INVESTIGATING THE ROLES OF PHENYLPROPANOID PATHWAY IN PLANT DEFENSE AGAINST PATHOGEN ATTACK

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By
TAO SONG

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ABSTRACT

The plant phenylpropanoid pathway is initiated from deamination of phenylalanine to form cinnamic acid followed by hydroxylation and methylation of the aromatic ring to generate a variety of phenolic compounds including lignin monomers, flavonoid compounds and sinapate esters. The incorporation of phenylpropanoid metabolism served as a key step in the early land-colonization of plants from aqueous environment since phenolic compounds play important roles in plant development and abiotic/biotic stress responses. Lignin is a heteropolymer of hydroxycinnamyl alcohols that are derived from the major branch of plant phenylpropanoid pathway. The main function of lignin is to enhance the strength of plant cell wall and waterproof the vascular system for long-distance transportation of water and solutes. In addition, lignin is also involved in protecting plants against pathogen attack. My Ph.D. research is to investigate how lignin biosynthesis contributes to plant immunity. The results showed that the expression of major lignin biosynthetic genes was induced upon host fungal pathogen infection. Moreover, a mutant disrupted in the lignin gene *F5H1* showed enhanced susceptibility when challenged with several fungal pathogens. *F5H1* encodes a ferulic acid 5-hydroxylase that is uniquely present in angiosperm plants, leading to the biosynthesis of syringyl lignin monomer, which is not present in gymnosperm plants. Subsequent research demonstrated that *f5h1* mutation impaired the penetration (pre-invasion) resistance but did not impact post-invasion resistance. Furthermore, the pathogen-induced expression of lignin genes was independent of well-characterized defensive signaling pathways, and regulated by a novel regulating mechanism. *F5H1* contributes to *pmr2*-mediated resistance but acts independently of other molecular components of penetration resistance including *PEN1*, *PEN2*, and *PEN3*. In contrast to *f5h1*, a knockout mutant of flavonoid pathway gene *chalcone isomerase* (*CHI*/*TT5*) showed enhanced resistance to host anthracnose pathogen *Colletotrichum higginsianum* in a salicylic acid (SA)-dependent manner. Taken together, our results for the first time provide genetic evidence demonstrating that lignin biosynthetic gene *F5H1* plays critical roles in plant penetration resistance and that an uncharted pathway in flavonoid metabolism confers an SA-dependent resistance pathway in Arabidopsis.

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LIST OF ABBREVIATIONS

ABBREVIATION	FULL NAME
ABA	abscisic acid
ABC	ATP-binding cassette
ABRC	Arabidopsis biological resource center
ANOVA	analysis of variance
BAP	6-benzylaminopurine
BLAST	Basic Local Alignment Search Tool
cDNA	complementary DNA
CHR	chalcone reductase
CWA	cell wall appositions
CWDEs	cell wall degrading enzymes
DAMPs	damage-associated molecular patterns
DFRC	derivatization, followed by reductive cleavage
dpi	days post inoculation
ER	endoplasmic reticulum
EST	expression sequence tag
ET	ethylene
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
f.sp.	fomae speciales
G	guaiacyl
GA	gibberellic acid
GUS	β-glucuronidase
H	ρ-hydroxyphenyl
hpi	hours post inoculation
HPLC-MS	high performance liquid chromatography-mass spectrometry
hr	hour
HR	hypersensitive response
IAA	3-indole acetic acid
IFS	isoflavone synthase
JA	jasmonic acid
LSD	Least Significant Difference
MAMPs	microbe-associated molecular patterns
MAPK	mitogen associated protein kinase
min	minute
NASC	Nottingham Arabidopsis Stock Centre
PAMPs	pathogen-associated molecular patterns
PCD	programmed cell death
PCR	polymerase chain reaction
PDA	potato dextrose agar
PG	polygalacturonase
PGIP	polygalacturonase inhibiting protein
PTI	pattern-triggered immunity
PRR	pattern recognition receptors

R-gene	resistance-gene
ROS	reactive oxygen species
RT-PCR	reverse-transcription polymerase chain reaction
S	syringyl
SA	salicylic acid
SAS	Statistics Analysis System
SD	standard deviation
SNP	sodium nitroprussid
UV	ultraviolet
var.	variety

CHAPTER 1

INTRODUCTION

1.1 Plant disease resistance

1.1.1 Overview of plant-pathogen interactions.

Plants constantly confront numerous pathogens in nature, ranging from bacteria, fungi to nematodes. Different from animals, plants lack of mobile defender cells and rely on innate immunity that is gradually reinforced during the evolution of plant-pathogen interactions. Plant immunity consists of both constitutive defenses and inducible defenses, the latter of which is activated upon the successful elimination of constitutive defenses by a potential pathogen and mediated by non-self recognitio (Lipka et al., 2008). This non-self recognition is accomplished by either direct recognition of the pathogen itself, or indirectly by its infectious activities. Direct recognition requires membrane-bound pattern recognition receptors (PRRs) of host plant cell to recognize highly conserved microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) of pathogens and activate PAMP-triggered immunity (PTI; Schwessinger and Zipfel, 2008). Several PRR-MAMP/PAMP pairs have been characterized in various plant pathosystems, including Arabidopsis Flagellin Sensitive 2 (FLS2)/bacterial flagellin (Gomez-Gomez and Boller, 2000), EF-Tu Receptor (EFR)/bacterial elongation factor EF-Tu (Zipfel et al., 2006) and LysM DOMAIN Receptor-like Kinase 1 (LysM-RLK1)/fungal chitin (Wan et al., 2008).

Indirect non-self recognition usually detects fungal pathogenic activities; especially the debris generated from pathogen penetration activities. Many plant pathogens secrete multiple cutinases and cell wall degrading enzymes (CWDEs) to degrade the plant epidermal cuticle and cell wall. As a result, derivatives generated from the degradation of cuticle and cell wall will be recognized by host cell as damage-associated molecular patterns (DAMPs) and further activate defensive responses. A well-characterized example is the oligogalacturonides, fragments of pectin derived from polygalacturonase (Ridley et al., 2001), which can be detected by Wall-Associated Kinase 1 (WAK1; Brutus et al., 2010). Either type of non-self recognition will subsequently activate various signaling transmissions and finally induce defensive responses to prevent further colonization.

Successful pathogens are able to suppress PTI by employing effector molecules to target distinct plant metabolism to either suppress defensive reactions or hijack plant cell metabolisms for their own benefits (O'Connell and Panstruga, 2006; Speth et al., 2007). This results in effector-triggered-susceptibility (ETS). Correspondingly, plants have evolved resistance (R) genes that can specially recognize effector molecules and trigger effector-triggered immunity

(ETI), which is usually featured by hypersenstitive response (HR)-cell death at the infected cell (Spoel and Dong, 2012). In this scenario, natural selection will force the pathogen to adapt new mechanisms to overcome ETI. Such evolutionary steps occur back and forth between plants and pathogens *ad infinitum*.

Due to innate plant immunity, only a small group of pathogens (referred to as host/compatible/adapted pathogens) are able to cause disease on plants. Most potential pathogens (referred to as nonhost/incompatible/non-adapted pathogens) fail to cause disease due to unique plant nonhost resistance.

Nonhost resistance refers to the ability of one entire plant species to resist all genetic variants of one pathogen species (Heath, 1997). In general, nonhost resistance can be divided into penetration (or pre-invasion) resistance and post-invasion resistance (Thordal-Christensen, 2003; Lipka et al., 2005). Upon the arrival at a host plant cell, many pathogens, especially fungal pathogens, will have to obtain nutrients from host cells to support subsequent development and reproduction. However, the rigid plant cell wall separates pathogens from host cell metabolites. Therefore, plant pathogens deploy various strategies to break this obstacle and obtain access to intracellular nutrients. Fungal pathogens usually directly penetrate through plant cell walls to form intracellular hyphae (such as anthracnose pathogens) or feeding organ haustoria followed by development of extracellular hyphae on the top of infected cells (such as powdery mildew; Jones and Dangl, 2006). Penetration resistance is mainly focused against this positive penetration process and results in a failure of entry into the host cell. For example, the nonhost powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* displays as low as a 5% entry rate on the model plant Arabidopsis (Lipka et al., 2005). For the 5% successfully penetrated *B. graminis* f.sp. *hordei* spores, post-invasion resistance including HR-like cell death will eventually terminate further fungal development.

In contrast, host pathogens are able to overcome both penetration and post-invasion resistance and subsequently complete their life cycles on host plants. For example, the host powdery mildew pathogen *Golovinomyces orontii* achieves 70% entry frequency on Arabidopsis, indicating the failure of penetration resistance (Lipka et al., 2005). Post-invasion resistance of Arabidopsis such as jasmonic acid/ethylene-mediated resistance is not activated upon the infection of host powdery mildew as well (Zimmerli et al., 2004). In the subsequent sections,

common mechanisms of plant disease resistance, including both constitutive and inducible defenses, will be discussed in detail.

1.1.2 Constitutive defenses.

The first layer of plant disease resistance is pre-formed or constitutive defenses, including physical barriers such as cuticular layers and plant cell wall and toxic chemical compounds such as phytoanticipins to kill the pathogens (Dixon, 2001; Heath, 2000; Kamoun, 2001; Numberger et al., 2004; Curvers et al., 2010). Although little evidence is available, pre-formed defenses are capable of terminating infection of nonhost pathogens without further activation of inducible defenses. A well-characterized example is avenacin, a phytoanticipin identified from oat plants (Papadopoulou et al., 1999). Wheat root pathogen *Gaeumannomyces graminis* var. *tritici* is unable to infect oat, while a closely related pathogen *Gaeumannomyces graminis* var. *avenae* is a host pathogen to oats. Research points out that *G.graminis* var. *tritici* is unable to detoxify avenacin (Bowyer et al., 1995).

1.1.3 Penetration resistance.

If a potential pathogen overcomes or evades constitutive defenses, a host cell will first recognize the intruder and subsequently activate inducible defense reactions, including penetration and post-invasion resistance. Penetration resistance mainly occurs in the apoplastic region of epidermal cells since its major task is to prevent the entry of pathogens into the host cell. Three main strategies are employed by plants for penetration resistance: inhibiting CWDEs secreated by pathogens, “poisoning” the apoplastic region and finally, forming cell wall appositions (CWAs; papillae) as a reinforcement of host cell wall at specific plant-pathogen interaction sites (Hückelhoven, 2007).

Plant pathogens are able to secrete multiple enzymes to hydrolyze the structural polymers of cell wall to generate an ingress port. The cell-wall degrading secretome includes cellulases, polygalacturonases (PGs), xylanases and proteinases (Juge, 2006). One of the most characterized examples is the fungal PGs degrading pectin polymers. Pectin is a major constituent in the middle lamella, which holds neighboring plant cells together. Hydrolysis of pectin demolishes the integrity of plant cell wall and exposes other structural polymers such as cellulose and hemicelluloses to other hydrolytic enzymes. PG is able to hydrolyze pectin to generate galacturonic acid oligomers with different chain lengths, depending on the different action

modes on the pectin substrates (Parenicova et al., 2000). PG inhibiting proteins (PGIP) are membrane-bound proteins which are specific to PG but not to other pectin degrading enzymes (De Lorenzo et al., 2001). The contribution of PGIP to plant penetration resistance has been characterized in various plants including Arabidopsis (Powell et al., 2000; Ferrari et al., 2003).

In addition to cell wall degrading enzyme inhibitors, plant cells are also able to secrete toxic compounds into the apoplastic region to attack potential intruders upon recognizing their presence. These toxic compounds, which are termed as antimicrobial agents, include low-molecule-weight chemical compounds and antimicrobial proteins. The low-molecular-weight compounds, or phytoalexins, are synthesized *de novo* and accumulate in plants in response to infection or stress due to wounding, freezing, ultraviolet light exposure, and exposure to microorganisms (Darvil and Albersheim, 1984; Graham and Graham, 1991; Paxton, 1991). For example, the powdery mildew resistant barley mutant *mlo* accumulates a higher amount of ρ-coumaroyl-hydroxyagmatine, a type of phenolic phytoalexin, at the infection site and, thererby, is able to inhibit the formation of haustorium *in vivo* (von Röpenack et al., 1998). In addition to phenolic phytoalexins, Arabidopsis also accumulates tryptophan-derived phytoalexins such as camalexin that confer nonhost resistance against necrotrophic fungi *Plectosphaerella cucumerina* (Sanchez-Vallet et al., 2010). Antimicrobial proteins are mainly the enzymes that can influence the integrity of pathogen cell wall or plasma membrane, such as chitinase, glucanases, thionins, osmotins, proteases and defensins (Hükelhoven, 2007). Additionally, pathogenesis related protein 1 (PR-1) also shows antifungal activity by inhibiting the differentiation of the *Uromyces fabae* infection hyphae in resistant broad bean plants (Rauscher et al., 1999).

The focused formation of papilla at the infection site is considered to be the most important strategy in penetration resistance. The formation of papilla occurs in both compatible and incompatible plant-pathogen interactions, indicating its common role in plant-pathogen interactions (Aist, 1976). The main structural components of papilla are callose and lignin or lignin-like phenolic compounds that confer autofluorescence (McLusky et al., 1999). Moreover, papillae also contain other cell wall polymers (cellulose and pectin), phytoalexins, silicon, reactive oxygen species (ROS), and proteins such as peroxidase and hydroxyproline-rich glycoproteins (Aist, 1976; Schmelzer, 2002; Zeyen et al., 2002; An et al., 2006). Lignin, callose and other cell wall polymeric deposits in papillae might function together as a physical barrier

against physical forces generated by pathogens (Bechinger et al., 1999). In addition, lignin could also generate a hydrophobic environment in papilla to reduce the activities of CWDEs secreted by pathogens (Hückelhoven, 2007). As result, treatment with phenylpropanoid enzyme inhibitors that suppress lignin formation always impairs the papilla-based penetration resistance (Carver et al., 1992; Kruger et al., 2002).

The generation and accumulation of ROS molecules, such as hydrogen peroxide (H_2O_2), and nitric oxide (NO), occur at papillae and serve multiple purposes (Hüeckelhoven et al., 1999; 2000; Prats et al., 2005; Trujillo et al., 2006). Firstly, they facilitate the hardening of papillae structure by enhancing the cross-linking of lignin precursors and cell wall associated proteins (Hüeckelhoven et al., 1999). Secondly, they might also cause direct toxic effects on invading pathogens (such as inhibiting spore germination; Peng and Huc, 1992). Finally, ROS could also function potentially as signaling molecules in the activation of downstream defense genes (Desikan et al., 2000).

A noticeable observation is that all defensive reactions described in penetration resistance are focused beneath the attempted penetration sites of pathogens. Therefore, directed transport is required to convey various molecules (from phytoalexins to building blocks of papillae) to the right site for proper function. Such directed intracellular transport requires the cytoskeleton system, including actin filaments and microtubules (Schmelzer, 2002). Similar activities, including cytoplasmic streaming and aggregation underneath the plant-pathogen interaction site and translocation of cellular organelles are correlated with the polarized re-organization of cytoskeleton components (Kobayashi, et al., 1992, 1997a, b; McLusky, et al., 1999; Lipka and Panstruga, 2005; Takemoto et al., 2003; 2006). Interference with actin dynamics disrupts penetration resistance and enhances the entry of fungal pathogen in various plant species (Kobayashi, et al., 1997a, b; Yun et al., 2003). In comparison with actin filaments, the role of microtubules in penetration resistance is not clear. However, Lee et al. (2012) characterized a type III secreted effector from *Pseudomonas syringae*, HopZ1, which is able to interfere the microtubule network in Arabidopsis and block the deposition of callose, suggesting that microtubules might be responsible for the transport of callose in defensive responses.

Recent forward genetic analyses have already characterized a number of molecular components contributing to plant penetration resistance, including *PENETRATION 1* (*PEN1)*, *PEN2*, *PEN3*, as well as *MILDEW RESISTANCE LOCUS O 2* (*MLO2*)/*POWERY MILDEW*

RESISTANCE 2 (*PMR2*) (Jorgensen, 1992; Vogel and Somerville, 2000; Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). *PEN1* encodes an Arabidopsis syntaxin (SYP121), which is required for the fusion of vesicle membrane with plasma membrane, with partner proteins SOLUBLE N-ETHYLMALEIMIDE-SENSITIVE FACTOR ADAPTOR PROTEIN 33 (SNAP33) and VESICLE-ASSOCIATED MEMBRANE PROTEIN 721/722 (VAMP721/722; Kwon et al., 2008) and plays an indispensable role in the timely formation of papillae during fungal penetration activities (Assaad et al., 2004). Noticeably, PEN1 protein only accumulates at attempted penetration sites by powdery mildew pathogens, but not by other ascomycete fungi (Meyer et al., 2009). *PEN2* encodes a peroxisome-localized glycoside hydrolase belonging to the Arabidopsis family 1 glycosyl hydrolase group of enzymes (myrosinases; Lipka et al., 2005) and *PEN3* encodes an ATP-binding cassette (ABC) transporter (Stein et al., 2006). Further research pointed out that both *PEN2* and *PEN3* are required for the bacterial flagellin Flg22-induced callose deposition (Clay et al., 2009): PEN2 catalyzes the hydrolysis of 4-methoxy-indole-3-ylmethylglucosinolate (4-methoxyl-I3G; Bednarek et al., 2009) and PEN3 is responsible for directing the hydrolytic products generated by PEN2 to the apoplastic region (Stein et al., 2006). Different from *PEN1*, *PEN2* and *PEN3* is required for resistance against a broad spectrum of pathogens (Lipka et al., 2005; Stein et al., 2006).

The *MLO* locus was first identified in barley and mediates resistance against powdery mildew (Jorgensen, 1992; Piffanelli et al., 2004). Later, *MLO* loci with similar functions were also identified from Arabidopsis, tomato and pea (Chen et al., 2006; Bai et al., 2008; Humphry et al., 2011). In the Arabidopsis, several mutants with enhanced resistance against powdery mildew have been characterized (*pmr*; Vogel and Somerville, 2000). The *PMR2* is allelic to *MLO2*, and mutants of this gene display enhanced penetration resistance (Consonni et al., 2006). The specific function of MLO2/PMR2 proteins remains unknown, but *pmr2*/*mlo2*-mediated resistance in Arabidopsis requires *PEN1*, *PEN2*, *PEN3*, and tryptophan-derived metabolism, but not the SA/JA/ET signaling pathway (Consonni et al., 2006; 2010). Upon challenge of host powdery mildew, barley MLO proteins are accumulated underneath the pathogen penetration sites, suggesting its contribution to the successful entry of pathogen into host plant cell (Bhat, et al., 2005). Based on these observations, *PMR2*/*MLO2* is proposed to be a negative regulator controlling *PEN* gene function in penetration resistance through an unknown mechanism (Underwood and Somerville, 2008).

1.1.4 Post-invasion resistance.

Usually, penetration resistance cannot completely block pathogen entry. For example, nonhost powdery mildew *B. graminis* f.sp. *hordei* is able to successfully penetrate to form haustoria on Arabidopsis. In this circumstance, post-invasion resistance will be induced to restrict post-haustorial fungal development. The induction of post-invasion resistance can be characterized by the expression of marker genes, including *Pathogenesis Related Gene-1* (*PR-1*; At2g14610) for the salicylic acid (SA) signaling pathway and *Plant Defensin 1.2* (*PDF1.2*; At5g44420) for the jasmonic acid/ethylene (JA/ET) signaling pathway (Niu et al., 2011). Both the SA and JA/ET signaling pathways will further induce various defense responses such as a HR-like programmed cell death (PCD) in penetrated cells (Lipka et al., 2005). Various components of the SA/JA/ET metabolism/signaling pathways and their corresponding genes have been identified, including *SALICYLIC ACID INDUCTION DEFICIENT 2* (*SID2*), *PHYTOALEXIN DEFICIENT 4* (*PAD4*)/*SENESCENCE-ASSOCIATED GENE 101* (*SAG101*), *JASMONATE RESISTANT 1* (*JAR1*) and *ETHYLENE INSENSITIVE 2* (*EIN2*; Wildermuth et al., 2001; Gfeller et al., 2010; Lu, 2009; Stepanova and Alonso, 2009). Arabidopsis lines disrupted in the SA/JA/ET signaling pathways either by gene mutations or expression of a bacterial gene *NahG* to degrade SA molecule in Arabidopsis displayed enhanced suscpetibility against various plant pathogens (Lawton et al., 1995; Zimmerli et al., 2004; Lipka et al., 2005; Guo and Stotz, 2007). In summary, both penetration and post-invasion resistance play essential roles in nonhost resistance of Arabidopsis against powdery mildew since a triple mutant *pen2*/*pad4*/*sag101* disrupted in both penetration and post-invasion resistance allowed the establishment of basic compatibility of nonhost powdery mildew *B. graminis* f.sp. *hordei* on Arabidopsis (Lipka et al., 2005).

1.2 Plant phenylpropanoid pathway.

Approximately 450 million years ago, plants began to migrate from water to the land. During this process, plants have incorporated various molecular, physiological and morphological adaptations to accommodate to the terrestrial environment (Waters, 2003). The phenylpropanoid pathway, which gives rise to a large group of secondary metabolites with common aromatic rings and hydroxy groups, is a critical evolutionary strategy during the "landing" of plants due to the significant contribution of phenylpropanoid compounds in plant

growth and development. For example, the major products of the phenylpropanoid pathway, hydroxycinnamyl alcohols, serve as the building units of lignin polymers that provide plants with strong mechanical support and waterproofing barriers (Boerjan et al., 2003). Phenolic compounds play indispensable roles in the survival of land plants.

Phenylpropanoid metabolism is initiated with phenylalanine (Figure 1.1). Phenylalanine is derived from shikimate pathway that also generates other aromatic acids, tyrosine and tryptophan (Hermann and Weaver, 1999; Triz and Galili, 2010). The initial three steps, which are catalyzed sequentially by phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-hydroxycinnamoyl CoA ligase (4CL) to form ρ-coumaroyl CoA, comprise the general pathway of phenylpropanoid metabolism (Figure 1.1). Subsequently, ρ-coumaroyl CoA is fluxed into both the flavonoid pathway and the lignin pathway, catalyzed by chalcone synthase (CHS) and hydroxycinnamoyl-CoA shikimate/quinate hydroxyxinnamoyl transferase (HCT), respectively (reviewed by Fraser and Chapple, 2011). The third branch of the phenylpropanoid pathway, the sinapate esters pathway, originates from intermediates of lignin pathway, coniferaldehyde and sinapaldehyde, which are catalyzed by an aldehyde dehydrogenase *REDUCED EPIDERMAL FLUORESCENCE1* (REF1; Vogt, 2010).

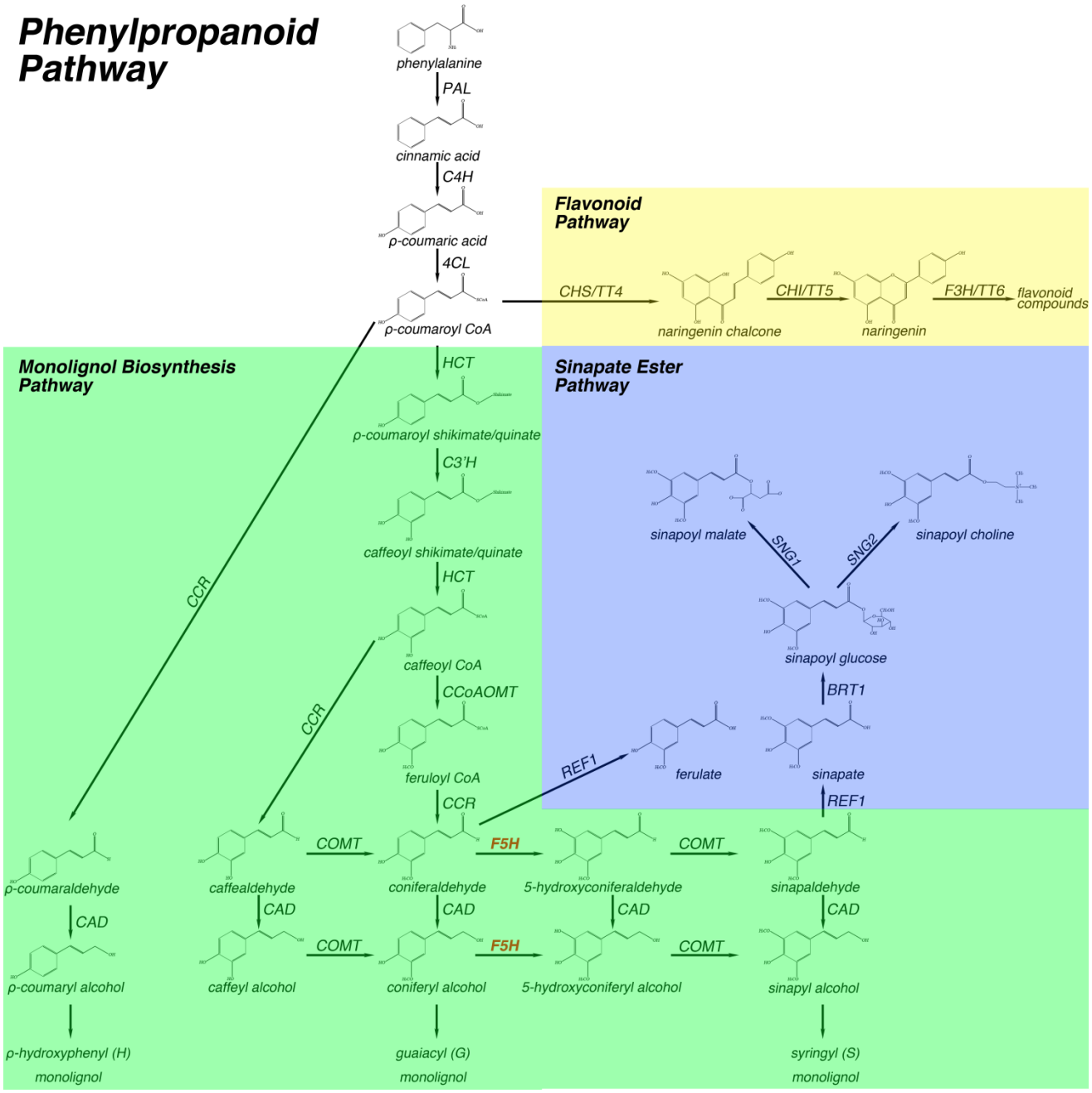
Phenylpropanoid Pathway
phenylalanine
PAL
cinnamic acid
C4H
p-coumaric acid
4CL
p-coumaroyl CoA
Flavonoid Pathway
CHS/TT4
naringenin chalcone
CHI/TT5
naringenin
F3H/TT6
flavonoid compounds
Monolignol Biosynthesis Pathway
Sinapate Ester Pathway
HCT
p-coumaroyl shikimate/quinate
C3'H
caffeoyl shikimate/quinate
HCT
caffeoyl CoA
CCoAOMT
feruloyl CoA
CCR
CCR
CCR
sinapoyl malate
SNG1
SNG2
sinapoyl choline
sinapoyl glucose
BRT1
REF1
ferulate
sinapate
REF1
p-coumaraldehyde
CAD
p-coumaryl alcohol
p-hydroxyphenyl (H) monolignol
COMT
caffealdehyde
CAD
COMT
caffeyl alcohol
F5H
coniferaldehyde
CAD
F5H
coniferyl alcohol
guaiacyl (G) monolignol
COMT
5-hydroxyconiferaldehyde
CAD
COMT
5-hydroxyconiferyl alcohol
COMT
sinapaldehyde
CAD
sinapyl alcohol
syringyl (S) monolignol

Figure 1.1 Plant phenylpropanoid pathway.

Schematic image of plant phenylpropanoid pathway including the chemical structures of each metabolite and the genes encoding the enzymes involved in this metabolism. Three branches that are derived from the general phenylpropanoid pathway are highlighted by color shadows: green shadow for monolignol biosynthetic pathway, blue for sinapate ester pathway and yellow for flavonoid pathway. The major research object gene of this project, *F5H*, is highlighted by red font. Gene abbreviations: *PAL, phenyalanine ammonia-lyase; 4CL, 4-hydroxy cinnamoyl CoA ligase; C4H, cinnamate 4-hydroxylase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxyxinnamoyl transferase; C3'H, p-coumaroyl shikimate/quinate 3'-hydroxylase; CCoAOMT, caffeoyl CoA O-methyl transferase; CCR, cinnamoyl CoA reductase; F5H, ferulic acid 5'-hydroxylase; COMT, caffeic acid/5-hydroxyferulic acid O-methyl transferase; CAD, cinnamyl alcohol dehydrogenase; CHS, chalcone synthase; TT4, transparent testa 4; CHI, chalcone isomerase; TT5, transparent testa 5; F3H, flavanone 3-hydroxylase; TT6, transparent testa 6; REF1, Reduced Epidermal Fluorescence 1; SNG1, sinapoylglucose 1, SNG2, sinapoylglucose 2, BRT1, bright trichome 1*. Gene *REF1* encodes an Arabidopsis aldehyde dehydrogenase. This figure is drawn by the author of this thesis based on information collected from (Liu, 2012); Buer and Muday, (2004); Shirley et al., (2001).

1.2.1 Plant lignification process.

1.2.1.1 Lignin: form and functions.

Lignin is a generic term for a heteropolymer which is mainly composed of three types of hydroxycinnamyl alcohols (monolignols): ρ-coumaryl, coniferyl and sinapyl alcohols, which are converted to ρ-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units respectively when incorporated into lignin polymers during the polymerization process (Figure 1.2A; Boerjan et al., 2003). Lignin polymers are mainly deposited in the secondary plant cell walls of various tissues including xylem vessels, fibers and sclereids. The composition of lignin monomers varies among different plant species. Generally, gymnosperm plants mainly consist of G units with minor amount of H units while angiosperm plants contain both G and S lignin monomers with variable ratios (Baucher et al., 1998). The deposition and composition of lignin are also under developmental and environmental regulations (Campbell and Sederoff, 1996). In addition to these three major monolignols, other phenolic compounds, such as 5-hydroxyconiferyl alcohols, hydroxyl-cinnamaldehyde, and hydroxycinnamic acids, are also incorporated into lignin polymers (Boerjan et al., 2003, Leple et al., 2007, Morreel et al., 2004, Ralph et al., 2008).

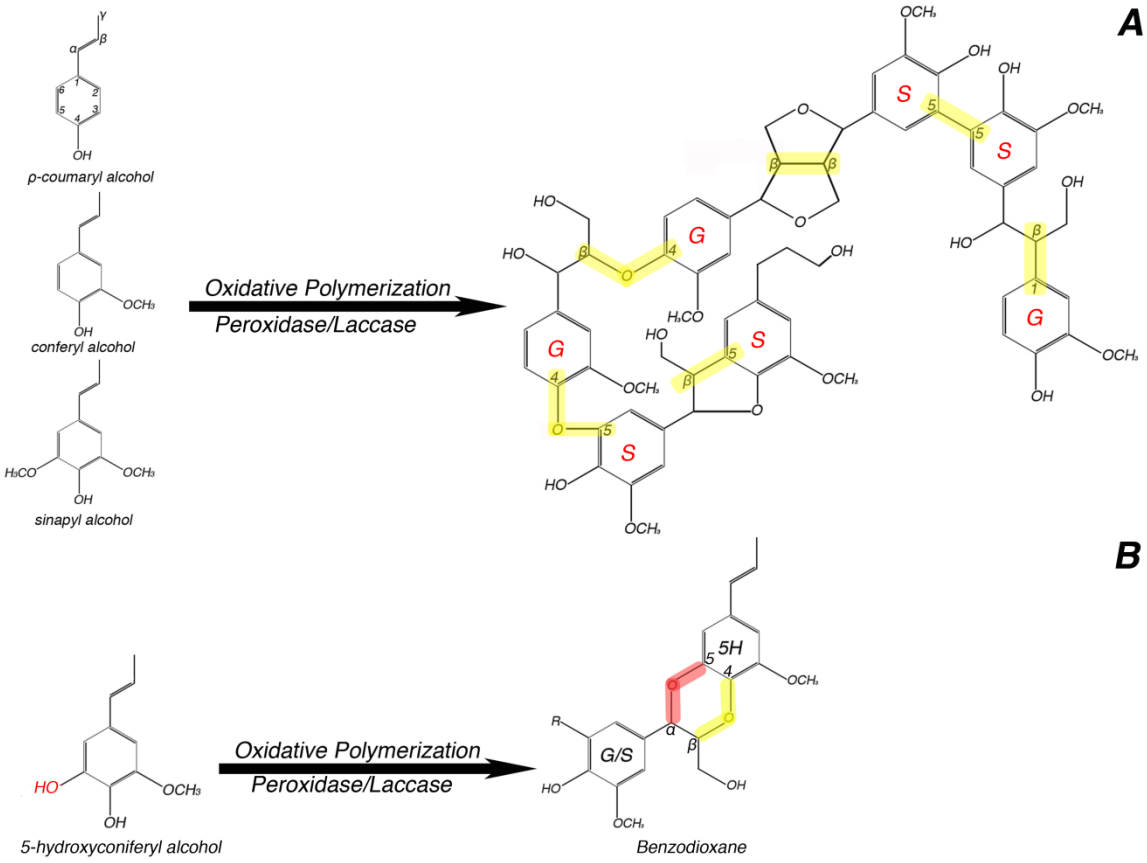

Figure 1.2 Chemical structures of monolignol and lignin polymerization bonds.

(A) Chemical structures of classical monolignols. Numerical designations of carbon atoms are labeled in ρ-coumaryl alcohol. Coniferyl alcohol and sinapyl alcohol share the same numerical designation as ρ-coumaryl alcohol. Peroxidase and laccase are involved in the oxidative polymerization process. G, guaiacyl unit; S, syringyl unit. The polymerization bonds between monolignols are highlighted by yellow shadows. (B) Chemical structures of 5-hydroxyconiferyl alcohol and the dimer of benzodioxane formed by 5-hydroxyconiferyl alcohol with either G or S monolignols. The hydroxyl group at 5-position is labeled by red font. Red shadow highlights the special α-O-5 bond in benzodioxane. The oxidative polymerization process is catalyzed by peroxidase and laccase. R=H for G unit; R=OCH_3 for S unit. The image is adapted from Albersheim et al., 2010.

As the second most abundant biopolymer in nature, lignin plays critical roles in resistance against environmental stresses. Firstly, it provides rigid mechanical support required for upright growth. Arabidopsis mutants with a significantly reduced level of lignin deposition, such as *irx4* and *ref3* mutants, result in developmental deficiencies, including slower growth rate, failure of maintaining upright growth, dwarfism, and male sterility (Jones et al., 2001; Schilmiller et al., 2009). Secondly, lignin generates a waterproofing surface for vascular elements, which enables the long-distance transportation of water and soluble nutrients from roots to aboveground organs. Finally, lignin is an extremely degradation-resistant and stress-inducible polymer that makes it an ideal barrier against various types of environment stresses, especially plant pathogens and herbivores.

The entire lignification process consists of three stages: biosynthesis of monolignols, intracellular transportation of monolignols to the cell wall, and oxidative dehydrogenation and polymerization to form heterogeneous macromolecules. Decades of research on plant lignification process have accumulated intensive knowledge of the biosynthesis and regulation of monolignols by characterization of participating gene members as illustrated in Figure 1.1. However, our understanding of subsequent transportation of monolignols to the plant cell wall and the polymerization remains fragmentary.

1.2.1.2 Biosynthesis of monolignols.

Biosynthesis of monolignols comprises the major branch of plant phenylpropanoid pathway and involves more than ten metabolic enzymes (Table 1.1; Figure 1.1; Costa et al., 2003). Following the general phenylpropanoid pathway, ρ-coumaric CoA serves as the common precursor of all three types of monolignols; firstly, hydroxycinnamoyl CoA reductase (CCR) leads to the biosynthesis of ρ-coumaraldehyde which will be used by (hydroxy)cinnamyl alcohol dehydrogenase (CAD) to generate ρ-coumaryl alcohol; secondly, ρ-coumaroyl-CoA can also be catalyzed by hydroxycinnamoyl-CoA shikimate/quinate hydroxyxinnamoly transferase (HCT) and ρ-coumaroylshikimate 3'-hydroxylase (C3'H) to from caffeoyl CoA. Caffeoyl CoA can be either converted to feruloyl CoA by a caffeoyl CoA O-methyltransferase (CCoAOMT), or to caffeoyl CoA by CCR and subsequently to caffeoyl alcohol by CAD. Feruloyl CoA will be converted to coniferaldehyde by CCR and subsequently to coniferyl alcohol by CAD. Moreover, caffeoyl CoA and caffeoyl alcohol can be also converted to coniferaldehyde and coniferyl alcohol by caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT), respectively. Both

coniferaldehyde and coniferyl alcohol can be catalyzed by ferulic acid 5'-hydroxylase (F5H) to form 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol, respectively. 5-Hydroxyconiferaldehyde can be converted to sinapaldehyde by COMT. The final monolignol unit, sinapyl alcohol can be either directly generated from 5-hydroxyconiferyl alcohol by COMT, or from sinapaldehyde by CAD (Fraser and Chapple, 2011; Liu, 2012).

Table 1.1 Monolignol biosynthetic genes in Arabidopsis. (Adapted from Coasta et al., 2003)

Enzymes	Gene redundancy in Arabidopsis Genome	Reference
Phenylalanine ammonia lyase (PAL)	Multi gene family; 4 members	Koukol and Conn, 1961
Cinnamate-4-hydroxylase (C4H)	Single copy	Benveniste et al., 1986
4-Coumarate CoA ligase (4CL)	Multi gene family; 14 members	Lee et al., 1995
Hydroxycinnamoyl-Coenzyme A shikimate/quinate hydroxycinnamoyltransferase (HCT)	Single copy	Hoffmann, et al., 2004
ρ-Coumaroylshikimate 3'-hydroxylase (C3H)	Single copy	Schoch et al., 2001
Caffeoyl-CoA O-methyltransferase (CCoAOMT)	Multi gene family; 4 members	Ye et al., 1994
Cinnamoyl CoA reductase (CCR)	Multi gene family; 12 members	Jones et al., 2001
Cinnamyl alcohol dehydrogenase (CAD)	Multi gene family; 9 members	Baucher et al., 1995
Ferulate 5-hydroxylase (F5H)	Multi gene family; 2 members	Meyer et al., 1998
Caffeic acid O-methyltransferase (COMT)	Multi gene family; 17 members	Muzac et al., 2000

Although monolignol biosynthetic genes have defined above, the specific subcellular locations of monolignol biosynthesis remain contentious. Phenylalanine is generated by the plastid-localized shikimate pathway (Herrmann and Weaver, 1999; Rippert et al., 2009), but the subsequent lignin biosynthetic enzymes are located in two different cellular locations: the three cytochrome P450 proteins, C4H, C3'H and F5H, are predicted to be anchored at the outer surface of endoplasmic reticulum (ER) membranes (Dunkley et al., 2006), while other enzymes are soluble proteins in the cytosol (Takabe et al., 1985, Nakashima et al., 1997, Chen et al., 2000). Recently, ER-localizations of C4H and C3'H have been confirmed in *Populus* by fluorescence fusion proteins, which is consistent with previous publications (Ro, et al., 2001; Chen et al., 2011). The two different subcellular locations of monolignol biosynthetic enzymes, as well as the plastid localization of the original substrate phenylalanine, suggest a possible sequential translocation of intermediates between enzymes that are located in different cellular organelles. This translocation procedure might result in an efficient metabolism process. For

example, it may help to reduce the potential toxicity of intermediate phenolic metabolites to the plant and/or to enhance enzymatic reaction efficiencies (Srere, 1987; Hrazdina and Jensen, 1992; Burbulis and Winkel-Shirley, 1999; Winkel-Shirley, 1999). In fact, several cytosolic lignin biosynthetic enzymes, including PAL, CAD, COMT and 4CL have been shown to transiently associate with ER-Golgi derived vesicles and disperse into cytosol (Takabe, et al., 2001; Takeuchi, et al., 2001). Moreover, immunolocalization analysis of the poplar PAL enzyme and a fluorescence fusion protein of the tobacco C4H enzyme further demonstrated that PAL is in close physical association with C4H on ER microsomes (Achnine et al., 2004; Sato et al., 2004). Together with the observation that PAL enzyme activities are reduced in transgenic tobacco with down-regulated C4H (Blount et al., 2000), all of the evidence implies that the three cytochrome P450 monooxygenases C4H, C3'H and F5H may coordinate the entire lignin biosynthesis by anchoring other soluble lignin biosynthetic enzymes to the outer-surface of ER for specific reactions. However, a massive amount of work is required in the future to fully demonstrate this hypothesis, such as determination of F5H subcellular location, a dynamic analysis of whether spatial and physical locations change for all monolignol biosynthetic enzymes, and how this process affects biosynthesis, intracellular transportation and eventual deposition of monolignols.

1.2.1.3 Intracellular transportation and polymerization of monolignols.

Monolignols must be transported to the cell wall where oxidative polymerization of the lignin polymer occurs. The molecular mechanism of intracellular transportation remains unclear. Previous research has revealed that the hydroxycinnamyl alcohols undergo a glycosylation process in plant cell: the ρ-coumaryl, coniferyl and sinapyl alcohols were glucosylated to form 4-O-β-D-glucosides, named as ρ-coumaryl alcohol glucoside, coniferin and syringin, respectively. Coniferin and syringin have been identified mostly in gymnosperm and some angiosperm plants (Terazawa and Miyake, 1984; Whetten and Sederoff, 1995; Hemm et al., 2004). This glucosylation process is catalyzed by coniferyl/sinapyl alcohol glucosyltransferase (Steeves et al., 2001). Previous research pointed out that these glucocojugates possibly exist in the vacuoles of cambial cells. Therefore, it was thought that they function in storage and subsequent transport of monolignols at least in these gymnosperm species and relatively less advanced angiosperm plants such as *Magnoliaceae* and *Oleaceae* families (Leinhos and Savidge, 1993; Dharmawardhana et al., 1995; Steeves et al., 2001; Tsuji and Fukushima, 2004). From vacuoles, it was thought that monolignol-glucosides are exported to the cell wall through an unknown

mechanism and undergo deglucosylation that is catalyzed by specific β-glucosidases before polymerization occurs. Such a glucosidase has been characterized from various plant species, including Arabidopsis (Marcinowski and Grisebach, 1978, Leinhos et al., 1994, Dharmawardhana et al., 1995; Escamilla-Trevino et al., 2006). The coniferyl/sinapyl alcohol glucosyltransferase and the corresponding glucosidase had given rise to the hypothesis of monolignol regulation of storage and transportation for lignin biosynthesis.

