the upload of amino acid to the leaf phloem.

3.1 Abstract

The concentration of apoplastic amino acids is defined by the UMAMIT-mediated passive secretion from photosynthetic cells and the active proton-coupled uptake into photosynthetic cells and phloem parenchyma cells mediated by ATP-dependent amino acid transporters. To directly test the potential role of amino acid importers in plant immunity, the resistance to *Pst* DC3000 infections was tested in loss-of-function mutants for COR-responsive genes. Mutants for two COR-suppressed importers, *AAP6,* and *LHT7*, were more susceptible to bacterial infections than wild-type plants, suggesting that COR suppresses their plant immune function. While *AAP6* is known to upload amino acids to the phloem, the localization and function of *LHT7* are still unknown. To address potential connections between COR-induced responses, plant amino acid transport activity, phloem loading, and bacterial infections, I focused my effort on *AAP6* and left *LHT7* aside for future studies. Expression analysis of AAP transporters showed that all AAPs were downregulated in the *aap6* mutant. To address potential connections between phloem loading and immunity, I assessed the concentrations of amino acids in *aap6* leaf apoplast and petiole phloem sap. While the concentration of total amino acids in leave tissue and leaf apoplast was similar in *aap6* and wild-type naïve plants, the concertation of total amino acids in the petiole phloem sap of naïve *aap6* was lower than in naïve wild-type plants. In addition, while total amino acid concentrations in the phloem sap of wild-type plants dropped 6HAI and increased 24HAI with *Pst* DC3000, the concentration of total amino acid remained unchanged in the *aap6* mutant. In addition, in spite of having a fully

functional PTI signaling pathway, *aap6* fails to accumulate amino acids in the leaf tissue and leaf apoplast 24h post flg22 treatment, suggesting that key responses that suppress bacterial infections are compromised in this mutant. Despite having concentrations of total amino acids similar to those of wild-type plants, *aap6* plants had a higher relative level of Glu in the leaf apoplast and allowed *Pst* DC3000 to express higher levels of virulence genes than wild-type plants, again suggesting that the compromised transport of amino acids in *aap6* negatively impact plant immunity. Overall, these findings suggest that the COR-mediated suppression of *AAP6* impairs the execution of changes in leaf apoplastic concentrations of amino acids that are needed to restrict bacterial infections. Further studies will be required to address if the *aap6* phenotypes described in this chapter can be attributed to the loss of *AAP6* or to the combined activity of several AAPs whose expression is downregulated in the *aap6* mutant.

3.2 Introduction

Amino acids play a crucial role as building blocks of proteins and secondary metabolites essential for various physiological processes in plants. Amino acids also act as signaling molecules and indirectly regulate plant gene expression. Additionally, they are involved in the synthesis of phytohormones, which play a key role in plant growth, development, and immunity (Trovato et al., 2021). Plants take up inorganic nitrogen from the soil in the form of nitrate or ammonium and synthesize amino acids in photosynthetically active cells from where they are transported to the rest of the plant to support growth and reproduction. Plants regulate the distribution of amino acids through their tissues to meet the demands of stress responses, growth, and reproduction. This is accomplished through the regulation of amino acid metabolism and the expression of transporters that allow amino acids to move in and out of

cells. AATs are integral membrane proteins that play a critical role in the secretion, uptake, translocation, and distribution of amino acids in plants. Based on protein sequence similarity and transport activity, AATs can be organized into three major families: the Amino Acid/Auxin Permease (AAAP) family, the Amino Acids/Polyamine organocation transporter (APC) family, and the Usually Multiple Amino acids Move In and out Transporter (UMAMIT) family. AAAPs can be further divided into general Amino Acid Permeases (AAPs), Lysine and Histidine Transporters (LHTs), Gamma-aminobutyric Acid Transporters (GATs), Proline Transporters (ProTs), Like-Auxin transporters (LAXs), and Aromatic/Neutral amino acid Transporters (ANTs). The APC family consists of three sub-families: Cationic Amino acid Transporters (CATs), Amino acid/Choline transporters (ACTs), and polyamine H+-symporters (PHSs) (Yao et al., 2020). Differences in substrate specificities, transport activity, and tissue expression patterns contribute to distributing amino acids through the plant body.

Plant-pathogen interactions are complex and dynamic processes that involve numerous molecular players and biochemical events. Amino acids have emerged as important players that regulate plant immunity and bacterial virulence. Several amino acids, including glutamate, arginine, and lysine are involved in the regulation of plant immune responses (Goto et al., 2020; Yang & Ludewig, 2014; Y. Zheng et al., 2011). Phenylalanine serves as a precursor for the biosynthesis of intracellular signaling module salicylic acid (H. Lee et al., 1995), a key player in plant defense against biotrophic pathogens. Tryptophan and methionine are precursors for the synthesis of secondary metabolites with antimicrobial activity (Ahuja et al., 2012). On the other hand, amino acids are a preferred source of nitrogen and carbon for bacteria that colonization of the leaf apoplast. In *Arabidopsis* and most dicotyledonous crops, newly synthesized amino acids move out of the photosynthetic cells and into the leaf apoplast

via UMAMIT facilitator transporters that localize to the plasma membrane. While a fraction of the apoplastic amino acids is taken back into the photosynthetic cells, another fraction is uploaded into the phloem parenchyma cells for long-distance translocation. The function of amino acids as nutrients and signaling molecules has a profound impact on bacterial metabolism and virulence. High levels of Gln and Ser, that accumulate at the leaf apoplast after the perception of PAMPs, delay the onset of bacterial virulence and compromise the ability of *Pst* DC3000 to colonize *Arabidopsis* leaves (X. Zhang et al., 2022; X. Zhang et al., 2023). An indirect role of AATs in plant defense against microbes has been previously reported. For instance, the *LHT1* loss-of-function mutant plants develop spontaneous cell death, progressively accumulate salicylic acid, and become hyper-resistant to bacterial infections (A. Hirner et al., 2006). Similarly, the ectopic overexpression of *CAT1* produces spontaneous cell death and enhanced resistance to bacterial infections (Yang, Postel, et al., 2014). These pleiotropic phenotypes complicate the analysis of *LHT1* and *CAT1* role in plant immunity. Several *AAPs* are differentially regulated during biotrophic pathogen infections. More direct roles have been proposed for some AAPs whose loss-of-function mutants do not seem to have obvious pleiotropic effects as those described for *lht1* and *CAT1*-OX lines. For instance, *Arabidopsis* roots infested with the Cyst nematode *Heterodera schachtii* express high levels of several AAPs, and loss-of-function mutants for *AAP1*, *AAP2*, and *AAP6* negatively impacted root colonization by cyst nematodes (Elashry et al., 2013). Similarly, the reproduction of the root-knot nematode *Meloidogyne incognita* was compromised in the *Arabidopsis aap3* and *aap6* loss-of-function mutants, suggesting that the activity of these transporters supports plant infestation with nematodes (Marella et al., 2013). UMAMIT transporters, on the other hand, can have a detrimental impact on plant disease as they have been shown to support infections

with biotrophic bacterial and oomycete pathogens (Denancé et al., 2013). Hence, the ability of pathogens to manipulate plant amino acid transporters has a profound impact on the outcome of plant pathogen interaction.

In Chapter 2, I demonstrated that COR induces changes in plant metabolism and transport that favor the secretion of amino acids. To further understand the role that COR plays in regulating amino acid transport and bacterial infections, I tested the transcriptional changes of AATs after COR treatment of mature leaves. I found that several AATs responded transcriptionally to COR treatment. Among other AAT mutants with altered pathogen's phenotypes *aap6* was compromised to upload amino acids into the phloem in response to infections and showed enhanced susceptibility to *Psm* ES4326 and *Pst* DC3000. This study provides molecular and physiological details to understand the role of COR on modulating plant amino acids transport to support bacterial growth during infections.

3.3 Results

3.3.1 COR induces the expression of the AATs in mature leaves.

The concentration of free amino acids in the apoplast results from opposing transport processes that move solutes in opposite directions: passive secretion, and active uptake. Since COR induces changes in amino acid concentrations in the leaf apoplast (Chapter 2), I assessed the expression of the AATs in the leaves of six-week-old plants 3h post COR infiltration using nanoString® RNA hybridization. The rapid detoxification of infiltrated COR that takes place in *Arabidopsis* leaves between 2-6 hours after infiltration (de Torres Zabala et al., 2016) hinders the analysis of gene expression beyond 3h post-treatment. The analyses identified

several AATs whose mRNA levels change significantly 3h post COR treatment in leaves (Table 3-1). If these transporters are important in defining *Arabidopsis-P. syringae* interactions, the growth of the pathogen should be altered in the loss-of-function mutants of these genes. T-DNA insertional lines that knock out the expression of these genes were identified (Figure 3-1). Most of the mutant lines supported bacterial infection levels similar to those of wild-type plants (Figure 3-2). While mutants of *aap3* and *lht1* had enhanced resistance to *Psm* ES4326 infection, *aap6* and *lht7* mutants had enhanced susceptibility phenotypes (Figure 3-3). The enhanced resistance phenotype of *lht1* is attributed to the increased salicylic acid levels and was previously described (Liu et al., 2010). Unlike LHT1, the function of LHT7 and its tissue-specific expression or subcellular localization has not been reported so far. Similarly, the tissue-specific expression of *AAP3* in leaves is still unknown. Although interesting, the in-depth study of *LHT7* and *AAP3* would require molecular genetics and cellular biology tools that are currently unavailable and out of the scope of my dissertation. Unlike *LHT7* and *AAP3*, *AAP6* is expressed in the leaf vascular system and has been previously described to play a role in the loading of amino acids to the leaf phloem (Hunt et al., 2010). Thus, I chose to study *AAP6* further to unveil its potential contributions to plant immunity against bacteria.

