Studies on postinvasive resistance of *Arabidopsis thaliana* against multiple fungal pathogens

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CONTENTS

GENERAL INTRODUCTION 1

CHAPTER I 7

CYP71A12-dependent biosynthesis of indole-3-carboxylic acids is involved in the postinvasive resistance against *Colletotrichum tropicale*

CHAPTER II 31

bak1–5 mutation uncouples tryptophan-dependent and independent postinvasive immune pathways triggered in *Arabidopsis thaliana* by multiple fungal pathogens

CHAPTER III 73

EDR1- and ORA59-dependent expression of plant defensin contributes to the postinvasive resistance against fungal pathogen

SUMMARY 94

REFERENCES 97

ACKNOWLEDGEMENTS 112

GENERAL INTRODUCTION

Plant developed a sophisticated immune system as an outcome of co-evolution with plant pathogens over millions of years. Plant immune system can be categorized into host resistance and nonhost resistance. Strong host resistance is mostly mediated by resistant (R) proteins. R proteins are cytoplasmic localized sensors (nucleotide-binding and LRR domain, called NLR) that recognize pathogen effectors (specific proteins expressed by plant pathogens to aid infection) and initiate defense signaling, which often causes cell death at the site of infection. Therefore, R protein-mediated plant immunity is effective to terminate the spreading of biotrophic and hemibiotrophic pathogens, whereas it is often ineffective to necrotrophic pathogens (Jones and Dangl, 2006). Nonhost resistance can be defined as the immunity of an entire plant species against all tested isolates of a particular pathogen. Nonhost resistance confers a durable defense mechanism to plants against the majority of potential pathogens (Heath, 2000). In nonhost resistance, plant recognizes pathogens, in part, by using extracellular oriented transmembrane protein (protein with leucine-rich repeat domain) and initiates the downstream defense signaling including callose and phytoalexin accumulation around the infection sites (Jones and Dangl, 2006). Because of its durability and broad range activity, nonhost resistance is thought to be a valuable strategy to improve crop resistance. Thus, the study of nonhost resistance provides better understanding of this resistance and chances to utilize obtained knowledge to develop novel crop protection technologies.

Nonhost resistance of a model plant *Arabidopsis thaliana* is extensively studied now. Studies on *Arabidopsis* nonhost resistance to its nonadapted powdery mildew fungi *Blumeria graminis* f. sp. *hordei* (called *Bgh*) and *Erysiphe pisi* revealed that this immune system is composed by two layers of resistance: (a) preinvasive resistance and (b) postinvasive resistance (Lipka *et al*. 2005, 2008; Nürnberger and Lipka 2005). *Arabidopsis PENETRATION 1* (*PEN1*), *PEN2* and *PEN3* genes are essential for full preinvasive resistance against nonadapted powdery mildew pathogens. *PEN1* encodes a

plasma membrane-localized synthaxin, which forms complex with the SNAP33 adaptor and VAMP721/722 and functions in the execution of secretory immune responses at the invasion site of powdery mildew fungi in leaf epidermal cells (Collins *et al*. 2003; Assaad *et al*. 2004; Kwon *et al*. 2008; Johansson *et al*. 2014). *PEN2* encodes the atypical myrosinase that hydrolyzes 4-methoxynidol-3-ylmethylglucosinolates (4MI3G), leading to the production of an unidentified antifungal compound (Bednarek *et al*. 2009; Clay *et al*. 2009; Fuchs *et al*. 2016; Lipka *et al*. 2005). *PEN3*/*ABCG36* encodes an ATP-binding cassette transporter, which is believed to export antifungal compounds, including PEN2-derived metabolites, to the apoplast and prevents the entry of fungal pathogens (Stein *et al*. 2006).

Colletotrichum tropicale, formerly *Colletotrichum gleosporioides,* isolate S9275 is nonadapted pathogen to its nonhost *Arabidopsis thaliana*. This fungal pathogen, hereafter called *Ctro,* is known to cause anthracnose disease on mulberry (Agrios 2004; Cannon *et al*., 2012). In contrast to the adapted pathogen *Colletotrichum higginsianum* that causes necrotic lesions on inoculated *Arabidopsis* leaves, nonadapted *Ctro* fails to induce lesions, indicating that *Arabidopsis* exhibits nonhost resistance to *Ctro* (Shimada *et al*., 2006). *PEN2* and *PEN3* are involved in the entry control (preinvasive resistance) to *Ctro* (Hiruma *et al*., 2010; Kosaka and Takano, 2018), whereas *PEN1* is dispensable unlike the case of *Bgh* (Shimada *et al*., 2006). In addition to *PEN2*, nonhost resistance toward *Ctro* also involves *EDR1* (*ENHANCED DISEASE RESISTANCE 1*) (Hiruma *et al.,* 2011), whereas the *edr1* mutants exhibit enhanced resistance toward the adapted powdery mildew *Golovinomyces cichoracearum* (formerly named *Erysiphe cichoracearum*) (Frye *et al.,* 2001), suggesting the presence of diverse plant strategies for controlling the entry attempts of pathogens showing distinct infection modes. Even though *Ctro* successfully invades *Arabidopsis* mutants with defect in *PEN2* and *EDR1*, these mutants are still not fully susceptible to *Ctro*, because postinvasive resistance is newly activated and then terminates further pathogen growth inside plant tissues (Hiruma *et al*., 2011). This suggests the importance of postinvasive resistance for plant survival against attack of

numerous pathogenic fungi. CYP79B2 and CYP79B3 are monooxygenases crucial for biosynthesis of multiple secondary metabolites derived from tryptophan (Trp) (Zhao *et al*., 2002). In contrast to *pen2* mutant or WT plant (Col-0), *Arabidopsis cyp79B2 cyp79B3* double mutant is fully susceptible to the hemibiotrophic nonadapted pathogen *Ctro* (Hiruma *et al*., 2013), necrotrophic adapted and nonadapted *Plectosphaerella cucumerina* isolates (Sanchez-Vallet *et al*., 2010), and oomycete *Phytophtora brassicae* (Schlaeppi *et al*., 2010). *Ctro* is able to invade both *pen2* and *cyp79B2 cyp79B3* mutant. However, after the invasion, *Ctro* expands invasive hyphae in the *cyp79B2 cyp79B3* mutant, whereas its invasive hyphal growth is terminated in the *pen2* mutant (Hiruma *et al*., 2013). The result indicates that the *cyp79B2 cyp79B3* mutant is defective in both preinvasive and post invasive resistance. It has also been shown that the *pen2 pad3* mutant is partially defective in postinvasive resistance to *Ctro*, indicating that PAD3-dependent synthesis of *Arabidopsis* phytoalexin, called camalexin, is a critical factor for this immunity (Hiruma *et al.,* 2013). Importantly, the *cyp79B2 cyp79B3* mutant plant exhibits a more severe defect in this second-defense layer than the *pen2 pad3* plant (Hiruma *et al.,* 2013). This enhanced susceptibility in the *cyp79B2 cyp79B3* plants compared with the *pen2 pad3* plants was also reported for the interactions of *Phytophthora brassicae* and *Plectosphaerella cucumerina* with *Arabidopsis* (Sanchez-Vallet *et al.,* 2010; Schlaeppi *et al.,* 2010). These findings suggested the presence of additional Trp-derived secondary metabolites crucial for postinvasive resistance against fungal pathogens, but the detailed information on this point is lacking.

In addition to *PEN2*, *EDR1* mentioned above is involved in the preinvasive resistance against *Ctro*. *EDR1* encodes a protein kinase homologous to the mitogen-activated protein kinase kinase kinase (MAPKKK) belonging to the Raf family (Frye *et al*., 2011). It was reported that EDR1 positively regulates the expression of antimicrobial plant defensins (PDFs) in response to the entry trial of *Ctro* (Hiruma *et al*., 2011). Currently, it remains to be elucidated (i) whether EDR1 also regulates the expression of

PDFs during postinvasive defense and (ii) whether the expression of *PDFs* is involved in postinvasive defense against fungal pathogens such as *Ctro*.

In this thesis, I studied *Arabidopsis* postinvasive resistance against multiple fungal pathogens with different infection styles. In Chapter I, I investigated Trp-derived secondary metabolites that can explain the different phenotypes upon *Ctro* inoculation between the *pen2 pad3* mutant and the *cyp79B2 cyp79B3* mutant. CYP79B2/CYP79B3 converts Trp into indole-3-acetaldoxime (Zhao *et al.,* 2002), and this precursor is then converted into multiple compounds for antimicrobial immunity, such as PEN2-related indole-glucosinolates (IGs), PAD3-related camalexin, and CYP82C2-related 4-hydroxy-ICN (4-OH-ICN) (Böttcher *et al.,* 2009; Bednarek, 2012; Rajniak *et al.,* 2015). In addition to these compounds, indole-3-acetaldoxime is known to be used for the biosynthesis of indole-3-carboxylic acid derivatives (ICAs) (Müller *et al*., 2015). I here revealed that the biosynthesis of ICAs is activated when *Ctro* attacks and is dependent on CYP71A12. Furthermore, CYP71A12-dependent production of ICAs is involved in the postinvasive resistance to *Ctro*. I also revealed that CYP71A13- and PAD3-dependent biosynthesis of camalexin is involved in the postinvasive resistance to *Ctro*, whereas CYP82C2-dependent biosynthesis of 4-OH-ICN was dispensable. While camalexin and ICAs are important for the postinvasive defense to *Ctro*, they are dispensable for the preinvasive defense that controls the entry of this pathogen.

In Chapter II, I asked whether ICAs and camalexin are involved in postinvasive resistance against other fungal pathogens exhibiting different infection strategies, in addition to *Ctro*. The study showed that, in contrast to *Ctro*, *PEN2* is not required for the entry control of a necrotrophic pathogen *Alternaria brassicicola* (here after *Ab*). However, similar to *Ctro*, both CYP71A12-dependent synthesis of ICAs and CYP71A13- and PAD3-dependent synthesis of camalexin are essential for the postinvasive defense against *Ab*. Then I asked how *Arabidopsis* recognizes invasion by fungal pathogens to activate its postinvasive resistance. I hypothesized that *Arabidopsis* might recognize PAMPs (pathogen-associated molecular patterns) derived from invasive hyphae, or

DAMPs (damage-associated molecular patterns) generated by pathogen invasion to activate these Trp-related antifungal pathways upon pathogen invasion. To assess this point, I focused on components of pattern recognition receptor (PRR) complexes because PRRs are involved in the recognition of PAMPs and DAMPs (Jones and Dangl, 2006; Saijo *et al*., 2018). BAK1 is a conserved co-receptor for multiple PRRs involved in the recognition of PAMPs and DAMPs (Heese *et al.,* 2007; Postel *et al.,* 2010; Roux *et al.,* 2011). The study revealed that a particular mutation on *BAK1* (*bak1–5* mutation) reduced the postinvasive resistance against fungal pathogens, suggesting the involvement of PRRs in the postinvasive resistance. Unexpectedly, the *bak1–5* mutation had no clear effects on the induced expression of *PAD3* and *CYP71A12* upon pathogen invasion, indicating that the activation of Trp-metabolism pathway does not depend on BAK1. Furthermore, the microarray analysis revealed that the expression of defense-related genes upon *Ab* invasion, including *Apoplastic EDS1-Dependent 1* (*AED1*), *beta-1,3-glucanase 2*/*Pathogenesis related protein 2* (*BGL2*/*PR2*), *GDSL esterase*/*lipase 1* (*GLIP1*) and *RECEPTOR LIKE PROTEIN 23* (*RLP23*), are down-regulated in the presence of the *bak1-5* mutation. These findings suggested that pathogen invasion in *Arabidopsis* activates (i) *bak1–5* insensitive Trp-metabolism that leads to antimicrobial small molecules, and (ii) a *bak1–5* sensitive immune pathway that activates the expression of antimicrobial proteins.

In Chapter III, I investigated a possible link between plant defensins (PDFs) and postinvasive resistance in *Arabidopsis*. I found that the expression of the *PDF* genes was highly induced upon invasion by fungal pathogens. However, the pathogen invasion resulted in the transient expression of *PDFs* where it resulted in the sustained expression of the Trp-metabolism pathway genes. I also revealed that *EDR1* is required for the induced expression of the *PDF* genes upon invasion of the fungal pathogens. The AP2/ERF domain transcription factor ORA59 is required for the induced expression of the *PDF* gene under the jasmonic acid (JA) treatment (Pré *et al*., 2008). Here, I also found that *ORA59* is critical for the induced expression of the *PDF* genes upon pathogen

invasion. Importantly, the inoculation assay of *Ab* revealed that *ORA59* is involved in the postinvasive resistance against *Ab*, suggesting the involvement of PDFs in the *Arabidopsis* postinvasive resistance in addition to the Trp-related secondary metabolites.

CHAPTER I

CYP71A12-dependent biosynthesis of indole-3-carboxylic acids is involved in the postinvasive resistance against *Colletotrichum tropicale*

INTRODUCTION

Plants are constantly targeted by pathogen attack which leaded the development of sophisticate defense strategis to defend from pathogen. In *Arabidopsis*, one of the strategies is the synthesis of secondary metabolites derived from tryptophan (Trp) metabolism, including phytoalexin camalexin and indoleglucosinolates (Glawischnig *et al*., 2007; Pastorczyk and Bednarek, 2016). In addition to these, indole-3-carboxylic acid derivatives (ICAs) and 4-hydroxy-ICN (4-OH-ICN) were also reported to be induced in response to pathogen attack (Hagemeier *et al*., 2001; Tan *et al*., 2004; Bednarek *et al*., 2005; Rajniak *et al*., 2015). These Trp-derived secondary metabolites are synthesized from common precursor indole-3-acetaldoxime (IAOx). Redundant P450 monooxygenases CYP79B2 and CYP79B3 converts Trp into IAOx (Mikkelsen *et al*., 2000; Zhao *et al*., 2002; Glawischning *et al*., 2004; Böttcher *et al*., 2009). IAOx represents the metabolic branching point for IG biosynthesis and pathways leading to ICAs, ICNs and camalexin.

In *Arabidopsis* CYP71A13 and CYP71A12 enzymes show 89 % identity on the amino acid level and the corresponding genes are located in tandem on the chromosome II. Metabolic phenotypes of single, and double *cyp71A12* and *cyp71A13* mutant lines revealed that both monooxygenases contribute redundantly to camalexin biosynthesis during the infection with *Pseudomonas syringae* and *Alternaria brassicicola* as well as in response to microbe-associated molecular pattern (MAMP) flg22 (bacterial flagellin epitope), silver nitrate (AgNO3) and UV treatment (Nafisi *et al*., 2007; Millet *et al*., 2010; Müller *et al*., 2015). The last two steps in the formation of this phytoalexin are catalyzed

by the multifunctional PHYTOALEXIN DEFICIENT 3 (PAD3) CYP71B15 monooxygenase (Schuhegger *et al.,* 2006; Böttcher *et al.,* 2009).

Accumulation levels of ICAs in single and double *cyp71A12* and *cyp71A13* mutant lines suggested that biosynthesis of these compounds during the response of *Arabidopsis* to AgNO3 is dependent predominantly on CYP71A12 monooxygenase, while upon UV-treatment specific ICAs can be produced with additional contribution of CYP71A13 (Müller *et al.,* 2015). However, so far, their role in plant immunity has been not experimentally verified.

Plant immunity to all tested isolates of a particular pathogen is called nonhost resistance (Heath 2000). *Colletorichum tropicale* (here after *Ctro*) is a hemibiotrophic pathogen that naturally infects its host mulberry but not its nonhost *Arabidopsis thaliana*. The study focusing on *Arabidopsis* immunity towards *Ctro* and powdery mildews pathogen *Blumeria graminis* f. sp. *hordei* (called as *Bgh*) discovered that *Arabidopsis* nonhost resistance is composed by two layers: preinvasive resistance and postinvasive resistance (Lipka *et al.,* 2005; Hiruma *et al.,* 2013). *Arabidopsis pen2* mutants permit *Ctro* to entry, which results in lesion development on the inoculated leaves. However, these lesions are not expanded from inoculated area, indicating that the *pen2* mutant plants are defective in preinvasive resistance but still retains its postinvasive resistance (Hiruma *et al.,* 2010). *Arabidopsis pen2 pad3* double mutant is defective in the synthesis of both indoleglucosinolates and camalexin. Toward *Ctro* challenge, the *pen2 pad3* mutant showed lesion expansion in contrast to the *pen2* plant, indicating that this double mutant is defective in postinvasive defense and that camalexin is involved in the postinvasive defense to *Ctro* (Hiruma *et al.,* 2013). As mentioned above, CYP79B2 and CYP79B3 are key enzymes for Trp-metabolism pathway (Zhao *et al.,* 2002). *Arabidopsis* defective in both *CYP79B2* and *CYP79B3* showed more severe defects in postinvasive resistance to *Ctro* compared with *pen2 pad3* (Hiruma *et al.,* 2013), suggesting that, in addition to camalexin, other Trp-derived metabolites are involved in the postinvasive resistance against *Ctro*.

In this study, I focused on possible contribution of other Trp-derived metabolites, ICAs and 4-OH-ICN, to the postinvasive defense to *Ctro*. First, I studied the contribution of CYP71A12 and CYP71A13 to the postinvasive defense to *Ctro*. For this evaluation, leaves inoculated with *Ctro* were collected and stained with tryphan blue solution. The plants having a defect in postinvasive defense showed expansion of fungal hyphae from the inoculated area, whereas the expansion of invasive hyphae was limited to the inoculated area in plants having no defects in postinvasive defense. *pen2 pad3* and *pen2 cyp71A12* double mutant permitted comparable level of *Ctro* hyphal expansion in contrast to the *pen2* mutant, suggesting that both camalexin and ICAs are involved in this second layer defense. Also, *pen2 cyp71A12 cyp71A13* triple mutant plant showed higher hyphal expansion than *pen2 pad3* and *pen2 cyp71A12*, indicating that these metabolites contribute independently to the postinvasive defense against *Ctro*. Importantly, the accumulation data of ICAs support this idea.

CYP82C2-dependent biosynthesis of 4-OH-ICN is involved in the immunity to *Botrytis cinerea*, *Alternaria brassicicola* and *Pseudomonas syringae* (Rajniak *et al*., 2015). However, the *pen2 cyp82C2* mutant showed no difference towards *Ctro* inoculation compared with the *pen2* mutant, suggesting that 4-OH-ICN is not essential for the postinvasive defense to *Ctro*. These results indicate that ICAs are critical factors for the postinvasive defense to *Ctro*.