However, recent research has obtained negative evidence regarding this hypothesis and has complicated the issue. The most surprising observation is that ectopic expression of glucosyltransferses only affects the content of soluble monolignol glucosides, but does not alter overall lignin deposition (Lanot et al., 2006; Vanholme et al., 2008). Moreover, feeding radioisotope labeled phenylalanine showed specific incorporation of ^{3}H-Phe into monolignols and lignin constituents, but not monolignol glucosides (Kaneda et al., 2008). Therefore, monolignol glucosylation-deglucosylation is believed to be part of a regulation or storage mechanism for monolignols preventing free diffusion of monolignols, and monolignol aglycone is the chemical form of monolignols for transport to the cell wall.

Another critical step of lignin transportation is the cross-membrane transfer of monoligols to reach the apoplastic region. Three hypothetical mechanisms regarding the cross membrane transport of monolignols have been proposed: exocytosis via ER-Golgi derived vesicles, passive diffusion, and active transportation via membrane-located transporters (as reviewed by Liu et al., 2011). Other cell wall materials, such as pectin and hemicellulose, are synthesized in the Golgi and exported to the apoplastic region by ER-Golgi-mediated exocytosis (Lerouxel et al., 2006; Sandhu et al., 2009). Early feeding experiments with radioisotope-labeled phenylalanine and autoradiographic and immunochemical studies also supported the ER-Golgi-mediated exocytosis of monolignols (Pickett-Heaps, 1968; Fujita and Harada, 1979; Takbe et al., 1985). However, recent research pointed out that observed isotope labels in the ER-Golgi are derived from phenylalanine incorporated into proteins, rather than monolignols (Kanede et al., 2008). Although subcellular locations of monolignol biosynthetic enzymes C3'H and C4H had suggested ER as the location of lignin biosynthesis (Chen et al., 2011), it may not play significant roles in monolignol transportation.

The passive fusion hypothesis originated from the facts that "non-traditional" monolignols (rather than H, G and S units) can be detected in lignin polymers both in natural

plants and in transgenic plants with altered lignin biosynthesis (reviewed by Vanholme et al., 2008; 2010). Furthermore, lignin precursor analogs are easily partitioned into artificial membranes such lipid bilayer discs *in vitro* (Bojia et al., 2007, 2008). However, the selection mechanism is not clear regarding how the monolignols and derives are sorted and incorporated into the apoplastic region.

Finally, recent research highlights the hypothesis that transporter-mediated active trans-membrane transport may play a significant role in transportation of monolignols across the plasma membrane. This hypothesis is based on the observation of a highly spatial deposition pattern of various monolignols in plant cells. For example, in birch woods, coniferyl alcohols are deposited in both vessel and fiber cell walls while sinapyl alcohols are only deposited in the latter (Fergus and Goring, 1970). A similar distribution is also reported in *Arabidopsis thaliana* (Chapple et al., 1994). Even within one type of cell, the middle lamella region and the secondary cell wall consist of different monolignols (Terashima et al., 1993) and the S1, S2 and S3 layers of secondary cell wall consist of sequentially deposited ρ-coumaryl alcohols, coniferyl alcohols and sinapyl alcohols (Terashima et al., 1993; Terashima et al., 1986a; 1986b). Such a highly organized distribution of different monolignols suggests that the deposition of lignin monomers in the plant cell wall is a highly regulated process in which an active transport mechanism is possibly involved.

ABC transporters represent a large, ubiquitous superfamily with up to 120 putative members in Arabidopsis (Sanchez-Fernandez et al., 2001). Most of the ABC members are membrane-bound proteins that participate in translocation of a wide range of molecules, including mineral ions, lipids, and peptides (Higgins, 1992; Sanchez-Fernandez et al., 2001; Rea, 2007; Verrier et al., 2008). Moreover, ABC transporters ABCG11/WBC11 and ABCG12/CER5 export wax and cutin precursors to the apoplast region to generate cuticle, the lipid layer covering plant epidermal cells (Pighin et al., 2004; McFarlane et al., 2010).

Numerous reports suggest the possible role of ABC transporters in lignin export. For example, expressed sequence tags (ESTs) encoding putative ABC transporters are frequently identified in lignifying or lignified tissues, such as xylem and tracheary elements, as measured by transcriptome profiling conducted on gymnosperm and angiosperm plants (Allona et al., 1998; Hertzberg et al., 2001; Kirst et al., 2003; Egertsdotter et al., 2004; Paux et al., 2004; Pesquet et al., 2005). Proteomic analysis also showed tissue-specific distribution of ABC transporter

subfamilies B and G in cambium/phloem (Nilsson et al., 2010). In Arabidopsis, seven ABC transporter genes were co-expressed with lignin biosynthetic genes in the tissues described above (Ehlting et al., 2005). The maize mutant *bm2,* which deposits a reduced level of guaiacyl lignin also displays decreased transcription of one ABC transporter gene. All of these results suggested that ABC transporters might play a significant role in transporting monolignols to the cell wall (Samuels et al., 2002; Douglas and Ehlting, 2005). Recently, Miao and Liu (2010) showed that the uptake of monolignol coniferyl alcohol by prepared plasma and vacuolar membrane vesicles from Arabidopsis is ATP-dependent and inhibited by ABC transporter inhibitor, demonstrating the participation of ABC transporter in the cross-membrane transportation of monolignols. Moreover, their work also demonstrated that monolignol aglycones and glucocojugate can be recognized by and taken up by plasma membrane and vacuole membranes, respectively. Such selective uptake of monolignols by different membrane preparations further supported the opinion that glucocojugate is for storage of monolignol in central vacuoles while monolignol aglycones for transmembrane export.

It remains a challenge to identify the particular ABC transporter(s) for lignin export due to the natural properties of lignin and ABC transporters. Many ABC transporter genes showed correlated expression with lignification process (Ethlting et al., 2005), but none of the mutants disrupted in these genes shows altered lignin deposition in xylem vessels (Kaneda et al. 2011). As described, the lignification process is complicated and highly organized in a "cell type-specific" manner. Therefore, distinct membrane transporters might be involved. Since many ABC transporters displayed overlapping transcription patterns, functional redundancy may have resulted in the unaltered lignin depositions in single mutants when the lignin profiling is performed as pooled samples from various tissues. In addition, some ABC transporters, such as ABCB14, show low substrate specificity and are able to carry unrelated molecules in order to execute various biological processes, and this low specificity enhances the complexity of the ABC transporter story in lignin biosynthesis (Verrier et al., 2008; Ruzicka et al., 2010; Lee et al., 2008; Kaneda et al., 2011). Therefore, mutants disrupted in multiple ABC transporter genes and a precise "cell type-specific" lignin profiling are required for future research in this area of lignin deposition.

The final step of lignification is the polymerization of lignin monomers by oxidative-coupling process catalyzed by perxidases, laccases and other phenol oxidases (Freudenberg,

1959; Ralph et al., 2004; Davin et al., 2008). Various bonds are formed to construct a heteropolymer of lignin, with the most abundant, but least strong interaction bond being β-O-4 (Figure 1.2A). Other bonds, including β-O-5, β-β, β-1, 5-5, 4-O-5, are present in less amount but are more resistant to chemical degradation (Figure 1.2A; Boerjan et al., 2003). Peroxidase uses hydrogen peroxide (H_2O_2) as its electron receptor to oxidize various phenolic compounds, preferrably the coniferyl alcohol to generate a monolignol radical (Harkin and Obst, 1973). In contrast, sinapyl alcohol is a poor substrate for oxidation and only specific peroxidases are able to directly oxidase sinapyl alcohols (Sasaki et al., 2006; 2008). Thus, a radical-mediated lignin polymerization model was initially described by Hatfield and Vermerris (2001), such that peroxidase will first oxidize a coniferyl alcohol to form a highly oxidative coniferyl alcohol radical. Subsequently, the coniferyl alcohol radical, in one aspect, can be directly polymerized to lignin monomer at a higher oxidation state through a coupling reaction; in the other aspect, the highly oxidative state of the coniferyl alcohol radical can be transferred to sinapyl alchol or to a lignin polymer with a basic oxidative state to generate sinapyl alcohol radicals and high-oxidative state lignin polymers, respectively. Later a diffusible redox shuttle-peroxidase system was proposed regarding the mechanism of lignin monomer/polymer oxidation as a supplement to the model. In this case, a manganese ion will be oxidized by a membrane-bound peroxidase and diffuse through small pores in the plant cell wall to the apoplastic region, where the oxidative ion will subsequently oxidize both monolignols and polymers (Onnerud et al., 2002). However, the finding of a sinapyl alcohol-specific peroxidase that is able to directly oxidase sinapyl alcohol and lignin polymer strongly challenges the hypothetical radical-/redox-shuttle-mediated polymerization model described above (Sasaki et al., 2004).

Laccases are a group of copper-containing enzymes that oxidize phenolics and inorganic and aromatic amines using O_2 as an electron receiver (Reinhammar and Malmstroem, 1981). The ability of laccase to oxidize monoligols has been demonstrated *in vitro* (Freugdenberg, 1959; Sterjiades et al., 1992; Bao et al., 1993; Takahama, 1995; Richardson et al., 1997). Laccase-like activities are also detected in various lignifying tissues (Driouich t al., 1992; Bao et al., 1993; Liu et al., 1994; Richardson et al., 2000). The most well studied examples are Arabidopsis *LAC4* and *-17* genes, the double mutant of which displays a 30~40% reduction of lignin content (Berthet et al., 2011). Like most of the peroxidases, LAC17 shows a preference for conferyl alcohols as substrates. Currently, no sinapyl alcohol-specific LAC members are known. Moreover, the

double mutant *lac4lac17* displays unique suppression of lignin biosynthetic gene transcriptions suggesting the existence of a feedback regulation mechanism (Berthet et al., 2011).

Generally, peroxidase/laccase-mediated polymerization process results in a "random-coupled", optically inactive and racemic lignin heteropolymer (Vanholme et al., 2010). The characterization of non-enzymatic dirigent protein challenges this hypothesis. Dirigent protein was originally identified as a guide protein for the biosynthesis of pinoresinol, an optically active dimer of coniferyl alcohols through β-β bond (Davin, et al., 1997). This guiding function of dirigent protein was then further expanded to lignin polymerization where it was thought to guide the coupling process of phenolic radicals and serve as the initiation site of lignin polymerization (Davin and Lewis, 2000; 2005; Davin et al., 2008; Donaldson, 2001). However, this hypothesis remains imperfect since no biochemical or genetic evidence is available to define the roles of characterized dirigent proteins.

1.2.1.4 Regulation of lignin metabolism in plants.

As described above, the deposition of lignin occurs at specific developmental stages and displays complicated spatial deposition patterns, which together imply the existence of a sophisticated regulation system controlling the entire lignification process. How lignin genes are coordinately regulated remained a mystery until the characterization of AC-elements in the promoter region of the bean *PAL2* gene (Hatton et al., 1995). The presence of AC-I (ACCTACC), AC-II (ACCAACC), and AC-III (ACCTAAC) is indispensable for the xylem-specific expression pattern of the *PAL2* promoter by a GUS-reporter gene assay (Seguin et al., 1997). AC-elements have been discovered in the promoter regions of most of the lignin genes, including *PAL*s, *4CL*, *C3'H*, *CCoAOMT*, *CCR* and *CAD* (Raes et al., 2003). None of the AC-elements were discovered from the promoter regions of *C4H* and *COMT1* genes. However, they may contain degenerate AC-elements since they are responding to the same type of MYB transcription factors as the lignin genes containing AC-elements (Zhou et al., 2009). The only exception is the S-lignin-specific gene *F5H1,* since no AC-element is present in its gene sequence and it is not regulated by lignin specific transcription factors *MYB58* and *-63* (Zhou et al., 2009), suggesting a differential regulation of *F5H1* from other lignin biosynthetic genes (Figure 1.3). Zhao et al. (2010) demonstrated that the *Medicago F5H* gene was regulated by the Arabidopsis secondary cell wall master switch *NST3*/*SND1* by direct binding of *SND1* to the promoter of *Medicago F5H*. However, in Arabidopsis, no interaction between *F5H1* and *SND1* was reported (Ohman et al.,

2012). Zhong et al. (2010) also did not report the activation of Arabidopsis *F5H* as a direct target of *SND1*. Instead, the decrease of both S monolignols and *F5H1* transcription were reported in mutant *myb103* (Ohman et al., 2012), suggesting the Arabidopsis *F5H1* is under regulation of this transcription factor *MYB103*. However, *MYB103* failed to transactivate *F5H1* promoter; other MYB and SND transcription factors were also down regulated in *myb103* mutant (Ohman et al., 2012). Taken together, these observations suggest the existence of additional transcription factor(s) as the target of *MYB103* and that further activate *F5H1* (Figure 1.3). The direct binding of Arabidopsis *SND1* with *Medicago F5H* might be a species-specific observation and may not be a general regulation pattern among other plant species.

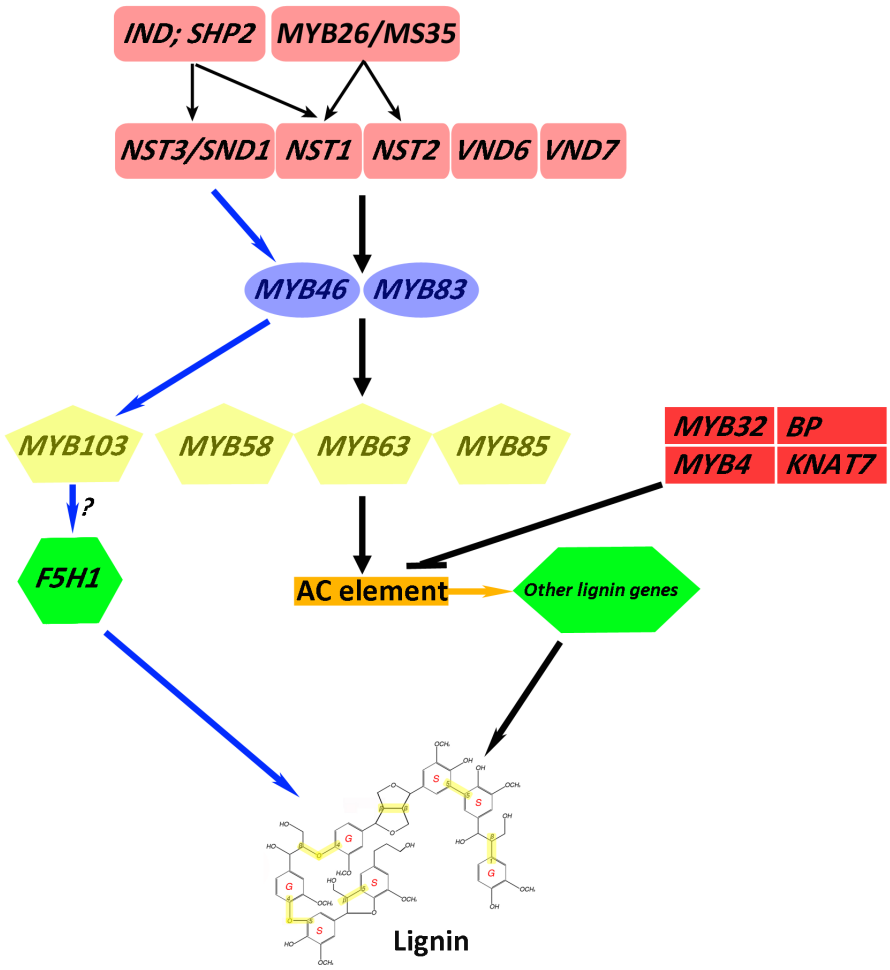

Figure 1.3 Network of transcription factors regulating lignification in Arabidopsis.

The schematic image of the Arabidopsis genetic regulation of lignification; The first level of "master switch" transcription factors *NST3*/*SND1* and *NST1* are regulated by organ identity genes *IND* and *SHP2* (Mitsuda and Ohme-Takagi, 2008); while *NST1* and *NST2* are regulated by *MYB26*/*MS35* (Yang et al., 2007). The upstream regulating of *VND6* and *-7* are not reported. First level of "master switch" transcription factors will further activate second level of "master switch" transcription factors, *MYB46* and *MYB83*. The second level of "master switch" transcription factors will further activate lignin specific transcription factors *MYB58*, *-63* and *-85*, which will subsequently activate the lignin biosynthetic genes containing AC-elements, except gene *F5H1* which is under regulation of *MYB103*. The interaction between *MYB103* and *F5H1* are not clearly identified (as indicated by "?"). Negative regulators of lignin biosynthetic genes include *MYB32*, *-4*, *KNAT7* and *BP*. The figure is adapted from Zhao and Dixon, 2011 with new published data. The regulations of other secondary cell wall polymers by the two levels of "master switch" are omitted. Gene Abbreviations: *IND, INDEHISCENT; SHP, SHATTERPROOF; MS, MALE STERILE; NST, NAC SECONDARY WALL THICKENING PROMOTING; SND, SECONDARY WALL-ASSOCIATED NAC DOMAIN; BP, BREVIPENDICELLUS; KNAT7, KNOTTED-LIKE HOMEOBOX OF ARABIDOPSIS THALIANA; F5H, FERULIC ACID 5'-HYDROXYLASE*.

The ability of MYB transcription factors to activate phenylpropanoid genes was identified even before the discovery of AC elements in the bean *PAL2* gene (Sablowski et al., 1994; 1995). Subsequent research conducted on transgenic tobacco plants over-expressing *Antirrhinum MYB308* and *MYB330*, as well as MYB proteins identified from *Arabidopsis thaliana*, *Pinus taeda*, *Eucalyptus gunnii* and *Populus trichocarpa*, reinforced the observation that MYB transcription factors regulate lignin biosynthetic genes (Tamagnone, et al., 1998; Borevitz et al., 2000; Patzlaff et al., 2003; Zhou et al., 2009; Legay et al., 2007; Lacombe et al., 2000; Bomal et al., 2008; Anjanirina et al., 2010; Goicoechea et al., 2005; McCarthy et al., 2010; McCarthey et al., 2009; Zhong et al., 2007). Therefore, MYB transcription factors are the direct regulators of plant secondary cell wall lignification. Lignin-specific transcription factors were first characterized in Arabidopsis, including *MYB85*, *MYB58* and *MYB63* (Zhong et al., 2008; Zhou et al., 2009; Figure 1.3). Later *Pinus taeda PtMYB1* was demonstrated to be a homologue of Arabidopsis *MYB85* (Bomal et al., 2008). Based on their functions, these transcription factors are classified as negative repressors including *MYB4*, *MYB32* and *BREVIPEDICELLUS* (*BP*), and positive regulators including *MYB83*, *MYB58*/*63*, *MYB85* and *MYB61* (Zhao and Dixon, 2011; Figure 1.3). In addition to MYB transcription factors, LIM domain containing transcription factors have been identified from tobacco plants were also shown to regulate lignin genes *PAL*, *4CL* and *CAD* and affect lignin deposition (Kawaoka and Ebinuma, 2000; 2001).

Some of the MYB transcription factors are able to control the formation of entire secondary cell walls by regulating the biosynthesis of other polymers such as cellulose and hemicellulose. Typical examples of this type of MYB transcription factors include Arabidopsis *MYB46* and its homologues *MYB83* in Arabidopsis; *EgMYB2*, *PtMYB4*, *PtMYB8*, *PtrMYB3*, and *PtrMYB20* in other plant species (Bomal et al., 2008). Due to the ability to regulate entire secondary cell wall metabolism and activate other MYB transcription factors such as *MYB58* and *MYB63* (Zhou et al., 2009), *MYB46*/*MYB83* is considered as a "master switch" of secondary cell wall biosynthesis program. Similarly, another group of Arabidopsis transcription factors containing NAC domain, including *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*), *NST2*, *SECONDARY WALL-ASSOCIATED NAC DOMAIN 1* (*SND1*)/*NST3*, *VASCULAR-RELATED NAC-DOMAIN 6* (*VND6*) and *-7* are also controlling the entire secondary cell wall metabolism (Kubo et al., 2005; Zhong et al., 2006; 2007; Mitsuda et al., 2007; Zhao et al., 2010). Subsequent research demonstrated that *MYB46*/*MYB83*, together with

SND3, *MYB103*, and *KNOTTED-LIKE HOMEOBOX OF ARABIDOPSIS THALIANA 7* (*KNAT7*) are direct targets of *SND1*/*NST3* (Zhong et al., 2008). *VND6* and -*7* are also able to up-regulate *MYB46* directly (McCarthy et al., 2009). Based on this observation, *SND1*/*NST3*, *NST1*, *VND6* and -*7* are classified as first-level master switch and *MYB46* and its homologue *MYB83* as "second-level master regulator". The master regulators activate down-stream transcription factors that will eventually activate lignin biosynthetic genes. All of the transcription factors described above form a complicated, multi-layered regulation network which is capable of coordinately regulating lignin metabolism in secondary cell wall metabolism (Zhao and Dixon, 2011; Figure 1.3). However, experimental data suggests that such a network is not complete. For example, *NST3* itself cannot activate lignification in developing interfascicular fiber cells (in which no *MYB46* activity is detected), suggesting that an unknown negative repressor is present and able to suppress the expression of *MYB46* and further prevent lignification at improper timing points of fiber cell development (Zhong et al., 2006; 2007).

Many lignin transcription factors display unique expression patterns and are responding to phytohormones and environmental stimuli. First-level master switches display tissue-specific expression patterns. *SND1* specially controls the secondary cell wall metabolism in fibers while *VND6* and -*7* are indispensable to the secondary cell wall thickening in vessels (Kubo et al., 2005; Zhong et al., 2006; Mitsuda and Ohme-Takegi, 2008; Mitsuda et al., 2008; Yamaguchi et al., 2008; Zhong et al., 2008). Both fiber-specific and vessel-specific first-level master switches can activate *MYB46/83*. In the case of other lignin activating transcription factors, most are expressed in lignifying tissues, while lignin repressors are expressed in tissues with little or no lignin deposition as well as at particular times in lignifying tissues to prevent lignification at improper developmental stages. For example, the Arabidopsis lignin repressor *MYB32* is highly expressed in flowers rather than in stems and greatly activated by auxin (Preston et al., 2004). The aspen lignin repressor *MYB21* is also induced by auxin, but highly expressed in lignifying zones (Karpinska et al., 2004). In addition to auxin, gibberellin, cytokinin, jasmonic acid, ethylene and brassinosteroid are also involved in regulating lignification process (Ellis et al., 2002; Zhong et al., 2002; Karpinska et al., 2004; Burx et al., 2008; Hossain et al., 2012). However, the evidence is fragmentary and the connecting signaling components linking phytohormones and lignification remain to be identified. A mitogen-activated protein kinase

(MAPK) cascade is very likely to play a role in this connection due to the fact that *PtMAPK6* is able to phosphorylate *PtMYB1* and *-4* (Morse et al., 2009).

1.2.2 Flavonoid metabolism.

In addition to the monolignol pathway, the flavonoid pathway generates another group of important secondary metabolites, which have essential physiological functions in plants (Ververidis et al., 2007; Buer et al., 2010). For instance, some metabolites of flavonoid pathway, such as anthocyanins, serve as visual attractors of pollinator insects to facilitate pollination process (Mol et al., 1998). They can also influence the transport of auxin (Peer and Murphy, 2007). In legume plants, flavonoids are known to provide critical contributions in the legume-bacterium symbiosis (Redmond et al., 1986; Djordjevic et al., 1987; Wasson et al., 2006; Zhang et al., 2009). In addition, flavonoid compounds also contribute to plant immunity. For instance, flax over-expressing a GT Family 1 *Solanum sogarandinum* Glycosyltransferase (*SsGT1*) displays enhanced resistance against fungal pathogen *Fusarium* and such enhance resistance is dependent on an increase of flavonoid glycoside (Lorenc-Kukuła et al., 2009).

Recently, plant flavonoids are receiving more and more attention due to their therapeutic effects in animals. The diversities of flavonoid biochemical structures allow them to interact with different target molecules identified from animals, including humans (William and Grayer, 2004; Taylor and Grotewold, 2005; Peer and Murphy, 2007). Flavonoids act as anti-oxidant, anti-proliferative, anti-tumor, anti-inflammatory and pro-apoptotic agents in animals and are potentially benefical at contributing to cure of many human diseases such as cancer, Alzheimer's disease and cardiovascular disease (Williams et al., 2004; Taylor and Grotewold, 2005; Garcia-Mediavilla et al., 2007; Pandey et al., 2007; Sung et al., 2007; Kim et al., 2008; Singh et al., 2008). Some of the molecular targets of flavonoids in animals have been characterized in the work described above (eg. quercetin affects cancer by inhibiting a group of kinases; Boly et al., 2011), which opens the gate for genetic engineering of plant flavonoids for pharmaceutical purposes. Taken together, a comprehensive understanding of plant flavonoid metabolism can benefit to both agriculture and other related industries, such as the health of human beings.

The biosynthesis of flavonoids is branched from the general phenylpropanoid pathway from ρ-coumaroyl-CoA (Figure 1.1). The subsequent biosynthetic process has been extensively studied in the model plant Arabidopsis (Figure 1.4), due to the availability of mutants disrupted in each individual flavonoid biosynthetic gene, as well as transcription factors regulating

flavonoid metabolism (Buer et al., 2010). Most of these mutants, commonly known as *transparent testa* (*tt*) lines, can be easily identified by their seed testa colors; wild type seeds display a dark red-brown color while flavonoid mutant seed shows a range from lighter brown, tan colors through to the lemon-yellow color of mutant *tt4* seeds due to the different levels of seed coat proanthocyanidin (Debeaujon et al., 2000).

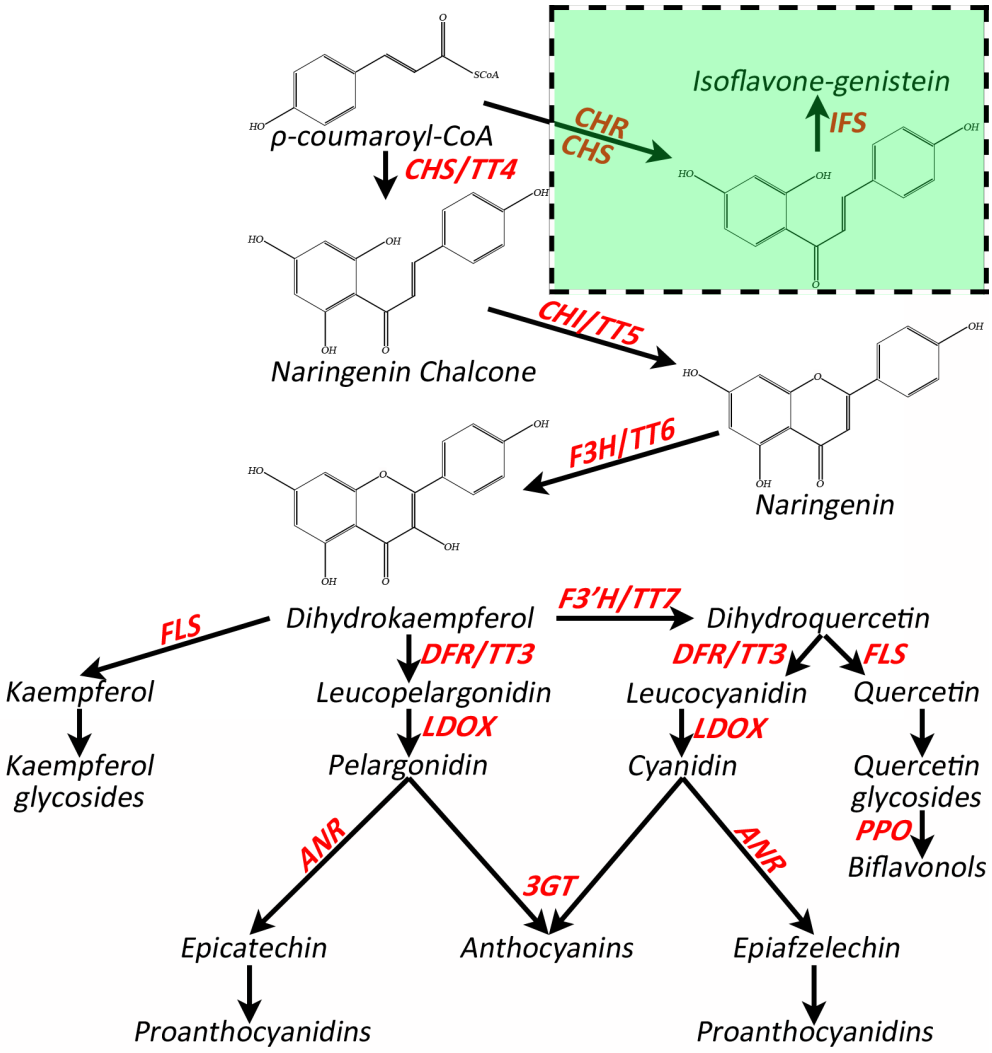

Figure 1.4 Flavonoid biosynthesis pathway.

The schematic image of plant flavonoid biosynthesis. Image is adapted from Buer et al., (2010). Only the chemical structures of metabolites involved in early steps are shown. Green shadow box with dashed line highlights the isoflavonoid branch, which is not normally in Arabidopsis and is mainly restricted to legume plants. All genes are labeled with red fonts. Gene abbreviations: *TT, transparent testa; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavonone 3-hydroxylase; F3'H, flavonol 3'-hydroxylase; DFR, dihydroflavonol reductase; PPO, polyphenol oxidase; 3GT, anthocyanidin 3-O-glycosyltransferase; ANR, anthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase. FLS, flavonol synthase; CHR, chalcone reductase; IFS, isoflavone synthase.*

Flavonoid pathways in other plant species have also been analyzed, especially in legume plants, which lead to the discovery of isoflavone metabolism (Figure 1.4, green box with dash line; Buer et al., 2010). Isoflavone metaoblism is mediated by chalcone reductase (CHR) and isoflavone synthase (IFS; Akashi, et al., 1999; Steele et al., 1999; Jung et al., 2000; Overkamp et al., 2000). It is commonly believed that Arabidopsis does not possess CHR and IFS enzymes (Aoki et al., 2000). However, transgenic Arabidopsis expressing the legume gene *IFS* is able to produce isoflavone metabolites, suggesting that isoflavone metabolism may exist in Arabidopsis at an undetectable level (Jung et al., 2000; Yu et al., 2000; Liu et al., 2002). In fact, isoflavone metabolites have been detected at low level by high performance liquid chromatography-mass spectrometry (HPLC-MS) assay in wild type Arabidopsis Col-0 and *Lepidium sativa* (Lapcik et al., 2006). Therefore, it has been hypothesized that Arabidopsis might be able to generate isoflavone through putative *CHR*-like genes and another unknown gene for the construction of the isoflavone skeleton (Lapcik et al., 2006).

1.2.3 Roles of phenolic metabolites in plant defense.

Phenylpropanoid compounds also play essential roles in plant defense against pathogen attacks (Dixon et al., 2002). As described in section 1.1, the plant defense system consists of both constitutive and inducible defenses. Phenylpropanoid compounds have been commonly identified in all defensive reactions, including constitutive phytoanticipin, inducible phytoalexins, and the signaling molecules, which regulate the plant defense system (VanEtten et al., 1994; Naoumkina, et al., 2010).

As a major branch of phenylpropanoid metabolism, lignin has been shown to be part of plant defense against plant pathogens. Pathogen-induced up-regulation of lignin biosynthetic genes has been characterized in various pathosystems (such as wheat-*Blumeria. gramins* f.sp. *tritici*; Bhuiyan et al., 2009). Lignin also accumulates at pathogen-induced hypersensitive reaction regions (Reimers and Leach, 1991; Lange et al., 1995; Bhuiyan et al., 2009) and serves as an important structural component of papillae in penetration resistance against fungal pathogens (McLusky et al., 1999). The major function of lignin is assumed to be its physical barrier and structural property (Moerschbacher et al., 1990), although evidence also implies other functions in plant defense. For example, lignan, a dimer of cinnamic alcohols, functions in plant defense system as a phytoanticipin and phytoalexin with anti-bacterial and anti-fungal activities (Carpinelle et al., 2005; Ralph et al., 2006; Akiyama et al., 2007). Coumarins comprise another

group of phenolic compounds derived from intermediates of lignin metabolism (including caffeoyl CoA and feruloyl CoA) and are induced under stress conditions (Gachon et al., 2004; Shimizu et al., 2005). Some coumarin compounds, such as scopoletin and its glucose derivatives, show special effects on plant viruses by possibly regulating the accumulation of reactive oxygen intermediates (Chong, et al., 2002; Matros and Mock, 2004).

The major function of flavonoid metabolites in plant defense is as anti-microbial compounds (Treutter, 2005). Different flavonoid compounds target different functions within microbial cells to achieve their anti-microbial activities. For example, quercetin is able to inhibit *Escherichia coli* DNA gyrase by binding to the GyrB subunit (Plaper et al., 2003) while sophoraflavanone and catechins can disturb the normal function of bacterial plasma membrane (Tsuchiya and Iinuma, 2000; Tamba et al., 2007).

Isoflavonoid compounds uniquely exist in legume plant and many of them also have shown antimicrobial activities. Pterocarpans characterized from various species of legume plants represent a major class of isoflavonoid phytoalexins. For instance, disruption of pisatin biosynthesis in pea results in enhanced susceptibility against the fungal pathogen *Nectria haematococca* (Wu and VanEtten, 2004).

1.3 Overview of research hypothesis and objectives.

Although it is commonly agreed that lignin polymers may serve as physical barriers against pathogen infection, the role of lignin in plant immunity has not been proven with direct genetic evidence. Especially, the role of the lignin gene, *F5H1* that mediates S lignin biosynthesis is unclear. S lignin is uniquely present in angiosperm plants among spermatophytes. It is a relatively recent innovation (~125 million years ago) during the long period of land plant evolution (~450 million years ago), although the lycophyte *Selaginellales* incorporates S lignin metabolism via a route distinct from angiosperm plants (Weng et al., 2010a). The biosynthesis of the S lignin unit is initiated by the cytochrome P450-dependent monooxygenase ferulic acid 5‘ hydroxylase (F5H; Figure 1.1). Mutant lines with lignin gene alleles that result in reduced deposition of total lignin, such as *irx4*/*ccr1*, usually have a collapsed form of vascular tissue (Jones et al., 2001). However, elimination of S lignin by knockout of the *F5H1* gene does not significantly affect the normal growth of plants nor the formation of the vascular system (Meyer et al., 1998; Weng et al., 2010a) suggesting that the S lignin unit may not be indispensable to the

functioning of lignin polymers as a mechanical support. Moreover, previous research conducted on wheat revealed the pathogen-induced transcription of all lignin genes with a particularly strong induction at 24 hours post inoculation (hpi) of *F5H* by *B. graminis* f.sp. *tritici* in a CWA-based resistant cultivar (Bhuiyan et al., 2009). This observation suggests that *F5H* gene may play a role in plant immunity system. Considering the fact that broad leaf morphology of angiosperm plants should receive more microbial pathogens (fungal conidia, bacterial, *etc*.) than needle-shape leaves of gymnosperm plants, I hypothesized that *F5H* plays a critical role in angiosperm plant immunity system against pathogen infections.

The putative roles of S lignin in plant defense have been investigated in several plant-pathogen interaction studies. A sinapyl alcohol-specific cinnamyl alcohol dehydrogenase (CAD) is induced in elicitor-treated wheat leaves (Mitchell et al., 1999), and S lignin is accumulated in wheat leaves challenged by rice rust or fungal elicitors (Menden et al., 2007). An Arabidopsis transgenic line with an enhanced level of S lignin restricts the reproduction of the nematode pathogen *Moleoidegyne incognita* (Wuyts et al., 2006). Moreover, disease screening with host pathogen *Colleotrichum higginsianum* on Arabidopsis mutants disrupted in each lignin genes has shown that only the *f5h1*-1 mutant was more susceptible than the wild type Col-0; mutants disrupted in other lignin genes displayed similar level of susceptibility as wild type Col-0 (Liu G and Wei Y, unpublished data). Based on all the knowledge and experimental results as described above, the hypothesis is proposed that *F5H1* might play critical role in the plant immunity system against pathogen infections.

In addition to the lignin branch study, mutants of flavonoid genes are also examined for their resistance against pathogens. Since flavonoid compounds might contribute to plant immunity as anti-microbial agents, Arabidopsis mutants eliminating the biosynthesis of majority of flavonoid compounds, including mutants of *tt4*, *tt5* and *tt6* are tested in this project for their resistance against pathogens. Since they eliminate the generation of downstream flavonoid compounds by blocking the initial three metabolic steps of Arabidopsis flavonoid pathway (Figure 1.4), an enhanced susceptibility is expected upon host pathogens due to the absence of potential flavonoid anti-microbial agents.

The goal of this project was to investigate the roles of phenylpropanoid pathway, especially the lignin branch and part of the flavonoid pathway, in plant immunity using molecular genetic tools. In particular, the project was divided into four aspects described below:

1. Characterizing the transcriptional responses of lignin biosynthetic genes to pathogen attack within a complete pathogen infection cycle;
2. Identifying the role of *F5H1* in Arabidopsis defense against pathogen attack;
3. Revealing the interaction between *F5H1* and other defensive components such as *pen1, pen2, pen3, pmr2* and *pmr4*;
4. Investigating the role of loci in the upstream flavonoid pathway in the Arabidopsis defense system.

Two different types of fungal pathogens were used in this project, the anthracnose pathogen *Colletotrichum* and powdery mildew. *Colletotrichum* is a large group of ascomycete genus causing plant anthracnose disease or blight worldwide on a broad spectrum of host plants and many of them are important economic crops such as grain, legumes, cereals and fruits (Bailey and Jeger, 1992). As a haploid organism in most stages of its life cycle, *Colletotrichum* can be cultured axenically and is easily manipulated by transformation to generate mutants. Moreover, many *Colletotrichum* species undergo a hemibiotrophic life cycle: pathogens begin their infection process in a biotrophic stage on living host cells and subsequently switch to a necrotrophic stage to eventually kill host cells (O'Connell et al., 2004). Therefore, *Colletotrichum* has been widely employed together with Arabidopsis as a model pathosystem to analyze plant defense responses.

Different species of *Colletotrichum* possess distinct levels of pathogenicity on Arabidopsis. One host species *Colletotrichum higginsianum* and one nonhost species *Colletotrichum destructivum* were used in this project. *C. higginsianum* and Arabidopsis have been widely used as a compatible pathosystem (Liu et al., 2007). *C. destructivum* usually fails to infect Arabidopsis and instead induces callose-containing papillae at attempted penetration sites similar to other nonhost species of *Colletotrichum* (O'Connell, et al., 2004; Shimada, et al., 2006). In general, after landing on the plant epidermis, the *Colletotrichum* conidia first germinate and form melanized appressoria on the surface of plant cell. The appressoria further penetrate through the host epidermal cuticle and cell wall by two alternative penetration models: turgor-mediated invasion and hyphal tip-based entry (Hiruma et al., 2010). Successful penetration will facilitate the development of primary hyphae in the living cell, the typical hallmark of biotrophic stage. The development of secondary hyphae indicates the switch from biotrophic to necrotrophic stage, and the pathogen will eventually kill infected host cells.

Powdery mildew pathogens are obligate biotrophic pathogens with a broad host spectrum on both dicot and monocot plant species (Inuma et al., 2007). They feed on living host cells by direct penetration through the host cell wall and formation of feeding organs, haustoria, to obtain nutrients from host cell. Subsequently, they extend hyphae over the surface of infected cells and accomplish their life cycle by formation of conidiophores (Lipka et al., 2008). Two species of powdery mildew pathogens, *Blumeria graminis* f.sp. *hordei* (barley powdery mildew) and *Erysiphe cichoracearum* (cucumber powdery mildew), were used in this study. *B. graminis* f.sp. *hordei* is a non-host pathogen to Arabidopsis, while *E. cichoracearum* is a host pathogen. Most *B. graminis* f.sp. *hordei* conidia fail to penetrate through host cell wall. The post-invasive growth of occasionally successful *B. graminis* f.sp. *hordei* conidia will be restricted by post-invasion resistance of the infected plant cells. *E. cichoracearum* is able to complete the entire life cycle on Arabidopsis (Lipka et al., 2005). Both *B. graminis* f.sp. *hordei* and *E. cichoracearum* have been used by many researchers to study Arabidopsis immunity, especially penetration resistance (Lipka et al., 2005, Mang et al., 2009).

CHAPTER 2

MATERIALS & METHODS

2.1 Plant materials and growing conditions.

All of the *Arabidopsis thaliana* lines used in the experiments described in this thesis are summarized in Table 2.1. All of these mutant alleles were created in Arabidopsis ecotype Col-0 background as described in each corresponding reference. Plants were grown at 22˚C with a 16-hr light/8-hr dark photoperiod and a light intensity at 100 μmol m^{-2} s^{-1} in a controlled-environment growing chamber. Sunshine® Mix 1 professional growing mix was purchased from Sun Gro Horticulture (Seba Beach, CA) and used to prepare the growth bed in pots. The seeds were initially sown out in round pots (4-inch diameter; Kord Product, Toronto, CA) for bulk growth. After sowing seeds, the pot was covered by a piece of plastic wrap and incubated in 4˚C fridge for 3 days to break dormancy and synchronize germination (Weigel and Glazebrook, 2002). When plants reached the 4-leaf stage (the development of the first pair of true leaves), seedlings were transferred manually to square pots (3.5-inch by 3.5-inch; Kord Product, Toronto, CA) at a density of 5 plants per pot for all subsequent experiments.