3.3.2 Characterization of *aap6* mutants

To further analyze the function of *AAP6,* two independent homozygous T-DNA insertional lines were identified within the *AAP6* locus. The *aap6-1* (SALK_140384) and *aap6-2* (SALK_013231) were isolated and analyzed. The *aap6-1* line contains T-DNA insertions in the 3rd intron while *aap6-2* harbors T-DNA insertions in the 5th intron The T-DNA insertions

were confirmed by PCR-genotyping (Figure 3-4A). Expression analysis of *AAP6* in source leaves of six-week-old plants was analyzed using RT-PCR and confirmed that the two T-DNA insertional lines are *AAP6* knockout mutants (Figure 3-4B).

In *Arabidopsis*, most AAPs are expressed in the vascular tissues where they play an important role in shuttling amino acids from the xylem to the phloem (Y.-H. Lee et al., 2007; Okumoto et al., 2002; Santiago & Tegeder, 2016). Previously published studies have shown that the expression of the eight AAP genes in *Arabidopsis* is coordinated via unknown signaling mechanisms likely involving the sensing of AA concentrations across various tissues (Santiago & Tegeder, 2016). To account for similar changes in gene expression that could contribute to the phenotypes observed in *aap6*, I tested the expression of AAPs in *aap6* mutant and wild-type plants using RT-qPCR. The analysis showed that the expression of *AAP1*, *AAP2*, *AAP3*, *AAP4*, *AAP5*, and *AAP8* was significantly lower in *aap6* compared to wild-type leaves (Figure 3-5). These data suggests that the expression of other AAPs is co-regulated to compensate for the lack of AAP6 activity and shows that the *aap6* phenotypes are the result of a concerted lower activity in most members of the AAP family.

3.3.3 *AAPs* serve a defense function against *Pst* DC3000 colonization.

Like COR-treated leaves, *Arabidopsis* leaves infected with *Pst* DC3000 expressed less *AAP6* (www.bar.utoronto.ca), suggesting that a low *AAP6* expression facilitates leaf colonization. To assess the potential role of AAP6 in plant immunity, I quantified AAP6 expression in wild-type Col-0 plants 6 HAI and 24 HAI with *Pst* DC3000. The expression of *AAP6* remained unchanged at 6 HAI but was significantly suppressed 24 HAI with *Pst* DC3000 (Figure 3-6A).

These results suggest that *AAP6* plays a positive role in plant defense. To test this hypothesis, I infected plants with *Pst* DC3000 to assess bacterial infections. Two independent *aap6* loss-of-function mutants showed enhanced susceptibility phenotype to *Pst* DC3000 infection (Figure 3-6B), again suggesting that the COR and bacterial elicited suppression of *AAP6* in wild-type plants could facilitate *P. syringae* infections.

Since the expression of all AAPs was also downregulated in the *aap6* loss-of-function mutant (Figure 3-5), I sought to address the expression of each *AAP* in response to pathogen infection 6 HAI and 24 HAI with *Pst* DC3000. The expression of *AAP2* was suppressed, while *AAP1*, *AAP3*, and *AAP4* were induced at 6HAI. While the expression of *AAP1*, *AAP3*, *AAP4*, and *AAP8* was induced 24 HAI, *AAP2*, *AAP5*, and *AAP6* expression was suppressed (Figure 3-7). These data suggest that the enhanced susceptibility phenotype of *aap6* could be attributed to the lower levels of *AAP1*, *AAP3*, *AAP4*, and *AAP8*, whose expression is induced in wild-type plants in response to *Pst* DC3000 infection. Alternatively, the enhanced susceptibility to bacterial infection of *aap6* could be attributed to the loss of *AAP6* activity in conjunction with the low expression of *AAP2* and *AAP5*, whose expression is suppressed in *Pst* DC3000-infected wild-type plants.

3.3.4 PTI and ETI defense programs remain intact in the *aap6* mutant.

Plant resistance to bacterial pathogens depends on the timely perception of invading microbes and the induction of immune programs. Both PTI and ETI, the two microbial-induced immune programs that coordinately protect plants from infections, are partially controlled by salicylic acid-mediated signaling. Since previous studies showed that mutants (Liu et al., 2010) or

transgenic lines (Yang, Postel, et al., 2014) with modified amino acids transport activity had pleiotropic phenotypes attributed to the dysregulation of salicylic acid homeostasis, I sought to address if the enhanced susceptibility of *aap6* could stem from a defective salicylic acid signaling. To that end, I inoculated plants with *Pst*-AvrRpt2, a bacterial strain that elicits strong and fast salicylic acid-mediated defense responses and thus is unable to produce infections in wild-type plants (Z. Chen et al., 2004). The induction of the salicylic acid signaling marker gene *PR1* was significantly lower in *aap6* than in wild-type plants 6 HPI but similarly high 24 and 48 HPI (Figure 3-8B). In addition, the expression of the salicylic acid synthesis gene *Iso-Chorismate Synthase-1 (ICS1)* was similarly induced in both *aap6* and wild-type *Pst*-AvrRpt2-inoculated plants (Figure 3-8C). Importantly, *aap6* suppressed the growth of *Pst*-AvrRpt2 to the same extent as wild-type plants 72 HPI (Figure 3-9), suggesting that the delayed onset of *PR1* induction does not impact salicylic acid-mediated immunity and ETI in *aap6*.

A defective early onset of salicylic acid-mediated immunity could originate from a compromised PAMP perception or signaling (Tsuda et al., 2008). To test this hypothesis, I inoculated plants with *Pst* Δ*hrcC* and *Pst* Δ*CEL,* two *Pst* DC3000-derivative strains that fail to assemble the T3SS protein complex and lack essential T3Es, respectively. The compromised virulence in these strains hinders their ability to suppress the early onset of salicylic acid signaling and PTI. Because of their compromised virulence, these strains fail to proliferate in the leaf apoplast (Badel et al., 2003; Yuan & He, 1996). After inoculation, both strains produced similarly mild infections in wild-type and *aap6* plants, suggesting that *aap6* possesses an intact and fully functional PTI (Figure 3-10). In addition, the flg22-mediated induction of PTI markers *WRKY29* and *MYB51* in *aap6* was similar in timing and magnitude to wild-type plants (Figure 3-11). Since flg22 perception and signaling lead to changes in the

concentration of apoplastic amino acids that suppress bacterial infections (X. Zhang et al., 2023), I tested if such changes still happen in the *aap6* mutant. While the concentration of total amino acids increased in leaf tissue and leaf apoplast of wild-type plants 24 h post flg22 treatment, *aap6* concentrations remained constant (Figure 3-12B). Overall, these data show that the enhanced susceptibility of *aap6* to *Pst* DC3000 and *Psm* ES4326 is unrelated to immune signaling defects, and suggests that *AAP6*, and perhaps other AAP transporters, contribute to plant immunity by executing changes in amino acid concentrations in the leaf apoplast elicited by PAMPs.

3.3.5 AAPs are necessary to execute changes in the loading of amino acids to the leaf phloem in response to both PAMPs and bacterial infection.

In *Arabidopsis*, AAP6 localizes to the plasma membrane of xylem parenchyma cells, where its transport activity contributes to loading amino acids to the phloem (Hunt et al., 2010; Okumoto et al., 2002). To assess potential defects of *aap6* in modulating amino acids transport activity during a bacterial infection, I assessed the concentration of total amino acids in the petiole phloem sap under infection conditions. While the total concentration of amino acids in the petiole phloem sap of wild-type plants decreased 6 HPI with *Pst* DC3000 compared to non-infected leaves, naïve or inoculated *aap6* plants had phloem concentrations of amino acids lower than wild-type plants and did not respond to *Pst* DC3000 infection by decreasing amino acid concentrations (Figure 3-13A). The drop in petiole phloem sap concentrations was reverted at later time points. While the concentrations of total amino acids in phloem sap were higher in infected than in mock-treated wild-type plants 24HAI, the concentrations in *aap6* phloem sap remained unchanged (Figure 3-13B). Overall, the data presented in this section

shows that *app6* is unable to execute changes in amino acid concentrations in the leaf apoplast needed to suppress bacterial infections. The data also provide insights into how COR could alter *Arabidopsis* amino acid transport to create leaf apoplast conditions that promote bacterial infections, an entirely new facet in the virulence role of COR.

3.3.6 *AAPs* are necessary to restrict *Pst* DC3000 virulence.