RESULTS

CYP71A12 and PAD3 contribute to the postinvasive defense against *Ctro*

Ctro is a hemibiotropic pathogen that naturally infects its host mulberry but is not able to infect its nonhost *Arabidopsis thaliana*. *Arabidopsis* immunity to *Ctro* is called nonhost resistance and it is composed by preinvasive resistance and postinvasive resistance. *PEN2* is essential for the preinvasive resistance to *Ctro* and its defect permits *Ctro* invasion, but not their expansion from the inoculated area (Hiruma *et al*., 2010; 2013; Fig. I-1). PAD3 is responsible for camalexin biosynthesis in response to pathogen attack. *Arabidopsis pad3* mutant showed susceptibility to multiple fungal pathogens such as *Alternaria brassicicola*, *Botrytis cinerea*, *Plectosphaerella cucumerina*, *Blumeria graminis* and *Erysiphe pisi* (Kliebenstein *et al*., 2005; Nafisi *et al*., 2007; Sanchez-Vallet *et al*., 2010). The *pen2 pad3* double mutant was not only defective in preinvasive resistance but also permitted fungal hyphal expansion by *Ctro* from inoculated area, suggesting that the mutant was also defective in its postinvasive resistance (Bednarek *et al*., 2009; Hiruma *et al*., 2010; 2013; Fig. I-1). The *cyp79B2 cyp79B3* double mutant is defective in all Trp-derived metabolites and showed more severe defect in postinvasive resistance to *Ctro* than in *pen2 pad3* (Hiruma *et al*., 2013). The difference in susceptibility to *Ctro* between *pen2 pad3* and *cyp79B2 cyp79B3* indicated the presence of additional Trp-derived metabolites required for the postinvasive defense. In *Arabidopsis*, in addition to PEN2-dependent glucosinolates and PAD3-dependent camalexin, the indole-3-carboxilic acids (ICAs) and 4-hydroxy-ICN were synthesized though Trp metabolism pathway (Rajniak *et al*., 2015; Fig. I-2). To reveal additional Trp-derived metabolites involved in the postinvasive defense, *Ctro* was inoculated onto series of *Arabidopsis* mutants with defects in Trp-metabolism pathway, the *pen2* mutant, the *pen2 pad3* mutant, the *pen2 cyp71A12* mutant, the *pen2 cyp71A12 cyp71A13* mutant, the *pen2 cyp82C2* mutant, and the *cyp79B2 cyp79B3* mutant (Fig. I-3). As a result, both *pen2 pad3* and *pen2 cyp71A12* double mutant plant showed defects in postinvasive defense, suggesting the

contribution of PAD3-dependent camalexin and CYP71A12-dependent metabolites for this second layered defense (Fig. I-3). Also, both *pen2 pad3* and *pen2 cyp71A12* allowed similar degree of hyphal expansion, indicating that PAD3 and CYP71A12 contribute independently to the postinvasive defense to *Ctro* (Fig. I-3B).

CYP82C2-dependent biosynthesis of 4-OH-ICN is dispensable for the postinvasive defense to *Ctro*

CYP82C2 is essential for biosynthesis of 4-OH-ICN (Rajniak *et al.,* 2015). To investigate possible contribution of 4-OH-ICN to the postinvasive resistance to *Ctro*, I inoculated *Ctro* on the *pen2 cyp82C2* mutant. As a result, the *pen2 cyp82C2* plant showed similar level of lesion development and hyphal expansion as *pen2*, suggesting that CYP82C2-dependent biosynthesis of 4-OH-ICN is dispensable for the postinvase defense to *Ctro* (Fig. I-3). I also inoculated *Ctro* on *pen2 pad3 cyp82C2* triple mutant plant and compared with the phenotype of *pen2 pad3* to investigate potential contribution of 4-OH-ICN in the absence of camalexin. However, the lesion development and hyphal expansion in this triple mutant was comparable to *pen2 pad3*, indicating that 4-OH-ICN is not essential for the postinvasive defense to *Ctro* even in the absence of camalexin (Fig. I-4).

CYP71A12, PAD3 and CYP82C2 are dispensable for the preinvasive defense to *Ctro*

PEN2-derived indoleglucosinolate is one of the Trp-derived metabolites and is involved in the preinvasive resistance to fungal pathogens including *Ctro* (Lipka *et al.,* 2005; Hiruma *et al.,* 2010; 2013). Here the study evaluated the contribution of CYP71A12-derived metabolites, camalexin, ICAs and 4-OH-ICN, to the preinvasive defense against *Ctro*. *Ctro* was inoculated onto series of *Arabidopsis* mutants, the *pen2* mutant, the *pad3* mutant, the *cyp82C2* mutant, and the *cyp71A12 cyp71A13* double mutant. As a result, in contrast to the *pen2* mutant, none of the tested mutants showed lesion development, suggesting that *PAD3, CYP82C2*, *CYP71A12,* and *CYP71A13* are

dispensable for preinvasive defense to *Ctro* (Fig. I-5). This idea was further supported by the microscopic observation of *Ctro* entry in each *Arabidoipsis* line, i.e., *Ctro* formed invasive hyphae in the *pen2* mutants effectively, but did not entry in the other mutant lines (Fig. I-5).

Invasion by *Ctro* triggered the expression of *CYP71A12*, *PAD3* and *CYP82C2*

Since *CYP71A12*, *PAD3* and *CYP82C2* are dispensable for the entry control of *Ctro* (Fig. I-5), it was assumed that mentioned genes are activated after the pathogen entry. To asses this point, the expression of *CYP71A12*, *PAD3* and *CYP82C2* was investigated in WT (Col-0) plants and *pen2* plants when *Ctro* was inoculated. In contrast to the *pen2* mutant plant, the WT plants exhibit preinvasive defense, thus *Ctro* is not able to invade. As a result, the tested genes were strongly induced in the *pen2* plants after *Ctro* inoculation. In contrast, this induced expression was not observed in the WT plants (Fig. I-6). I also found higher expression of the tested genes at 48 hpi than at 24 hpi, it might be due to the increased invasion of *Ctro* at later time point (Fig. I-6).

CYP71A12 is important for the *Ctro*-triggered accumulation of ICAs

Previous study using *cyp71A12* single mutant suggested that CYP71A12 has a contribution to the accumulation of ICAs in response to UV, AgNO3 and pathogen (Müller *et al.,* 2015; Rajniak *et al.,* 2015). Additionally, the study with *cyp71A12 cyp71A13* double mutant indicated the minor contribution of CYP71A13 towards the UV-treatment (Müller *et al.,* 2015). Here, the contribution of CYP71A12 and CYP71A13 for the accumulation of ICAs in response to *Ctro* was studied. For this propose, the accumulation of ICAs was investigated in WT (Col-0), the *pen2* mutant, the *pen2 pad3* mutant, the *pen2 cyp71A12* mutant and the *pen2 cyp71A12 cyp71A13* mutant at 48 and 96 hours after the inoculation of *Ctro*. The result showed significant reduction of ICAs accumulation in the *pen2 cyp71A12* mutant in comparison with the *pen2* mutant (Fig. I-7). However, the accumulation level of camalexin in *pen2 cyp71A12* was comparable to

pen2 (Fig. I-7). *Ctro*-triggered accumulation of ICAs in the *pen2 cyp71A12 cy71A13* triple mutant was comparable to those accumulated in *pen2 cyp71A12*, suggesting that CYP71A13 has no clear contribution to *Ctro*-triggered accumulation of ICAs (Fig. I-7). As for the accumulation of camalexin, the *pen2 cyp71A12* mutant phenotype is comparable to the *pen2* phenotype. However, the *pen2 cyp71A12 cyp71A13* mutant drastically reduced the accumulation of camalexin upon *Ctro* inoculation, which is reminiscent of the *pen2 pad3* mutant, suggesting the importance of CYP71A13 for camalexin biosynthesis in response to *Ctro* invasion (Fig. I-7).

RAR1, *SGT1*, *PAD4* and *SAG101* are dispensable for the postinvasive resistance against *Ctro*

Plant utilizes (i) transmembrane proteins containing leucine rich repeat domain (LRR) for the recognition of pathogen external molecules and (ii) cytoplasmic resident nucleotide-binding (NB) LRR sensors (NLRs) for the recognition of intracellular pathogen molecules (Jones and Dangl, 2006). REQUIRED FOR MLA12 RESISTANCE 1 (RAR1) and SUPPRESSOR OF THE G2 ALLELE OF SKP1 (SGT1, also known as EDM1, enhanced downy mildew) are essential for the stabilization of NLR proteins and required for the proper function of several resistance (*R*) genes that encode NLRs in plants (Shirasu *et al.,* 2009). *Arabidopsis* mutants defective in *RAR1* or *SGT1* are more susceptible to pathogen having particular avirulence effectors because of the instability of corresponding NLRs (Peart *et al.,* 2002; Holt *et al.,* 2005; Jarosch *et al.,* 2005) and fail to activate hypersensitive response (HR). When postinvasive resistance is activated in *Arabidopsis* by *Ctro* entry, HR-related cell death is detected around pathogen entry sites (Hiruma *et al.,* 2013). Here, to investigate the possible contribution of NLRs for the postinvasive resistance to *Ctro*, *Ctro* was inoculated onto the *pen2 rar1* mutant and *pen2 sgt1* (*pen2 edm1*) mutant plants. As a result, I did not find enhanced lesion development in *pen2 rar1* and *pen2 sgt1* compared with *pen2* (Fig. I-8). *Ctro* hyphal expansion from the inoculated area was also determined. The hyphal expansion in both tested double

mutants was comparable to that in the *pen2* mutant (Fig. I-8B). Also, it was likely that HR cell death was not canceled in *pen2 rar1* and *pen2 sgt1*. These results indicate that *RAR1* and *SGT1* are not essential for *Arabidopsis* postinvasive defense to *Ctro*, suggesting no involvement of NLR machineries in this second layered defense (Fig. I-8).

In *Arabidopsis*-powdery mildews fungi interaction, PEN2 is required for the preinvasive defense, and the postinvasive defense is associated with HR cell death (Lipka *et al*., 2005). In this plant-pathogen interaction, this HR cell death mainly depends on two lipase-like proteins, phytoalexin deficient 4 (PAD4) and senescence associated gene 101 (SAG101) (Lipka *et al*., 2005). Thus, I also investigated the involvement of *PAD4* and *SAG101* in postinvasive defense to *Ctro*. As a result, I found that *Ctro* hyphae were not expanded from the inoculated area in the *pen2 pad4 sag101* triple mutant, showing no difference from *pen2* (Fig. I-8). The result suggested that PAD4 and SAG101 are dispensable for postinvasive defense to *Ctro*.

DISCUSSION

Different susceptibility between the *pen2* mutant and the *pen2 pad3* mutant indicated the contribution of camalexin to the immunity to *Ctro* (Hiruma *et al*., 2013). Also, higher susceptibility of the *cyp79B2 cyp79B3* mutant than the *pen2 pad3* mutant strongly suggested the requirement of additional Trp-derived metabolites for the postinvasive defense against *Ctro* (Hiruma *et al*., 2013). In this chapter, I investigated the relation of Trp-derived metabolites to the postinvasive defense against *Ctro*, especially focusing on the identification of additional Trp-derived metabolites required for this second layered defense. For this purpose, I performed *Ctro* inoculation and subsequent microscopic observation of hyphal expansion by *Ctro* toward series of *Arabidopsis* mutants defective in Trp-metabolism pathway. The assay revealed that, in addition to *PAD3*, *CYP71A12* is also involved in the postinvasive defense to *Ctro*. The *pen2 pad3* mutant and the *pen2 cyp71A12* mutant showed comparable level of susceptibility towards *Ctro*, indicating that *PAD3* and *CAYP71A12* independently contribute to this defense.

Further study focused on investigating the contribution of CYP71A12 and CYP71A13 to the accumulation of ICAs upon *Ctro* invasion. CYP71A12 and CYP71A13 shows highly similarity sharing 89% identity on the amino acids level, and both genes are located as tandem copies on chromosome 2 (Nafisi *et al*., 2007). The analysis on the accumulation of ICAs (6-OGlc-indole-3-carboxylic acid and indole-3-carboxylic acid glucose ester) upon *Ctro* invasion in the *cyp71A12* mutant, the *cyp71A13* mutant and the *cyp71A12 cyp71A13* double mutant suggested the contribution of CYP71A12 but not CYP71A13 to biosynthesis of ICAs (Fig. I-7). The findings suggested that, despite highly similarities of CYP71A12 and CYP71A13, these monooxygenases showed different contribution to the biosynthesis of ICAs in response to pathogen entry. This difference might result from their different subcellular colocalization and enzymatic activity, or different ability to form complexes with the downstream enzymes involved in the synthesis of ICAs.

Also, the analysis of camalexin accumulation upon *Ctro* invasion in the *cyp71A12* mutant, the *cyp71A13* mutant and the *cyp71A12 cyp71A13* double mutant showed the contribution of CYP71A13, but not CYP71A12, to the accumulation of camalexin when *Ctro* invaded. Consistent with this, *CYP71A13* was reported to be highly transcriptionally co-regulated with *CYP71B15/PAD3* (Nafisi *et al.,* 2007). Furthermore, *CYP71A13* is involved in camalexin biosynthesis in response to multiple pathogen such as *Alternaria brassicicola* (necrotrophic fungi) and *Pseudomonas syringae* (biotrophic bacteria) (Nafisi *et al.,* 2007; Müller *et al.,* 2015). These results suggest the importance of *CYP71A13* for camalexin biosynthesis activated by pathogen infection.

The analysis of CYP71A12-deficient mutants proved that this monooxygenase has an important function in *Arabidopsis* postinvasive resistance against *Ctro*. Infection phenotypes observed in the *cyp82C2* lines indicated that a defect in synthesis of 4-OH-ICN is not related to a defect of the CYP71A12-deficient mutants in postinvasive resistance against *Ctro*. For this reason, it is very likely that ICAs contribute to *Arabidopsis* postinvasive immunity towards *Ctro*. However, the roles of ICAs for this resistance remains obscure, because in vitro assays show no direct antifungal activity of ICAs against *P. cucumerina* hyphae development (Gamir *et al.,* 2014). Recent published results indicated that exogenously applied ICA induces abscisic acid (ABA)-dependent callose deposition upon *P. cucumerina* infection (Gamir *et al.,* 2018). For this reason, it was postulated that ICAs might function as regulatory or signaling molecules in *Arabidopsis* immunity. However, even if this is the case the importance of different substitutions observed within identified ICA derivatives remains obscure (Böttcher *et al.,* 2014). For instance, IG function in preinvasive resistance and in aphid deterrence requires substitution at the position 4 of the indole ring (Bednarek *et al.,* 2009; Pfalz *et al.,* 2009), suggesting that particular substitution(s) of ICA could be also of functional significance. Moreover, it is difficult to predict whether ICA role is linked with the soluble or with the cell wall-bound fraction of these compounds (Hagemeier *et al.,* 2001; Tan *et al.,* 2004).

These questions could be only answered if enzymes involved in ICA metabolism and extracellular transport are identified.

The postinvasive resistance to *Ctro* was associated with plant cell death similar to HR cell death (Fig. I-8). To assess whether plant cell death observed in postinvasive resistance to *Ctro* is mediated by NLR, I utilized mutations in *RAR1* and *SGT1*. RAR1 interacts with SGT1 and forms complexes with NLR and contributes for the stability of NLR (Shirasu *et al*., 2009). I detected strong cell death responses in the *pen2 rar1* mutant and the *pen2 sgt1* mutant similar to the *pen2* single mutant when *Ctro* was inoculated. Furthermore, fungal hyphae were not expanded form the inoculated area in these double mutants, indicating that *RAR1* and *SGT1* have no clear contribution for postinvasive resistance and HR-like cell death in response to the invasion by *Ctro*. I also found that PAD4 and SAG101 were not essential for postinvasive resistance to *Ctro*. These results suggest that HR-like cell death triggered by *Ctro* invasion is unlikely mediated by NLR machineries (Fig. I-8).

MATERIALS AND METHODS

Fungal material

C. tropicale (*Ctro*) (formerly *Colletotrichum gleosporioides*) S9275 was provided by Shigenobu Yoshida (National Institute for Agro-Environmental Sciences, Japan). *Ctro* was cultured on 2.5% (w/v) PDA (Difco, Detroit, MI, USA) at 24˚C under a cycle of 16 h black light (FS20S/BLB 20W; Toshiba, Tokyo, Japan) illumination and 8 h dark.

Arabidopsis lines and growth conditions

The *A. thaliana* accession Col-0 was used as the WT plant. The mutants *pen2-1*, *pen2-2* (Lipka *et al.,* 2005), *pad3-1* (Glazebrook and Ausubel, 1994), *cyp71A12*, *cyp71A12*/*cyp71A13* (Müller *et al.,* 2015), *cyp82C2* (Rajniak *et al.,* 2015), *cyp79B2 cyp79B3* (Zhao *et al.,* 2002), *pen2-1 pad3-1* (Bednarek *et al.,* 2009), *pen2 rar1* (Maeda *et al.,* 2010), *pen2 sgt1* (Maeda *et al.,* 2010) and *pen2 pad4 sag101* (Lipka *et al.,* 2005) were used in this study. *pen2 cyp82C2*, *pen2 cyp71A12* and *pen2 cyp71A12 cyp71A13* were obtained from Dr. Paweł Bednarek as collaboration study. *Arabidopsis* seeds were sown on rockwool (Grodan; http://www.grodan.com) and kept at 4 °C in the dark for 2 days, and later grown at 25 °C with a cycle of 16 h light and 8 h dark in Hoagland medium.

Pathogen inoculation and trypan blue staining assay

To investigate the contribution to postinvasive defense, *Ctro* hyphal expansion from the inoculated area was determined. For this assay, *Ctro* conidial suspension (2.5 x 10^5 conidia/mL with 0.1% Glc) was inoculated onto 4 to 5-week-old *Arabidopsis* plants and the inoculated plants were kept at 100% relative humidity. At 4 days postinoculation (dpi), the inoculated leaves were collected and stained with Trypan blue solution according to Koch and Slusarenko (1990). For the trypan blue assay, at least 50 lesions were investigated in each experiment. The *Ctro* invasion ratio (%) was calculated by using the following formula: *Ctro* invasion ratio (%) = (number of germinating conidia that

developed invasive hyphae / number of germinated conidia) x 100. *Arabidopsis* lines used in this study was listed on Table I-2.

RT–qPCR analysis

Seven *Arabidopsis* leaves inoculated with *Ctro* (5 x 10^5 conidia/mL) were collected from each of seven different plants of either WT Col-0 or tested mutant plants at corresponding time points. Total RNA was extracted using PureLink (TRIzol plus RNA purification kits, Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA) and treated with DNase (RQ1 RNase-free DNase; Promega, Madison, WI, USA; http://www.promega.com) to remove DNA contamination. Takara Prime Script™ RT kits (Takara Bio Inc., Shiga, Japan; http://www.takara-bio.com) was used for the cDNA synthesis. Takara TB Green™ Premix Ex Taq™ I was used for RT–qPCR with the primers listed in Table I-1. *Arabidopsis UBC21* (At5g25760) was used as an internal control for normalizing the level of cDNA (Czechowski *et al.,* 2005). RT–qPCR analysis was performed using a Thermal Cycler Dice Real Time System TP800 (Takara). The expression levels of genes of interest were normalized relative to those of *UBC21*. Relative expression (log2) was calculated by subtracting Ct values of genes of interest from those of *UBC21*. Fold change was based on values of relative expression (log2), which was calculated by two to the power of relative expression (log2). For the statistical analysis, relative expression values (in log2 scale) were used, whereas the graphs of the RT–qPCR analysis in figures were represented using fold change values (for easy observation).

Metabolite analysis

Conidial suspensions (1 x 10^6 conidia/mL with 0.1% Glc) of *Ctro* were spray-inoculated onto 4–5-week-old plants and kept at 100% relative humidity. Leaf samples (100–200 mg fresh weight) were collected at corresponding time points and frozen immediately in

liquid nitrogen. The plant extracts containing Trp derivatives were extracted using DMSO and metabolite analyses were performed as described (Bednarek *et al*., 2009)

Fig. I-1. Enhanced susceptibility of *Arabidopsis pen2 pad3* and *cyp79B2 cyp79B3* mutants towards *Ctro*.