Table 2.1 List of plant materials.

Mutant Name	Corresponding locus	Type of plant	TAIR annotation of corresponding gene	Commet/Source	Reference
f5h1-1	At4g36220	SNS	Encodes ferulate 5-hydroxylase (F5H). Involved in lignin biosynthesis.	Used as the major mutant in this study	Meyer et al., 1998
f5h1-2	At4g36220	SNS	Same as above	Originally identified as *fah1*-7; ABRC stock CS8604	Chapple et al., 1992
f5h1-3	At4g36220	T-DNA	Same as above	SALK_063792 from ABRC	This project
f5h2-1	At5g04330	T-DNA	Cytochrome P450 superfamily protein	SALK_064404c from ABRC	This project
C4H-F5H	n/a	TP	n/a	Overexpression of *F5H1* under *C4H* promoter.	Meyer et al., 1998
sid2-1	At1g74710	SNS	Encodes a protein with isochorismate synthase activity.		Wildermuth et al., 2001
pad4-1	At3g52430	SNS	Encodes a lipase-like gene that is important for salicylic acid signaling and function in resistance (R) gene-mediated and basal plant disease resistance.	CS3806 from ABRC	Glazerbrook et al., 1997
sag101	At5g14930	T-DNA	Encodes an acyl hydrolase involved in senescence.		Fey et al., 2005
NahGox	n/a	TP	n/a	Transgenic plant expressing Pseudomonas gene NahG; disrupted in SA signaling pathway by degrading SA;	Lawton et al., 1995
jar1-1	At2g46370	SNS	Encodes a jasmonate-amido synthetase that is a member of the GH3 family of proteins.	CS8072 from ABRC	Staswick et al., 1992
ein2-1	At5g03280	SNS	Involved in ethylene signal transduction. Acts downstream of CTR1. Positively regulates ORE1 and negatively regulates mir164A,B,C to regulate leaf senescence.	CS3071 from ABRC	Guzmán and Ecker, 1990
pmr2-1	At1g11310	SNS	A member of a large family of seven-transmembrane domain proteins specific to plants, homologs of the barley mildew resistance locus o (MLO) protein.		Vogel and Somerville, 2000

pmr4-1	At4g03550	SNS	Encodes a callose synthase that is required for wound and papillary callose formation in response to fungal pathogens *Erysiphe* and *Blumeria*		Vogel and Somerville, 2001
pen1-1	At3g11820	SNS	Encodes a syntaxin localized at the plasma membrane (SYR1, Syntaxin Related Protein 1, also known as SYP121, PENETRATION1/PEN1).		Collins et al., 2003
pen2-1	At2g44490	SNS	Encodes a glycosyl hydrolase that localizes to peroxisomes and acts as a component of an inducible preinvasion resistance mechanism. Required for mlo resistance.		Lipka et al., 2005
pen3-1	At1g59870	T-DNA	ATP binding cassette transporter.	SALK_000578 from ABRC	Stein et al., 2006
tt4-T1	At5g13930	T-DNA	Encodes chalcone synthase (CHS), a key enzyme involved in the biosynthesis of flavonoids. Required for the accumulation of purple anthocyanins in leaves and stems.	GK_304D03 from NASC	Shirley et al., 1995
tt5-T1	At3g55120	T-DNA	Catalyzes the conversion of chalcones into flavanones. Required for the accumulation of purple anthocyanins in leaves and stems.	SALK_034145 from ABRC	Shirley et al., 1995
tt6-T1	At3g51240	T-DNA	Encodes flavanone 3-hydroxylase that is coordinately expressed with chalcone synthase and chalcone isomerases. Regulates flavonoid biosynthesis.	SALK_113321 from ABRC	Wisman et al., 1998

SNS: single nucleotide substitution; SND: single nucleotide deletion; T-DNA: T-DNA insertion; TP: transgenic plant. Seeds were purchased from ABRC if there is a stock number assigned in the "Source" column (*tt4*-T1 is from Nottingham Arabidopsis Stock Centre/NASC). Other seeds were provided by the original authors who characterized the mutant as listed in the reference column.

2.2 Pathogenic fungal materials, cultivation and inoculation protocol.

Colletotrichum higginsianum: *C. higginsianum* (IMI349061) was obtained from CABI Bioscience (Egham, UK) and maintained in Dr. Yangdou Wei's lab. *C. higginsianum* was cultured on potato dextrose agar (PDA) plates. PDA medium was prepared by mixing 39 g of Difco® PDA (Becton Dickinson and Company; Sparks, US) in 1 L of distilled water. The medium was autoclaved at 121˚C/15 psi for 25 min and poured into 100 mm×15 mm polystyrene petri dish (Fisher Scientific; Ottawa, CA) to make a PDA plate. To prepare fresh plate inoculum, an inoculation loop was used to collect conidia from a stock plate and, subsequently, the surface of the newly prepared PDA plates were touched in five evenly distributed spots to inoculate the conidia from the stock plate. After inoculation, the PDA plate was incubated at room temperature until the generation of mature conidia, which normally show a pink color on the plates from 10 to 14 days after inoculation. The conidia were later collected by an inoculation loop and suspended in sterile water. After gently centrifuging at 5000 revolutions per min (rpm) in an Eppendorf 5415R centrifuge equipped with an Eppendorf F45-24-11 rotor (Mississauga, CA) for 1 min, the supernatant was carefully removed by a micropipette and the conidia were re-suspended in 1 ml of sterile water and further diluted with desired amount of water, depending on the number of plants to be inoculated: for 12 pots of Arabidopsis with 5 plants per pots, 45 ml of conidia suspension was sprayed evenly to cover the surface of rosette leaves. The density of conidia was measured by a hemocytometer to maintain 1×10^6 conidia per ml. The prepared inoculum solution was sprayed on 3~4 weeks old Arabidopsis plants with a Continental E-Z sprayer (Brampton, CA). To maintain a high humidity for better pathogen growth, the tray containing inoculated plants was covered by a transparent plastic lid and sealed with tape. For the experiments shown in Figure 3.4a, 11a, 15, 23 and 31, the lid was maintained on the inoculated plants until the time of photography. The plants shown in Figure 3.9, after photography and sampling for microscopic analysis at 2 dpi, were returned to the growing chamber for another two days without plastic lid covered on the plants. The plants were further photographed at 4 dpi. The leaf tissue leision spots were photographed by a Nikon D70 digital camera (Mississauga, CA).

Colletotrichum destructivum: *C. destructivum* isolate AJ558106 was originally obtained from Dr. Gary Peng's lab in Agriculture and Agri-Food Canada, Saskatoon, Saskatchewan and maintained in the lab. The culture and preparation of *C. destructivum* inoculum were performed

in same method as *C. higginsianum*. The conidia suspension of *C. destructivum* was inoculated by placing a 15-µl droplet on the center of Arabidopsis rosette leaves (approximately 3~4 weeks old before the bolting of the floral stem). After inoculation, the tray containing inoculated plants was covered by a transparent plastic lid and sealed with tape to maintain humidity required for the fungal development.

Magnaporthe oryzae: *M. oryzae* isolate P131 was kindly provided by Dr. Youliang Peng (China Agricultural University) and cultured on oatmeal medium plates. The oatmeal medium was prepared by mixing 30 g of No Name® natural oat bran (Loblaws Inc.; Calgary, CA) and 15 g of agar (EMD Chemicals; San Diego, US) in 1 L of distilled water. The medium was autoclaved at 121˚C/15 psi for 25 min and poured into 100 mm×15 mm polystyrene petri dish (Fisher Scientific; Ottawa, CA) to make oatmeal medium plates. Six agar blocks (1-cm by 2-cm) covered with *M. oryzae* mycelium were cut from a stock plate with a sterile scalpel and placed evenly on 1 fresh made oat meal medium plate and the plate was incubated at room temperature with light for 1~2 weeks. To prepare inoculum, 1 ml of sterile distilled water was added to the surface of the oatmeal plate and a spreader was used to suspend the fungal material by gently scrapping the entire surface without damaging the medium below. The suspension of fungal materials was collected and filtered through a piece of Miracloth (EMD Chemicals; San Diego, US) to separate conidia from other fungal materials. The suspension with conidia was gently centrifuged at 5000 rpm for 1 min and the supernatant was removed. The resulting *M. oryzae* conidia were further suspended in sterile distilled water to reach a density of 1×10^4 conidia per ml (Koga and Nakayashi, 2004) and sprayed on Arabodposis plants as above. To maintain a high humidity for better pathogen growth, the tray containing inoculated plants was covered by transparent plastic lid and sealed with tape.

Powdery mildew: two different species *Blumeria graminis* f.sp. *hordei* and *Erysiphe cichoracearum* were used in the experiment described in this thesis. Both of these powdery mildew pathogens had been previously isolated from field in Saskatoon and maintained in Dr. Yangdou Wei's lab. *B.graminis* f.sp. *hordei* was cultured on six-row spring hulless barley "CDC Silky" and *E. cichoracearum* was cultured on cucumber "National Pickling" (Early's Farm & Garden Center; Saskatoon, CA). Inoculation was performed by shaking heavily infected barley/cucumber leaves within a paperboard box containing Arabidopsis plants (appximately 3~4 weeks old with development of matured rosette leaves). After inoculation, the box was sealed

and left still for at least 3 hours (hrs) to facilitate the settlement of conidia onto the surface of Arabidopsis plant leaves. The inoculated Arabidopsis plants were then transferred to their original growing environment and samplings were performed at specific timing points for each experiment described.

2.3 Genetic crossing of Arabidopsis.

In order to investigate the interaction between *F5H1* and other characterized components of plant defense system, as well as the putative *F5H* gene *F5H2*, the *f5h1*-1 mutant was crossed with *f5h2*-1 and other single mutants and transgenic plant disrupted in the SA signaling pathway (*sid2*, *pad4*/*sag101* and NahGox), the JA/ET signaling pathway (*jar1* and *ein2*), in penetration resistance (*pen1*, *pen2* and *pen3*) or with Arabidopsis *pmr* mutants (*pmr2* and *pmr4*), respectively. Mutant *f5h1*-1 plants were used as female parental plants for all crossings since the F1 generation of a successful cross can be verified by the fluorescence response under UV radiation. F1 generation of successful crossings should display a blue-green fluorescence similar to wild type Col-0 since it is heterozygous of *f5h1* mutation, while unsuccessful crossing, which results from self-fertilized female *f5h1*-1 plants, should show a red fluorescence.

All crossings were performed with 5-6 week-old Arabidopsis plants. The double/triple progeny mutant lines are summarized in Table 2.2. To perform crosses, unopened buds of female parental plants were selected and opened with sharp tweezers under a dissection microscope (Wild of Canada; Ottawa, CA). Sepals, petals and stamens were carefully removed by fine tweezers without damaging the pistil. For each inflorescence, 3 buds were treated as described and other remaining buds were removed without damaging the stem. Opened flowers were selected from male parental plants (pollen donors). The bottom of the flower was held by tweezers to further widen the flower in order to expose the anthers. Carefully holding the tweezers with the opened flower, the exposed anthers were used to brush the stigma of the emasculated female flower to initiate fertilization. The coverage of pollen on the stigma was verified by viewing under dissection microscope. After fertilization, each pistil was wrapped with a small piece of plastic wrap to avoid cross contamination of pollen from other plants in the same growing chamber. Successfully fertilized pistils elongated and finally formed mature siliques. F1 seeds were collected and F1 plants were grown and self-fertilized to produce F2 seeds for the subsequent screening work to identify homozygous mutants.

All homozygous double and triple mutants were identified in the F2 generation by either PCR for all T-DNA insertions (*f5h2*-1, *sag101* and *pen3*-1) or by sequencing PCR fragments of mutated gene containing mutation site (*f5h1*-1, *sid2*-1, *pad4*-1, *jar1*-1, *ein2*-1, *pmr2*-1, *pmr4*-1, *pen1*-1 and *pen2*-1). The identified homozygous plants were self-fertilized to generate F3 seeds, the homozygosity of which was further verified by the same method as the F2 generation. All experiments were performed on F3 generation plants.

Table 2.2 Double/triple mutants generated in this study.

Lignin-related Mutants					
f5h1/f5h2	*f5h1/sid2*	*f5h1/*NahGox	*f5h1-pad4/sag101*	*f5h1/jar1*	*f5h1/ein2*
f5h1/pmr2	*f5h1/pmr4*	*f5h1/pen1*	*f5h1/pen2*	*f5h1/pen3*	*f5h1/tt4*
f5h1/tt5	*f5h1/tt6*				
Flavonoid-related Mutants					
tt4/tt5	*tt5/sid2*	*tt5/pad4/sag101*	*tt5/*NahGox	*tt5/jar1*	*tt5/ein2*

All mutants were generated by crossing; *f5h1*-1 is uses as female (pollen receiver) in all lignin-related mutants and *tt5*-T1 is used as female (pollen receiver) for all flavonoid-related mutants. See Figure 2.1 for an example for pedigree charts of crossing and screening for homozygous double/triple mutants.

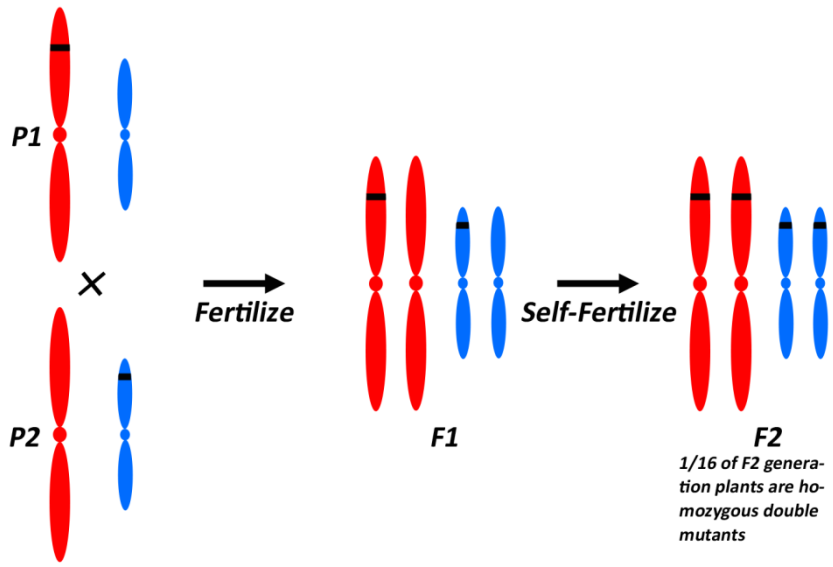

Figure 2.1 Schematic diagram of the creation of Arabidopsis double mutant.

Flowchart of the generation of Arabidopsis double mutant using genetic crossing. Assume that the two mutated genes are localized at different chromosomes, which are distinguished by red and blue colors. Two parental gametes (P1, P2) fuse to generate F1 generation. F1 plants are allowed to self-fertilize and generate F2 generation. Segretation occurs in F2 generation following Mendel's second law and 1/16 of F2 plants are target homozygous double mutant. For the creation of a triple mutant *f5h1pad4sag101*, one of the parental plants (either P1 or P2) is a homozygous double mutant *pad4sag101* and the ratio of homozygous triple mutants in the F2 population is 1/64. Black line on the upper arm of chromosome represents the mutation site. In F2 generation, other genotype combinations are omitted.

2.4 Determination of UV-responses of *f5h1* mutant alleles.

To analyze the UV-responses of *f5h1* mutant alleles, plants were grown as described in section 2.1 until the development of the 2nd pair of true leaves (the Arabidopsis seedling should have 6 leaves at this stage). Fresh leaves were collected and immediately mounted in water and examined on an Axioplan epifluorescence microscope with a FluoArc UV generator (Carl Zeiss Canada; Toronto, CA). Images were captured by an AxioCam ICc1 digital CCD camera equipped with the epifluorescence microscope.

2.5 Isolation of plant genomic DNA for polymerase chain reaction (PCR).

All chemicals and organic reagents described in this section and below were purchased from Fisher Scientific (Ottawa, CA) unless specially addressed. All sterile solutions were autoclaved at 121˚C/15 psi for 25 min unless specified.

The genomic DNA samples from plants were isolated following a quick DNA miniprep method kindly provided by Dr. Tamara Western (McGill University). One piece of a 3-week old plant leaf was used for each DNA isolation. The rosette leaf was removed from a plant by scissor and ground into a homogenate liquid by a plastic pestle in a 1.5-ml eppendorf microcentrifuge tube (Fisher Scientific; Ottawa, CA). A 400 µl aliquote of sterile DNA extraction buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS; SDS was added after autoclaving) was added into the ground plant tissue and the mixture was vortexed to form a homogenous solution. After centrifugation at 13,200 rpm for 5 min in a Beckman-Coulter Allegra 25R centrifuge equipped with a Beckman-Coulter TA-15-1.5 rotor (Mississauga, CA), 300 µl of supernatant was transferred to a new microcentrifuge tube and 300 µl of isopropanol was added to precipitate DNA. After incubation at room temperature for 10 min, the tube was centrifuged as described above for 2 min. The supernatant was carefully removed and the DNA sample was washed with 500 µl of 70% ethanol. After air-drying for 20 min, the DNA pellet was dissolved in 50 µl of sterile TE buffer (100 mM Tris-Cl pH 7.5, 10 mM EDTA pH 8.0) and was be directly used for PCR reactions. All DNA samples were stored in -20 ˚C freezer for long term storage.

2.6 PCR reactions and DNA precipitation for sequencing.

Most of the PCR reactions were used to screening for T-DNA insertions or to amplify target gene fragments for sequencing. PCR reactions were conducted using a home-made Taq

DNA polymerase recipe from Dr. Boham-Smith's lab, Department of Biology, University of Saskatchewan. The general PCR reaction system is as below:

10× PCR buffer (homemade; 500 mM KCl, 100 mM Tris-Cl pH 8.3)	2 µl
50 mM $MgCl_2$	0.6 µl
10 mM dNTPs (Invitrogen; Burlington, CA)	0.25 µl
Taq DNA polymerase	0.1 µl
H_2O	12.05 µl
Forward Primer (5µM)	2 µl
Reverse Primer (5µM)	2 µl
Template DNA	1 µl
Total	20 µl

The PCR reaction was performed in an Eppendorf Mastercycler (Westbury, US). The program was initiated with incubation at 94 ˚C for 3 min, followed by the three-step amplification cycle: 94 ˚C for 45 s, 57 ˚C for 30 s and 70 ˚C for 1 min. The extension time was adjusted according to the size of target product based. A 1 min extension time usually amplifies a 1000 bp PCR product. The three-step amplification cycle was repeated for 35 times and a final incubation was performed at 70 ˚C for 10 min to ensure the PCR products were amplified in full length.

For T-DNA insertion screening, two types of primer pairs were used and then the PCR products were electrophoresed on a 0.8% agarose gel to check band patterns (see Figure 2.2 for an example of the strategy and results using *pen3*-1 mutant as an example). The primers were designed by a web-based tool iSct-Primers provided by the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/ tdnaprimers.2.html; Sequences of all primers used in this project was listed in Appendix Table 1). For sequencing reactions, the PCR product solution was transferred to new 1.5-ml eppendorf microcentrifuge tube (Fisher Scientific; Ottawa, CA); 1/10 volume of NaAc (3 M, pH 5.2) and 2.5 volume of 100% ethanol were added into the PCR solution to precipitate DNA. After precipitation at -20˚C overnight, the tube was centrifuged as described above for 10 min to collect precipitated DNA. After a wash with 70% ethanol, the DNA samples were air-dried and dissolved in 30 µl of distilled water. The concentrations of DNA samples were measured at 260/280 nm by a UV spectrophotometer (Beckman-Coulter;

Mississauga, CA). Finally sample was then diluted to desired concentration depending on the size of PCR products and sent to the DNA lab at the National Research Council-Plant Biotechnology Institute (NRC-PBI) for sequencing.

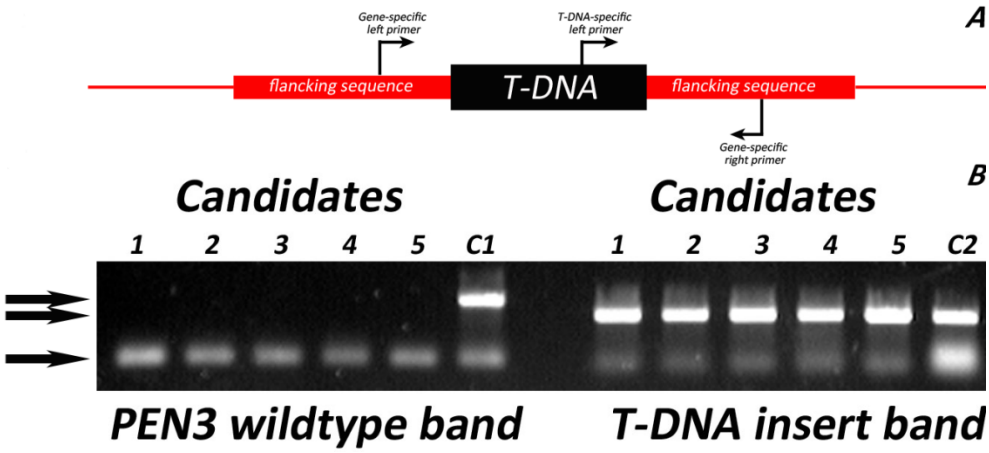

Figure 2.2 Example of T-DNA insert verification in insertion lines.

Verification of T-DNA insert mutation by PCR. (A) Schematic image of T-DNA insertion screening method. For each candidate of an insertion mutant line, 2 PCR reactions were set up, using gene-specific left and right primers for one reaction, and T-DNA-specific left primer and a gene-specific right primer for the other one. The presence of T-DNA will interfere with the PCR amplification reaction containing gene-specific left and right primers. Therefore, homozygous mutants should have only T-DNA insert bands; heterozygous mutants should have both wild type and T-DNA insert bands. Wild type plants should have wild type band only. (B) An example of PCR electrophoresis result from a *pen3* T-DNA insert mutant screening PCR results. C1, wild type band amplified from control plant Col-0 genomic DNA with gene-specific left and right primer; C2, T-DNA insert band amplified from verified control plant *pen3*-1 single mutant genomic DNA with T-DNA specific left primer and gene-specific right primer. Failure to amplify the wild type band and success to amplify T-DNA insert band was the marker of homozygous T-DNA insert mutants. All 5 candidates were homozygous *pen3*-1 mutants. The bands at bottom were primer dimers. Black arrows indicate the position of DNA bands.

2.7 RNA isolation.

The pathogen-challenged and hormone-treated samples for RNA isolation were collected and frozen in -80˚C freezer for subsequent RNA isolation. All operations and centrifugation described below were conducted on ice or at 4˚C, respectively. Tissue collected from 15 plants was first ground into a fine powder by mortar and pestle with liquid nitrogen. After the nitrogen was completely evaporated, the powder was transferred into a new 50 ml centrifuge tube (Fisher Scientific; Ottawa, CA) and 15 ml of extraction buffer (150 mM LiCl, 5 mM EDTA, 50 mM Tris-Cl pH 9.0, 5% SDS) was added. The tube was capped and shaken to form a homogenous solution. A 15 ml aliquot of phenol-chloroform (1:1. v:v) was then added and tube was capped and shaken vigorously ~100 times. After centrifuge at 1328g in a Beckman-Coulter Allegra 25R centrifuge (Mississauga, CA) for 20 min, the aqueous supernatant was transferred to a new 50ml centrifuge tube with 15ml of phenol-chloroform. After shaken for ~100 times, the tube was centrifuged at 850g for 20 min. Aqueous phase was transferred to a new 50ml centrifuge tube with 15 ml of chloroform. After shaken ~100 times, the tube was centrifuged at 850g for 20 min. 15 ml of aqueous phase was transferred to corex glass centrifuge tubes (Krackeler Scientific; Albany, US). A 1.5 ml aliquot of NaAc (4 M, pH 5.3) and 12 ml of cold isopropanol were added to precipitate the DNA/RNA mixture. After overnight precipitation in -20˚C freezer, the tube was centrifuged at 6869g in the same centrifuge as described above for 30 min. The supernatant was removed and the pellet was re-dissolved in 6 ml of sterile water, then 2 ml of LiCl (8 M) was added and the tube was placed on ice for 2 hrs to precipitate RNA. The tube was subsequently centrifuged at same condition for 10 min. The supernatant was removed and pellet was washed with 1 ml of 70% ethanol and transferred into cold 1.5-ml microcentrifuge tube, which was centrifuged at 21913g in a the same centrifuge for 5 min. The supernatant was discarded and RNA pellet was air-dried for 20 min, and then finally dissolved in 80 µl of sterile water. The concentration was determined by a UV spectrophotometer (Beckman-Coulter; Mississauga, CA). The quality of RNA samples was verified by electrophoresis in agrose gel. All RNA samples were stored in -80 ˚C freezer until use.

2.8 Synthesis of cDNA.

Synthesis of cDNA was carried out by Superscript II reverse transcriptase (Invitrogen, Burlington, CA) following the manufacturer's protocol. One µg of RNA was used to synthesize

cDNA. After synthesis, 5 µl of cDNA was used to make 20 times dilution for the synthesis of northern blotting probes.

2.9 Northern blotting.

For northern blotting probe synthesis, PCR reactions were carried out using the condition described above for normal PCR using 1 µl of diluted cDNA template. To obtain enough PCR products for purification, 35 cycles were used. The resulting PCR product was examined by agarose electrophoresis and the gel block containing the probe band was cut out using a new sterile scalpel and collected into a new 1.5-ml microcentrifuge tube (Fisher Scientific; Ottawa, CA). The volume of the gel block was determined at an approximate density of 1 g/ml. Three volumes of 6 M NaI and 1/10 volume of 3 M NaAc (pH 5.5) were added to the tube containing the gel block, which was subsequently incubated at 50 ˚C for 5 min or until the agarose block melted. Ten µl of well-suspended glassmilk ((1000 mg/ml of silica (Sigma-Aldrich, Oakville, CA) suspended in 3 M NaI)) was added to the melted agarose solution and vortexed to form an evenly suspended solution. The solution was incubated at room temperature for 10 min with vortexing every 2 min to maintain a glass milk suspension and then centrifuged in an Eppendorf 5415R centrifuge equipped with an Eppendorf F45-24-11 rotor at room temperature for 15 sec. The supernatant was removed completely and the pellet was washed twice with wash buffer (50 mM NaCl, 10 mm Tris-Cl pH 7.0, 2.5 mM EDTA and 50% v/v ethanol). The pellet was air-dried and DNA was eluted by incubating pellet suspended in 50 µl of sterile water for 10 min at 50 ˚C. The purified probe was quantified at 260/280 nm by UV spectrophotometer (Beckman-Coulter, Mississauga, CA) to determine its concentration and then sequenced as above to verify its correctness.

RNA samples from plants were separated on formaldehyde agarose gel. The gel was prepared by mixing 5.4 g of agarose in 393.75 ml of sterile water and 45 ml of 10×MOPS buffer (200 mM MOPS, 50 mM NaAc, 10 mM EDTA). The agarose was dissolved by heating in a commercial Panasonic microwave oven. After cool down to approximately 60˚C, 13.5ml of formaldehyde was added into the gel solution and thoroughly mixed by shaking the flask. Eventually, 281 µl of 200 µg/ml ethidium bromide was added and the gel solution was poured into a casting tray to stand at room temperature for at least 45 min until fully solidified.

To pre-aliquoted RNA samples (6 μg per mutant line per blot), 1.5 μl of loading buffer was added to make a total volume of 15~16 μl. The samples were vortexed, centrifuged to collect liquid and heated in a PCR machine at 55˚C for 15 minutes. Samples were then immediately cooled down on ice for 2 minute, then the liquid was collected as above collect and loaded onto the solidified gel, which were electrophoresed at 70 volts for 3 hrs.

After washing in distilled water for 15 min twice, the gel was trimmed to remove area without RNA samples and transferred to a solid support covered with a filter paper bridge soaked with 20×SSc buffer (175.3 g of sodium chloride and 88.2 g of sodium citrate dissolved in 1 L of distilled water). The support was placed in a tray filled with 20×SSc buffer and the 4 ends of the paper bridge were immersed in 20×SSc buffer. A piece of Amersham Hybond-XL membrane (GE Healthcare; Mississauga, CA) and 3 pieces of Waterman filter paper were cut according to the size of the gel, then membrane was wet with sterile water and placed over the gel without trapping any air bubbles. The 3 pieces of filter paper were also wet and placed onto the membrane one by one, and any observed air bubbles removed by rolling a 15-ml centrifuge tube on the surface of the filter paper. Tissue paper was cut according to the size of gel and piled on top up to 5~8 cm, then a glass plate and a 400 g weight were used to compress the whole blotting sandwich. Blots were left overnight at room temperature, and then the membrane was transferred with RNA side up to a baking plate with sufficient 2×SSc buffer to cover the membrane. The membrane was washed in 2×SSc buffer twice for 10 min of each wash, laid on a transparent glass plate and exposed to UV for 1 min 30 sec, and then stained in 0.02% methylene blue for 3 min and distained the membrane in 1×SSc buffer for no more than 10 min with at least 1 change of fresh buffer. The stained membrane was photographed in an Alphaimager 2200 gel-doc system and finally baked for 1 hr at 80˚C.

Each gene-specific probe was amplified from cDNA as described above and for radiation labeling and hybridization. ^{32}P was purchased from Perkin Elmer (Waltham, USA). The Prime-It random primer labeling kit and Quickhyb hybridization solution were purchased from Stratagene (Mississauga, CA). The labeling of probes was carried out following manufactures' instruction. The hybridization was performed according to the manufacturer's instruction with minor changes: the hybridization time was increased to 3 hr rather than 1 hr as described in the manual. Hybridized membranes were exposed overnight and image was captured in a phosphorimager system (GE healthcare; Mississauga, CA).

2.10 Histochemical staining.

Aniline blue staining

The inoculated leaves were collected and fixed in a fixation solution (methanol : chloroform : acetic acid = 6 : 3 : 1) (with an occasional change of fresh fixation solution) until the original green color was completed faded. The fixation solution was then removed and the samples were incubated in 100% (or 95%) ethanol overnight. Rehydration of fixed samples was carried out by incubating leaves 2 hrs in each of five gradient concentrations of ethanol starting from 95%, 80%, 70%, 50% and finally in 25% ethanol. For callose staining, residual ethanol was removed and samples were rinsed with pure water and stained in 150 mM K_2HPO_4 (pH 9.5) containing 0.05% aniline blue (Fisher Scientific; Ottawa, CA) overnight. For fungal hyphae staining, residual ethanol was removed by washing with acidic water (pH 2.0; adjusted with hydrochloric acid) and sample was stained in acidic water containing 0.05% aniline blue for 5 min. After staining, the plant samples were rinsed to remove excessive staining solution in K_2HPO_4 (150 mM pH 9.5) for callose staining or acidic water (pH 2.0) for fungal hyphae staining, respectively. To view callose staining, the samples were mounted in 30% glycerol and photographed under an Axioplan epifluorescence microscope (Carl Zeiss Canada, Toronto, CA) with UV application. To view fungal hypha staining, the samples were mounted in acidic water (pH 2.0) and photographed using the same microscope without UV application.

Trypan blue staining

For trypan blue staining, samples were collected and treated as described in aniline blue staining until the final rehydration step with 25% ethanol. Residual ethanol was removed and samples treated with pure water overnight. Subsequently, samples were stained with 0.05% trypan blue (Hartman-Leddon; Philadelphia, US) dissolved in pure water overnight. Stained samples were washed in pure water and observed under microscope after mounted in water.

In situ histochemical detection of H_2O_2

The *in situ* histochemical detection of H_2O_2 was characterized by a 3,3'-diaminobenzidine (DAB; Sigma-Aldrich; Oakville, CA) uptake experiment (Liu et al., 2010). DAB uptake was carried out by incubating fresh collected inoculated Arabidopsis leaves in 1 mg/ml DAB solution for 8 hrs. After incubation, the samples were fixed in fixation solution as described in section 2.10 for microscopy analysis. The brownish color indicates the accumulation of H_2O_2 molecule.

2.11 Plant hormone treatments.

The concentration of each hormone used was: salicylic acid (SA), 1 mM; jasmonic acid (JA), 50 mM; sodium nitroprussid (SNP; NO releasing agent), 10mM; ethephon (ethylene releasing agnet), 100 μM; H_2O_2, 10 mM (SA to H_2O_2; Liu G, personal communication); abscisic acid (ABA), 50 μM (Yang et al., 2009); 3-indole acetic acid (IAA), 100 μM (Arteca and Arteca, 2008); gibberellic acid (GA), 100 μM (Day et al., 2004); 6-benzylaminopurine (BAP), 20 μM (Yang et al., 2009).

Plant hormone treatments (including water treatment as a mock control) were carried out by spraying hormone solution on 3 weeks old Col-0 Arabidopsis plants. For each hormone treatment, 3 pots (5 plants per pot) were treated. To facilitate the absorbance of sprayed hormone, sterile water was sprayed to all plants 1 hr after hormone solution was sprayed to maintain humidity at the leaf surface. At 24 hrs after treatment, the total above-ground tissues were harvested and immediately frozen in -80˚C freezer for subsequent RNA isolation as described in section 2.7.

2.12 Transmission electron microscopy.

All steps, unless specified, were performed at room temperature. Col-0 and *f5h1*-1 plants were grown and *B. gramins* f.sp. *hordei* inoculation was performed as described above with high conidia density. At 48 hpi, inoculated leaves were harvested and cut into 1-mm × 2-mm pieces, which were immediately fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) in 4˚C overnight. After three washes of 0.1 M phosphate buffer (pH 7.2), samples were post-fixed in osmium tetroxide (1% in phosphate buffer) at room temperature for 3 hrs. Following three washes of phosphate buffer (pH 7.2), the samples were dehydrated in an ethanol series (15%, 30%, 45%, 60%, 70%, 85%, 95%, 100%, 100%) for 10 min per level. After dehydration, samples were incubated twice in acetone for 10 min and infiltrated with acetone/LR White resin (3:1, 30 min; 1:1, 1~2 hr; London Resin Company; Reading, UK) and eventually 100% resin overnight. Finally, samples were embedded in a mold in 100% resin and incubated at 50˚C for polymerization.

Ultra-thin sections (60nm) were generated with a Microstar diamond knife (Huntsville, US) on a Reichert-Jung microtome (Reichert microscopic service; Depew, US) and captured onto single slot copper grids (Ted Pella; Redding, US) coated with Formvar. Grids were stained

with 2% uranyl acetate and Reynold's lead citrate solution (Reynolds, 1963), and observed with a Philip CM10 transmission electron microscope (Netherlands).

2.13 Data collection and statistical analysis.

Statistical analysis was performed on all penetration efficiencies data, hyphae development data and total haustoria formation per colony data to compare the significant differences between mutant lines and wild type Col-0. To collect data for statistical analysis, Arabidopsis plants were placed in growing chamber and selected for pathogen inoculation, following the manner of "Completely Random Design". When collecting samples for microscopic analysis, fully matured leaves in same developmental stages were collected from different plants within one pot at the specific timing post pathogen inoculation as indicated in the corresponding figures. The penetration frequencies of *B. graminis* f.sp. *hordei* and *E. cichoracearum* were determined by the ratio of successful penetrations per 100 germinated conidia. The formation of fungal haustoria was counted as a "successful" penetration. The entire leaf was divided into three equal areas from the tip to the petiole. For each area, a value of penetration frequency was determined as described and the average of the 3 values from the three areas was used to represent the penetration frequency of this leaf. This is to avoid the different penetration resistance of different areas within the leaf. The value of 1 leaf was considered as 1 observation and 5 observations were obtained for each plant line in each replicate of experiment. The total hyphae length of *E. cichoracearum* colony was measured with Image J software (http://rsbweb.nih.gov/ij/) on the digital images photographed after staining with aniline blue. At least 20 colonies were randomly chosen from collected leaves of each plant line in each replicate of experiment. The total haustoria formation per colony of *E. cichoracearum* at 7 dpi was directly counted on leaves stained with aniline blue as described above. At least 15 colonies that were randomly selected from 5 different sampled leaves were examined for each plant line in each replicate of experiment. All of the data were summarized, and the mean values and standard deviations were calculated in Excel of Microsoft Office software (Microsoft Company; Mississauga, CA). Analysis of variance (ANOVA) test ($P < 0.05$ and $P < 0.01$) with Fisher's Least Significant Difference (LSD) was performed to compare the mean values in IBM Statistical Product and Service Solutions (SPSS; Ver. 20.0) package

licensed to University of Saskatchewan (IBM; Markham, CA). The output tables of ANOVA analyses were listed as Appendix Table 2.

All experiments described in this chapter were repeated three times except northern blotting experiments. Northern blotting experiments shown in the Chapter 3 below were repeated biologically twice except the experiment shown in panel B of Figure 3.15 that was performed only once.

CHAPTER 3

RESULTS

3.1 Expression of lignin genes is induced by pathogen in Arabidopsis.

The expressions of five Arabidopsis lignin genes upon pathogen infection were examined. Wild type Arabidopsis ecotype Col-0 was challenged with host pathogen *C. higginsianum*. Expression was determined at 7 different timing points post inoculation using northern blotting. Key lignin genes that were tested are as follows: *phenylalanine lyase 1* (*PAL1*), *cinnamic acid 4-hydroxylase* (*C4H*), *hydroxycinnamoyl CoA shikimate/quinate hydroxyl-cinnamoyltransferase* (*HCT*), *ferulic acid 5'-hydroxylase 1* (*F5H1*) and *caffeic acid/5-hydroxyferulic acid O-methyltransferase 1* (*COMT1*). *PAL1* and *C4H* were chosen since they catalyze the first two steps of the phenylpropanoid pathway; *HCT* was chosen since it catalyzes the first step of the monolignol pathway; *F5H1* and *COMT1* were chosen since they are involved in the metabolism of the S lignin monomer (Figure 1.1). As shown in Figure 3.1, all the lignin genes tested, together with two marker genes of plant defense *PR-1* and *PDF1.2* displayed an induced expression pattern upon the infection of host pathogen *C. higginsianum*, suggesting that the entire lignin pathway was induced by host pathogen infection. Noticeably, all lignin genes tested showed a similar increasing trend in expression, which reached to a maximum level at 96 hpi, was maintained at 120 hpi and decreased at 144 hpi except for *PAL1* that remained high at 144 hpi.

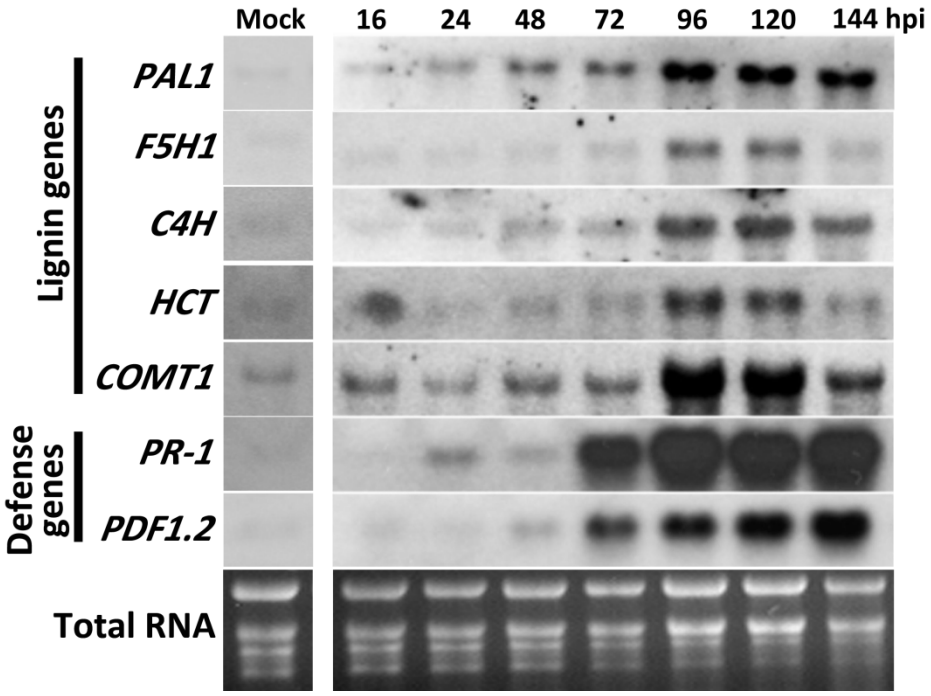

Figure 3.1 Expression of lignin genes upon host pathogen inoculation.

Northern blotting analysis of lignin genes' expression upon infection of host anthracnose pathogen *Colletotrichum higginsianum*. 3-week-old wild type Arabidopsis Col-0 plants were inoculated with *C. higginsianum* and total above-ground tissues were collected at specific timing points as indicated above for RNA isolation. Non-inoculated sample was labeled as "Mock". Mock samples were also collected at the 7 time points as inoculated samples. The gene expression patterns were consistent at all 7 mock samples. Thus, only one of them is presented here to avoid redundancy. Loading of total RNA was viewed by ethidium bromide staining of the RNA gel. The sequences of primers used to synthesize probes can be found in Appendix Table 1. Gene abbreviations: *PAL1, phenylalanine ammonia lyase 1; F5H1, ferulic acid 5-hydroxylase 1; C4H, cinnamate 4-hydroxylase; HCT, hydroxycinnamoyl-Coenzyme A shikimate/quinate hydroxycinnamoyltransferase; COMT1, caffeic acid O-methyltransferase 1; PR-1, pathogenesis related gene-1; PDF1.2, plant defensing 1.2*.