Large-scale bacterial transcriptomic analysis revealed that early infection-phase bacterial transcriptome patterns may provide insight into how bacteria proliferate. In PTI-elicited plants, expression of T3SS-related genes was suppressed at earlier time points after inoculation, which correlated with reduced bacterial growth at later time points (Nobori et al., 2018). Considering that several AA found in the leaf apoplast of *Arabidopsis* regulate the expression of bacterial genes encoding T3SS virulence components and COR biosynthesis, I reasoned that apoplastic AA in *aap6* could modify the expression of bacterial virulence genes. To test the possibility that *AAP6* contributes to suppressing bacterial virulence, I inoculated wild-type and *aap6* plants with *Pst* DC3000 and checked the expression of virulence genes at different time points. A previous study from our lab showed that an increased concentration of Gln and Ser in the leaf apoplast of flg22-treated plants delays the onset of bacterial virulence and compromises the ability of *Pst DC*3000 to establish robust infections. Thus, I hypothesize that *aap6*, being compromised to execute changes in concentrations of apoplastic amino acids in response to flg22 and bacterial inoculation, could fail to suppress bacterial virulence at the early stages of infection. Bacterial gene expression data showed that *Pst* DC3000 was able to express higher levels of the virulence genes *hrpL, hrpA, HopM1,* and *cfl* in *aap6* than in wild-type plants 3h after inoculation (Figure 3-14) suggesting that *aap6* enhanced-susceptibility could stem from

its inability to suppress bacterial virulence. In addition to Gln and Ser (X. Zhang et al., 2023) other amino acids have been reported to modulate the expression of bacterial virulence genes. For instance, glutamic acid (Glu) supplemented with 10 mM fructose, induces the expression of *hrpL* in *Pst* DC3000 in minimal medium (Yan et al., 2020). In naïve *aap6* leaves, the concentration of Glu in the leaf apoplast was modestly but significantly higher than in the wild type (Table 3-2). To assess the contribution of Glu on *Pst* DC3000 virulence induction *in planta*, I co-infiltrated *Pst* DC3000 and 10 mM Glu and assessed the expression of bacterial genes encoding T3SS and COR biosynthesis genes. The expression of *hrpL* and *cfl* was significantly induced in *Pst* DC3000 2.5 HAI of wild-type leaves (Figure 3-15). This result suggests that the COR-mediated suppression of *AAP6* expression could compromise the plant ability to suppress virulence by increasing apoplastic amino acids concentrations in response to PAMP's perception, at the same time that induces the accumulation of Glu, a bacterial virulence-inducing amino acid.

3.4 Discussion

COR suppresses *AAP6* expression and induces the relative accumulation of glutamic acid in the leaf apoplast.

In this study, I showed that COR suppresses the expression of several plant amino acid influx transporters (Table 3-1), including *AAP6*. Most AAP transporters localize to the plasma membrane of phloem parenchyma cells or the integuments of the developing seeds where they

directly upload amino acids to the phloem for long-distance translocation to sink tissues or to the developing embryo, respectively (B. Hirner et al., 1998; Sanders et al., 2009; Schmidt et al., 2007). Unlike other AAPs, *AAP6* localizes to the plasma membrane of xylem parenchyma cells where its transport activity contributes to transferring amino acids from the xylem tubes to the sieve elements of the phloem (Hunt et al., 2010; Okumoto et al., 2002). Like COR-treated leaves, *Pst* DC3000-infected leaves express lower levels of *AAP6* than mock-treated leaves. In addition, *Pst* DC3000-inoculated leaves slow down the upload of amino acids to the phloem at early time points and enhanced the phloem upload at later time points (Figure 3-13B). Since the loss of AAP6 function compromises both phloem loading and resistance to *Pst* DC3000 infections, I hypothesize that COR contributes a virulence function by suppressing *AAP6* and producing a leaf apoplastic environment that allows *Pst* DC3000 to growth to a larger extent than they do in wild-type plants (Figure 3-6). Importantly, the pathogens phenotype of *aap6* is not related to altered salicylic acid signaling as previously described in plants with altered amino acid transport (Liu et al., 2010). Indeed, my data show that PTI and ETI, two salicylic acid-signaling dependent programs that coordinately protect plants from infections, remain fully functional in *aap6* (Figure 3-8A, 3-11). Having ruled out the possibility that the enhanced susceptibility of *aap6* to *Pst* DC3000 and *Psm* ES4326 could derive from a compromised salicylic acid signaling or defective PTI or ETI (Figure 3-10), it becomes apparent that a compromised phloem loading (Figure 3-13) could lead to enhanced susceptibility to bacterial infections. Since immune signaling pathways remain fully operational in *aap6*, the enhanced susceptibility to bacterial infections of this mutant must relate to compromised defense mechanisms that operate downstream of defense signaling. Changes in the composition and concentration of apoplastic amino acids have emerged as

important players in plant immunity (X. Zhang et al., 2022, 2023). While the elicitation of PTI increases the concentration of Gln and Ser in the leaf apoplast to delay the onset of bacterial virulence at the early stages of infection (X. Zhang et al., 2023), COR induces the accumulation of apoplastic amino acids that allow *Pst* DC3000 to sustain fast growth at later stages of infection (Chapter 2). The relative concentration of Glu, a bacterial virulence-inducing amino acid (Anderson et al., 2014), was higher in the leaf apoplast of *aap6* than in wild-type plants (Table 3-2). When wild-type leaves were infiltrated with *Pst* DC3000 supplemented with Glu, bacteria expressed higher levels of virulence (Figure 3-15), suggesting that the increased relative concentration of Glu in the *aap6* leaf apoplast could contribute to the higher levels of bacterial virulence expression in the mutant (Figure 3-14). The higher levels of bacterial virulence at early stages of infection could explain not only the enhanced susceptibility of the *aap6* to bacterial infections but also the delayed induction of the salicylic acid signaling marker gene *PR1* (Figure 3-8). Since Avr*Rpt2* suppresses salicylic acid responses in wild-type plants (Z. Chen et al., 2004), the delayed expression of *PR1* in *Pst* Avr*Rpt2*-inoculated *aap6* plants could stem from the high expression of the T3SS secretion apparatus (Figure 3-14) that would accelerate the delivery of Avr*Rpt2* into *aap6* cells. In addition, the higher level of COR synthesis gene expression in *aap6*-inoculated *Pst* DC3000 (Figure 3-14) could more rapidly suppress salicylic acid responses in *aap6*, a well-documented effect of COR on plant immunity (Brooks et al., 2005; X. Y. Zheng et al., 2012) and delay the induction of *PR1*.

Overall, the data presented in Chapter 3 supports a model (Figure 3-16) where COR suppresses *AAP6* and promotes the accumulation of Glu in the leaf apoplast. As COR reaches high concentration in infected leaves as *Pst DC*3000 colonizes the leaf apoplast, it seems reasonable to propose that, in addition to providing amino acids to support bacterial growth, COR

promotes the accumulation of Glu in the leaf apoplast to secure adequate levels of virulence expression throughout the entire leaf apoplast colonization.

3.5 Future directions

The study found that AAP6 is used by the plant to move nutrients away from the reach of invading pathogens via phloem loading. Future directions for this project should be focusing on how exactly the mechanism of phloem loading strategy could be used efficiently to engineer pathogen resistant plants without costing N mobilization in the plants.

Tissue specific *AAP6* overexpressor line could be used to support the hypothesis that AAP6 functions as starvation gene during pathogen infection. Dexamethasone inducible overexpressors of *AAP6* was used for the project which generated positive results (Data not shown) as these could be due to pleiotropic effect.

Another important aspect of study could be further exploration of subcellular localization of these transporters and their regulation under infection conditions. In this study, I attempted to cross the *AAP6*-GFP lines with plasma membrane marker fluorescent and vacuole marker fluorescent line without successful outcome.

Previous studies have suggested that induction of salicylic acid induces the expression of *AAP6* gene in Brassica napus (Q. Zhang et al., 2015) and in this study I found that early onset of salicylic acid is delayed in aap6 mutant. Thus, studying the regulation of the *AAP6* by plant hormones or vice versa could add another piece of information.

The study found that elevated *ICS1* levels alone were not sufficient to activate PR1 expression in aap6 mutants. Further studies could be conducted to determine if the reduced expression of *PR1* is due to changes in the level of total salicylic acid in aap6 mutants under infection conditions.

This study focused on the role of *AAP6* in plant defense. In my initial screening, I found that *AAP3*, *LHT1*, and *LHT7* also play a role in plant defense. As mentioned above, *LHT1* is extensively studied. *AAP3* is also studied for their role in root-nematode interaction but the role of *LHT7* is not studied. Further studies could be conducted to investigate the expression of these AATs in response to pathogen infection, and to determine their role in plant fitness.

	COR/Mock 3h	
Gene	Fold change	P-value
AAP3	1.62	0.01398
AAP4	1.72	0.02128
AAP6	-2	0.04346
CAT1	-1.85	0.03859
CAT3	-1.41	0.03121
LHT1	-1.57	0.04997
LHT2	-2.1	0.04819
LHT4	-2.09	0.03501
LHT7	-2.04	0.01845
ProT2	-1.39	0.01292
ProT4	-1.72	0.00873
PUT4	1.38	0.00976
UMAMIT10	1.47	0.01177
UMAMIT12	-4.65	0.02408
UMAMIT20	-1.7	0.00253
UMAMIT21	2.2	0.02310
UMAMIT28	-3.25	0.00255
UMAMIT30	-1.68	0.00140
UMAMIT33	-1.63	0.04242
UMAMIT42	2.47	0.01607
VAAT1	-2.47	0.01050
VAAT2	1.98	0.00408
VAAT3	-3.58	0.00492
VAAT8	3.25	0.00032

Table 3-1. Transcriptional changes of AATs after COR elicitation

Log2 fold changes of the transcriptional levels of amino acid transporter (AAT) genes that respond in adult leaves 3 hours after COR treatment. Gene expression was assessed via nanoString® hybridization. Data analysis: Student's t-test (two tails) was used to obtain the P values for comparisons. Statistically significant differences between mock and treatment for each AATs are highlighted in red ($P < 0.05$).

