AN ARABIDOPSIS *PURPLE ACID PHOSPHATASE5* (*PAP5*) IS ESSENTIAL FOR MAINTAINING BASAL RESISTANCE AGAINST *PSEUDOMONAS SYRINGAE*

by

Sridhar Ravichandran

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Abstract

Plants have evolved an array of constitutive and inducible defense strategies to restrict pathogen ingress. Despite this, some pathogens still invade plants and impair growth and productivity. Previous studies have revealed key regulators of defense responses, and efforts have been made to develop disease resistant crop plants. These attempts are hampered by the complexity of defense signaling pathways. To further elucidate the complexity of defense responses, a population of T-DNA mutants in Colombia-0 background were screened for altered defense responses to virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). This study demonstrated that the Arabidopsis *Purple Acid Phosphatse5* (*PAP5*) gene, normally induced only under prolonged phosphate (Pi) starvation, was required for maintaining basal resistance to certain pathogens. The expression of *PAP5* was distinctly induced only under prolonged Pi starvation and during the early stages of *Pst* DC3000 infection (6 h.p.i). T-DNA tagged mutant, *pap5* displayed enhanced susceptibility to the virulent bacterial pathogen *Pst* DC3000. The *PAP5* mutation greatly reduced the expression of the pathogen inducible gene *PR1,* compared to wild-type plants. Other defense related genes, including *ICS1* and *PDF1.2,* were impaired in *pap5* plants. Similarly, overexpression of *PAP5* impaired *PR1*, *ICS1* expression and salicylic acid (SA) accumulation. Moreover, application of benzothiodiazole (BTH), an analog of SA restored *PR1* expression in *pap5* plants. These results provide evidence that PAP5 acts upstream of SA accumulation to regulate the expression of other defense responsive genes. Optimal levels of PAP5 are crucial for mounting complete basal resistance. SA accumulation in transgenic plants (*35S:PAP5*), following *Pst* DC3000 infection, was only ~ 60% of the wild-type plants. PAP5 was also found to be peroxisomal localized and aid the generation of reactive oxygen species for activation of defense responses. These results suggest that optimal levels of PAP5 are required for induction of PR genes and SA accumulation, demonstrating the requirement of PAP5 for maintaining basal resistance against *Pst* DC3000.

List of abbreviations used

AMF	Arbuscular mycorrhizal fungi
AP	Acid phosphatase
B. cinerea	*Botrytis cinerea*
BTH	Benzothiodiazole
CaMV	Cauliflower mosaic virus
DAB	3-3 Diaminobenzidine
EDS	Enhanced disease susceptibility
ET	Ethylene
ETI	Effector triggered immunity
GAPDH	*Glyceraldehyde 3-phosphate dehydrogenase*
H_2O_2	Hydrogen peroxide
HMW	High molecular weight
HR	Hypersensitive response
h.p.i	Hours post inoculation
IAA	Indole acetic acid
ICS1	*Isochorismate synthase1*
JA	Jasmonic acid
LMW	Light molecular weight
MAMP	Microbe associated molecular patterns
MJ	Methyl jasmonate
MS	Murashige and Skoog media
NOD	Nodulation
P. putida	*Pseudomonas putida* WCS 358
PAD	Phytoalexin deficient
PAMP	Pathogen associated molecular patterns
PAP	Purple acid phosphatase

PDF1.2	*Plant defensin1.2*
PGPR	Plant growth promoting rhizobacteria
Pi	Phosphate
PR	Pathogenesis related
PR1	*Pathogenesis related gene1*
Pst DC3000	*Pseudomonas syringae* pv. *tomato* DC3000
PTI	PAMP triggered immunity
ROI	Reactive oxygen intermediates
SA	Salicylic acid
SAR	Systemic acquired resistance

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Chapter 1 Introduction

Plants are exposed to a diverse array of microorganisms, including beneficial mutualists, commensals and potential pathogens in their environments. In nature, plants establish multiple interactions with many different microorganisms. Interestingly, most microorganisms associated with the plants result in commensalistic or mutualistic interactions (Schenk et al., 2012). However, a small fraction of these microbial encounters gives rise to symbiotic or pathogenic interactions. These complex microbial activities undoubtedly affect plant health and productivity. For example, the beneficial interactions that are frequent in nature, improve plant nutrition and aid plants to overcome biotic and abiotic stresses (Geurts et al., 2012). On the other hand, all crop plants are vulnerable to various diseases that can reduce both the quality and quantity of yield.

In modern agriculture, numerous efforts have been made to develop disease tolerant crops. Plant breeding strategies have been successful for developing disease resistant cultivars. Since plant breeding strategies are time-consuming, genetic engineering enables the generation of crops with desirable traits in a relatively shorter time period (Gust et al., 2010). However, the genetic engineering approach requires a better understanding of how plant microbe interactions lead to physiological changes in plants. Although previous studies have revealed a number of key regulators of plant microbe interactions (Van et al., 2008; Gust et al., 2010), it is still unclear how plants integrate and differentiate the signals that arise from symbiotic or pathogenic interactions.

Plants support a vast array of positive interactions, including mycorrhizal, rhizobial and plant growth promoting rhizobacterial (PGPR) associations. The rhizosphere associated microbial communities directly influence plants' productivity by fixing atmospheric nitrogen and by inducing systemic resistance (ISR) to enhance host disease resistance (Choong-Min Ryu et al., 2004). The establishment of beneficial associations requires mutual recognition and coordination between the plant and its microbial partner (Zamioudis and Pieterse, 2012). Even though the interactions between plants and microbes are diverse, the rhizosphere, encompassing the first few millimeters of the root, supports a discrete group of microorganisms. The microbial community in the rhizosphere is different from that in the bulk soil. Moreover, it is evident that microbes colonizing the rhizosphere are distinct to every plant species, suggesting that the plants are able to modulate their microbial association (Mendes et al., 2011). Previously, investigations into plant associated microbial communities were often hampered since most microbes are not culturable. In recent years, metagenomic approaches have been successfully adopted to functionally characterize the rhizosphere microbiome (Mendes et al., 2011).

Plants recognize and respond to phytopathogens by activating a series of defense responses to restrict microbial attack. Even though plants possess innate immunity to resist most pathogens (Chisholm et al., 2006; Jones and Dangl, 2006), a proportion of pathogens are able to invade and impair plant growth (Baker et al., 1997). As plants lack the specialized immune cells which mammals have acquired, they rely on the innate immunity of each cell and on systemic signaling to induce disease resistance (Dangl and Jones, 2001). Activation of defense response in plants involves the initial recognition of

potential pathogens by chemical cues (elicitors), referred to as Pathogen Associated Molecular Patterns (PAMP). Recognition of PAMP on the plasma membrane leads to activation of defense responses in both basal and non-host disease resistance. However, a successful pathogen can overcome PAMP triggered immunity by evading detection and the hosts defense to cause a disease (Niks and Marcel, 2009).

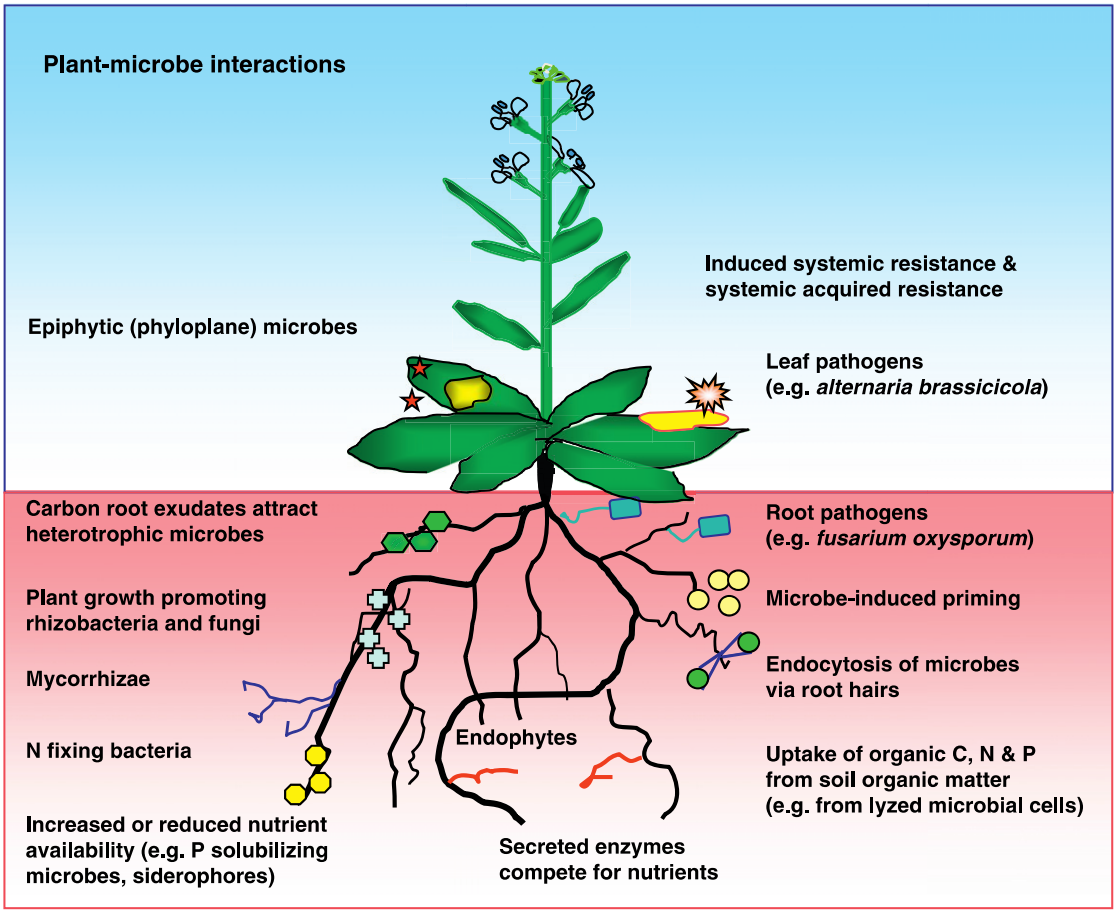
Genetic screening of mutant plant populations has proven useful for the functional analysis of plant defense responses (Ausubel et al., 1995; Alonso and Ecker, 2006; Petersen et al., 2009). In Arabidopsis, genetic screening has revealed a large number of mutants that exhibit altered responses to salicylic acid (SA), jasmonic acid (JA) and/or ethylene (ET) and are more susceptible to virulent pathogens (Volko et al., 1998). Identification and characterization of enhanced disease susceptibility (EDS) mutants including a series of phytoalexin deficient (PAD), mutants has helped to elucidate a number of defense signaling pathways involving both basal and non-host defense responses (Glazebrook and Ausubel, 1994; Rogers and Ausubel, 1997; Wiermer et al., 2005). Similarly, Arabidopsis mutants have been previous used to study root-microbe interactions (Persello-Cartieaux et al., 2001).

To date, plant responses to beneficial and pathogenic microbes have been largely studied as separate events. Recent research suggests that numerous commonalities occur in both types of plant-microbe interactions (reviewed by Zamioudis and Pieterse, 2012). Like PAMPs in microbial pathogens, molecular patterns of beneficial microbes are recognized by plants to induce systemic resistance to enhance defense (Van et al., 2008). A deeper understanding of plant microbe interactions may offer exciting novel

opportunities to control devastating plant diseases for sustainable agriculture (Bisseling et al., 2009).

Figure 1. Overview of interactions between plant and microbe.

Plants inhabit a complex microbial community of beneficial symbionts, commensals and potential pathogens which colonize on and within tissues and vasculature (image adapted from Schenk et al., 2012).



1.1 Beneficial root-microbe interactions

Beneficial interactions between plants and microorganisms are common in nature. Specifically, the rhizosphere, which encompasses the first few millimeters of the root, supports a discrete group of microorganisms (also known as rhizosphere effect). The microbes in the rhizosphere constitute 10^9 to 10^{12} cells per gram of soil (Lynch and Whipps, 1990) and they influence carbon sequestration and nutrient recycling (Singh et al., 2004), predominantly affecting plant health and quality. A multitude of biotic and abiotic factors, including plant genotype and soil type, influence the structure and functional diversity of rhizosphere associated microorganisms (Berendsen et al., 2012). Recent advances in root-microbe interactions have revealed that the microbes which colonize the rhizosphere are distinct to every plant species, implicating the role of plant roots in selecting and shaping their microbial associations (Bisseling et al., 2009).

Beneficial plant-microbe interactions include mycorrhizal, rhizobial and plant growth promoting rhizobacterial (PGPR) associations. Rhizosphere associated microbial communities directly influence plants' productivity by fixing atmospheric nitrogen and by mycorrhizal associated phosphate release, reducing the net input cost of chemical fertilizers. Purified isolates of rhizospheric bacteria stimulate plant growth and a subset of these bacteria, free living diazotrophs, can fix atmospheric nitrogen (Steenhoudt and Vanderleyden, 2000). Plant growth promoting rhizobacteria (PGPR) enhance nutrient uptake in plants and stimulate growth by the secretion of precursors of phytohormones, such as tryptophan for synthesis of indole acetic acid (IAA) (Gray and Smith, 2005). Strains of rhizobacteria are potential biocontrol agents, competing for nutrients and secreting antibiotic metabolites to suppress soil borne pathogens. Strains of *Pseudomonas*

synthesize several antibiotic chemicals that are activated and regulated by the signaling molecules of root exudates (Nielsen et al., 2000). *Pseudomonas* spp and *Bacillus* spp in the rhizosphere induce systemic resistance (ISR) thus enhancing host disease resistance (Ryu et al., 2004).

1.1.1 Nitrogen fixation

Root-microbe interactions can positively influence plant growth through a variety of mechanisms, including nitrogen fixation by legume-rhizobial symbiosis and by a class of free living proteobacteria (Diazotrophs). Legume-rhizobial symbiosis is one the best studied root-microbe interactions, explaining legume specificity over its nodulation. The interaction between nitrogen fixing microsymbiont (rhizobial bacteria) and leguminous plants starts with a specific exchange of signals. A class of flavonoids secreted by the host plant to the rhizosphere has been identified as the regulator of host specificity in legumes (Peters et al., 1986). Flavonoids induce (Nodulation) *nod* gene expression and chemotaxis in rhizobial strains. The expression of *nod* genes leads to the synthesis of lipochitooligosaccharides (LPS, symbiotic exopolysaccharides) that are recognized by the host legume for nodulation. Nod factors and symbiotic exopolysaccharides bind to specific receptor kinases in the host plant, which contain LysM motifs (Limpens et al., 2003). This induces the root hair to curl and form an infection thread that traps rhizobia which then penetrate into the plant cortical tissue (Gage, 2004). With the release of rhizobia into the cortical cells, the host cell wall is disrupted and the rhizobial cells come into contact with the host cell plasma membrane (Brewin, 2004). Later, the plant cell takes up the rhizobia by endocytosis to form a host membrane bound structure known as

the symbiosome (Figure 2). The internalized rhizobia differentiate into bacteriods which are capable of nitrogen fixation (Jones et al., 2007).

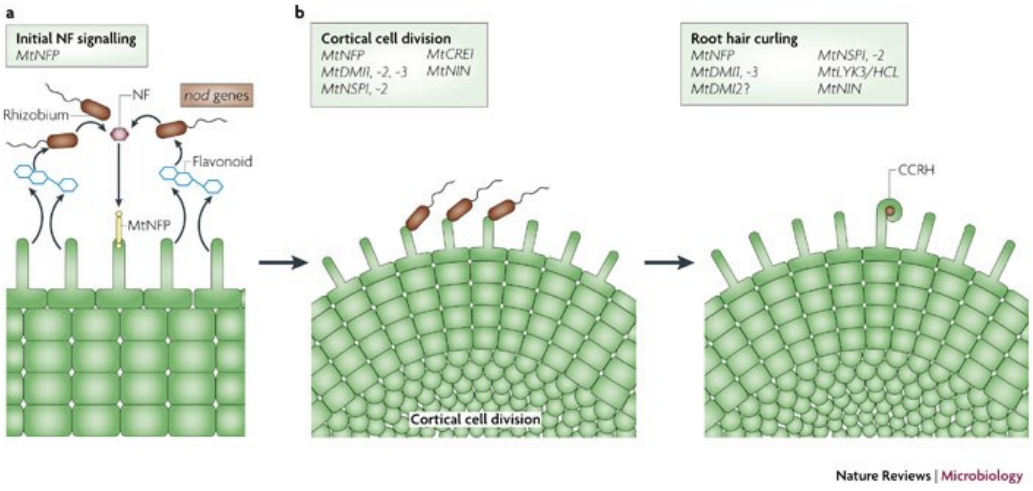
1.1.2 Mycorrhizal associations

Mycorrhizal associations occur in most beneficial root-microbe interactions. Unlike the legume-rhizobial association, mycorrhiza association occurs in over 80% of terrestrial plants. It is also evident that arbuscular mycorrhizal fungi (AMF) association is one of the oldest and most ecologically widespread (Corradi and Bonfante, 2012). AMF symbiosis is attributed to improved acquisition of nutrients, including phosphate and nitrates. These associations also sink atmospheric carbon dioxide and mediate the transfer of nutrients among neighboring plant and microbial communities (He et al., 2003). Recent studies suggest that AMF infection attracts specific bacteria to the rhizosphere, which enhances plant growth and suppresses diseases. Mycorrhizal symbiosis also modulates complex microbial communities called mycorrhiza helper bacteria (Frey-Klett et al., 2007; Cameron et al., 2013).

The branch-inducing factor is exuded from the root to trigger hyphal morphogenesis that is essential to root colonization (Buee et al., 2000). Branch-inducing factors are found in root exudates of all mycotrophic plants and phosphate deficient plants. It is also evident that stringolactones, a class of terpenoid lactones, exuded from the roots stimulate hyphal branching in AMF (Akiyama et al., 2005). The mycorrhizal colonization exhibits several commonalities to fungal plant pathogens. For example, pathogenic rust fungi and the beneficial AMF develop feeding structures similar to the haustoria, which allow movement of nutrients. Transcriptional profiling has revealed high similarity between

haustorial and arbusculated cells, irrespective of their nutritional strategies (Micali et al., 2011).

Figure 2. Signal exchange between *Sinorhizobium meliloti* and *Medicago trancatula.* Flavonoids secreted by the host plant induce rhizobial *nod* genes. Induction of *nod* results in secretion of Nod Factors (NF), which bind to specific receptor kinase MtNFP. These signaling events initiate root hair curling and cortical cell division that leads to colonized curled root hair formation (CCRH) (image adapted from Jones et al., 2007).



1.1.3 Plant growth promoting rhizobacteria (PGPR)

Apart from symbiotic associations, various other nitrogen fixing microorganisms have been identified. For example, the soil actinomycetes *Frankia* fixes nitrogen (N_2) under both symbiotic and free living aerobic conditions (Benson and Silverster, 1993). *Frankia* sp. and rhizobia are the two major groups of nitrogen-fixing symbionts and they are phylogenetically distant (Normand et al., 1996). In general, only the prokaryotes called diazotrophs have been found to mediate biological nitrogen fixation (BNF) (Gaby and Buckley, 2011). The diazotrophs encode nitrogenase enzyme (Nif) complex that catalyzes the conversion of atmospheric N_2 to ammonia. Several genera of bacteria, including *Acetinobacter*, *Azotobacter*, *Burkholderia*, *Klebsiella* and *Pseudomonas,* have been reported to possess nitrogen-fixing properties (Reviewed by Dobbelaere et al., 2003).

Beneficial rhizobacteria that stimulate plant growth are referred to as plant growth promoting rhizobacteria (PGPR). The plant-rhizobacteria interactions are mediated by root exudates, where PGPR are stimulated and attracted towards the root mucilage. Bacteria may locate plant roots through cues of root exudates, thereby influencing flagellar motility (de Weert et al., 2002). The major outer membrane protein (MOMP) of bacteia also plays an important role in *Azospirillum* spp host recognition. Some PGPRs fix atmospheric nitrogen in addition to secreting phytohormones such as auxins, cytokines and gibberllins (Steenhoudt and Vanderleyden, 2000). Interestingly, diazotrophic PGPRs deficient in nitrogenase (Nif^-) activity were still able to promote plant growth. Roots exude a pool of precursors that allow the PGPR to continuously produce the phytostimulatory compounds such as tryptophan, a major precursor of indole

3-acetic acid (Jaeger et al., 1999). Also, the production of phytohormones by *Azospirillum,* rather than nitrogen fixation, is considered to be a major factor for plant growth promotion in wheat (Fulchieri et al., 1993).

PGPRs positively interact with plants by secreting plant growth promoting substances, as well as protective biofilm and antibiotics which are used as biocontrol agents against potential pathogens (Moulin et al., 2001; Gray and Smith, 2005). PGPR strains are also attributed to the production of siderophores and solubilization of phosphorous. Production of siderophores stimulates plant growth by increasing the bioavailability of iron in the soil (Saha et al., 2013). Competition for nutrients and secretion of antimicrobial metabolites are characteristic of biocontrol activity; thus, rhizobacteria control plant diseases on suppressive soils. Strains of the genus Pseudomonas are best characterized as biocontrol agents, secreting phenazine, pyrrolnitrin and pyoluteorin, which are cyclic lipopeptides that are antifungal in nature (Nielsen et al., 2000). PGPR *Serratia* spp, *Pseudomonas* spp and *Bacillus* spp have been reported to induce resistance in wild type *Arabidopsis thaliana* against cauliflower mosaic virus (CaMV) and gram negative bacterial pathogens, such as *P. syringae* pv. *tomato* DC3000 (Ryu et al., 2004). It has also been speculated that root exudates mimic quorum sensing molecules to enhance PGPR colonization. These molecules are known to interfere with specific metabolic events of bacterial populations (Bauer and Mathesius, 2004).

1.2 Pathogenic root-microbe interaction

Root cells are constantly in contact with pathogenic microorganisms and plants overcome pathogenesis through root derived defense chemicals, such as phytoalexins and defense proteins. Antimicrobial indole, terpenoid, flavonoids and benzoxazione are the most common chemicals reported from root exudates that negatively regulate root-microbe interactions (Bais et al., 2004).

Plants constitutively synthesize metabolites, such as phytoanticipins, prior to pathogenesis, while phytoalexins are produced in response to pathogenesis. Root exudates of *Arabidopsis thaliana* have been found to constitute higher concentrations of indolic and polyproponoid secondary metabolites upon *Pythium sylvaticum* infection (Bednarek et al., 2005). Similarly, levels of phenylpropanoid were reported to be higher in roots challenged with non-host pathogens, compared to host bacterial pathogens (Bais et al., 2006).

Cell-to-cell communication in many bacteria is density dependent, involving quorum-sensing molecules, acyl homoserine lactones (AHL). Components of root exudates overlap with virulence factors of bacterial pathogens. Quorum sensing (QS) inhibitors can also serve as valuable tools in treatment of bacterial infection in animal systems (Hentzer et al., 2002). Quorum sensing was first described in aquatic bacteria *Vibrio fischeri* mediated by the induction of *lux* genes (Eberhard et al., 1981). Cell-to-cell communication is mediated by autoinducer molecules, which coordinate plant associated bacteria. N-acyl homoserine lactones are the most common autoinducer molecule. Typically, a basal level of AHLs is constitutively synthesized until a threshold population

of bacteria has been achieved by regulation of LuxR and Lux R-like proteins (Elasri et al., 2001).

1.3 Root exudates and their role in root colonization

Root colonization is the initial step in both soil-borne pathogenesis and beneficial associations with microorganisms. In addition to water and nutrient uptake, roots synthesize, accumulate and secrete a vast array of metabolites to support a diverse microbial community in the rhizosphere. The "rhizosphere effect," first described by Hiltner in 1904, postulated that many microorganisms are attracted to nutrients exuded by plant roots. The communication between the root and microbes is constant and is mediated by chemicals and signaling molecules in root exudates (Bais et al., 2004). Root exudates require 20-40 % of the photosynthetically fixed carbon in plants, contributing to rhizodeposition and carbon influx (Ramey et al., 2004; Danhorn and Fuqua, 2007). In addition to root exudates, a considerable quantity of carbon is released into the soil in the form of root debris and root border cells. The relative quantity and composition of root exudates vary with plant species, developmental stage, soil type and biotic and abiotic stress factors.

A chemotactic response towards root-secreted organic and amino acids is the initial step in root-microbe interaction. Motility is an important trait for competitive pathogens and beneficial microorganisms, enabling efficient colonization (Lugtenberg et al., 2001; de Weert et al., 2002). Chemical attraction of soil microbes to plant roots, or chemotaxsis, is a well understood mechanism, initiating cross-talk between plant roots and microbes (Reviewed in Bais et al., 2006). Root exudates influence flagellar motility in some rhizospheric bacteria. De Weert et al. (2002) demonstrated that rhizosphere

colonization differs between wild type *Pseudomonas fluorescens* and its mutant, defective in flagella-driven chemotaxis (*CheA*). Relative to wild-type bacteria, *CheA* mutants had a strongly reduced ability to competitively colonize roots.

1.3.1 Composition of root exudates

Roots exude carbon-containing primary and secondary metabolites, in addition to ions, free oxygen, enzymes, and mucilage providing nutrients to surrounding microflora (Bertin et al., 2003). Root exudates are transported across the cellular membrane and secreted into the rhizosphere in a gradient dependent process. Root border cells and root border-like cells contribute a major portion of root exudation and carbon influx (Bais et al., 2004). Border cells and their associated products can contribute up to 98% of carbon rich material as root exudates, which has profound impacts on root-microbe interaction (Griffin et al., 1976).

Root exudates are divided into two classes of compounds. The low molecular weight compounds include sugars, amino acids, lignins, organic acid, phenolics and other secondary metabolites accounting for the diversity of root exudates. Over 100 000 secondary metabolites have been identified and a few classes of secondary metabolites are known to occur only in a few species (Dixon, 2001). Classes of flavonoids, tannins, terpenoids, alkaloids, polyacetylene and simple phenols are among the diverse secondary metabolites which have been reported (Badri and Vivanco, 2009). High molecular weight compounds of root exudates, including mucilage and proteins, are less diverse, but often compose a larger proportion of the root exudates by mass (Brimecombe et al., 2001).