3.2 Characterization of *f5h1* and *f5h2* mutant alleles.

Previous disease tests of Arabidopsis mutants disrupted in individual lignin genes revealed that only mutant *f5h1*-1 showed enhanced susceptibility when inoculated with the host pathogen *C. higginsianum* (Liu G and Wei Y, unpublished data). To verify this observation, two additional alleles of *f5h1* mutants were obtained in a Col-0 background from ABRC. Allele *f5h1*-1 carries a single nucleotide mutation "T→C" at position 292 (from the ATG start codon) and was confirmed by sequencing PCR-amplified *F5H1* gene fragments (data not shown). Mutant allele *f5h1*-2 was originally characterized as *fah1*-7 (Chapple et al., 1992), but the specific *f5h1*-2 mutation site was not available from the ABRC database and original publication. As described by Chapple et al. (1992), this mutation of gene *F5H1* resulted in a red fluorescence of leave cells under ultraviolet (UV) light. Therefore, the fluorescence response upon UV application was determined. As shown in Figure 3.2C, the red fluorescence was observed in both *f5h1*-1 and *f5h1*-2 mutants. Thus, *f5h1*-2 was considered to be a homozygous mutant line since plants carrying a heterozygous *f5h1* mutation displayed a blue-green fluorescence indistinguishable from the wild type Arabiodpsis ecotype Col-0 (Figure 3.2C). Allele *f5h1*-3 was identified from SALK T-DNA insertion mutant SALK_063792 by PCR verification following the seed provider's instruction (Figure 3.2A). Red fluorescence phenotype was also observed for the *f5h1*-3 allele (Figure 3.2C).

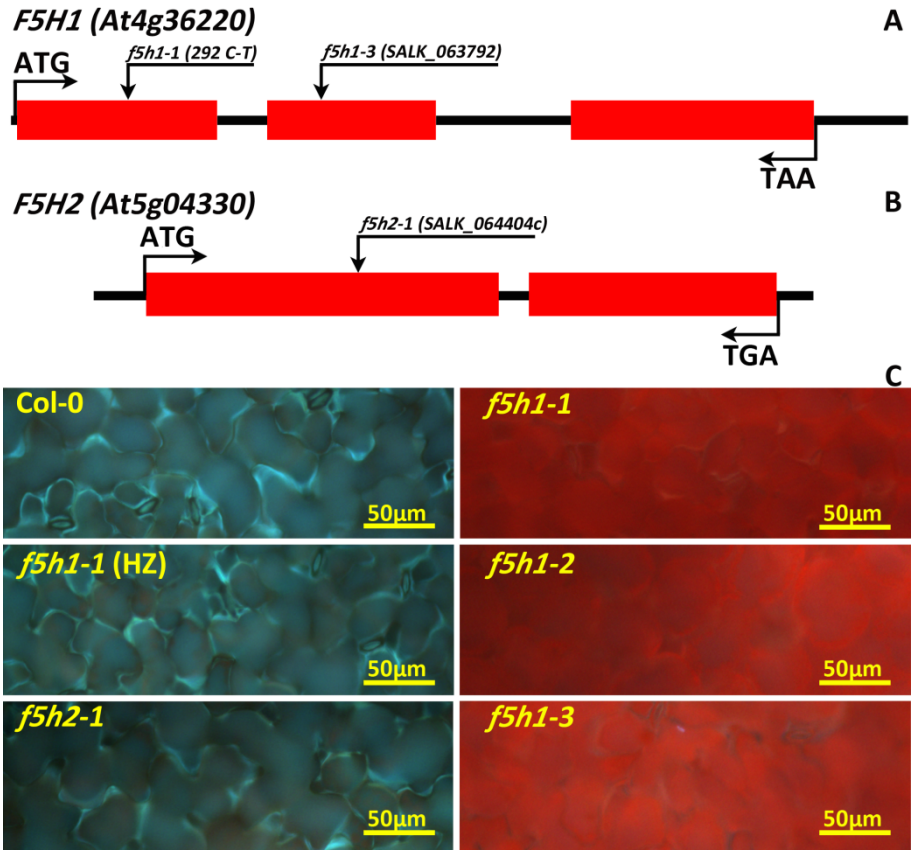

Figure 3.2 Gene structures and phenotype of mutant *f5h1* and *f5h2*.

Schematic diagrams of gene *F5H1* (At4g36220; A) and *F5H2* (At5g04330; B). Red boxes represent exons and horizontal black lines represent 5' untranslated region (UTR) before the ATG start codon, introns and 3'UTR after the TAA/TGA stop codons. The lengths of each red box and segment of black line were scaled to match the sizes of actual gene sequences based on the information of TAIR database (http://www.arabidopsis.org). Arrows indicate the position of mutation site (*f5h1*-1) and insertion flanking sequences (*f5h1*-3 and *f5h2*-1). The mutation site of *f5h1*-2 (*fah1*-7) was not available and not annoted in image. (C) The fluorescence responses of leaf cells of *f5h1* and *f5h2* mutants, wild type Col-0 and plant carrying both wild type and mutant *F5H1* alleles (heterozygous; HZ) under exogenous UV application. Scale bar=50μm.

In the Arabidopsis genome, there is another putative gene encoding ferulic acid 5' hydroxylase, At5g04330, which is named as *F5H2* in this thesis. *F5H1* and *F5H2* showed 76.6% similarity in the nucleotide sequence (Costa et al., 2003; see Figure 3.3 for gene alignment). Mutant *f5h2*-1 (SALK_064404c) was obtained from ABRC and homozygosity was verified by PCR. Homozygous *f5h2*-1 mutant did not show enhanced red fluorescence (Figure 3.2C).

To investigate the interactions between *F5H1* and other genes involved in plant immunity, various double/triple mutants were generated as described in "Chapter 2 Materials and Methods". Under ordinary growing condition without pathogen challenge, all of these double/triple mutants displayed normal growth similar to wild type Arabidopsis Col-0 (data not shown).


At4g36220 ATGGAGTCTTCTATATCACAAACACTAAGCAAACTATCAGATCCCACGACGTCTCTTGTC 60
At5g04330 ATG----CTT----------ACTCTAAT--GACTCTCAT---------CGT-TCTTGTC 33
At4g36220 ATCGTTGTCTCTCTTTTCATCTTCATCAGCTTCATCACACGGCGGCGAAGGCCTCCA--- 117
At5g04330 --CCTCTTCT-TCTTTTTCTCTTCCCCCACCTCCTTTTACGGCGGCAGATGCTTCTGAAA 90
At4g36220 ---TATCCTCCCGGTCCACGAGGTTGGCCCATCATAGGCAACATGTTAATGATGGACCAA 174
At5g04330 CCCTACCCTCCTGGCCCCAAAGGCTTACCCGTCATTGGAAATATTCTCATGATGAACCAG 150
At4g36220 CTCACCCACCGTGGTTTAGCCAA-TTTAGCTAAAAAGTATGGCGGATTGTGCCATCTCCG 233
At5g04330 TTTAACCACCGGGGTCTGGCCAAGCTCAGCCGTATA-TACGGTGGGTTGCTCCATCTCCG 209
At4g36220 CATGGGATTCCTCCATATGTACGCTGTCTCATCACCCGAGGTGGCTCGACAAGTCCTTCA 293
At5g04330 CCTCGGATTTTCCCACATTTTCGTCGTTTCTTCTCCGGACATTGCGCGTCAAGTCCTCCA 269
At4g36220 AGTCCAAGACAGCGTCTTCTCGAACCGGCCTGCAACTATAGCTATAAGCTATCTGACTTA 353
At5g04330 AGTCCAAGACCACGTTTTCTCAAACCGTCCCACCACAATCGCAATCCGATACTTGACCTA 329
At4g36220 CGACCGAGCGGACATGGCTTTCGCTCACTACGGACCGTTTTGGAGACAGATGAGAAAAGT 413
At5g04330 CGGAGGATCCGATCTTGCATTCTGCAATTACGGCCCGTTTTGGCGTCGGATGAGAAAACT 389
At4g36220 GTGTGTCATGAAGGTGTTTAGCCGTAAAAGAGCTGAGTCATGGGCTTCAGTTCGTGATGA 473
At5g04330 CTACGTCATGATGCTCTTTAGCCGTAAACGGGCCGAGTCGTGGGTCTCTGTTGATGAAGA 449
At4g36220 AGTGGACAAAATGGTCCGGTCGGTCTCTTGTAACGTTGGTAAGCTACTTCACATATTCAC 533
At5g04330 GGTCCACAAATCGGTCCGTTTAGTGGCTTCCAACGTCGGAAAACCACT------------ 497
At4g36220 CACTCTTGCTATATATATGTGCAATTAAACAAATATGTAAAAAGTGAAAGTACTCATTTC 593
At5g04330 ----------AAACATATGT------AAACTAGCAT------------------------ 517
At4g36220 TTCTTTCTTTAGTATGTACTTTAACATTTAACCAAAACAATTGTAGGTAAGCCTATAAAC 653
At5g04330 ---TTTCCCTATCAAGAGACATAACGTT----CCGAGCAGCTTTCGGT------------ 558
At4g36220 GTCGGGGAGCAAATTTTTGCACTGACCCGCAACATAACTTACCGGGCAGCGTTTGGGTCA 713
At5g04330 -------------TCCTC---CTCATCCACA------TCTGATGAAAGCCGTTTGG---- 592
At4g36220 GCCTGCGAGAAGGGACAAGACGAGTTCATAAGAATCTTACAAGAGTTCTCTAAGCTTTTT 773
At5g04330 --------------------ACGAGTTCCTTGAGATCATTCAAGAGTTCTCTAAGCTCTTT 633
At4g36220 GGAGCCTTCAACGTAGCGGATT---TCATACCATATTTCGGGTGGATCGATCCGCAAGGG 830
At5g04330 GGTGAGTTTAACGTAGCGGATTATGTCCCGTCGTGGCTCAGTTGGATTGACCCGCAAGGG 693
At4g36220 ATAAACAAGCGGCTCGTGAAGGCCCGTAATGATCTAGACGGATTTATTGACGATATTATC 890
At5g04330 ATAAACGGGCGAGTTGAGAAAGCCCGAAAATCTCTAGACGGTTTCATTGAGTCCGTCATC 753
At4g36220 GATGAACATATGAAGAAGAAGGAGAATCAAAACGCTGTGGATGATGGGGATGTTGTCGAT 950
At5g04330 GATGATCACTTGCACAAGAAGAAGAG--AGAAC-------ATGATAACGTTGACGAAGAG 804
At4g36220 ACCGATATGGTTGATGATCTTCTTGCTTTTTACAGTGAAGAGGCCAAATTAGTCAGTGAG 1010
At5g04330 ACTGATATGGTAGATCAATTACTTGCGTTCTACGAAGAAGAAGTCAAA---GTCA----- 856
At4g36220 ACAGCGGATCTTCAAAATTCCATCAAACTTACCCGTGACAATATCAAAGCAATCATCATG 1070
At5g04330 ACAAC------TCAG----TCACCAAAATTAATCTCGATAACATAAAAGGCATCATCATG 906

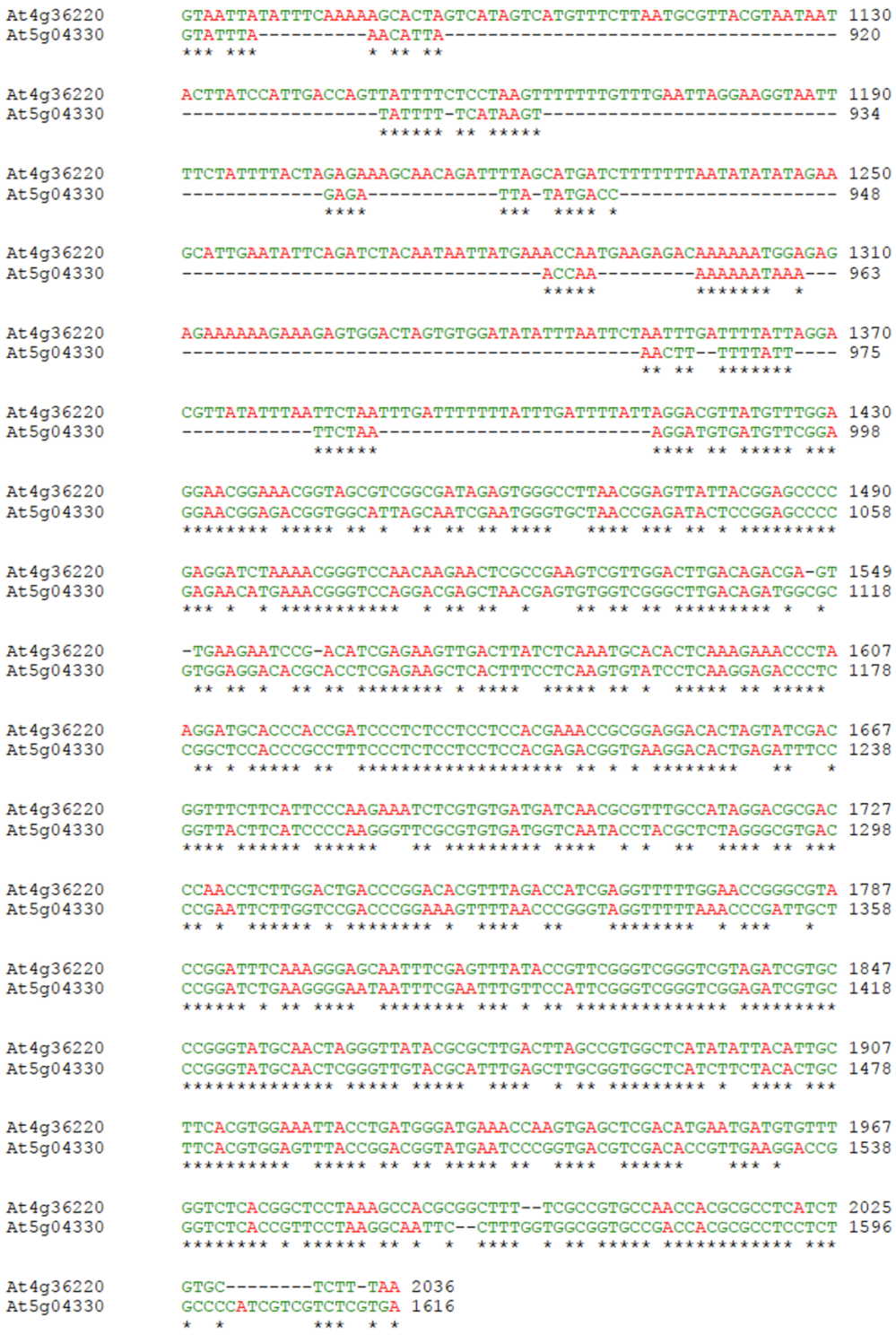

Figure 3.3 Alignment of *F5H1* (At4g36220) and *F5H2* (At5g04330).

Performed by ClustalW2 - Multiple Sequence Alignment tool of The European Bioinformatics Institute (EBI), part of the European Molecular Biology Laboratory (EMBL; http://www.ebi.ac.uk/Tools/msa/clustalw2/). Asterisks indicate the identical sequence between *F5H1* and *F5H2*.

3.3 Knockout of *F5H1* results in enhanced susceptibility.

Since only the mutant *f5h1*-1 was susceptible to the host pathogen *C. higginsianum* among all lignin gene mutants previously tested (Liu G and Wei Y, unpublished data), two additional *f5h1* allelic mutants were tested with host pathogens *C. higginsianum* and *E. cichoracearum* together with *f5h1*-1 and Col-0 to identify their response toward host fungal pathogens. As shown in Figure 3.4A, all three *f5h1* mutant alleles showed similar levels of disease symptoms (tissue collapse) as early as 48 hpi after exposure to *C. higginsianum*, while wild type Arabidopsis Col-0 still maintained a healthy appearance. Further microscopic analysis revealed that this pathogen had switched from biotrophic stages to necrotrophic stages due to the development of secondary hyphae on *f5h1* mutant alleles (indicated by red arrows in Figure 3.4A) as described by Kleemann et al., 2008; while remaining at a biotrophic stage on Col-0 wild type plants. Consistent with the *C. higginsianum* disease assay, *E. cichoracearum* formed significantly more conidiophores on all three allelic *f5h1* mutants tested, suggesting that *f5h1* mutants were also susceptible to host powdery mildew in addition to the anthracnose pathogen (Figure 3.4B). This result confirmed that the *f5h1* mutation impaired immunity of Arabidopsis and conferred enhanced susceptibility against two host pathogens.

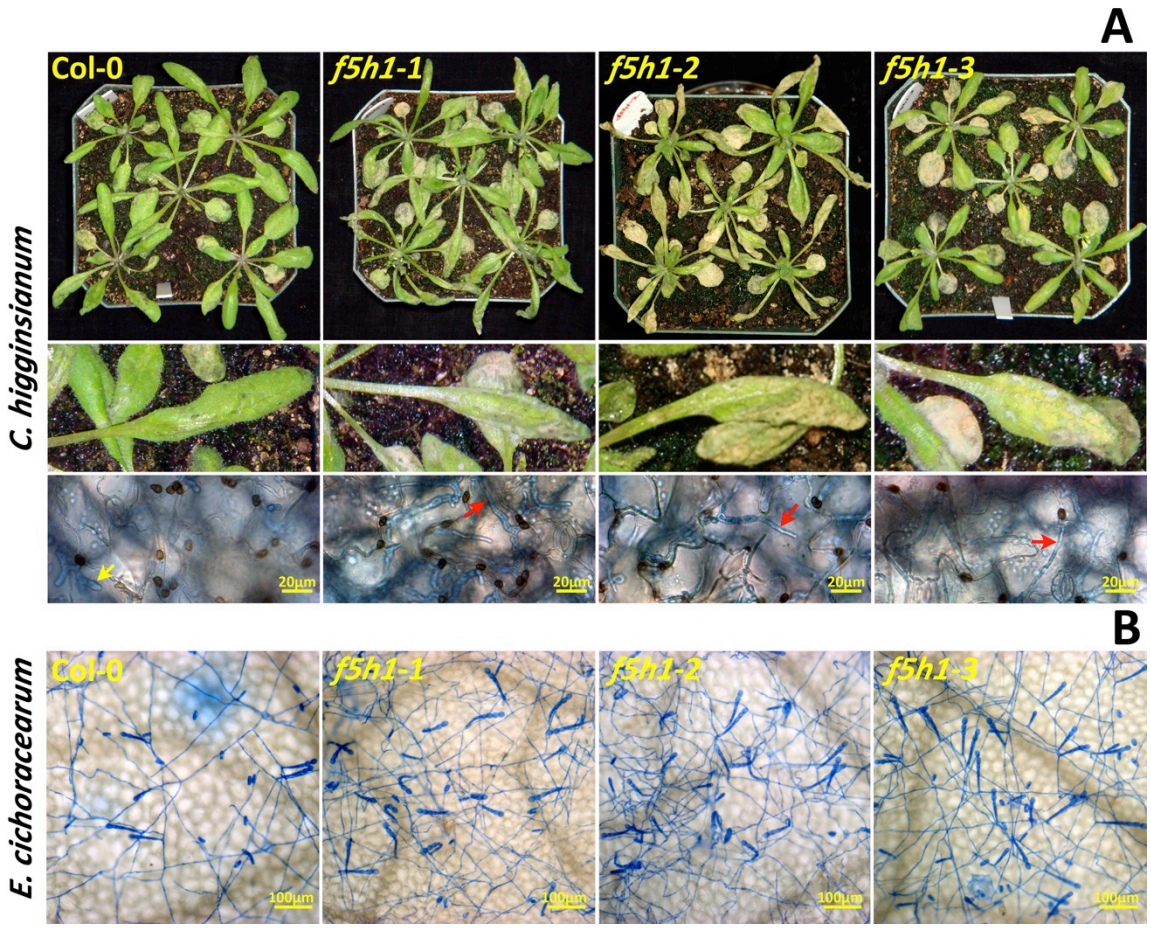

Figure 3.4 Enhanced pathogen susceptibilities in *f5h1* mutant alleles.

Disease symptoms of host fungal pathogens on *f5h1* mutant alleles and wild type Col-0. (A) Disease symptoms at 3 days post inoculation (dpi) with *Colletotrichum higginsianum*. Representative leaves of each allele were selected and enlarged to show the collapse of plant tissue. Microscopic images show the development of fungal hyphae. Yellow arrow indicates primary hyphae on Col-0 and red arrows indicate secondary hyphae on *f5h1* mutant alleles. Scale bar=20μm. (B) *Erysiphe cichoracearum* conidiophore formation was photographed at 7 dpi after acidic aniline blue staining. The density of fungal conidiophore reflects the severity of disease on the plant lines tested. Scale bar=100μm. Images are representative of 3 independent experiments and 5 observations of each independent experiment.

In addition to *f5h1*-1 mutant alleles, *f5h2*-1 and the double mutant *f5h1*/*f5h2* were tested with *C. higginsianum* to investigate the possible roles of *F5H2* in plant immunity. As shown in Figure 3.5, *f5h2*-1 did not show a significantly susceptible phenotype compared with Col-0 wild type. Moreover, double mutants *f5h1*/*f5h2* showed a similar level of susceptibility as the *f5h1*-1 single mutant. Taken together, *F5H2* did not contribute in plant immunity. Therefore, all subsequent experiments, except where stated, were performed on *f5h1*, rather than on the *f5h1*/*f5h2* progeny line.

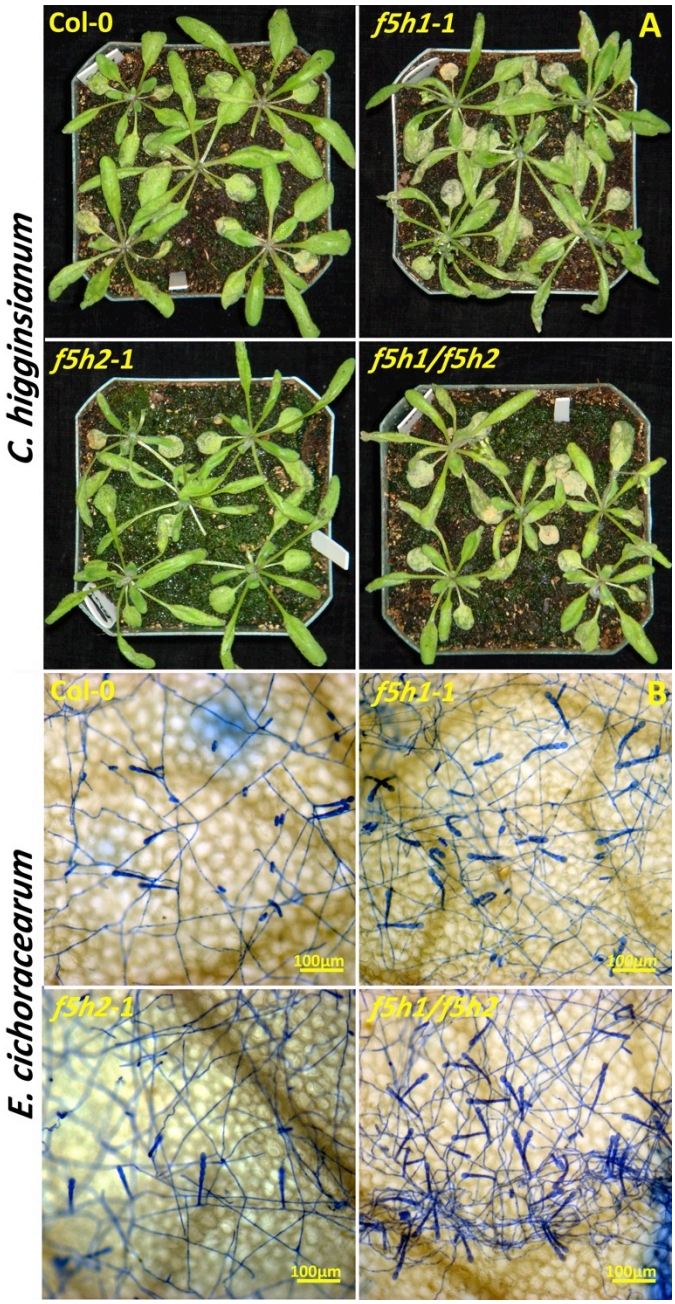

Figure 3.5 *F5H2* is not involved in plant disease resistance.

Disease symptoms of host fungal pathogens infected on *f5h1* and *f5h2* mutant lines and wild type Col-0. (A) Disease symptoms, as indicated by tissue collaps, of *Colletotrichum higginsianum* at 3 days post inoculation (dpi). (B) Conidiation of *Erysiphe cichoracearum* at 7 dpi was photographed after aniline blue staining. The density of fungal conidiophore reflects the severity of disease on the plant line tested. Scale bar=100µm Images are representative of 3 independent experiments and 5 observations of each independent experiment.

3.4 Plant penetration resistance is disrupted by mutation of *F5H1*.

Penetration resistance represents the first level of plant nonhost resistance aiming to prevent the entry of pathogen infection structures into host cell. Therefore, penetration resistance was analyzed in the *f5h1*-1 mutant. First, penetration frequency of a nonhost pathogen *B. graminis* f.sp. *hordei* on the *f5h1* mutant was determined by measuring the proportion of successful entries per 100 interaction sites. At 48 hpi, a 2.5-fold increase of the penetration frequency of *B. graminis* f.sp. *hordei* was observed on *f5h1*-1 mutant in comparison with Col-0, suggesting that penetration resistance was impaired by *f5h1*-1 mutant (Figure 3.6A). Successful penetrations were characterized by the formation of unilateral haustoria (uh) while unsuccessful penetration was prevented by papillae (pa; Figure 3.6B). Further development of hyphae was also observed on the *f5h1*-1 mutant but not on Col-0 (Figure 3.6B). However, *B. graminis* f.sp. *hordei* could not complete its entire life cycle to form conidiophore on either Col-0 or *f5h1*-1 mutant, since further post-invasion growth of *B. graminis* f.sp. *hordei* was inhibited by a hypersensitive reaction (HR) responses in mutant *f5h1*-1 (Figure 3.6C). Moreover, no significant difference in penetration ratio was determined for the *f5h2*-1 mutant and a similar penetration ratio was observed for the double mutant *f5h1*/*f5h2* as for the single mutant *f5h1*-1 (Figure 3.6A), which further supported the previous observation that *F5H2* gene was not involved in plant disease resistance.

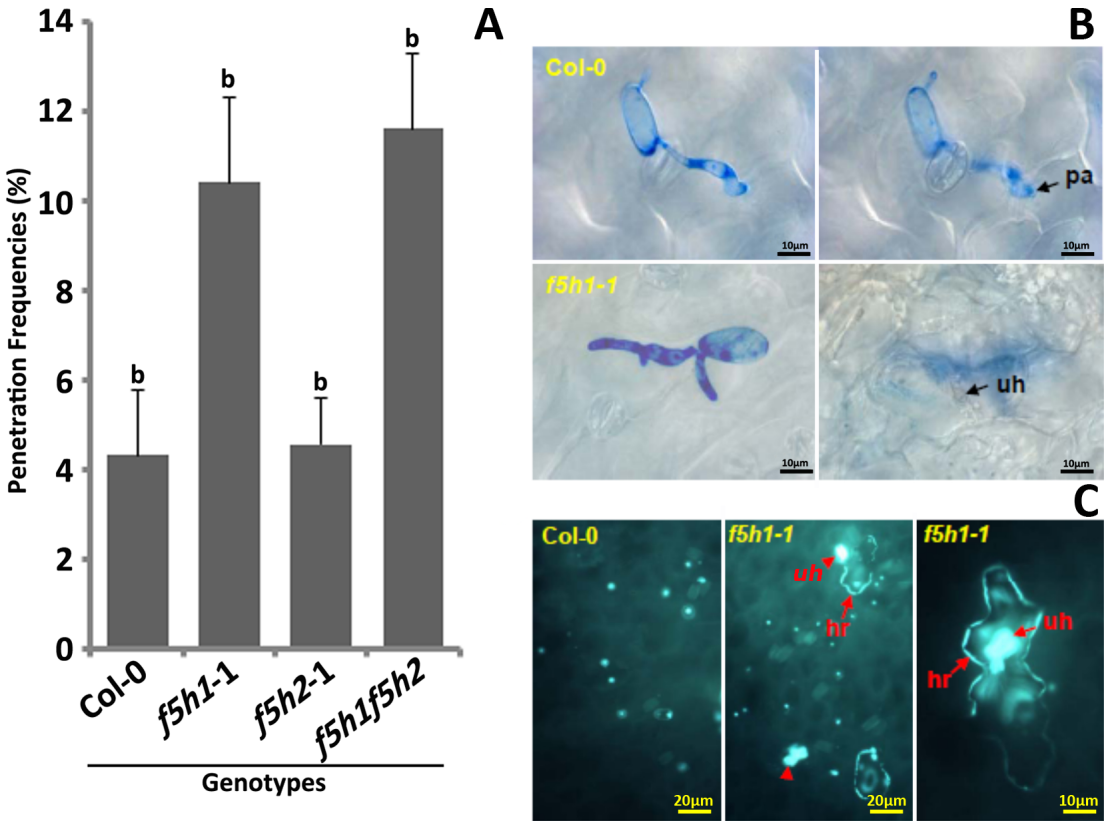

Figure 3.6 Penetration frequencies of nonhost powdery mildew for various mutant lines and wild type plants at 48 hpi.

(A) Penetration frequency of nonhost powdery mildew *Blumeria graminis* f.sp. *hordei* was determined by measuring the ratio of successful penetrations per 100 interaction sites. The formation of haustoria was counted as a successful penetration. Plant samples were collected at 48 hpi of nonhost powdery mildew *B. graminis* f.sp. *hordei* and stained with aniline blue before collecting the penetration frequency data. The mean and standard deviation (SD; as indicated by error bar) were calculated from 3 independent experiments (n=3). Different letters denoted significant differences of the means as determined by ANOVA with Fisher's Least Significant Difference (LSD; $P < 0.01$). (B) Acidic aniline blue (pH=2.0) staining showing the fungal structures. pa, papillae; uh, unilateral haustoria. (C) Alkaline aniline blue (pH=9.5) staining showing deposition of callose at cells with a hypersensitive response (HR). hr, hypersensitive response; uh, unilateral haustoria. Scale bar=10µm.

To further test the penetration resistance of the *f5h1* mutant, three additional pathogens were employed to evaluate the penetration resistance of this mutant allele, including host powdery mildew *E. cichoracearum*, nonhost *Colletotrichum destructivum* and host *C. higginsianum*. Consistent with nonhost powdery mildew *B. graminis* f.sp. *hordei*, *E. cichoracearum* also displayed a higher penetration frequency on *f5h1*-1 than on Col-0 (Figure 3.7). On other mutants disrupted in post-invasion signaling pathways such as *sid2*, *pad4*/*sag101*, *jar1*, *ein2* and transgenic line NahGox, *E. cichoracearum* displayed similar levels of penetration frequency as on wild type Arabidopsis Col-0. Knockout of *F5H1* in these mutant backgrounds enhanced the penetration frequencies to the same extent as *f5h1* single mutant compared with wild type Col-0. Taken together, penetration resistance against host powdery mildew was also impaired by *f5h1* mutation.

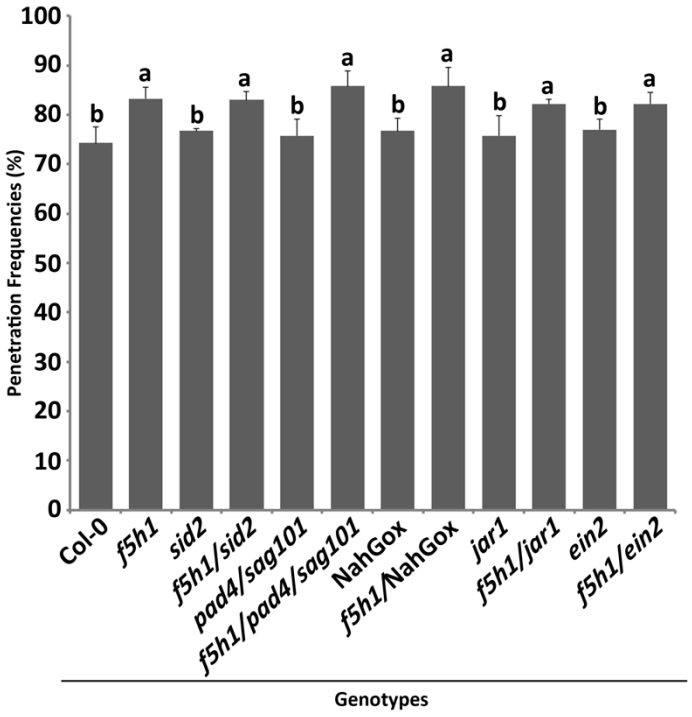

Figure 3.7 Penetration frequency of host powdery mildew.

Penetration efficiencies of host powdery mildew *Erysiphe cichoracearum* were determined as the proportion of successful penetrations per 100 interaction sites at 2 dpi of *E. cichoracearum*. The mean and standard diviation (SD; as indicated by error bar) were calculated from 3 independent experiments (n=3). Different letters denoted significant differences of the means as determined by ANOVA with Fisher's Least Significant Difference (LSD; $P < 0.01$).

Different species of *Colletotrichum* have different levels of pathogenicity on Arabidopsis (O'Connell, et al., 2004). Contrasting nonhost and host *Colletotrichum* species were applied to test the penetration resistance of Arabidopsis against anthracnose pathogens. The nonhost *C. destructivum* usually cannot penetrate through the Arabidopsis cell wall and complete its entire pathogenicity cycle. Germination of conidia and formation of appressoria of *C. destructivum* were as successful on all mutants tested as a wild type Arabidopsis Col-0 (Figure 3.9). However, for single *f5h1* mutant and double/triple mutants carrying the *f5h1* mutation, successful penetrations resulting in the formation of primary hyphae (pointed by red triangles) were widely observed at 3 dpi, while Col-0 and mutants carrying wild type allele *F5H1* and disruption in post-invasion resistance were strongly resistant to penetration of *C. destructivum* (Figure 3.9). This result further confirms that penetration resistance against nonhost *C. destructivum* was significantly impaired by the *f5h1* mutation.

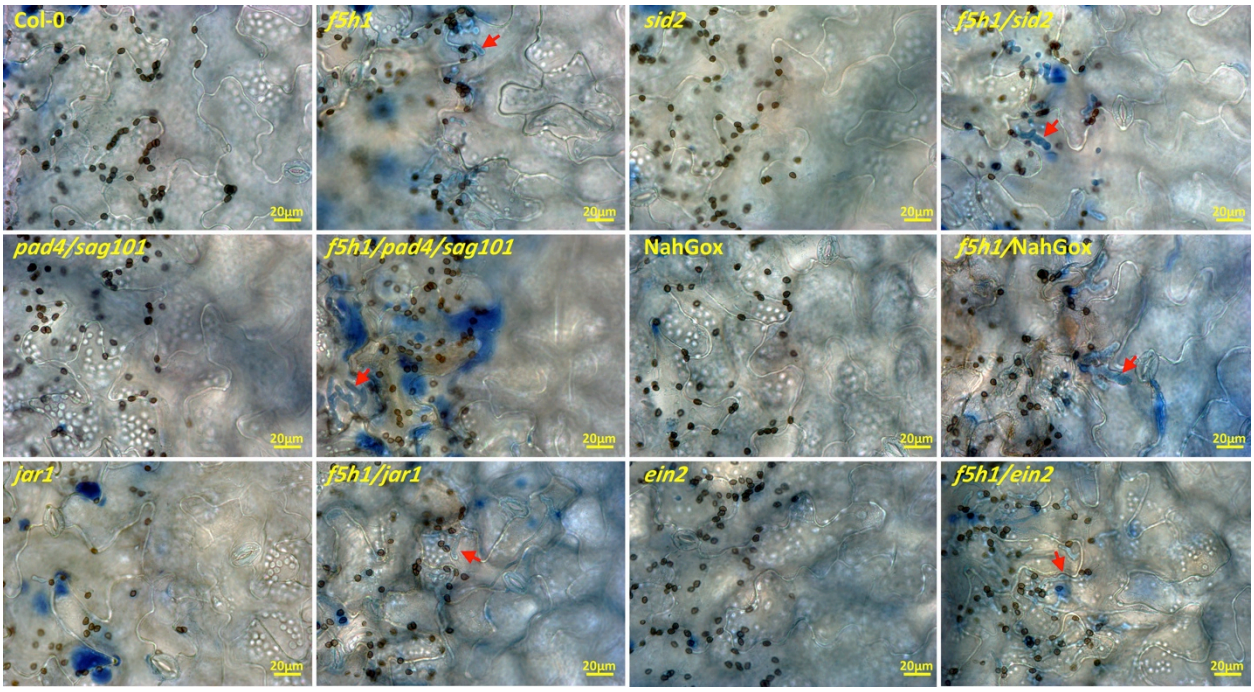

Figure 3.8 Mutant *f5h1* results in penetration of nonhost fungal pathogen.

Successful penetration of *Colletotrichum destructivum* was observed on single *f5h1* mutant and double/triple mutants carrying *f5h1* mutation. *C. destructivum* was inoculated on mature rosette leaves by a single droplet (15µl) of conidia suspension. Samples were collected at 3 days post inoculation (dpi) and photographed after trypan blue staining. Red triangles point the primary hyphae. Scale bar=20µm. Images are representative of 3 independent experiments and 5 observations of each independent experiment.

Finally, the host pathogen *C. higginsianum* was used to challenge the same collection of mutants used with *C. destructivum*. Since *C. higginsianum* is able to complete its pathogenicity cycle and cause disease symptoms in Arabidopsis, the *f5h1* mutant might accelerate the development of disease due to more and faster penetrations. As shown in Figure 3.9, significant disease symptoms of tissue collapse were clearly observed on all mutant lines containing the *f5h1* mutation as early as 2 dpi while Col-0 and other mutants with the wild type *F5H1* allele maintained a healthier appearance at the same time point. The tissue collapse within the lesion indicated the switch in *C. higginsianum* from a biotrophic stage to a necrotrophic stage, which was verified by microscopic analysis showing the existence of secondary hyphae at 48 hpi (Figure 3.9). Taken together, a wild type *F5H1* gene contributes to plant penetration resistance against a broad spectrum of pathogens, including both host and nonhost pathogens of powdery mildew and anthracnose pathogens.

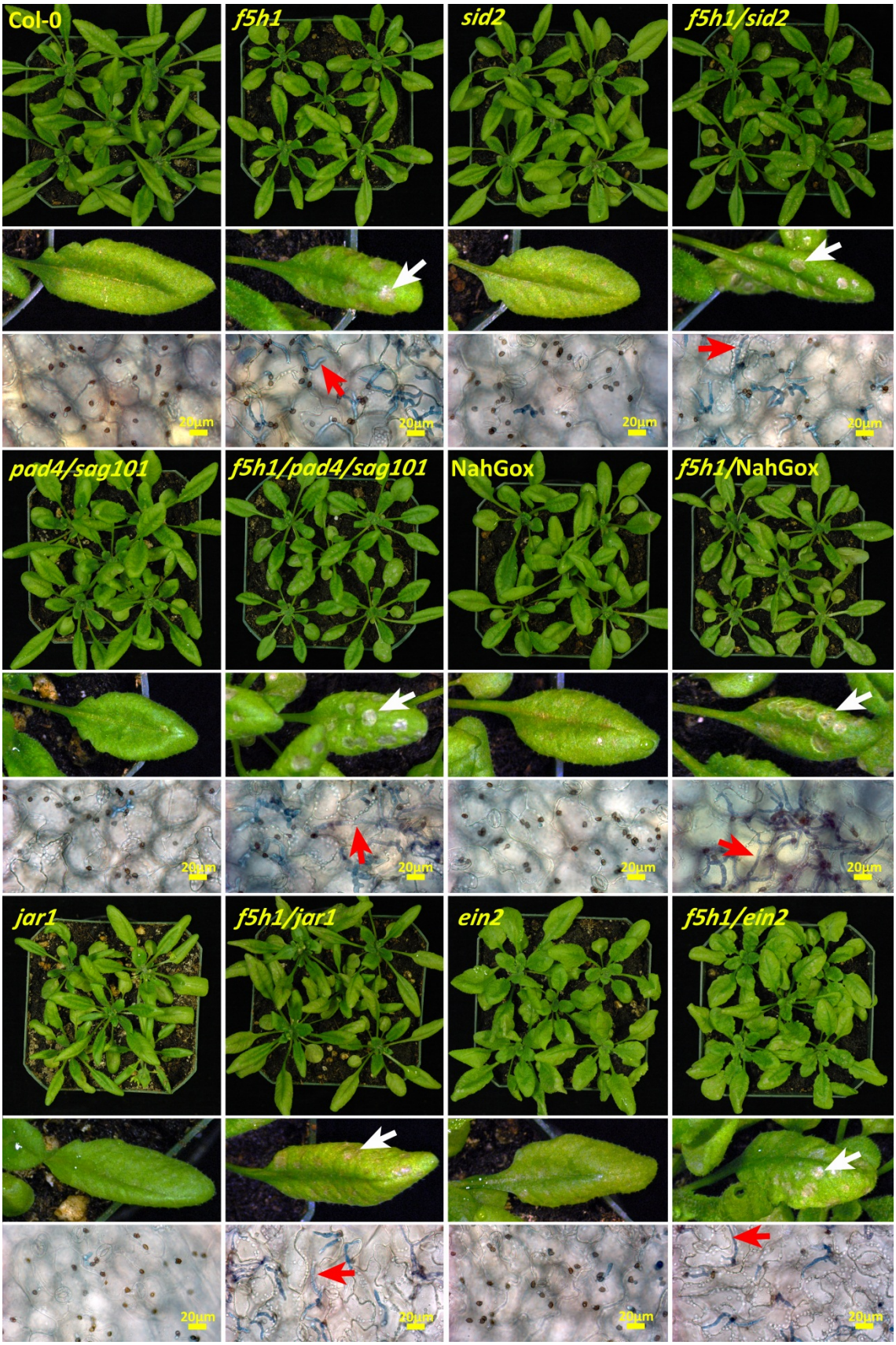
Col-0
f5h1
sid2
f5h1/sid2
pad4/sag101
f5h1/pad4/sag101
NahGox
f5h1/NahGox
jar1
f5h1/jar1
ein2
f5h1/ein2
20µm

Figure 3.9 Mutant *f5h1* promotes disease development of host fungal pathogen.