Ctro was inoculated with 0.1% Glc on tested *Arabidopsis* plants. Enhanced lesion development was observed in *Arabidopsis* mutants *pen2 pad3* (with defect in camalexin) and *cyp79B2 cyp79B3* (with defect in all Trp-derived metabolites) in comparison with the *pen2* mutant. The photo was taken at 4 dpi.

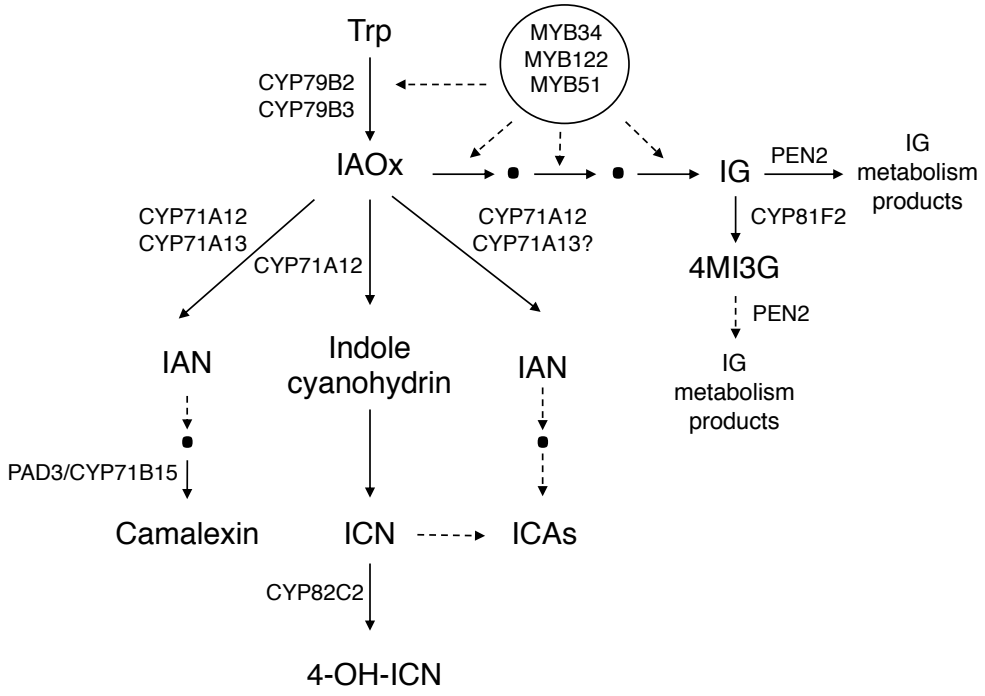

Fig. I-2. Simplified scheme of tryptophan metabolism pathways.
Trp-derived metabolites are synthesized from a common amino acid tryptophan (Trp). First step is the synthesis of a common precursor named indole-3-aldoxime (IAOx) via monooxygenases CYP79B2 and CYP79B3. From this precursor, camalexin (via CYP71A12/CYP71A13 and PAD3), 4-hydroxy-ICN (4-OH-ICN) (via CYP71A12 and CYP82C2), indole-3-carboxylic acid and derivatives (ICAs) (via CYP71A12 mainly) and 4-methoxyindol-3-ylmethylglucosinolate (4MI3G) (via CYP81F2) are produced. Subsequently, 4MI3G are subjected to PEN2 to produce unidentified indole glucosinolate (IG) metabolism products. IAN, indole-3-acetonictrile.

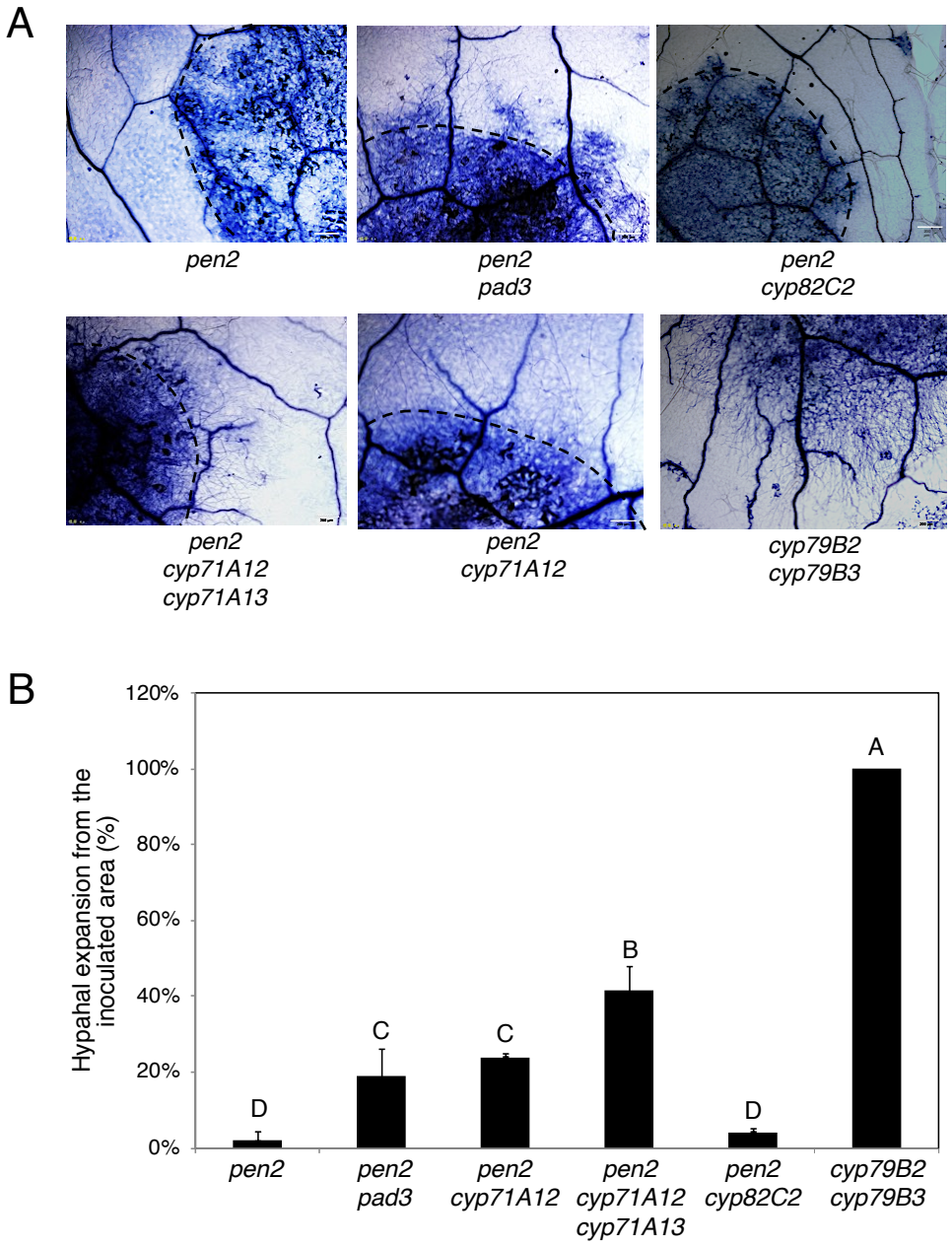

Fig. I-3. Trp-derived secondary metabolites, camalexin and ICAs, are required for postinvasive defense against *Ctro*.

(A) *Ctro* hyphal expansion in the Trp-pathway defective mutants. *Ctro* was inoculated with 0.1% Glc on the tested plants. The inoculated leaves were collected at 4 dpi, and after the staining with trypan blue solution, *Ctro* hyphal expansion was observed under light microscopy. The area out of the dot line indicates the area outside of the inoculation. Bars indicate 200 μm.

(B) Quantitative analysis of *Ctro* hyphal expansion. *Ctro*-inoculated leaves were collected at 4 dpi and stained with trypan blue solution. The *Ctro* hyphae was observed under light microscopy. The statistical significance of differences between means was determined by Tukey's honestly significant difference (HSD) test. Means not sharing the same letter are significantly different ($P < 0.01$).

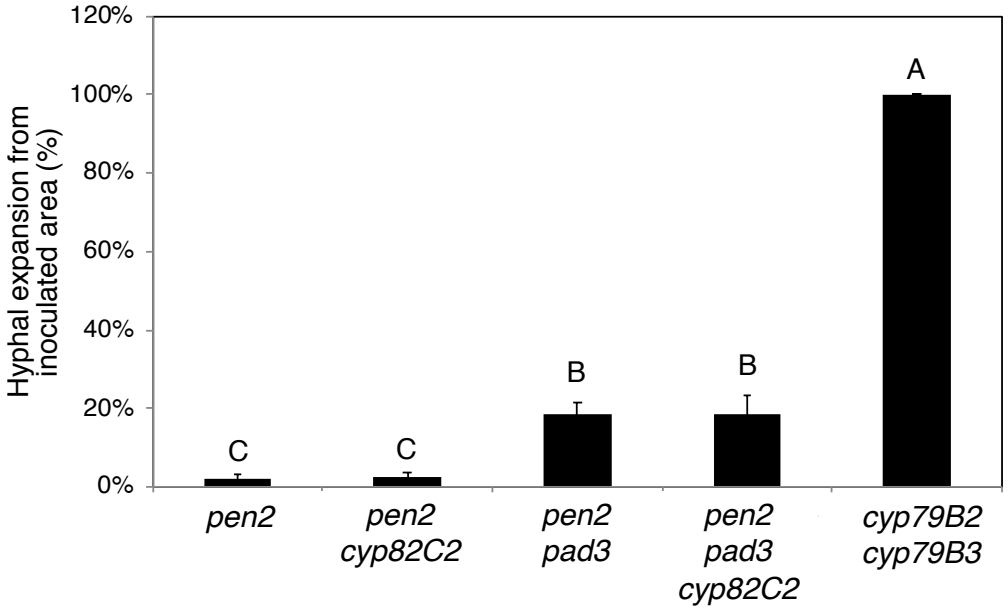

Fig. I-4. CYP82C2-dependent biosynthesis of 4-OH-ICN is dispensable for postinvasive defense to *Ctro* even in the absence of camalexin.

Quantitative analysis of *Ctro* hyphal expansion. *Ctro*-inoculated leaves were collected at 4 dpi and stained with trypan blue solution. The *Ctro* hyphae was observed under light microscopy. The statistical significance of differences between means was determined by Tukey's honestly significant difference (HSD) test. Means not sharing the same letter are significantly different ($P < 0.01$).

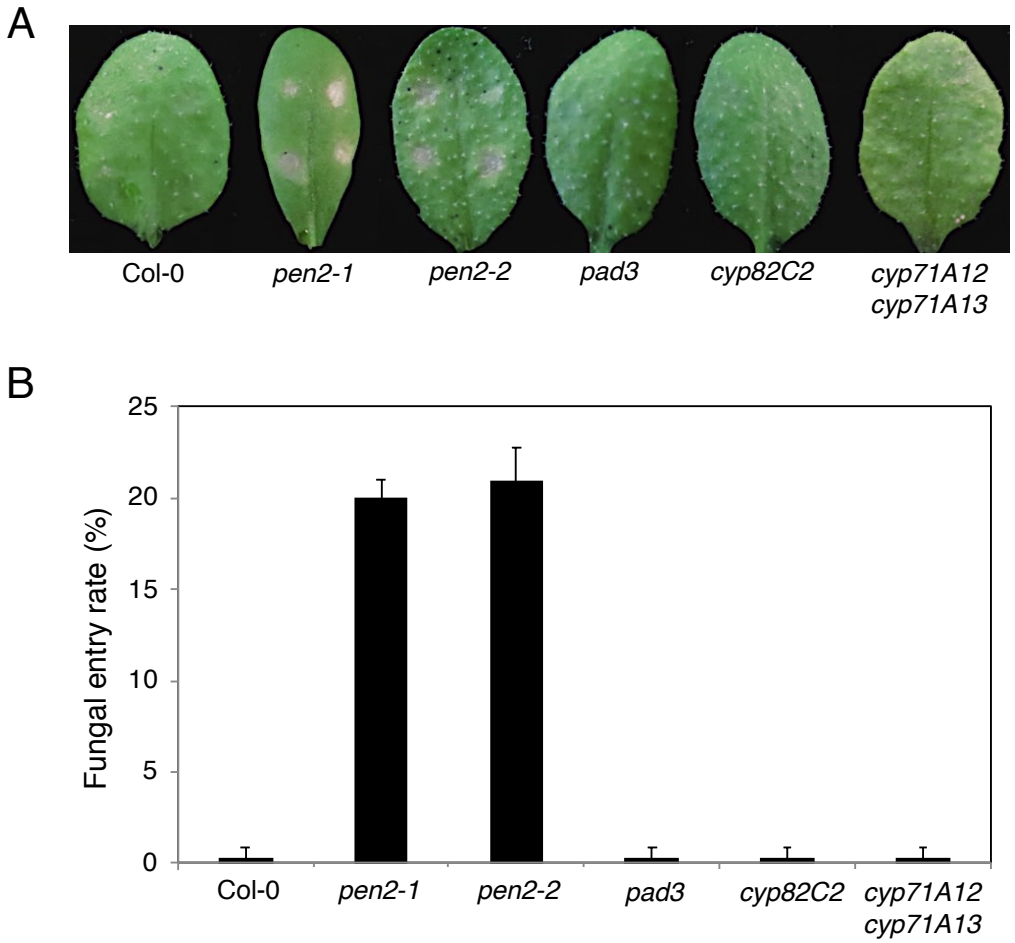

Fig. I-5. Camalexin and ICAs are not required for the entry control (preinvasive defense) to *Ctro*.

(A) Lesion development caused by *Ctro*. *Ctro* (2.5 x 10^5 conidia/mL with 0.1% Glc) was inoculated onto 4 to 5-week-old plant. The photograph was taken at 4 dpi.

(B) Analysis of *Ctro* entry rate. Aliquots (5 μL each) of *Ctro* conidial suspension with 0.1% Glc were drop-inoculated onto *Arabidopsis* cotyledon. At 14 hpi, the fungal entry rate was determined by using light microscopy. At least 100 germinating conidia were counted in each experiment. The means and SDs were calculated from three independent experiments.

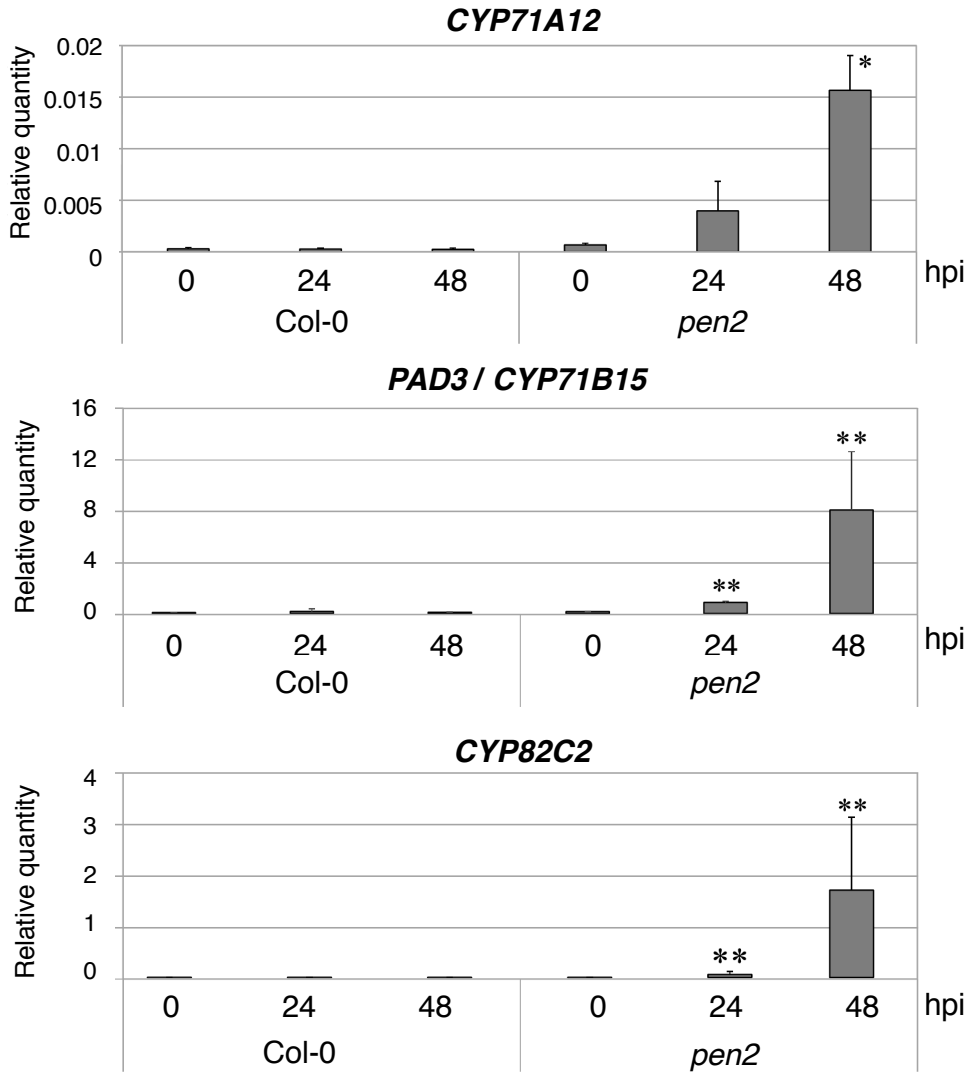

Fig. I-6. The invasion of *Ctro* activates the expression of *CYP71A12*, *PAD3* and *CYP82C2*.

Conidial suspension of *Ctro* ($5x10^5$ conidia/mL with 0.1% Glc) was spray-inoculated onto 4 to 5-week-old *Arabidopsis* plant. The leaf sample were collected at 0, 24 and 48 hours post inoculation (hpi). Each gene transcript was quantified by quantitative polymerase chain reaction (qPCR) using the gene specific primers listed in Table I-1. Values were normalized to the expression level of *UBC21*. The means and SDs were calculated from three independent experiments. For statistical analysis, 0 hpi samples were compared with 24 and 48 hpi samples using two-tailed Student's *t* tests (*$P < 0.05$ and **$P < 0.01$).