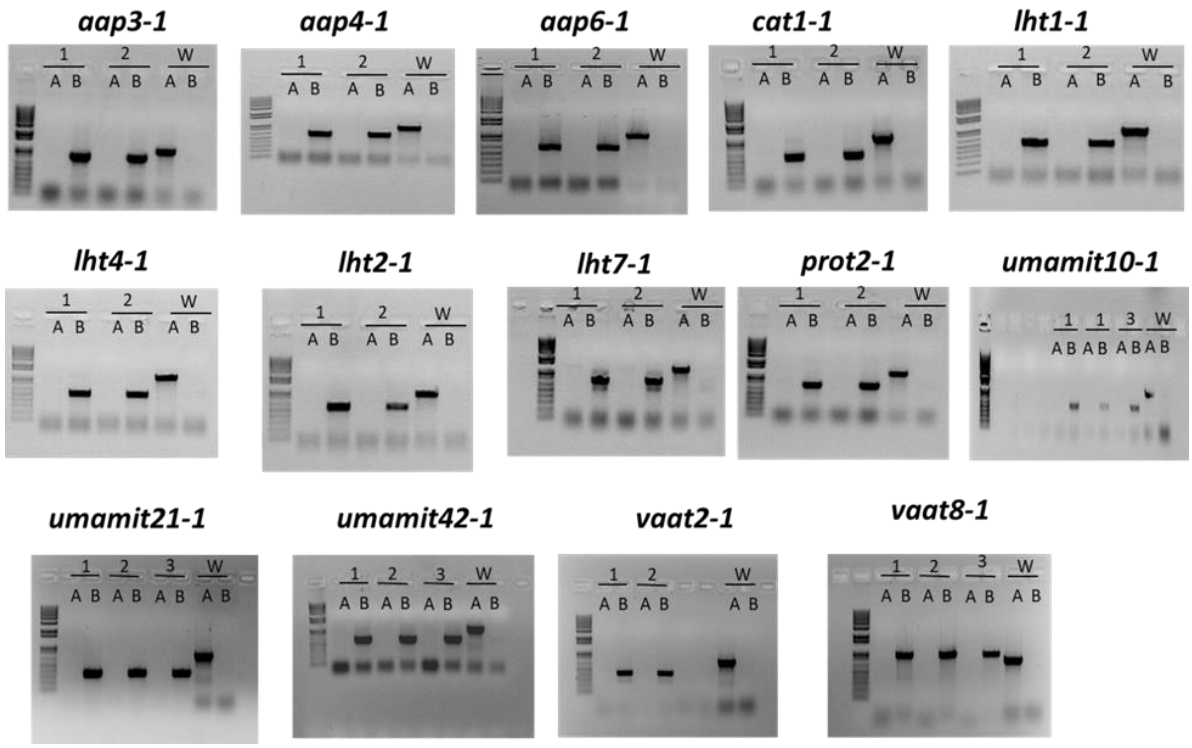

Figure 3- 1. PCR-Genotyping of Arabidopsis insertion lines for AATs that respond to COR treatment.

Genomic DNA was isolated from 2 to 3 independent plants per T-DNA insertional line, or wild-type plants. T-DNA zygosity was assessed via PCR using gene-specific primers to detect the wild-type allele (A) of each gene analyzed, or a primer sets that amplify the T-DNA flanking region in each gene under study to detect the T-DNA insertion (B). Agarose gel images show DNA ladder (left) and PCR products obtained from genomic DNA of plants with T-DNA insertions (1, 2, or 3 plants in some cases) or wild-type plants (W). Results clearly show that all T-DNA insertional lines and plants used in this study have the corresponding T-DNA insertion in homozygosity: while wild-type alleles were only amplified from W genomic DNA and not from mutant's genomic DNA, T-DNA flanking regions were amplified from genomic DNA of T-DNA insertional lines but not from wild type.

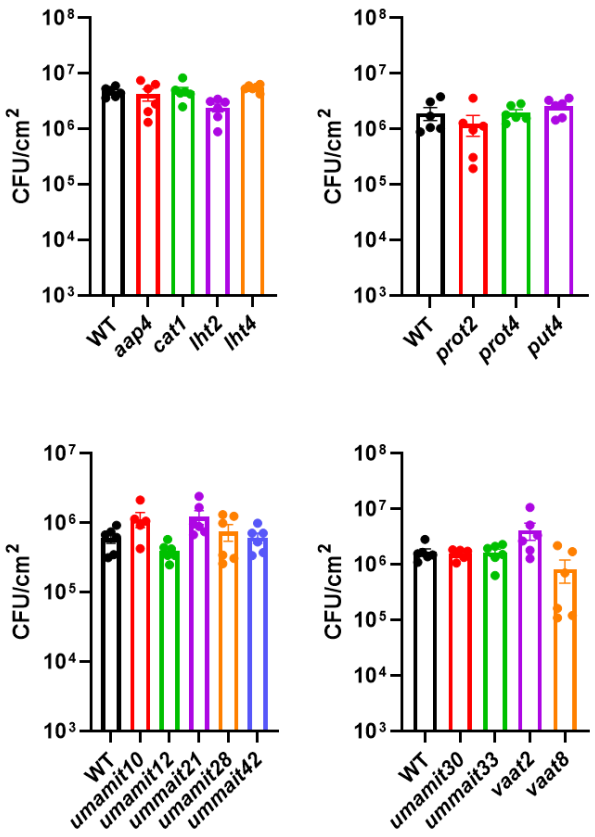

Figure 3- 2. Screening of COR-responsive AAT mutants for infection phenotype.

Infection titer in leaves of wild-type (WT) and insertional mutants of COR-responsive AATs genes 72HAI with *Psm* ES4326 (OD_{600nm} = 0.0002). Mean ± SEM (n = 5). Data analysis: Student's t-test ($P < 0.05$). As infection titers vary across experiments for the wild-type genotype, the data presented in each figure panel correspond to one infection experiment. Similar results were obtained in three out of three independent experiments.

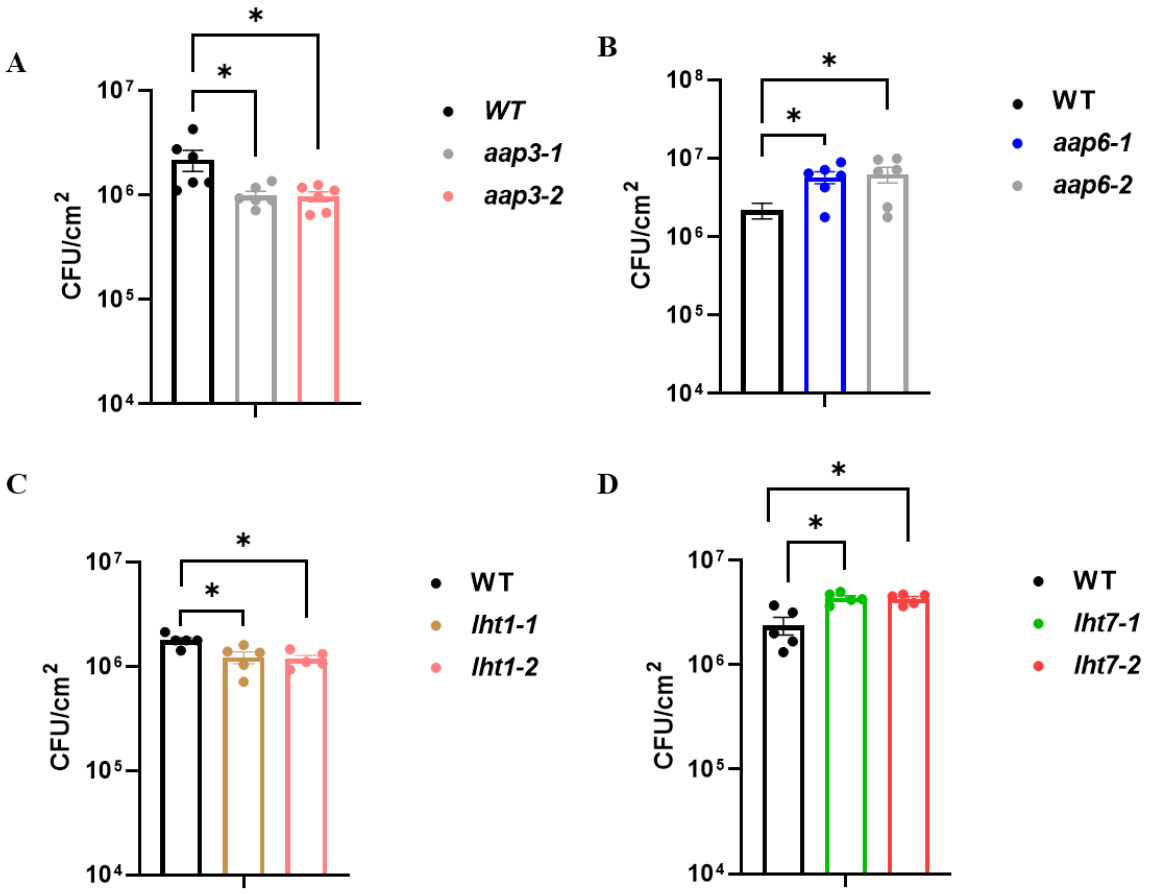

Figure 3-3. COR-responsive AATs play a role in plant defense.

Bacterial infection titer of wild-type plants and AATs mutants 72HAI with *Psm* ES4326 (OD_{600nm} = 0.0002). **A**) *aap3* mutant. **B**) *aap6* mutant. **C**) *lht1* mutant. **D**) *lht7* mutant. Mean ± SEM (n = 5). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*) are shown. Similar results were obtained in three out of three independent experiments.