The disease symptom of *Colletotrichum higginsianum* at 2 days post inoculation (dpi). All plant lines as labeled in the image were inoculated with *C. higginsianum* by spraying conidia suspension at density of 1×10^6 spore/ml. Whole plants were photographed at 2 dpi. Leaves were also collected at 2 dpi and microscopic images photographed after trypan blue staining. White arrows indicate the tissue lesion spots caused by *C. higginsianum* on leaves. Red arrows indicate the development of secondary hyphae. Scale bar=20μm. Images are representative of 3 independent experiments and 5 observations of each independent experiment.

3.5 Papillae structure is altered by the *f5h1* mutation.

The formation of fortified cell wall structures in papillae is the major strategy of penetration resistance. In general, callose and lignin have been considered as major structural components of pathogen induced papillae. Reduction of callose deposition by knocking out the *PMR4* gene does not alter the overall size and shape of papillae but changes the internal structures of papillae (Nishimura et al., 2003). Therefore, the internal structure of papillae formed by the infection of *B. graminis* f.sp. *hordei* pathogen on the *f5h1* mutant was examined by transmission electron microscopy (TEM). As shown in Figure 3.10, the general size and shape of papillae on mutant *f5h1*-1 were not significantly altered in comparison with the papillae formed on wild type Arabidopsis Col-0. However, the layered structures observed in papillae of Col-0 wild type leaves were impaired within papillae in the *f5h1* mutant line. Thus, a wild type *F5H1* gene is required for the construction of functional papillae structures.

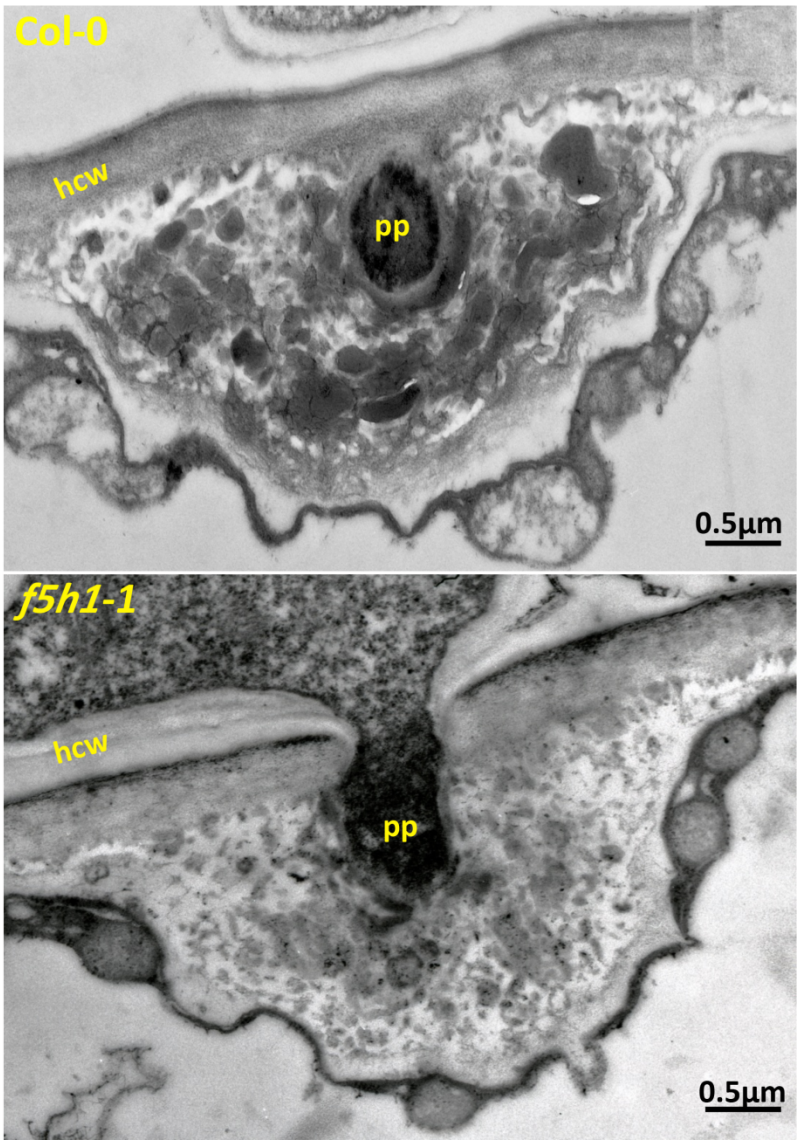

Figure 3.10 Transmission electron microscopy analysis of papillae induced by nonhost powdery midlew.

The ultra structure of papillae formed upon inoculation of nonhost powdery mildew *Blumeria. graminis* f.sp. *hordei* were analyzed by transmission electron micrscope (TEM). Wild type Arabiopsis Col-0 and mutant *f5h1* were inoculated with *B. graminis* f.sp. *hordei*. Samples were collected at 48 hpi, fixed and embeded in resin, which will be subsequently sectioned and observed using TEM after staining. hcw, host cell wall; pp, penetration peg. Scale bar=0.5µm. Images are representative of 2 independent experiments and 4 observations of each independent experiment.

3.6 Plant post-invasion resistance is not affected by the mutation in *f5h1*.

In addition to penetration resistance, plant cells also possess post-invasion resistance against the successful entry of fungi and such post-invasion resistance is regulated by SA, JA and ET signaling pathway (Dodds and Rathjen, 2010). Therefore, subsequent research was to investigate if *F5H1* is involved in post-invasion resistance.

Several disease tests described above in this thesis had provided clues about the relationship of *F5H1* and post-invasion resistance. If *F5H1* was also involved in post-invasion resistance, the knockout mutant *f5h1*-1 should impair both penetration and post-invasion resistance. Therefore, the mutant *f5h1*-1 should display more severe disease symptoms to host pathogens than other mutants which have a wild type *F5H1* allele and are only disrupted in post-invasion resistance such as *pad4*/*sag101* and NahGox. The host pathogen *C. higginsianum* generated severe disease symptoms on several single mutants compared with Col-0, including *pad4*/*sag101* and NahGox (Figure 3.11). Even though the disease symptom observed on *sid2*, *jar1* and *ein2* appeared similar as *f5h1*-1, double mutants *f5h1*/*sid2*, *f5h1*/*jar1* and *f5h1*/*ein2* displayed more severe disease symptoms than the single parental mutants (Figure 3.11). Similarly, the conidiophore formation of *E. cichoracearum* on the *f5h1*-1 single mutant was much less than *pad4*/*sag101* and NahGox lines (Figure 3.12). All of these results strongly suggest that *F5H1* might not be associated with post-invasion resistance.

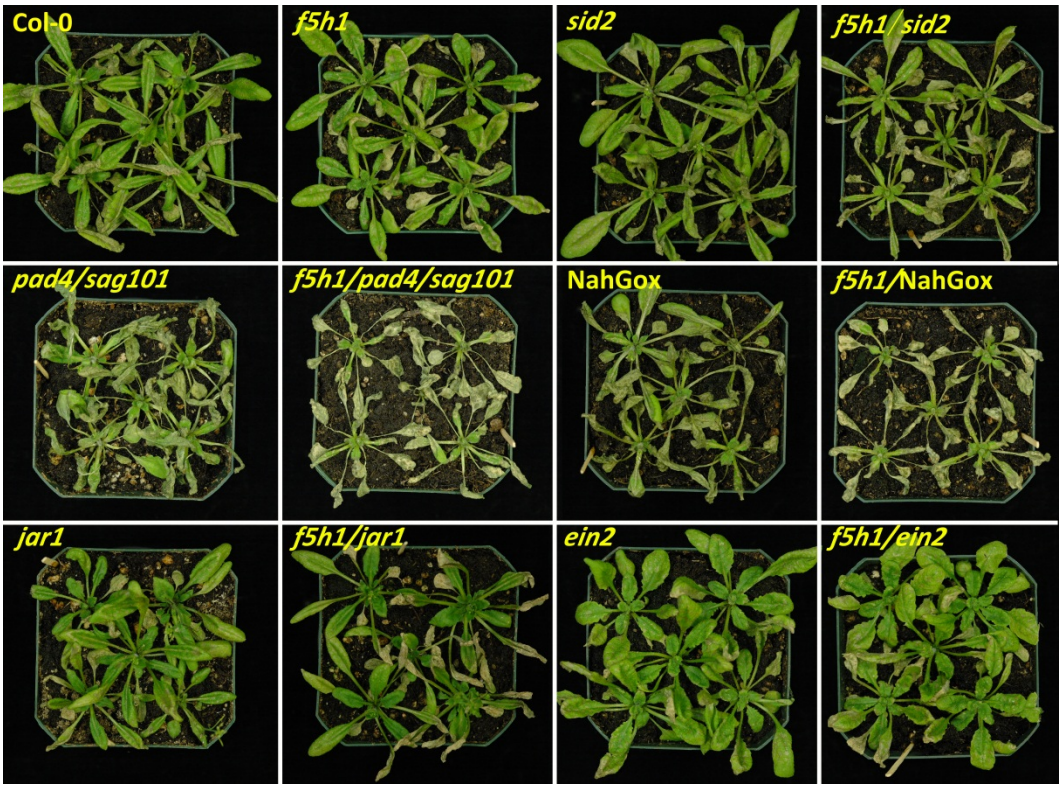

Figure 3.11 Disease symptoms of host fungal pathogen at 4 dpi.

After photographed at 2 dpi, as shown in Figure 3.9, the plants were kept in the same growing conditions for additional two days without lids covered. Removal of lids' cover will reduce the humidity and slow down the impairment of disease caused by *Colletotrichum higginsianum*. In this condition, the pathogenicity of *C. higginsianum* is slightly weakened, which will allow the generation of distinguishable responses from mutant with various levels of disease resistance. Subsequently, images were photographed at 4 dpi. Images are representative of 3 independent experiments.

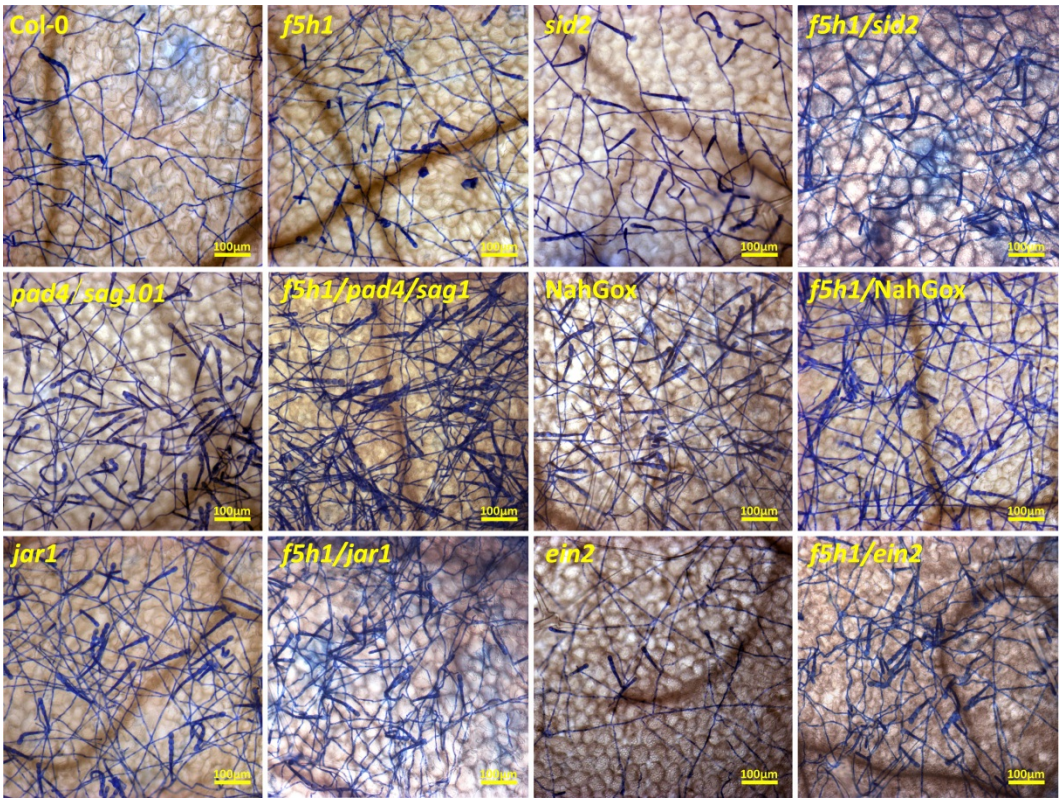

Figure 3.12 Conidiophore formation of host powdery mildew.

Conidiophore formation of host powdery mildew *Erysiphe cichoracearum* was photographed at 7 days post inoculation (dpi) after acidic aniline blue staining (pH=2.0). The density of fungal conidiophore reflects the severity of disease on the plant line tested. Scale bar=100µm. Images are representative of 3 independent experiments and 5 observations of each independent experiment.

To further test this possibility at a molecular level, the expression of marker genes of the SA and JA/ET signaling pathway mediating post-invasion resistance, *PR-1* and *PDF1.2* (Niu et al., 2011) were characterized by northern blotting. As shown in Figure 3.13, both *PR-1* and *PDF1.2* were induced upon *C. higginsianum* infection on the wild type Col-0 and such induction was not altered in *f5h1*-1 mutant, indicating that neither signaling pathway was affected by the knockout of *F5H1*. Together with the disease test analysis, all of the evidence demonstrates that mutation of the *F5H1* gene does not affect post-invasion resistance.

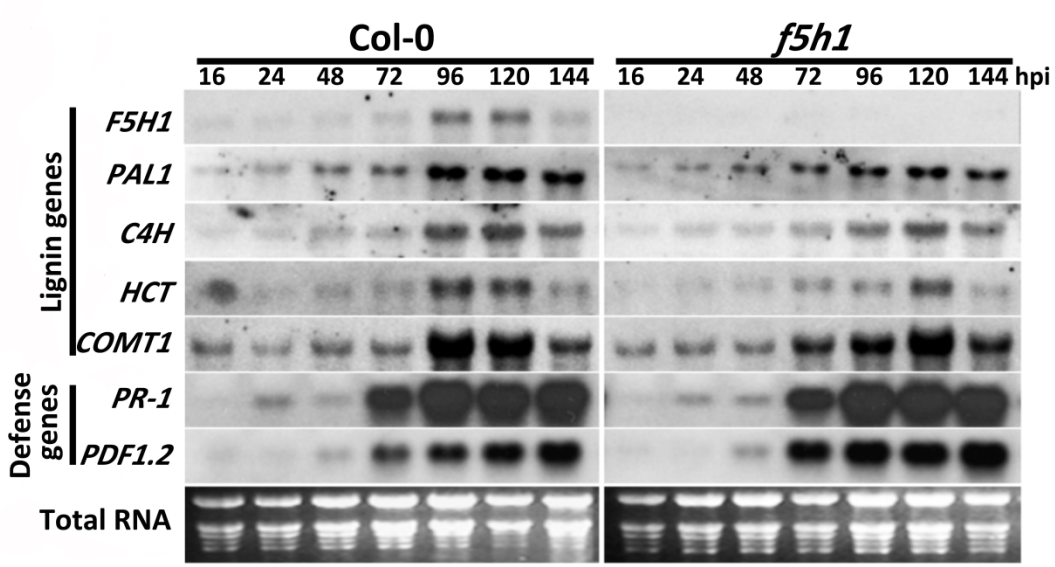

Figure 3.13 Expression of defensive genes and lignin genes in the *f5h1* mutatn during pathogen infection for 12 dpi.

Expression of lignin genes and defensive genes were determined by northern blotting in Col-0 wild type and a *f5h1* mutation background after inoculation with *Colletotrichum higginsianum*. Total aboveground tissues were collected at specific timing points as indicated for RNA isolation. Images were captured by a phosperimager system. Loading of total RNA was visualized under UV light after ethidium bromide staining. Images are representative of 2 independent experiments. Gene abbreviations: *PAL1, phenylalanine ammonia lyase 1; F5H1, ferulic acid 5-hydroxylase 1; C4H, cinnamate 4-hydroxylase; HCT, hydroxycinnamoyl-Coenzyme A shikimate/quinate hydroxycinnamoyltransferase; COMT1, caffeic acid O-methyltransferase 1; PR-1, pathogenesis related gene-1; PDF1.2, plant defensing 1.2.*

3.7 The induction of lignin biosynthetic genes by pathogen is regulated by novel signaling pathway(s)

Previous research had pointed out that almost all of the lignin biosynthetic genes displayed an upregulated expression pattern upon fungal pathogen infection in wheat (Bhuiyan et al., 2009). However, the relevant signaling pathway controlling this reaction remains unknown. Since SA, JA and ET are well-studied hormones regulating plant resistance, the induction of lignin biosynthetic gene expression was tested for dependence on any of the characterized defensive signaling pathways. The expression pattern of *F5H1* and other lignin genes in mutants disrupted in the SA, JA or ET signaling pathways was determined by northern blotting. As shown in Figure 3.14, the induced expression of *F5H1* was slightly increased in transgenic Arabidopsis NahGox, mutant *sid2* and *ein2* while not affected in *pad4sag101* and *jar1*. *PAL1* displayed similar pattern as *F5H1*. Both *C4H* and *COMT1* were not significantly altered in any mutant tested. *HCT* was not affected in mutant *sid2*, *f5h1sids*, transgenic Arabidopsis NahGox and Arabidopsis line *f5h1*/NahGox but slightly down-regulated in other mutants. Wild type Arabidopsis Col-0 was also treated with SA, JA, ET and two defense signaling molecules as well as four developmental hormones including abscisic acid (ABA), auxin (IAA), gibberellin (GA) and cytokinin (BAP). The expression of lignin genes was determined by northern blotting. This experiment showed that exogenous application of SA/JA/ET hormones was not able to affect the expression of lignin genes (Figure 3.15A). Treatment of SNP and H_2O_2 and other hormones failed to induce lignin genes in a pattern that mimicked pathogen-induced expression of lignin genes. Although auxin appeared to induce *F5H1* and *COMT1*, such induction was not observed in a subsequent experiment with a gradient of different auxin concentrations (Liu X, unpublished data).

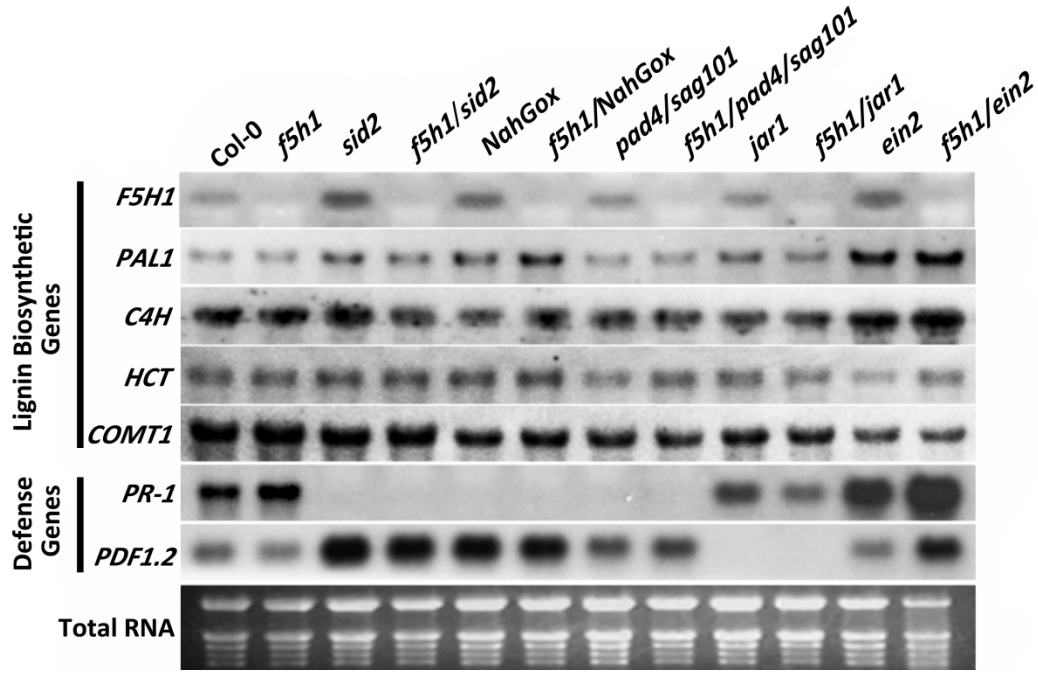

Figure 3.14 Expression of lignin genes and defensive genes in hormone signaling pathway mutants.

All plant lines were inoculated with the host pathogen *Colletotrichum higginsianum* and total above-ground tissues were collected at 3 dpi for RNA isolation. Expression level of each gene was determined by northern blotting and images were captured by a phosphorimager system. Loadings of total RNA was determined by ethidium bromide staining of RNA gel and visualized under UV light. The less intense RNA in Col-0 and *f5h1* samples was due to uneven UV light application during photography, not to lower RNA loading. Images are representative of 2 independent experiments. Gene abbreviations: *PAL1, phenylalanine ammonia lyase 1; F5H1, ferulic acid 5-hydroxylase 1; C4H, cinnamate 4-hydroxylase; HCT, hydroxycinnamoyl-Coenzyme A shikimate/quinate hydroxycinnamoyltransferase; COMT1, caffeic acid O-methyltransferase 1; PR-1, Pathogenesis Related Gene-1; PDF1.2, Plant defensing 1.2.*

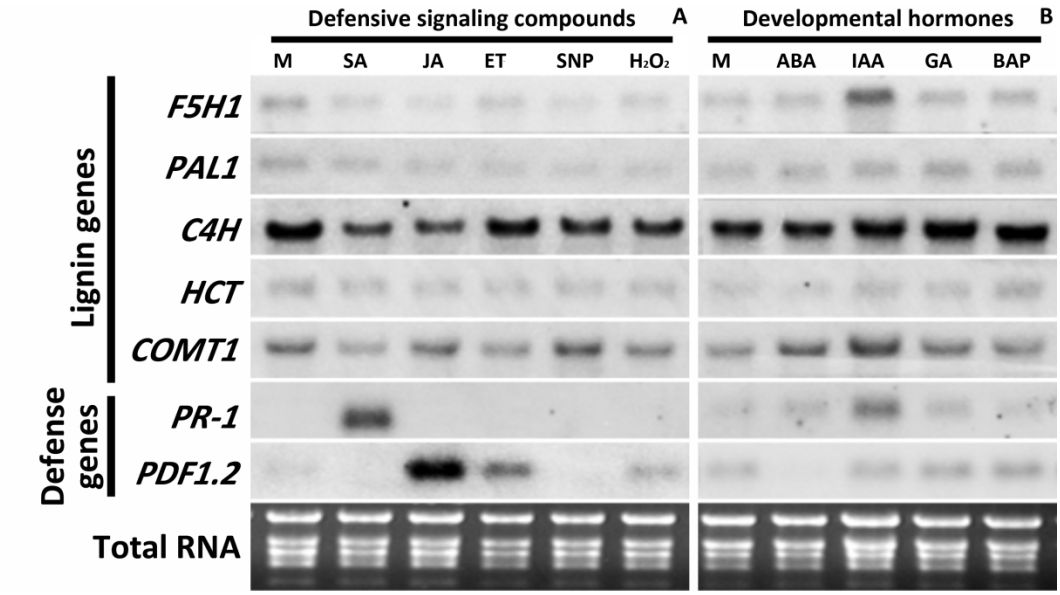

Figure 3.15 Expression of lignin genes and defensive genes after exogenous treatment of signaling molecules.

Expression of lignin genes and defensive genes were characterized by northern blotting. Total above-ground tissues were collected 24 hours post treatment of defensive signaling molecules (A) and developmental hormones (B) for RNA isolation and northern blotting. Treatment abbreviations: M, solvent control treatment; SA, salicylic acid; JA, jasmonic acids; ET, ethephon (ethylene releasing agents); SNP, sodium nitro prussid (NO releasing agent); H_2O_2, hydrogen peroxide; ABA, abscisic acid; IAA, indole-3 acetic acid; GA, gibberellic acid; BAP, 6-benzylaminopurine. Gene abbreviations: *PAL1, phenylalanine ammonia lyase 1; F5H1, ferulic acid 5-hydroxylase 1; C4H, cinnamate 4-hydroxylase; HCT, hydroxycinnamoyl-Coenzyme A shikimate/quinate hydroxycinnamoyltransferase; COMT1, caffeic acid O-methyltransferase 1; PR-1, pathogenesis related gene-1; PDF1.2, plant defensing 1.2.*

3.8 *F5H1* acts independently of *PEN1*, *PEN2* and *PEN3* in penetration resistance.

PEN1, *PEN2* and *PEN3* are well-characterized plant penetration resistance components by the failure of penetration resistance against varieties of powdery mildew pathogens after *PEN* gene mutation (Collins, et al., 2003; Lipka et al., 2005; Stein et al., 2006). Therefore, it is important to analyze the interactions between *F5H1* and *PEN* genes in penetration resistance. To achieve this purpose, double mutants carrying mutations in *f5h1* and individual *pen* mutants were created. Nonhost powdery mildew *B. graminis* f.sp. *hordei* displayed increased penetration frequencies on all *f5h1/pen* double mutants in comparison with corresponding single *pen* mutants (Figure 3.16), suggesting that *F5H1* acts independently of *PEN* genes during penetration resistance against powdery mildew.

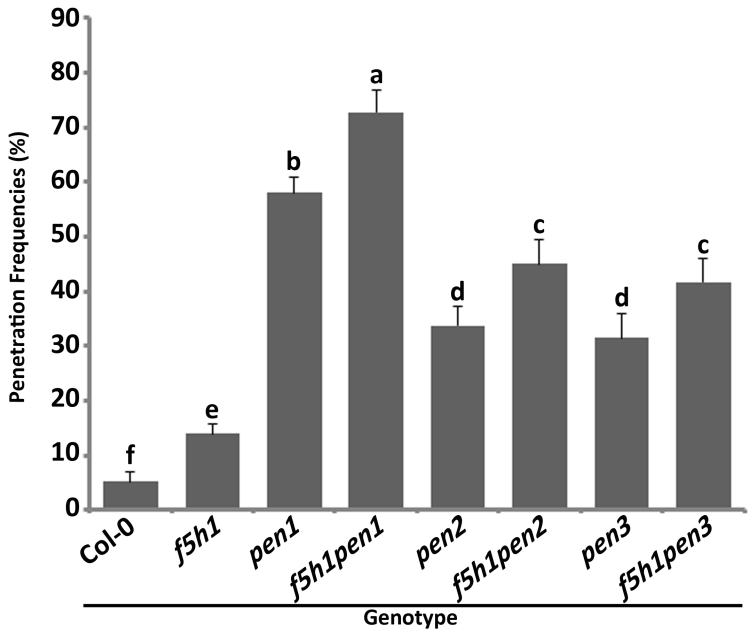

Figure 3.16 *F5H1* acts independently of *PEN*s in penetration resistance against nonhost powdery mildew.

Penetration frequencies of *Blumeria graminis* f.sp. *hordei* at 40 hpi on wild type Arabidopsis Col-0 and mutants were determined by ratio of successful penetrations per 100 plant-pathogen interaction sites. The mean and standard deviation (SD; as indicated by error bar) were calculated from 3 independent experiments (n=3). Different letters denoted significant differences of the means as determined by ANOVA with Fisher's Least Significant Difference (LSD; $P < 0.01$).

Subsequently, the host anthracnose pathogen *C. higginsianum* was employed to verify the additive effect of *f5h1* towards *pen* mutants against other fungal pathogens. At 2 dpi, mutant *f5h1*, *pen2* and *pen3* displayed increased penetration frequencies compared with *Col-0* and the *pen1* mutant (Figure 3.17A) and as expected, all three double mutants showed more severe impairment of penetration resistance as indicated by enhanced penetration frequencies of the pathogen (Figure 3.17A). Moreover, at 3 dpi, this pathogen had already switched to a necrotrophic stage on all double mutants as shown by the development of secondary hyphae (red arrows) while remaining in a biotrophic stage on Col-0 as well as all single mutants (Figure 3.17B). Consistently, this pathogen had caused significant disease symptoms (lesion pointed by white arrows) on all *f5h1-pen* double mutants.

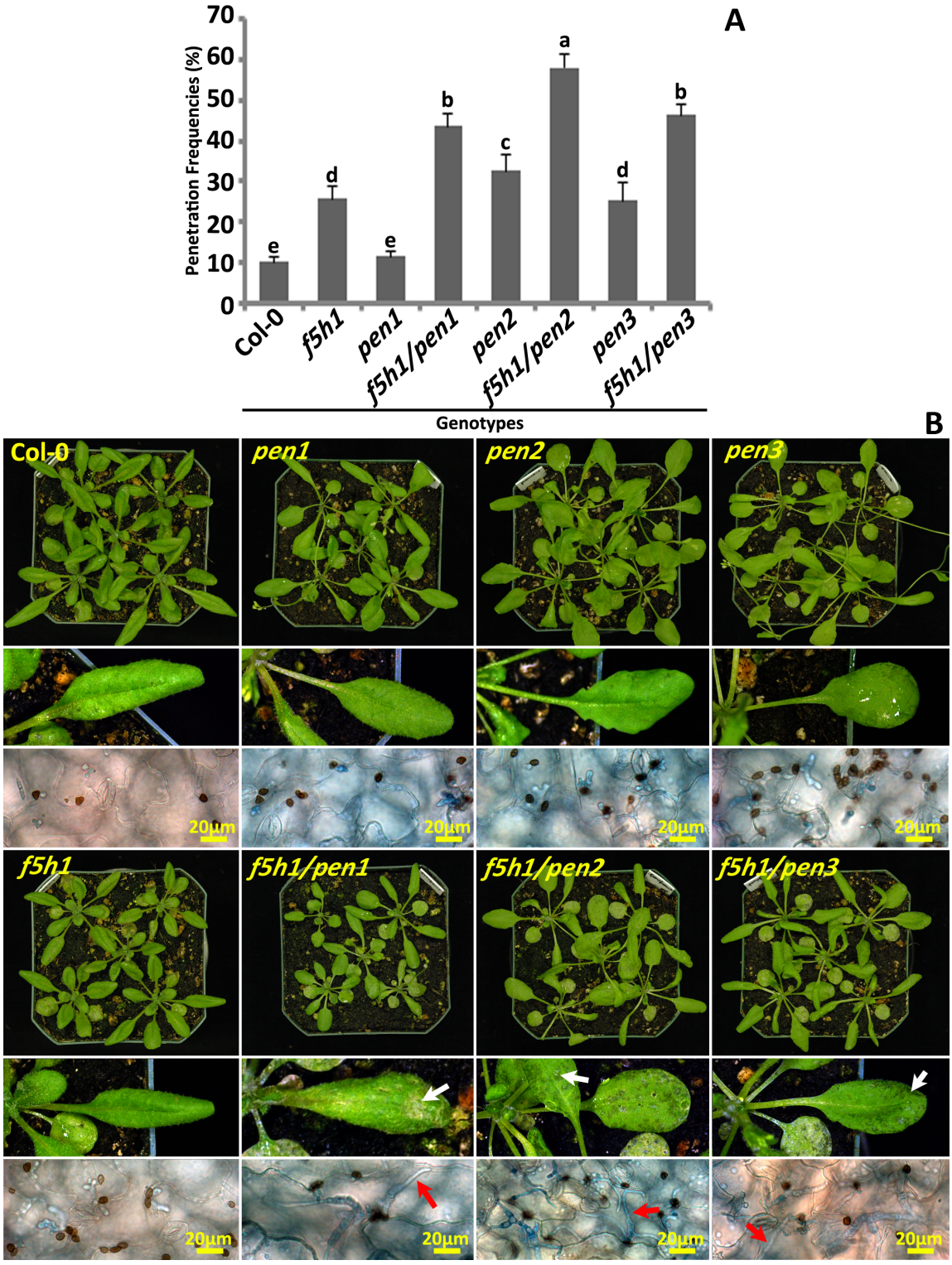
A
Penetration Frequencies (%)
70
60
50
40
30
20
10
0
e
d
e
b
c
a
d
b
Col-0
f5h1
pen1
f5h1/pen1
pen2
f5h1/pen2
pen3
f5h1/pen3
Genotypes
B
Col-0
pen1
pen2
pen3
f5h1
f5h1/pen1
f5h1/pen2
f5h1/pen3
20μm

Figure 3.17 Disease test of host fungal pathogen on *f5h1*/*pen* mutants.

(A) Penetration frequencies of *Colletotrichum higginsianum* at 2 dpi. Value is determined by ratio of successful penetrations per 100 germinated conidia. The mean and standard deviation (SD; as indicated by error bar) were calculated from 3 independent experiments (n=3). Different letters denoted significant differences of the means as determined by ANOVA with Fisher's Least Significant Difference (LSD; $P < 0.01$). (B) Disease symptom of *C. higginsianum* at 3 dpi. *C. higginsianum* was inoculated at Col-0 wild type, *f5h1*, *pen* single mutants, and all double mutants as indicated in images. Plants were photographed at 3 dpi and leaves were collected and fixed for histochemical analysis. Microscopic images were taken after trypan blue staining. White arrows indicate the tissue lesions. Red arrows indicate the secondary hyphae. Scale bar=20μm. Images are representative of 3 independent experiments and 5 observations of each independent experiment.

Finally, the rice blast pathogen *Magnaporthe oryzae* was applied to further verify the relationship between *F5H1* and *PENs*. *M. oryzae* did not successfully penetrate on wild type Arabidopsis (Figure 3.18A) but occasionally penetrated on the *f5h1* mutant (Figure 3.17B). Surprisingly, a large number of successful penetration sites was observed on *f5h1*/*pen2* double mutants, but not on the *pen2* single mutant (Figure 3.18C, D). The germination of fungal conidia and formation of appressoria were not affected (Figure 3.18E). Since no penetration of *M. oryzae* was detected on other *pen* single mutants and *f5h1-pen* double mutants (data not shown), *F5H1* is likely the major component of penetration resistance against pathogen *M. oryzae*. Moreover, this fungal pathogen was able develop longer hyphae on the *f5h1/pen2* double mutant (Figure 3.18F). This suggested that *PEN2* might have additional roles that restrict the post-invasion growth of *M. oryzae* hyphae. The HR response due to the activation of post-invasion resistance was observed in the host cells that were penetrated by *M. oryzae* (Figure 3.18G). In sum, all of these data demonstrate that *F5H1* and *PEN* genes probably act independently in penetration resistance against fungal pathogens.

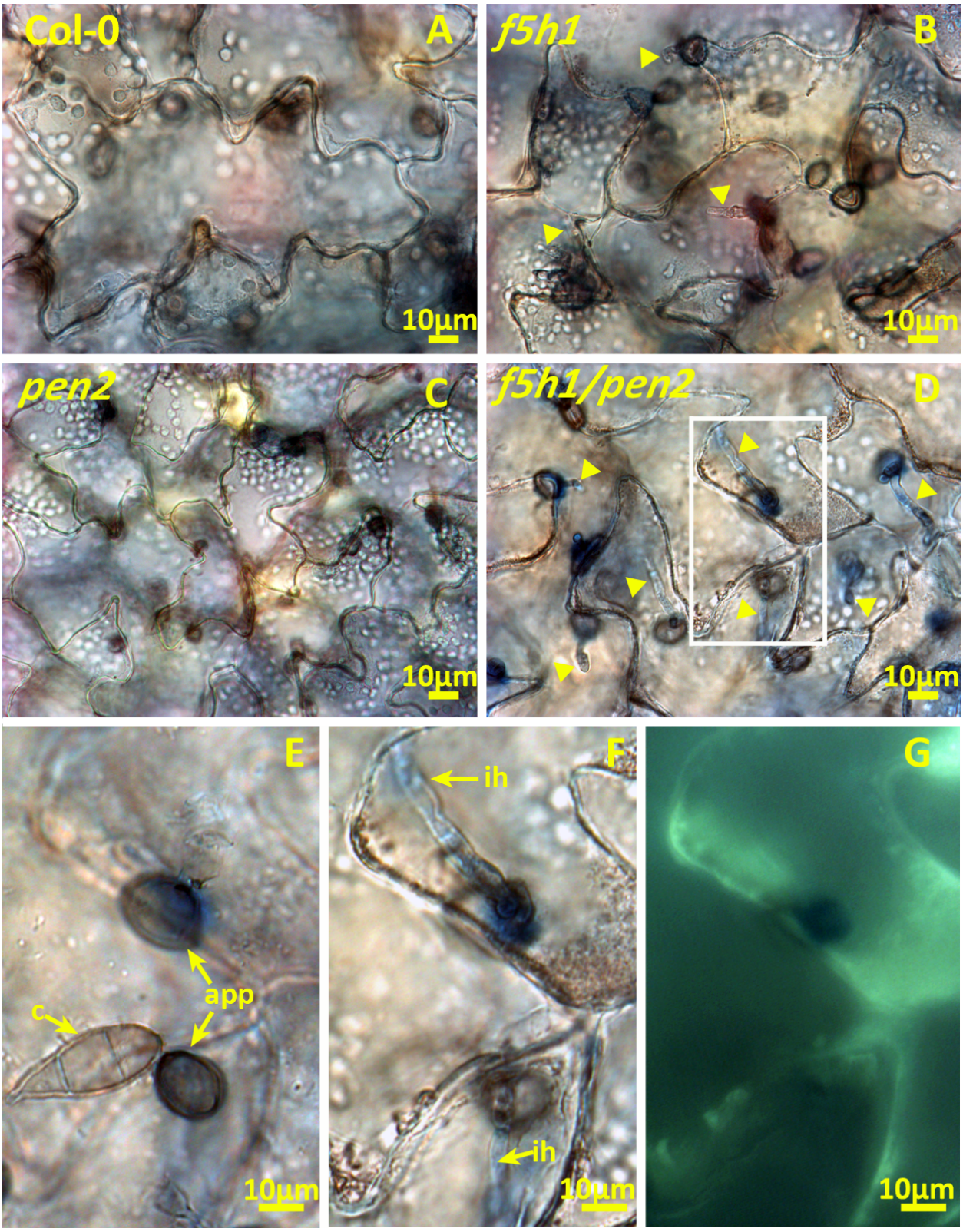
Col-0
A
10µm
f5h1
B
10µm
pen2
C
10µm
f5h1/pen2
D
10µm
E
app
c
10µm
F
ih
ih
10µm
G
10µm

Figure 3.18 Typical Penetrations of *Magnaporthe oryzae* on Arabidopsis *f5h1pen2* double mutants and wild type Col-0.

Magnaporthe oryzae was inoculated by droplet inoculation and leaves were collected at 2 dpi. After fixation and rehydration, leaves were stained with trypan blue and observed under microscope. Yellow triangles in panels B and D indicate successful penetration sites. E and F show enlarged views of the area labeled by the rectangle on D with various focusing depths. G shows the same area as E and F but stained with alkaline aniline blue staining to display callose deposition (blue-green color). c, conidia; app, appressorium; ih, intracellular hyphae. Scale bar=10μm. Images are representative of 3 independent experiments and 5 observations of each independent experiment.

Another unexpected observation was the ectopic deposition of callose on the *pen1* mutant upon pathogen infection. As shown in Figure 3.19A, after *B. graminis* f.sp. *hordei* inoculation at a lower density than disease tests described above, the *pen1* mutant and *f5h1/pen1* double mutant showed a much higher density of callose than the Col-0 wild type and the *f5h1* single mutant. Close investigation of callose deposition on the *pen1* single mutant and the *f5h1pen1* double mutant revealed that most of these callose depositions displayed an altered shape that was different from the circular papillae-associated callose deposition (Figure 3.19B). This data suggested that the ectopic callose deposition was associated with the *pen1* mutant, not *f5h1*.

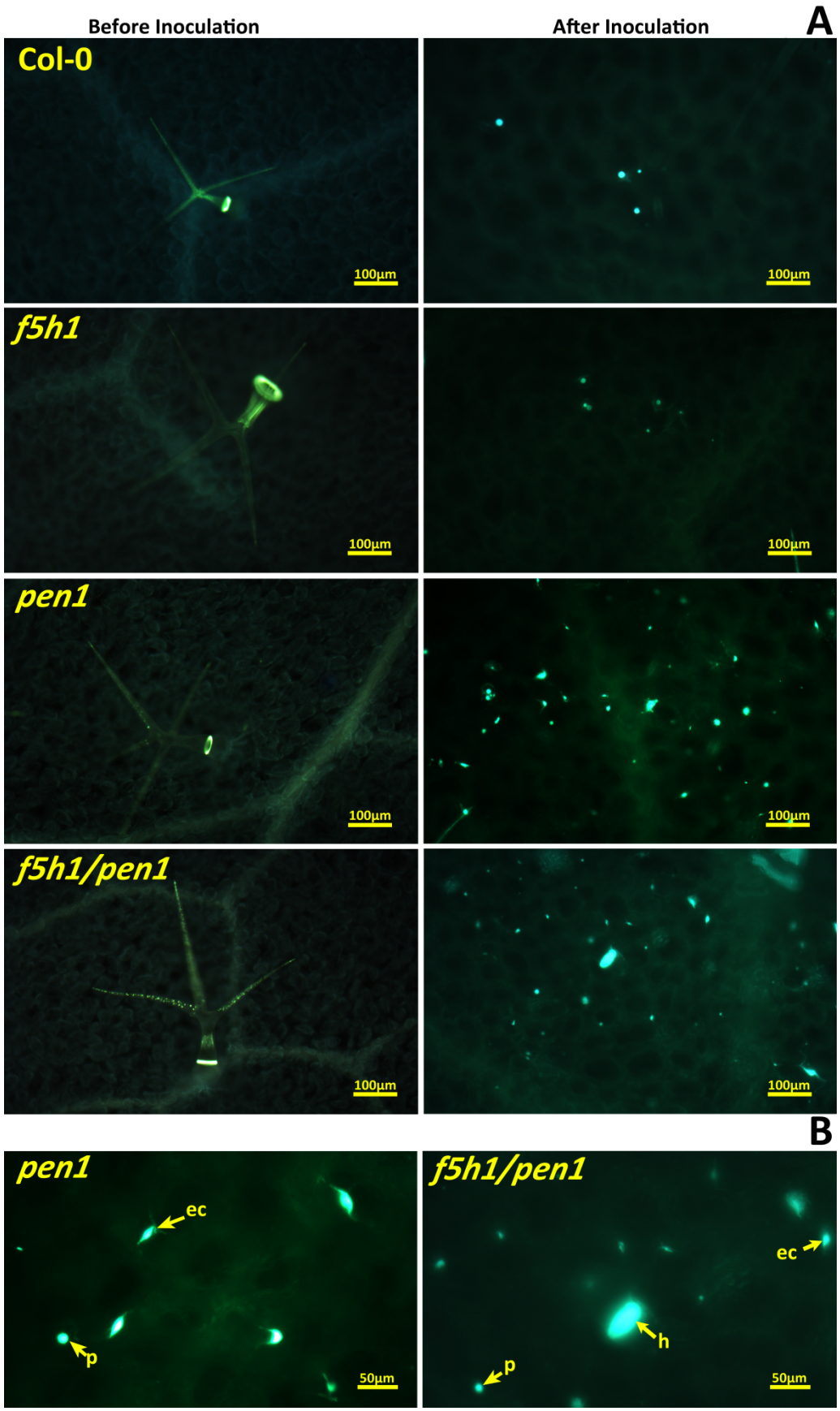
Before Inoculation
After Inoculation
A
Col-0
100µm
100µm
f5h1
100µm
100µm
pen1
100µm
100µm
f5h1/pen1
100µm
100µm
B
pen1
ec
p
50µm
f5h1/pen1
ec
h
p
50µm

Figure 3.19 *B. graminis* f.sp. *hordei* induced ectopic deposition of callose in *pen1* mutant.