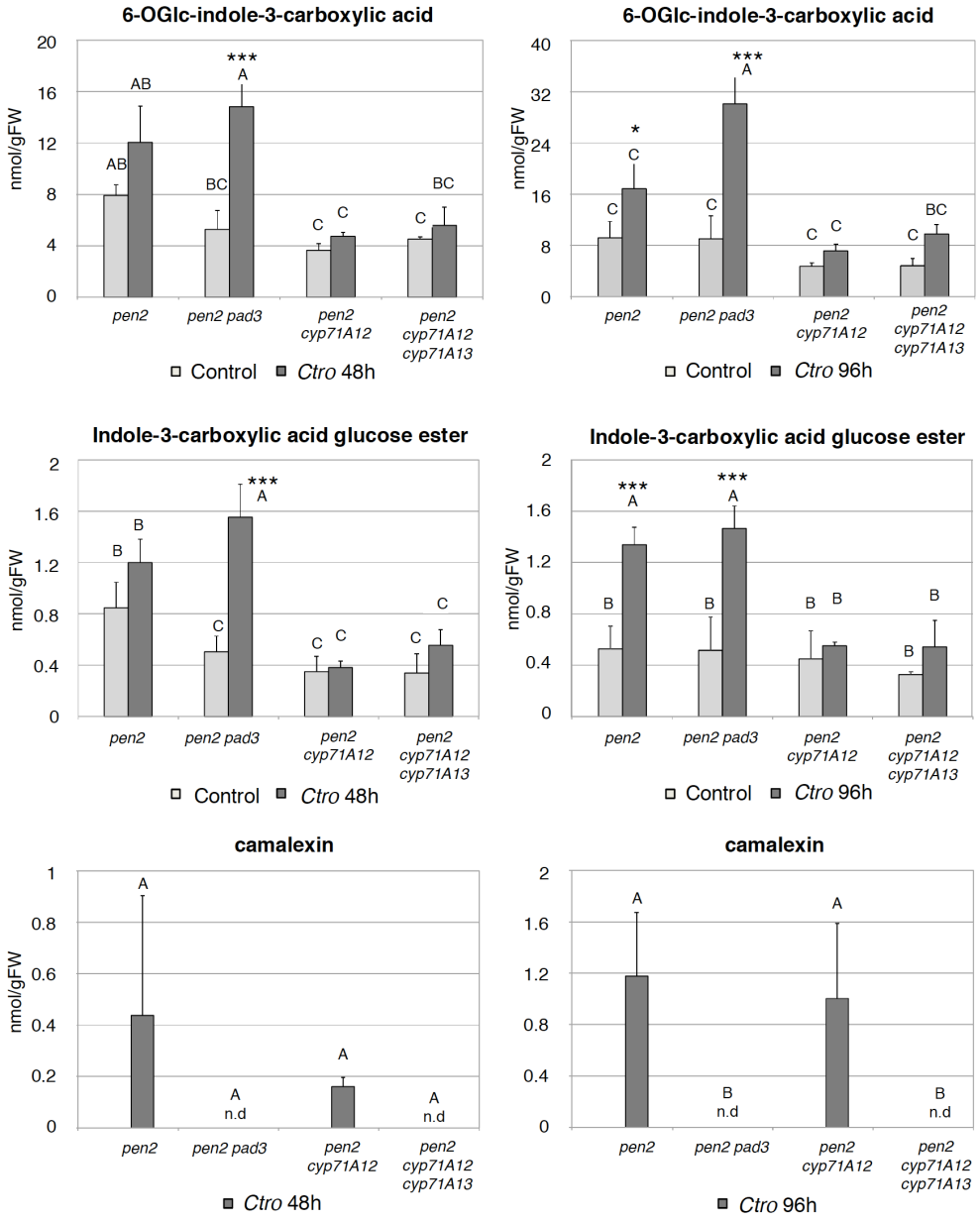

Fig. I-7. CYP71A12 and CYP71A13 contribute differentially to the biosynthesis of indole-3-carboxylic acid derivatives and camalexin in response to *Ctro* invasion.
Accumulation of 6-OGlc-indole-3-carboxylic acid, indole-3-carboxylic acid glucose ester and camalexin was determined in tested mutant lines at 48 and 96 hpi of *Ctro* inoculation. Results are means ± SD from two independent experiments with four biological replicates in each (n=8). Significantly different statistical groups indicated by the two-way analyses of variance (ANOVA) followed by post hoc Tukey's honest significance test are shown with different letters. Values marked with asterisks are significantly different from respective controls (* $P < 0.05$; *** $P < 0.0001$). FW, fresh weight

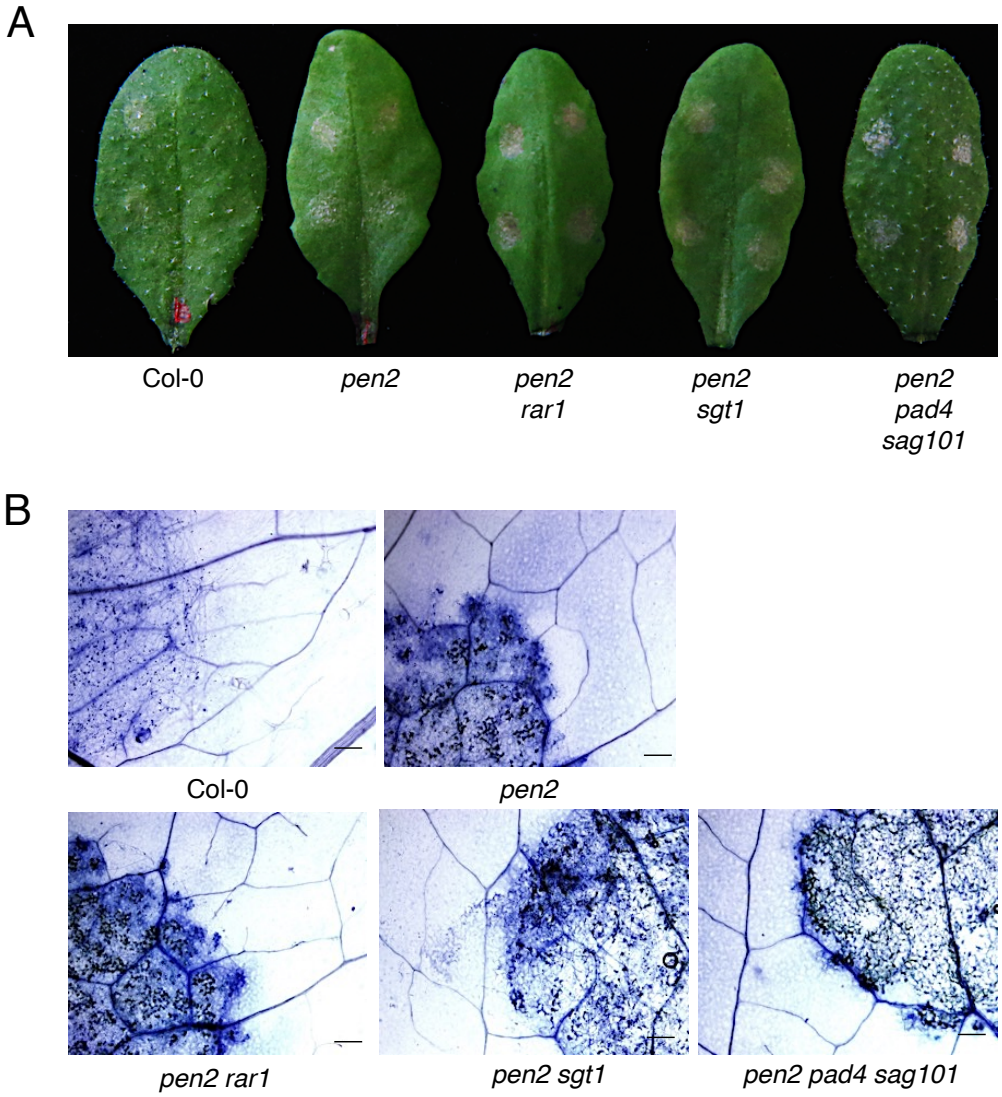

Fig. I-8. *RAR1* and *SGT1* are dispensable for the postinvasive defense to *Ctro*.
(A) Lesion development caused by *Ctro*. Conidial suspension of *Ctro* ($5x10^5$ conidia/mL, with 0.1% Glc) were drop-inoculated onto 4 to 5-week-old plant. The picture was taken at 4 dpi. Lesion development by *Ctro* in all tested mutants was comparable to that in *pen2*. **(B)** Observation on *Ctro* hyphal expansion. *Ctro*-inoculated leaves were collected at 4 dpi and stained with trypan blue solution. The *Ctro* hyphae was observed under light microscopy. The hyphal expansion on tested mutants were similar to the hyphal expansion observed on *pen2* mutant. Bars indicate 200 μm.

Table I-1. List of primers used in this study.

Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
CYP71A12 (At2g30750)	CATTCCCTAAGCCTTCGGTAC	CTTGGAGTTTCTTCATAACA
CYP82C2 (At4g31970)	CATTTGGTTCGGGAAGAAGA	AGCCAGGGCTCTCAGTCATA
PAD3 (At3g26830)	TGCTCCCAAGACAGACAATG	GTTTTGGATCACGACCCATC
UBC (At5g25760)	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC

Table I-2. List of *Arabidopsis* lines used in this study

Arabidopsis thaliana line	Reference
Col-0 (WT)	
pen2-1	Lipka *et al.,* 2005
pen2-2	Lipka *et al.,* 2005
pad3-1	Glazebrook and Ausubel, 1994
cyp71A12	Müller *et al.,* 2015
cyp82C2	Rajniak *et al.,* 2015
cyp71A12 cyp71A13	Müller *et al.,* 2015
cyp79B2 cyp79B3	Zhao *et al.,* 2002
pen2 pad3	Bednarek *et al.,* 2009
pen2 cyp82C2	Obtained from Dr. Paweł Bednarek as collaboration study
pen2 cyp71A12	
pen2 cyp71A12 cyp71A13	
pen2 rar1	Maeda *et al.,* 2010
pen2 sgt1 (*pen2 edm1*)	Maeda *et al.,* 2010
pen2 pad4 sag101	Lipka *et al.,* 2005

CHAPTER II

bak1–5 mutation uncouples tryptophan-dependent and independent postinvasive immune pathways triggered in *Arabidopsis thaliana* by multiple fungal pathogens

INTRODUCTION

In Chapter I, I have shown that CYP71A12 has an important contribution to the accumulation of ICAs upon the *Ctro* infection. On the other hand, the loss of *CYP71A13* reduced camalexin accumulation upon infection by multiple pathogens (Nafisi *et al.,* 2007), whereas I have revealed that a single loss of *CYP71A12* did not reduce camalexin accumulation upon infection with *Ctro*. These findings suggest distinct roles of these two homologous P450 monooxygenases in the immune responses. Importantly, I also revealed that the *pen2 cyp71A12* double mutant exhibits a partial reduction in postinvasive resistance to *Ctro*, which was similar to that of the *pen2 pad3* plants. Furthermore, the *pen2 cyp71A12 cyp71A13* plants exhibited enhanced susceptibility compared with the *pen2 pad3* and *pen2 cyp71A12* plants. These findings suggest that CYP71A12-dependent synthesis of ICAs as well as camalexin synthesis is critical for postinvasive resistance to *Ctro*. It was reported that PAD3-dependent camalexin synthesis is involved in the immunity of *Arabidopsis* to multiple fungal pathogens, indicating the common roles of camalexin for antifungal defense, including postinvasive defense, in this plant species (Thomma *et al.,* 1999; Sellam *et al.,* 2007; Schlaeppi *et al.,* 2010; Hiruma *et al.,* 2013). However, it remains unclear whether CYP71A12 and ICAs are commonly involved in the postinvasive resistance to fungal pathogens other than *Ctro*.

In this chapter, I first investigated whether CYP71A12-dependent synthesis of ICAs might be involved in postinvasive resistance to a necrotrophic fungal pathogen *Alternaria brassicicola* (hereafter called *Ab*) that infects Brassicaceae plants. I found that invasion by *Ab* triggers the CYP71A12-dependent accumulation of ICAs as well as camalexin,

and that postinvasive resistance to *Ab* needs both CYP71A12 and PAD3. I found the same situation in terms of postinvasive resistance to the adapted hemibiotrophic fungus *Colletotrichum higginsianum* (hereafter called *Ch*), suggesting common roles of CYP71A12 and ICAs for this invasion-triggered defense against diverse fungal pathogens with distinct infection modes. I also found that PEN2 is not essential for the immunity of *Arabidopsis* to *Ab*, in contrast to *Ctro* and *Ch* (Hiruma *et al.,* 2010, 2011). Thus, *Arabidopsis* utilizes conserved mechanisms for postinvasive resistance against diverse fungal pathogens, whereas it has developed various ways to control the entry of different fungal pathogens. Subsequently, I investigated how *Arabidopsis* recognizes pathogen invasions to trigger postinvasive resistance. I assessed a possible involvement of pattern recognition-receptor (PRR) complexes by focusing on BAK1 (BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1), which is known to act as a co-receptor with multiple PRRs, including FLS2 and EFR, via ligand-induced heteromerization (Chinchilla *et al.,* 2007; Heese *et al.,* 2007; Roux *et al.,* 2011; Ma *et al.,* 2016). BAK1 was initially identified as a positive regulator of the brassinosteroid response (BR) (Li *et al.,* 2002; Nam and Li, 2002). Therefore, the *bak1* null mutants are not only defective in FLS2 and EFR-dependent immune responses, but also hyposensitive to BR. In contrast to the null allele, the *bak1–5* allele is impaired in pathogen-associated molecular pattern (PAMP)-triggered immunity, but not in BR signaling (Schwessinger *et al.,* 2011). I found that the *bak1–5* mutation reduced postinvasive resistance to *Ab*, revealing the involvement of a PRR system in the recognition of pathogen invasion for the activation of defense. Surprisingly, I found that the *bak1–5* mutation had no negative impacts on the invasion-triggered activation of camalexin or ICA biosynthesis, suggesting that the corresponding metabolic pathways are controlled by an unknown mechanism that (i) senses pathogen invasion, and (ii) is insensitive to the *bak1–5* mutation. I also showed that the pathway sensitive to *bak1–5* activates the expression of distinct defense-related genes such as *GLIP1* (*GDSL LIPASE1*) encoding a secreted protein, which was previously shown to be involved in

Arabidopsis immunity to *Ab* (Oh *et al.,* 2005; Kwon *et al.,* 2009). Notably, the activation of the Trp-related pathway in the postinvasive resistance against *Ctro* as well as *Ab* is also not canceled by the *bak1–5* mutation.

RESULTS

CYP71A12 contributes to the immunity of *Arabidopsis* against the necrotrophic pathogen *Alternaria brassicicola* independently of CYP71A13 and PAD3

Ab is a necrotrophic fungal pathogen that infects several Brassicaceae spp., including cabbage and canola. It was reported that the inoculation with several *Ab* strains, including the strain Ryo-1 used in this study, produced lesions in the wild-type (WT) of *A. thaliana*, Col-0. However, the lesions developed by the *Ab*-infected strains did not expand, indicating that the WT plant is able to activate immunity to *Ab* (Thomma *et al.,* 1999; Narusaka *et al.,* 2003; Hiruma *et al,* 2011). Here, to investigate the roles of Trp metabolism for the immunity of *Arabidopsis* against *Ab*, the strain Ryo-1 of *Ab* was inoculated onto a series of *Arabidopsis* mutants related to Trp metabolism: *pen2*, *pen2 pad3*, *pen2 cyp82C2*, *pen2 cyp71A12*, *pen2 cyp71A12 cyp71A13*, and *cyp79B2 cyp79B3*. At 4 days postinoculation (dpi), lesion development was evaluated. It was first found that lesion development in the *pen2* mutant was not significantly different from that in the WT Col-0 plant (Fig. II-1), suggesting that PEN2 has no detectable contribution to the immunity of *Arabidopsis* against *Ab*, whereas PEN2 is known to be critical for preinvasive resistance toward multiple fungal pathogens, especially those nonadapted to *Arabidopsis* (Lipka *et al.,* 2005; Hiruma *et al.,* 2010). It was also found that both the *pad3* and *pen2 pad3* mutants increased susceptibility to the *Ab* strain Ryo-1 compared with the WT plant (Fig. II-1 and Fig. II-3), indicating the importance of PAD3-dependent camalexin synthesis, consistent with previous reports on *pad3* susceptibility toward different *Ab* strains (Thomma *et al.,* 1999; Narusaka *et al.,* 2003) and other fungal pathogens (Schlaeppi *et al.,* 2010; Hiruma *et al.,* 2013). The assay results also suggested that CYP82C2 is involved in the immunity to the *Ab* strain Ryo-1 (Fig. II-1 and Fig. II-3), consistent with a previous report (Rajniak *et al.,* 2015). However, lesion development in the *pen2 cyp82C2* mutant was less severe than in the *pen2 pad3* mutant, suggesting a relatively minor contribution of CYP82C2 to *Arabidopsis* immunity to *Ab*.

Finally, it was unable to perform quantitative analysis on lesion development in the *cyp79B2 cyp79B3* mutant at 4 dpi because the lesions had already merged. However, quantitative analysis at 3 dpi revealed that the *cyp79B2 cyp79B3* mutant exhibited the most severe phenotype against *Ab* compared with the other tested *Arabidopsis* lines, including the *pen2 cyp71A12 cyp71A13* triple mutant (Fig. II-4).

Biosynthesis of ICAs and camalexin occurs during postinvasive resistance toward *Ab*

In the case of the inoculation assay with the nonadapted pathogen *Ctro*, the expression of *CYP71A12* and *PAD3* was strongly induced in the *pen2* mutant, but not in the WT Col-0 (Fig. I-6). Because PEN2 is critical for preinvasive resistance against *Ctro* (Hiruma *et al*., 2010; 2011), the result indicated the importance of *CYP71A12* and *PAD3* for postinvasive resistance against such fungal invasion. To assess whether *CYP71A12* and *PAD3* are expressed during postinvasive resistance against *Ab*, similar as against *Ctro*, it was first investigated the expression pattern of these genes at several time points after *Ab* inoculation (at 4, 12, 24, and 48 h postinoculation, hpi). To compare these precisely with present findings on postinvasive resistance against *Ctro* (Fig. I-3), hereafter it was mainly used mutants with the *pen2* background in this study focusing on immunity to *Ab*, although the lesion development assay revealed that PEN2 is not essential for this (Fig. II-1). It was found that the expression of *CYP71A12* and *PAD3* started to be induced at 12 hpi, and these expression levels were elevated at later time points (Fig. II-2A). By contrast, it was not detected any induction at 4 hpi (Fig. II-2A). In parallel, it was also investigated the temporal infection behavior of *Ab* on *Arabidopsis*. The conidia of *Ab* had already germinated on *Arabidopsis* at 4 hpi, however, it was not detected any host invasion at this time (Fig. II-2B, C). By contrast, it was found that *Ab* started to invade the plants at 12 hpi and the invasion ratio was elevated at later time points (Fig. II-2B, C). These findings indicate a link between *CYP71A12* and *PAD3* induction and the initiation of host invasion in the *Ab-Arabidopsis* interactions, strongly suggesting that the

expressions of *CYP71A12* and *PAD3* are triggered by *Ab* invasion. Furthermore, it was also revealed that simultaneous loss of both *CYP71A12* and *CYP71A13* produced no detectable effects on the preinvasive resistance against *Ab* (Fig. II-2D), suggesting that *CYP71A12* and *CYP71A13* are involved in the postinvasive resistance against *Ab*. It was also found that PEN2 is dispensable for the preinvasive resistance against *Ab* (Fig. II-2D), in contrast to *Ctro* (Hiruma *et al*., 2010).

Then, it was assessed whether present observations on the gene expression level could be supported with metabolite profiles. The result showed the pathogen-induced accumulation of two ICAs, glucoside of 6-hydroxy-indole-3-carboxylic acid and glucose ester of indole-3-carboxylic acid, was detected at 24 hpi, but not at 4 hpi of *Ab* (Fig. II-5A and Fig. II-6), which matched strongly with the gene expression data (Fig. II-2A). Similar results were also obtained in the analysis of camalexin accumulation (Fig. II-5A). Therefore, it was concluded that the synthesis of ICAs and camalexin is triggered by *Ab* invasion.