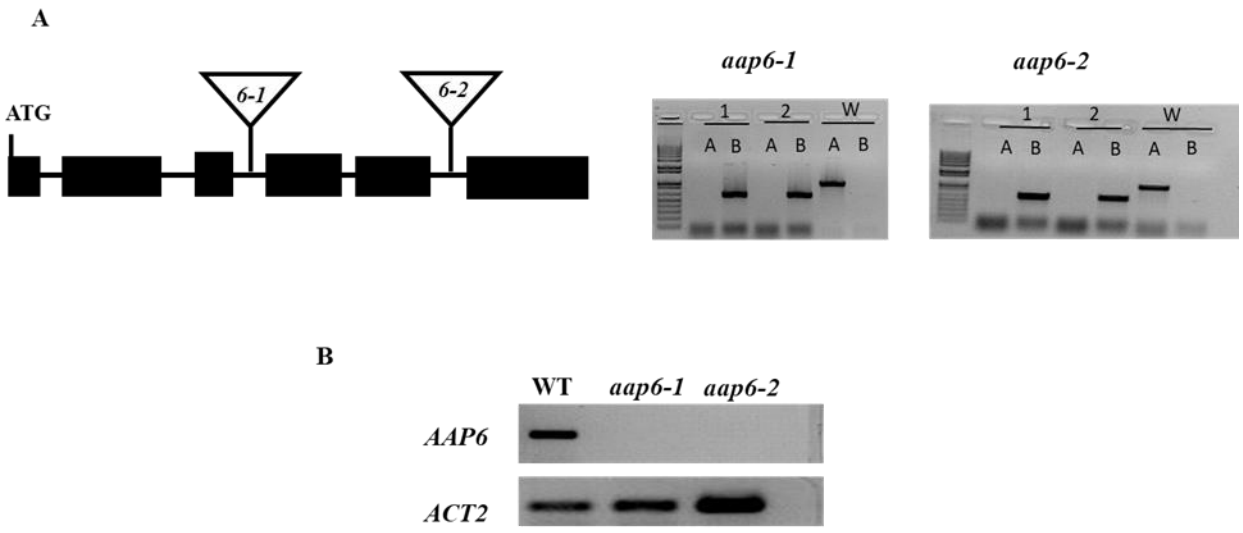

Figure 3- 4. Molecular characterization of *aap6* mutants.

A) Schematic diagram of T-DNA insertions (6-1 and 6-2) in AAP6 and PCR genotyping of the T-DNA insertions in two independent insertional mutants. Boxes and lines represent exons and introns, respectively. Inverted triangles indicate the T-DNA insertion site. PCR fragments amplified from genomic DNA using *AAP6*-specific primers in combination with T-DNA left border primers. Agarose gel image show DNA ladder on the left and PCR products obtained from genomic DNA of plants with T-DNA insertions (1, 2, or 3 plants in some cases) or wild-type plants (W). B) RT-PCR products amplified from wild-type plants (WT), aap6-1, and aap6-2 mutant RNA using *AAP6* gene-specific primers. *ACTIN2 (ACT2)* was amplified as an internal control.

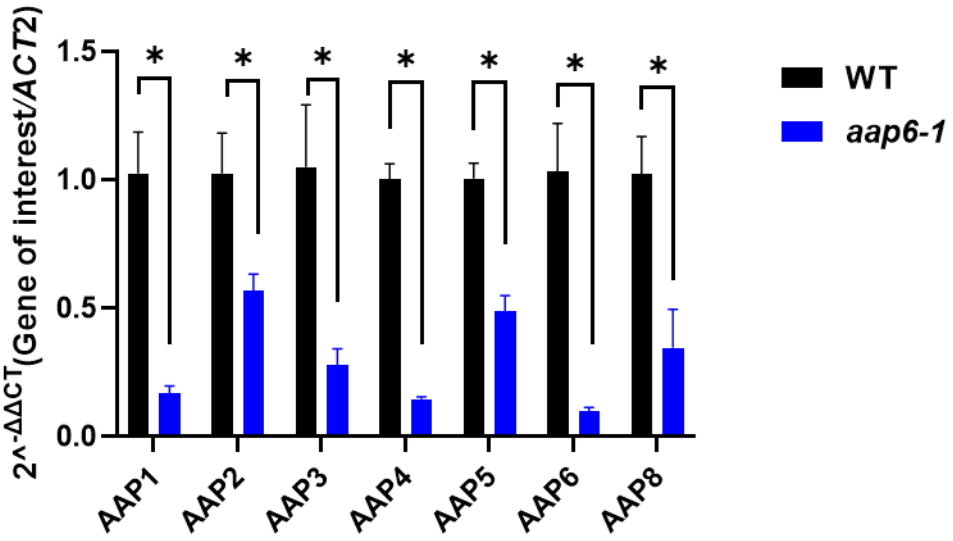

Figure 3- 5. The *aap6* knock out expresses low levels of close homologs in the AAP family.

Gene expression analysis of AAPs in mature leaves of the aap6-1 knock out mutant. RT-qPCR was performed on rosette leaf RNA of six-week-old aap6-1 and wild-type (WT) plants as control. Mean ±SEM (n = 3). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*) are shown. Similar results were obtained in three out of three independent experiments.

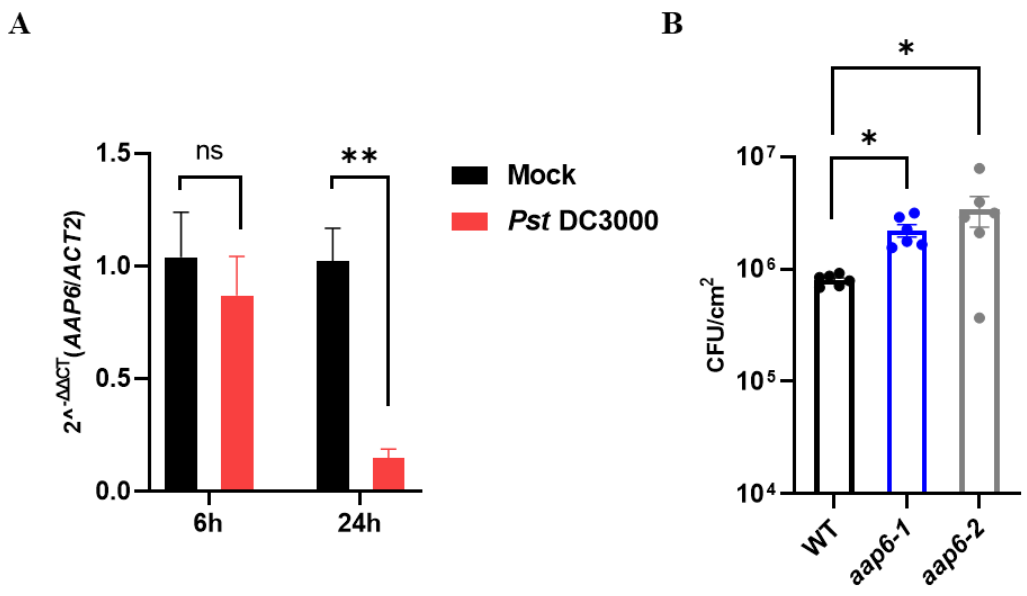

Figure 3- 6. Compromised ability of *aap6* is to restrict *Pst* DC3000 infections.

A) *AAP6* expression in leaves of wild-type plants infiltrated with *Pst* DC3000 (O.D. $_{600}$ = 0.02). Mean ± SEM (n = 3). B) Bacterial infection titter in the leaves of wild-type (WT) and the aap6 knock out mutants 72HAI with *Pst* DC3000 (OD_{600nm} = 0.0002). Mean ± SEM (n = 6). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), < 0.01(**), < 0.001 (***), or non-significant (ns) differences are shown. Similar results were obtained in three out of three independent experiments.

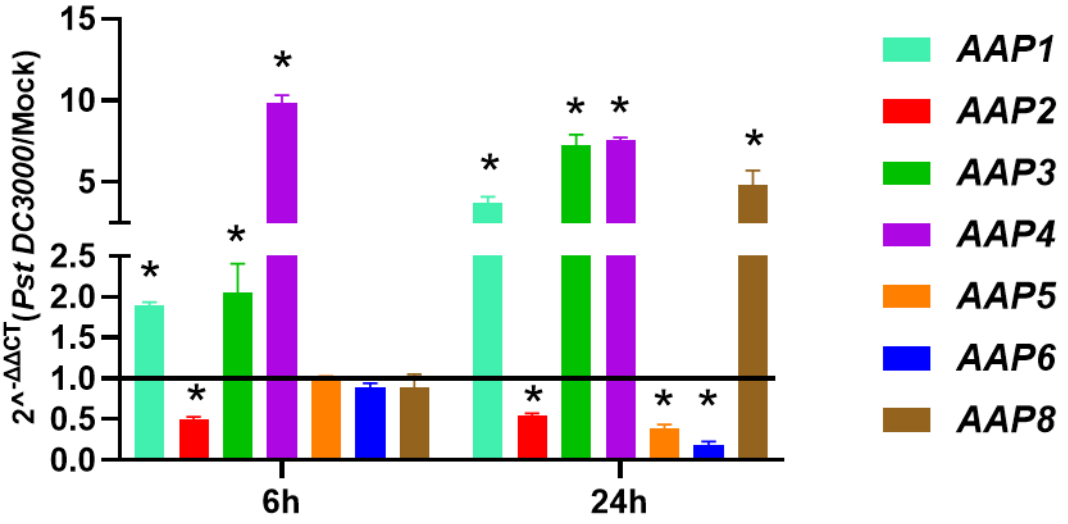

Figure 3- 7. The expression of all *AAPs* significantly changes with *Pst* DC3000 infection in wild-type plants.