Secondary metabolites in root exudates potentially perform numerous functions in the rhizosphere. Plant secondary metabolites were initially presumed to be waste products, but recently, they have been reported to regulate antibiosis, induce disease resistance, mediate symbiosis and repel pathogens, thus, influencing the microbial population and nutrient recycling (Sudha and Ravishankar, 2002; Bertin et al., 2003). Most antimicrobial metabolites are active against a broad range of pathogens; however those microbes which possess mechanisms to overcome toxicity are able to thrive (Bouarab et al., 2002). The antimicrobial indole, terpenoid, benzoxazione, flavonoids and isoflavonoids are the most commonly reported root derived metabolites (Dixon, 2001). As well, a vast array of secondary metabolites are synthesized and secreted by soil microbes.

Phytotoxins are another class of compounds found on root exudates. Plants secrete phytotoxins such as catechin, benzoflavones and hydroxyquinoline to compete with other plants in their environment. Phytotoxic root exudations significantly influence neighboring plants and microbial communities. Toxins of *Carduus nutans* are known to inhibit nodulation, affecting nitrogen fixation in white clover and catechin inhibits root growth of more than 20 grass species (Wardle et al., 1994).

1.4 Pathogenic plant-microbe interactions

A plant responds to a pathogen by activating a series of diverse and remarkably complex defense mechanisms (Glazebrook, 2005). Structural alteration in the cell wall, including the presence of a waxy cuticle layer and deposition of callose, suberin and lignifications of cell membranes, provide passive protection, contributing to non-host disease resistance (Dangl and Jones, 2001). Synthesis and secretion of anti-microbial compounds confer a selective advantage over pathogen invasion. Over 100,000 low-

molecular-mass compounds, derived from isoprenoid, polypropanoid, polyketide pathways, are known to enhance defense against microbial infections (Dixon, 2001).

Plant pathogens are often grouped as biotrophs, necrotrophs and hemi-biotrophs, based on their life styles and infection strategies. Biotrophic pathogens derive nutrients from living tissues, while necrotrophs feed on dead tissues; hemi-biotrophs behave as both biotroph and necrotroph, depending on the stage of their life cycles (Dixon, 2001). The response to a specific pathogen is governed in the host by gene-for-gene recognition for the activation of resistance (R) genes or, as it has been recently termed, effector-triggered immunity (ETI) (Nimchuk et al., 2003; Chisholm et al., 2006). To constrain the pathogen, activation of the R gene(s) is usually accompanied by the production of reactive oxygen species (ROS), leading to hypersensitive responses (HR) which result in localized cell death (Glazebrook, 2005). Localized cell death, in turn, triggers systemic acquired resistance (SAR) to confer protection throughout the plant (Baker et al., 1997). The hypersensitive response is also associated with the induction of a diverse group of defense related and pathogenesis related (PR) genes. *In vitro* experiments and overexpression of PR2 and PR3 proteins in transgenic plants exhibited enhanced ß-1, 3 glucanase and chitinase activity, respectively (Jörg Durner et al., 1997).

1.4.1 Role of plant hormones in defense response

Primarily, regulation of plant defense responses is mediated through the phytohormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Ton et al., 2002; Robert-Seilaniantz et al., 2007). However, in recent years, other phytohormones, including abscisic acid (ABA), auxins, gibberellins (GA), cytokines (CK) and brassinosteriods (BR), have been shown to mediate specific plant defense responses (reviewed in Bari and Jones, 2009; Pieterse et al., 2009). As plants are exposed to pathogens with diverse infection strategies, activation of appropriate pathogen specific defense responses is vital for plant growth and productivity (Glazebrook, 2005).

Defense against biotrophs involves SA-dependent responses, whereas nectroph resistance is SA-independent, relying primarily on JA/ET-dependent pathways (Robert-Seilaniantz et al., 2007). The SA signaling pathway is associated with transcriptional activation of pathogenesis related (PR) genes and the establishment of systemic acquired resistance (SAR), to provide enhanced long lasting resistance to secondary infections (Grant and Lamb, 2006; Vlot et al., 2009). By contrast, JA/ET signaling pathways are associated with resistance against necrotrophic pathogens and rhizobacteria mediated induced systemic resistance (ISR) not typically associated with PR gene expression (Grant and Lamb, 2006; Ton et al., 2002). However, a complex regulation and cross talk exist between the SA-dependent and -independent pathways (Vlot et al., 2009).

SA is synthesized from the primary metabolite chorismate via two distinct enzymatic pathways involving phenylalanine ammonia lyase (*PAL*) (MauchMani and Slusarenko, 1996) and/or Isochorismate synthase (*ICS*) (Wildermuth et al., 2002). Arabidopsis

encodes two ICS enzymes, among which *ICS1* is responsible for ~90% of pathogen induced SA production (Wildermuth et al., 2002). SA biosynthesis is triggered on perception of PAMPs and effectors of pathogen. The two lipase like proteins; enhanced disease susceptibility1 (EDS1) and phytoalexin deficient4 (PAD4), act upstream of SA to activate the biosynthesis SA (Glazebrook and Ausubel, 1994). Signaling downstream of SA is largely regulated by non-expressor of PR genes1 (NPR1) (Durrant and Dong, 2004). NPR1 exists as an oligomer through intermolecular disulfide bond and occurs in the cytoplasm. When SA levels increase, NPR oligomers disassociate into monomers and enter the nucleus where they interact with TGA type transcription factors to activate the expression of SA dependent PR1 (Figure 3). Immediate early response genes, including glutathione s transferase 6 (GST) and immediate early induced glucosyltransferase (IEGT), detoxify and protect cells from oxidative stress (references in Uquillas et al., 2004). SA-marker genes, such as *PR1*, are induced later during pathogenesis, involving the action of the key signal transducer NPR1 (Schenk et al., 2000). JA-dependent genes include PDF1.2, Thionin2.1(THI2.1), Hevein-like protein (HEL) and Chitinase B (Kunkel and Brooks, 2002).

Apart from plant hormones, a class of secondary metabolites, including phenyl proponoid, glucosinolates, terpenoids and phytoalexins, aid in the protection against most biotic stressors (Kliebenstein, 2004). In Arabidopsis, plants deficient in PAD4 (encoding camalexin) display enhanced susceptibility to pathogens *Psm* ES4326, as well as reduced SA and PR1 expression (Jirage et al., 1999).

1.4.2 Molecular mechanism of plant defense

Activation of defense responses in plants involves the initial recognition of potential pathogens by chemical cues (general elicitors) or microbe associated molecular patterns (MAMP). Fungal chitins, ergosterols, bacterial lipopolysaccharides and flagellin are the most well-known MAMPs, or elicitors of defense responses (Zipfel and Felix, 2005). MAMPs are recognized by specific pattern recognition receptors, often comprised of leucine-rich repeats and an intracellular ser/thr kinase signaling domain (He et al., 2006). Recognition of MAMP leads to rapid ion fluxes, generation of reactive oxygen species (ROS), nitrous oxide (NO), ethylene and synthesis of anti-microbial compounds (Zipfel and Felix, 2005). Other early responses include the activation of calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) signaling cascades (Shan et al., 2007). Activation of MAPK leads to phosphorylation of transcription factors to induce the expression of defense related genes (Zhang and Klessig, 2001).

To colonize and reproduce within the intracellular space (apoplast), a successful pathogen can suppress both basal plant defense and pathogen specific HR responses to cause disease symptoms. Gram negative bacteria have evolved the Type 3 secretion systems to inject effector molecules which subvert host immune response (Alfano and Collmer, 2004). Effector screening on Arabidopsis leaf cells has shown that AvrPtoA and AvrPtoB suppress MAMP triggered defense genes and MAPK signaling (He et al., 2006).

1.4.3 Reactive oxygen intermediates and plant immunity

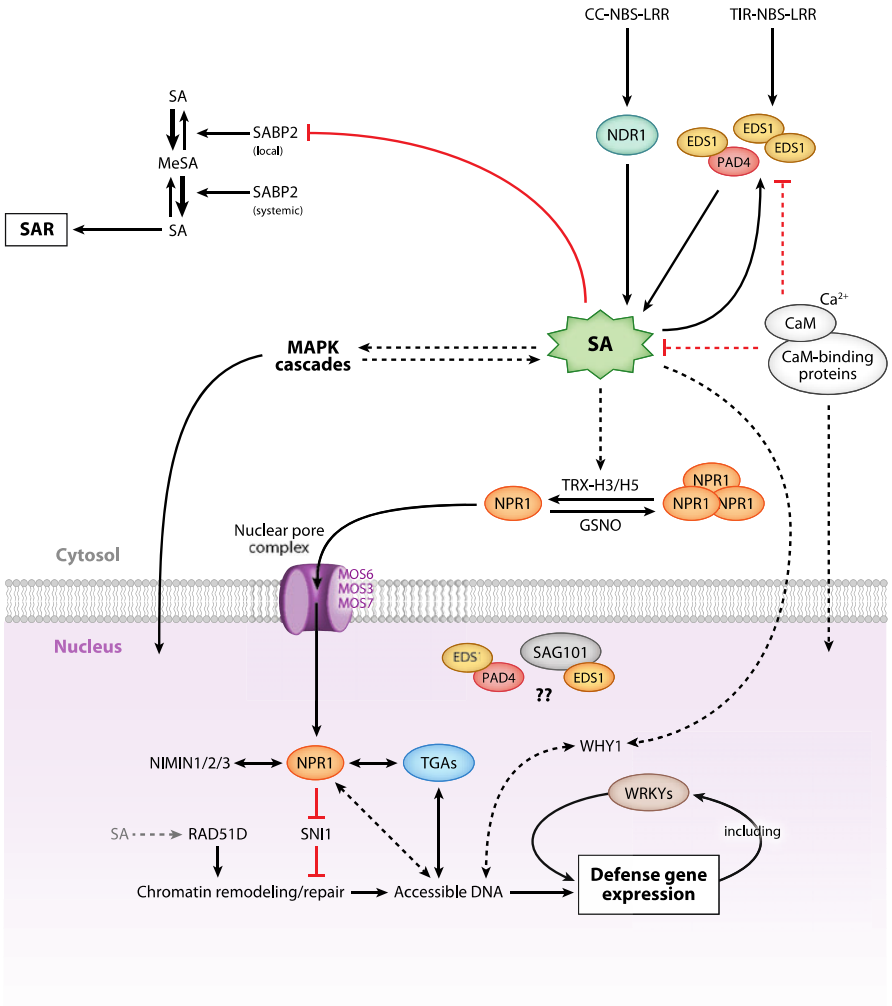
One of the earliest responses to pathogen infection is the generation of reactive oxygen intermediates, ROIs (O_2^- and H_2O_2). Although, ROIs such as hydroxyl radical (.OH), superoxide radical ($.O_2^-$) and hydrogen peroxide (H_2O_2) are produced under normal metabolic processes, plant cells produce a burst of reactive oxygen species (ROS) that is primarily made of H_2O_2 in response to pathogen infection. The rapid accumulation of ROS (also known as oxidative burst) is accompanied by oxidative cross linking of cell wall, activation of cellular signaling (protein phosphorylation) and induction of pathogenesis related (PR) genes (Alvarez et al., 1998).

It is widely assumed that ROS production after pathogen recognition is associated with membrane bound NADPH oxidase in the apoplast (Lamb and Dixon, 1997). NADPH oxidases, also known as respiratory burst oxidases (RBOs) in plants have been identified to be analogous to those present in mammalian phagocytes (Torres et al., 2002b). Arabidopsis genome enocodes10 sequence homologs of respiratory burst NADPH oxidase (*Atrboh*). *AtrbohD* and *AtrbohF* are required for the generation of H_2O_2 and to elevate levels of Ca^{2+} (Kwak et al., 2003; Torres and Dangl, 2005). H_2O_2 generated in response to pathogen recognition induces salicylic acid (SA) and PR protein accumulation (Chamnongpol et al., 1998). Mutation in *Atrboh* genes eliminates ROS production and compromises SA dependent cell death (Torres et al., 2005). A number of studies have indicated that ROS produced in response to pathogen recognition are located in the apoplast (Reviewed by Torres and Dangl, 2005). It is also evident that plants can produce ROS in other inter-cellular organelles including chloroplasts, mitochondria and peroxisomes. However, the cellular homeostasis and concentration of ROS is highly

regulated by enzymes such catalase, peroxidase and superoxide dismutase (Foyer and Noctor, 2003).

Figure 3. Schematic overview of SA signaling pathway.

Upon perception of PAMPs and effectors of pathogen, SA biosynthesis is triggered where EDS1 and PAD4 activate SA accumulation. An increase in SA level disassociates the key regulator NPR1 and the monomers of NPR1 translocate from the cytosol to the nucleus to activate the transcription of defense related genes (image adapted from Vlot et al., 2009)



1.5 Purple Acid Phosphatase

Purple Acid Phosphatases (PAPs) belong to a family of binuclear metalloenzymes and have been identified and characterized in numerous plants, animals and a limited number of microorganisms (Schenk et al., 2008; Mitić et al., 2009). All PAPs contain dinuclear metal ions and a characteristic set of seven invariant residues, which coordinate the metal ions within the active site (reviewed by Schenk et al., 1999). All mammalian PAPs that have been characterized exist as monomers with redox-active Fe(III)-Fe(II/III) (Oddie et al., 2000; Flanagan et al., 2006). Plant PAPs are larger, compared to mammalian PAPs, and exist as monomers (Bozzo et al., 2002), homodimers (Schenk et al., 1999; Veljanovski et al., 2006) or heterodimers (Olczak and Wątorek, 2003; Tran et al., 2010b) with Fe(III)-Zn(II) and Fe(III)-Mn(II) catalytic centers (Schenk et al., 1999). Despite the presence of highly conserved sequences around the catalytic centre (Figure 4), plant and animal PAPs share less than 20% amino acid identity, overall (Flanagan et al., 2006).

PAPs have been implicated in an array of biological functions. Most PAPs have been classified as non-specific acid phosphatases that catalyze the hydrolysis of inorganic phosphate (Pi) from various monoesters and anhydrides substrates (Olczak et al., 2003). The physiological role of plant PAPs is predominantly associated with the regulation of Pi uptake and recycling (Li et al., 2002; Veljanovski et al., 2006). However, recent studies have also revealed roles for plant PAPs in other biological functions, including peroxidation (Del Pozo et al., 1999), ascorbate recycling (Zhang et al., 2008), mediation of salt tolerance (Liao et al., 2003) and regulation of cell wall carbohydrate biosynthesis (Kaida et al., 2009).

Figure 4. Partial sequence alignment of selected PAPs from various kingdoms. Only the five conserved motifs are shown containing all seven metal-coordinating amino acids (in bold) are shown. The number of amino acid residues between the motifs and total length are indicated (Reproduced from Mitic et al., 2006)).

	Source	Motif 1		Motif 2		Motif 3		Motif 4		Motif 5	Length
Plants	Red kidney beans	GDLG	25	GDLS**Y**	32	G**N**HE	79	VLM**H**	35	G**H**V**H**	459
	Sweet potato	GDIG	25	GDLS**Y**	32	G**N**HE	79	VLV**H**	35	G**H**V**H**	473
	Sweet potato[a]	GDWG	29	GDNS**Y**	33	G**N**HD	89	VIG**H**	33	G**H**D**H**	313
Animals	Human	GDWG	34	GDNS**Y**	34	G**N**HD	87	VAG**H**	33	G**H**D**H**	325
	Rat	GDWG	34	GDNS**Y**	34	G**N**HD	89	VAG**H**	33	G**H**D**H**	327
	Pig	GDWG	34	GDNS**Y**	34	G**N**HD	89	VAG**H**	33	G**H**D**H**	338
Fungi	*A. ficuum*	NDMG	25	GDLS**Y**	84	G**N**HE	151	VMS**H**	33	G**H**I**H**	614
Bacteria	*M.tuberculosis*	GDQG	40	GDLC**Y**	31	G**N**HE	89	VCM**H**	35	G**H**E**H**	529
Metal binding site[b]		1		X 1		2		2		2 1	

[a]Mammalian-like plant PAP.[18] [b]Numbers 1 and 2 indicate ligands that coordinate to the metal ion in site M1 or M2, respectively. X indicates the metal ion-bridging ligand.

Mammalian PAPs (also known as tartarate-resistant acid phosphatases) are associated with bone resorption (Ekrylander et al., 1994), iron transport (Nuttleman and Roberts, 1990) and in the generation of reactive oxygen species for microbial killing (Kaija et al., 2002). In humans, PAP expression is restricted to activated macrophages, where they aid in the generation of free radicals to enhance microbial killing (Hayman and Cox, 1994). Recent studies have attributed PAPs to diverse biological functions and it has been difficult to assign a general physiological purpose for plant PAPs.

1.5.1 Characteristics of plant and animal purple acid phosphatases

All PAPs are characterized by several distinct features, including the pink/purple color of the enzyme in solution, that is due to a charge transfer transition (λ_{max}=510-560 nm) from the conserved metal coordinating tyrosine to the metal ligand Fe(III) (Antanaitis et al., 1983). In mammals, PAPs exist in two interconvertible redox states: the purple (oxidized) diferric form, which is enzymatically inactive and the pink (reduced) form, which is enzymatically active (Schlosnagle et al., 1976). PAPs are activated by various thiol containing reagents, such as L-ascorbate, L- cysteine and 2-mercaptoethanol. The activation of PAPs yields a pink coloration (λ_{max}=515 nm), while treatment with oxidants, such as hydrogen peroxide and ferricyanide, leads to the purple (oxidized) form (λ_{max}=560 nm) (Schlosnagle et al., 1976). Other characteristics are distinguished by glycosylation (5-10% of their molecular mass) (Wang et al., 2005) and resistance to inhibition by L(+) tartrate (Jemtland et al., 1998; Oddie et al., 2000). Resistance to inhibition by L(+) tartrate distinguishes PAPs from other acid phosphatases (APs) of lysosomal and osteoclast origin in mammalian cells (Jemtland et al., 1998). Despite the

conserved catalytic domains, plant and mammalian PAPs differ in their metal ion composition and oligomeric structures (Schenk et al., 1999; Olczak and Wątorek, 2003).

In general, mammalian and plant PAPs are distinguished by their molecular weight. All mammalian PAPs that have been characterized exist as monomers of ~35 kDa (Mitic et al., 2006). Plants encode a relatively large family of High Molecular Weight (HMW) homodimeric and oligomeric PAPs (~45-74 kDa), where their subunits are linked via disulphide bridges. The Arabidopsis genome contains 29 *PAP* encoding genes, mostly HMW oligomerics composed of 45-74 kDa subunits (Li et al., 2002). However, PAPs, including SAP1 and SAP2 which are secreted by Pi starved tomato (*Solanum lycopersicum*) cell cultures (Bozzo et al., 2002), and AtPAP26 which is secreted by Pi starved *Arabidopsis thaliana* suspension cells, have been identified to be monomeric enzymes (Tran et al., 2010b).

Although mammalian and plant PAPs are primarily distinguished by their molecular weights, bioinformatics tools, such as PSI-BLAST, have also identified larger HMW PAP isoforms in mammals, insects and nematodes that are similar to the HMW PAPs characterized from plants (Flanagan et al., 2006). Similarly, a recent study has identified a mammalian-like low molecular weight PAP (~34 kDa) from the roots of Pi starved bean plants (Liang et al., 2010a). The three dimensional structures of mammalian and plant PAPs shows that there is a high degree of conservation among the seven invariant amino acids around the catalytic center of the enzyme (Flanagan et al., 2006). These seven metal ligating residues (**D**xG-G**D**XX**Y**-G**N**H(D/E)-VXX**H**-G**H**X**H)** shown in bold letters are required for metal coordination to form the dimetallic active site (Figure 4). This highly conserved sequence motif has facilitated the detection of numerous novel

PAPs and PAP like sequences (Schenk et al., 2013). Multiple isoforms of both low and high molecular weight PAPs have been identified in plants, in contrast to only a single copy of low molecular weight PAP detected in mammalian genomes (Flanagan et al., 2006). A comparison of 35 kDa mammalian and plant PAP amino acid sequences shows that the plant Light Molecular Weight (LMW) PAP is more closely related to the 35 kDa mammalian PAPs than to the larger plant PAPs (Schenk et al., 2000). Detailed analyses of the general structures, active sites and enzymatic properties of PAPs have been previously presented (Olczak et al., 2003; Plaxton and Tran, 2011; Tran et al., 2010a), most recently, in 2013 (Schenk et al., 2013).

1.6 Biological function of mammalian purple acid phosphatases

Mammalian PAPs, which have a role in regulating bone resorption, are proteins produced by osteoclasts (Li et al., 2002). The phosphatase activity of PAPs plays a vital role in dephosphorylating bone matrix proteins, such as osteopontin (Ekrylander et al., 1994). Osteoponotin is required for the adhesion of osteoclasts to the bone surface and upon dephosphorylation, osteopontin no longer supports adhesion (Ekrylander et al., 1994). Transgenic mice overexpressing PAPs showed increased bone turnover and displayed symptoms of mild osteoporosis (Angel et al., 2000).

In humans, PAP expression is associated with activated macrophages where they aid in the generation of free radicals as an immune response, to enhance microbial killing (Hayman and Cox, 1994). Mammalian PAPs are abundantly expressed in dendritic cells and have been shown to catalyze the formation of reactive oxygen species (ROS) (Hayman et al., 2000). Overexpression of PAP in mice increased both ROS and

superoxide production. Moreover, transgenic mice overexpressing PAPs showed enhanced bacterial killing in the presence of hydrogen peroxide (Räisänen et al., 2005).

1.7 Role of plant purple acid phosphatases

1.7.1 Function of plant PAPs in responses to Pi starvation

Phosphate (Pi) is an essential, but limiting, macronutrient required for various physiological and metabolic processes. Pi is integral to several biomolecules, including ATP, NADPH, nucleic acids and phospholipids. Despite the abundance of organic Pi reserves in the soil, the bioavailability of assimilable inorganic Pi is often suboptimal for plant growth and productivity (Nagarajan et al., 2011). Soluble Pi concentrations in most soils range from 1-10 μM, while cellular Pi concentrations are greater than 10 mM (Vance et al., 2003). Fertilizer is often used to alleviate the low availability of Pi in agricultural soil. However, the fertilized crop does not readily assimilate Pi and the majority of applied Pi becomes insoluble in the soil (Tran and Plaxton, 2008). Additionally, insoluble Pi and runoffs from soil leads to eutrophication of surface water.

Plants have evolved an array of adaptive mechanisms to enhance Pi acquisition and survival under Pi starvation. These adaptations include, alteration in root architecture (profuse lateral roots), increased root-to-shoot ratio, and the accumulation of anthocyanin pigments (reviewed in Raghothama, 2000). Other biochemical/metabolic adaptations are the induction of intra-cellular and extra-cellular acid phosphatases (APases) to catalyze the hydrolysis of Pi from various monoesters and anhydrides substrates (Tran et al., 2010b). PAPs represent a specific class of Pi starvation-inducible (PSI) phosphohydrolase (Hurley et al., 2010; Plaxton and Tran, 2011). PAPs have been

characterized from various tissues and cellular compartments and are primarily implicated in facilitating Pi uptake and recycling (Plaxton and Tran, 2011; Vance et al., 2003). Intracellular forms of PAPs are predominantly found in vacuoles and the cytoplasm, where they remobilize and recycle Pi from intracellular Pi monoesters (Duff et al., 1994). Extracellular PAPs are secreted by Pi starved plant roots and cell cultures to hydrolyze Pi from external organophosphates (Tran et al., 2010a).

A number of intracellular and secreted PAPs that are up-regulated under Pi starvation have been biochemically characterized. A marked up-regulation of some PAPs under Pi starvation has been reported in several plant species, including tomato (Bozzo et al., 2002), tobacco (Lung et al., 2008a), Arabidopsis (Hurley et al., 2010; Tran et al., 2010b), white lupin (Miller et al., 2001), common bean (Liang et al., 2010b) and rice (Zhang et al., 2011). It is also evident that the 55-60 kDa subunits of plant PAPs share 70% sequence identity (Li et al., 2002; Flanagan et al., 2006). For example, the PAP secreted by the white lupin, that is expressed in proteoid roots under Pi starvation, shares about 65% amino acid sequence identity with Arabidopsis PAP12 (AtPAP12). Moreover, this similarity extends to the putative promoter sequence of the gene, which has 72% sequence in common with its Arabidopsis counterpart AtPAP12 (Miller et al., 2001).

Pi starved tomato suspension cells express a heterodimeric intracellular PAP and two monomeric secreted PAPs which hydrolyze a broad range of substrates (Bozzo et al., 2006). The biochemical characterization of these PAP isoforms indicated both temporal, as well as tissue specific synthesis for Pi scavenging and recycling (Bozzo et al., 2006). Similarly, other studies have reported both temporal and tissue specific synthesis of PAPs in *Arabidopsis thaliana* under Pi starvation (Wu et al., 2003; Lung et al., 2008). Liang et

al. (2010) identified a 34 kDa PAP (PvPAP3) from the common bean plant (*Phaseolus vulgaris*) grown under Pi starvation. PvPAP3 had a broad pH activity profile and demonstrated high heat stability. Transient expression of a *35S:PvPAP3-GFP* reporter gene in onion (*Allium cepa*) indicated that it might be anchored to the plasma membrane and secreted into the apoplast (Liang et al., 2010).