Reduced accumulation of ICAs correlates with the breakdown of the postinvasive resistance toward *Ab*

The results revealed that both the *cyp71A12* and *pen2 cyp71A12* mutants showed enhanced susceptibility to *Ab*, suggesting the importance of CYP71A12-dependent production of ICAs (Fig. II-1 and Fig. II-3). Furthermore, the loss of CYP71A13, a close CYP71A12 homolog, reduced the immune response to *Ab* in both the *cyp71A12* and *pen2 cyp71A12* mutants (Fig. II-1 and Fig. II-3). I revealed that the accumulation of ICAs triggered by inoculation with *Ctro* reduced in the *cyp71A12* mutant (Fig. I-7). By contrast, the *Ctro*-triggered accumulation of camalexin was not reduced in *pen2 cyp71A12* but reduced in *pen2 cyp71A12 cyp71A13* (Fig. I-7). Therefore, it was assumed that the CYP71A12-dependent production of ICAs contributes to the *Arabidopsis* immunity to *Ab* independently of CYP71A13-dependent camalexin production, which is also consistent with the finding that the phenotype of the *pen2 pad3* mutant was less severe

than that of the *pen2 cyp71A12 cyp71A13* mutant after *Ab* inoculation (Fig. II-1). To validate this hypothesis, it was investigated the Trp-related metabolite profiles of the aforementioned mutants: *pen2*, *pen2 pad3*, *pen2 cyp71A12*, *pen2 cyp71A12 cyp71A13*, and *cyp79B2 cyp79B3*. Obtained results revealed that the simultaneous loss of *CYP79B2* and *CYP79B3* completely abolished the *Ab*-triggered accumulation of ICAs and camalexin, whereas the loss of *PAD3* canceled camalexin accumulation, but had no effects on the accumulation of ICAs (Fig. II-5B and Fig. II-7). In the *pen2 cyp71A12* mutant, the *Ab*-triggered accumulation of ICAs was reduced significantly compared with the *pen2* mutant, opposite with camalexin accumulation that was rather increased compared with *pen2* (Fig. II-5B and Fig. II-7). These results further strengthen the idea that the accumulation of ICAs triggered by the *Ab* invasion is critical for the postinvasive resistance of *Arabidopsis* against this necrotrophic fungal pathogen. In the *pen2 cyp71A12 cyp71A13* mutant, the accumulated levels of ICAs upon *Ab* invasion were similar to those of the *pen2 cyp71A12* mutant, but camalexin accumulation in the triple mutant was completely diminished, in contrast to *pen2 cyp71A12* (Fig. II-5B and Fig. II-7), further supporting the importance of CYP71A13 for the pathogen-induced accumulation of camalexin, but not ICAs.

It is noteworthy that the *pen2 cyp71A12 cyp71A13* mutant still accumulated ICAs upon *Ab* inoculation to some degree, whereas the accumulated level of camalexin in the triple mutant was under the detection limit (Fig. II-5B and Fig. II-7). As mentioned above, the *cyp79B2 cyp79B3* mutant exhibited a more severe phenotype to *Ab* inoculation compared with the *pen2 cyp71A12 cyp71A13* mutant (Fig. II-1 and Fig. II-4) being entirely defective in the production of not only camalexin, but also ICAs (Fig. II-5B and Fig. II-7). These data suggested that the different levels of susceptibility to *Ab* between *pen2 cyp71A12 cyp71A13* and *cyp79B2 cyp79B3* is likely caused by the different accumulated levels of ICAs in these two mutants. Consistent with this idea, the *pen2 cyp71A12* mutant was more susceptible to *Ab* than the *pen2* mutant (Fig. II-1) additionally

supporting the correlation between the reduced levels of ICAs and the breakdown of the postinvasive resistance toward *Ab*.

CYP79B2/CYP89B3 is essential for biosynthesis of other Trp-derived metabolites including IGs, in addition to ICAs and camalexin (Frerigmann *et al*., 2016). The *pen2 cyp71A12 cyp71A13* mutant lacks the PEN2-dependent IG-hydrolysis products, but retains the ability to produce IGs, which can be hydrolyzed by other myrosinases. It was reported that the transcription factors MYB34, MYB51, and MYB122 regulate IG biosynthesis in *Arabidopsis* (Frerigmann and Gigolashvili, 2014; Frerigmann *et al*., 2016). Thus, to assess the possibility that the different susceptibility to *Ab* between *pen2 cyp71A12 cyp71A13* and *cyp79B2 cyp79B3* mutants might be linked to IG biosynthesis, it was compared *pen2 cyp71A12 cyp71A13* with *cyp71A12 cyp71A13 myb34 myb51 myb122* following *Ab* inoculation. As a result, lesion development in the *cyp71A12 cyp71A13 myb34 myb51 myb122* mutants was similar to that in the *pen2 cyp71A12 cyp71A13* mutant, suggesting that PEN2-independent IG hydrolysis is likely not involved in the postinvasive resistance against *Ab* (Fig. II-8). Thus, the different susceptibility between the *pen2 cyp71A12 cyp71A13* mutant and the *cyp79B2 cyp79B3* mutant is not linked to IG-deficiency. Together with the involvement of CYP71A12-dependent ICAs synthesis in the postinvasive resistance of *Arabidopsis* against *Ab*, it was considered that the remaining ICAs in *pen2 cyp71A12 cyp71A13* are still effective against *Ab*, i.e., the ICAs contribute to the postinvasive resistance of *Arabidopsis* in a dose-dependent manner.

ICAs function commonly in the postinvasive resistance of *Arabidopsis* against multiple fungal pathogens with different infection modes

It was further investigated the possible contributions of ICAs to the immune response of *Arabidopsis* against the hemibiotrophic fungus *C. higginsianum* (*Ch*), which is adapted to this plant species. Conidial suspension of *Ch* was inoculated on the aforementioned *Arabidopsis* mutants with altered Trp metabolism. As a result, the degree of lesion development by *Ch* in the *pen2* mutant was similar to that in the WT Col-0 (Fig. II-9A),

whereas PEN2 was reported to be involved in preinvasive resistance against *Ch* to some degree (Hiruma *et al.,* 2011). Compared with *pen2*, I found the increased lesion development in both the *pen2 pad3* and *pen2 cyp71A12* mutants (Fig. II-9A). Furthermore, there was an additive effect in *pen2 cyp71A12 cyp71A13* compared with *pen2 pad3* and *pen2 cyp71A12* (Fig. II-9A). These tendencies are clearly consistent with the phenotypes observed for both *Ab* and *Ctro* inoculation (Fig. I-3, and II-1). It was also found that the *pen2 cyp71A12 cyp71A13* plants had no obvious defects in preinvasive resistance against *Ch* (Fig. II-9B). Therefore, it was concluded that ICAs and camalexin function commonly in the postinvasive resistance of *Arabidopsis* against multiple fungal pathogens, the necrotrophic fungus *Ab*, and the two hemibiotrophic fungi, *Ch* and *Ctro*. By contrast, PEN2-dependent preinvasive resistance is critical to *Ctro* and is partially effective to *Ch* (Hiruma *et al.,* 2010, 2011), but is dispensable to *Ab* (Fig. II-2D). Therefore, *Arabidopsis* has evolved to use conserved and common mechanisms related to Trp metabolism for postinvasive resistance against a broad range of fungal pathogens, whereas it has developed distinct mechanisms for controlling entry by the pathogens. It was also found that CYP82C2 has a minor role in immunity to *Ch*, likely consistent with immunity to *Ab* (Fig. II-9A).

The *bak1–5* mutation reduces the postinvasive resistance of *Arabidopsis* to *Ab*, independently of pathogen-triggered ICAs and camalexin biosynthesis

Next, it was investigated the molecular mechanisms of how *Arabidopsis* recognizes the invasion of pathogens such as *Ab* to activate the biosynthesis of ICAs and camalexin. The candidate mechanism that is critical for this process is the PRRs-dependent PAMP recognition machinery (Couto and Zipfel, 2016; Saijo *et al.,* 2018). When *Arabidopsis* recognizes PAMPs, at least some of the cognate PRRs, including FLS2 and EFR receptor-like kinases (RLKs), form complexes with co-receptors such as BAK1 (Chinchilla *et al.,* 2007; Heese *et al.,* 2007; Roux *et al.,* 2011; Ma *et al.,* 2016). Therefore, it was decided to assess the possible involvement of BAK1 in the invasion-triggered accumulation of

ICAs and camalexin. For this purpose, two mutant alleles of *BAK1*, *bak1–4* and *bak1–5* were used; *bak1–4* is a BAK1 null allele (Chinchilla *et al.,* 2007), and *bak1–5* is a semi-dominant allele with a specific phenotype related to PAMP responsiveness (Schwessinger *et al.,* 2011). To precisely compare the results in this assay using *Ab* with the assay using *Ctro*, I used the *pen2 bak1–4* mutant (Takahashi *et al.,* 2016) and the newly generated *pen2 bak1–5* mutant. First, *Ab* was inoculated on these plant lines to assess immunity to *Ab*. It was found that the *pen2 bak1–5* mutant significantly reduced immunity to *Ab*, whereas the *pen2 bak1–4* mutant reduced immunity, but without significance (Fig. II-11A). The effect of *bak1–5* on susceptibility to *Ab* infection was also observed in the WT background (Fig. II-10).

I also investigated the possible involvement of SOBIR1 (SUPPRESSOR OF BIR1-1) because SOBIR1 is reported to be essential for triggering defense responses by certain leucine-rich repeat receptor-like proteins (LRR-RLPs) that, together with BAK1, act as immune receptors (Zhang *et al.,* 2013; Albert *et al.,* 2015; Postma *et al.,* 2016). The *Ab* inoculation assay on the *pen2 sobir1* mutant suggested that this line slightly reduced the immunity to *Ab* (Takahashi *et al.,* 2016) (Fig. II-11A), although a statistical test failed to detect significant differences between *pen2* and *pen2 sobir1*.

Further it was evaluated whether the *bak1–5* mutation would reduce postinvasive resistance to *Ab*. To assess this, the invasion behavior of *Ab* in the *pen2* mutant was compared with that in the *pen2 bak1–5* mutant. The invasion ratio of *Ab* in the *pen2 bak1–5* mutant was similar to that in the *pen2* mutant, suggesting that the *bak1–5* mutation did not have detectable impacts on preinvasive resistance to *Ab* (Fig. II-11B). These results indicate that the *bak1–5* mutation reduces postinvasive resistance to *Ab*, i.e., PRR systems likely function in the recognition of *Ab* invasion. PEPR1 and PEPR2 are LRR-RLKs that recognize the endogenous plant elicitor peptides called Peps (Yamaguchi *et al.,* 2010). Peps such as AtPep1 are classified as danger-associated molecular patterns (DAMPs) (Boller and Felix, 2009). To assess whether the plant recognition of *Ab* invasion might be related to the possible generation of DAMPs via the pathogen invasion, it was generated

a *pen2 pepr1 pepr2* mutant and *Ab* inoculation was performed on this triple mutant; however, the assay did not detect reduced immunity in this triple mutant (Fig. II-11A).

Because it was found that the *bak1–5* mutation reduced postinvasive resistance to *Ab*, it was next investigated whether *bak1–5* would have negative effects on the *Ab* invasion-triggered activation of camalexin and ICAs biosynthesis. First, I checked the gene expression of *PAD3* and *CYP71A12* in the *pen2* and *pen2 bak1–5* mutants after *Ab* invasion. Surprisingly, both *PAD3* and *CYP71A12* were similarly expressed upon *Ab* invasion in both mutant lines (Fig. II-12). Furthermore, I found that the accumulation of camalexin and ICAs upon *Ab* invasion was not reduced in the *pen2 bak1–5* mutant compared with the *pen2* mutant (Fig. II-13 and Fig. II-14). Or rather, the accumulation level of ICAs was higher in *pen2 bak1–5* than *pen2*, which might be due to enhanced infection of *Ab* in *pen2 bak1–5* (Fig. II-13 and Fig. II-14). Collectively, these results indicate that the *bak1–5* mutation does not reduce the *Ab*-triggered activation of camalexin and ICAs biosynthesis, although this mutation reduces postinvasive resistance to *Ab*. These findings suggest that (i) the *bak1–5* mutation impairs or reduces antifungal pathways distinct from Trp metabolism, and that (ii) camalexin and ICAs biosynthesis upon *Ab* invasion depend on an unknown pathogen recognition mechanism that is not impaired by *bak1–5*.

The *bak1–5* mutation reduces the *Ab* invasion-triggered expression of defense-related genes including *GLIP1*

I further investigated the *bak1–5* sensitive pathways for postinvasive resistance against *Ab*. Comparative expression profiling experiments in *pen2* and *pen2 bak1–5* plants following *Ab* invasion were performed using microarray analysis. *Ab* was inoculated to each plant, and RNAs isolated from inoculated leaves at 24 hpi were subjected to microarray analysis. It was then focused on differentially expressed genes associated with the immune response based on Gene Ontology (GO) data (GO term 0006955: http://www.informatics.jax.org). As a result, it was found that 14 genes had

greater than a 2.5-fold change in expression. Interestingly, 11 were down-regulated, and three were up-regulated (Table II-1), implying that, compared with the *pen2* plants, the *pen2 bak1–5* plants exhibited a trend for reduced immune responses upon *Ab* invasion. Among the 11 down-regulated genes, four were shown previously to be involved in the *Arabidopsis* immune system using functional analyses such as the analysis of corresponding knockout mutants, including *AED1* (*APOPLASTIC, EDS1-DEPENDENT 1*) (Breitenbach *et al.,* 2014), *BGL2*/*PR2* (Leubner-Metzgerand and Meins, 1999; Oide *et al.,* 2013), *GLIP1* (Oh *et al.,* 2005; Kwon *et al.,* 2009), and *RLP23* (*RECEPTOR-LIKE PROTEIN 23*) (Albert *et al.,* 2015). Notably, the *glip1* plants were reported to be more susceptible to *Ab* than the WT plant (Oh *et al.,* 2005). Subsequently, RT–qPCR analyses were performed to investigate the expression levels of these four genes (*AED1*, *BGL2*/*PR2*, *GLIP1*, and *RLP23*) at 4, 12, 24, and 48 hpi with *Ab* in the *pen2* and *pen2 bak1–5* plants (Fig. II-15). As a control, I also investigated gene expression in the mock-treated plants. It was first confirmed that these four genes exhibited lower expression in the *pen2 bak1–5* than in the *pen2* mutant at 24 hpi, consistent with the array data. Furthermore, the RT–qPCR analysis revealed that the expression of *AED1*, *BGL2*/*PR2*, *GLIP1*, and *RLP23* was lower at 4 hpi than at 12, 24, and 48 hpi (Fig. II-15). As showed above *Ab* did not invade at 4 hpi, but started to invade at 12 hpi (Fig. II-2B), indicating that these genes are induced upon *Ab* invasion, probably to function in postinvasive resistance. Such induced expression was continuously suppressed in the *pen2 bak1–5* plants (Fig. II-15), further suggesting that this invasion-triggered expression depends on putative PRRs with function impaired in the *bak1–5* mutant. It is also noteworthy that the invasion-triggered expressions of *AED1*, *BGL2*/*PR2*, *GLIP1*, and *RLP23* were time-dependent, i.e., the induced expression was down-regulated at 24 hpi (*RLP23*) or at 48 hpi (*AED1*, *BGL2*/*PR2*, and *GLIP1*) (Fig. II-15), which was in contrast to the invasion-triggered expressions of *PAD3* and *CYP71A12* that exhibited sustained elevations for up to 48 hpi (Fig. II-2A and Fig. II-12).

Conserved machinery in the immunity of *Arabidopsis* toward invasion of multiple fungal pathogens with distinct infection modes

Up to here, it has revealed that postinvasive resistance toward *Ab* involves (i) a Trp-related secondary metabolism including the biosynthesis of both camalexin and ICAs, and (ii) a Trp-unrelated defense pathway that is sensitive to the *bak1–5* mutation. *Arabidopsis* exhibits robust nonhost resistance against the nonadapted hemibiotroph *Ctro*, and full postinvasive resistance against *Ctro* requires camalexin and ICAs synthesis together with the *Ctro*-induced expression of *CYP71A12* and *PAD3* (Fig. I-6). Therefore, it was tested whether the postinvasive resistance against *Ctro* would also be affected by the *bak1–5* mutation. It was reported previously that the *bak1–5* mutation reduced preinvasive resistance to *Ctro* (Irieda *et al*., 2019). Here, a trypan blue staining assay revealed that the *bak1–5* mutation also reduced postinvasive resistance to this nonadapted pathogen (Fig. II-16A). I can exclude the possibility that the reduced preinvasive resistance in the *pen2 bak1–5* mutant results in the decreased postinvasive resistance to *Ctro*, because the *pen2 edr1* plants exhibited a more severe reduction in preinvasive resistance, but still had no detectable reduction in postinvasive resistance to *Ctro* (Hiruma *et al*., 2013). Thus, *bak1–5* reduced postinvasive resistance toward both *Ab* and *Ctro*.

It was further investigated whether the *bak1–5* mutation would have negative effects on the expression of *CYP71A12* and *PAD3* triggered by *Ctro* invasion. It was observed that the expressions of *CYP71A12* and *PAD3* were induced in *pen2* but not in WT plants, indicating that their expressions were triggered by *Ctro* invasion (Fig. II-16B). Notably, it was found that the *bak1–5* mutation had no clear effect on this aspect (Fig. II-16B). Thus, postinvasive resistance against *Ctro* also requires (i) a *bak1–5*-insensitive Trp-metabolism including the synthesis of camalexin and ICAs, and (ii) a *bak1–5*-sensitive defense response uncoupled from Trp-metabolism, which is quite similar to postinvasive resistance against *Ab*. Collectively, these results represent unexpectedly strong conservation of machineries for postinvasive resistance against two distantly related

fungal pathogens having distinct infection strategies, i.e., a brassica-adapted necrotrophic fungus and a nonadapted hemibiotrophic fungus.

DISCUSSION

In Chapter I, I revealed that CYP71A12 is indispensable for postinvasive resistance to the hemibiotrophic pathogen *Ctro* that is not adapted to *Arabidopsis* (Fig. I-3). However, there is no information on whether CYP71A12 is also involved in this invasion-triggered resistance of *Arabidopsis* against other fungal pathogens. In this chapter, I found that the necrotrophic pathogen *Ab* enhanced lesion development in the absence of functional CYP71A12, indicating that this enzyme is required for the immune response of *Arabidopsis* against *Ab*. *CYP71A12* was not induced at 4 hpi with *Ab* when the pathogen had not yet invaded, but it started to be induced upon *Ab* invasion. It was also found that *CYP71A12* was dispensable for preinvasive resistance against *Ab*. Collectively, these results demonstrate that CYP71A12 is required for postinvasive resistance against *Ab* as well as *Ctro*. Metabolic analyses showed that the enhanced accumulation of ICAs triggered by *Ab* invasion was reduced in the absence of functional CYP71A12. Thus, this enzyme is involved in the accumulation of ICAs following *Ab* invasion (Fig. II-5B and Fig. II-7). Therefore, it was considered that the CYP71A12-dependent synthesis of ICAs or the synthesis of their derivatives upon *Ab* invasion is critical for postinvasive resistance to *Ab*.

I also showed that CYP71A13 contributes to the postinvasive resistance against *Ab* via synthesis of camalexin, but not of ICAs. The result suggests the importance of camalexin for this second layer of defense, which is further supported by the phenotypic analyses on the mutants defective in *PAD3* (Fig. II-1 and Fig. II-3). Remarkably, it was also found that both CYP71A12, CYP71A13 and PAD3 are involved in the postinvasive resistance of *Arabidopsis* against *Ch*, an adapted hemibiotrophic fungus. These results strongly suggest the broad importance of camalexin and ICAs for postinvasive resistance against fungal pathogens with distinct infection strategies.