RT-qPCR expression analysis of *AAP1*, *AAP2*, *AAP3*, *AAP4*, *AAP5*, *AAP6*, and *AAP8* in wild-type leaves 6HAI and 24HAI with *Pst* DC3000 (OD_{600nm} = 0.02). Mean ± SEM, (n = 3). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*) are shown. Similar results were obtained in three out of three independent experiments.

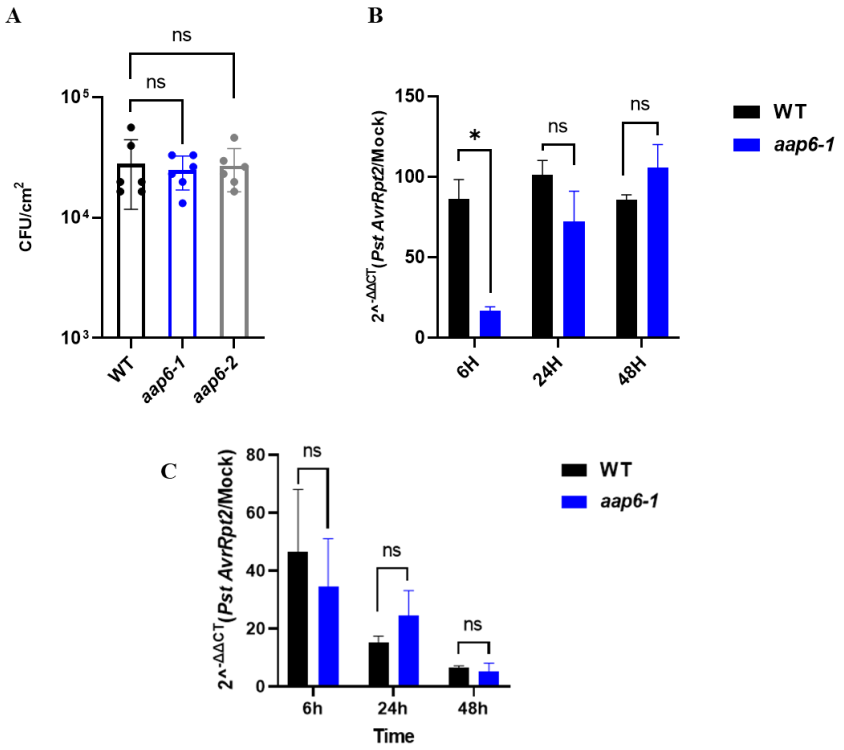

Figure 3- 8. The ability of *aap6* to initiate salicylic acid signaling in response to the T3E Avr*Rpt2* is delayed without affecting ETI.

A) Infection titer in the leaves of wild-type (WT) and the aap6 mutants 72HAI with the salicylic acid defense-inducing strain *Pst*-AvrRpt2 (OD_{600nm} = 0.001). Mean ± SEM (n = 6). B) *PR1* expression in *Pst*-AvrRpt2-infected leaves. Mean ± SEM (n = 3). C) *ICS1* expression in *Pst*-AvrRpt2-infected leaves. Mean ± SEM (n = 3). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), or non-significant (ns) differences are shown. Similar results were obtained in three out of three independent experiments.

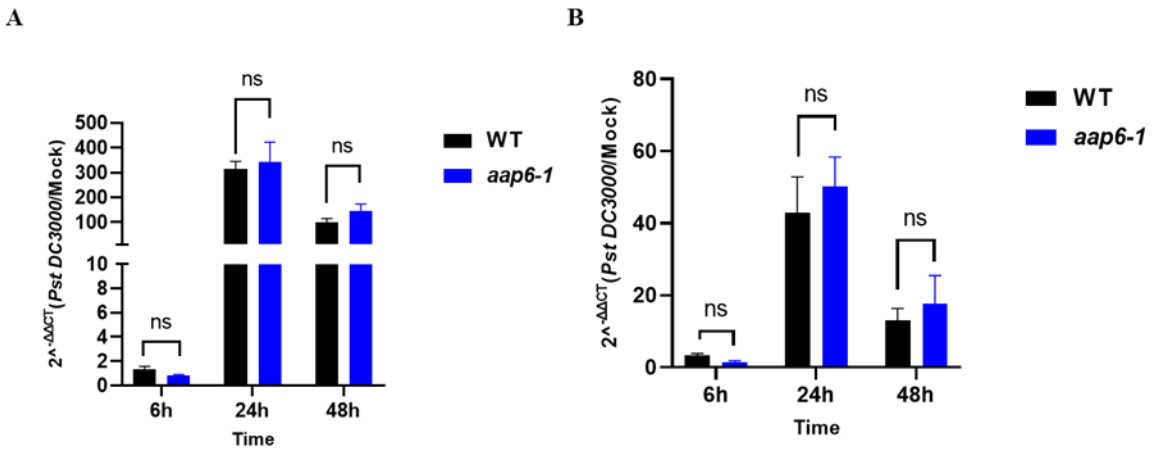

Figure 3- 9. The ability of *aap6* to initiate salicylic acid signaling in response to *Pst* DC3000 infection remains unaffected.

A) *PR1* expression in *Pst* DC3000 (OD_{600nm} = 0.001)-inoculated plants. Mean ± SEM (n = 3). B) *ICS1* expression in *Pst* DC3000-inoculated plants. Mean ± SEM (n = 3). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), or non-significant (ns) differences are shown. Similar results were obtained in three out of three independent experiments.

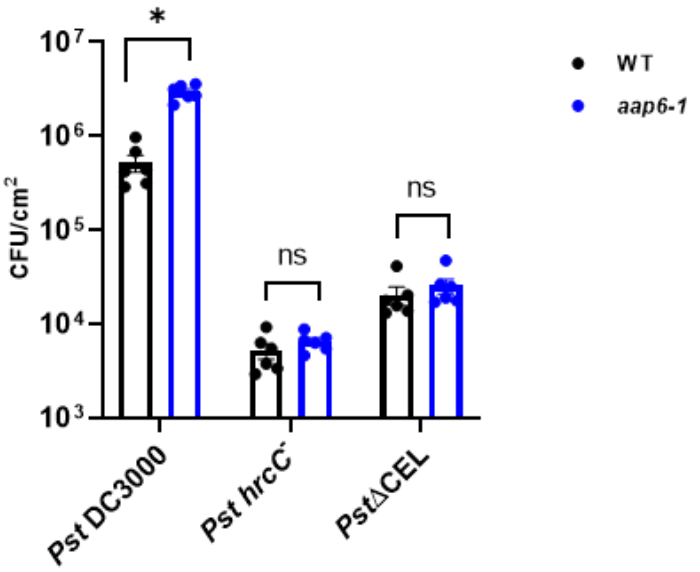

Figure 3- 10. The ability of *aap6* to restrict infections by non-virulent *Pst* DC3000 strains remains unaffected.

Bacterial infection titer in the leaves 72HAI infiltration with virulent the virulent strain *Pst* DC3000 (OD_{600nm} = 0.0002), or the hrp-deficient non-virulent strains *Pst* ΔhrcC (OD_{600nm} = 0.0002), or the attenuated strain *Pst* ΔCEL (OD_{600nm} = 0.0002). Mean ± SEM (n = 6). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), or non-significant (ns) differences are shown. Similar results were obtained in three out of three independent experiments.

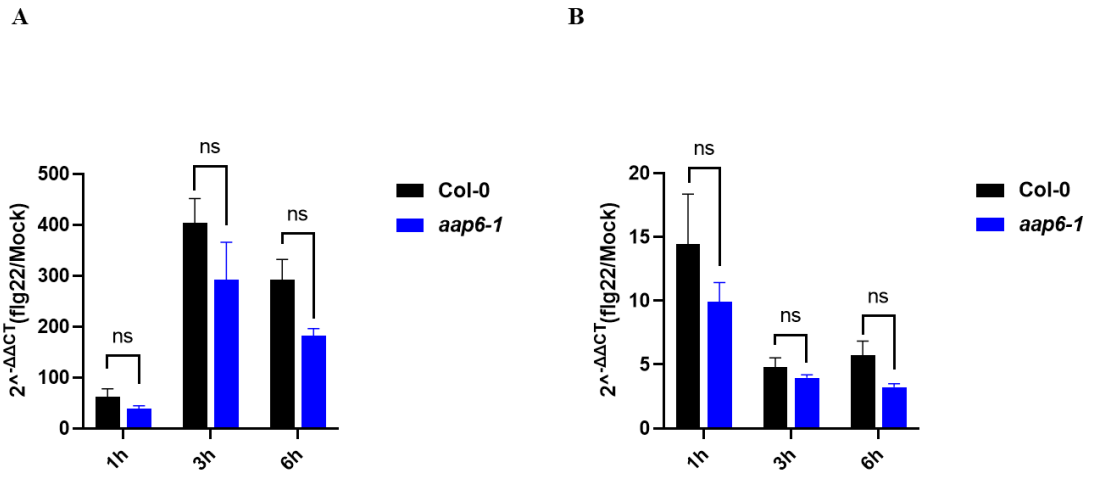

Figure 3- 11. The ability of *aap6* to initiate PTI signaling remains unaffected.