The Arabidopsis genome encodes 29 PAPs, which have been classified into three distinct groups, according to their amino acid sequences (Li et al., 2002). A few of the Arabidopsis PAPs that are induced under Pi starvation have been biochemically characterized (Reviewed by Tran et al., 2010a). AtPAP17, also known as AtACP5, was purified to homogeneity and identified to be a 35 kDa, low molecular weight monomeric PAP (LMW) (Del Pozo et al., 1999). AtPAP12 (60 kDa) and AtPAP26 (55 kDa) were predominantly expressed in Pi-starved Arabidopsis suspension cultures (Tran et al., 2010b). Both PAPs scavenge Pi from a wide range of Pi containing monoesters over a broad pH activity profile. AtPAP26 was also identified in both the cell vacuole and secretome, under Pi starvation (Tran et al., 2010b). In a separate study, Wang et al. (2011) reported AtPAP10 to be specifically induced with Pi limitation and were predominantly associated with the root surface. Although some PAPs are induced by Pi limitation, the cell specific expression pattern and sub-cellular localization of these proteins remain elusive (Tran et al., 2010a).

1.7.2 Role of PAPs in abiotic stresses

The role of PAPs under Pi starvation has been well documented in many plant species (Reviewed by Tran et al., 2010a). However, emerging evidence suggests that PAPs may be involved in other biological functions. For example, Pi starvation inducible AtPAP17 exhibited both acid phosphatase and alkaline peroxidase activity (Del Pozo et al., 1999). A novel PAP from soybean (*Glycine max*; GmPAP3) was identified from salt stressed plants, but without Pi limitation (Liao et al., 2003). GmPAP3 was also induced by oxidative stress and was shown to carry a putative mitochondrion targeting transit peptide (Li et al., 2008). AtPAP15, soybean PAP (GmPhy) and tobacco (*Nicotiana tabacum*) PAP (NtPAP) share a higher degree of amino acid identity and have been shown to hydrolyze phytate (*myo*-inositol *hexakis*phosphate) (Hegeman and Grabau, 2001; Lung et al., 2008; Kuang et al., 2009). Phytases are classified within the class of phosphatases that initiate the sequential hydrolysis, to release free myoinositol, from phytate, a major form of phosphorous in plant seeds and pollen grain. Previously Zhang et al., (2008) have identified myoinositol, derived from phytate, to be an initial substrate for ascorbate (AsA) biosynthesis. Recently, PAPs exhibiting phytase activity were also described as *PAPhy* (Madsen et al., 2013). Transgenic Arabidopsis plants expressing a AtPAP15 promoter::GUS transgene showed strong expression in the vasculature, pollen grains, and roots (Kuang et al., 2009). In a separate study Zhang et al. (2008), reported that overexpression of *AtPAP15* resulted in a 2-fold increase in foliar ascorbate via *myo*-inositol. Overexpression of *AtPAP15* also enhanced salt tolerance and reduced sensitivity to abscisic acid. Similarly, overexpression of *AtPAP15* in soybean significantly

increased phytase activity in root exudates and improved yield, with increased pod and seed number per plant (Wang et al., 2009). Recently, loss of *PAP26* was reported to result in delayed senescence and reduced phosphorous levels in seeds (Robinson et al., 2012).

1.7.3 Dephosphorylation of wall proteins and carbon metabolism

PAPs exist in an array of tissue and/or cellular compartments. Although most PAPs carry predicted signal peptides and ser/thr phosphatase activity, the role of these proteins in the apoplastic space is unknown. The dephosphorylation of proteins, catalyzed by a native wall associated PAP, has been shown to be involved in cell wall regeneration in protoplast prepared from tobacco cells (Sano et al., 2003). Transgenic tobacco (*Nicotiana tabacum*) cells overexpressing NtPAP12 exhibited enhanced phosphatase activity, with higher β-glucan synthesis and cellulose deposition (Kaida et al., 2009). Most recently, NtPAP12 was found to catalyze the dephosphorylation of apoplastic proteins, α-xylosidase, and β-glucosidase. Overexpression of NtPAP12 in tobacco cells decreased the activity of α-xylosidase and β-glucosidase activity and also increased xyloglucanoligosaccharide and cello-oligosaccharide in the apoplast. Glucosidase degrade xyloglucanoligosaccharide to remove xylose (Xyl) and glucose (Glc) residues. Similarly, β-glucosidase acted on cello-oligosaccharide to release Glc residues. Previously, cello-oligosaccharides were identified as effectors for callose and cellulose synthase (Hayashi et al., 1987). These results suggest that NtPAP12 regulates α-xylosidase and β-glucosidase to control the degradation of xyloglucan oligosaccharides and cello-oligosaccharide in the cell wall (Kaida et al., 2010).

Recent studies have shown that some PAPs regulate carbon metabolism, and overexpression of these proteins has been implicated in improving plant growth and productivity. For example, overexpression of *AtPAP2* in Arabidopsis resulted in enhanced plant growth and a higher seed yield (Sun et al., 2012). Transgenic plants overexpressing *AtPAP2* enhanced sucrose phosphate synthase (SPS), sucrose synthase and invertase activity, promoting higher levels of sugar (sucrose and hexose sugar) and tricarboxylic acid (TCA) metabolites. *AtPAP2* was also identified to be dually targeted to plastids and mitochondria (Sun et al., 2012). Similarly, overexpression of AtPAP2 in *Camelina sativa* improved growth rate and seed yield (Zhang et al., 2012). Interestingly, *Camelina sativa* plants overexpressing *AtPAP2* also showed enhanced sucrose phosphate synthase (SPS) activity, as observed previously in transgenic Arabidopsis (Zhang et al., 2012).

1.7.4 PAPs in symbiosis and pathogenesis

Although the roles of PAPs in response to Pi starvation and other abiotic stresses are well documented, in recent years, the roles of PAPs in biotic interactions have also emerged. Kaffarnik et al.(2009) first reported the accumulation of PAP10 and decreased abundance of PAP14 in the secretome of Arabidopsis cells in culture following *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) infection. Interestingly, PAP10 was reported to accumulate only in response to *Pst* DC3000 *AvrRpm1,* but not in response to virulent *Pst* DC3000 and *Pst* DC3000 *hrpA*$^{-}$. In contrast, the abundance of PAP14 decreased in response to all strains of *Pst* DC3000. These results suggest that the PAP10 plays a role in gene-for-gene resistance (Kaffarnik et al., 2009). It is also evident that PAP10 and PAP14 are differentially expressed, in response to *Pst* DC3000 infection.

Under Pi starvation, an increased phosphatase and phytase activity was observed in nodules of the common bean plant (*Phaseolus vulgaris*), providing an adaptive mechanism for nitrogen fixation (Araujo et al., 2008). Similarly, a mycorrhiza (*Glomus etunicatum*) responsive PAP was identified from the dwarf marigold (*Tagetus patula*) plants (Ezawa et al., 2005). *TpPAP1* was also induced when *T. patula* plants were inoculated with symbiotic mycorrihzae *Archaeospora leptoticha*. Phylogenetic analysis revealed that *TpPAP1* is associated with HMV PAP. Recently, Li et al. (2012) also reported a role of PAPs in symbiosis in soybeans, under Pi starvation where nine out of 35 *GmPAPs* were highly expressed in nodules. However, most *GmPAPs* showed reduced expression when soybean seedlings were inoculated with arbuscular-mycorrhyza (Li et al., 2012).

1.8 Arabidopsis thaliana (L.) Heynh (Colombia-0)

Arabidopsis thaliana is a small dicotyledonous plant belonging to the genus Brassicaceae. Arabidopsis possess a small genome compared to other model plants (125 mega base pairs), produces numerous progeny, requires limited space requirement and can be rapidly manipulated through genetic engineering. These advantages have made Arabidopsis an important model for the study of various aspects of plant biology. (Meinke et al., 1998; The Arabidopsis Initiative 2000).

1.9 The *Pseudomonas syringae* pv. *tomato* DC3000 pathosystem

Pseudomonas syringae infects hundreds of taxonomically diverse plant species and undergoes host-specific interactions comprising about 50 pathovars (Hirano and Upper, 2000). *Pst* DC3000 has emerged as an important model organism in molecular plant pathology because of its genetic tractability (Fouts et al., 2002). The *Pseudomonas*

syringae pv *tomato* DC3000 used in this study is known to infect tomato and Arabidopsis, causing necrotic lesions surrounded by chlorotic halos (Whalen et al., 1991).

The pathogen *Pst* DC3000 has evolved a variety of virulence factors to subvert host defense mechanisms. About 298 virulence genes of *Pst* DC3000 have been identified thatencodes protein that are involved in motility, adhesion, injection of effector proteins into host cells, degradation of host cell walls, production of phytotoxic compounds, iron acquisition, and those that interference with host defenses. This may promote epiphytic growth, as well as actual disease expression. The *hrp*-gene of *Pst* DC3000 encodes the Type III protein secretion system, allowing pathogens to inject more than 40 virulence effector proteins into the host cells (Collmer et al., 2002). The polyketide toxin, coronatin (COR), and phytotoxins produced by *Pst* DC3000 are also known to enhance pathogen virulence in plants (Bender et al., 1999; Brooks et al., 2004).

The Arabidopsis-*Pst* DC3000 is a suitable system to study plant-pathogen interaction, since the infection mechanisms are similar to other phytopathogenic bacteria of the genera *Xanthomonas*, *Ralstonia* and *Erwinia* (Collmer et al., 2002). The pathogenicity of *Pst* DC3000 also resembles many important bacterial and fungal pathogens of plants where host specificity is controlled by ‘‘gene-for-gene’’ interactions, in which a dominant allele in the host and a dominant allele in the pathogen condition the outcomes of the plant-pathogen interaction (Buell et al., 2003).

1.10 *Pseudomonas putida* WCS358

Pseudomonas putida WCS358 (*P. putida*) is a PGPR isolated from the rhizosphere of potato (Geels and Schippers, 1983). *P. putida* antagonize soil borne pathogens by siderophore-mediated competition for iron (VanWees et al., 1997). *P. putida* induces systemic resistance in Arabidopsis against *P. syringae* pv. *tomato*, in the bean against *Colletotrichum lindemuthianum* and *B. cinerea,* and in the tomato, against *Botrytis cinerea* (Meziane et al., 2005). This strain is reported not to induce resistance in carnation and radish against *Fusarium* wilt (Duijff et al., 1993; Leeman et al., 1996). Hence, the induction of systemic resistance by *P. putida* WCS358 is variable and complex among plants. Meziane et al. (2005) reported that the effect of flagella, pseudobactin and LPS on Arabidopsis is complementary, rather than additive, since all the mutants of *P. putida* WCS358 induced resistance to the same level as the wild type.

1.11 Purpose of study

In this study Arabidopsis T-DNA mutants in Colombia-0 (Col-0) background were screened to identify genes that affect pathogen tolerance/susceptibility and those that alter microbial colonization of root. This screen led to the identification of a mutant with an insertion in purple acid phosphatase5 gene, which was hypersusceptible to *Pst* DC3000. In the present study I carried out molecular and functional characterization of PAP5.

Chapter 2 Materials and Methods

2.1 Biological materials and growth conditions

Arabidopsis thaliana (L.) Heynh, ecotype Columbia (Col-0) seeds were purchased from Lehle seeds (Round Rock, TX, USA) and T-DNA insertion mutant lines were obtained from Arabidopsis Biological Resource Center (ABRC) (Columbus, OH, USA). Seeds were surface sterilized with NaOCl 2% (v/v), rinsed five times with sterile water and stratified at 4 °C for 3 days. Seeds were planted either in Jiffy peat pellets (Halifax seeds, Canada) or on plates with 0.5X Murashige and Skoog (MS) media (Murashige and Skoog, 1962). Plants were grown at 22 ± 2 °C with a photoperiod of 16 h light at 125 µmol $m^{-2}s^{-1}$ and 8 h dark cycle.

Virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) was kindly gifted by Dr. Diane Cuppels, Agriculture and Agri Food Canada (AAFC), ON, Canada. *Pseudomonas syringae* pv. *tomato* DC3000 strains was maintained on King's medium B, supplemented with rifampicin (50µg ml^{-1}). PGPR *Pseudomonas putida* WCS 358 was maintained on King's medium B. *Botrytis cinerea* was cultured on modified King's medium B (10 g peptone, 1.5 g potassium phosphate monobasic, 15 g dextrose, pH 5.5, 5 ml of 1 M MgSO4/l).

For plant treatment, Benzothiodiazole (Actigard®; active ingradient 50% w/v BTH) was a gift from Syngenta Corp., USA. Methyl jasmonate, glufosinate-ammonium and other microbiological media were purchased from Sigma Aldrich, Oakville, Canada. Hygromycin for antibiotic selection was purchased from Bioshop, Canada.

2.2 Genetic screening, root colonization assay and pathogen inoculation

Surface sterilized, stratified seeds were germinated on 0.5X Murashige and Skoog (MS) media, containing 0.5% sucrose, pH 5.7 at 22 ± 2 °C, with a photoperiod of 16 h light at 125 µmol $m^{-2}s^{-1}$ and 8 h dark cycle. After ten to twelve days, the plants were rinsed with sterile distilled water and transferred to 24 well plates containing 1 ml of *Pseudomonas syringae* pv *tomato* DC3000 (OD_{600} 0.01) suspension. MS (0.5X) basal media without sucrose was used to prepare bacterial suspension to restrict *Pst* DC3000 from utilizing exogenous sucrose. Wild type (Col-0) plants on 0.5X MS media without sucrose were used as the control, to compare disease symptoms and mortality.

For root colonization assays, ten to twelve day old seedlings were transferred to 24 well plates containing bacterial suspension of *P. putida* (OD_{600} 0.01). An aliquot of the suspension was read spectrophotometrically to determine growth of *P. Putida*. Leaf and root tissues of mutants showing altered phenotype was macerated and diluted in sterile distilled water to determine the colony forming units (CFUs) of *Pst* DC3000 and *P. putida.*

2.3 Pathogen inoculation

Plants exhibiting altered disease tolerance/susceptibility were tested further. Surface sterilized, stratified seeds were planted in Jiffy peat pellets. For pathogenicity assay, 4 to 5 week old plants underwent spray inoculation with bacterial suspension of virulent *Pst* DC3000. Plant inoculation and bacterial growth in plant apoplasts was determined, as described by Zipfel et al. (2004). In brief, strains of *Pst* DC3000 were cultured in King's medium B, supplemented with rifampicin (50µg ml^{-1}), at 28 ºC until optical density (OD) OD_{600} of 0.8. Bacterial cells were collected by centrifugation and resuspended in water

containing 0.02% Silwet L-77 (Lehle seeds, USA), to a final concentration of 10^8 c.f.u ml^{-1}. Plants (4-5 weeks old) were spray inoculated and kept under high humidity for disease development. Leaves were excised (8-10 replicates) from different infected plants and were surface sterilized with ethanol (75% v/v). Four to five samples were made by pooling two leaf discs (0.5 cm^2) and the samples were ground in sterile water with a microfuge tube pestle. The ground tissues were serially diluted and plated on King's B medium containing rifampicin (50µg/ml). The plates were incubated at 28 ºC and colonies were counted after 48 hours. For *Pst* DC3000 induced gene expression analysis, plants were spray inoculated with bacterial suspension (10^8 c.f.u ml^{-1}) and leaf tissues were frozen in liquid nitrogen at the time points indicated.

For *Botrytis cinerea* (*Bcr*) inoculation, spore suspension ($1x10^5$ conidia mL^{-1}) was prepared in potato dextrose broth (PDB), as described by (Ferrari et al., 2007). Four to five week old plants were inoculated by placing 5µl of the spore suspension on either side of the mid vein of fully expanded leaves. Inoculated plants were covered with a transparent plastic dome to maintain high humidity for disease development. For all gene expression analysis, leaf tissues were harvested from four individual plants for each biological replicate and were snap frozen in liquid nitrogen for RNA extraction.

Benzothiozidole (BTH) and methyl jasmonate (MJ) treatments were performed by spraying 4-5 week old plants with solutions containing 0.06% w/v Actigard® (Active ingradient: 50% w/v BTH) or 50µM methyl jasmonate (MJ) with 0.02% Silwet L-77.

2.4 Confirmation of T-DNA insertion

T-DNA insertion and homozygosity of mutant line were confirmed as described by Alonso et al. (2003). The left and right primers flanking the T-DNA insertion were designed using the SALK T-DNA Primer Design tool (http://signal.salk.edu/tdnaprimers.2.html). The T-DNA insertion on salk_126152 was confirmed by PCR, using *AtPAP5* gene specific primers generated from SALK T-DNA verification primer design tool LP 5'-TTCACGGTTTTGTTGTTAGACG-3', RP 5'-TCGTTGAAAACTACACTCGATTTAAC-3' and left border primer LBb1.3 5'-ATTTTGCCGATTTCGGAAC-3'.

2.5 Phosphate starvation

Sterile, stratified seeds (20-25 per jar) were dispensed into 50 ml of liquid 0.5X MS medium containing Pi (1.25mM) or with reduced Pi (0.2mM). The seedlings were grown under constant shaking (85 rpm) at 22 ± 2 °C, in the continuous illumination at 100 µmol $m^{-2}s^{-1}$. After 9 days, the seedlings were rinsed thrice with sterile distilled water and transferred to 0.5X MS medium containing +Pi (1.25mM) or –Pi (0 mM) (Morcuende et al., 2007). Plants were harvested after 11 days for RNA extraction. Whenever Pi was reduced from growth medium, equivalent amounts of sulphate salts were added to maintain the concentration of conjugate cations.

2.6 RNA extraction and quantitative Real-time PCR

Total RNA was extracted from frozen tissues using a monophasic extraction method (Chomczynski and Sacchi, 1987). Reverse transcription was performed with 2µg of total RNA using Quantiscript RTase (Qiagen, ON, Canada). Relative transcript levels

were assayed by Real-Time PCR using gene specific primers (Table 2) on a StepOnePlus Real-Time PCR system (Applied Biosystems, ON, Canada), using SYBR Green reagent (Applied Biosystems, ON, Canada). To determine relative expression levels, the amount of target gene signal was normalized over the abundance of constitutive *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) or *Actin* as endogenous controls. Primers were generated using the Roche Universal Probe Library assay design centre.

2.7 DAB staining

To visualize H_2O_2 production *in situ*, plants were inoculated with a suspension of *Pst* DC3000, as described in earlier in section 2. 3. Leaves were excised at 24 and 48 h.p.i and stained with 3-3 Diaminobenzidine (DAB), as described by Torres et al. (2002). Excised leaves were placed in DAB (1mg/ml) solution for 8-12 hours and the tissues were soaked in ethanol (95%, v/v) to remove chlorophyll. For H_2O_2 quantification, the excised leaf tissues were frozen and ground in liquid nitrogen. To 50 mg of ground frozen tissue, 500 µl of phosphate buffer (50 mM, sodium phosphate, pH-7.4) was added. The samples were centrifuged and 50 µl of the aliquot was used for H_2O_2 quantification, using an Amplex red hydrogen peroxide/peroxidase assay kit (Molecular Probes, Life Technologies, Canada).

Table 1. List of primer sequences used in RT-qPCR

Gene	Locus	Primer sequences (5'-3')
GAPDH	At1g13440	TTGGTGACAACAGGTCAAGCA AAACTTGTCGCTCAATGCAATC
ICS1	At1g74710	GCGTCGTTCGGTTACAGG ACAGCGAGGCTGAATATCAT
PAP5	At1g52940	AACAGGTCGCTCCACTAGACA TGGTTAGAGGCATATGTTTGTCC
PDF1.2	At5g44420	GTTCTCTTTGCTGCTTTCGAC GCAAACCCCTGACCATGT
PR1	At2g14610	TGATCCTCGTGGGAATTATGT TGCATGATCACATCATTACTTCAT

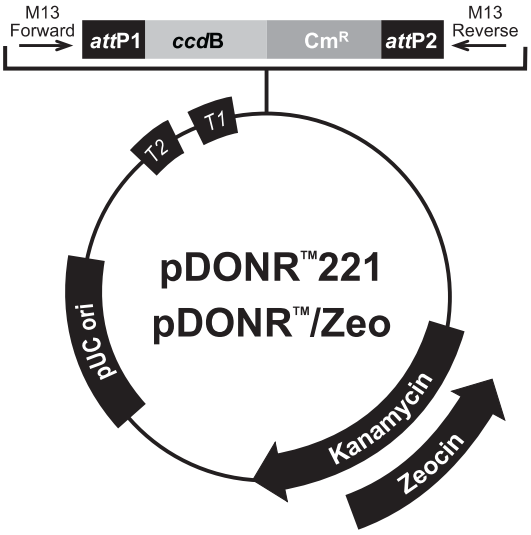
2.8 Cloning and Plant Transformation

The clone of interest was obtained from the ABRC and cloned in to gateway compatible expression vectors (Earley et al., 2006) using LR Clonase II Gateway Technology (Invitrogen, ON, Canada). Briefly, the clone DQ459170, containing full length *PAP5* (At1g52940) cDNA, was obtained from the ABRC. The full length cDNA was amplified without the stop codon via polymerase chain reaction (PCR), using TaKaRA Ex Taq® Polymerase (Clontech, USA). The PCR primers were designed to contain *att*B sites to enable Gateway® technology compatible for cloning (Gateway® Technology, Life Technologies, Canada). Shine-Dalgarno and Kozak consensus sequences were included between the *att*B1 site and the start codon to allow protein expression in *E. coli* and

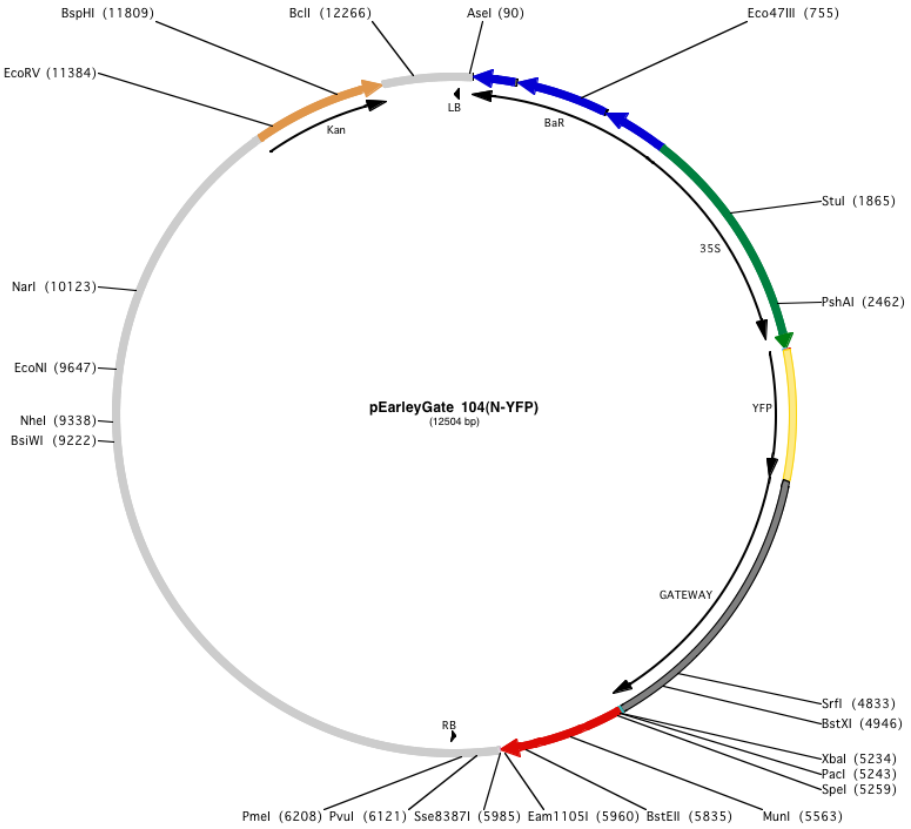
mammalian cells. The PCR product was purified and introduced to pDONR^{TM} 221 vector (Figure 5A), as per the manufacturer's instructions, to generate entry clones (Gateway® Technology, Life Technologies, Canada). The recombinant plasmids were sequenced, using the M13 sequencing primers, to confirm the insert position and orientation. The entry clone, containing the full length *PAP5,* was introduced to the expression vector pEarleyGate 104 (Earley et al., 2006) and pMDC32 (Curtis et al., 2003), using LR clonase (Gateway® Technology, Life Technologies) to generate *35S:YFP-PAP5* and 35S:PAP5 fusion constructs, respectively. The recombinant plasmids were introduced into the *Agrobacterium* strain GV310 (pMB90), using the freeze thaw method (Weigel, D and Glazebrook, J 2002). The *Agrobacterium* strain, carrying the fusion construct, was used to transform Arabidopsis plants by the floral dip method (Clough and Bent, 1998) or infiltrated into tobacco plants for subcellular localization studies (Sparkes et al., 2006). The floral dip inoculation medium contained 0.5X Murashige and Skoog medium, with 5.0% sucrose and 0.05% Silwet (Lehle seeds, TX, USA).

Figure 5. A schematic view of Gateway-compatible cloning vectors.
A. Plasmid map of pDONR^{TM}221 used for cloning *PAP5*. Vector detail of expression vectors pEarleyGate 104 (B) and pMDC32 (C).