By contrast, PEN2 is involved in preinvasive resistance against *Ctro* and *Ch* (Hiruma *et al.,* 2010, 2011), but dispensable for preinvasive resistance against *Ab* (Fig. II-2D).

Furthermore, PEN1 is known to be required for preinvasive resistance against nonadapted powdery mildews, but dispensable for the *Colletotrichum* fungi and nonadapted *A. alternata* (Shimada *et al.,* 2006; Egusa *et al.,* 2013). Thus, the molecular components that underlie preinvasive resistance vary between fungal pathogens, probably because pathogens have evolved various strategies for plant entry. By contrast, once the pathogens enter *Arabidopsis*, they commonly grow invasive fungal structures inside the plants; thus, the hosts might deploy common defense systems to terminate further fungal growth.

It was found that the *cyp79B2 cyp79B3* mutant is more susceptible to *Ab* than the *pen2 cyp71A12 cyp71A13* mutant (Fig. II-1 and Fig. II-4). Interestingly, this phenomenon is also observed in the infection by *Ch* (Fig. II-9A) and *Ctro* (Fig. I-3). Metabolite analyses of plants upon *Ab* invasion revealed that camalexin accumulation in the *pen2 cyp71A12 cyp71A13* plants was almost the same as that in the *cyp79B2 cyp79B3* plants. Importantly, the *pen2 cyp71A12 cyp71A13* plants reduced their accumulation of ICAs, but still produced them to some degree, whereas the *cyp79B2 cyp79B3* plants were entirely defective in this regard. I also compared the phenotype of *pen2 cyp71A12 cyp71A13* mutants with that of *myb34 myb51 myb122 cyp71A12 cyp71A13* following *Ab* inoculation and found no detectable differences in either mutants in terms of postinvasive resistance against *Ab*, indicating that the difference between *pen2 cyp71A12 cyp71A13* and *cyp79B2 cyp79B3* mutants is not caused by PEN2-unrelated IG metabolism products (Fig. II-8). Collectively, these findings suggest that the lower susceptibility in the former mutants is caused by residual ICAs, i.e., ICAs contribute to postinvasive resistance in a dose-dependent manner. This supports the idea that ICAs or their derivatives work as antifungal compounds as opposed to functioning as signaling molecules for plant immune responses. However, it cannot exclude that the accumulation of so far not-reported Trp-derivatives whose biosynthesis is not dependent on CYP71A12 and CYP71A13 contributes to the difference observed in susceptibility of *pen2 cyp71A12 cyp71A13* and *cyp79B2 cyp79B3* plants.

It was also investigated how *Arabidopsis* recognizes the invasion of fungal pathogens and then mounts its postinvasive resistance. It was found that the *bak1–5* mutation significantly reduced postinvasive resistance against *Ab* (Fig. II-10 and Fig. II-11A). Surprisingly, it was found that the *bak1–5* mutation did not inhibit the invasion-triggered expressions of *PAD3* and *CYP71A12* and the subsequent accumulation of ICAs and camalexin (Fig. II-12; Fig. II-13 and Fig. II-14). Thus, I postulated the existence of another defense mechanism that is sensitive to the *bak1–5* mutation and required for postinvasive resistance against *Ab*. The further analyses revealed that this pathway activates the expression of distinct defense-related genes, including *AED1*, *BGL2*/*PR2*, *GLIP1*, and *RLP23* (Table II-1 and Fig. II-15). Notably, the expressions of these genes were induced following *Ab* invasion, and these were canceled in *bak1–5* mutants (Fig. II-15). Therefore, it was suggested that *Arabidopsis* deploys a common PRR system to sense the invasion of multiple fungal pathogens and subsequently activate antifungal defense pathways that are uncoupled from Trp-metabolism. It was hypothesized that the *bak1–5*-sensitive pathway involves the recognition of DAMP. It is known that *Arabidopsis* perceives endogenous Pep peptides by two redundant PRRs: PEPR1 and PEPR2 (Yamaguchi *et al*., 2010). Moreover, the PEPR1/PEPR2 pathway is sensitive to *bak1–5* mutation (Yamada *et al*., 2016). However, it was found that immunity to *Ab* was not significantly reduced in the *pepr1 pepr2* mutant (Fig. II-11A). It will therefore be important to identify the corresponding PRR in the *bak1–5*-sensitive pathway for postinvasive resistance.

Notably, it has been reported that the *glip1* mutant plants exhibit enhanced susceptibility to *Ab* (Oh *et al*., 2005). The recombinant GLIP1 protein exhibits antimicrobial activity that disrupts the *Ab* spores and hyphae (Oh *et al.,* 2005) and triggers systemic acquired resistance (SAR) against bacterial pathogens (e.g., *Erwinia carotovora* and *Pseudomonas syringae*) as well as *Ab* (Kwon *et al*., 2009). Thus, the enhanced susceptibility of *Arabidopsis* to *Ab* in the presence of *bak1–5* might be partially caused by the reduced expression of *GLIP1*. *BGL2* (*PR2*) and *Apoplastic EDS1-Dependent 1*

(*AED1*) were also down-regulated in the *pen2 bak1–5* plants following *Ab* invasion. *BGL2* (*PR2*) is a well-known salicylic acid (SA) signaling marker together with *PR1* and *PR5*. The infection of both bacterial and fungal pathogens triggers expression of the aforementioned genes followed by the establishment of SAR (Uknes *et al*., 1992). AED1 is a protein secreted in common with GLIP1 and BGL2 (Breitenback *et al*., 2014), although it remains to be elucidated whether AED1 itself has antimicrobial activity. Interestingly, (i) accumulation of the *AED1* transcript is tightly correlated with the extent of SAR, and (ii) AED1 has a role in suppressing SAR (Breitenback *et al*., 2014). Collectively, the invasion recognition system exhibiting *bak1–5* sensitivity activates the expression of multiple secreted protein genes that function in the regulation of SAR. It was suggested that this *bak1–5*-sensitive pathway activates local resistance against invasion by fungal pathogens such as *Ab*, and in systemic leaves, the pathway also might activate a form of SAR that is SA-dependent. The enhanced expression of *PDF1.2a* in the *pen2 bak1–5* plants (Table II-1) is likely consistent with the positive role of BAK1 in SA signaling that antagonizes the *PDF1.2a*-related JA signaling pathway. Furthermore, transgenic *Arabidopsis* plants overexpressing *BAK1* exhibited enhanced SA accumulation compared with WT Col-0 plants (Kim *et al*., 2017).

It is also noteworthy that the expression of *RLP23* was down-regulated in *pen2 bak1–5* plants following *Ab* invasion. Compared with *AED1*, *BGL2*, and *GLIP1*, the peak of *RLP23* expression was quicker (it peaked at 12 hpi), whereas those of *AED1*, *BGL2*, and *GLIP1* peaked at 24 hpi (Fig. II-15). RLP23 is a PRR that recognizes 20–24 amino acid peptides of NLP proteins (Albert *et al*., 2015). Because a broad range of fungal pathogens possesses *NLP* genes, the RLP23-dependent recognition of NLPs might contribute to postinvasive resistance toward fungal pathogens in *Arabidopsis*.

It remains unclear how this plant recognizes pathogen invasion and then activates Trp-related metabolite accumulation as key immune responses in postinvasive resistance. Present data suggest that the corresponding pathway is not sensitive to the *bak1–5* mutation. Because *PAD3* and *CYP71A12* were commonly induced by the invasion of

diverse fungal pathogens such as *Ab* and *Ctro*, it was suggested that *Arabidopsis* probably recognizes the cell damage that is commonly caused by pathogen invasion and then mounts its immune responses. For example, *Arabidopsis* recognizes released extracellular ATP or fragments of plants cell walls as DAMPs (Quintana-Rodriguez *et al*., 2018), which are possibly generated by fungal pathogen invasion. The *dorn1 (does not respond to nucleotides 1*) mutant plants are deficient in their response to ATP (Choi *et al*., 2014). *DORN1* encodes LecRK-I.9, which has a binding affinity for ATP and is the first identified plant receptor for extracellular ATP (Choi *et al*., 2014). Although it is unknown whether the DORN1 pathway is sensitive to *bak1–5* (Choi and Klessig, 2016), *BAK1* is dispensable for the immunity of *Arabidopsis* mediated by LORE, a G-type LecRK that responds to bacterial lipopolysaccharides (Ranf *et al*., 2015; Couto and Zipfel, 2016). A LecRK-dependent perception of unknown or known DAMP(s) generated by pathogen invasion might activate Trp-related antimicrobial pathways; however, further studies are needed to explore this aspect.

MATERIALS AND METHODS

Fungal materials

C. tropicale (*Ctro*) (formerly *Colletotrichum gleosporioides* S9275) was provided by Shigenobu Yoshida (National Institute for Agro-Environmental Sciences, Japan); *C. higginsianum* (*Ch*) isolate MAFF305635 was obtained from the Ministry of Agriculture, Forestry and Fisheries (MAFF) Genebank, Japan; and *A. brassicicola* (*Ab*) strain Ryo-1 was provided by Akira Tohyama. Cultures of *Ch* and *Ab* were maintained on 3.9% (w/v) potato dextrose agar medium (PDA; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 24 °C in the dark. *Ctro* was cultured on 2.5% (w/v) PDA (Difco, Detroit, MI, USA) at 24 °C under a cycle of 16 h black light (FS20S/BLB 20W; Toshiba, Tokyo, Japan) illumination and 8 h dark.

Arabidopsis lines and growth conditions

The *A. thaliana* accession Col-0 was used as the WT plant. The mutants *pen2-1*, *pen2-2* (Lipka *et al.,* 2005), *pad3-1* (Glazebrook and Ausubel, 1994), *cyp71A12*, *cyp71A12 cyp71A13* (Müller *et al.,* 2015), *cyp82C2* (Rajniak *et al.,* 2015), *bak1–4* (Chinchilla *et al.,* 2007), *bak1–5* (Schwessinger *et al*, 2011), *cyp79B2 cyp79B3* (Zhao *et al.,* 2002), *pen2-1 pad3-1* (Bednarek *et al.,* 2009), *pepr1 pepr2* (Yamaguchi *et al.,* 2010), *pen2 sobir1* (Takahashi *et al.,* 2016), and *myb34 myb51 myb122* (Frerigmann and Gigolashvili, 2014) were used in this study. *pen2 cyp82C2*, *pen2 cyp71A12*, *pen2 cyp71A12 cyp71A13*, and *cyp71A12 cyp71A13 myb34 myb51 myb122* were obtained from Dr. Paweł Bednarek as collaboration study. *Arabidopsis* seeds were sown on rockwool (Grodan; http://www.grodan.com) and kept at 4 °C in the dark for 2 days, and later grown at 25 °C with a cycle of 16 h light and 8 h dark in Hoagland medium. *Arabidopsis* lines used in this study was listed on Table II-3.

Pathogen inoculation, lesion development analysis and trypan blue staining assay

For spray inoculation assays of *Ab*, *Ch*, and *Ctro*, 5 x 10^5 conidia/mL of conidial suspension was spray-inoculated on 4–5-week-old plants. For drop-inoculation, 5 μL of conidial suspensions of *Ab* (1 x 10^5 conidia/mL), *Ch* (1 x 10^5 conidia/mL) or *Ctro* (2.5 x 10^5 conidia/mL) were placed onto each leaf. Conidia of *Ctro* were inoculated with 0.1% (w/v) glucose (Glc). The inoculated plants were kept at 25 °C with a cycle of 16 h light and 8 h dark and maintained at 100% relative humidity. For analysis of lesion development following the inoculation of *Ab* or *Ch*, four drops of 5 μL conidial suspension of each pathogen were drop-inoculated on each leaf, and 24–50 lesions were evaluated in each experiment. The developed lesions were quantified using ImageJ image analysis software (http://imagej.net) and relative values to WT (Col-0) plants were calculated. To measure lesion areas, yellowish areas were included as lesions. Trypan blue staining was conducted according to Koch and Slusarenko (1990). For the trypan blue assay, at least 50 lesions were investigated in each experiment. The *Ab* invasion ratio (%) was calculated by using the following formula: *Ab* invasion ratio (%) = (number of germinating conidia that developed invasive hyphae / number of germinated conidia) × 100. For the *Ch* invasion assay, 2 μL aliquots of 5 x 10^5 conidia/mL of the *Ch* conidial suspension were drop-inoculated onto *Arabidopsis* cotyledons (14 days old). At 60 hpi, invasive hyphae were investigated using light microscopy. The invasion ratio of *Ch* was calculated as described previously (Irieda *et al.,* 2019).

Generation of mutant plants

The generation of *pen2 bak1–4*, *pen2 bak1–5* and *pen2 pepr1 pepr2* lines used in this study were generated by crossing the *pen2–1* mutant with *bak1–4*, *bak1–5*, or *pepr1 pepr2* plants. Each genotype was checked with the corresponding specific primers for the derived cleaved-amplified polymorphic sequence (dCAPS) markers using dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html), and the PCR products (WT or mutant types) were cleaved with appropriate restriction enzymes (Table II-2).

RT–qPCR analysis

RT–qPCR analysis was performed in according to the methods described in Chapter I. The primers used in this study are listed in Table II-2.

Metabolite analysis

Conidial suspensions (5 x 10^5 conidia/mL) of *Ab* were spray-inoculated onto 4–5-week-old plants and kept at 100% relative humidity. Leaf samples (100–200 mg fresh weight) were collected at corresponding time points and frozen immediately in liquid nitrogen. The plant extracts containing Trp derivatives were extracted using DMSO and metabolite analyses were performed as described (Bednarek *et al.,* 2009)

Microarray analysis

Ab conidial suspensions (5 x 10^5 conidia/mL) were spray-inoculated onto 4–5-week-old plants of the *pen2* and *pen2 bak1–5* mutants. For each sample, five leaves were collected at 24 hpi and frozen immediately in liquid nitrogen. In total, eight samples (four biological replicates each of the mock and *Ab*-treated samples) were used for RNA extraction. Total RNA was extracted using Plant RNA Isolation Mini kits (Agilent Technologies., Santa Clara, CA, USA). Aliquots of 200 ng of total RNA were used to prepare Cy3-labeled cRNA using Agilent Low Input Quick Amp labeling kits. The labeled samples were hybridized onto an Agilent *Arabidopsis thaliana* microarray (ver. 4.0; 4 × 44 K format). After hybridization and washing, the arrays were scanned using an Agilent microarray scanner (G2565BA). The images were analyzed using Agilent Feature Extraction software (ver. 10.7.3.1), and further analysis was performed using Agilent GeneSpring GX12.1 software. Signal normalization was based on the expression ratio of *pen2 bak1–5* to *pen2*. Differentially upregulated genes were defined as having a greater than 2.5-fold increase in expression, and differentially downregulated genes were defined as having at least a 0.4-fold decrease in expression. Microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database GSE 124921.

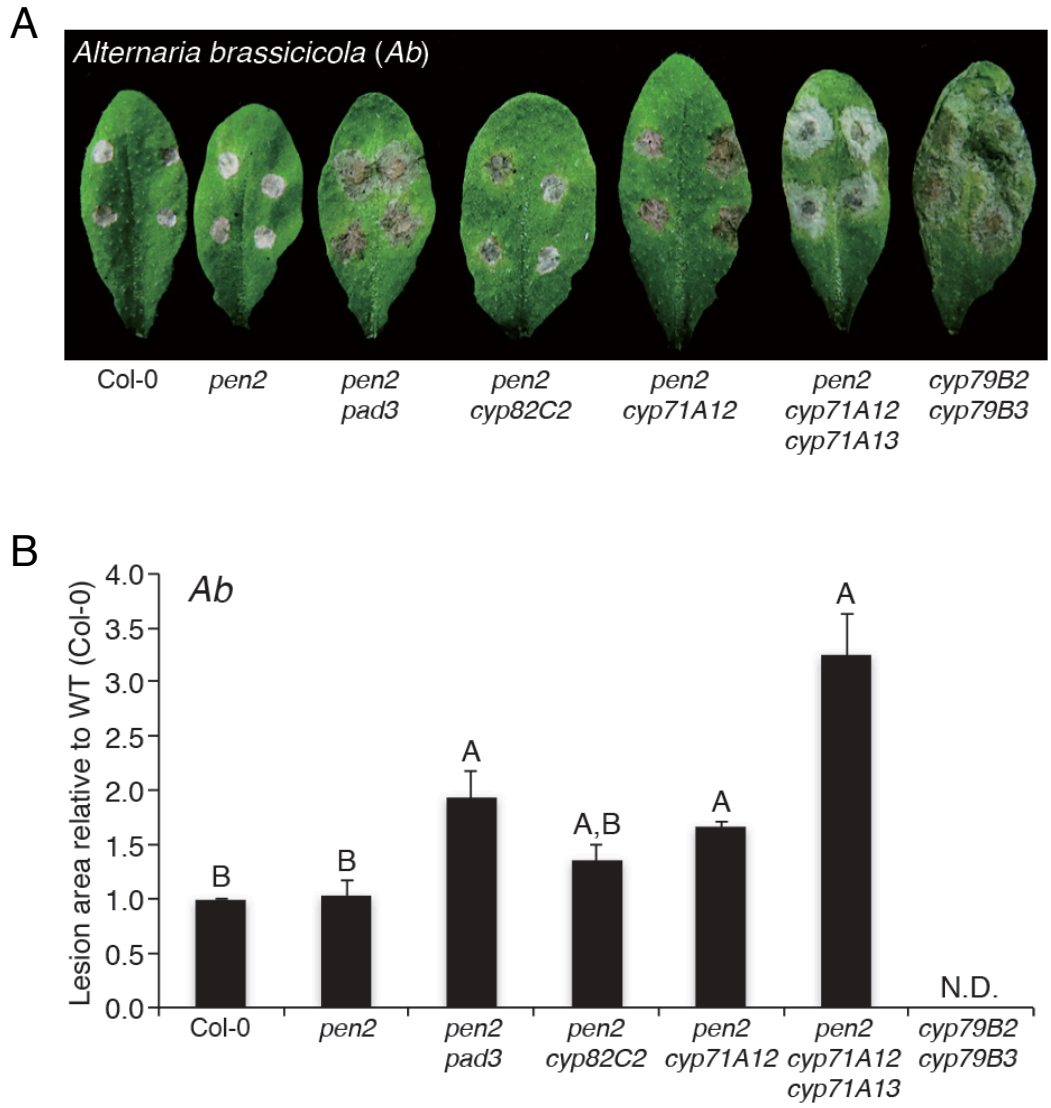

Fig. II-1. *PAD3* and *CYP71A12* are involved in the immunity of *Arabidopsis* against the necrotrophic pathogen *Alternaria brassicicola* (*Ab*).

(A) Lesion development caused by *Ab* on *Arabidopsis* mutant plants with defects in Trp metabolism pathways. Conidial suspensions (1 x 10^5 conidia/mL) of *Ab* were drop-inoculated onto mature leaves of 4–5-week-old plants. The photograph was taken at 4 days postinoculation (dpi).

(B) Quantification of lesion development. Conidial suspensions of *Ab* were drop-inoculated onto tested plants. At 4 dpi, lesion areas were measured and the relative values to Col-0 (WT plants) were calculated. Means and standard deviations (SDs) were calculated from three independent experiments. The statistical significance of differences between means was determined by Tukey's honestly significant difference (HSD) test. Means not sharing the same letter are significantly different ($P < 0.01$).