(A) Callose deposition (blue-green color spots) before/after inoculation on Col-0, *f5h1*, *pen1* and *f5h1pen1* after alkaline aniline blue (pH=9.5) staining. Scale bar=100μm (B) Enlarged images of *pen1* and *f5h1pen1* showing the deposition of callose with three different shapes: papillae shape (p), hasustorium shape (h; wrapping up fungal haustorium) and ectopic shape (ec). Scale bar=50μm. All samples were collected at 2 days post inoculation.

3.9 *F5H1* is involved in *pmr2*-mediated resistance but not *pmr4*.

Arabidopsis *PMR2* is an ortholog of the barley *MLO* gene that negatively modulates resistance against powdery mildew (Consonni et al., 2010). Similar to barley loss-of-function *mlo* resistance, Arabidopsis *pmr2*-mediated resistance is characterized by enhanced penetration resistance that requires *PEN1*, *PEN2* and *PEN3* gene functions as well as a tryptophan-derived metabolism pathway (Consonni et al., 2006; 2010). Since previous results described in this thesis demonstrated that *F5H1* contributed to penetration resistance independently of *PEN* genes, *pmr2*-mediated resistance may also require the participation of *F5H1* for its enhanced penetration resistance. Therefore, a double mutant carrying *pmr2* and *f5h1* alleles was generated and challenged with both host and nonhost powdery mildew to investigate the interaction between *pmr2* and *F5H1*. The disease symptoms caused by this fungal pathogen were analyzed at 40 hpi for penetration efficiencies by identifying micro-colonies with extended hyphae, at 72 hpi for hyphae development and at 168 hpi for conidiation.

The disease development of *E. cichoracearum* was monitored at the 3 time points described on Col-0 wild type (Figure 3.20A to C), the *f5h1* single mutant (Figure 3.20D to F), the *pmr2* single mutant (Figure 3.20 G to I) and the *f5h1/pmr2* double mutant (Figure 3.20J to L). At 40 hpi, this fungal pathogen was able to develop micro-colonies with short hyphae on wild type Col-0, the *f5h1* single mutant and the *f5h1/pmr2* double mutant but not on the *pmr2* single mutant. The development of fungal hyphae on the *f5h1/pmr2* double mutant but not on the *pmr2* single mutant implied that the *f5h1* mutation impaired the penetration resistance of the *pmr2* single mutant. This was verified by the quantification of penetration frequencies at 40 hpi (Figure 3.20 M). *E. cichoracearum* achieved significantly higher penetration frequency on the *f5h1/pmr2* double mutant in comparison with the *pmr2* single mutant, but such penetration frequency remained significantly lower than the value obtained on wild type Col-0 and the single mutant *f5h1* (Figure 3.20M).

At 3 dpi, this fungal pathogen developed very tiny colonies with fewer branches on both the *pmr2* single mutant and the *f5h1/pmr2* double mutant compared with wild type Col-0 and *f5h1* single mutant. This was further confirmed by the quantification of total hyphae length per colony (Figure 3.20N). However, at 7 dpi, *E. cichoracearum* colonies observed on the *f5h1/pmr2* double mutant were significantly bigger and had more hyphal branches than the colonies observed on *pmr2* single mutants. Since the *E. cichoracearum* underwent secondary penetration

during the hyphae development, the total haustoria formed per colony were counted (Figure 3.20O). The reduced penetration resistance of the *f5h1/pmr2* double mutant resulted in an increase of the total haustoria formed per colony than found on single mutants. Although the *f5h1/pmr2* double mutant showed a weaker penetration resistance than *pmr2* single mutant, the overall resistance was not affected since no conidiophore was formed. These results demonstrated that knockout of *F5H1* in *pmr2* mutant partially impaired the penetration resistance but did not affect overall resistance against host powdery mildew.

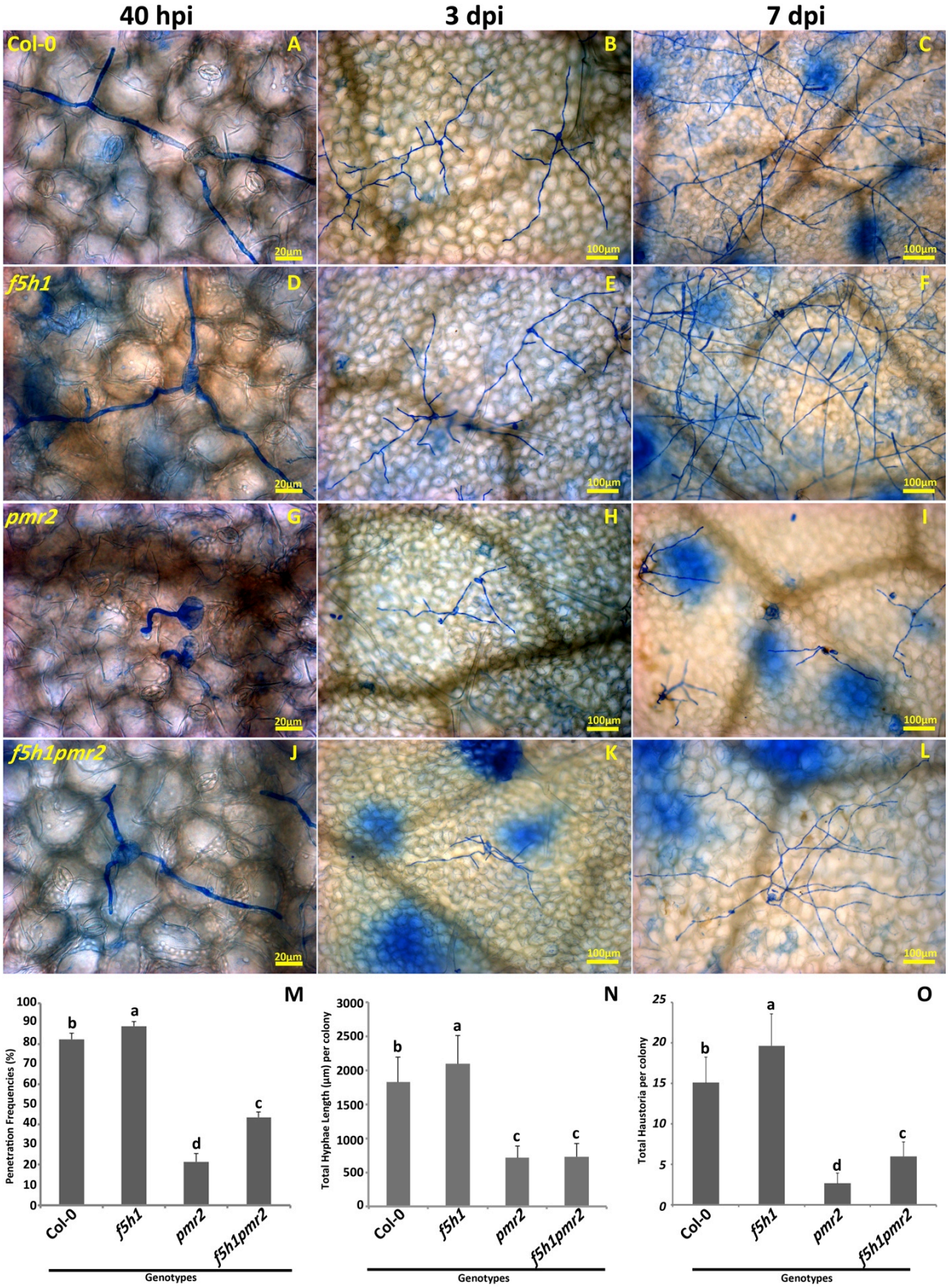
40 hpi
3 dpi
7 dpi
Col-0
A
B
C
f5h1
D
E
F
pmr2
G
H
I
f5h1pmr2
J
K
L
20µm
100µm
M
N
O
Penetration Frequencies (%)
Total Hyphae Length (µm) per colony
Total Haustoria per colony
Col-0
f5h1
pmr2
f5h1pmr2
Genotypes

Figure 3.20 *F5H1* is involved in *pmr2*-mediated resistance against host powdery mildew.

Histochemical analysis of typical *Erysiphe cichoracearum* colony development at 40 hours post inoculation (hpi), 3 and 7 days post inoculation (dpi) on Col-0 (A)-(C); *f5h1*-1 (D)-(F); *pmr2* (G)-(I) and *f5h1/pmr2* (J)-(L). Samples were collected at appointed timing points as shown in image and stained with acidic aniline blue (pH=2.0) after fixation treatment. (M) Penetration frequencies at 40 hpi. The value is determined by successful penetrations per 100 interaction sites. (N) Total hyphae length per colony of *E. cichoracearum* at 3 dpi. (O) Numbers of haustoria formed per colony of E.cichoracearum at 7 dpi. For panel M, N and O, the mean and standard deviation (SD; as indicated by error bar) were calculated from 3 independent experiments (n=3). Different letters denoted significant differences of the means as determined by ANOVA with Fisher's Least Significant Difference (LSD; $P < 0.05$).

However, when challenged with nonhost pathogen *B. graminis* f.sp. *hordei*, the *f5h1* mutation in the *pmr2* background did not significantly enhance fungal entry ratio (Figure 3.21). This was probably due to the weak infection capacity of nonhost pathogen *B. graminis* f.sp. *hordei* that could not the overcome partially impaired penetration resistance resulting from the *f5h1* mutation in a *pmr2* background. Moreover, the over accumulation of hydrogen peroxide at papillae formed on the *pmr2* single mutant (indicated by the enhanced brownish color post DAB staining) remained unaltered on the *f5h1/pmr2* double mutant (Figure 3.22). All of these results pointed out that *F5H1* was only contributing to the penetration resistance of the *pmr2* mutant and was not associated with its other resistance mechanisms.

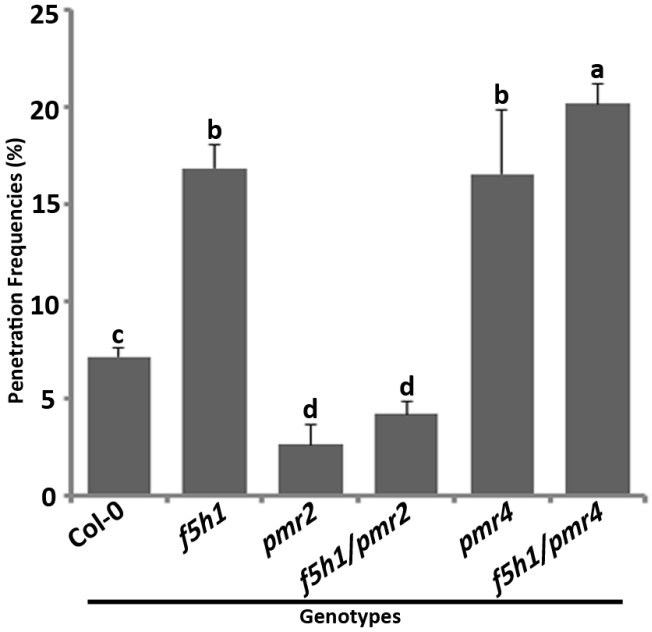

Figure 3.21 Penetration efficiencies of nonhost powdery mildew on *pmr*-related mutants.

Penetration efficiencies of *Blumeria graminis* f.sp. *hordei* on wild type Arabidopsis Col-0 and mutants of *f5h1*, *pmr2*, *f5h1*/*pmr2*, *pmr4* and *f5h1/pmr4*. Values were determined as successful penetration/100 interaction sites. The mean and standard deviation (SD; as indicated by error bar) were calculated from 3 independent experiments (n=3). Different letters denoted significant differences of the means as determined by ANOVA with Fisher's Least Significant Difference (LSD; $P < 0.05$).

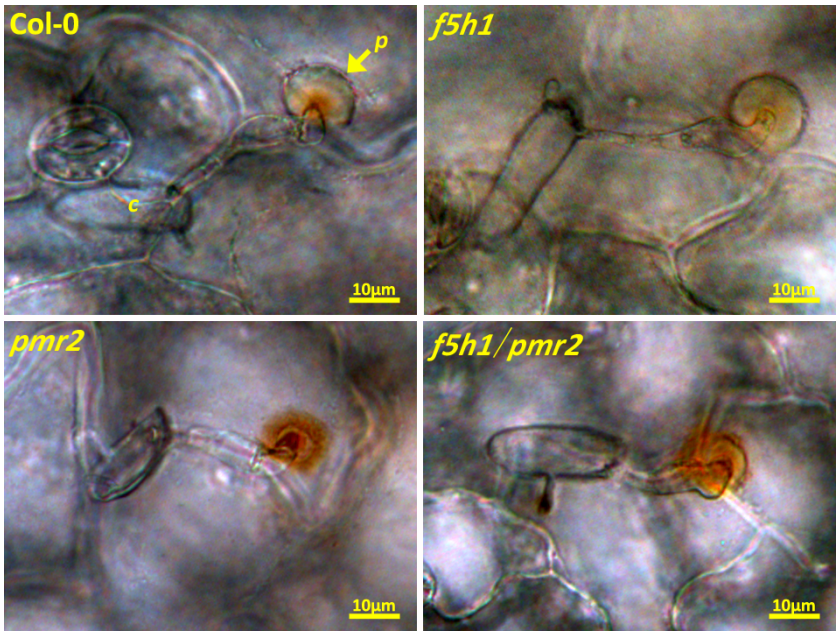

Figure 3.22 Accumulation of hydrogen peroxide at papillae site induced by nonhost powdery mildew infection.

Leaves of Arabidopsis wild type Col-0 and mutants *pmr2*, *f5h1*, *f5h1/pmr2* were inoculated with the nonhost pathogen *Blumeria graminis* f.sp. *hordei*. Inoculated leaves at 60 hpi were detached for 3,3'-Diaminobenzidine (DAB) uptake. After 6 hr incubation, the leaves were fixed for microscopy analysis. The brownish color reactions indicated the accumulation of hydrogen peroxide. c, conidia; p, papilla; Scale bar=10µm. Images are representative of 3 independent experiments and 5 observations of each independent experiment.

Similarly, double mutant *f5h1pmr4* was also analyzed using both nonhost and host powdery mildews. In Arabidopsis, *PMR4* encodes pathogen-induced callose synthase (Nishimura et al., 2003). Since callose is also important structural component of papillae, the penetration resistance was tested to see if it was affected by the *pmr4* mutation. Single *pmr4* mutant resulted in a significant increase in the penetration ratio when challenged with nonhost *B. graminis* f.sp. *hordei,* suggesting that loss of callose impaired penetration resistance (Figure 3.21). Double mutant *f5h1/pmr4* allowed a significantly higher penetration ratio of nonhost powdery mildew pathogen, suggesting the independency of *F5H1* and *PMR4* (Figure 3.21).

When challenged with host powdery mildew *E. cichoracearum*, the disease development was recorded at 40 hpi, 3 dpi and 7 dpi on Col-0 (Figure 3.23A to C), *f5h1* single mutant (Figure 3.23D to F), *pmr4* single mutant (Figure 3.23G to I) and *f5h1/pmr4* double mutant (Figure 3.23J to L). At 40 hpi, the morphologies of *E. cichoracearum* fungal colonies observed on all of the four plant lines were comparable (Figure 3.23A, D, G). Quantification of penetration resistance at this time point also revealed that values obtained for the *pmr4* single mutant and the *f5h1/pmr4* double mutant were comparable to wild type Col-0 and the *f5h1* single mutant. This observation implied a similar level of penetration resistance of *pmr4* single mutants and *f5h1/pmr4* double mutant. However, the colonies of host powdery mildew *E. cichoracearum* observed on the *f5h1/pmr4* double mutant displayed more aggressive hyphae development at 3 dpi and 7 dpi compared to their development on the *pmr4* single mutant, and even occasional conidiation (data not shown), suggesting a possibly weaker penetration resistance of the *f5h1/pmr4* double mutants in comparison with the *pmr4* single mutant. In addition, previous reports have characterized that loss-of-function *pmr4*-mediated resistance was dependent on the SA signaling pathway (Nishimura et al., 2003). The SA-dependent pattern conferred by *pmr4* mutant, such as HR responses associated with penetrated epidermal cell and constitutive expression of *PR-1*, was not affected by *f5h1* mutation (Figure 3.23I; L; O). Therefore, the *f5h1* mutation in the *pmr4* mutant background must allow more secondary penetrations and resulted in enhanced disease development, but does not affect the mechanism of *pmr4*-mediated resistance.

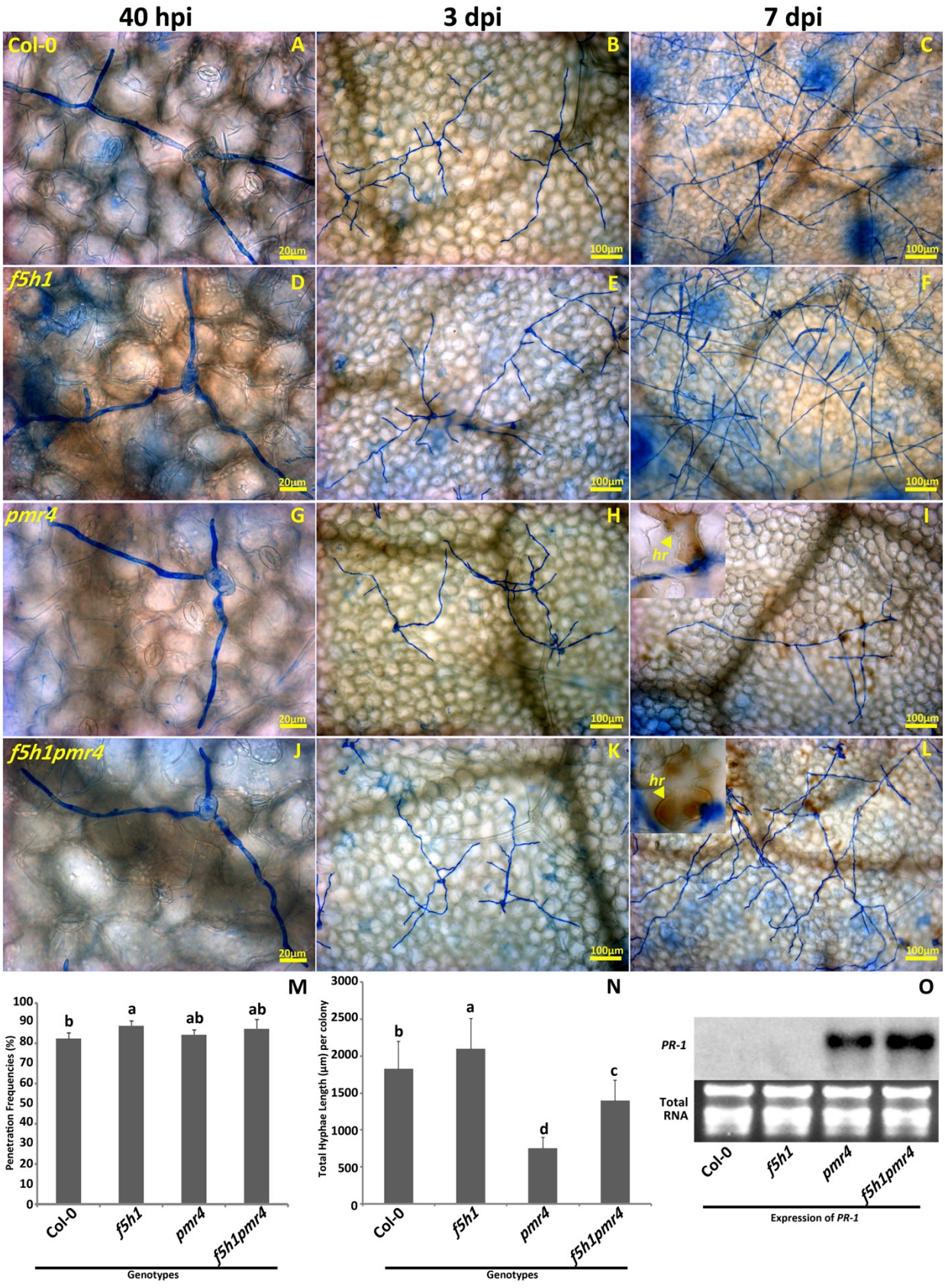
40 hpi
3 dpi
7 dpi
Col-0
A
B
C
f5h1
D
E
F
pmr4
G
H
I
hr
f5h1pmr4
J
K
L
hr
20µm
100µm
M
Penetration Frequencies (%)
b
a
ab
ab
Col-0
f5h1
pmr4
f5h1pmr4
Genotypes
N
Total Hyphae Length (µm) per colony
b
a
d
c
Col-0
f5h1
pmr4
f5h1pmr4
Genotypes
O
PR-1
Total RNA
Col-0
f5h1
pmr4
f5h1pmr4
Expression of PR-1

Figure 3.23 *F5H1* is not involved in *pmr4*-mediated resistance against host powdery mildew.

Histochemical analysis of *Erysiphe cichoracearum* colony development at 40 hours post inoculation (hpi), 3 and 7 days post inoculation (dpi) on Col-0 (A)-(C); *f5h1*-1 (D)-(F); *pmr4* (G)-(I) and *f5h1pmr4* (J)-(L). The panel A to F was reproduced from Figure 3.21 since the experiment was conducted at the same time. Samples were collected at appointed timing points listed in image and stained with acidic aniline blue (pH=2.0) after fixation treatment. Scale bars are labeled in the images. Inserts of panel I and L show cells with hypersensitive response (HR) as indicated by the brown color reaction. hr, hypersensitive response. (M) Penetration frequencies of *E. cichoracearum* at 40 hpi. (N) Total hyphae length per colony of *E. cichoracearum* at 3 dpi For panel M and N, the mean and standard deviation (SD; as indicated by error bar) were calculated from 3 independent experiments (n=3). Different letters denoted significant differences of the means as determined by ANOVA with Fisher's Least Significant Difference (LSD; $P < 0.05$). (O) Expression of *PR-1* gene was determined by northern blotting with phosphorimager system. Equal loading of total RNA was determined by ethidium bromide staining and photographed in Alphaimager 2200 system.

3.10 Characterization of Arabidopsis mutants disrupted in flavonoid.

As described in Chapter 1, a few legume flavonoid compounds show anti-microbial activities and function in disease resistance. Such anti-microbial flavonoids were not commonly identified in the model plant Arabidopsis. To investigate the role of flavonoid compounds in immunity of Arabidopsis, the mutants disrupted in genes encoding chalcone synthase (CHS), chalcone isomerase (CHI) and flavonone 3-hydroxylase (F3H) were analyzed for their resistance against host fungal pathogen. CHS, CHI and F3H catalyze the initial three steps of the flavonoids pathway and generate dihydrokaempferol and dihydroquercetin, the general precursors of other flavonoids compounds (Figure 1.3 for detailed metabolism pathway). The three corresponding genes, *TT4* (At5g13930; CHS; Koornneef, 1990), *TT5* (At3g55120; CHI; Shirley et al., 1995) and *TT6* (At3g51240; F3H; Pelletier and Shirley, 1996), exist as single copy genes in the Arabidopsis genome. Therefore, one mutant of each gene was obtained from the SALK T-DNA insertion collection (Figure 3.24A, B and C). Homozygous lines for mutants were verified by PCR according to the instructions of the seed supplier. All three mutants lacked anthocyanin pigment at petioles, which is consistent with previous phenotype reports (Figure 3.24D; Dong et al., 2001). Moreover, mutants *tt4*-T1 and *tt6*-T1 showed leaf and whole plant developmental phenotypes indistinct from Col-0 wild type plants, while *tt5*-T1 displayed a reduction in plant size under normal “long day” photoperiod conditions as well as etched leaf edges (Figure 3.24D; 32).

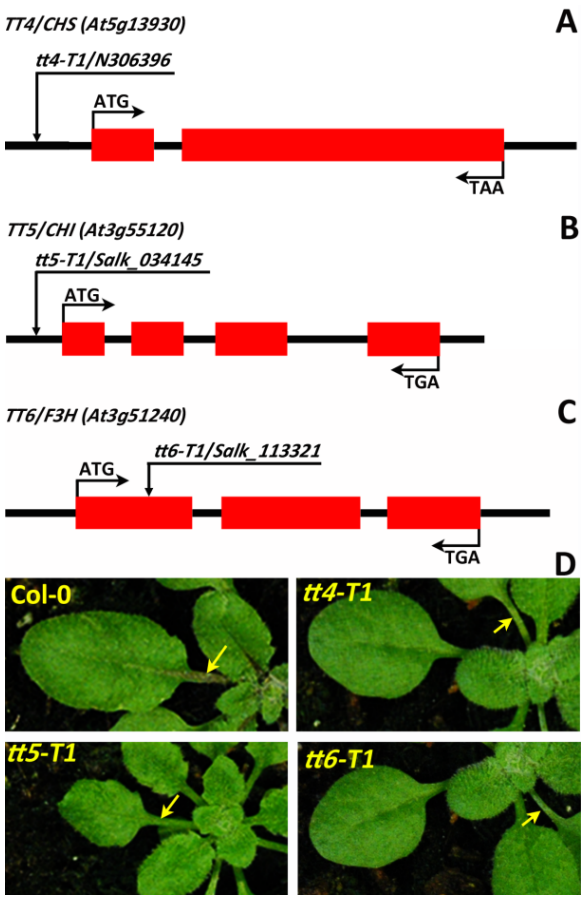

Figure 3.24 Evaluation of flavonoid upper pathway mutants.

(A)-(C) Gene structures of *TT4*, *TT5* and *TT6*. Red boxes represent excons. Black lines represent 5'-UTR and promoter region if prior to the ATG start codon, 3'-UTR if after the TAA/TGA stop codons and introns if between ATG and TAA/TGA. Arrows indicate the position of the T-DNA insertion. (D) Developmental phenotypes of mutants *tt4*-T1, *tt5*-T1 and *tt6*-T1 under a long day photoperiod. Plants shown in images were two weeks old. One representative leaf of each mutant was selected and magnified to show the morphology and the absence of pigment at petioles of the mutants, as indicated by yellow arrows.

3.11 Mutation of *TT5* confers enhanced resistance in Arabidopsis against host pathogen.

Based on current knowledge of Arabidopsis flavonoid metabolism, mutants of *tt4*, *tt5* and *tt6* should eliminate the large proportion of downstream metabolic steps (Figure 1.4). Due to the putative anti-microbial activities of flavonoid compounds, an enhanced susceptibility should be expected upon challenge with a host pathogen. To analyze the disease resistance of these mutants, host pathogen *C. higginsianum* was applied to all three Arabidopsis flavonoid mutants to determine their responses. By 5 dpi, more tissue lesions were observed on *tt4*-T1 and *tt6*-T1 than on the Col-0 wild type (Figure 3.25), suggesting that both mutations conferred enhanced susceptibility against host pathogen *C. higginsianum* on Arabidopsis. In contrast, more healthy tissues were observed for *tt5*-T1, especially in the mature leaf tissues, suggesting that this mutant was more resistant to *C. higginsianum* than Col-0 wild type (Figure 3.25).

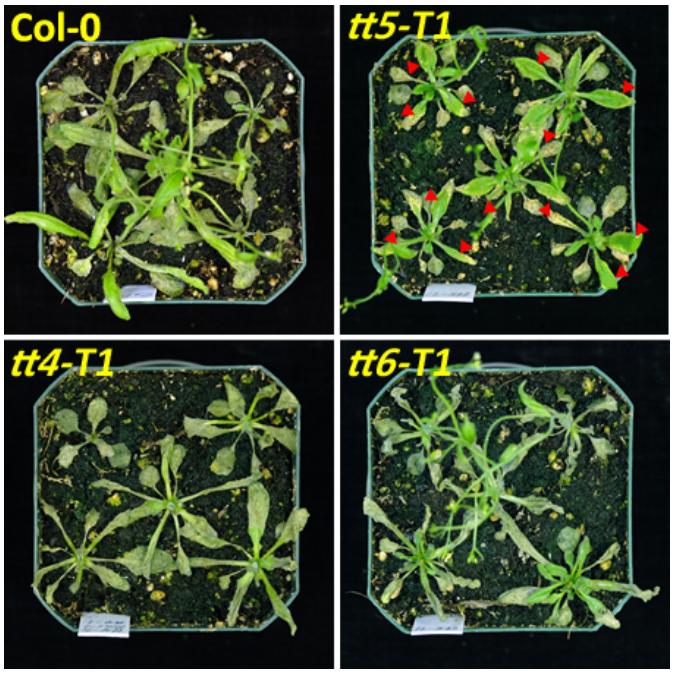

Figure 3.25 Distinct disease responses of flavonoid mutants challenged with host fungal pathogen.

Three weeks old plants were inoculated with host pathogen *Colletotricum higginsianum*. Plants were photographed at 5 days post inoculation (dpi). The healthy tissues on *tt5*-T1 mutant were indicated by the red triangles. Images are representative of 3 independent experiments and 2 observations of each independent experiment.

3.12 The *tt5*-mediated phenotype is associated with salicylic acid signaling pathway.

To analyze the molecular mechanism of this unique *tt5*-mediated enhanced disease resistance, *tt5* single mutants were crossed with various mutants that showed more susceptible phenotype against host pathogen *C. higginsianum*, including *tt4*-T1, *pad4*/*sag101*, *jar1* and *ein2*. As shown in Figure 3.26, the developmental phenotypes including small plant size and altered leaf morphology of *tt5*-T1 were rescued by knockout of the up-stream gene *TT4* and in a mutant of the SA signaling pathway, *pad4/sag101*, suggesting that *tt5*-mediated developmental phenotype required the generation of naringenin chalcone and depended on a functional SA-signaling pathway. Mutation of *jar1* and *ein2* that are disrupted in JA and ET signaling pathway did not affect the developmental phenotype of the *tt5* mutant, suggesting that the phenotype of the *tt5* mutant is independent of the JA and ET signaling pathways.

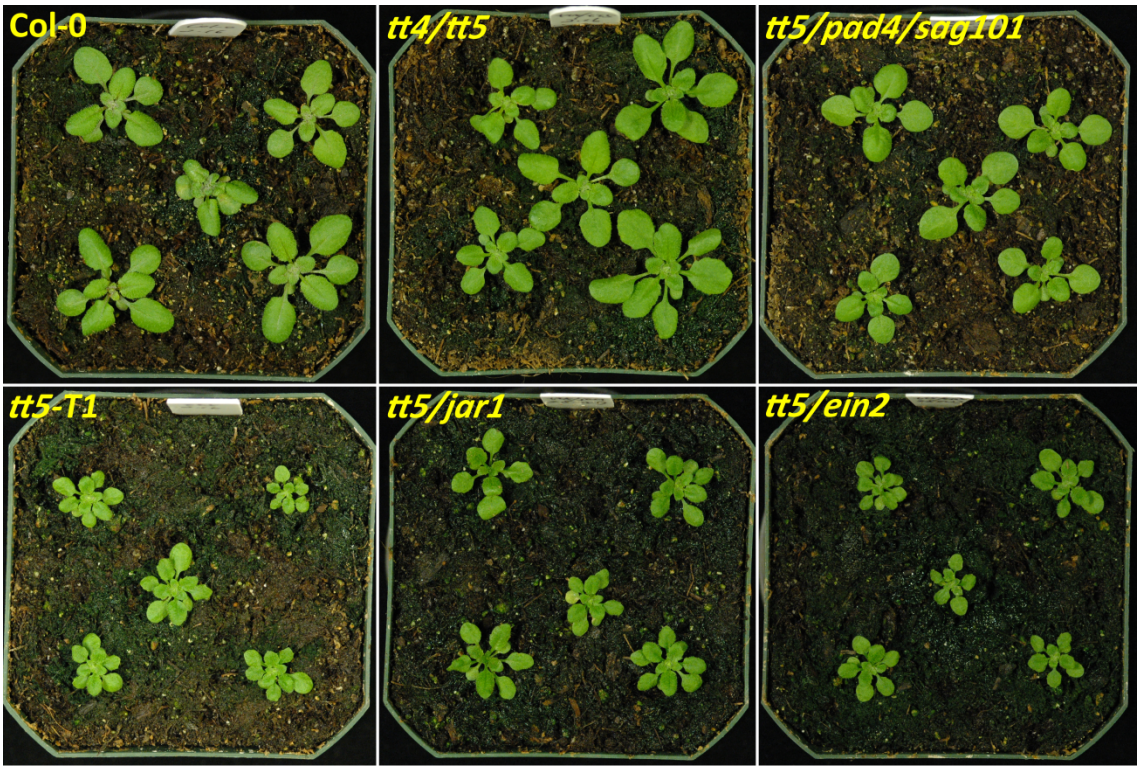

Figure 3.26 Restoration of developmental phenotype of *tt5*-T1 mutant.

Plants shown in images were two weeks old under normal growing environment with a light periode of (16-hr light/8-hr dark). One representative pot of each mutant was selected. Images are representative of 3 independent experiments and 2 observations of each independent experiment.

CHAPTER 4

DISCUSSION & FUTURE PERSPECTIVES

4.1 Induced expression of lignin genes is a common plant defensive response.

As the major metabolites of the phenylpropanoid pathway, lignin polymers reinforce plant cell walls for upright growth and waterproof vascular tissues for water and soluble nutrient transportation (Boerjan et al., 2003). Moreover, lignin also contributes to plant immunity. The earliest report addressing the role of lignin in plant disease resistance can be traced back to the 1970s (Ride, 1975). However, the specific role of lignin polymers in plant immunity and the importance of specific lignin composition remain a mystery even after decades of research. This Ph.D. study mainly provided genetic evidence that clearly demonstrates that a functional *F5H1* is the key lignin biosynthetic gene required for plant immunity, and further defines its specific roles in plant defense. Pathogen-induced expression of lignin genes has been found in various plant-pathogen interactions, including *Triticum monococcum* challenged by *B. graminis* f.sp. *tritici* (Bhuiyan et al., 2009), *Camelina sativa* by *Sclerotinia sclerotiorum* (Eynck et al., 2012), and *Gossympium barbadense* by *Verticillium dahlia* (Xu et al., 2011). This Ph.D. work demonstrated that induced expression of lignin genes was also observed for Arabidopsis challenged by *C. higginsianum*. Subsequently, Arabidopsis lignin biosynthetic genes were also induced upon inoculation of *B. graminis* f.sp. *hordei* (Liu X and Wei Y, unpublished data), suggesting that up-regulation of lignin genes is a common defensive response among angiosperm plants against pathogens. Moreover, this fungus-induced up-regulation of lignin genes may also represent a broader resistance mechanism against non-fungal pathogens. For example, 3 members of the Arabidopsis CAD gene family were induced upon inoculation of bacterial pathogen *Pseudomonas syringae* (Tronchet et al., 2010). The rice lignin gene, *Snl6*, also displays enhanced susceptibility against bacterial pathogens (Bart et al., 2010). Since bacteria and fungi employ distinctly different infection strategies, this study was limited to demonstrate the role of *F5H1* in defense only against fungal pathogens.

4.2 *F5H1* plays critical roles in plant defense against fungal pathogens.

A preliminary screening of mutants of lignin genes with pathogen *C. higginsianum* indicated that *f5h1* was the only mutant showing enhanced susceptibility (Liu G and Wei Y, unpublished data). Two *F5H* genes exist in Arabidopsis genome, *F5H1* (*CYP84A1*; At4g36220) and *F5H2* (*CYP84A4*; At5g04330; Raes et al., 2003). The Arabidopsis mutant *f5h1* (also known as *sin1*, *fah1*) was characterized 20 years ago by its reduced level of sinapoylmalate accumulation (Chapple et al., 1992). *F5H1* encodes a class of cytochrome P450-dependent

monoxygenases (CYP84A1) that is able to catalyze the C5-hydroxylation of coniferaldehyde and coniferyl alcohol to 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol leading to the biosynthesis of syringyl (S) lignin monomer biosynthesis and sinapate ester metabolism (Landry et al., 1995; Meyer et al., 1995; Humphreys et al., 1999). BLAST search of *F5H1* nucleotide sequence retrieved from Arabidopsis ecotype Col-0 in other 4 ecotypes' assembled genome sequences, including L*er*-1, Bur-0, C24 and Kro-0, revealed the existence of same genee with >99% nucleotide identities (http://1001genomes.org/cgi-bin/blast/blast.cgi). Interestingly, the single mutant *f5h1* shows undetectable level of syringyl lignin monomer in spite of the presence of the *F5H2* gene (Meyer et al., 1998). This observation suggested that *F5H2* might not be a functional *F5H* gene in S lignin metabolism. To demonstrate the roles of F5H genes in plant immunity, 3 alleles of *f5h1* mutant and *f5h2*-1 single mutant were obtained and challenged with host pathogen *C. higginsianum* and *E. cichoracearum*. Double mutant *f5h1*/*f5h2* was generated by genetic crossing of *f5h1*-1 with *f5h2*-1 to investigate the redundancy between *F5H1* and *F5H2*. Disease tests revealed that all 3 alleles of *f5h1* mutant displayed enhanced susceptibility against the two host fungal pathogens tested. This result demonstrated that *F5H1* plays a critical role in plant immunity against fungal pathogens. In contrast, the *f5h2*-1 mutant displayed a level of susceptibility comparable to that of Arabidopsis wild type Col-0. Moreover, the double mutant *f5h1f5h2* showed a similar level of disease symptom as *f5h1*-1 single mutants, suggesting that *F5H2* does not contribute to plant immunity. Considering it is undetectable in most of the organs except in the 2nd internode and hypocotyl (by Arabidopsis eFP browser; Winter et al., 2007), *F5H2* might not be a functional gene in Arabidopsis, or it might be responsive only to a specific stimulus that has not been determined yet.

Mutant *f5h1* disrupted not only S lignin metabolism, but also sinapate ester accumulation that is responsible for ultraviolet (UV) responses (Landry et al., 1995). Enzyme REF1 catalyzes sinapaldehyde, an intermediate metabolite derived from the *F5H1*-catalyzed reaction and is involved sinapate ester biosynthesis (Figure 1; Chapple et al., 1992; Nair, et al., 2004). S lignin composition is not dependent on the sinapate ester pathway, since mutant *ref1* with similar phenotype accumulates normal level of syringyl lignin monomer (Ruegger and Chapple, 2001). The *ref1* mutant also did not show altered disease resistance when inoculated with *C. higginsianum* (Liu G and Wei Y, unpublished data). Thus, all of the results described above

demonstrate the critical role of *F5H1* and not *F5H2*, other lignin genes, or other sinapyl-related gene in plant resistance against fungal pathogens.

4.3 Mutation *f5h1* affects penetration resistance, but not post-invasion resistance.

Plant penetration resistance represents the first layer of inducible plant defense to prevent the direct penetration activities of pathogens in their effort to reach host intracellular nutrients. Previous research had only implied a potential role for the *F5H* gene in penetration resistance against fungal pathogens, but it was only correlative evidence. For instance, the wheat *F5H* gene displayed distinct expression patterns that differed between a susceptible cultivar and the cell wall appositions (CWAs)-based resistant cultivar (Bhuiyan et al., 2009). In particular, the expression of *F5H* is transiently and highly induced at 24 hpi during very early fungal penetration, but not detected at other pathogen progression stages in the resistance cultivar. Such a unique pathogen-induced expression pattern was not observed for other wheat lignin genes.

To identify the specific role of *F5H1* in disease resistance, powdery mildew pathogens and mutant *f5h1* were used to directly examine the penetration resistance of this Arabidopsis mutant line. Mutants disrupted in penetration resistance components, such as *pen1*, *pen2* and *pen3*, allow more entries of powdery mildew conidia, especially nonhost powdery mildew *B. graminis* f.sp. *hordei* that normally cannot invade (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). Consistently, the *f5h1* mutant allowed significantly higher penetration frequencies of nonhost powdery mildew *B. graminis* f.sp. *hordei* (a 2.5-fold increase over wild type Col-0), confirming the role of *F5H1* in penetration resistance of Arabidopsis to a nonhost pathogen. Entry frequency on *f5h2*-1 was comparable to wild type, which further confirmed that *F5H2* plays no role in plant immunity. Moreover, *f5h1*-1 also showed reduced penetration resistance against a host pathogen *E. cichoracearum* as well: a ~10% increase of penetration frequency at 48 hpi and 30% more formation of haustoria (a hallmark of secondary penetrations during hyphae development) compared with the Col-0 wild type plant.