(A)


M13
Forward
attP1
ccdB
CmR
attP2
M13
Reverse
T2
T1
pDONR™221
pDONR™/Zeo
pUC ori
Kanamycin
Zeocin

(B)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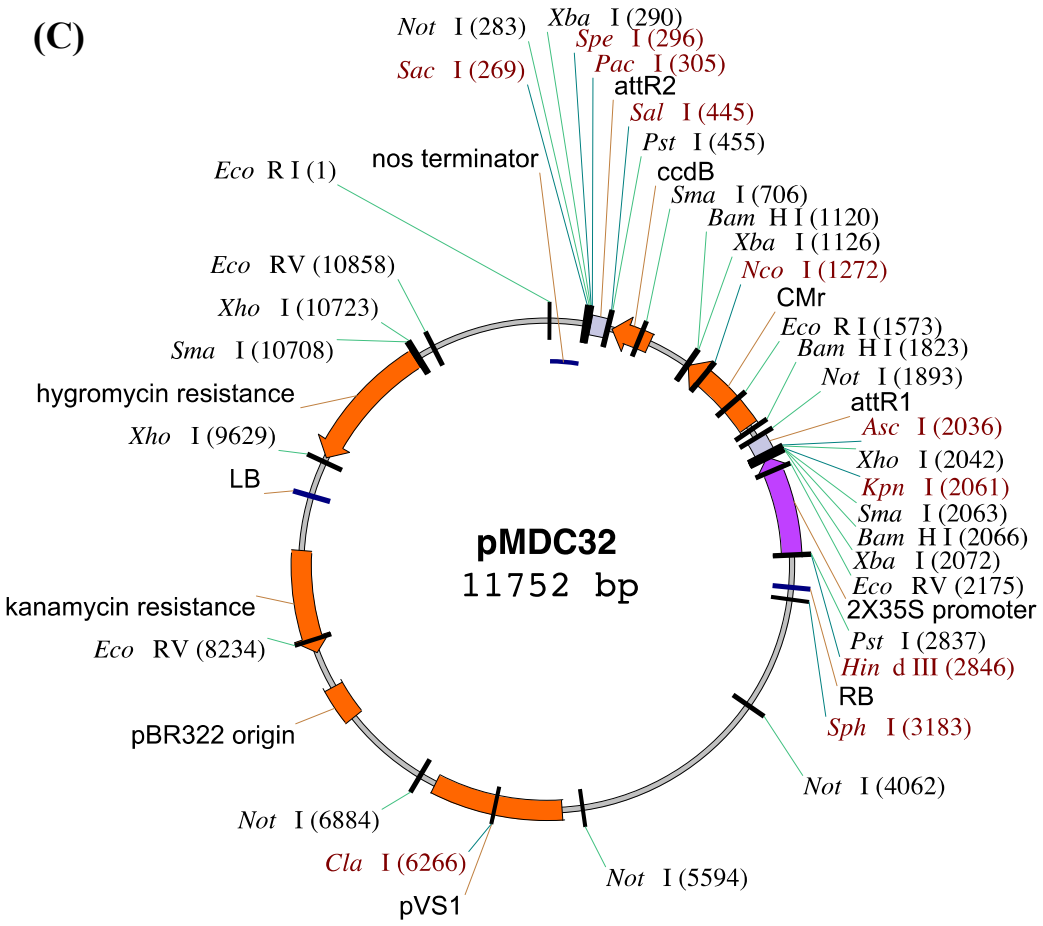
BclI (12266)
AseI (90)
Eco47III (755)
EcoRV (11384)
LB
BaR
Kan
StuI (1865)
35S
NarI (10123)
PshAI (2462)
EcoNI (9647)
pEarleyGate 104(N-YFP)
(12504 bp)
YFP
NheI (9338)
BsiWI (9222)
GATEWAY
SrfI (4833)
BstXI (4946)
RB
XbaI (5234)
PacI (5243)
SpeI (5259)
PmeI (6208)
PvuI (6121)
Sse8387I (5985)
Eam1105I (5960)
BstEII (5835)
MunI (5563)



2.9 Transient protein expression and subcellular localization

For subcellular localization, six week old *Nicotiana benthamiana* (tobacco) plants were used. Tobacco plants were grown at 22 ± 2 °C with a photoperiod of 16 h light at 125 µmol $m^{-2}s^{-1}$ and 8 h dark cycle. Plant organelle specific markers were obtained from the ABRC (Nelson et al., 2007) and the plasmids were transformed to *Agrobacterium* strain GV310 (pMB90), using the freeze thaw method (Weigel and Glazebrook 2002). Agrobacterium strains, carrying the recombinant plasmids, were grown in liquid Luria-Bertani (LB) media supplemented with appropriate antibiotics. Cells were harvested by

centrifugation (5500 g for 10 minutes) and resuspended in infiltration medium to OD_{600} of 0.8. The infiltration medium contained 0.5% glucose, 50 mM MES, 2 mM Na_3PO_4, 0.1 mM acetosyringone (Sparkes et al., 2006). For co-expression of different constructs, *Agrobacterium* suspension was mixed in equal proportion and the *Agrobacterium* suspension mixtures were infiltrated to the tobacco leaves, using a needleless syringe. The leaf samples were harvested at different time points for microscopic imaging.

2.10 Quantification of salicylic acid (SA) and jasmonic acid (JA)

Leaves were excised at 24 and 48 h.p.i and were snap frozen in liquid nitrogen. Leaf tissues (250mg) were ground with liquid nitrogen and extracted with 5-10 ml of MeOH-H_20-HOAc (90:9:1). After 15 minutes, the samples were centrifuged at 12000xg for 10 mins and the supernatant was collected. The extraction procedure was repeated twice. The pooled supernatant was dried under a stream of N_2 and suspended in 5 ml of 0.05% HOAc in H_2O-MeCN (85:15). The samples were then strained through 0.45µm filter and meanwhile, the SampliQ SAX (Aligent technologies, USA) cartridge was conditioned with 2 ml of MeOH. The cartridge was then equilibrated with 5 ml of water. The filtered samples were loaded onto SampliQ SAX cartridge and were washed with 5 ml of 50 mM sodium acetate in 5% methanol. The interphase (IP) was removed with 5 ml of methanol. SA and JA were eluted with 5 ml of 2% formic acid in methanol and dried under N_2.

After optimization of the liquid chromatography conditions and the tuning of the Orbitrap mass spec for negative mode ionization, a dilution series of jasmonic acid (JA) and salicylic acid (SA) were injected. The detection was performed by negative mode monitoring for the exact mass of the pseudomolecular ion $[M-H]^-$, which is 209 amu and

137 amu for JA and SA, respectively. The samples (500ug/ml) were analysed with the same optimized LC/MS method, using a Agilent analytical C18 (3.5 µm, 2.1x100 mm), with a gradient elution from 5% ACN in water to 100% ACN, using 0.1% formic acid in both solvents.

2.11 Yeast two-hybrid screen

The Matchmaker GAL4-based two-hybrid system with Gal4 DNA binding domain (DNA-BD; pGBKT7) and Gal4 activation domain (DNA-AD) were obtained from Clontech, USA. The full length open reading frame of *PAP5* was introduced into the Gal4 DNA-Binding Domain (BD) and was used as bait. A cDNA library (prey) for the screen was generated from total RNA, extracted from *Pst* DC3000 infected wild-type plant. cDNA (prey) library was generated using Make Your Own "Mate & Plate™" (Clontech, USA), using Gal4 activation domain (DNA-AD). The bait and prey plasmids were cotransformed into Y2HGold Yeast strain and Y187 Yeast Strain, respectively, using lithium acetate method (Schiestl and Gietz, 1989). Transformants were plated on growth media without leucine (Leu)/Tryptophan (Trp) and the plates were incubated at 30 °C for 3 days. Positive clones were spot inoculated on selective media containing X-α-gal and Auerobasidin A (AbA). Positive clones were amplified using Activation Domain (AD) insert screening amplimer sequence 5'-CTATTCGATGATGAAGATACCCCACCAAACCC– 3' and 5'–GTGAACTTGCGGGGTTTTTCAGTATCTACGAT–3' to eliminate duplicate clones. Plasmids from the positive clones were also purified and sequenced to identify the interacting partners.

Chapter 3 Results

3.1 Isolation of mutants that exhibited altered root colonization and disease susceptibility/resistance

To identify the genes that affect root colonization and disease susceptibility/resistance, over 960 T-DNA insertions lines (stock number CS27951), generated in Colombia-0 (Col-0), were tested. The root colonization pattern and disease susceptibility/resistance were tested using the virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and beneficial PGPR *Pseudomonas putida* WCS358 (*P. putida*). The plants' responses to *Pst* DC3000 and *P. putida* were monitored and compared, by visual examination, to wild type plants. In addition, the growth of bacteria in media was monitored spectrophotometrically (A_{600}). This preliminary screening revealed several T-DNA lines which showed enhanced/reduced growth compared to wild type plants. Among these T-DNA lines, salk_113904, salk_150259, salk_137036 and salk_091744, carrying a T-DNA insertion in the gene encoding *Flavone 3-hydroxylase* (At3g51240), the *Temperature induced lipocalin* gene (At5g58070), a *multidrug* transporter gene (At4g21910), and an RNA *binding protein* gene (At3g12640) respectively, enhanced the growth of *P. putida*. In addition to these classes of proteins, salk lines corresponding to other genes, including At2g27820 (*Cyclic nucleotide gated channel*), At2g33500 (Zn finger family protein), At2g22680 (Zn finger, ubiquitin protein ligase) and At2g20240 (unknown protein), enhanced the growth of both *Pst* DC3000 and *P. putida*. Visual examination also revealed that salk lines, corresponding to *At2g44450* (beta glucosidase 15) and *At4g28020* (unknown protein), were susceptible to *P. putida.*

As shown in

Table 2, among the screened population, six of the ~ 960 T-DNA insertional lines exhibited enhanced disease susceptibility (EDS) to *Pst* DC3000 and were also susceptible to the beneficial bacteria *P. putida*. Interestingly, salk_129827 (Aspartate aminotransferase; At2g22250) was resistant to *Pst* DC3000 and these plants also enhanced the growth of *P. putida*. Twenty-one of the T-DNA lines exhibited greater disease susceptibility with extensive chlorosis, while thirteen of the T-DNA lines exhibited enhanced disease resistance to *Pst* DC3000 (Table 2). The analysis also revealed that the salk lines 095998, 143601, 152141, 152145, 127112, 123169 and 123960 failed to geminate.

Table 2. Summary of T-DNA lines that exhibited altered root colonization and diseasesusceptibility/resistance

No	Acc No	salk	Locus	Phenotype	Spectophotometric assay	Annotation detail	Polymorphism site
1	97	126152	At1g52940	EDS to *Pst* DC3000 and enhanced *P. putida* growth		Purple acid phosphatase with ser/thr phosphatase activity	Intron
2	147	118554	At5g18720	EDS to *Pst* DC3000		Molecular chaperon, similar to heat shock protein Hsp40	Promoter
3	148	116446	At4g12570	EDS to *Pst* DC3000		Ubiquitin protein ligase	Exon
4	151	115326	At1g15990	EDS to *Pst* DC3000	Enhanced *Pst* DC3000 and *P. putida* growth	Cyclic nucleotide gated channel family	Promoter
5	154	113904	At3g51240	EDS to *Pst* DC3000	Enhanced *P. Putida* growth	Encode Flavone 3 hydroxylase. Regulates flavonoid biosynthesis	Exon
6	173	111051	At2g38870	Resistant to *Pst* DC3000		Encode PR peptide, serine protease inhibitor	Exon
7	195	110573	At4g32820	EDS to *Pst* DC3000		Unknown protein with binding function	Exon
8	207	114468	At3g51230		Enhanced *Pst* growth	Promoter of unknown protein	Promoter
9	263	133208	At1g49470	EDS to *Pst* DC3000	Enhanced *Pst* growth	Unknown protein located in endomembrane	Promoter
10	273	129987	At4g27290		Enhanced (4 fold increase) Pst growth	Sugar and ATP binding with ser/thr kinase activity	Exon
11	285	129827	At2g22250	Resistant to *Pst* DC3000, enhanced growth with *P. Putida*	*P. putida*, *Pst* DC300 growth suppressed	Aspartate amino transferase	Exon
12	286	127970	At1g25460	Resistant to *Pst* DC3000	Same as wild type	Oxidoreductase, Flavonoid biosynthesis	Promoter
13	314	132462	At1g27180	EDS to *Pst* DC3000		Putative disease resistance protein, transmembrane receptor	Exon
14	315	129666	At5g22690	EDS to *Pst* DC3000		Putative disease resistance protein, transmembrane receptor	Exon

No	Acc no	salk	Locus	Phenotype	Spectophotometric assay	Annotation detail	Polymorphism site
15	393	091744	At3g12640		Enhanced *P putida* growth	RNA binding family protein	Intron
16	445	137578	At2g33500		Enhanced *Pst* DC3000 and P putida growth	Zn finger family protein, transcription factor activity	Promoter
17	460	137036	At4g21910		Enhanced *P putida* growth	Multidrug transport, antiporter activity, transporter activity	Exon
18	527	144012	At2g20240		Enhanced *Pst* DC3000 and P putida growth	Unknown protein	Exon
19	532	149664	At2g22680		Enhanced *Pst* DC3000 and *P putida* growth	Zn finger, ubiquitin protein ligase	Exon
20	548	150259	At5g58070		Enhanced *P putida* growth	Temprature induced lipocalines, transporter activity	Exon
21	557	147447	At3g23390	EDS to *Pst* DC3000 and susceptible *to P putida*		Zinc binding ribosomal protein family	Exon
22	572	146748	At2g01290	EDS to *Pst* DC3000 and susceptible to *P putida*		Ribose 5 phosphate isomerase 2	Exon
23	580	146583	At5g57420	Resistant to *Pst* DC3000		Indole 3 acetic acid inducible 33	Intron
24	600	144747	At1g05790	Resistant to *Pst* DC3000		Lipase class 3 family protein	Intron
25	627	074606	At5g07780	EDS to *Pst* DC3000 and susceptible *P putida*		Formin homolog 19	Exon
26	638	073671	At3g25860	EDS to *Pst* DC3000 and susceptible *P putida*		Encodes pyruvate decaboxylase E2 subunit	Exon
27	648	077252	At5g38460	EDS to *Pst* DC3000		ALG8 glucosyl transferase family	Promoter

No	Acc no	salk	Locus	Phenotype	Spectophotometric assay	Annotation detail	Polymorphism site
29	660	078875	At1g17500	EDS to *Pst* DC3000		ATPase E1-E2 type family protein	Promoter
30	663	077716	At5g65830	Resistant to *Pst* DC3000		Receptor like protein 57	Unknown
31	667	070963	At2g32010	EDS to *Pst* DC3000		Inositol polyphosphate 5 phosphatase	Exon
32	668	071663	At1g75400	EDS to *Pst* DC3000		RING/U box super family	Exon
33	685	070362	At1g50090	Resistant to *Pst* DC3000		Branched chain amino acid transferasaminase 7	Intron
34	695	073602	At3g15550	EDS to *Pst* DC3000		Unknown pritein	Exon
35	701	078355	At2g45790	Resistant to *Pst* DC3000		Phosphomannomutase	Promoter
36	709	078780	At2g02800	Resistant to *Pst* DC3000		Protein kinase, APK2B	Exon/Intron
37	711	076344	At5g43500	Resistant to *Pst* DC3000		Actin related protein 9	Exon
38	719	077422	At3g19680	EDS to *Pst* DC3000		Unknown protein	Exon
39	720	073248	At4g36840	EDS to *Pst* DC3000		Galactose oxidase	Exon
40	721	100969	At4g33625	EDS to *Pst* DC3000		Unknown pritein	Exon
41	729	098122	At1g69260	EDS to *Pst* DC3000		ABI5 binding protein	Exon
42	730	096057	At4g28020	Susceptible to *P putida*		Unknown protein, Biological process and cellular component unknown	Promoter

No	Acc no	salk	Locus	Phenotype	Spectophotometric assay	Annotation detail	Polymorphism site
43	736	197633	At2g44450	Susceptible to *P putida*		beta glucosidase 15	Exon
44	739	101638	At4g12460	Resistant to *Pst* DC3000		Oxysterol related protein 2B	Exon
45	775	097966	At5g21960	EDS to *Pst* DC3000		DREB subfamily A-5 of ERF/AP2 transcription factor family	Exon
46	829	108078	At1g65590	EDS to *Pst* DC3000 and susceptible *to P putida*		Beta hexosaminidase activity	Intron
47	835	105951	At3g22090	Resistant to *Pst* DC3000		Unknown protein	Exon
48	842	107491	At1g73370	EDS to *Pst* DC3000		Sucrose synthase 6	Exon
49	843	105371	At1g29530	EDS to *Pst* DC3000		Unknown protein	Promoter
50	849	108290	At1g03600	EDS to *Pst* DC3000		PSB27	Promoter
51	854	105251	At1g03280	Resistant to *Pst* DC3000		Transcription factor TFIIE	Intron

3.2 Identification of mutants exhibiting altered defense responses

Selected T-DNA lines were retested for their response to *Pst* DC3000. A total of 21 T-DNA insertion lines exhibited enhanced disease susceptibility. The mutant line salk_126152C (*pap5-1*), which exhibited enhanced susceptibility to *Pst* DC3000 with extensive chlorosis on leaf tissues, was selected for further analysis (Figure 6). Salk_126152C carried a T-DNA insertion in the gene coding for *Purple Acid Phosphatase 5* (*PAP5*; *At1G52940*) (Genome-Wide Insertional Mutagenesis of *Arabidopsis thaliana*, 2003). The enhanced susceptibility phenotype of *pap5-1* plants was confirmed by assessing bacterial growth in leaf tissues, post inoculation. As shown in figure 6, *pap5-1* plants had greater titers of bacteria at 48 and 72 hours post inoculation (h.p.i), compared to the wild-type plant. To ensure that the altered responses to the pathogen were caused by disruption of the *PAP5* gene and not by an unlinked mutation, a second knockout mutant line salk_081481C (*pap5-2*), carrying a T-DNA insertion in *PAP5* (*At1g52940*), was tested. *pap5-2* plants also exhibited the extensive chlorosis and higher titer of bacteria, similar to that of *pap5-1* plants (**Figure S1**).

3.3 Further characterization of *pap5-1* mutant plants

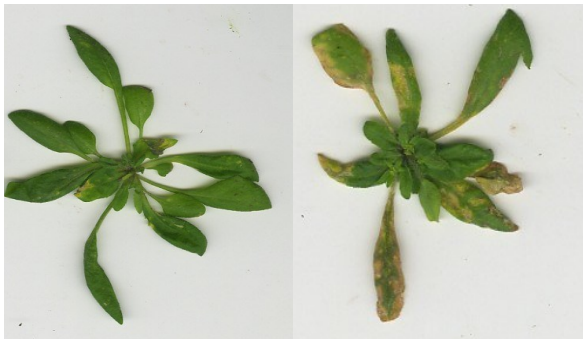
Genotyping, via polymerase chain reaction (PCR), confirmed that *pap5-1* (salk_126152C) carries a T-DNA insertion within the first intron of the *PAP5* gene (Figure 7A and B). To determine the impact of T-DNA insertion on transcript levels of *PAP5*, Reverse Transcription-quantitative PCR (RT-qPCR) was performed, using gene specific primers (Figure 7A). Most PAPs are reported to be highly inducible under phosphate starvation (Pi). In these experiments, 5 day old wild-type seedling showed no induction of *PAP5* when grown in the presence of phosphate (1.25mM) or under

phosphate starved conditions (-Pi, 0 mM) (data not shown). It was also observed that the expression of *PAP5,* under optimal growing conditions, was very low and this was confirmed with the *PAP5* expression profile in the comprehensive microarray site https://www.genevestigator.com/gv/ (Appendix Figure S2). Interestingly, a marked increase in the expression *PAP5* was observed when wild type seedlings were grown under prolonged phosphate starvation (Figure 7C). For prolonged Pi starvation, wild-type seedlings were germinated in media containing reduced Pi (0.25mM) for seven days and then transferred to media with no Pi (0 mM). After 9 days, the seedlings were harvested for gene expression analysis. RT-qPCR analysis revealed an increase in transcript levels of *PAP5* in wild-type seedlings grown under prolonged phosphate starvation (-Pi), compared to seedlings grown in the presence of phosphate (+Pi) (Figure 7C). An increase in *PAP5* transcript levels was not observed in *pap5* seedlings grown under prolonged phosphate starvation (-Pi). No major alteration in germination, growth and development of *pap5* mutant plants was observed, compared to wild-type, under optimal growth conditions (data not shown).

Figure 6. *pap5-1* plants exhibit enhanced susceptibility to *Pst* DC3000.

A. The phenotype of *pap5-1* plants, exhibiting extensive chlorosis and enhanced susceptibility to *Pst* DC3000. Plants were spray inoculated with 10^8 c.f.u ml $^{-1}$ and photographed after 5 days of inoculation. B. Growth of virulent *Pst* DC3000 in wild type (Col-0) and *pap5-1* leaves. Plants were spray inoculated with *Pst* DC3000 (10^{-8} c.f.u ml^{-1}) and bacterial growth in plant apoplasts was determined as described in the materials and methods section. The bars represent the mean and standard deviation from values of six to eight replicate samples. The experiment was repeated three times with similar results. An asterisk indicates significance (Student's *t*-test; $P<0.05$).

(A)



WT *pap5-1*

(B)

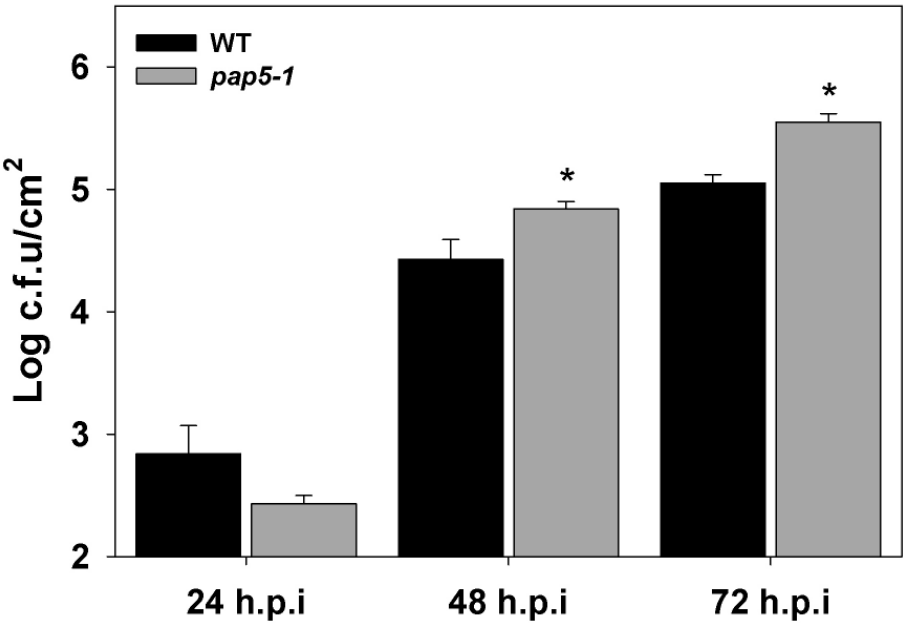
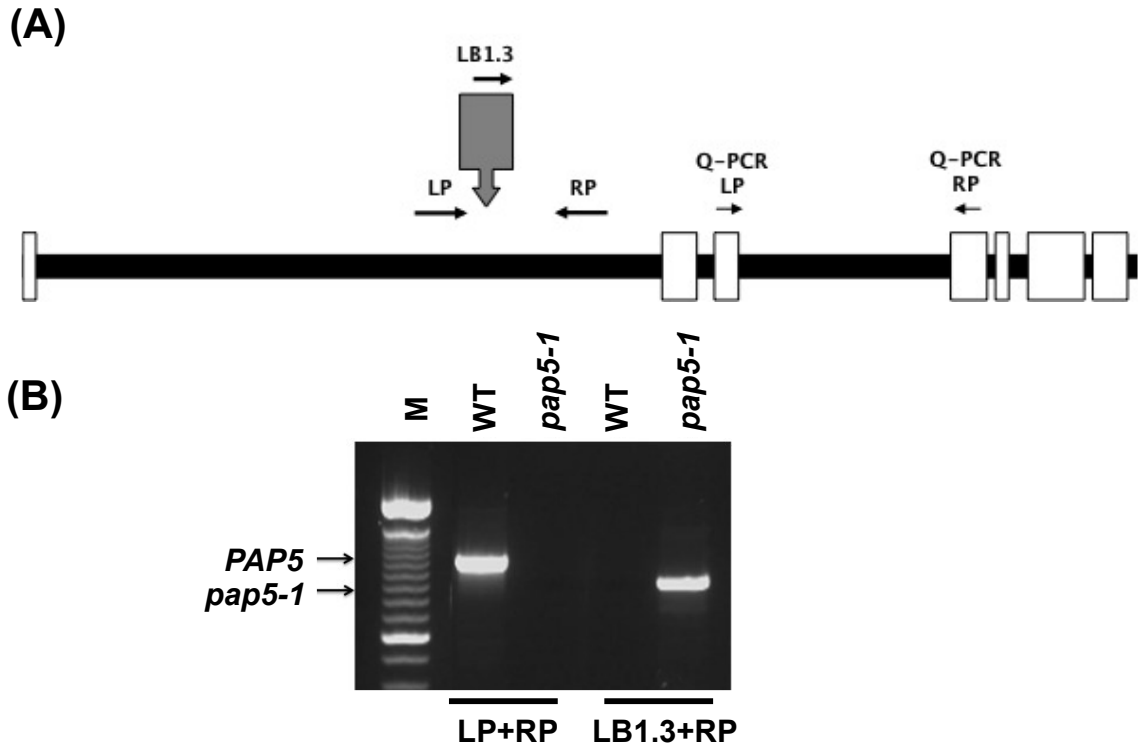
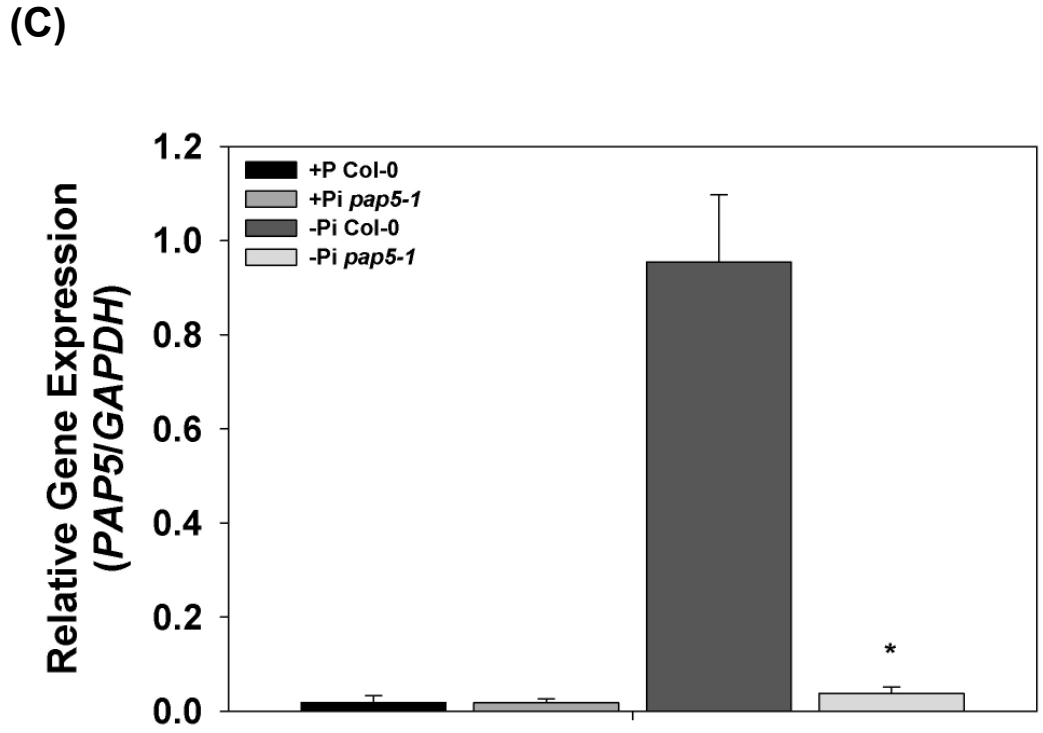


Figure 7. Validation of T-DNA insertion in *pap5-1* plants.