A) *WRKY29* expression in response to flg22 infiltration of leaves. Mean ± SEM (n = 3). B) *MYB51* expression in response to flg22 infiltration of leaves. Mean ± SEM (n = 3). Both genes were similarly induced by flg22 in both genotypes. Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), or non-significant (ns) differences are shown. Similar results were obtained in three out of three independent experiments.

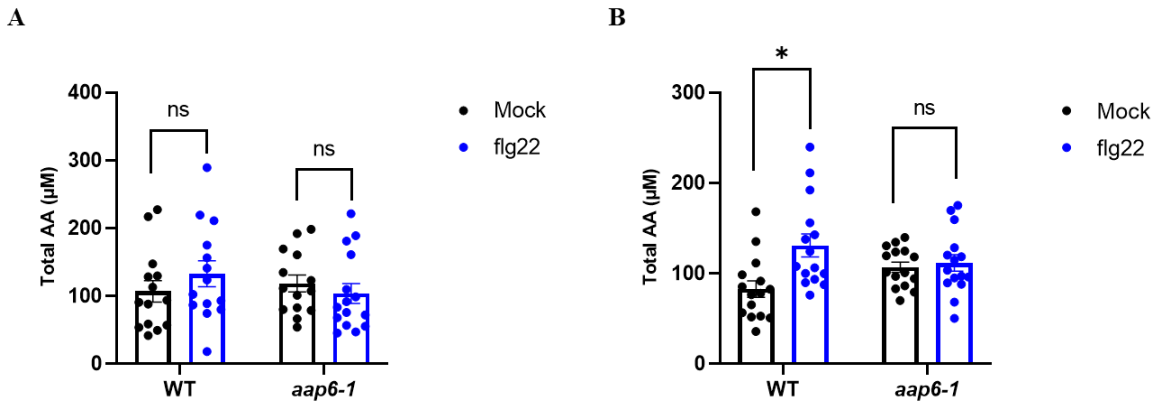

Figure 3- 12. Compromised ability of *aap6* to execute leaf apoplast amino acid concentration changes in response to PTI elicitation with flg22.

A) Amino acids concentration in the AWF 6HAI of leaves with water (mock) or flg22. B) Amino acids concentration in the AWF 24HAI of leaves with water (mock) or flg22. Amino acid content was normalized to sample dry weight. Mean ± SEM (n = 15). Asterisks denote statistically significant differences ($P < 0.05$; Student's *t*-test) Results are combination of three independent experiments. Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), or non-significant (ns) differences are shown. Data is combination of three separate experiments with similar results.

	WT		*aap6*		
Amino acid	**Mean (µM)**	**SEM**	**Mean (µM)**	**SEM**	**P-value**
Alanine	203.141	21.290	358.663	57.524	0.044
Arginine	2.569	0.277	3.493	0.374	0.094
Asparagine	0.581	0.098	0.528	0.114	0.740
Aspartic acid	450.186	24.069	455.603	39.536	0.911
Cysteine	0.050	0.050	0.036	0.036	0.832
Glutamic acid	388.195	29.369	527.634	38.562	0.028
Glutamine	4.493	0.349	4.906	0.522	0.535
Glycine	0.051	0.051	0.053	0.053	0.976
Histidine	4.603	0.691	3.940	0.348	0.424
Isoleucine	19.423	1.885	30.498	3.455	0.031
Leucine	21.235	1.734	33.502	6.070	0.100
Lysine	243.504	30.499	315.787	65.712	0.357
Methionine	0.611	0.133	0.648	0.099	0.831
Phenylalanine	18.442	1.295	35.498	4.356	0.009
Proline	40.979	2.689	51.370	3.469	0.056
Serine	266.218	9.543	249.720	21.998	0.517
Threonine	89.273	10.334	149.637	8.422	0.004
Tryptophan	4.546	0.552	6.695	0.973	0.103
Tyrosine	15.138	2.191	28.984	5.135	0.048
Valine	51.980	3.639	67.349	8.400	0.144
Total	1825.219	112.199	2324.547	189.929	0.064

Table 3- 2. Concentration of amino acids in AWF of six-week-old plants

Concentrations of individual amino acids in apoplastic washing fluid of naïve wild type and *aap6* plants, Mean ± SEM (n=4). Data analysis: Student's t-test. Statistically significant differences between mock and flg22 treatment for each amino acid are highlighted in red ($p < 0.05$).

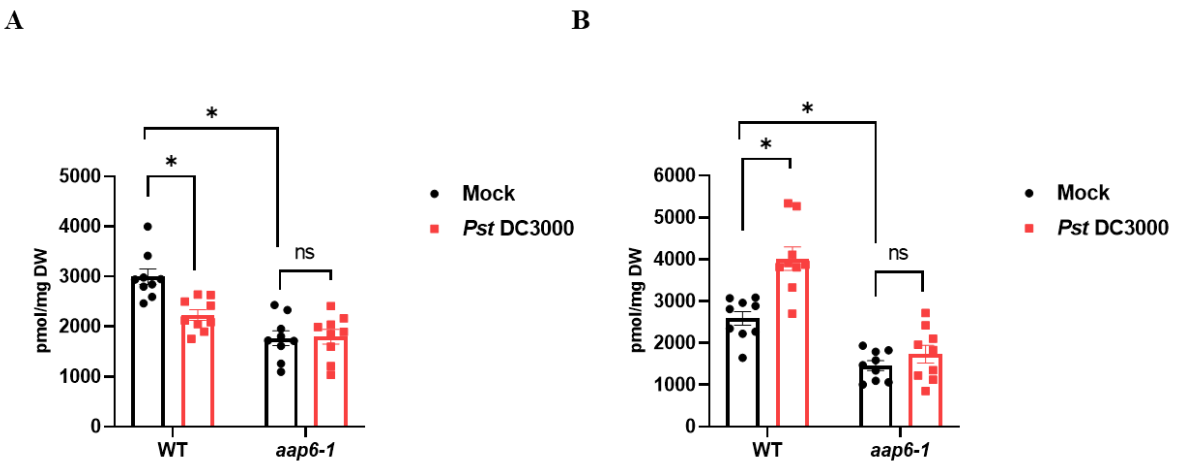

Figure 3- 13. Compromised ability of *aap6* to load amino acids to the leaf phloem in *Pst* DC3000-infected leaves.

A) Amino acids concentration in the phloem sap 6HAI with *Pst* DC3000 (OD_{600nm} = 0.02). B) Amino acids concentration in the phloem sap 24HAI with *Pst* DC3000 (OD_{600nm} = 0.02). Leaves were infiltrated with *Pst* DC3000 (OD_{600nm} = 0.02). Amino acid content was normalized to sample dry weight. Mean ± SEM (n=9). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*), or non-significant (ns) differences are shown. Data is combination of three separate experiments with similar results.

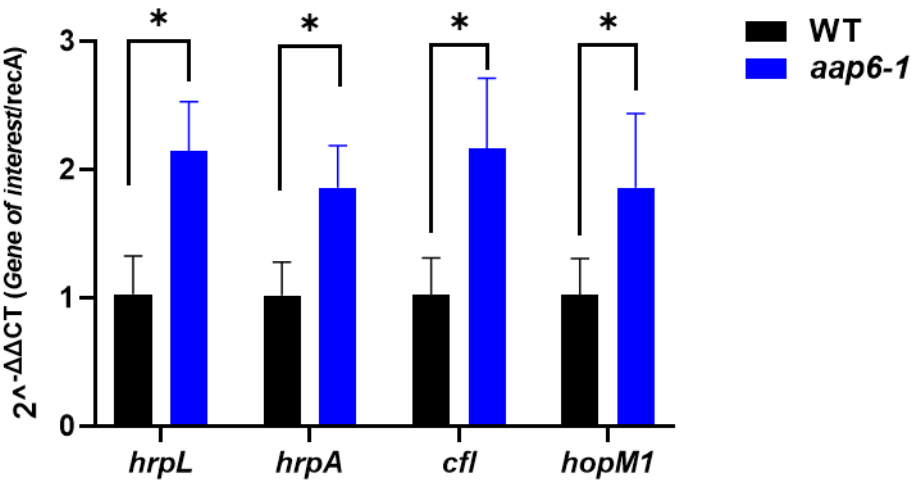

Figure 3- 14. Compromised ability of *aap6* to restrict *Pst* DC3000 expression of virulence genes.

The expression of the bacterial virulence and metabolic genes in aap6 mutant. Col-0 and *aap6* mutant leaves were infiltrated with *Pst* DC3000 strain (OD_{600nm} = 0.02). Samples were collected 3HAI. Mean ± SEM (n = 3). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*) are shown. Similar results were obtained in three out of three independent experiments.

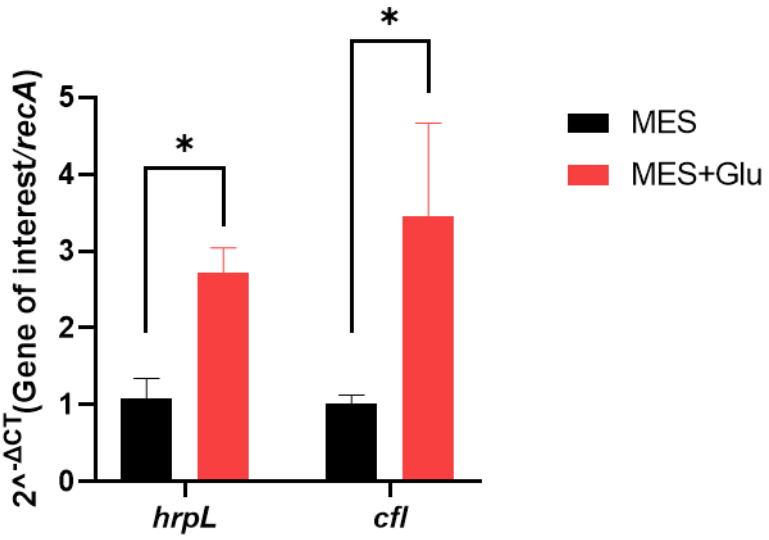

Figure 3- 15. Glu induces the virulence of *Pst* DC3000.