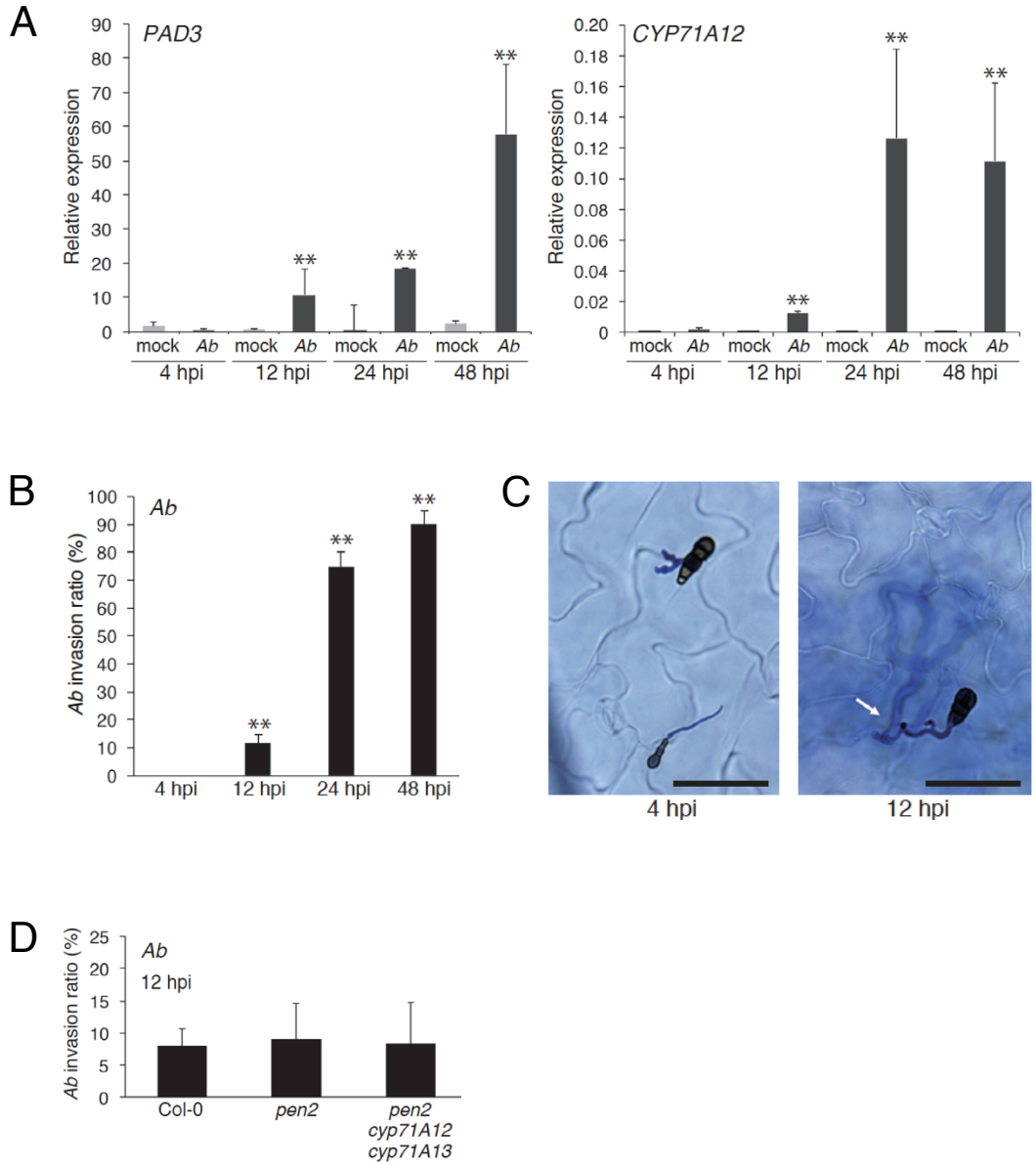

Fig. II-2. The invasion of *Ab* activates the expression of *PAD3* and *CYP71A12*.
(A) Expression of *PAD3* and *CYP71A12* following *Ab* inoculation. Conidial suspensions (5 x 10^5 conidia/mL) of *Ab* were spray-inoculated onto 4–5-week-old *pen2* plants and kept at 100% humidity. The samples were collected at 4, 12, 24, and 48 hpi. Each gene transcript was quantified by quantitative polymerase chain reaction (qPCR) using the gene-specific primers listed in Table II-2. Values were normalized to the expression level of *UBC21*. The means and SDs were calculated from three independent experiments. Statistical comparisons between mock and *Ab* treated samples were conducted using two-tailed Student's *t* tests (**$P < 0.01$).

(B) Quantitative analysis of the *Ab* invasion ratio among *pen2* plants. Conidial suspensions (1 x 10^5 conidia/mL) of *Ab* were drop-inoculated onto *pen2* plants and kept at 100% humidity. The inoculated leaves were collected at 4, 12, 24, and 48 hpi, and then subjected to a trypan blue viability staining assay. The presence or absence of invasive hyphae from at least 50 germinating conidia were counted in each experiment. The means and SDs were calculated from three independent experiments. Statistical comparisons of the *Ab* invasion ratios at 12, 24, and 48 hpi against that at 4 hpi were conducted using two-tailed Student's *t* tests (**$P < 0.01$).
(C) Light microscopy observations. At 4 hpi, most of the *Ab* conidia had germinated and started to elongate hyphae, but had not developed invasive hyphae. At 12 hpi, some of the germinating conidia developed invasive hyphae (arrow) inside the plants. Bars = 50 μm.
(D) *PEN2*, *CYP71A12*, and *CYP71A13* are dispensable for preinvasive resistance against *Ab*. Aliquots of 5 μL of *Ab* conidial suspension were drop-inoculated onto leaves of 4–5-week-old plants. At 12 hpi, the inoculated leaves were collected and stained with trypan blue, and then invasive hyphae were observed by light microscopy. At least 50 germinating conidia were counted in each experiment. The means and SDs were calculated from three independent experiments. Statistical analysis using two-tailed Student's *t* tests showed no significant differences among the genotypes.

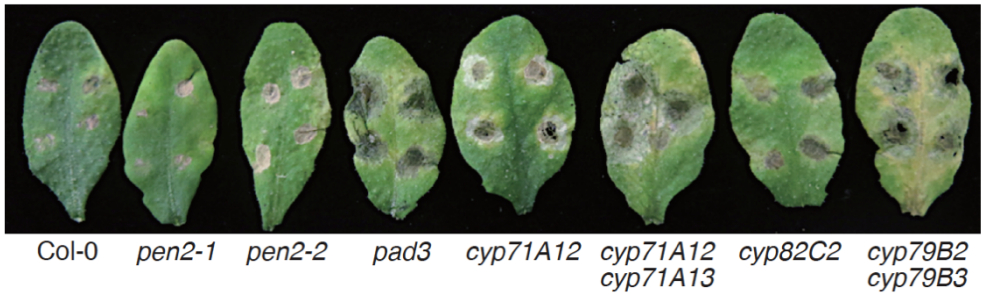

Fig. II-3. Lesion development caused by *Ab* on *Arabidopsis* mutant plants.
Conidial suspensions (1 x 10^5 conidia/mL) of *Ab* were drop-inoculated onto the tested mutant lines. This photograph was taken at 4 days after the inoculation.

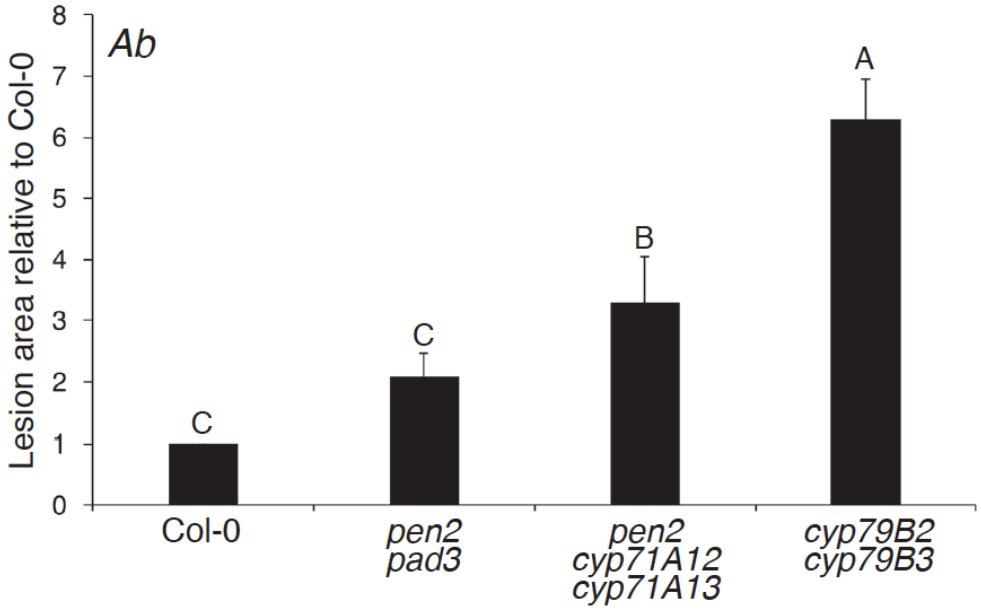

Fig. II-4. The *cyp79B2 cyp79B3* mutant was more susceptible to *Ab* than the *pen2 cyp71A12 cyp71A13* mutant.

Quantification of lesion development in the *Ab*-inoculated leaves of the tested *Arabidopsis* lines at an early time point (3 dpi). Conidial suspensions of *Ab* (1 x 10^5 conidia/mL) were drop-inoculated onto true leaves of 4–5-week-old plants. At 3 dpi, lesion areas were measured, and then the relative values to those in Col-0 (WT plants) were calculated. The means and SDs were derived from three independent experiments. The statistical significance of differences between means was determined by Tukey's honestly significant difference (HSD) test. Means not sharing the same letter are significantly different ($P < 0.01$).

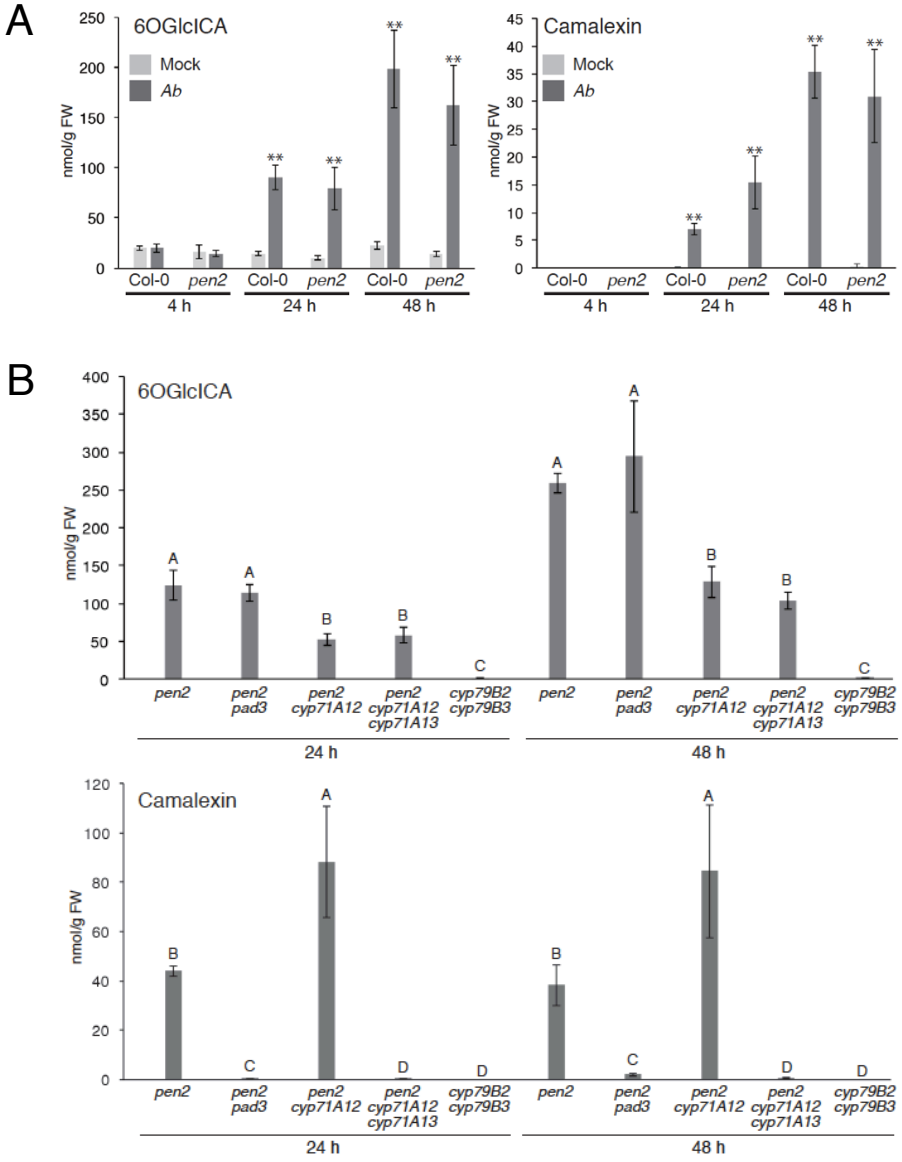

Fig. II-5 Invasion by *Ab* induced CYP71A12-dependent biosynthesis of 6-hydroxy-ICA and PAD3-dependent biosynthesis of camalexin.

(A) The accumulation of 6-hydroxy-ICA (6OGlcICA) and camalexin in *Arabidopsis* plants inoculated with *Ab*. Conidial suspensions (5 x 10^5 conidia/mL) of *Ab* were spray-inoculated onto Col-0 (WT) and *pen2* plants. As a control, water was sprayed as a mock treatment. The means of metabolites (nmol/g fresh weight, FW) and SDs from four biological replicates are shown in the graph. The statistical analysis between mock and *Ab* treated samples was conducted using two-tailed Student's *t* tests (*$P < 0.05$; **$P < 0.01$).

(B) *CYP71A12* is required for the *Ab*-triggered accumulation of 6OGlcICA, whereas *PAD3* and *CYP71A13* are required for the accumulation of camalexin. Conidial suspensions (5 x 10^5 conidia/mL) of *Ab* were spray-inoculated onto tested mutant lines. The statistical significance of differences between means was determined by Tukey's honestly significant difference (HSD) test. Means not sharing the same letter are significantly different ($P < 0.01$).

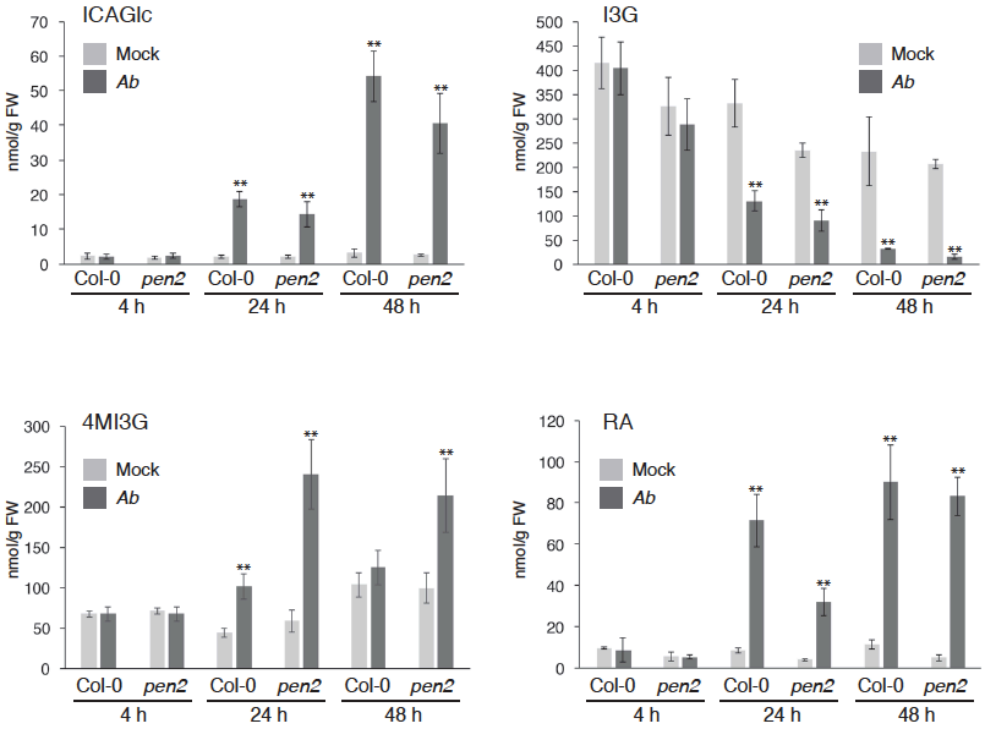

Fig. II-6. The analysis of ICAGlc, I3G, 4MI3G, and RA accumulation in WT (Col-0 plants) and the *pen2* mutant inoculated with *Ab*.

Conidial suspensions (5 x 10^5 conidia/mL) of *Ab* were spray-inoculated onto WT and *pen2* plants. As a control, water was sprayed as a mock treatment. The samples were collected at 4, 24, and 48 hpi. The accumulation of indole-3-carboxylic acid glucose ester (ICAGlc), indole-3-ylmethylglucosinolate (I3G), 4-methoxyindol-3-ylmethylglucosinolate (4MI3G), and raphanusamic acid (RA) were determined. The means of metabolites (nmol/g FW) and SDs from four biological replicates are shown in the graph. The statistical analysis between mock- and *Ab*-treated samples at each time point was conducted using two-tailed Student's *t* tests (**$P < 0.01$).