A. Schematic representation of *AtPAP5* (At1G52940); white boxes and solid lines represent exons and introns respectively. T-DNA insertion is designated with a grey arrow and the solid arrows indicate the primers used for genotyping and quantitative RT-qPCR. B. Location of the T-DNA insertion and homozygosity of *pap5* was confirmed by PCR using the gDNA from wild-type and *pap5-1* plants (M, 100bp marker). Thirty cycle PCR reactions were performed, with the primer pairs indicated. C. Relative expression of *PAP5* transcripts, in response to prolonged Pi starvation; For prolonged Pi starvation, wild type and *pap5-1* seedlings were germinated and grown in 0.5X MS media containing reduced Pi (0.25mM). After seven days, the seedlings were washed with sterile water and transferred to 0.5X MS with no Pi (0 mM). After 9 days, the seedlings were harvested for gene expression analysis. Total RNA was extracted from wild-type and *pap5-1* plants, as described in materials and methods. Transcript levels of *PAP5* were normalized to the expression of *GAPDH* in the same samples and expressed relative to the normalized transcript levels of Pi starved wild-type plants. The bars represent the mean and standard deviation from two independent experiments. Asterisks represent data sets significantly different from the wild-type data sets ($P<0.05$ using one-tailed Student's *t*-test).


(A)
LB1.3
LP
RP
Q-PCR LP
Q-PCR RP
(B)
M
WT
pap5-1
WT
pap5-1
PAP5
pap5-1
LP+RP
LB1.3+RP


(C)
Relative Gene Expression (PAP5/GAPDH)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+P Col-0
+Pi pap5-1
-Pi Col-0
-Pi pap5-1
*

3.4 Mutation in *PAP5* alters expression of host defense responsive genes and ROS production

To explore the enhanced susceptibility of *pap5-1* plants and to determine the role of PAP5 in host defense responses, plants were inoculated with the virulent isolate *Pst* DC3000 (10^8 c.f.u ml^{-1}) and the transcript abundances of selected defense responsive genes, including the pathogenesis-related gene1 (*PR1*), were determined. Infection of wild-type plants with the virulent isolate *Pst* DC3000 resulted in ~10-fold induction of the *PR1* transcript 24 h.p.i, while an increase of only ~2-fold was observed in *pap5-1* plants (Figure 8). The level of *PR1* transcripts in *pap5* plants, following *Pst* DC3000 infection, was variable at 48 h.p.i. However, the expression of *PR1* was less induced in *pap5-1* plants, compared to wild-type (Figure 8). Expression of *isochorismate synthase1* (*ICS1*) was induced in wild-type plants (~2-fold), while no increase in transcript levels was observed in *pap5-1* plants. Although, expression of *plant defensin1.2* (*PDF1.2*) was induced (~2-fold higher) in wild-type plants, expression of *PDF1.2* was suppressed in *pap5-1* plants (Figure 9A). The expression pattern of these pathogenesis related genes was also confirmed using *Actin* as the internal control.

A marked increase in the expression of *PAP5* at 6 h.p.i was observed in wild-type plants (Figure 9B). However, this difference was not noted at 24 and 48 h.p.i. Induction of *PAP5* was not observed in mock infected and *Pst* DC3000 inoculated *pap5-1* plants. The expression profile of *PAP5* was further verified from the comprehensive microarray site http://bar.utoronto.ca/ using Arabidopsis eFP Browser (Appendix Figure S4)(Winter et al., 2007). Although, *PAP5* was not strongly upregulated following *Pst* DC3000

infection, these results suggest that a basal level of *PAP5* is required for maintaining resistance against virulent *Pst* DC3000.

To further explore the mechanism of enhanced susceptibility, hydrogen peroxide (H_2O_2) accumulation was investigated using 3-3'-Diaminobenzidine (DAB) staining. As shown in Figure 10A, accumulation of H_2O_2, was reduced in *pap5-1* leaves at 24 and 48 h.p.i. in response to *Pst* DC3000. In contrast, there was an accumulation of H_2O_2 in the wild-type plants.

Figure 8. Expression of *PR1* in wild-type and *pap5-1* plants after *Pst* DC3000 infection. Transcript levels of *PR1* in wild-type and *pap5-1* plants were quantified after infection with virulent *Pst* DC3000 (10^8 c.f.u ml^{-1}). Total RNA was extracted from leaf tissues sampled 24 and 48 h.p.i. Transcript levels were normalized to the expression of *GAPDH* in the same samples. The transcript levels were expressed relative to the normalized transcript levels of mock infected wild-type plants. The bars represent the mean and standard deviation from two independent experiments. Significant differences ($P<0.05$) are indicated by different letters.

(A)

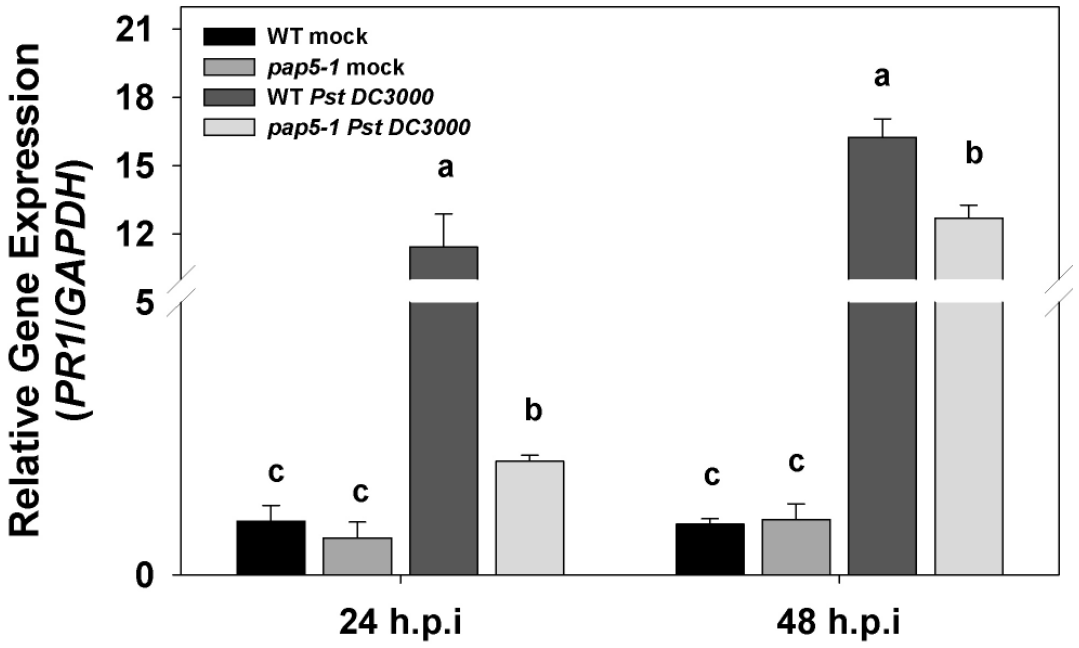

Relative Gene Expression
(PR1/GAPDH)
21
18
15
12
5
0
WT mock
pap5-1 mock
WT Pst DC3000
pap5-1 Pst DC3000
a
b
c
c
b
c
c
a
b
24 h.p.i
48 h.p.i

Figure 9. Expression of *ICS1*, *PDF1.2* and *PAP5* in wild-type and *pap5-1* plants after *Pst* DC3000 infection.

Transcript levels of *ICS1*, *PDF1.2* and *PAP5* in wild-type and *pap5-1* plants after infection with virulent *Pst* DC3000 (10^8 c.f.u ml^{-1}). Total RNA was extracted from leaf tissues 6 h.p.i for *PAP5* expression (A) and 24 h.p.i for *ICS1* and *PDF1.2* expression (B). Transcript levels were normalized to the expression of *GAPDH* in the same samples. The transcript levels were expressed relative to the normalized transcript levels of infected wild-type plants for *PAP5* expression and mock infected wild-type plants for *ICS1* and *PDF1.2* expression. The bars represent the mean and standard deviation from two independent experiments. Significant differences ($P<0.05$) are indicated by different letters. Asterisks indicate significant difference in transcript levels compared to wild-type (Students *t*-test; $P<0.05$).

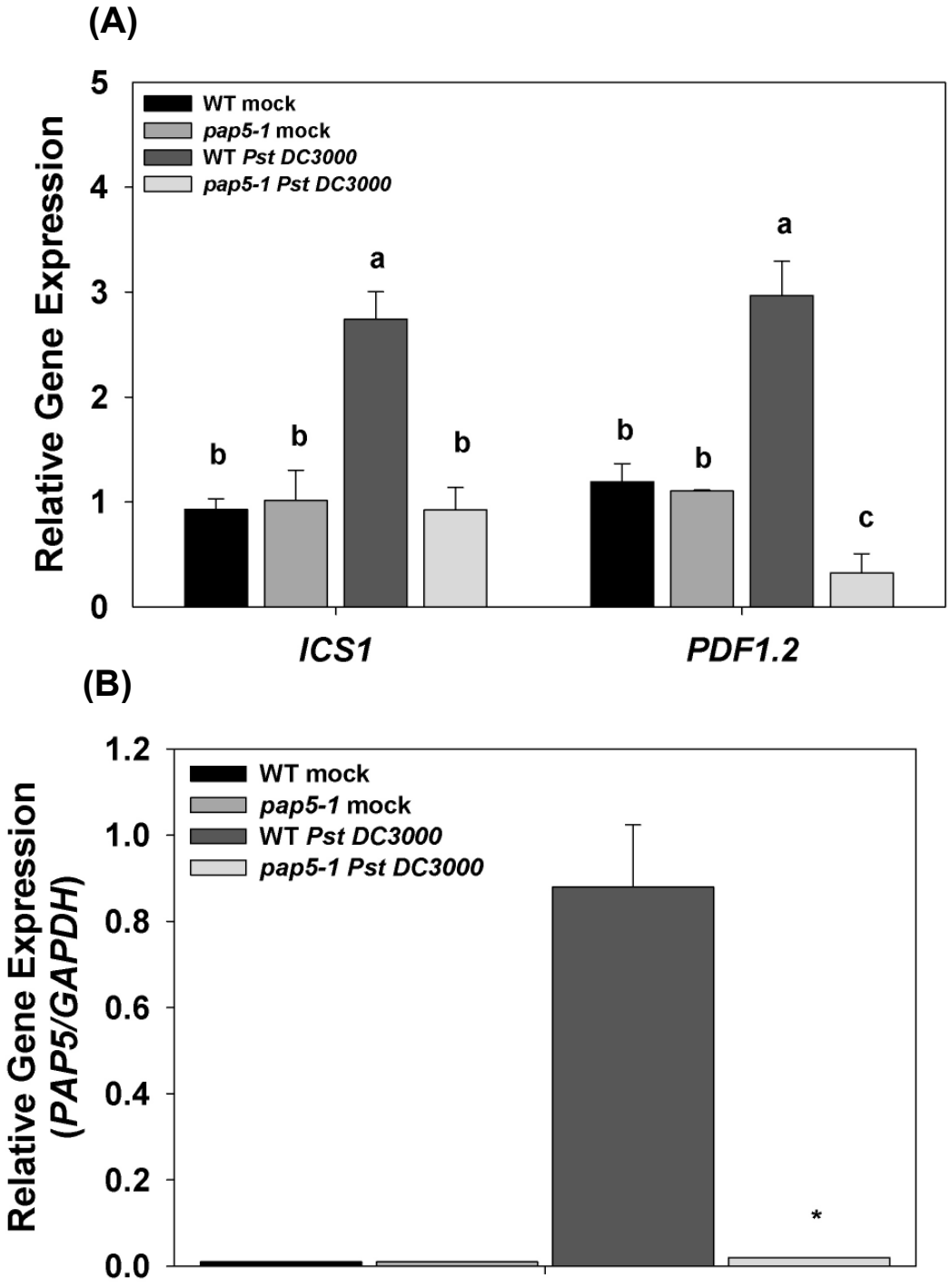
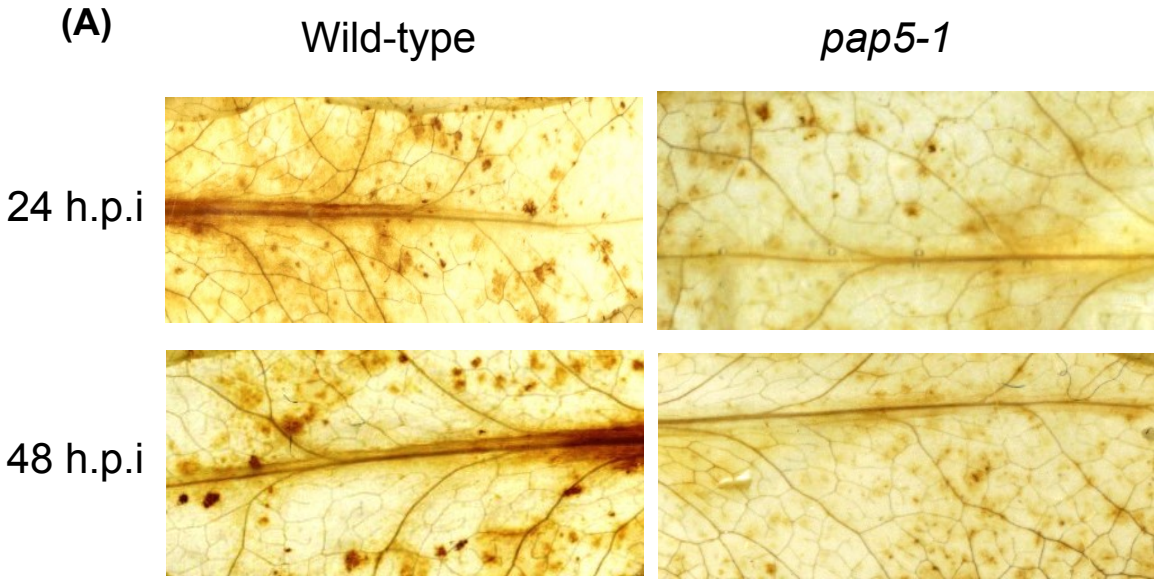
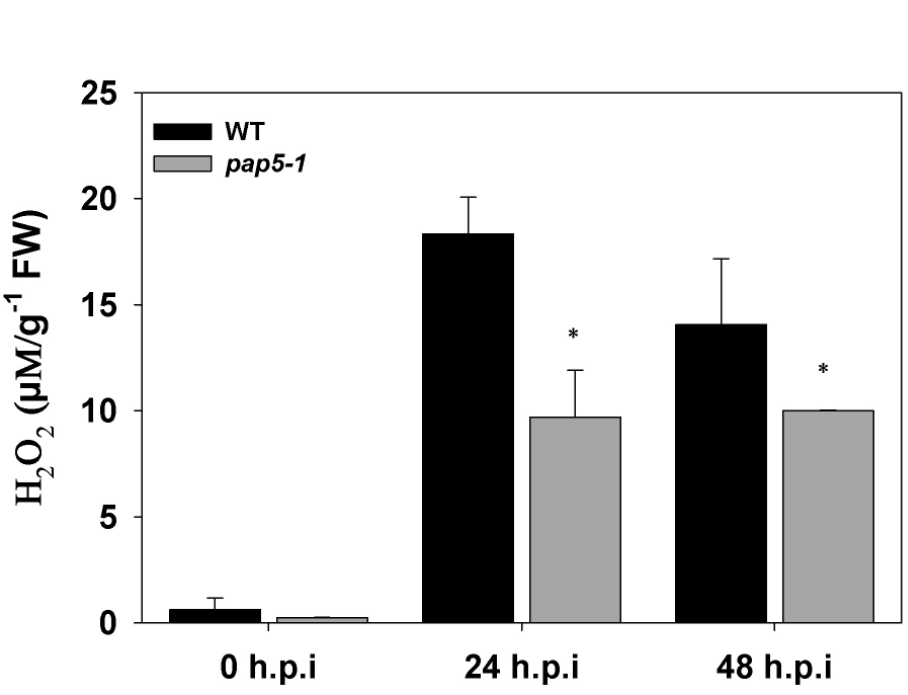

(A)
Relative Gene Expression
WT mock
pap5-1 mock
WT Pst DC3000
pap5-1 Pst DC3000
0
1
2
3
4
5
b
b
a
b
b
b
a
c
ICS1
PDF1.2
(B)
Relative Gene Expression
(PAP5/GAPDH)
WT mock
pap5-1 mock
WT Pst DC3000
pap5-1 Pst DC3000
0.0
0.2
0.4
0.6
0.8
1.0
1.2
*

Figure 10. Histochemical detection and quantification of H_2O_2.

A. Wild-type and *pap5-1* leaves were excised following *Pst* DC3000 infection and stained with DAB for hydrogen peroxide. B. Quantification of H_2O_2 following *Pst* DC3000 infection. The bars represent mean and SD of H_2O_2 accumulation. Asterisks represent significant difference in H_2O_2 production compared to wild type (Student's *t*-test; $P<0.05$).


(A)
Wild-type
pap5-1
24 h.p.i
48 h.p.i


(B)
25
20
15
10
5
0
WT
pap5-1
H_2O_2 (μM/g^{-1} FW)
*
*
0 h.p.i
24 h.p.i
48 h.p.i

3.5 Resistance to *Botrytis cinerea* is affected in *pap5* plants

Having demonstrated the enhanced susceptibility of *pap5-1* plants to the hemi-biotrophic pathogen *Pst* DC3000, the level of resistance of *pap5-1* plants to the necrotrophic pathogen *Botrytis cinerea,* was tested. Four week old plants were inoculated with a spore suspension of *B. cinerea,* and lesion size was measured three days later. As shown in Figure 11, *pap5-1* plants developed a significantly larger lesion (5.4±0.3 mm) than the wild-type (3.9±0.2 mm). The greater lesion size on *pap5-1* plants, in response to *B. cinerea* infection, suggests that the role of *PAP5* is also important in limiting fungal growth.

To identify the role of *PAP5* in the resistance against *B. cinerea,* the transcript abundance of *PR1* and *PDF1.2* was assessed. As shown in Figure 12A, *B. cinerea* strongly induced the expression of *PR1* in both wild-type and *pap5*. In contrast, the level of the *PDF1.2* transcript at 24 h.p.i was only half of that observed in wild-type plants (Figure 12B). By 48 h.p.i., however, the transcript levels of *PDF1.2* were similar in both wild-type and *pap5* plants. No significant differences in *PAP5* transcripts with *B. cinerea* infection were observed (Figure 12B).

3.6 Responses to exogenous application of BTH, a salicylic acid analog, and methyl jasmonate (MJ) are unaffected in *pap5* plants

Since *pap5-1* plants exhibited enhanced susceptibility to *Pst* DC3000 and *B. cinerea,* the role of PAP5 in response to BTH and MJ, was investigated. Exogenous application of BTH induced higher levels of *PR1* in wild-type and *pap5-1* plants (Figure 13A). A

slightly higher increase in the expression *PR1* in *pap5-1* plants 24 h after BTH treatment was observed. Similarly, application of MJ strongly induced the expression of *PDF1.2* in both wild-type and *pap5-1* plants. No significant difference in expression of *PDF1.2* between wild-type and pap5-1 plants was not noted, following application of MJ (Figure 13B). Application of BTH and JA induced expression of *PR1* and *PDF1.2*, respectively, indicative of an intact JA signaling pathway in *pap5* plants. Based on these experiments, it was clear that *pap5-1* plants were not defective in responding to exogenously applied BTH or MJ.

Figure 11. *pap5-1* plants show increased susceptibility to necrotrophic pathogen *B. cinerea.*

A. Size of lesion in wild type and *pap5-1* plants inoculated after *B. cinerea* infection. Leaves were inoculated by placing 5µl of the *B. cinerea* spore suspension ($1x10^5$/ml) on either side of the mid vein and the lesion size was measured after 3 days. The bars represent mean and SD of 20 individual lesions. Asterisks represent significant difference in lesion size compared to wild-type (Student's *t*-test; $P<0.05$). B. *PAP5* expression in response to *B. cinerea* infection. Leaf tissues were harvested 48 h.p.i for RNA extraction. . Transcript levels of *PAP5* were normalized to the expression of *GAPDH* in the same samples. The transcript levels were expressed relative to the normalized transcript levels of infected wild-type plants.

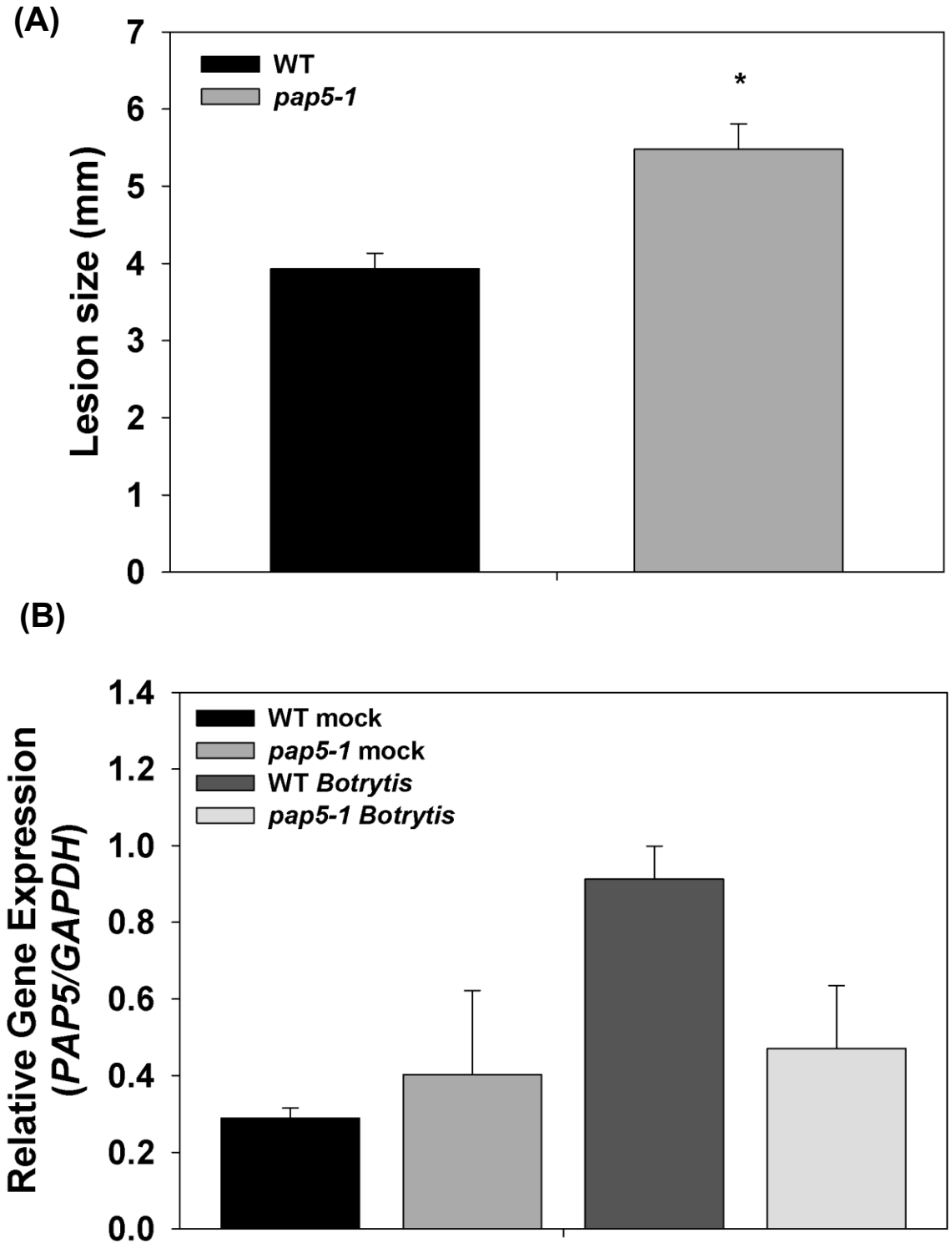

(A)
Lesion size (mm)
0
1
2
3
4
5
6
7
WT
pap5-1
*
(B)
Relative Gene Expression
(PAP5/GAPDH)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
WT mock
pap5-1 mock
WT Botrytis
pap5-1 Botrytis

Figure 12. Expression of *PR1* and *PDF1.2* in response to *B.cinerea* infection.

Plants were spray inoculated with spore suspension of *B.cinerea* ($1x10^5$) and leaf tissues were harvested for total RNA extraction. Transcript levels of *PR1* (A) and *PDF1.2* (B) was normalized to the expression of *GAPDH* in the same samples and expressed relative to the normalized transcript levels of mock infected wild-type plants. The bars represent the mean and standard deviation from two independent experiments. Asterisks indicate significant difference in transcript levels compared to wild-type (Students *t*-test; $P<0.05$).