Previously characterized penetration resistance genes contribute to resistance against pathogens with varied resistance spectra. *PEN1* is particularly focused against nonhost powdery mildew pathogens while *PEN2* affects a much broader spectrum of pathogens, including the oomycete *Phytophthora infestans*, *Pythium irregulare*, *Plectosphaerella cucumerina* and the anthracnose pathogen *Colletotrichum* species (Hiruma et al., 2010). Therefore, the *f5h1* mutant was challenged with several additional fungal pathogens to investigate its penetration resistance

spectrum. Consistent with the powdery mildew tests, *f5h1* mutant also affected penetration resistance against two other nonhost fungal pathogens *C. destructivum* and *M. oryzae*. Moreover, *f5h1* mutation was also able to accelerate the disease symptom development of host anthracnose pathogen *C. higginsianum* and attenuate resistance against host powdery mildew *E. cichoracearum*. Taken together, the mutant *f5h1* showed impaired penetration resistance against five species of fungal pathogens, each with distinct compatibilities and infection strategies, indicating that *F5H1* is involved in a general penetration resistance mechanism that is active against a broad spectrum of fungal pathogens.

All of the results described above clearly demonstrate the vital role of *F5H1* in penetration resistance. However, it was not clear whether impaired penetration resistance in the *f5h1* mutant was the sole reason causing the enhanced susceptibility against host pathogens *C. higginsianum* and *E. cichoracearum* since the role of *F5H1* in post-invasion resistance was not fully clarified in these experiments. To test this, the occasional penetrations of nonhost powdery mildew *B. graminis* f.sp. *hordei* were exploited, since they should induce subsequent post-invasion resistance featured by hypersensitive reaction (HR) responses, which will ultimately terminate the further development of fungi. Disrupted post-invasion resistance by knockout of the *PAD4*/*SAG101* signaling pathway allows the development of large colonies of *B. graminis* f.sp. *hordei* and the triple mutant *pen2*/*pad4*/*sag101* increases the frequency of such large colonies (Lipka et al., 2005). In this thesis work, HR responses were commonly observed without such large colonies on the *f5h1* mutant infected with *B. graminis* f.sp. *hordei*, suggesting that post-invasion resistance remains functional in the *f5h1* mutant plant. Moreover, *C. destructivum* was not able to cause substantial disease symptoms on the *f5h1* mutant but only on *pad4*/*sag101* and *f5h1*/*pad4*/*sag101* mutants (Yang L and Wei Y, unpublished data), which also implied a functional post-invasion resistance.

When challenged with *C. higginsianum* and *E. cichoracearum*, the *f5h1* single mutant should display much more severe disease symptoms than other single mutants disrupted only in post-invasion resistance at later disease progression points if *F5H1* is involved in both penetration and post-invasion resistance. However, such a severe disease phenotype did not appear in the *f5h1* mutant at 4 dpi. To confirm this, the expression patterns of two marker genes of SA/JA/ET-mediated post-invasion resistance, including *PR-1* and *PDF1.2*, were determined by northern blotting in *f5h1* mutant. As expected, no altered expression pattern was observed,

which confirmed that the *f5h1* mutation does not affect post-invasion resistance mediated by SA, JA and ET. In sum, all of the results above demonstrate the critical role of *F5H1* in penetration resistance but not in post-invasion resistance.

A noticeable observation is that when analyzing the gene expression of Arabidopsis upon challenge with *C. higginsianum*, the expression level of *F5H1* peaked at a maximum level at 96 hpi, which is far later than the timing of early penetration activities. Such delayed induction of *F5H1* gene is possibly due to secretion of effector molecules of host pathogen *C. hissginsianum* to suppress host defense responses, including the induction of lignin biosynthetic genes.

Secretion of effector molecules that manipulate and re-programme metabolism of host cells is critical to the pathogenesis of host pathogens. One of the most important functions of effectors is to defeat PAMP-triggered immunity (PTI) responses. For instance, multiple effectors characterized from oomycete *Hyaloperonospora arabidopsidis* are able to suppress host plant immunity when they are delivered into host cells via bacterial type-three secretion system or by constitutive expression in Arabidopsis (Fabro et al., 2011). The bacterial effector HopZ1 can interfere with plant microtubule networks and suppresses cell wall-mediated defense (Lee et al., 2012).

Although such specific effectors have not been isolated from *C. higginsianum*, Kleemann et al. (2012) reported a collection of candidate plant-inducible effector genes from this host anthracnose pathogen. These genes are expressed in a biotrophic stage and their proteins are secreted from appressoria and intracellular hyphae. Moreover, Shimada et al. (2006) also demonstrated that *C. higginsianum* is able to suppress Arabidopsis papilla formation at entry sites by monitoring the formation of papillary callose deposition. Knockout of these candidate genes in *C. higginsianum* and infection on the host plant Arabidopsis may reveal their specific functions and whether these genes actually specify effector molecules, including whether they delay the timing of pathogen-induced up-regulation of lignin genes, and impair the pathogenesis of *C. higginsianum*. Futhermore, it is also important to test the expression pattern of *F5H1* using incompatible Arabidopsis pathosystem, such as the *Arabidopsis thaliana-B. graminis* f.sp. *hordei* pathosystem, to determine if *F5H1* is induced at an early time corresponding to the penetration activities. The peak expression at 24 hpi of wheat *F5H* reported by Bhuiyan et al. (2009) was observed on a resistant cultivar upon infection of host powdery mildew; in other words, in an incompatible pathosystem. Therefore, the pathogen-induced expression of the *F5H1* gene in an

incompatible pathosystem may differ from the compatible pathosystem, such as the *Arabidopsis thaliana-C. higginsianum* used in this thesis.

4.4 Papillae morphology is involved in penetration resistance.

The formation of papillae upon fungal penetration is a major step in penetration resistance. Papillae play critical a role in successful penetration resistance against nonhost powdery mildew *B. graminis* f.sp. *hordei.* A 2-hr delay of papilla formation is observed in the *pen1* mutant, which is hypersusceptible to penetration of the nonhost pathogen *B. graminis* f.sp. *hordei* (Assaad et al., 2004). Since lignin is an important structural component of papillae (McLusky et al., 1999), the morphology of papillae in the *f5h1* mutant was examined by transmission electron microscopy. Although the general shape and size of papillae were indistinguishable between wild type and *f5h1* mutant, the highly organized layered structures of papillae formed on Col-0 was absent in the *f5h1* mutant. Such internal structure may be indispensable for the normal function of papillae during penetration resistance. This data is reminiscent of the *pmr4* mutant, which lacks papillary callose and the lacks same layered internal structure of papillae without change of size and shape (Nishimura et al., 2003). After challenging the *pmr4* mutant, *B. graminis* f.sp. *hordei* also showed a similar level of impaired penetration resistance as that seen on the *f5h1* mutant. Thus, the internal structures of papillae appear critical to their functions in penetration resistance against powdery mildew functions and both *PMR4* and *F5H1* genes are required for the generation of such internal structures.

4.5 The unique 5-hydroxyconiferyl alcohol might be the real contributor of *F5H1*-dependent penetration resistance.

Based on the available knowledge of lignin monomer polymerization, a model regarding how *F5H1* contributes to the organization of papillae internal structure is proposed. During plant growth, lignin polymers provide enhanced mechanical support and act as barriers in various tissues in order to satisfy their specific physiological functions (Moerschbacher et a., 1990). Therefore, a specific lignin deposited in papillae might also strengthen the papillae as a physical barrier against fungal penetration. As described in section 1.3, the unique existence of the syringyl unit in angiosperm lignin suggests that it might play a role in strengthening the defense system of angiosperm plants. Since the *F5H1* gene is the only key step in lignin biosynthesis involved in penetration resistance, it is reasonable to deduce that the syringyl lignin monomer is

responsible for a more rigid structure that enhances the lignin barrier against the physical penetration of fungal pathogens. In the development of lignin polymer, the β-O-4 linkage is the most abundant polymerization bond, suggesting that the active C4 site is the preferred position for the oxidative coupling process (as reviewed by Boerjan et al., 2003). However, if we compare the chemical structures of guaiacyl and syringyl lignin monomers, both of them have the same unmethylated (active) hydroxyl group attached at the C4-position of the benzene ring (Figure 1.2). Moreover, lignin composed mainly of G monomers contains more chemically resistant bonds than S/G lignin polymers likely since the C5-position of S lignin is occupied by a methyl group which would prevent the formation of bonds with strong chemical resistance (Boerjan et al., 2003).

Based on our current knowledge of *F5H1*-mediated lignin biosynthesis, the model would be better served by the immediate products of the *F5H*-catalyzed reaction, which are 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol (the latter of which is known as a 5-hydroxy-guaiacyl unit; Figure 1; 2B). The 5-hydroxyconiferaldehydes can be converted to the 5-hydroxyconiferyl alcohols by a CAD enzyme. The 5-hydroxyconiferyl alcohols contain two active hydroxyl groups at C4- and C5-position of benzene ring. In *comt* mutants of poplar and Arabidopsis, 5-hydroxyconiferyl alcohol can be incorporated into a natural lignin polymer to generate this unique 5-hydroxy-guaiacyl monolignol (Lu et al., 2010; Weng et al., 2010b). Moreover, a special benzodioxane structure can be formed between the 5-hydroxy-guaiacyl unit and G or S lignin monomers through both 5-O-α and 4-O-β ether linkages (Figure 1.2B), and this "bridge" is too strong to be cleaved by a traditional DFRC method, the "derivatization, followed by reductive cleavage" method which is able to degrade the usual angiosperm lignin polymer to release G and S units (Lu and Ralph, 1998). Therefore, it is highly likely that the 5-hydroxy-guaiacyl monolignol is the real contributor to a reinforced papillary structure, not the syringyl lignin monomers. In support of this hypothesis, the Arabidopsis mutant *comt1*, which does not generate syringyl lignin monomer but is still able to generate 5-hydroxy-guaiacyl monolignols (Weng et al., 2010b), showed no reduction in disease resistance against host anthracnose pathogen *C. higginsianum* (Liu G and Wei Y, unpublished data).

In future research, a series of experiments should be performed to confirm the chemical components of papillary lignin. The most direct evidence would be to demonstrate the lignin deposition pattern in papillae upon pathogen infection. This could be achieved by immuno-

histochemical labeling of lignin epitopes that would determine visually the distribution pattern of different lignin units (Ruel et al., 2002) and chemical analysis to determine the composition of lignin monomers. Due to the tiny size of papillae, special techniques such as laser-micro dissection will be required to separate papillae from host cell wall for subsequent chemical analysis with additional methods such as a revised DFRC method and NMR spectroscopy to verify the existence of the 5-hydroxy-guaiacyl unit and the benzodioxane structure in papillae. Moreover, Weng et al. (2010b) demonstrated that the Arabidopsis line *comt1*/C4H-F5H (crossing of mutant *comt1* and transgenic plant overexpressing *F5H* under a *C4H*-promoter) deposits a much higher proportion of 5-hydroxy-guaiacyl derived benzodioxane than Arabidopsis wild type Col-0. Thus, if 5-hydroxy-guaiacyl units are able to enhance penetration resistance, such an Arabidopsis line *comt1*/C4H-F5H should be tested for enhanced penetration resistance with fungal pathogens that use direct penetration strategies, such as *B. graminis f.sp. hordei* and *E. cichoracearum*.

In addition to the hypothetical model described above, other possibilities should also be considered in tfuture research to illustrate the mechanism of *F5H1*-dependent penetration resistance. Secretion of anti-microbial agents is another important strategy of penetration resistance besides the formation of papillae (Hückelhoven, 2007). As described in section 1.2.3, lignan and coumarins are derived from intermediates of lignin biosynthesis and function in plant immunity against bacteria, fungi and virus. Therefore, it is also possible that there is an unknown pathway derived from 5-coniferaldehyde and 5-hydroxyconiferyl alcohols, the immediate products of the *F5H*-catalyzed reaction, to generate defensive compounds involved in plant penetration resistance. A metabolites profiling of phenolic compounds deposited at papillae formed on both wild type Col-0 and *f5h1* mutant upon challenge of nonhost pathogen such as *B. graminis* f.sp. *hordei* would provide biochemical evidence to identify if any new phenolic compounds are generated and deposited in papillae against fungal penetration.

4.6 Unknown signaling pathway regulates expression of Arabidopsis lignin genes in defense.

Lignin metabolism plays such critical roles in plant defense, but the signaling pathway responsible for the activation of lignin metabolism for defensive purpose remains mystery. Several defensive signaling molecules have been characterized in plants, such as SA, JA, ET, ROS molecules, *etc*. ROS is involved in signaling pathway of early defensive responses and able to induce a serie of disease resistance responses including formation of papillae (Hückelhoven,

2007) while SA, JA and ET are functional in post-invasion resistance (Niu et al., 2011). The expression of lignin genes in mutants disrupted in SA/JA/ET signaling pathways displayed confusing patterns in this thesis. For example, the expression of *F5H1* seemed to be up regulated in transgenic Arabidopsis NahGox and mutant *sid2*, but in *pad4*/*sag101*, no up-regulation of *F5H1* was observed. All of these three Arabidopsis lines are disrupted in SA metabolism. Importantly, exogenous application of SA, JA and ET, as well as the ROS signaling molecules NO and H_2O_2, also failed to induce the expression of lignin genes in a pattern that mimiced the inducible pattern by the pathogen. Therefore, traditional plant defensive signaling pathways are not responsible for pathogen defensive lignin biosynthesis.

In addition to defense-associated phytohormones, plants also produce various hormones that regulate plant development, including abscisic acid, auxin, gibberellin, cytokinin, *etc*. Many of these hormones are associated with developmental lignification processes (see section 1.2.1.4 "Regulation of lignin metabolism in plants"). The traditional "growth regulator" auxin is associated with plant defense as well (as reviewed by Kazan and Manners, 2009). For example, the mutant of auxin signaling component, *SGT1b*, confers enhanced resistance to Arabidopsis against the fungal pathogen *Fusarium culmorum* (Cuzick et al., 2009). Gene Ontology (GO) analysis revealed that cytokinin, gibberellin and abscisic acid responses are modulated during the incompatible interactions between *Phaseolus vulgaris* L. and *Colletotrichum lindemuthianum* (Oblessuc et al., 2012). Thus, they are also great candidates to regulate defensive lignin metabolism. Unfortunately, treatments of abscisic acid, auxin, gibberellin and cytokinin on Col-0 wild type failed to induce the expression of all lignin genes in a pattern similar to the induction during pathogen inoculation. The induction of *F5H1* and *COMT1* by auxin were also not reproduced in another independent experiment with application of auxin at various concentrations on wild type Arabidopsis Col-0 for northern blotting analysis (Liu X, unpublished data). In future research, the exogenous treatments of other hormones need to be repeated to obtain solid evidence in addition to this preliminary experiment described in this thesis. For example, each hormone needs to be tested with different concentrations since their functional concentrations in defense might not be the same as their physiological functional concentrations.

In addition, we obtained a collection of Arabidopsis mutants disrupted in lignin-associated transcription factors, including *nst1*, *nst2*, *snd1*-1, *snd1*-2, *myb46*, *myb83*, *myb63*, and a *nst1*/*snd1* double mutant. None of these mutants displayed enhanced susceptibility similar to

the *f5h1* mutant after a *C. higginsianum* disease test; northern blotting also showed that the expression pattern of lignin genes upon pathogen infection was not affected by any of these mutants as well (Liu X and Wei Y, unpublished data). Taken together, all of these results suggest that defensive lignification and development lignification are independently regulated. In the future research, this possibility should also be considered that a novel signaling pathway might be responsible for the pathogen-induced up-regulation of lignin genes.

What, then, is a suitable candidate(s) to regulate the defensive activation of lignin metabolism? Considering the fact that *F5H1* contributes to papillae-based penetration resistance, the early signaling events soon after the recognition of pathogens are likely involved in the stimulation of lignin biosynthesis in plant penetration resistance.

Fungal pathogens usually secrete a set of cell wall degrading enzymes (CWDEs) to damage the integrity of host cell wall. One of the well-characterized CWDEs is polygalacturonase (PG), which degrades pectin polymers. It has been demonstrated that cell wall debris derived from pathogen penetrations activities, such as oligogalacturonides (OGs), can be recognized as damage associated molecular patterns (DAMPs) and signal the further activating of defensive signaling pathways (D'Ovidio et al., 2004). In addition, PG itself displays elicitor effects regardless of its enzymatic activities (Poinssot et al., 2003). Recently, an increased level of lignification has been characterized in tobacco plants over-expressing a grapevine polygalacturonase-inhibiting protein (PGIP) in the absence of pathogen infection (Alexandersson et al., 2011). Thus, a PG/OGs/PGIP-mediated signaling pathway might be responsible to induce lignification for defensive purposes.

Recently, wall associated kinase 1 (WAK1) has been demonstrated as a receptor of OGs (Brutus et al., 2010). In addition, among three defense-related MAPKs, OGs induce phosphorylation of MPK 3 and -6 (Asai, et al., 2002; Galletti et al., 2011). Since protein kinase signaling has been well studied in plant innate immunity (reviewed by Tena et al., 2011), the MAPK kinase signaling pathway is likely involved in regulating lignification in plant defense by PG/OGs/PGIP. However, future work needs to fill in the gaps between MAPK cascade and lignin biosynthetic genes. Currently, *F5H1* is thought to be regulated by the master switch of secondary cell wall *SND1*/*NST3* (Zhao et al., 2010). Recently, *MYB103* was shown to regulate the expression of *F5H1* (Ohman et al., 2012). However, *MYB103* might not be the immediate transcription factor to directly induce expression of *F5H1* since *MYB103* failed to transactivate

F5H1 promoter (Ohman et al., 2012). Thus, identification of the immediate transcription factors of *F5H1* in plant defensive responses is critical. This could be achieved by a transcriptome analysis, such as by using microarrays to identify novel genes induced upon pathogen infection. The mutant of such a new regulatory gene should show a reduced or eliminated expression of *F5H1* and enhanced susceptibility upon pathogen challenge. Once the immediate regulator of *F5H1* is identified, research could be further conducted to link this immediate regulator to the possible MAPK cascade and finally to the initial receptor of DAMPs.

4.7 Relationships between *F5H1* with other molecular components involved in penetration resistance.

4.7.1 *F5H1* and *PEN* genes.

Penetration resistance represents the first layer of Arabidopsis nonhost resistance and several molecular components involved have been determined other than *F5H1*, including *PEN1*, *PEN2* and *PEN3* (Collins et al*.,* 2003; Lipka et al., 2005; Stein et al., 2006). To investigate the relationships between *F5H1* and *PEN* genes, double mutants were generated and tested with various plant pathogens. Disease tests with the nonhost powdery mildew *B. graminis* f.sp. *hordei* and host anthracnose pathogens *C. higginsianum* demonstrated the lack of interaction between *F5H1* and *PEN* genes in penetration resistance since in general both pathogens displayed more aggressive infections on double mutants than the parental single mutants. However, the responses varied somewhat among the 3 different pathogens tested.

B. graminis f.sp. *hordei* displayed significantly higher penetration frequencies on *pen1* than an *f5h1*, suggesting that *PEN1* contributes more than *F5H1* to penetration resistance against nonhost powdery mildew. However, when challenged with anthracnose pathogen *C. higginsianum*, *F5H1* is playing a more important role than *PEN1* since the pathogen displays more enhanced penetration frequencies on *f5h1* mutant than *pen1* mutant. Moreover, the higher penetration frequencies on double mutant *f5h1pen1* than single mutant *f5h1* might suggest a minor function of *PEN1* in penetration resistance against *C. higginsianum*. The role of *F5H1* in penetration resistance against *C. higginsianum* is more like *PEN2*/*PEN3*, since *f5h1*, *pen2* and *pen3* single mutants shows similar susceptiblities than wild type Col-0. This observasion is consistent with previous reports that also demonstrated the enhanced susceptibility of the *pen2* and *pen3* mutants against host and nonhost *Colletotrichum* species (Huser et al., 2009; Hiruma et a., 2010).

The *F5H1*-dependent in penetration resistance was further examined by a disease test with the rice blast pathogen *Magnaprothe oryzae*. *M. oryzae* is a nonhost pathogen to the Arabidopsis ecotype Col-0, and this host restriction is mainly based on a complete block of *M. oryzae* penetration (Maeda et al., 2009). Penetrations were not observed on any *pen1* or *pen3*-related mutants (including both single and double mutants with *pen1* mutation; data not shown), as well as *pen2* single mutants. But, successful penetrations were occasionally observed on *f5h1* single mutant. However, *pen2* is known to be susceptible to penetration by *M. oryzae* (Maeda et al., 2009). This inconsistency is probably due to the relatively weaker pathogenicity of the *M. oryzae* isolate used in this study, rather than the lack of involvement of *PEN2* in penetration resistance against *M. oryzae*.

Successful penetration sites with significantly extended intracellular hyphae were observed more frequently for *M. oryzae* on *f5h1/pen2* double mutants than on *f5h1* single mutant. Furthermore, no extended hyphae were observed at successful penetration sites on *f5h1* mutant. This result further confirms that *F5H1* acts independently of *PEN2* in penetration resistance against this pathogen and *F5H1* is likely the critical determinant of penetration resistance against *M. oryzae* rather than *PEN2*. Moreover, the extended hyphae implies that *PEN2* plays additional roles in post-invasion resistance against *M. oryzae* to restrict the hyphae development after penetration, which is distinct from the *F5H1*. Cell death accompanied the penetrated *f5h1/pen2* cell, which suggests that other components (such as *PMR5*, *AGB1*, and SA signaling pathway) may also be functioning to terminate further aggressive growth of *M. oryzae* in these cells (Nakao et al., 2011).

Taken together, *F5H1* represents a unique component of Arabidopsis resistance combining characteristics of both *PEN1* and *PEN2*. In one aspect, *F5H1* is only involved in penetration resistance as *PEN1* (Consonni et al., 2006) but not in post-invasion resistance. In the other aspect, *F5H1*-mediated resistance is against broad spectrum of fungal pathogens as *PEN2* while *PEN1* is highly specialized against to nonhost powdery mildew pathogens.

A surprising observation in the *F5H1-PEN* interaction experiment is the abnormal callose deposition pattern in pathogen-challenged *pen1* and *f5h1pen1* mutants. Based on the shape of alkaline aniline blue stained callose deposition, it is clear that such callose deposition is neither localized in papillae nor wrapping aroud the haustoria. In other words, the localized deposition of callose normally underneath the plant pathogen interaction sites in Col-0 was disrupted in

pathogen-challenged *pen1* and *f5h1/pen1* mutants and resulted in ectopic callose deposition at locations which were devoid of pathogen-response plant structure. This observation seems to imply the dependence of callose deposition on the PEN1 syntaxin-mediated secretary pathway, which is inconsistent with a previous report suggesting that callose deposition and PEN1 are independent (Meyer et al., 2009). This might be due to the existence of a homologue of PEN1, SYNTAXIN PROTEINS OF PLANTS 122 (SYP122) and the different time points of observation in this work (at 2 dpi) and in Meyer et al. (2009)'s work (at 7 dpi). SYP122 is also induced upon pathogen infection but mutant *syp122* showed no effects on penetration resistance (Assaad et al., 2004). Although both PEN1 and SYP122 are accumulated in papillae, SYP122 shows much less degree of accumulation at papillae than PEN1 as indicated by their fluorescence signal ratio of papillae and plasma membranes. The signal of PEN1 at papillae is 6 times higher than its signal on plasma membrane upon pathogen challenge, while such ratio of SYP122 is only 2 (Assaad, et al., 2004). These results suggest that the response of SYP122 to pathogen-induced papillae formation might be slower than PEN1 and result in delayed formation of papillae in *pen1* mutant (Assaad et al., 2004). Therefore, in *pen1* mutant, since no PEN1 protein is incorporated into papillae, part of pathogen-induced callose may be guided by membrane-bound SYP122 and results in the ectopic deposition as observed. Meyer et al. (2009)'s results were observed at 7 dpi. Plants may have eliminated such ectopic callose deposition at such later timing through an unknown mechanism.

4.7.2 *F5H1* and *PMR4.*

Several Arabidopsis mutants showing enhanced resistance against powdery mildew (*powdery mildew resistance; pmr*) have been characterized (Vogel and Somerville, 2000). Among them, *PMR4* encodes a pathogen-inducible callose synthase that synthesizes papillary callose. Mutant *pmr4* show an abnormal fibrillar network of ultrastructure in powdery mildew-induced papillae (Nishimura et al., 2003) and impairs penetration resistance. Mutant *pmr4*-mediated resistance depends on the SA signaling pathway, and is not affected by the *f5h1* mutation, since HR responses and *PR-1* expression without pathogen infection remained unaltered in *f5h1/pmr4* double mutants in comparison with the single mutant *pmr4*. These results demonstrate that *F5H1* is not associated with SA-dependent *pmr4* resistance. Therefore, the enhanced hyphae development observed on the *f5h1/pmr4* double mutant compared with the

single mutant *pmr4* is likely due to the reduced penetration resistance of the *f5h1* mutation which allowed more secondary penetrations during hyphae development.

4.7.3 *F5H1* and *PMR2.*

MILDEW RESISTANCE LOCUS O (MLO) loci were first characterized from barley as a negative regulator of resistance against powdery mildew *B. graminis* f.sp. *hordei* (Jorgensen, 1992), and subsequently these loci were also found in the model plant Arabidopsis (Consonni et al., 2006). Subsequent research demonstrated that Arabidopsis *PMR2* is allelic to *MLO2* and is proposed to be a negative regulator of plant defense (Consonni et al., 2006). Arabidopsis *pmr2*-mediated resistance requires *PEN1*, *PEN2*, *PEN3* and tryptophan-derived metabolism (Consonni et al., 2006; 2010). In addition to these published observations, this work identified *F5H1* as a novel component contributing to the *pmr2*-mediated resistance independently of other components described above (Figure 4.1), since the enhanced penetration resistance against host powdery mildew *E. cichoracearum* of *pmr2* was partially impaired after mutation of *f5h1* in *pmr2* mutant background. However, knockout of *F5H1* in a *pmr2* background did not affect the overall resistance against powdery mildew because no conidiation was observed on double mutant *f5h1/pmr2*. Even within penetration resistance, *F5H1* might solely contribute to a physical barrier in papillae since the over-accumulation of ROS, as indicated by DAB staining in the *pmr2* mutant, was not affected by a mutation in *F5H1*.

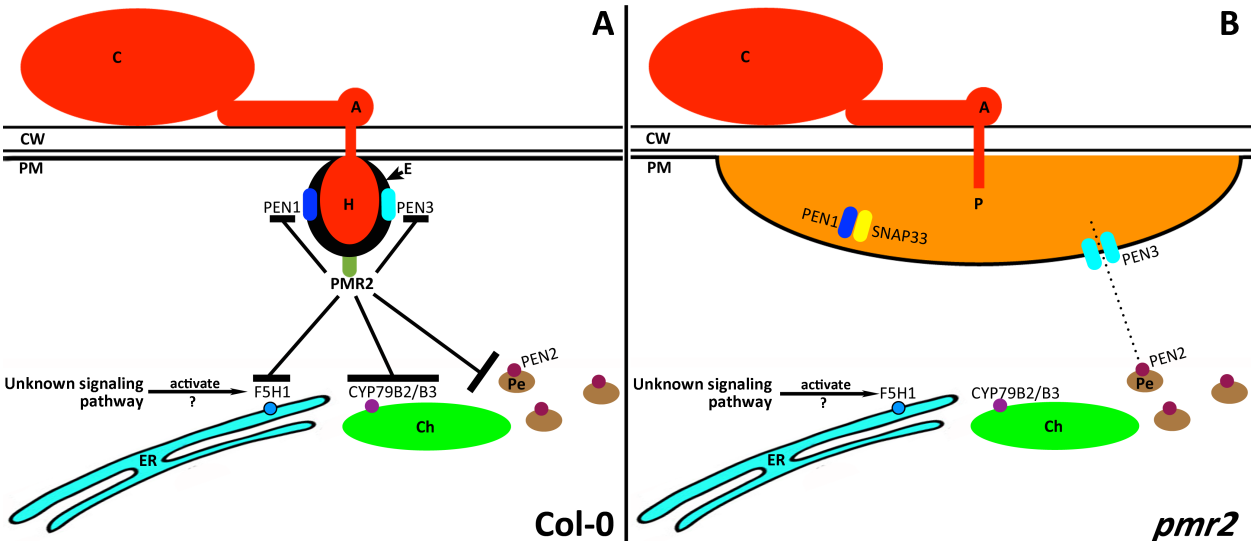

Figure 4.1 Current model of Arabidopsis *pmr2*-mediated penetration resistance against host powdery mildew.

Schematic images summarizing the characterized molecular components required for plant penetration resistance in both wild type (A) and *pmr2* mutant (B) of Arabidopsis against host powdery mildew *Erysiphe cichoracearum*. The images are adapted from Underwood and Somerville (2008). References for each components identified refers to the text. *F5H1* and *CYP79B2/B3* represent their corresponding metabolism pathways respectively. The subcellular localization of F5H protein is proposed to be ER (Dunkley et al., 2006). PEN1 syntaxin and its interacting partner SNAP33 are incorporated into extracellular encasements surrounding haustoria of the powdery mildew and entrapped into the papillary matrix (Meyer et al., 2009). Black dots represent the putative metabolites from *PEN2*-mediated metabolism and delivered by *PEN3*. The hypethatical inhibitory effects of PMR2 are illustrated by the thick black line against each target. In wild type plant (A), the presence of functional PMR2 will inhibit the functions of each individual component in penetration resistance and the pathogen is able to form haustorium. The molecular mechanism of PMR2's inhibitory effects on defensive responses of penetration resistance remained mystery; in *pmr2* mutant (B), the inhibitory effects of PMR2 on other penetration resistance components were not present. Therefore, the penetration resistance responses are induced to prevent the penetration of powdery mildew by the formation of papillae. C, conidia; A, appresorium; CW, cell wall; PM, plasma membrane; H, haustorium; E, encasement; P, papillae; Ch, chloroplast; Pe, peroxisome; ER, endoplasmic reticulum.

4.7.4 A hypothetical model of *PMR2* in plant immunity.

The full function of the *PMR2* protein remains a mystery even though the gene has been known for decades. *PMR2* gene encodes a plant-specific seven transmembrane-domain protein without characterized function (Chen et al., 2006). Current results implied that *PMR2* functions as a negative regulator of broad defense affecting both pre- and post-invasion resistance. Although *pmr2*-mediated resistance requires *PEN* genes, *F5H1* and tryptophan-derived metabolites, it does not directly regulate the expression of these genes, since PMR2 is a structural protein and none of these genes were induced in the Arabidopsis *pmr2* mutant in comparison with a wild type (Supplementary data of Consonni et al., 2010).

Recently, another MLO family member, MLO7/NORTIA (NTA), has been demonstrated to regulate plant pollen tube reception in the synergids together with a receptor like kinase FERONIA (FER; Kessler, et al., 2010; Escobar-Restrepo et al., 2007). Both pollen tube reception within the synergids and powdery mildew infection of an epidermal cell undergo a similar "physical piercing" process requiring communication between two cells and penetration of one cell into another with a tip-structure. NTA/MLO7 proteins are found throughout an unfertilized ovule and undergo a directed re-location beneath pollen tube tip (Kessler et al., 2010). Such redistribution is also reported for MLO2/PMR2 proteins. They are evenly distributed on plasma membrane until approximately 13 hours post inoculation of the powdery mildew, when they are accumulated beneath the fungal appressoria (Bhat et al., 2005). This observation implies possibly similar functions of MLO protein in these two seemingly irrelevant processes.

The focal localization of the NTA protein upon arrival of pollen tube tip requires the FER-mediated pathway and both genes are expressed in synergids (Kessler et al., 2010). Both *fer* and *nta* single mutants disrupt the reception of pollen tube by synergids, suggesting that the interaction between FER/NTA is indispensable for the successful communication between pollen tube and synergids. Unexpectedly, a homogyzgous *fer* mutant displayed similar resistance against powdery mildew as *pmr2* (Kessler et al., 2010). Therefore, an audacious, but reasonable model can be proposed to illustrate the role of PMR2/MLO2 in plant immunity: a MLO2/FER-mediated reception mechanism is hijacked by host powdery mildew pathogens to facilitate the entry of penetration pegs into host cell. FER and MLO members together may serve as a conserved recognition mechanism within plant cell in response to exogenous physical stimuli. In

Arabidopsis, the MLO family has 15 members that show tissue-specific expression patterns and are regulated by different stimuli, suggesting the common roles of MLO protein in diverse developmental and responses processes. In addition to MLO2 and MLO7, MLO4 and MLO11 are required for touch-induced root tropism (Chen et al., 2009). Mutant *mlo4* resulted in a tight curling root response to a tactile stimulus. Physical stimulus detection is the common characteristic of the functions of MLO2, MLO7 and MLO4/11, strongly implying that the MLO is involved in response against physical stimulus. In epidermal cells, FER/MLO2 may become repressed since most of physical stimuli from environment should be considered as dangerous signals indicating potential intruders or physical damages. Thus, epidermal cells tend to repel these exogenous physical touches, rather than prepare to accept them; this is opposite to the synergids that accept pollen tube tips. However, during the evolution of plant-pathogen interactions, some host powdery mildew pathogens, such as *E. cichoracearum*, might obtain the ability to disguise their penetration peg as a "pollen tip" to be recognized by FER and further activate the FER-MLO pathway to "positively accept" a penetration peg or suppress the defense responses. Thus, these host powdery mildew pathogens display extremely high penetration frequencies with the presence of powerful penetration resistance mediated by *PEN* genes, *F5H1* and other putative components. Nonhost powdery mildew pathogens, such as *B. graminis* f.sp. *hordei*, fail to activate the MLO/FER pathway to facilitate the invasion process. As a result, their penetration activities are largely terminated by penetration resistance.

Based on this hypothesis, both *fer* and *pmr2* mutants shut down this pathway and *E. cichoracearum* cannot bypass the penetration resistance. Therefore, the penetration frequency decreased to 25% of the wile type level on the *pmr2* mutant. This is probably due to the fact that other gene members within same clade of *PMR2*, such as *MLO6* or *MLO12* (Chen et al., 2006), may partially execute *PMR2*'s function. Thus, a triple mutant of *pmr2mlo6mlo12* even reduced the penetration frequency of host powdery mildew to an undetectable level (Consonni et al., 2006).

An immediate experiment to verify this hypothesis is to determine if the polarized re-localization of the PMR2 protein underneath fungal appressoria is altered in the *fer* mutant. If the hypothesis described above was true, the *fer* mutant should be able to disrupt such re-localization of PMR2/MLO2 similar to the disrupted re-localization of NTA/MLO7 in synergids of the *fer* mutant. Moreover, it is also important to determine the recognition mechanism between receptor-

like kinase FER and pollen tube tip. Is there any specific molecular pattern existing on the pollen tube tip, which can be recognized by the FER, or simply is the physical force detected by FER? If the former exists, does the penetration peg generated by host powdery mildew possess a similar molecular pattern that mimics a pollen tube tip? The answer will provide strong evidence to support this hypothesis.

4.8 A preliminary investigation of the contribution of the flavonoid pathway in Arabidopsis disease resistance.

In addition to the physiological functions in plant development, flavonoid compounds provide resistance against environmental challenges, including drought, toxic metal contamination, ultraviolet radiation and excess light (reviewed by Treutter, 2005). In addition, many flavonoid compounds characterized in legume plants, such as luteolin, have shown anti-microbial activities: eg. in the susceptible sorghum cultivar BTx623, the replacement (by apigenin) of luteolin as the major flavone that inhibits germination of *Colletotrichum sublineolum* spores results in spore germination (Du et al., 2010).

Although most defense-related flavonoid compounds are characterized from legumes, a few reports indicating the role of flavonoids in Arabidopsis defense recently become available. For example, pre-treatment of Arabidopsis by spraying quercetin shows enhanced resistance against *Pseudomonas syringae* pv. *tomato DC3000*. Such resistance is attenuated by the presence of catalase to scavenge H_2O_2 or the blocking of the SA signaling pathway, suggesting that quercetin confers Arabidopsis resistance against this bacterial pathogen via the H_2O_2 burst and requires a functional SA signaling pathway (Jia et al., 2010). Therefore, in a preliminary investigation of the genetic components that impact on the Arabidopsis flavonoid-dependent defense pattern, pathogen susceptibility was tested using knockouts of several upstream genes in a Col-0 background, including *TT4*/*CHS*, *TT5*/*CHI* and *TT6*/*F3H*, to eliminate a large portion of antimicrobial compound variation (Figure 1.3). As expected, mutant *tt4*-T1 (which eliminates all flavonoids) and *tt6*-T1 (which eliminates flavonols and anthocyanins) display enhanced susceptibility to the host anthracnose pathogen *C. higginsianum* in comparison with wild type Col-0. This result revealed critical roles for Arabidopsis flavonoid compounds in the immunity system against fungal pathogens. However, the mutant *tt5*-T1 (which should accumulate chalcone based on current knowledge of flavonoid biosynthetic pathway in Arabidopsis) unexpectedly conferred enhanced resistance to Arabidopsis. The enhanced resistance against *C.*

higginsianum was also observed in previous experiments with another *tt5* mutant allele in a L*er* ecotype background (Wei Y, unpublished data). In addition, *tt5*-T1 also displays reduced plant size and altered leaf morphology. To analyze the molecular mechanism behind *tt5*-dependent disease resistance and developmental deficiencies, *tt5*-T1 was crossed with the *tt4*-T1. Knockout of gene *TT4*, the immediate upstream gene of *TT5*, in a *tt5* mutant background was able to completely restore the altered development of *tt5* mutant to a wild type appearance, suggesting the critical role of naringenin chalcone in *tt5*-dependent growth deficiencies. According to current understanding of Arabidopsis flavonoid biosynthesis, no branch pathway has been identified in Arabidopsis until the generation of dihydroflavonols (Figure 1.3). Therefore, the real contributor of *tt5*-mediated resistance remains a mystery until a comprehensive metabolite profiling is conducted in the future. Gene *TT5* encodes chalcone isomerase, catalyzing the generation of chalcone that serves as the precursor of all classes of flavonoids (Figure 1.4). If naringenin chalcone is accumulated without any new metabolites, then it must be responsible for the *tt5* mutant phenotype. Chalcone has shown inhibitory effects in animal cells on NF-κB-mediated inflammation and cancer (reviewed by Yadav et al., 2011), but it is not known if chalcone has any bioactivity on plant cells, which could be investigated in the future research.

Other pathways that could be initiated from naringenin chalcone or its upstream metabolite ρ-coumaroyl CoA in *tt5* mutant may result in accumulation of novel metabolites. This could be verified in the metabolite profiling as described above. Such branches have been reported in other plant species, including the aurone pathway in snapdragon (Ono et al., 2006) and chalcone reductase-isoflavone synthase (CHR-IFS) pathway in legume plants (Buer et al., 2010). Aurone confers a bright yellow color to some ornamental flowers, such as snapdragon (Schwarz-Sommer et al., 2003). In snapdragon, chalcone will be first glycosylated by the chalcone 4'-O glucosyltransferase (4'CGT) and then catalyzed in vacuole by aurone synthase to generate aurone, as represented by aureusidin 6-O-glucoside (Ono et al., 2006). Although such a chalcone glucosyltransferase is not reported in Arabidopsis, a functionally unknown Arabidopsis glucosyltranferase UDP-GLUCOSYL TRANSFERASE 88A1 (UGT88A1) shows 40% of its amino acid sequence identical to snapdragon 4'CGT (Ono et al., 2006). If a pathway similar to aurone synthesis of snapdragon is activated in Arabidopsis *tt5* mutant, UGT88A1 is likely involved in this process and generate novel metabolites in the mutant.

Although chalcone reductase (CHR) and isoflavone synthase (IFS)-mediated biosynthesis of isoflavonoid from naringenin chalcone is generally legume-specific (Buer et al., 2010), Arabidopsis still maintains the ability to metabolize the isoflavonoid genistein, which was generated after expressing the soybean isoflavone synthase in Arabidopsis (Yu et al., 2000). Moreover, Lapcik and associates (2006) recently detected low levels of isoflavones from Arabidopsis Col-0 by HPLC-MS and pointed out putative chalcone reductase genes in the Arabidopsis genome. These suggest a potential CHR pathway in Arabidopsis. Based on their results, the gene expression patterns of putative CHR genes were examined in *tt5*-T1 mutant background in this thesis. The candidate genes were selected from Lapcik's publication and a Basic Local Alignment Search Tool (BLAST) search of the Arabidopsis genome against a soybean (*Glycine max*) chalcone reductase (Liu, 2008). Surprisingly, one of the putative genes, At2g37760 (we named as *CHR3*), showed exclusive induction in *tt5*-T1 mutant but not in the *tt4*-T1 nor in the double mutant *tt4*/*tt5* and Arabidopsis wild type Col-0 (Yang L, Song T and Wei Y, unpublished data), suggesting that this gene might be involved in *tt5*-mediated resistance. Therefore, a T-DNA insertion mutant of this gene, SALK_065186C from ABRC, has been recently crossed with *tt5*-T1 to generate double mutants. If *CHR3* contributes to the phenotype of *tt5*, we should expect a wild type-like growth in the homozygous double mutant and a wild type-level of susceptibility to host anthracnose pathogen *C. higginsianum*.