Expression of virulence genes in *Pst* DC3000 (OD_{600nm} = 0.02) co-infiltrated with Glu 2.5HAI. Mean ± SEM (n = 3). Data analysis: Student's t-test. Statistically significant differences at P-values of < 0.05 (*) are shown. Similar results were obtained in three out of three independent experiments.

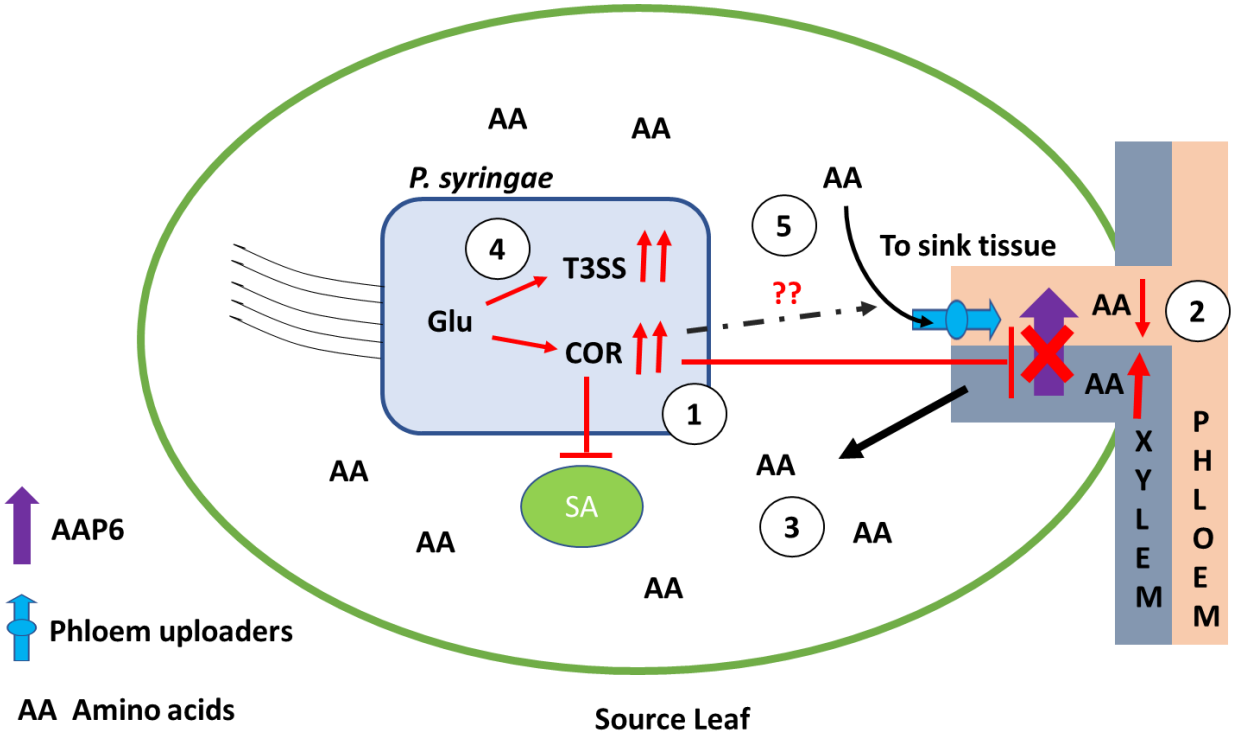

Figure 3- 16. Suppression of *AAP6* by COR compromises plant resistance against *P. syringae* infection.

1. COR suppresses the expression of *AAP6* and Salicylic acid mediated defense response, **2.** As the activity of *AAP6* is reduced, the xylem to phloem transport is suppressed which reduces the concentration of amino acids in phloem while increases the concentration in the xylem tissues, **3.** As the amount of amino acid increases in xylem, the concentration of amino acids also increases in the source leaf apoplast, **4.** Among the increased amino acids, Glu is utilized by *P. syringae* to induce the expression of T3SS (hrpL) and COR biosynthesis, **5.** The mechanism through which COR suppresses the expression of other phloem loaders to increase amino acid (Glu) concentration in apoplast is unknown.

3.6 Materials and methods

Plant lines and growth conditions

Arabidopsis plants were grown in peat pellets at 24 0C under a 15/9-hour day/night cycle. The wild type (Col-0), *aap6*-1 (SALK_140384), *aap6*-2 (SALK_013231) mutants were obtained from the *Arabidopsis* Biological Resource Center. *P. syringae* strains used are *Pst*DC3000, *Psm* ES4326, *Pst* DC3000 hrcC-, *Pst*-AvrRpt2, and *Pst* ΔCEL mutant. *P. syringae* cells were grown in low-salt Luria-Bertani (LB) medium plates with kanamycin (50µg/mL), streptomycin (50µg/mL), kanamycin (50µg/mL), and kanamycin (50µg/mL) respectively at 28 ^{0}C overnight.

Pseudomonas syringae infection

For bacterial growth assay, leaves of six-week-old plants were pressure-infiltrated using a needleless syringe with corresponding bacteria (OD_{600nm} = 0.0002) in sterile water. Six plants were assayed for each data point, and infected tissue was harvested after 72 hours. Two 6-mm leaf discs were collected per leaf. The leaf discs were placed in 400µL of sterile water in a 2mL centrifuge tube and ground using a metal bead in tissue lyzer (machine company and cat.) for 5 minutes. Serial 10-fold dilutions were made in sterile water. Aliquots (5µL) were placed in rows onto LB medium containing 50µg/mL antibiotics using a 12-channel multipipettor. The plates were incubated overnight at 28 ^{0}C before counting colonies. Statistical analyses were performed using Student's t-test (*, $p<0.05$).

For bacterial gene analysis, six-week-old plants were pressure-infiltrated using a needleless syringe with *Pst*DC3000 (OD_{600nm} = 0.02) in sterile water. A total of 4 leaves from 1 plant make one replicate and snap-frozen in liquid nitrogen.

For plant defense response assay, six-week-old plants were pressure-infiltrated using a needleless syringe with *Pst*-AvrRpt2 (OD_{600nm} = 0.001) in sterile water. A total of 8 leaves from 2 plants (4 leaves from each plant) were combined to make one replicate and snap-frozen in liquid nitrogen.

RNA extraction and qPCR analysis

Total RNA was extracted using Trizol reagent. RNA was treated with DNAse to remove any genomic DNA contamination using a DNA-free kit (Promega – Cat#M6101) according to the manufacturer's protocol. cDNA was synthesized using an M-MLV Reverse Transcriptase kit (Promega- Cat#M1705) according to the manufacturer's protocol. For the real-time PCR 20µL reactions consisting of 10µL of SYBR green master mix (CWBiosciences – Cat#CW2601H), 2µL of appropriate primer (each at 1µM) for respective genes, 2µL of a diluted template, and 6µL of ddH20.

Phloem sap collection

Phloem sap was collected as described in (Corbesier et al., 2001). *Arabidopsis* rosettes were cut at the base and placed in a 10mM EDTA solution. Four leaves per plant were cut at the base of the petiole with scissors, rinsed in 5 mM EDTA solution, and then placed in a 1.5 mL centrifuge tube containing 400 µL 5mM EDTA. This formed one replicate. Three such replicates were collected per genotype and treatment. Treatment or infection was done 6 hours before cutting rosettes.

Total amino acids quantification

Free amino acid concentrations were determined using UPLC. For AA extraction, 2 leaves were harvested into liquid nitrogen and freeze dried. Samples were next placed in a 2mL microfuge tube and grounded into a beat beater with 3mm metal beads at 25 Hz/s for 1 minute. 200µL of 10mM HCL and the internal standard norvaline at 0.1mM to 5 mg of lyophilized source tissue. 200 µL of chloroform was added and samples were mixed. Following the centrifugation at maximum speed for 5 min at room temperature, 120 µL of supernatant was transferred to a fresh tube. Pellets were reextracted with 200uL of HCL and 0.1mM norvaline along with 200µL of chloroform again. 170 µL of the supernatant was transferred to make total volume of 290 µL.

Apoplastioc washing fluid collection and quantification

Apoplastic washing fluid was extracted with infiltration and centrifugation method described by Lohaus et al (2001). The 4th and 5th pairs of leaves from the 6-week-old plants are treated as described in the text. The leaves were then cut and leaves from 2 plants were pooled together to make one replicate. The leaves are vacuum infiltrated with sorbitol (0.24M) and luciferase yellow (1mg/100mL) and then pat dried. The leaves are stacked together and then put in 5mL syringe without the plunger then put on 15mL conical tubes and sealed with parafilm to centrifuge for 5 mins at 1500rpm (Beckman Coulter). The apoplastic washing fluid are then collected in a new tube and stored at -20ºC. Total L-AA concentration in the exudates was quantified with the commercial L-AA Colorimetric/Fluorometic Kit (BioVision/abcam) or were analyzed in the University of Virginia-Biomolecular Analysis Facility Core using an UHPLC and a Q-Exactive HF-X mass spectrometer (Thermo Scientific).

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