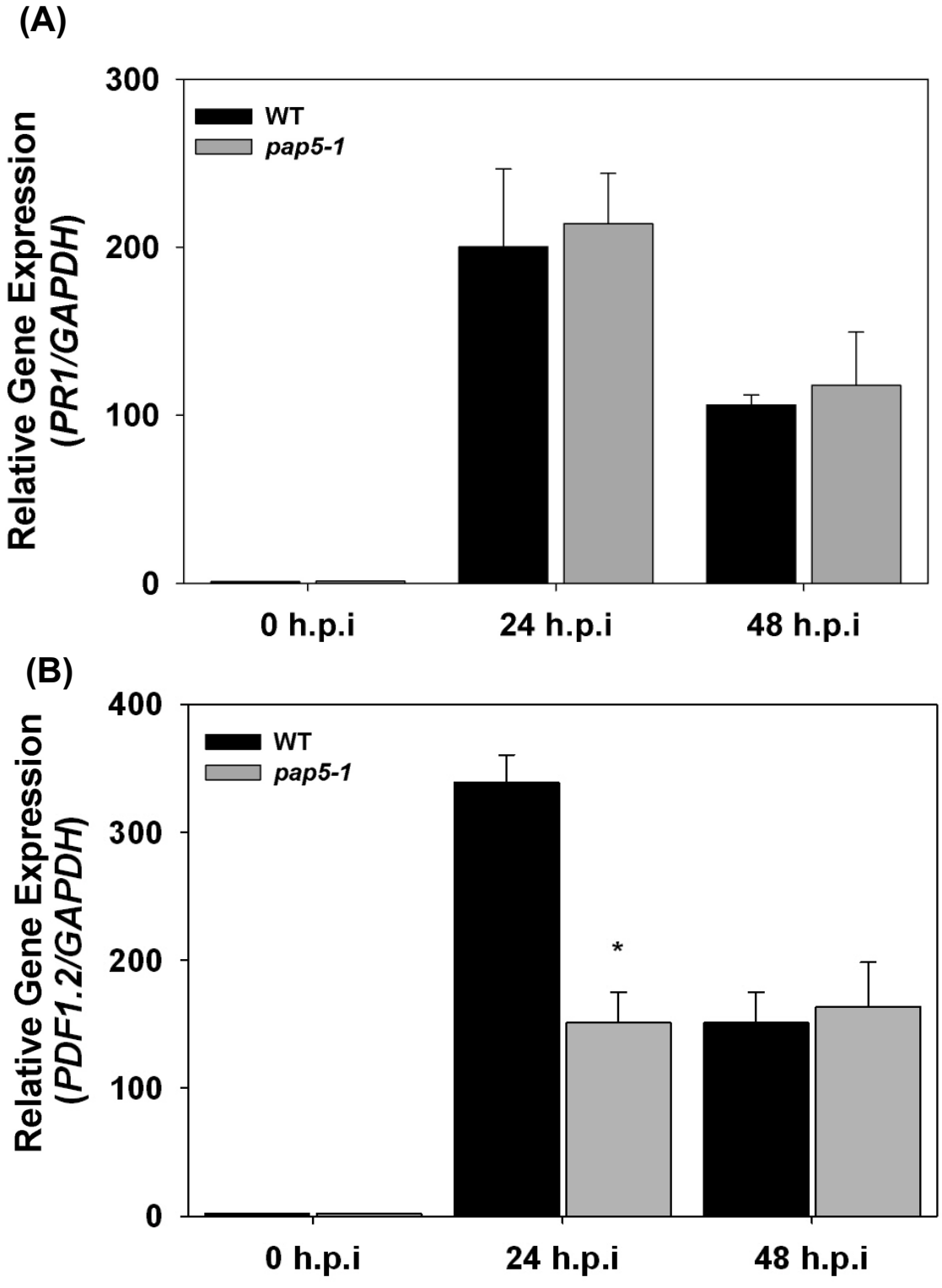
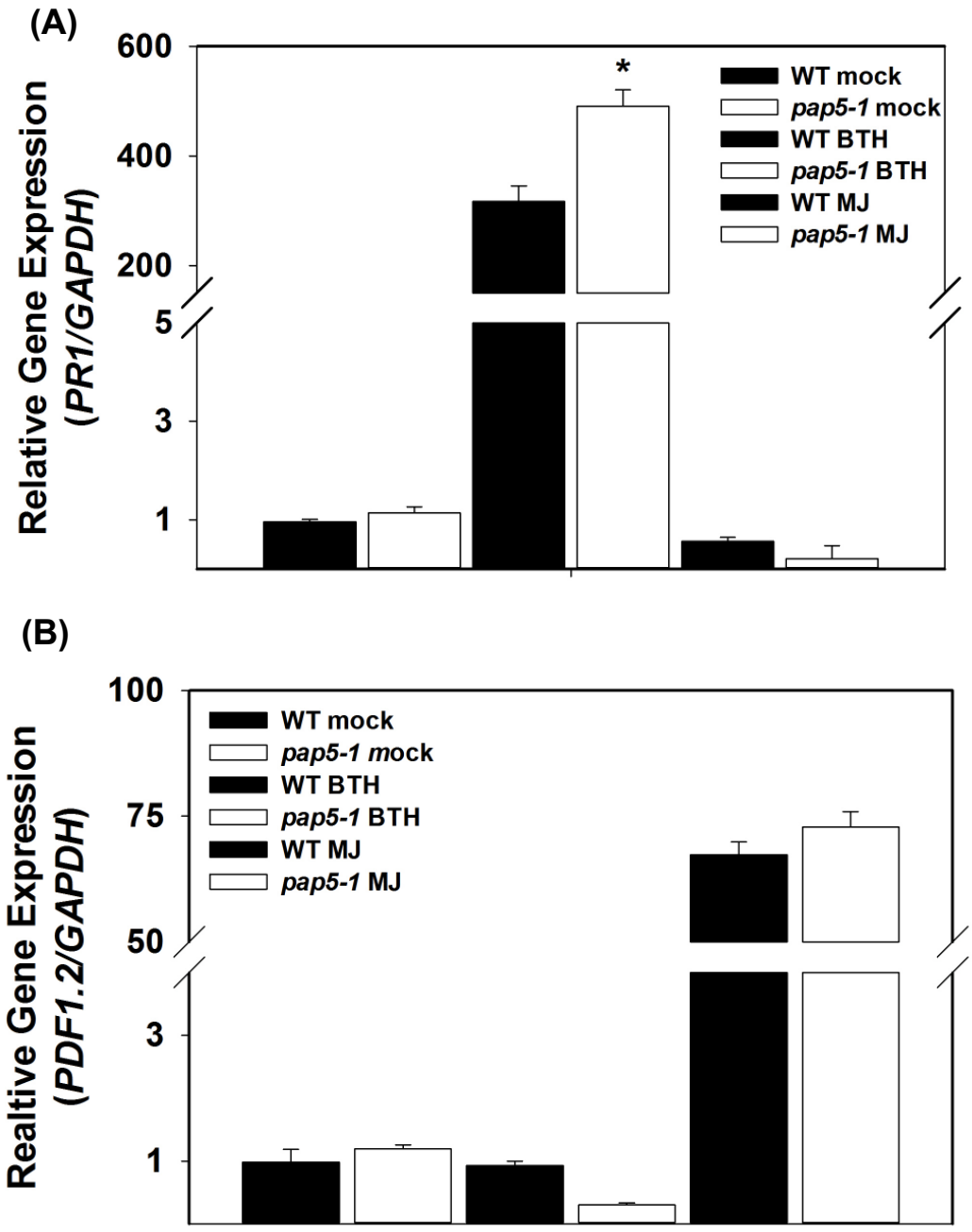

(A)
Relative Gene Expression
(PR1/GAPDH)
300
200
100
0
WT
pap5-1
0 h.p.i
24 h.p.i
48 h.p.i
(B)
Relative Gene Expression
(PDF1.2/GAPDH)
400
300
200
100
0
WT
pap5-1
*
0 h.p.i
24 h.p.i
48 h.p.i

Figure 13. Responses to exogenous application of BTH and MJ are affected in *pap5* plants.

A, Expression of *PR1* in response to benzothiodiazole (BTH) treatment. B, expression of *PDF1.2* in response to methyl jasmonate treatment. Plants were spray treated with either or 0.06% of Actigard® (Active ingradient: 50% w/w benzothiodiazole) or 50 μM methyl jasmonate. Leaf tissues were harvested after 24 h of spraying for total RNA extraction. Transcript levels of *PDF1.2* and *PR1* were normalized to the expression of *GAPDH* in the same samples and expressed relative to the normalized transcript levels of mock treated wild-type plants. The bars represent the mean and standard deviation from two independent experiments. Asterisks indicate significant difference in transcript levels compared to wild-type (Students *t*-test; $P<0.05$).


(A)
Relative Gene Expression
(PR1/GAPDH)
600
400
200
5
3
1
*
WT mock
pap5-1 mock
WT BTH
pap5-1 BTH
WT MJ
pap5-1 MJ
(B)
Realtive Gene Expression
(PDF1.2/GAPDH)
100
75
50
3
1
WT mock
pap5-1 mock
WT BTH
pap5-1 BTH
WT MJ
pap5-1 MJ

3.7 Optimal level of *PAP5* is required for complete resistance to *Pst* DC3000

Previously, in this study, loss of *PAP5* activity was observed to enhance susceptibility to virulent *Pst* DC3000 in Arabidopsis. Having demonstrated that the loss of *PAP5* led to enhanced susceptibility, a test was carried out to determine whether overexpression of *PAP5* would lead to enhanced resistance. Transgenic plants were generated which overexpressed PAP5 was generated under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. Plants exhibiting Basta resistance were chosen and from these, plants were picked randomly to verify the abundance of *PAP5* transcripts. Among the transgenic lines tested, two independent overexpressor lines, *35S:PAP5*-A and *35S:PAP5*-B, were chosen for further studies. Overexpressor lines *35S:PAP5*-A and *35S:PAP5*-B showed ~ 1 and 3 fold increase in *PAP5* transcripts respectively, compared to wild-type (Col-0) plants (Figure 14A). No major alteration in growth and development of either overexpressor lines compared to wild-type plants was observed.

For pathogenicity assay, plants were spray inoculated with a suspension of *Pst* DC3000, as described in the methods. Interestingly, the overexpressor lines exhibited extensive chlorosis and increased susceptibility to *Pst* DC3000, compared to wild-type plants. Assessment of *Pst* DC3000 growth in plant apoplasts revealed that both overexpressor lines contained higher bacterial titers, compared to wild-type plants (Figure 14B). Both overexpressor lines exhibited comparable levels of chlorosis and bacterial titers, suggesting that the enhanced susceptibility is not due to the insertion of the transgene. Although the overexpressor line *35S:PAP5*-B constitutively expressed higher levels (~ 3 fold) of *PAP5* compared to *35S:PAP5*-A plants (Figure 14A), no significant difference in susceptibility to *Pst* DC3000 was observed (Figure 14B).

3.8 Overexpression of *PAP5* impairs pathogenesis related (PR) gene expression and alters H_2O_2 and salicylic acid accumulation

Since transgenic plants overexpressing *PAP5* displayed enhanced susceptibility to *Pst* DC3000, tests were carried out to determine whether defense related genes are impaired in the overexpressor lines (*35:PAP5*-A and *35S:PAP5*-B). The overexpressor lines and wild-type plants were spray inoculated with a suspension of *Pst* DC3000 (10^8 c.f.u ml^{-1}) and leaf tissues were harvested for gene expression analysis. Expression of the pathogenesis related gene1 (*PR1*), a commonly used marker gene associated with *Pst* DC3000 infection and SA-mediated defense responses, was quantified. No significant differences were noted in the abundance of *PR1* between the mock infected, overexpressor lines and wild-type plants. The levels of *PR1* were strongly induced in infected wild-type plants 24 h.p.i., whereas both overexpressor lines infected with *Pst* DC3000 failed to induced *PR1* (Figure 15A). Although the expression of *PR1* was slightly higher (~ 1 fold) in both overexpression lines compared to mock treated control at 48 h.p.i., the levels of *PR1* were significantly lower compared to infected wild-type plants (Figure 15A).

To further establish the functiona of *PAP5*, the expression of the isocorismate synthase1 gene (*ICS1*), which is associated with defence related SA biosynthesis (Wildermuth et al., 2002), was tested. As shown in Figure 15B, accumulation of *ICS1* was ~ 4 fold higher in infected wild-type plants, whereas the expression of *ICS1* was strongly reduced in both overexpressor lines. However, the expression of *ICS1* in infected wild-type plants did not continue to 48 h.p.i. Moreover, the expression of *ICS1* correlated with the expression of *PR1*.

To determine whether the altered defense responses are associated with salicylic acid (SA), the accumulation of SA was quantified. SA accumulation was measured in wild-type, overexpressor lines (*35:PAP5*-A and *35S:PAP5*-B) and knockout line *pap5-1,* following *Pst* DC3000 infection. The level of SA increased in all *Pst* DC3000 infected plants. However, the SA levels in both overexpressor lines and *pap5-1* plants were only ~ 60% of the wild-type plants (Figure 16). These results suggest that the high abundance of *PAP5* impairs *ICS1*, *PR1* expression and SA accumulation subsequently similar to loss-of-function mutant *pap5*. Thus, PAP5 could be a part of a protein complex and constitutive production of PAP5 could result in the formation of inactive protein complexes. Taken together, it is evident that an optimal level of *PAP5* expression is required for the expression of *ICS1* and *PR1* and for the accumulation of SA after *Pst* DC3000 infection. Further, these results suggest that the enhanced growth of *Pst* DC3000 in *35S:PAP5*-A and *35S:PAP5*-B is dependent on reduced SA accumulation and a reduction in SA-mediated defense responses.

Figure 14. Transgenic plants overexpressing *PAP5* exhibit enhanced disease susceptibility.

A. Transcript levels of *PAP5* in transgenic plants. Total RNA was extracted from wild-type and transgenic plants, as described in materials and methods. PAP5 transcript levels were normalized to the expression of *GAPDH* in the same samples and expressed relative to the normalized transcript levels of wild-type plants. Significant differences ($P<0.05$) are indicated by different letters. B. Growth of *Pst* DC3000 in wild type and transgenic plants. Plants were inoculated with *Pst* DC3000 (10^{-8} c.f.u ml $^{-1}$) and bacterial growth in plant apoplasts was determined, as described in the materials and methods. The bars represent the mean and standard deviation from values of three separate trials with six to eight replicates each trial. An asterisk indicates a significant increase in *Pst* DC3000 growth compared to wild-type plants (Student's *t*-test; $P<0.05$).

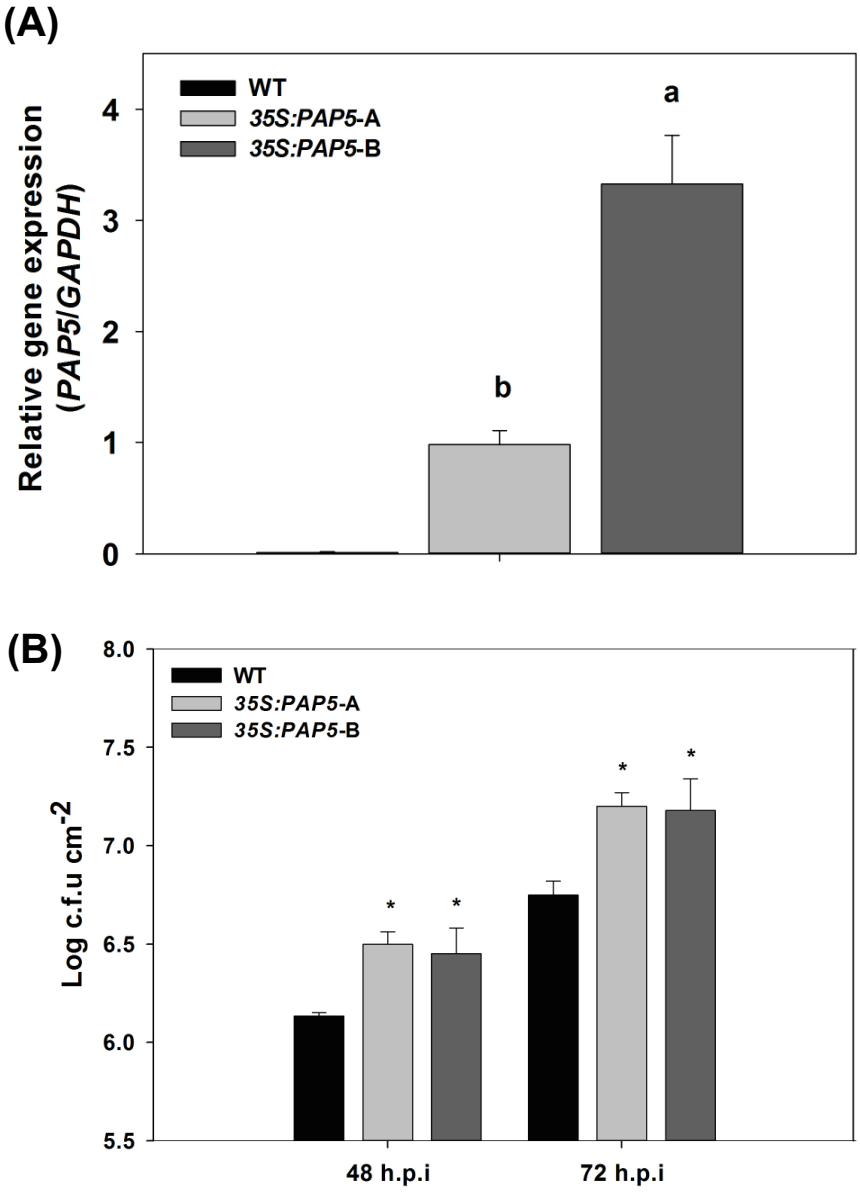

(A)
WT
35S:PAP5-A
35S:PAP5-B
Relative gene expression
(PAP5/GAPDH)
0
1
2
3
4
a
b
(B)
WT
35S:PAP5-A
35S:PAP5-B
Log c.f.u cm-2
5.5
6.0
6.5
7.0
7.5
8.0
*
*
*
*
48 h.p.i
72 h.p.i

Figure 15. Expression of *ICS1* and *PR1* is impaired in transgenic plants (*35S:PAP5*-A and *35S:PAP5*-B).

Plants were spray inoculated with *Pst* DC3000 (10^8 c.f.u ml^{-1}) and RNA extracted from leaf tissues sampled 24 and 48 h.p.i. Transcript levels were normalized to the expression of *GAPDH* in the same samples. The transcript levels were expressed relative to the normalized transcript levels of mock infected wild-type plants. The bars represent the mean and standard deviation from two independent experiments. Transcript levels of *ICS1* (A) and *PR1* (B) in mock infected and *Pst* DC3000 infection plants.

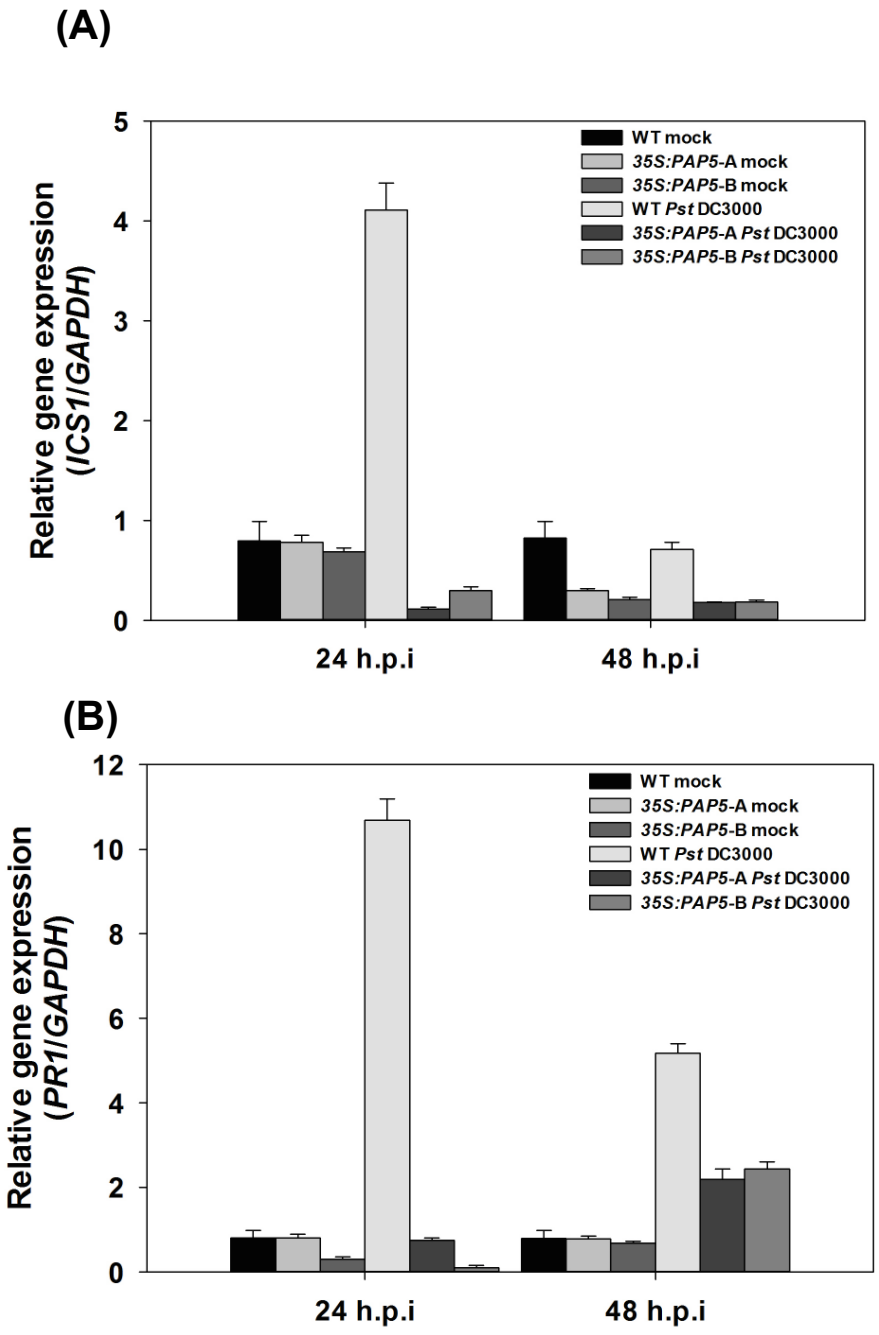
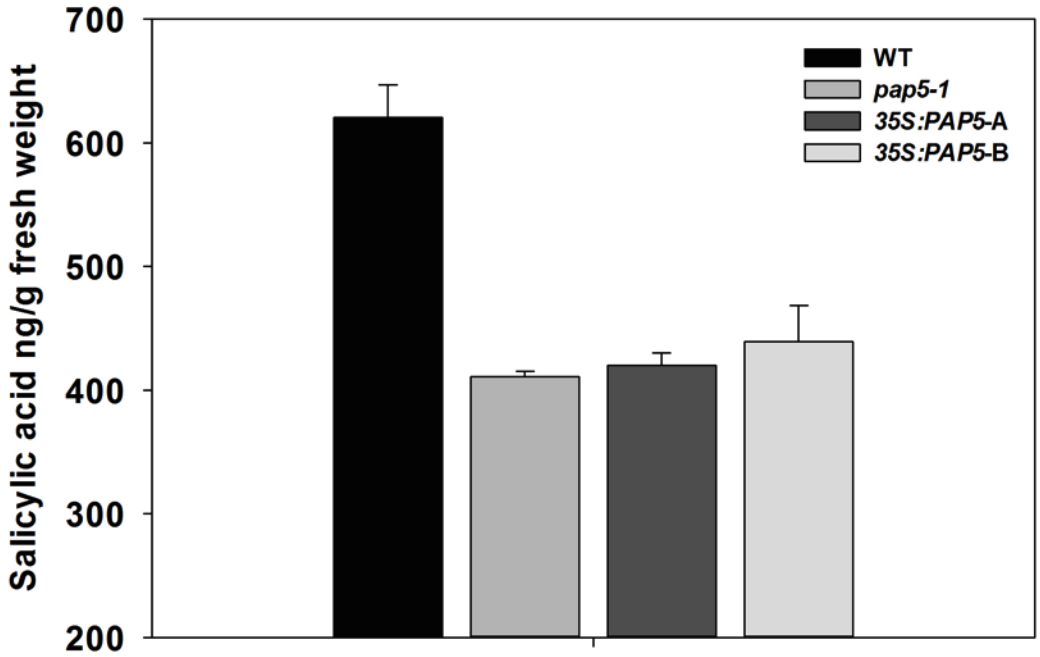

(A)
Relative gene expression
(ICS1/GAPDH)
5
4
3
2
1
0
WT mock
35S:PAP5-A mock
35S:PAP5-B mock
WT Pst DC3000
35S:PAP5-A Pst DC3000
35S:PAP5-B Pst DC3000
24 h.p.i
48 h.p.i
(B)
Relative gene expression
(PR1/GAPDH)
12
10
8
6
4
2
0
WT mock
35S:PAP5-A mock
35S:PAP5-B mock
WT Pst DC3000
35S:PAP5-A Pst DC3000
35S:PAP5-B Pst DC3000
24 h.p.i
48 h.p.i

Figure 16. Both loss and overexpression of *PAP5* affect salicylic acid (SA) accumulation in *Pst* DC3000 infected plants.

Quantification of SA in *pap5-1* and *35S:PAP5* plants following *Pst* DC3000 infection. Plants were spray inoculated with *Pst* DC3000 (10^8 c.f.u ml^{-1}) and leaf tissues were excised 48 h.p.i. The bars represent the mean and standard deviation from three replicates.