Some intermediate flavonoid compounds show an ability to induce defense responses and cell growth inhibition effects and may be present in low levels in *tt5*-T1 if the mutation is somewhat "leaky" due to the spontaneous isomerization reaction of naringenin chalcone to generate naringenin (Ralston, et al., 2005). One example of downstream flavonoid compounds is quercetin, which is able to induce resistance against *Pseudomonas syringae* pv. *tomato DC3000* in Arabidopsis if sprayed before pathogen inoculation (Jia et al., 2010). Transgenic flax plants expressing a *Solanum sogarandinum*-derived glucosyltransferase accumulate an increased level of a more stable quercetin glycoside and an enhanced resistance against *Fusarium* infection (Lorenc-Kukula, et al., 2009). Quercetin has been extensively studied as an anti-cancer agent since it is able to inhibit cell growth and cause apoptosis of cancer cells (Kuo et al., 2004; Vidya et al., 2010). Such cell growth inhibitory effects of quercetin may also function on plant cells and result in reduced plant development as observed in *tt5* mutant. Therefore, metabolic profiling is critical to conduct with the *tt5*-T1 line to illustrate how the flavonoid biosynthesis is affected by

the *tt5* mutation and to identify the metabolite(s) that is/are responsible for the developmental deficiency and enhanced disease resistance of the *tt5*-T1 mutant.

Genetic evidence confirming that the SA signaling pathway is affected in the *tt5* mutant was provided in this thesis by crossing of *tt5*-T1 with signaling mutants and tries to restore *tt5* mutant to a wild type phenotype. The developmental deficiencies of *tt5* mutant were partially restored to a wild type phenotype in a genetic background blocked in the SA signaling pathway but not affected in a disrupted JA/ET signaling background. Moreover, preliminary semi-quantitative RT-PCR has revealed that the marker gene of the SA signaling pathway, *PR-1*, is spontaneously expressed in the *tt5*-T1 mutant in the absence of pathogen infection (Yang L, Song T and Wei Y, unpublished data), suggesting that the whole SA signaling pathway might be turned on in the *tt5* mutant even without pathogen infection. Although time constraint on this thesis prevented the disease responses of all these double/triple mutants to be tested, it is very likely that the enhanced resistance of *tt5* mutant will be eliminated on the *tt4/tt5* double mutant and *tt5/pad4/sag101* triple mutant since the developmental change of *tt5* were overcome in these deficient backgrounds.

The roles of the SA pathway in plant defense have been extensively studied (reviewed by An and Mou, 2011), but its role in plant development is not clearly understood. However, genetic evidence has closely related the SA signaling pathway to plant development and senescence process. For example, Arabidopsis mutants *senescence-associated ubiquitin ligase 1* (*saul1*) and *necrotic spotted lesion 1* (*nsl1*) display early senescence and growth retardation phenotypes in a SA-dependent manner (Noutoshi et al., 2006; Vogelmann et al., 2012). A functional SA signaling pathway is also required for the *pmr2*-dependent early senescence but not its powdery mildew resistance phenotype (Consonni et al., 2006). The SA signaling pathway may affect plant growth through crosstalk with other plant hormones such as auxin and abscisic acid. Recently, numerous reports have been addressing the interactions between SA and other hormones in defense (reviewed by Robert-Seilaniantz et al., 2011b). Thus, in the mutants described above, such crosstalk between SA and other hormones may be responsible for the growth retardation phenotypes and auxin is the most promising candidate that is involved in the SA-dependency of *tt5* mutant phenotype.

Auxin is involved in almost every aspect of plant development and functions antagonistically to SA (Robert-Seilaniantz et al., 2011b). SA is able to negetively regulating the

auxin signaling pathway by stabilizing the auxin AUX-IAA negative regulators (Wang et al., 2007). Thus, the developmental deficiencies of the *tt5* mutant may be result from the inhibitory effects of increased level of SA on the auxin signaling pathway. Such reduced auxin signaling may subsequently result in an enhanced metabolism of indole-glucosinolate that is an important antimicrobial compounds. Auxin has four potential biosynthetical pathways and one of them is shared with the biosynthesis of indole-glucosinolate and camalexin (Mano and Nemoto, 2012). Inhibition of auxin signaling pathway by overexpression of microRNA miR393 results in the metabolic flux from camalexin to indole-glucosinolate throught tow auxin responsive factor *ARF1* and *-9* (Robert-Seilaniantz et al., 2011a). Such increased level of indole-glucosinolate might be responsible for the enhanced disease resistance of the *tt5* mutant. The antagonistic interactions between SA, auxin and glucosinolate metabolism could be another direction in the subsequent research regarding the molecular mechanism behind *tt5* mutant-mediated disease resistance.

In the future, the disease responses will need to be examined in all the *tt5* double/triple mutants above to confirm if the *tt5*-dependent enhanced disease resistance is also attenuated by knockout of *TT4*, *PAD4*/*SAG101*. Moreover, transcriptome and metabotome profiling in the *tt5* mutant should be carried out to determine the expression pattern of signaling and pathogen-related genes such as *PR-1* and *PDF1.2*, as well as any other gene with altered expression pattern in comparison with wild type, in case the metabolite profile within the *tt5*-T1 mutant contains signaling compounds themselves or compounds that bind to and inactivate a molecule that controls SA signaling pathway. Special attention should be focused on auxin-related genes, since flavonoid compounds are involved in auxin transportation (Peer and Murphy, 2007). SA and auxin signaling have antagonistic cross talk (Kazan and Manners, 2009) and the developmental deficiency of the *tt5* mutant could be a result of an altered or suppressed auxin metabolism/signaling pathway due to the alteration of the flavonoid composition.

4.9 Conclusion.

In this thesis, the roles of phenylpropanoid pathway in plant immunity system against pathogen attack were investigated with genetic materials disrupted in lignin pathway and flavonoid pathway. For the first time, genetic evidence is provided to demonstrate that the induced expression of lignin genes is part of plant resistance against fungal pathogens and might be regulated by a novel signaling pathway that has not been characterized before. Moreover,

F5H1 was shown to be a key gene particularly involved in resistance against penetration by a broad spectrum of pathogens, but with no impact on post-invasion resistance. Mutant *f5h1* accelerates penetration, the initial process of pathogen infection and thereby, facilitates pathogen adaptation on the host plant. The impaired penetration resistance of *f5h1* mutation was shown to be due to the loss of highly organized layered internal structures within pathogen-induced papillae. Lignin (formed through *F5H1*) and callose are important to the formation of such functional structures in papillae.

Arabidopsis requires many molecular components for its resistance against powdery mildew. In addition to the already characterized *PEN1*, *PEN2* and *PEN3* penetration resistance genes, and tryptophan-derived metabolism, this thesis further demonstrates that a functional *F5H1* gene has a role in penetration resistance of both wild type Arabidopsis and mutant *pmr2/mlo2*-dependent powdery mildew resistance. *F5H1* acts independently of all characterized molecular components described above and contributes only to enhanced penetration resistance but not to other phenotypes of the *pmr2* mutant.

In addition to the monolignol biosynthesis pathway, the flavonoid pathway is also of great importance to plant immunity. Results from this thesis indicate that post-F3H metabolites include fungal resistance compounds in wild type Arabidopsis, but also point to metabolites originating from naringenin chalcone that affect both plant development and disease resistance in a SA-dependent manner when the main flavonoid pathway is depressed in *tt5* mutant. The CHR pathway may be involved in these *tt5* mutant phenotypes since one putative Arabidopsis *CHR* gene displayed upregulated expression in the *tt5* mutant during a preliminary experiment. This observation suggests that CHR pathway that is currently believed to be restricted yo legume plants might also be active under some certain conditions in Arabidopsis.

While genetic evidence in this study confirms the roles in plant defense for several steps in the phenylpropanoid pathway, this thesis also raises several questions that remain to be answered in the future. Which form of *F5H1*-dependent lignin is the real contributor to papilla defensive structure and are any small metabolites also generated by *F5H1*? Which signaling pathway regulates the defensive lignification process in response to fungal pathogen? What is the real function of MLO protein in wild type Arabidopsis plants upon pathogen infection? What is the chalcone-dependent resistance branch pathway in Arabidopsis and exactly how is it regulated or exploited for plant defense? The answers to these questions will not only expand our

knowledge of phenolic compounds involved in plant defense, but also provide novel information to the signaling network regulating plant defense and development, the two critical aspects of plant physiology. Eventually, our knowledge of plant phenylpropanoid metabolism will contribute to the agricultural research and help to generate plants with enhanced disease without significant loss of biomass.

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APPENDIX TABLES

Appendix Table 1 List of primers sequences.

Primer Name	Purpose	Sequence (5' – 3')
F5H1-F1	Mutation verification	CACGACGTCTCTTGTCATCG
F5H1-R1	Mutation verification	TCTCGCAGGCTGACCCAAAC
F5H1-3F1	Mutation verification	CGTCGATACTAGTGTCCTCCG
F5H1-3R1	Mutation verification	GTAAGCCTATAAACGTCGGGG
F5H1-RT-F1	Amplification of hybridization probe	CTCTTGTAACGTTGGTAAGCC
F5H1-RT-R1	Amplification of hybridization probe	GGCAAACGCGTTGATCATCACACG
F5H2-1-F1	Mutation verification	GATGCTTCTGAAACCCTACCC
F5H2-1-R1	Mutation verification	CTCAGTGTCCTTCACCGTCTC
F5H2-RT-F1	Amplification of hybridization probe	GTCCAGGACGAGCTAACGCG
F5H2-RT-R1	Amplification of hybridization probe	AAAGGTGGAGGCTTCACGAGACGA
SID2-F1	Mutation verification	TGTCTGCAGTGAAGCTTTGG
SID2-R1	Mutation verification	TAGATCAATGCCCCAAGACC
PAD4-F1	Mutation verification	TTGTTCGGGCTCCTATTCT
PAD4-R1	Mutation verification	TGGCTCGGCTAAGAGTTGAT
SAG101-F3	Mutation verification	ATGCAAGGAGGTCAAGATCG
SAG101-R3	Mutation verification	TCTCGCAATGACACACTTTTG
dSpm11	Mutation verification	GGTGCAGCAAAACCCACACTTTTACTTC
SAG101-BF53	Mutation verification	ACTTCCGGGTGTTCATAAACTCGGTCAG
NahG-F1	Mutation verification	ACTCTGCCGCTACTCCCATA
NahG-R1	Mutation verification	CGAGCCCTAGGTACATCTGC
JAR1-F1	Mutation verification	GGAAACGCTACTGACCCTGA
JAR-R1	Mutation verification	TCGGGACTACAGGAAGGAGA
EIN2-F1	Mutation verification	TGGAACATGGATGCTCAAAA
EIN2-R1	Mutation verification	CTTAAGCTGCGGAATGAAGG
PMR2-F1	Mutation verification	CCTGAGAGGTTCAGGTTTGC
PMR2-R1	Mutation verification	TTGCGAGTAAGCAATGAACG
PMR4-F1	Mutation verification	AGATCAGGGACATGGGACAG
PMR4-R1	Mutation verification	TTACCAGCCCAACCAATTTC
PEN1-F1	Mutation verification	ACGGAGTTCAGATGGCGAAT
PEN1-R1	Mutation verification	CCCTCAATCGGTTGAAACTA

PEN2-F1	Mutation verification	CAGGTAAATCAGTTCGAATCAAG
PEN2-R1	Mutation verification	GGTAGAACTCGTGGCCAAGC
PEN3-F1	Mutation verification	TCACCCAACTAAATCCTCACG
PEN3-R1	Mutation verification	CCCAGCTAAAGCCAACAAAA
PAD3-F1	Mutation verification	CATCGGAAACTTACACCA
PAD3-R1	Mutation verification	CAAGATCCACGACTCTATCA
TT4-T1-F1	Mutation verification	TACAACAAAGCCCTTTGTTGG
TT4-T1-R1	Mutation verification	TACATCATGAGACGCTTGACG
TT5-T1-F1	Mutation verification	GGAGGGAATGTTTCTTCCTTG
TT5-T1-R1	Mutation verification	TTTTTCTTTGATTTTCCGAAAG
TT6-T1-F1	Mutation verification	AAACAGAACCAACGCAACAAC
TT6-T1-R1	Mutation verification	AAAGAGGAGAGATCTGCCGTC
PAL1-RT-F1	Amplification of hybridization probe	ACTTATTAGATTCCTTAACGCCGG
PAL1-RT-R1	Amplification of hybridization probe	GTTACCACCGTGAATCGCCTTGTT
C4H-RT-F1	Amplification of hybridization probe	GATGAGAGGAAGCAAATTGCGAG
C4H-RT-R1	Amplification of hybridization probe	GGAGTGGTTAAGGATGTGCAAGC
HCT-RT-F1	Amplification of hybridization probe	CTCGTTTTGCAGGTGACTTTC
HCT-RT-R1	Amplification of hybridization probe	TGTCCTGCAGCATACCATGT
COMT1-RT-F1	Amplification of hybridization probe	GAGAGAGAAATGGCGACGAC
COMT1-RT-R1	Amplification of hybridization probe	GGTTCTTCTCGTCAGCAACG
C3H-RT-F1	Amplification of hybridization probe	CTGTAACCTTCCTGAAAACAGAGC
C3H-RT-R1	Amplification of hybridization probe	GTGATCATATCCCATAGAAGACCA
PR-1-F2	Amplification of hybridization probe	ATAACACAACAATAACCATTATCAACTT
PR-1-R2	Amplification of hybridization probe	TTTTAAAAAGGCCACATATTTTACAT
PDF1.2-F2	Amplification of hybridization probe	ACACAACACATACATCTATACATTGAAA
PDF1.2-R2	Amplification of hybridization probe	AACAACAACGGGAAAATAAACA
T-DNA LBb1	Mutation verification (T-DNA specific)	GCGTGGACCGCTTGCTGCAACT

Appendix Table 2 ANOVA tables of statistical analysis

Figure 3.6A

ANOVA

Ratios

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.014	3	.005	167.947	.000
Within Groups	.000	8	.000		
Total	.014	11			

Post-Hoc Tests

Multiple Comparisons

Dependent Variable: Ratios

LSD

(I) Genotype	(J) Genotype	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
Col-0	f5h1	-.06333*	.00424	.000	-.0776	-.0491
	f5h2	-.00200	.00424	.650	-.0162	.0122
	f5h1/f5h2	-.07267*	.00424	.000	-.0869	-.0584
f5h1	Col-0	.06333*	.00424	.000	.0491	.0776
	f5h2	.06133*	.00424	.000	.0471	.0756
	f5h1/f5h2	-.00933	.00424	.059	-.0236	.0049
f5h2	Col-0	.00200	.00424	.650	-.0122	.0162
	f5h1	-.06133*	.00424	.000	-.0756	-.0471
	f5h1/f5h2	-.07067*	.00424	.000	-.0849	-.0564
f5h1/f5h2	Col-0	.07267*	.00424	.000	.0584	.0869
	f5h1	.00933	.00424	.059	-.0049	.0236
	f5h2	.07067*	.00424	.000	.0564	.0849

*. The mean difference is significant at the 0.01 level.

Figure 3.7

ANOVA

Ratios

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.055	11	.005	9.476	.000
Within Groups	.013	24	.001		
Total	.067	35			

Post-Hoc Tests

Multiple Comparisons

Dependent Variable: Ratios

LSD

(I) Genotypes	(J) Genotypes	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
	f5h1	-.10000*	.01871	.000	-.1523	-.0477
	sid2	-.02333	.01871	.224	-.0757	.0290
	f5h1/sid2	-.08667*	.01871	.000	-.1390	-.0343
	pad4/sag101	-.03000	.01871	.122	-.0823	.0223
	f5h1/pad4/sag101	-.10000*	.01871	.000	-.1523	-.0477
Col-0	NahGox	-.01333	.01871	.483	-.0657	.0390
	f5h1/NahGox	-.09667*	.01871	.000	-.1490	-.0443
	jar1	-.00333	.01871	.860	-.0557	.0490
	f5h1/jar1	-.08333*	.01871	.000	-.1357	-.0310
	ein2	-.02667	.01871	.167	-.0790	.0257
	f5h1/ein2	-.08333*	.01871	.000	-.1357	-.0310
	Col-0	.10000*	.01871	.000	.0477	.1523
	sid2	.07667*	.01871	.000	.0243	.1290
	f5h1/sid2	.01333	.01871	.483	-.0390	.0657
	pad4/sag101	.07000*	.01871	.001	.0177	.1223
	f5h1/pad4/sag101	.00000	.01871	1.000	-.0523	.0523
f5h1	NahGox	.08667*	.01871	.000	.0343	.1390
	f5h1/NahGox	.00333	.01871	.860	-.0490	.0557
	jar1	.09667*	.01871	.000	.0443	.1490
	f5h1/jar1	.01667	.01871	.382	-.0357	.0690
	ein2	.07333*	.01871	.001	.0210	.1257
	f5h1/ein2	.01667	.01871	.382	-.0357	.0690
	Col-0	.02333	.01871	.224	-.0290	.0757
	f5h1	-.07667*	.01871	.000	-.1290	-.0243
	f5h1/sid2	-.06333*	.01871	.002	-.1157	-.0110
	pad4/sag101	-.00667	.01871	.725	-.0590	.0457
	f5h1/pad4/sag101	-.07667*	.01871	.000	-.1290	-.0243
sid2	NahGox	.01000	.01871	.598	-.0423	.0623
	f5h1/NahGox	-.07333*	.01871	.001	-.1257	-.0210
	jar1	.02000	.01871	.296	-.0323	.0723
	f5h1/jar1	-.06000*	.01871	.004	-.1123	-.0077
	ein2	-.00333	.01871	.860	-.0557	.0490
	f5h1/ein2	-.06000*	.01871	.004	-.1123	-.0077
	Col-0	.08667*	.01871	.000	.0343	.1390
f5h1/sid2	f5h1	-.01333	.01871	.483	-.0657	.0390

	sid2	.06333*	.01871	.002	.0110	.1157
	pad4/sag101	.05667*	.01871	.006	.0043	.1090
	f5h1/pad4/sag101	-.01333	.01871	.483	-.0657	.0390
	NahGox	.07333*	.01871	.001	.0210	.1257
	f5h1/NahGox	-.01000	.01871	.598	-.0623	.0423
	jar1	.08333*	.01871	.000	.0310	.1357
	f5h1/jar1	.00333	.01871	.860	-.0490	.0557
	ein2	.06000*	.01871	.004	.0077	.1123
	f5h1/ein2	.00333	.01871	.860	-.0490	.0557
	Col-0	.03000	.01871	.122	-.0223	.0823
	f5h1	-.07000*	.01871	.001	-.1223	-.0177
	sid2	.00667	.01871	.725	-.0457	.0590
	f5h1/sid2	-.05667*	.01871	.006	-.1090	-.0043
	f5h1/pad4/sag101	-.07000*	.01871	.001	-.1223	-.0177
pad4/sag101	NahGox	.01667	.01871	.382	-.0357	.0690
	f5h1/NahGox	-.06667*	.01871	.002	-.1190	-.0143
	jar1	.02667	.01871	.167	-.0257	.0790
	f5h1/jar1	-.05333*	.01871	.009	-.1057	-.0010
	ein2	.00333	.01871	.860	-.0490	.0557
	f5h1/ein2	-.05333*	.01871	.009	-.1057	-.0010
	Col-0	.10000*	.01871	.000	.0477	.1523
	f5h1	.00000	.01871	1.000	-.0523	.0523
	sid2	.07667*	.01871	.000	.0243	.1290
	f5h1/sid2	.01333	.01871	.483	-.0390	.0657
	pad4/sag101	.07000*	.01871	.001	.0177	.1223
f5h1/pad4/sag101	NahGox	.08667*	.01871	.000	.0343	.1390
	f5h1/NahGox	.00333	.01871	.860	-.0490	.0557
	jar1	.09667*	.01871	.000	.0443	.1490
	f5h1/jar1	.01667	.01871	.382	-.0357	.0690
	ein2	.07333*	.01871	.001	.0210	.1257
	f5h1/ein2	.01667	.01871	.382	-.0357	.0690
	Col-0	.01333	.01871	.483	-.0390	.0657
	f5h1	-.08667*	.01871	.000	-.1390	-.0343
	sid2	-.01000	.01871	.598	-.0623	.0423
	f5h1/sid2	-.07333*	.01871	.001	-.1257	-.0210
	pad4/sag101	-.01667	.01871	.382	-.0690	.0357
NahGox	f5h1/pad4/sag101	-.08667*	.01871	.000	-.1390	-.0343
	f5h1/NahGox	-.08333*	.01871	.000	-.1357	-.0310
	jar1	.01000	.01871	.598	-.0423	.0623
	f5h1/jar1	-.07000*	.01871	.001	-.1223	-.0177
	ein2	-.01333	.01871	.483	-.0657	.0390
	f5h1/ein2	-.07000*	.01871	.001	-.1223	-.0177
	Col-0	.09667*	.01871	.000	.0443	.1490
	f5h1	-.00333	.01871	.860	-.0557	.0490
	sid2	.07333*	.01871	.001	.0210	.1257
	f5h1/sid2	.01000	.01871	.598	-.0423	.0623
	pad4/sag101	.06667*	.01871	.002	.0143	.1190
f5h1/NahGox	f5h1/pad4/sag101	-.00333	.01871	.860	-.0557	.0490
	NahGox	.08333*	.01871	.000	.0310	.1357
	jar1	.09333*	.01871	.000	.0410	.1457
	f5h1/jar1	.01333	.01871	.483	-.0390	.0657
	ein2	.07000*	.01871	.001	.0177	.1223
	f5h1/ein2	.01333	.01871	.483	-.0390	.0657

	Col-0	.00333	.01871	.860	-.0490	.0557
	f5h1	-.09667*	.01871	.000	-.1490	-.0443
	sid2	-.02000	.01871	.296	-.0723	.0323
	f5h1/sid2	-.08333*	.01871	.000	-.1357	-.0310
	pad4/sag101	-.02667	.01871	.167	-.0790	.0257
jar1	f5h1/pad4/sag101	-.09667*	.01871	.000	-.1490	-.0443
	NahGox	-.01000	.01871	.598	-.0623	.0423
	f5h1/NahGox	-.09333*	.01871	.000	-.1457	-.0410
	f5h1/jar1	-.08000*	.01871	.000	-.1323	-.0277
	ein2	-.02333	.01871	.224	-.0757	.0290
	f5h1/ein2	-.08000*	.01871	.000	-.1323	-.0277
	Col-0	.08333*	.01871	.000	.0310	.1357
	f5h1	-.01667	.01871	.382	-.0690	.0357
	sid2	.06000*	.01871	.004	.0077	.1123
	f5h1/sid2	-.00333	.01871	.860	-.0557	.0490
	pad4/sag101	.05333*	.01871	.009	.0010	.1057
f5h1/jar1	f5h1/pad4/sag101	-.01667	.01871	.382	-.0690	.0357
	NahGox	.07000*	.01871	.001	.0177	.1223
	f5h1/NahGox	-.01333	.01871	.483	-.0657	.0390
	jar1	.08000*	.01871	.000	.0277	.1323
	ein2	.05667*	.01871	.006	.0043	.1090
	f5h1/ein2	.00000	.01871	1.000	-.0523	.0523
	Col-0	.02667	.01871	.167	-.0257	.0790
	f5h1	-.07333*	.01871	.001	-.1257	-.0210
	sid2	.00333	.01871	.860	-.0490	.0557
	f5h1/sid2	-.06000*	.01871	.004	-.1123	-.0077
	pad4/sag101	-.00333	.01871	.860	-.0557	.0490
ein2	f5h1/pad4/sag101	-.07333*	.01871	.001	-.1257	-.0210
	NahGox	.01333	.01871	.483	-.0390	.0657
	f5h1/NahGox	-.07000*	.01871	.001	-.1223	-.0177
	jar1	.02333	.01871	.224	-.0290	.0757
	f5h1/jar1	-.05667*	.01871	.006	-.1090	-.0043
	f5h1/ein2	-.05667*	.01871	.006	-.1090	-.0043
	Col-0	.08333*	.01871	.000	.0310	.1357
	f5h1	-.01667	.01871	.382	-.0690	.0357
	sid2	.06000*	.01871	.004	.0077	.1123
	f5h1/sid2	-.00333	.01871	.860	-.0557	.0490
	pad4/sag101	.05333*	.01871	.009	.0010	.1057
f5h1/ein2	f5h1/pad4/sag101	-.01667	.01871	.382	-.0690	.0357
	NahGox	.07000*	.01871	.001	.0177	.1223
	f5h1/NahGox	-.01333	.01871	.483	-.0657	.0390
	jar1	.08000*	.01871	.000	.0277	.1323
	f5h1/jar1	.00000	.01871	1.000	-.0523	.0523
	ein2	.05667*	.01871	.006	.0043	.1090

*. The mean difference is significant at the 0.01 level.

Figure 3.16

ANOVA

Ratios

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.989	7	.141	124.620	.000
Within Groups	.018	16	.001		
Total	1.007	23			

Post-Hoc Tests

Multiple Comparisons

Dependent Variable: Ratios

LSD

(I) Genotypes	(J) Genotypes	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
	f5h1	-.07333	.02749	.017	-.1536	.0070
	pen1	-.52667*	.02749	.000	-.6070	-.4464
	f5h1/pen1	-.65000*	.02749	.000	-.7303	-.5697
Col-0	pen2	-.29000*	.02749	.000	-.3703	-.2097
	f5h1/pen2	-.39667*	.02749	.000	-.4770	-.3164
	pen3	-.23667*	.02749	.000	-.3170	-.1564
	f5h1/pen3	-.35333*	.02749	.000	-.4336	-.2730
	Col-0	.07333	.02749	.017	-.0070	.1536
	pen1	-.45333*	.02749	.000	-.5336	-.3730
	f5h1/pen1	-.57667*	.02749	.000	-.6570	-.4964
f5h1	pen2	-.21667*	.02749	.000	-.2970	-.1364
	f5h1/pen2	-.32333*	.02749	.000	-.4036	-.2430
	pen3	-.16333*	.02749	.000	-.2436	-.0830
	f5h1/pen3	-.28000*	.02749	.000	-.3603	-.1997
	Col-0	.52667*	.02749	.000	.4464	.6070
	f5h1	.45333*	.02749	.000	.3730	.5336
	f5h1/pen1	-.12333*	.02749	.000	-.2036	-.0430
pen1	pen2	.23667*	.02749	.000	.1564	.3170
	f5h1/pen2	.13000*	.02749	.000	.0497	.2103
	pen3	.29000*	.02749	.000	.2097	.3703
	f5h1/pen3	.17333*	.02749	.000	.0930	.2536
	Col-0	.65000*	.02749	.000	.5697	.7303
	f5h1	.57667*	.02749	.000	.4964	.6570
	pen1	.12333*	.02749	.000	.0430	.2036
f5h1/pen1	pen2	.36000*	.02749	.000	.2797	.4403
	f5h1/pen2	.25333*	.02749	.000	.1730	.3336
	pen3	.41333*	.02749	.000	.3330	.4936
	f5h1/pen3	.29667*	.02749	.000	.2164	.3770
	Col-0	.29000*	.02749	.000	.2097	.3703
	f5h1	.21667*	.02749	.000	.1364	.2970
	pen1	-.23667*	.02749	.000	-.3170	-.1564
pen2	f5h1/pen1	-.36000*	.02749	.000	-.4403	-.2797
	f5h1/pen2	-.10667*	.02749	.001	-.1870	-.0264
	pen3	.05333	.02749	.070	-.0270	.1336
	f5h1/pen3	-.06333	.02749	.035	-.1436	.0170

	Col-0	.39667*	.02749	.000	.3164	.4770
	f5h1	.32333*	.02749	.000	.2430	.4036
	pen1	-.13000*	.02749	.000	-.2103	-.0497
f5h1/pen2	f5h1/pen1	-.25333*	.02749	.000	-.3336	-.1730
	pen2	.10667*	.02749	.001	.0264	.1870
	pen3	.16000*	.02749	.000	.0797	.2403
	f5h1/pen3	.04333	.02749	.134	-.0370	.1236
	Col-0	.23667*	.02749	.000	.1564	.3170
	f5h1	.16333*	.02749	.000	.0830	.2436
	pen1	-.29000*	.02749	.000	-.3703	-.2097
pen3	f5h1/pen1	-.41333*	.02749	.000	-.4936	-.3330
	pen2	-.05333	.02749	.070	-.1336	.0270
	f5h1/pen2	-.16000*	.02749	.000	-.2403	-.0797
	f5h1/pen3	-.11667*	.02749	.001	-.1970	-.0364
	Col-0	.35333*	.02749	.000	.2730	.4336
	f5h1	.28000*	.02749	.000	.1997	.3603
	pen1	-.17333*	.02749	.000	-.2536	-.0930
f5h1/pen3	f5h1/pen1	-.29667*	.02749	.000	-.3770	-.2164
	pen2	.06333	.02749	.035	-.0170	.1436
	f5h1/pen2	-.04333	.02749	.134	-.1236	.0370
	pen3	.11667*	.02749	.001	.0364	.1970

*. The mean difference is significant at the 0.01 level.

Figure 3.17A

ANOVA

Ratios

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.596	7	.085	292.862	.000
Within Groups	.005	16	.000		
Total	.600	23			

Post-Hoc Tests

Multiple Comparisons

Dependent Variable: Ratios

LSD

(I) Genotypes	(J) Genotypes	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
	f5h1	-.15667*	.01392	.000	-.1973	-.1160
	pen1	-.01500	.01392	.297	-.0557	.0257
	f5h1/pen1	-.33333*	.01392	.000	-.3740	-.2927
Col-0	pen2	-.22500*	.01392	.000	-.2657	-.1843
	f5h1/pen2	-.47833*	.01392	.000	-.5190	-.4377
	pen3	-.15167*	.01392	.000	-.1923	-.1110
	f5h1/pen3	-.36167*	.01392	.000	-.4023	-.3210
	Col-0	.15667*	.01392	.000	.1160	.1973
	pen1	.14167*	.01392	.000	.1010	.1823
	f5h1/pen1	-.17667*	.01392	.000	-.2173	-.1360
f5h1	pen2	-.06833*	.01392	.000	-.1090	-.0277
	f5h1/pen2	-.32167*	.01392	.000	-.3623	-.2810
	pen3	.00500	.01392	.724	-.0357	.0457
	f5h1/pen3	-.20500*	.01392	.000	-.2457	-.1643
	Col-0	.01500	.01392	.297	-.0257	.0557
	f5h1	-.14167*	.01392	.000	-.1823	-.1010
	f5h1/pen1	-.31833*	.01392	.000	-.3590	-.2777
pen1	pen2	-.21000*	.01392	.000	-.2507	-.1693
	f5h1/pen2	-.46333*	.01392	.000	-.5040	-.4227
	pen3	-.13667*	.01392	.000	-.1773	-.0960
	f5h1/pen3	-.34667*	.01392	.000	-.3873	-.3060
	Col-0	.33333*	.01392	.000	.2927	.3740
	f5h1	.17667*	.01392	.000	.1360	.2173
	pen1	.31833*	.01392	.000	.2777	.3590
f5h1/pen1	pen2	.10833*	.01392	.000	.0677	.1490
	f5h1/pen2	-.14500*	.01392	.000	-.1857	-.1043
	pen3	.18167*	.01392	.000	.1410	.2223
	f5h1/pen3	-.02833	.01392	.059	-.0690	.0123
	Col-0	.22500*	.01392	.000	.1843	.2657
	f5h1	.06833*	.01392	.000	.0277	.1090
	pen1	.21000*	.01392	.000	.1693	.2507
pen2	f5h1/pen1	-.10833*	.01392	.000	-.1490	-.0677
	f5h1/pen2	-.25333*	.01392	.000	-.2940	-.2127
	pen3	.07333*	.01392	.000	.0327	.1140
	f5h1/pen3	-.13667*	.01392	.000	-.1773	-.0960
	Col-0	.47833*	.01392	.000	.4377	.5190
f5h1/pen2	f5h1	.32167*	.01392	.000	.2810	.3623

	pen1	.46333*	.01392	.000	.4227	.5040
	f5h1/pen1	.14500*	.01392	.000	.1043	.1857
	pen2	.25333*	.01392	.000	.2127	.2940
	pen3	.32667*	.01392	.000	.2860	.3673
	f5h1/pen3	.11667*	.01392	.000	.0760	.1573
	Col-0	.15167*	.01392	.000	.1110	.1923
	f5h1	-.00500	.01392	.724	-.0457	.0357
	pen1	.13667*	.01392	.000	.0960	.1773
pen3	f5h1/pen1	-.18167*	.01392	.000	-.2223	-.1410
	pen2	-.07333*	.01392	.000	-.1140	-.0327
	f5h1/pen2	-.32667*	.01392	.000	-.3673	-.2860
	f5h1/pen3	-.21000*	.01392	.000	-.2507	-.1693
	Col-0	.36167*	.01392	.000	.3210	.4023
	f5h1	.20500*	.01392	.000	.1643	.2457
	pen1	.34667*	.01392	.000	.3060	.3873
f5h1/pen3	f5h1/pen1	.02833	.01392	.059	-.0123	.0690
	pen2	.13667*	.01392	.000	.0960	.1773
	f5h1/pen2	-.11667*	.01392	.000	-.1573	-.0760
	pen3	.21000*	.01392	.000	.1693	.2507

*. The mean difference is significant at the 0.01 level.

Figure 3.20M

ANOVA

Ratios

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.929	3	.310	320.177	.000
Within Groups	.008	8	.001		
Total	.937	11			

Post-Hoc Tests

Multiple Comparisons

Dependent Variable: Ratios

LSD

(I) Genotypes	(J) Genotypes	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	f5h1	-.06983*	.02540	.025	-.1284	-.0113
Col-0	pmr2	.60220*	.02540	.000	.5436	.6608
	f5h1pmr2	.39870*	.02540	.000	.3401	.4573
	Col-0	.06983*	.02540	.025	.0113	.1284
f5h1	pmr2	.67203*	.02540	.000	.6135	.7306
	f5h1pmr2	.46853*	.02540	.000	.4100	.5271
	Col-0	-.60220*	.02540	.000	-.6608	-.5436
pmr2	f5h1	-.67203*	.02540	.000	-.7306	-.6135
	f5h1pmr2	-.20350*	.02540	.000	-.2621	-.1449
	Col-0	-.39870*	.02540	.000	-.4573	-.3401
f5h1pmr2	f5h1	-.46853*	.02540	.000	-.5271	-.4100
	pmr2	.20350*	.02540	.000	.1449	.2621

*. The mean difference is significant at the 0.05 level.

Figure 3.20N

ANOVA

Lengths

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4912945.967	3	1637648.656	141.721	.000
Within Groups	92443.753	8	11555.469		
Total	5005389.719	11			

Post-Hoc Tests

Multiple Comparisons

Dependent Variable: Lengths

LSD

(I) Genotypes	(J) Genotypes	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	f5h1	-342.74196*	87.77042	.005	-545.1409	-140.3430
Col-0	pmr2	1089.66209*	87.77042	.000	887.2631	1292.0610
	f5h1pmr2	1080.65951*	87.77042	.000	878.2606	1283.0585
	Col-0	342.74196*	87.77042	.005	140.3430	545.1409
f5h1	pmr2	1432.40404*	87.77042	.000	1230.0051	1634.8030
	f5h1pmr2	1423.40147*	87.77042	.000	1221.0025	1625.8004
	Col-0	-1089.66209*	87.77042	.000	-1292.0610	-887.2631
pmr2	f5h1	-1432.40404*	87.77042	.000	-1634.8030	-1230.0051
	f5h1pmr2	-9.00257	87.77042	.921	-211.4015	193.3964
	Col-0	-1080.65951*	87.77042	.000	-1283.0585	-878.2606
f5h1pmr2	f5h1	-1423.40147*	87.77042	.000	-1625.8004	-1221.0025
	pmr2	9.00257	87.77042	.921	-193.3964	211.4015

*. The mean difference is significant at the 0.05 level.

Figure 3.20O

ANOVA

Haustoria

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	529.690	3	176.563	154.429	.000
Within Groups	9.147	8	1.143		
Total	538.837	11			

Post-Hoc Tests

Multiple Comparisons

Dependent Variable: Haustoria

LSD

(I) Genotypes	(J) Genotypes	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	f5h1	-4.86667*	.87305	.001	-6.8799	-2.8534
Col-0	pmr2	11.80000*	.87305	.000	9.7867	13.8133
	f5h1pmr2	8.60000*	.87305	.000	6.5867	10.6133
	Col-0	4.86667*	.87305	.001	2.8534	6.8799
f5h1	pmr2	16.66667*	.87305	.000	14.6534	18.6799
	f5h1pmr2	13.46667*	.87305	.000	11.4534	15.4799
	Col-0	-11.80000*	.87305	.000	-13.8133	-9.7867
pmr2	f5h1	-16.66667*	.87305	.000	-18.6799	-14.6534
	f5h1pmr2	-3.20000*	.87305	.006	-5.2133	-1.1867
	Col-0	-8.60000*	.87305	.000	-10.6133	-6.5867
f5h1pmr2	f5h1	-13.46667*	.87305	.000	-15.4799	-11.4534
	pmr2	3.20000*	.87305	.006	1.1867	5.2133

*. The mean difference is significant at the 0.05 level.

Figure 3.21

ANOVA

Ratios

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.084	5	.017	66.526	.000
Within Groups	.003	12	.000		
Total	.087	17			

Post-Hoc Tests

Multiple Comparisons

Dependent Variable: Ratios

LSD

(I) Genotypes	(J) Genotypes	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	f5h1	-.09685*	.01297	.000	-.1251	-.0686
	pmr2	.04515*	.01297	.005	.0169	.0734
Col-0	f5h1pmr2	.02970*	.01297	.041	.0014	.0580
	pmr4	-.09382*	.01297	.000	-.1221	-.0656
	f5h1/pmr4	-.13019*	.01297	.000	-.1584	-.1019
	Col-0	.09685*	.01297	.000	.0686	.1251
	pmr2	.14200*	.01297	.000	.1137	.1703
f5h1	f5h1pmr2	.12656*	.01297	.000	.0983	.1548
	pmr4	.00304	.01297	.819	-.0252	.0313
	f5h1/pmr4	-.03333*	.01297	.025	-.0616	-.0051
	Col-0	-.04515*	.01297	.005	-.0734	-.0169
	f5h1	-.14200*	.01297	.000	-.1703	-.1137
pmr2	f5h1pmr2	-.01544	.01297	.257	-.0437	.0128
	pmr4	-.13896*	.01297	.000	-.1672	-.1107
	f5h1/pmr4	-.17533*	.01297	.000	-.2036	-.1471
	Col-0	-.02970*	.01297	.041	-.0580	-.0014
	f5h1	-.12656*	.01297	.000	-.1548	-.0983
f5h1pmr2	pmr2	.01544	.01297	.257	-.0128	.0437
	pmr4	-.12352*	.01297	.000	-.1518	-.0953
	f5h1/pmr4	-.15989*	.01297	.000	-.1881	-.1316
	Col-0	.09382*	.01297	.000	.0656	.1221
	f5h1	-.00304	.01297	.819	-.0313	.0252
pmr4	pmr2	.13896*	.01297	.000	.1107	.1672
	f5h1pmr2	.12352*	.01297	.000	.0953	.1518
	f5h1/pmr4	-.03637*	.01297	.016	-.0646	-.0081
	Col-0	.13019*	.01297	.000	.1019	.1584
	f5h1	.03333*	.01297	.025	.0051	.0616
f5h1/pmr4	pmr2	.17533*	.01297	.000	.1471	.2036
	f5h1pmr2	.15989*	.01297	.000	.1316	.1881
	pmr4	.03637*	.01297	.016	.0081	.0646

*. The mean difference is significant at the 0.05 level.

Figure 3.23M

ANOVA

Ratios

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.008	3	.003	2.643	.121
Within Groups	.008	8	.001		
Total	.016	11			

Post-Hoc Tests

Multiple Comparisons

Dependent Variable: Ratios

LSD

(I) Genotypes	(J) Genotypes	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	f5h1	-.06983*	.02628	.029	-.1304	-.0092
Col-0	pmr4	-.02347	.02628	.398	-.0841	.0371
	f5h1/pmr4	-.04790	.02628	.106	-.1085	.0127
	Col-0	.06983*	.02628	.029	.0092	.1304
f5h1	pmr4	.04637	.02628	.116	-.0142	.1070
	f5h1/pmr4	.02193	.02628	.428	-.0387	.0825
	Col-0	.02347	.02628	.398	-.0371	.0841
pmr4	f5h1	-.04637	.02628	.116	-.1070	.0142
	f5h1/pmr4	-.02443	.02628	.380	-.0850	.0362
	Col-0	.04790	.02628	.106	-.0127	.1085
f5h1/pmr4	f5h1	-.02193	.02628	.428	-.0825	.0387
	pmr4	.02443	.02628	.380	-.0362	.0850

*. The mean difference is significant at the 0.05 level.

Figure 3.23N

ANOVA

Lengths

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3206958.475	3	1068986.158	62.448	.000
Within Groups	136945.188	8	17118.148		
Total	3343903.662	11			

Post-Hoc Tests

Multiple Comparisons

Dependent Variable: Lengths

LSD

(I) Genotypes	(J) Genotypes	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	f5h1	-342.74196*	106.82743	.012	-589.0864	-96.3975
Col-0	pmr4	1049.36630*	106.82743	.000	803.0218	1295.7108
	f5h1/pmr4	385.13553*	106.82743	.007	138.7910	631.4800
	Col-0	342.74196*	106.82743	.012	96.3975	589.0864
f5h1	pmr4	1392.10826*	106.82743	.000	1145.7638	1638.4527
	f5h1/pmr4	727.87749*	106.82743	.000	481.5330	974.2220
	Col-0	-1049.36630*	106.82743	.000	-1295.7108	-803.0218
pmr4	f5h1	-1392.10826*	106.82743	.000	-1638.4527	-1145.7638
	f5h1/pmr4	-664.23077*	106.82743	.000	-910.5753	-417.8863
	Col-0	-385.13553*	106.82743	.007	-631.4800	-138.7910
f5h1/pmr4	f5h1	-727.87749*	106.82743	.000	-974.2220	-481.5330
	pmr4	664.23077*	106.82743	.000	417.8863	910.5753

*. The mean difference is significant at the 0.05 level.