3.9 Sub-cellular localization of *PAP5*

To localize PAP5 in plants, the coding region was fused to the YFP reporter gene under the control of CaMV 35S promoter. An Agrobacterium strain carrying the construct *35S:YFP-PAP5*, was transiently expressed in tobacco leaves, as described in materials and methods. Confocal microscopy revealed *YFP-PAP5* as rapidly moving punctate structures within the cytoplasm and they were faintly observed in the nucleus (Figure 17A). To identify the cellular compartment, Agrobacterium strains carrying organelle specific markers (Nelson et al., 2007); were infiltered with *35S:YFP-PAP5* and leaf tissues were then harvested at different time points for confocal microscopy. As shown in Figure 16B, *YFP-PAP5* demonstrated a strong colocalization pattern with the peroxisomal specific marker (PTS1-CFP). Interestingly, most of the subcellular localization prediction programs failed to detect the peroxisomal targeting signal.

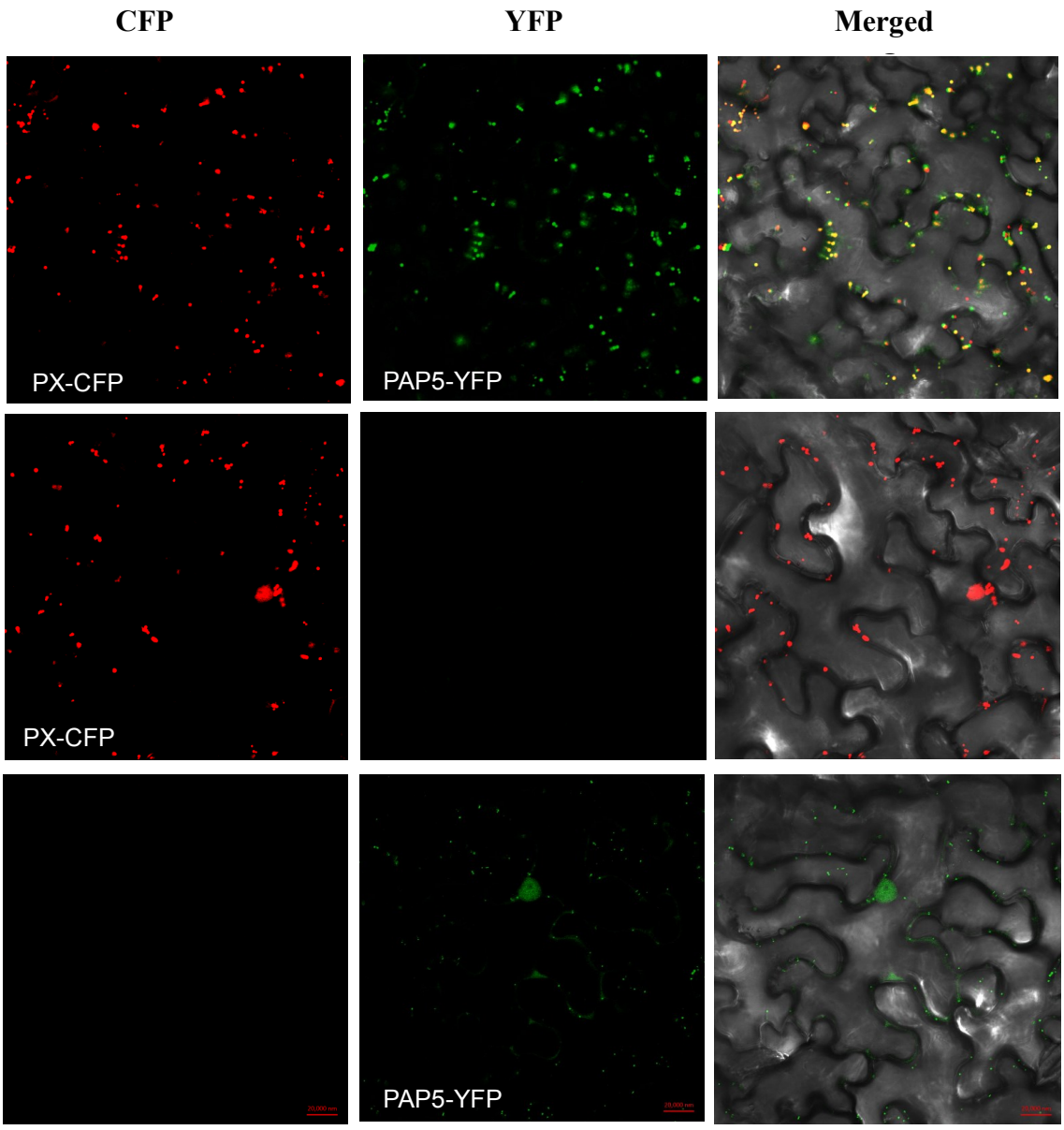
3.10 Yeast two hybrid screening

To identify cellular proteins that interact with PAP5, the full length open reading frame of *PAP5* was cloned into Gal4 DNA-Binding Domain vector (pGBKT7 DNA-BD) as bait. The pGBKT7 DNA-BD plasmid carrying *PAP5* was transformed to the yeast strain Y2HGold and tested for autoactivation by plating onto SD/-Trp/X-α-gal and SD/-Trp/X-α-gal/AbA. The prey library generated from *Pst* DC3000 infected wild-type plants was combined with the bait strain for yeast mating and plated on SD/-Leu/-Trp/X-α-gal/AbA (DDO/X/A). Initial selection on low stringency selection plates DDO/X/A (AbA; 100ng/ml) revealed over 120 potential binding partners. These colonies were subsequently patched on high stringency plates SD/-Ade/-His/-Leu/-Trp/X-α-gal/AbA (QDO/X/A). To increase the chance of rescuing the positive prey plasmid, the colonies

were streaked twice on QDO/X/A media and each time a single blue colony was picked for restreaking. Interestingly, only 40 colonies grew on the high stringency selection media QDO/X/A (AbA 100ng/ml). The positive clones were amplified using Activation Domain (AD) insert screening primer sequence to eliminate duplicate clones. The purified PCR products were sequenced to identify the potential interacting partners of PAP5.

The AD insert screening revealed that most cDNA clones contained the partial open reading frame of AtCG01180, encoding a 23S ribosomal RNA. Others sequences corresponded to Ribulose 5 phosphate carboxylase (At1g67090), Amine oxidase1 (At4g14940), abnormal suppressor 2 (At1g80070), ATPase (AtCG01180) and NADH dehydrogenase (AtMG01120). The list of the sequenced clones is shown in Table S1.

Figure 17. Subcellular localization of PAP5 in *N. benthamiana* leaves**.** Agrobacterium strain, carrying the fusion protein (35S:YFP-PAP5), was transiently expressed in tobacco leaves. For co-expression, *Agrobacterium* strains carrying the peroxisomal specific marker (PTS1-CFP) and 35S:YFP-PAP5 fusion protein, were mixed in equal proportion prior to infiltration. The left plane shows CFP fluorescence images, the middle plane shows YFP fluorescence and the right plane shows transmitted light images with merged CFP and YFP fluorescence.


CFP
YFP
Merged
PX-CFP
PAP5-YFP
PX-CFP
PAP5-YFP

Chapter 4 Discussion

Recent advancements in genome sequencing technologies and computational annotation tools have enhanced the identification of several genes and have facilitated the prediction of their function. For example, the Arabidopsis genome (120 Mb) encodes over 25,000 genes and approximately 55% of these genes have been annotated and their putative functions assigned (Arabidopsis Genome Initiative, 2000). Although these predicted functions are extremely useful, further validation and experimental evidences are required to confirm their biological function (Alonso and Ecker, 2006).

4.1 Genetic screening revealed diverse classes of candidate genes

A reverse genetics based approach is useful to identify genes that mediate altered root colonization, disease susceptibility/resistance (Table 2). Although the mutant analysis revealed several candidate genes, further characterization of these is required to validate their function. For example, salk_113904 (encoding *Flavone 3-hydroxylase* (F3'H), At3g51240) enhanced the growth of *P. putida* and also exhibited enhanced disease susceptibility to *Pst* DC3000. The role of flavones in legume-rhizobia symbiosis is well known, as reviewed by Jones et al. (2007). The present experiments further suggest that the loss of *Flavone 3-hydroxylase* (F3'H) might affect the phenylpropanoid pathway, altering defense responses. The phenylpropanoid pathway mediates the synthesis of various metabolites that regulate both symbiotic and pathogenic interactions (reviewed by Dixon et al., 2002; Vogt, 2010). A recent study has also shown that the F3H shares high similarity to salicylic acid 3-hydroxylase (S3H) which mediates the conversion of SA to 2,3-dihydroxybenzoic acid (2,3-DHBA) (Zhang et al., 2013).

T-DNA insertions in the genes encoding *Temperature induced lipocalin* (TIL; At5g58070), a *multidrug* transporter (MATE; At4g21910), and an RNA *binding protein* (At3g12640) also enhanced the growth of *P. putida* (Table 2). However, mutations in these genes did not affect growth of *Pst* DC3000, suggesting that these genes may not play a role in the establishment or growth of *Pst* DC3000. Previously, temperature induced lipocalin (TIL) has been shown to be essential for protection against oxidative (Charron et al., 2008), heat and cold stress (thermotolerance) (Chi et al., 2009). Similarly, RNA binding protein (At4g03110) was reported to be a positive regulator of SA mediated defense responses. The present mutant analysis shows that the loss of RNA binding protein (At3g12640) enhances the growth of *P. putida*. However, these plants did not exhibit enhanced disease susceptibility to *Pst* DC3000 (Table 2). Previous research indicated that multidrug transporter (MATE) family proteins can confer resistance to various antibiotics and antimicrobial agents (reviewed by Eckardt, 2001). However, it is unclear as to how the loss of the multidrug transporter could facilitate growth of *P. putida*. Visual examination also revealed that salk lines, corresponding to *At2g44450* (coding for a beta glucosidase 15) and *At4g28020* (unknown protein), were susceptible to *P. putida.* Beta-glucosidase has been previously shown to mediate defense against herbivory and the control of phytohoromones levels (Xu et al., 2004). Further, beta-glucisidase (*At2g44450*) was identified in duplicated chromosome 2 and 3 segments (Xu et al., 2004). Although, *P. putida* are beneficial PGPR (Meziane et al., 2005), plants carrying T-DNA insertion *At2g44450* and *At4g28020* were susceptible to *P. putida* at concentration OD_{600} 0.01.

Interestingly, At1g15990 (*Cyclic nucleotide gated channel*), At2g33500 (Zn finger family protein), At2g22680 (Zn finger, ubiquitin protein ligase) and At2g20240 (unknown protein) enhanced the growth of both *Pst* DC3000 and *P. putida*. These results suggest that the formerly mentioned genes are required to regulate the growth of both beneficial and pathogenic bacteria. The cyclic nucleotide-gated ion channel (CNGC) plays a crucial role in activation of several defense responses (Moeder et al., 2011). Similarly, the expression of the rice zinc finger family protein (*OsRHC*) has been shown to improve disease resistance to *Pst* DC3000 (Cheung et al., 2007).

Six T-DNA insertion lines that exhibited enhanced disease susceptibility (EDS) to *Pst* DC3000 were also susceptible to *P. putida*. At3g23390 (zinc-binding ribosomal protein) and At1g76880 (duplicated homeodomain like protein) were previously reported to be down regulated in *Agrobacterium tumefaciens* infected cell cultures (Ditt et al., 2006). Also, loss of At2g01290 (ribose-5-phosphate isomerase 2) has been reported to induce cell death and chloroplast dysfunction (Xiong et al., 2009). Thus, a marked reduction in chloroplasts partially explains the enhanced disease susceptibility phenotype to *Pst* DC3000 and susceptibility to *P. putida*. Similarly, loss of the chloroplast localized protein At3g25860 (pyruvate decoboxylase) led to EDS to *Pst* DC3000 and these plants were also susceptible to *P. putida*. Interestingly, At3g25860 has been reported to be expressed in response to salicylic acid (SA) (Rajjou et al., 2006). Moreover, chloroplastic protein, such as NRIP1, has been shown to play a key role in the recognition of pathogens and mounting defense responses (Caplan et al., 2008). These results further suggest that chloroplast associated genes may regulate various defense responses.

Loss of aspartate aminotransferase activity (At2g22250) differentially affected the growth of *Pst* DC3000 and *P. putida*. Although, enhanced resistance to pathogenic *Pst* DC3000 and enhanced growth of beneficial *P. putida* is a desired phenotype, the mechanism by which this differential colonization occurs is unknown. It is also evident that the biosynthesis of various amino acids is stimulated or repressed in response to stress (Less and Galili, 2008). Among the 21 T-DNA lines that exhibited enhanced disease susceptibility, genes (At4g12570, At1g27180, At5g22690, At1g75400, At3g19680, At4g33625, At1g69260, At1g69260, At5g21960, At1g29530) have been reported to be associated with plant defense responses. For example, both At1g27180 and At5g22690 have been shown to possess trans membrane receptor activity (Qutob et al., 2006; Tan et al., 2007). Similarly, At4g33625 has been identified to be responsive to SA (Krinke et al., 2007). However, several genes, including At5g18720, At4g32820, At1g49470, At5g38460, At1g17500, At2g32010, At3g15550, At4g36840, At1g73370 and At1g03600, have not been previously shown to play a role in disease resistance. Although plants carrying T DNA insertions in the formerly mentioned genes exhibited enhanced disease susceptibility, the corresponding salk lines should be further tested to validate their function.

Mutant analysis also revealed several salk lines which exhibited enhanced resistance to *Pst* DC3000. Previously, At2g38870(Osakabe et al., 2005), At5g57420 (Mutka et al., 2013), At2g45790 (induces cell death) (Hoeberichts et al., 2008), At2g02800 (Veronese et al., 2006), At5g43500 and At1g03280 (Ascencio-Ibanez et al., 2008) have been shown to be related to disease resistance. Interestingly, loss of At5g65830 (receptor like protein 57) led to enhanced susceptibility. Further, At1g25460,

At1g05790, At1g50090, At4g12460, At3g22090 and At3g22090 were identified to exhibit enhanced resistance to *Pst* DC3000, were not previously listed.

4.2 PAP5 is distinctly induced under prolonged Pi and during early stages of *Pst* DC3000 infection

Past studies have shown that *PAP5,* unlike *PAP12* and *PAP26,* is not abundantly expressed under normal phosphate starvation conditions (Zhu et al., 2005). The present results revealed that *PAP5* is induced only under prolonged Pi starvation (Figure 2C). A mutation in *PAP26* has been shown to impair growth and increase anthocyanin accumulation, in response to Pi starvation (Tran et al., 2010b). Despite the loss of *PAP5* expression, mutant plants did not show detectable phenotypic differences from those of wild-type plants. Both wild-type and *pap5-1* plants exhibited an increased root/shoot ratio under Pi starvation (data not shown). This finding also indicates that *PAP5* does not play a major role in Pi acquisition and is more likely to regulate other functions. The Arabidopsis genome contains 29 *PAP* encoding genes (Li et al., 2002) and this may lead to functional redundancy. This study suggests that the loss of *PAP5* results in impairment of the defense responsive genes which are expressed in response to *Pst* DC3000 infection. Further, it appears that other *PAP* genes do not compensate for the loss of *PAP5* function in response to pathogen attack.

Genetic analyses of Arabidopsis mutants have revealed the involvement of many key regulatory genes in plant defense responses. Enhanced disease susceptibility mutants, including *eds5*, *pad4*, *npr1* and *sid2,* have previously been reported to exhibit enhanced susceptibility and compromised defense responses to both virulent and avirulent isolates of *Pst* DC3000 (Glazebrook and Ausubel, 1994; Rogers and Ausubel, 1997; Dewdney et

al., 2000). Although the expression of *PR1* was slightly induced in *pap5* plants following *Pst* DC3000 infection, the relative transcript level of *PR1* was several fold lower, compared to the wild-type (Figure 8). The *PR1* transcripts at 48 h.p.i were slightly lower, compared to wild-type plants (Figure 8). Similar variability has been observed in MPK6 silenced plants that were susceptible to *Pst* DC3000 (Menke et al., 2004). It was observed, in this study, that *PAP5* was strongly induced in the early stages of infection (6 h.p.i). This induction was transient as no difference was noted at 24 and 48 h.p.i. One possible explanation of this observation is that the level of PAP5 induced during the early stages (6 h.p.i) of infection could be sufficient to dephosphorylate signaling proteins which are required for the activation of defense responses downstream of *PAP5*. Thus, it is also possible that *PAP5* might be involved in an early response to pathogens, in a manner similar to those of glutotione s-transferse (*GST6*) and glucosyltransferase (Uquillas et al., 2004). Moreover, members of the PAP family have been known to exhibit peroxidase activity, in addition to Pi acquisition and recycling (Del Pozo et al., 1999; Bozzo et al., 2002). Although the role of *PAP5,* with regard to peroxidase activity, has not been established, it can be hypothesized that *PAP5* might mediate the generation of ROS during *Pst* DC3000 infection. ROS was initially thought to mediate plant defense response, especially during an incompatible interaction (Torres and Dangl, 2005). Virulent pathogens, capable of evading pathogen recognition, are also known to induce ROS production at later stages of infection, but at lower levels (Jones and Dangl, 2006).

The importance of PAP5 in limiting the growth of the necrotrophic fungus, *B. cinerea* at the site of infection, was identified. The expression of *PDF1.2* was strongly suppressed in *pap5-1* plants at 24 h.p.i., resulting in an increase in lesion size. There were

no differences in *PDF1.2* transcripts between *pap5* and wild-type plants at 48 h.p.i. Similarly, *eds4-1* plants have been reported to exhibit enhanced susceptibility to *B. cinerea,* despite comparable expression of *PR1* and *PDF1.2* transcripts (Ferrari et al., 2007). These results also suggest that defense responsive genes, other than *PR1* and *PDF1.2,* are required to mount wild-type levels of resistance against *B. cinerea*. SA, synthesized in response to *B. cinerea* infection, was reported to be derived via phenylalanine ammonia lyase (*PAL*) and not via isochorismate synthase (*ICS*) (Ferrari et al., 2003). Since *pap5-1* plants induced levels of *PR1* comparable to wild-type plants following *B. cinerea* infection, it is possible that the effect of *PAP5* is restricted to SA derived via *ICS,* and not via *PAL*.

Application of BTH and MJ, in wild-type and *pap5* plants, induced expression of *PR1* and *PDF1.2,* respectively (Figure 13A and B). These results also suggest that *PAP5* is not required for the expression of SA dependent *PR1*. *PR1* expression in *pap5-1* plants appeared to be slightly higher than in wild-type plants after BTH treatment (Figure 13A). This slight increase in *PR1* expression and its significance is unclear. Similarly, application of SA on *pad4* plants showed a slight increase in *PR1* expression (Zhou et al., 1998). Application of MJ induced the expression of *PDF1.2,* indicating the regulatory function of *PAP5* to be upstream of SA and JA.

4.3 Optimal level of PAP5 is critical for mounting complete basal resistance

This study further revealed that an optimal level of PAP5 is critical for mounting appropriate defense responses. Previous studies have revealed several molecular cues that regulate plant defense responses. Often, genes identified as positive regulators of defense responses are overexpressed in order to generate disease tolerant crops (Zhang et al.,

2014). Interestingly, in some instances, both under- and over-expression of a particular gene could result in the same phenotype. For example, *OXI1,* encoding a serine/threonine kinase, has been shown to be essential for complete activation of mitogen-activated protein kinase (MAPKs). *oxi-1* null mutants demonstrated enhanced susceptibility to virulent *Hyaloperonospora arabidopsidis* (formerly *Paranospora parasitica*) (Rentel et al., 2004). Interestingly, transgenic plants overexpressing *OXI1* (*35S::OXI1*) displayed enhanced susceptibility to both virulent *Hyaloperonospora arabidopsidis* and *Pst* DC3000 (Petersen et al., 2009). Since both under- and over-expression of *PAP5* led to enhanced susceptibility to *Pst* DC3000, it is hypothesized that PAP5 could exist in a complex with other proteins. Thus, constitutive expression of PAP5 (*35S:PAP5*) alone may not be sufficient for complete resistance. It is also possible that the prolonged expression of *PAP5* could negatively affect basal resistance against *Pst* DC3000. Previously, *PAP5* was not known to be expressed under normal growth conditions; however, in this research, PAP5 was distinctly induced only during prolonged Pi starvation and the earlier stages of *Pst* DC3000 (6 hours post inoculation) (Ravichandran et al., 2013). Hence, constitutive overexpression of *PAP5* is not optimal and may impair its function following *Pst* DC3000 infection. Previous studies have also shown that both over-expression and loss of *FIP1* resulted in similar phenotypes (Chen et al., 2007). Similarly, loss and over-expression of *EBS* showed early flowering, a dwarf phenotype and reduced fertility (Pineiro et al., 2003).

Pathogen recognition triggers the generation of ROS, which is required for activation of the defense response (Torres et al., 2002b). It is also evident that the generation of ROS occurs within hours of pathogen infection (Alvarez et al., 1998). Interestingly, because,

PAP5 is induced only during the earlier stages (6 h.p.i) of *Pst* DC3000 infection, localization of PAP5 in peroxisome (Figure 17A) suggests that PAP5 may act as a component of ROS generation. Since it was hypothesized that PAP5 might exist in association with other proteins, a comprehensive in silico prediction was performed to identify proteins that may potentially interact with PAP5. Most in silico prediction (as of May 13 2014) searches on the Bio-Analytic Resource for Plant Biology (BAR), the Biological General Repository for Interaction Datasets (BioGRID) and GeneMANIA failed to detect any physical interaction. Moreover, most subcellular localization prediction tools including SUBA, TargetP and WoLF PSORT, failed to identify the peroxisomal targeting sequences of PAP5. Previous studies have also shown that most in silico prediction programs failed to identify signature peroxisomal targeting sequences (Nelson et al., 2007). In contrast, some in silico predictions showed that *PAP5* is targeted to the extracellular spaces (http://suba.plantenergy.uwa.edu.au/; http://wolfpsort.org/). Although the yeast two hybrid assays revealed few genes including Ribulose 5 phosphate carboxylase, ATPase and NADH dehydrogenase, their role in pathogen response is unclear. Further, other protein including 18S and 23S ribosomal RNA represents the housekeeping proteins that are primarily involved in translation.

In mammals, it has been demonstrated that a high level of expression of PAPs in macrophages and increased ROS production led to microbial killing (Kaija et al., 2002; Räisänen et al., 2005). From the present result, it is hypothesized that PAP5 can directly or indirectly play a role in microbial killing during pathogenesis. Since ROS are produced under normal metabolic processes, the role of ROS in signaling is largely dependent on the rate of synthesis and is controlled by antioxidative enzymes, such as catalase and

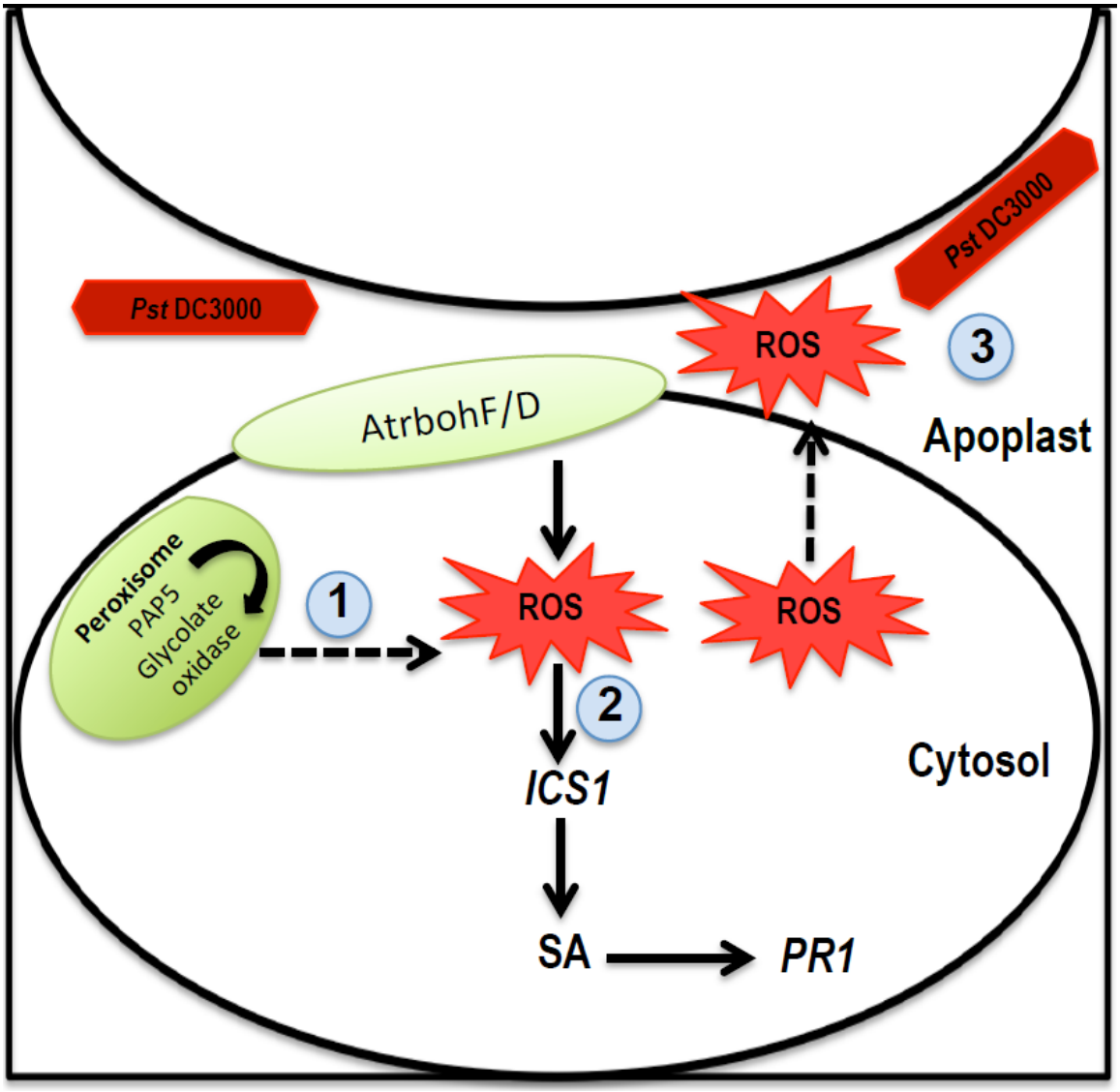
peoxidases in peroxisomes (Nyathi and Baker, 2006). Previously, catalase deficient plants have been shown to display marked perturbation under conditions of intracellular redox and cellular homeostasis (Vandenabeele et al., 2004; Queval et al., 2007). Such perturbation is associated with the accumulation of salicylic acid (SA) and the induction of pathogenesis related (PR) genes (Chamnongpol et al., 1998). Similarly, catalase deficient Arabidopsis (*cat2*) plants showed increased peroxisomal H_2O_2 (Chaouch et al., 2010). Moreover, *cat2* plants triggered pathogen defense responses and resistance in a daylength dependent manner. Peroxisomal β-oxidation is also attributed to the degradation of various straight and branched chain fatty acids (Baker et al., 2006). Derivatives of β-oxidation, such as cyclic oxylipins, also play a significant role in the synthesis of the plant hormones, jasmonic acid (JA) and salicylic acid (SA), which are important signaling molecules (Theodoulou et al., 2005). Following *Pst* DC3000 infection, the SA levels in both overexpressor lines and *pap5-1* plants were ~ 60% of the wild-type plants. These results suggest that SA accumulation is not completely abolished in either transgenic (*35S:PAP5*) or *pap5-1* plants.

It is well recognized that the majority of eukaryotic proteins undergo reversible phosphorylation via protein kinase (PK) and phosphatase (PP) to control major cellular processes. A large family of protein kinases has been characterized and their function in various cellular processes has been well established (Pais et al., 2009). However, the physiological role of the protein kinases' counter-partner protein phosphates is still poorly understood. Activation of sucrose phosphate synthase (SPS) and nitrate reduction (NR) has been associated with a decrease in the phosphorylation status of SPE and NR (Huber and Huber, 1996). Interestingly, phosphorylation of Ser158 is sufficient for

inactivation of spinach SPS *in vitro* (Huber and Huber, 1992). Phosphatase inhibitors, such as okadaic acid, prevent *in vivo* activation of SPS in spinach leaves (Huber and Huber, 1996). Similarly, PAP5 may be required for the complete activation of vital enzymes, such as glycolate oxidase in peroxisomes, that modulate H_2O_2 generation. Since ROS is generated under normal metabolic processes, a highly regulated mechanism must exist to control ROS generation on pathogen recognition.

Figure 18. Model for role of PAP5 during *Pst* DC3000 infection**.**

When plants are infected with virulent *Pst* DC3000; 1. Peroxisomal localized PAP5 may be required for activation of glycolate oxidase, which modulate H_2O_2 generation. 2. ROS induces defense responsive genes including *PR1* and *ICS1*. 3. ROS secreted to the apoplast may directly affect *Pst* DC3000. Recognition of *Pst* DC3000, induce expression of *PAP5* only during the early stages of infection (6h) and triggers ROS synthesis which subsequently activates other defense related signals down stream for complete resistance.


Pst DC3000
Pst DC3000
ROS
3
AtrbohF/D
Apoplast
Peroxisome
PAP5
Glycolate
oxidase
1
ROS
ROS
2
Cytosol
ICS1
SA
PR1

Several PAPs (SAP1, SAP2, AtPAP17 and AtPAP26) induced under Pi starvation are secreted to the extracellular space to hydrolyze Pi containing substrates and also exhibit peroxidase activity (Del Pozo et al., 1999; Bozzo et al., 2002; Hurley et al., 2010). However, the role of PAPs and their peroxidase activity in the extracellular space is not clear. Previously, PAPs exhibiting peroxidase activity has been speculated to function in ROS production similar to the oxidative burst that occurs in response to pathogen recognition (Del Pozo et al., 1999; Bozzo et al., 2002). It is possible that the PAPs secreted to the extracellular space have dual functions, i.e. the hydrolytic activity under Pi starvation and microbial killing during pathogenesis. The mechanism of resistance is associated with H_2O_2 production generated in response to *Pst* DC3000 infection. It is also propose that H_2O_2 produced in response to *Pst* DC3000 either directly or indirectly regulates salicylic acid (SA) signaling pathways (Figure 17). Taken together, the evidence suggests that peroxisomal localized PAP5 plays a vital role in the basal defense response. Moreover, an optimal level of PAP5 is critical for maintaining complete basal resistance during pathogenesis. It is evident that the isoform of PAP have evolved to attribute various biological functions in plants.

Chapter 5 Summary and Conclusion

In this study, PAP5 was found to be essential for maintaining basal defense responses against virulent *Pst* DC3000, suggesting a role for *PAP5* in pathogen triggered immunity (PTI). Further, loss of PAP5 impaired the expression of pathogenesis related genes including, *PR1*, *ICS1* and *PDF1.2*. *PAP5* was also found to act upstream of SA to affect the expression of *PR1,* and levels of *PAP5* did not affect both BTH and JA perception. Interestingly, it was evident that an optimal level of PAP5 is crucial for mounting complete basal resistance. Overexpression of *PAP5* impaired expression of *ICS1*, *PR1* and accumulation of salicylic acid (SA) similar to *pap5* mutant plants. Moreover, PAP5 was also identified to be peroxisomal localized. SA accumulation in transgenic plants (*35S:PAP5*) following *Pst* DC3000 infection was only ~ 60% of the wild-type plants. Hence, it is hypothesized that peroxisomal localized PAP5 may aid the generation of reactive oxygen species for activation of defense responses.

Previous studies have shown that *PAP5* is not expressed under normal and/or under Pi starvation. However, the mutant screening reveled that the T-DNA insertion in *PAP5* led to enhanced susceptibility. This indicates that certain genes such as PAP5 are induced during specific biotic (*Pst* DC3000 infection) or abiotic stresses (Pi starvation). Moreover, with *Pst* DC3000 *PAP5* was induced only at 6 h.p.i. In contrast, *PAP5* was induced only under prolonged Pi starvation. Hence it is essential to conduct gene expression analysis at different time points to validate the expression pattern. This study also demonstrated that the T-DNA insertion on the intron (non-coding) region could still disrupt the gene function. It is possible that the intron may carry regulatory elements.

Although introns have been considered as a non-coding part of a gene, emerging evidences suggest that the intron can affect gene expression.

This study also revealed that both under and overexpression of *PAP5* lead to enhanced susceptibility. Although, *in silico* predictions revealed several genes and their potential interacting partners, the existing bioinformatics tools failed to detect any positive interacting partners of PAP5. Previously, PAP5 have been shown to exist as homodimers, heterodimers or oligomers. Hence, PAP5 can exist in complex with other proteins. Separation, of protein on-denaturing gel and in-gel assay would likely reveal these isoforms and associated proteins. Moreover, PAP5 did not have signature signal peptide. It is evident that most PAPs are glycosylated in the endoplasmic reticulum. However, it is unclear how glycosylation can occur in a protein that lacks signature signal peptides. It is possible that PAP5 could be targeted to the endoplasmic reticulum and peroxisomes in a piggy-back fashion.

In this study yeast-two-hybrid analysis was performed to identify the potential interacting partners. Sequencing analysis revealed that most interacting partners were associated with house keeping genes, hence it is essential to optimize the prey library. Although the prey library was generated from cDNA synthesized from *Pst* DC3000 infected plants to maximize the identification potential interacting partners, the relative abundance of housekeeping genes such as rRNA masks other interacting partners. Commercial enterprises such as Clontech provide normalized prey libraries for human cells where highly abundant transcripts are selectively removed to enhance the representation of low-abundance and rare cDNAs. However, such prey libraries are not currently available for Arabidopsis.

Most PAPs have been shown to be secreted in to the extracellular spaces. However their role in the extracellular space is largely unknown. It is speculated that the PAPs secreted to the extracellular space have dual functions, i.e. the hydrolytic activity under Pi starvation and microbial killing during pathogenesis. The mechanism of resistance is associated with H_2O_2 production generated in response to *Pst* DC3000 infection. It is also evident that H_2O_2 produced in response to *Pst* DC3000 either directly or indirectly regulates salicylic acid (SA) signaling pathways. Taken together, the evidence suggests that peroxisomal localized PAP5 plays a vital role in basal defense response. Moreover, *PAP5* was induced only under prolonged Pi starvation suggesting that the PAP5 is primarily required for maintaining basal resistance during pathogenesis and is not primarily required under Pi starvation. It is evident that the isoform of PAP have evolved to attribute various biological functions in plants. This study also suggests that nutrients, such as Pi can influence disease susceptibility or resistance.

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Appendix

Figure S1. Enhanced susceptibility of *pap5-2* to *Pst* DC3000.

A. Phenotype of *pap5-2* plants exhibiting extensive chlorosis. Plants were spray inoculated with 10^8 c.f.u ml $^{-1}$ and photographed after 5 days of infection. B. Growth of virulent *Pst* DC3000 in wild type (Col-0) and *pap5-1* mutant leaves. Plants were spray inoculated with *Pst* DC3000 (10^{-8} c.f.u ml $^{-1}$) and bacterial growth in plant apoplast was determined. The bars represent the mean and standard deviation from values of six to eight replicate samples and the experiment was repeated two times with similar results. An asterisk indicates significant increase in *Pst* DC3000 growth compared to wild-type (Student's *t*-test; $P<0.05$).

(A)

WT *pap5-2*

(B)

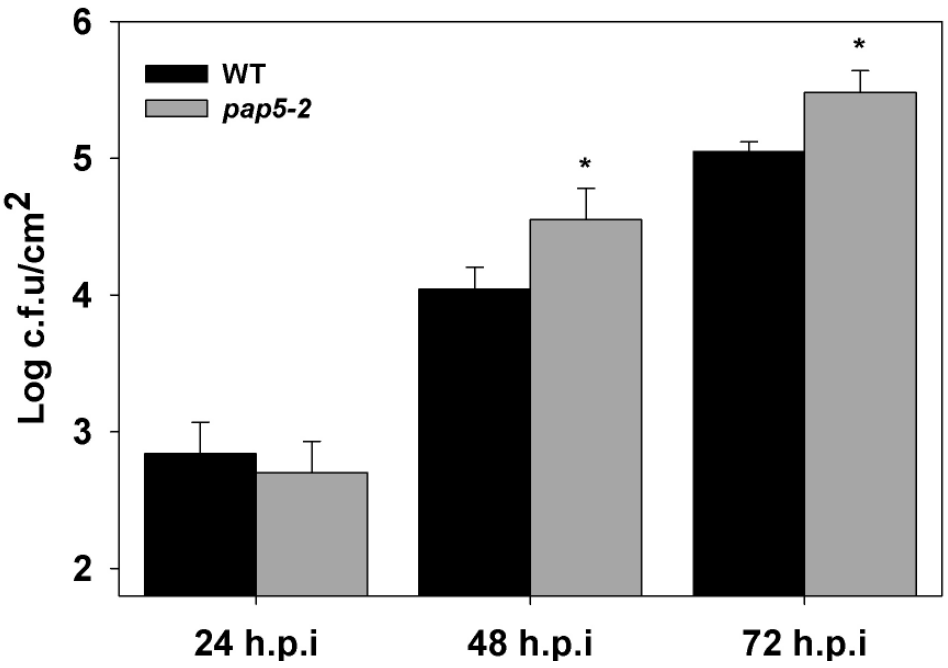


Figure S2. Expression profile of *PAP5* and *PR1*.

Expression of PAP5 (array element 261341_s_at) in comparison to *PR1* (array element 266385_at) from Genevestigator Expression Data.

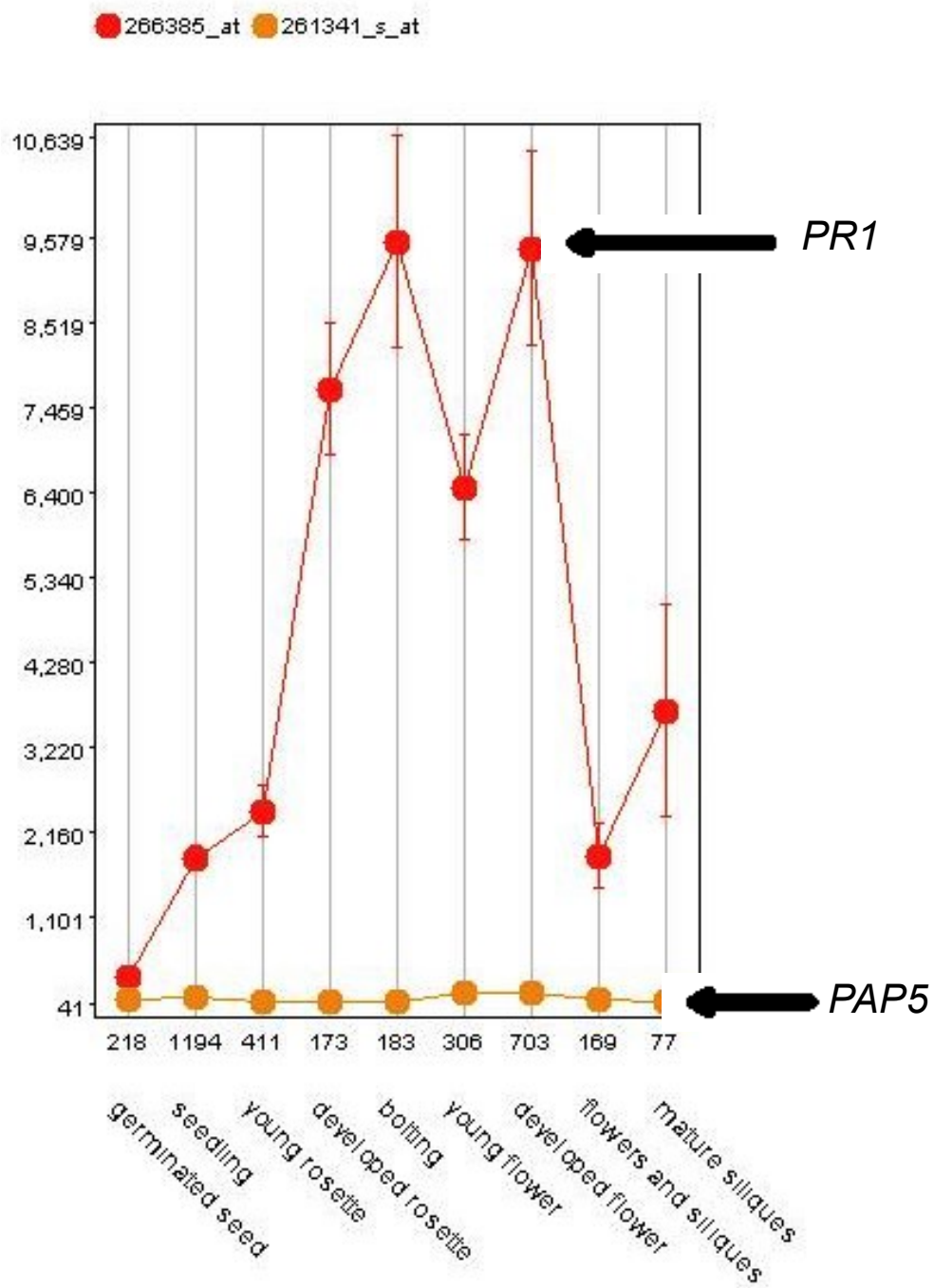
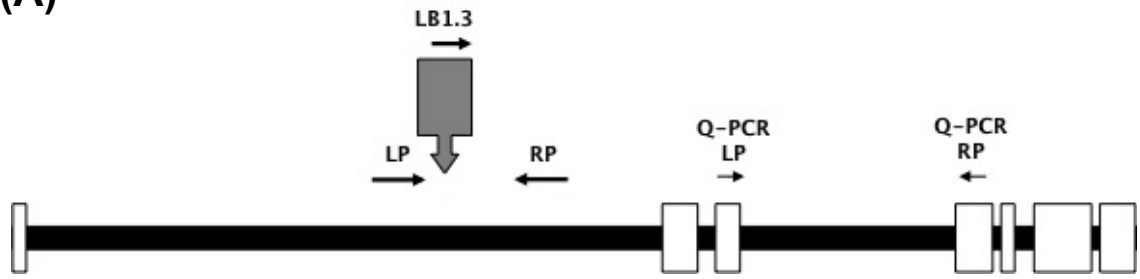


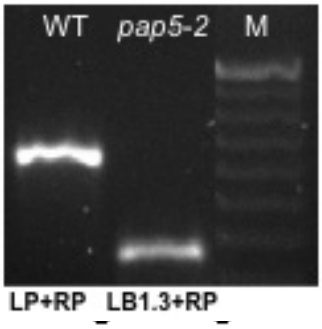
Figure S3. Validation of T-DNA insertion in *pap5-2* mutant plants.

A. Schematic representation of *AtPAP5* (At1G52940); white boxes and solid lines represent exons and introns respectively. T-DNA insertion is represented with a grey arrow and the solid arrows represent the primers used for genotyping and quantitative RT-qPCR. B. Location of the T-DNA insertion and homozygosity of *pap5-2* was confirmed by PCR using the gDNA from wild-type and *pap5-2* plants (M, 100bp marker). A 30 cycle PCR reactions was performed with the primer pairs indicated. C. Relative expression of *PAP5* transcripts in response to Pi starvation; Total RNA was extracted from wild-type and *pap5* plants as described in materials and methods. Transcript levels of *PAP5* was normalized to the expression of *GAPDH* in the same samples and expressed relative to the normalized transcript levels of Pi supplemented wild-type plants. The bars represent the mean and standard deviation from two independent experiments. Asterisks represents data sets significantly different from the wild-type data sets ($P<0.05$ using one-tailed Student's *t*-test).

(A)


LB1.3
LP
RP
Q-PCR
LP
Q-PCR
RP

(B)


WT
pap5-2
M
LP+RP
LB1.3+RP

(C)

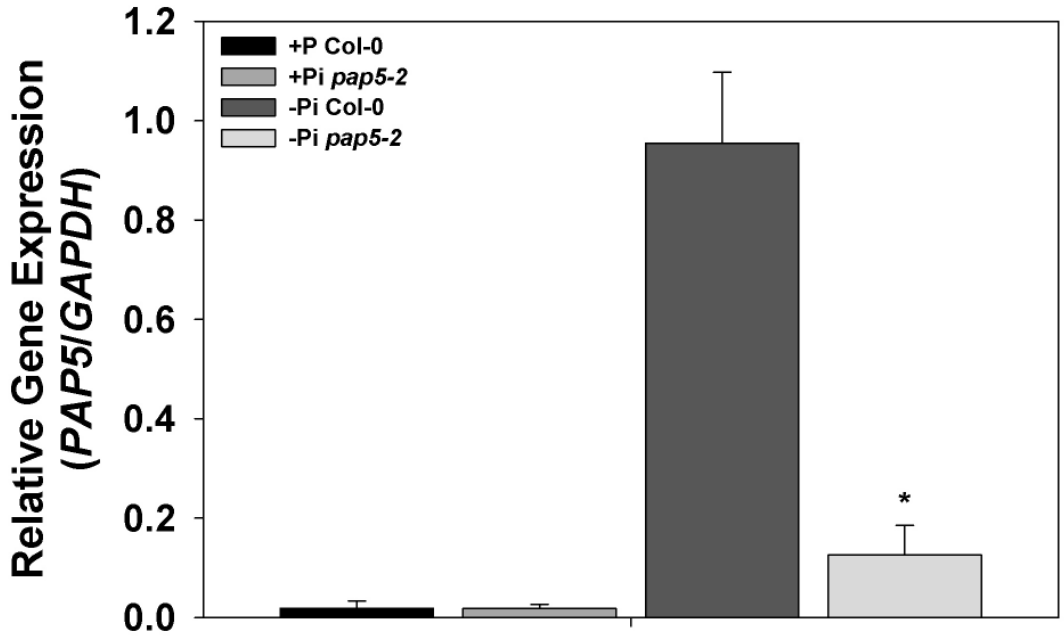

Relative Gene Expression
(PAP5/GAPDH)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+P Col-0
+Pi pap5-2
-Pi Col-0
-Pi pap5-2
*

Figure S4. Expression profile of *PAP5* (At1g52940) from the Arabidopsis eFP Browser.

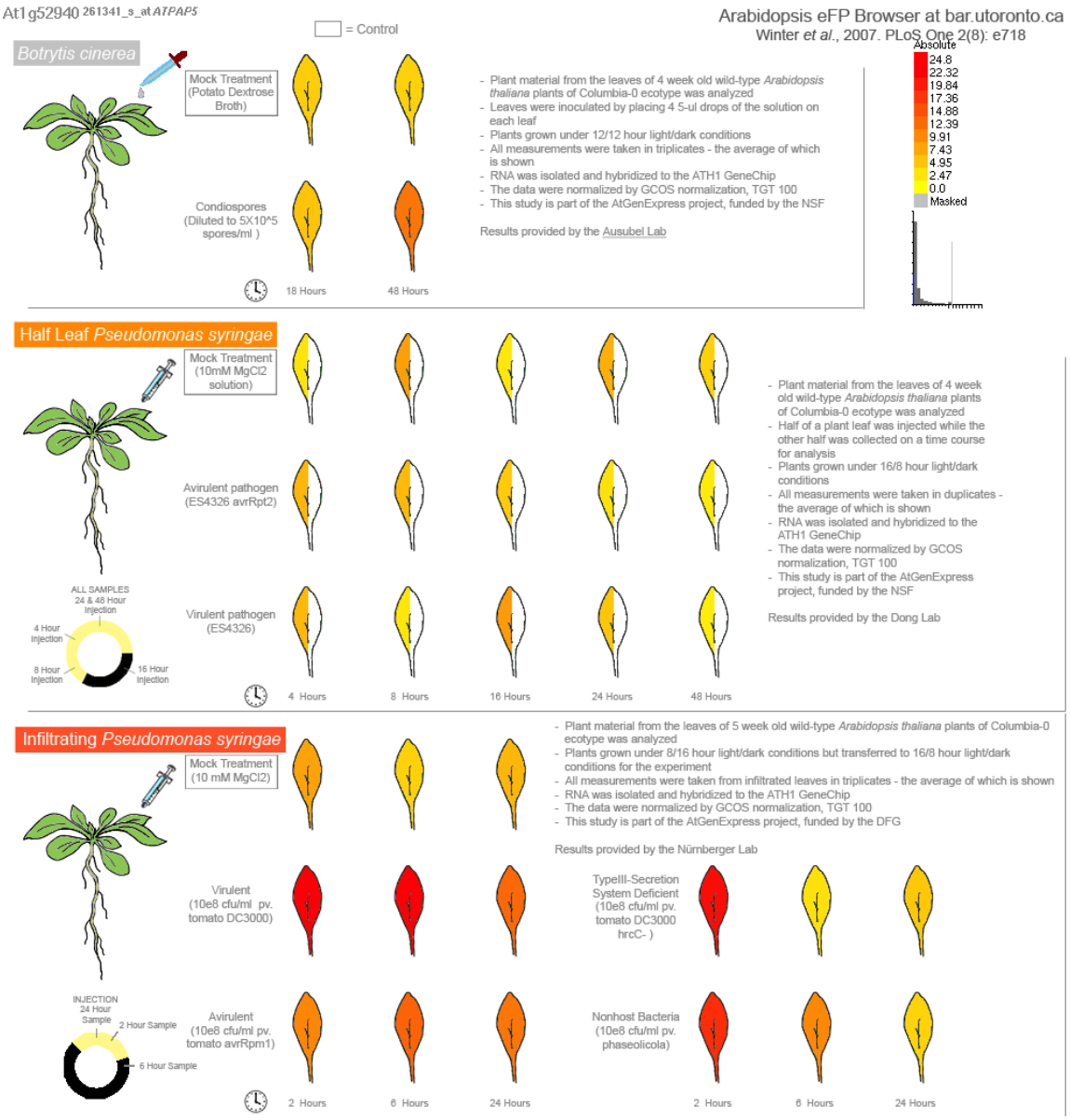


Table S1: List of sequenced clones with yeast two hybrid screening

C number	Annotation	GeneID
10F	23S Ribosomal RNA	AtCG01180
25F	23S Ribosomal RNA	AtCG01180
65F	23S Ribosomal RNA	AtCG01180
41F	23S Ribosomal RNA	AtCG01180
54F	Ribulose 5 phosphate carboxylase	
78F	23S Ribosomal RNA	AtCG01180
23F	23S Ribosomal RNA	AtCG01180
33F	Amine oxidase 1	
43F	Abnormal supressor 2	At1g80070
2F	23S Ribosomal RNA	
1F	23S Ribosomal RNA	AtCG01180
6aF	23S Ribosomal RNA	AtCG01180
8F	ATPase	AtCG00130
40F	23S Ribosomal RNA	AtCG01180
3F	23S Ribosomal RNA	AtCG01180
18F	23S Ribosomal RNA	AtCG01180
22F	23S Ribosomal RNA	AtCG01180
32F	23S Ribosomal RNA	AtCG01180
37F	23S Ribosomal RNA	AtCG01180
77F	23S Ribosomal RNA	AtCG01180
9F	23S Ribosomal RNA	AtCG01180
19F	23S Ribosomal RNA	AtCG01180
5F	23S Ribosomal RNA	AtCG01180
75F	23S Ribosomal RNA	AtCG01180

6F	23S Ribosomal RNA	AtCG01180
28F	23S Ribosomal RNA	AtCG01180
13F	23S Ribosomal RNA	AtCG01180
55F	23S Ribosomal RNA	AtCG01180
7F	23S Ribosomal RNA	AtCG01180
20F	23S Ribosomal RNA	AtCG01180
76F	18S rRNA	
51F	NADH dehydrogenase	AtMG01120
31F	23S Ribosomal RNA	AtCG01180