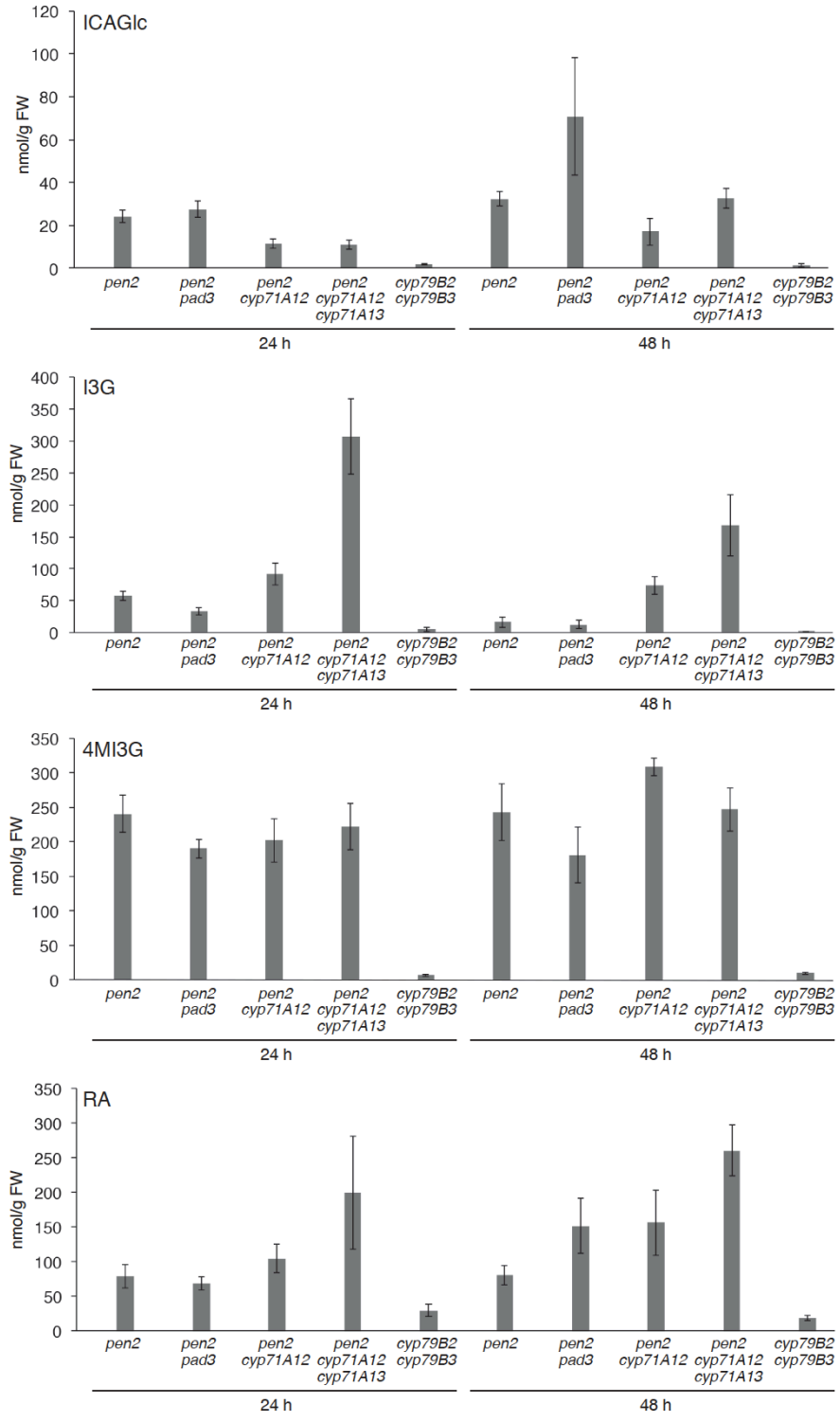

Fig. II-7. The analysis of ICAGlc, I3G, 4MI3G, and RA accumulation in a series of mutants defective in Trp metabolism.

Conidial suspensions (5 x 10^5 conidia/mL) of *Ab* were spray-inoculated onto the tested mutant plants. The samples were collected at 24 and 48 hpi. The accumulations of ICAGlc, I3G, 4MI3G, and RA were determined. The means of metabolites (in nmol/g FW) and SDs from four biological replicates are shown in the graph.

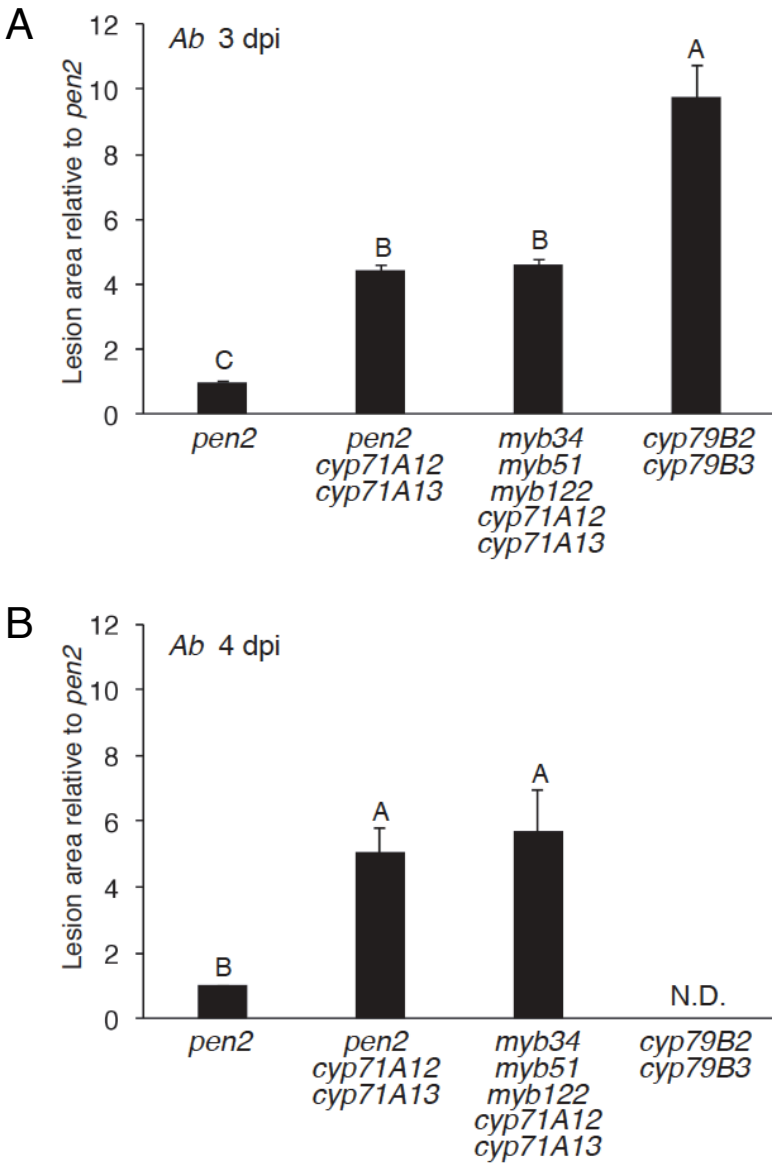

Fig. II-8. Higher susceptibility to *Ab* in *cyp79B2 cyp79B3* than in *pen2 cyp71A12 cyp71A13* mutants is not caused by the accumulation of PEN2-independent indole-glucosinolates.

Conidial suspensions of *Ab* (1 x 10^5 conidia/mL) were drop-inoculated onto true leaves of 4–5-week-old plants. At 3 dpi **(A)** and 4 dpi **(B)**, lesion areas were measured, and relative values to the *pen2* mutant were calculated. The means and SDs were derived from three independent experiments. The statistical significance of differences between means was determined by Tukey's honestly significant difference (HSD) test. Means not sharing the same letter are significantly different ($P < 0.01$). ND; not determined.

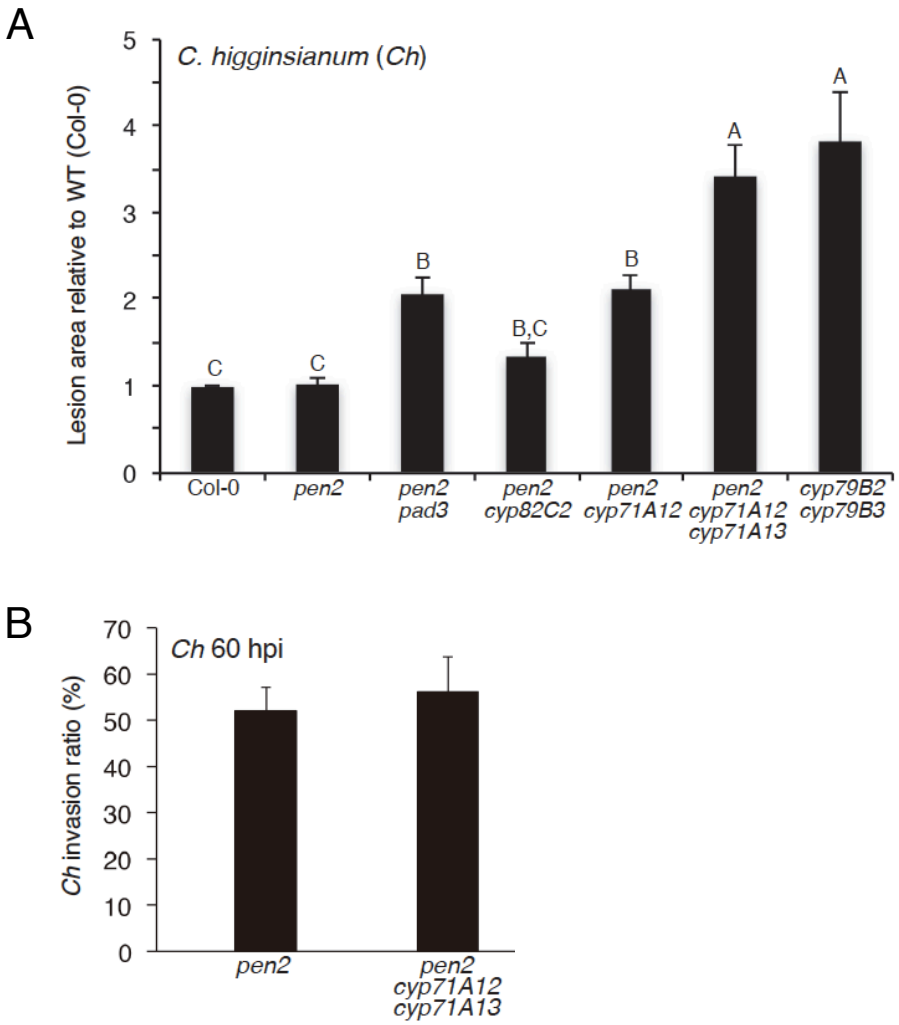

Fig. II-9. ICAs and camalexin are involved in the immunity of *Arabidopsis* against a hemibiotrophic pathogen *Colletotrichum higginsianum* (*Ch*).
(A) Quantitative analysis of lesion development produced by *Ch*. Conidial suspensions of *Ch* (1 x 10^5 conidia/mL) were drop-inoculated onto mature leaves of 4–5-week-old plants of the tested *Arabidopsis* lines. At 4 dpi, lesion development was measured. The means and SDs were calculated from three independent experiments. The statistical significance of differences between means was determined by Tukey's honestly significant difference (HSD) test. Means not sharing the same letter are significantly different ($P < 0.01$).
(B) *CYP71A12* and *CYP71A13* are dispensable for preinvasive resistance against *Ch*
Aliquots of 2 µL of a conidial suspension of *Ch* (5 x 10^5 conidia/mL) were drop-inoculated on cotyledons of the tested plants. At 60 hpi, invasive hyphae were observed using light microscopy. In each experiment, at least 50 appressoria were investigated to determine whether they had developed invasive hypha. The means and SDs were calculated from three independent experiments. Statistical analysis using two-tailed Student's *t* tests showed no significant differences between the genotypes.

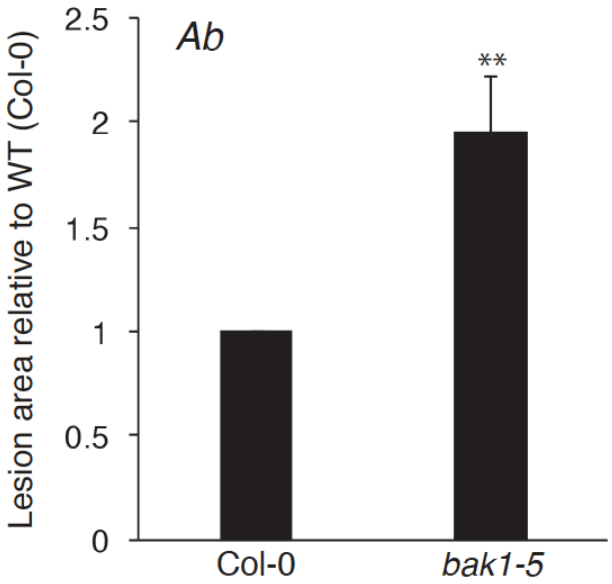

Fig. II-10. The *bak1–5* mutant showed enhanced susceptibility toward *Ab* compared with the WT plants (Col-0).

Conidial suspensions of *Ab* (1 x 10^5 conidia/mL) were drop-inoculated onto the tested plants. At 4 dpi, lesion development relative to WT plants was determined. The means and SDs were derived from three independent experiments. The statistical analysis between Col-0 and *bak1–5* was conducted using two-tailed Student's *t* tests (**$P < 0.01$).

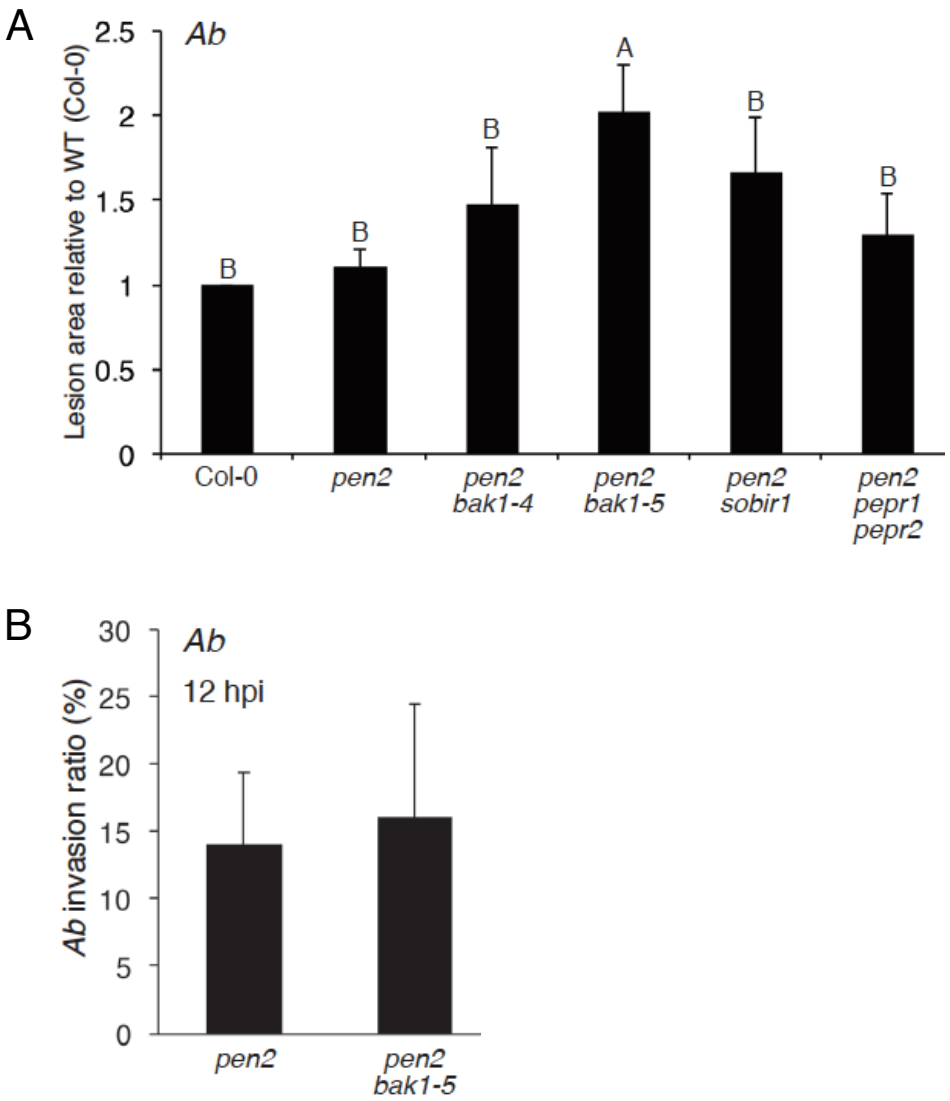

Fig. II-11. The *bak1–5* mutation reduced the immunity of *Arabidopsis* against *Ab*.
(A) Quantification of lesion development. Conidial suspensions of *Ab* (1 x 10^5 conidia/mL) were drop-inoculated onto true leaves of 4–5-week-old plants. At 4 dpi, lesion areas were measured and the relative values to Col-0 (WT plants) were calculated. The means and SDs were calculated from three independent experiments. The statistical significance of differences between means was determined by Tukey's honestly significant difference (HSD) test. Means not sharing the same letter are significantly different ($P < 0.01$).
(B) The *bak1–5* mutation did not reduce preinvasive resistance against *Ab* in the *pen2* mutant. Aliquots of 5 µL of conidial suspension (1 x 10^5 conidia/mL) of *Ab* were drop-inoculated onto leaves of 4–5-week-old plants of the tested mutants. At 12 hpi, the inoculated leaves were collected and stained with trypan blue, and then invasive hyphae were observed under light microscopy. At least 50 germinating conidia were counted in each experiment. The means and SDs were calculated from three independent experiments. Statistical analysis using two-tailed Student's *t* tests showed no significant differences between *pen2* and *pen2 bak1–5* mutants.

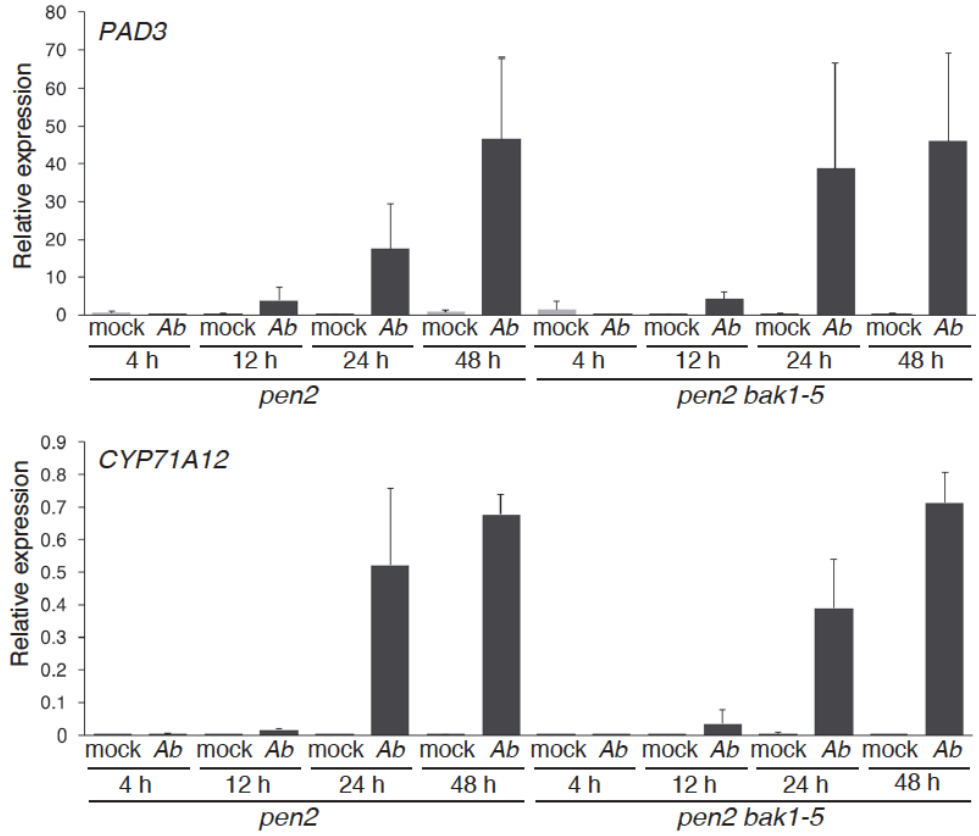

Fig. II-12. The *bak1–5* mutation did not reduce the *Ab*-invasion triggered expression of *PAD3* and *CYP71A12*.

The *Ab*-invasion triggered activation of *CYP71A12* and *PAD3* expression in the *pen2 bak1–5* mutant. Conidial suspensions (5 x 10^5 conidia/mL) of *Ab* were spray-inoculated onto 4–5-week-old plants. The samples were collected at 4, 12, 24, and 48 hpi. Each gene transcript was quantified by RT–qPCR using the gene-specific primers listed in Table II-2. Values were normalized to the expression level of *UBC21*. The statistical comparison between *Ab*-treated *pen2* and *Ab*-treated *pen2 bak1-5* at same time point samples was conducted using two-tailed Student's t tests and did not show significant differences.

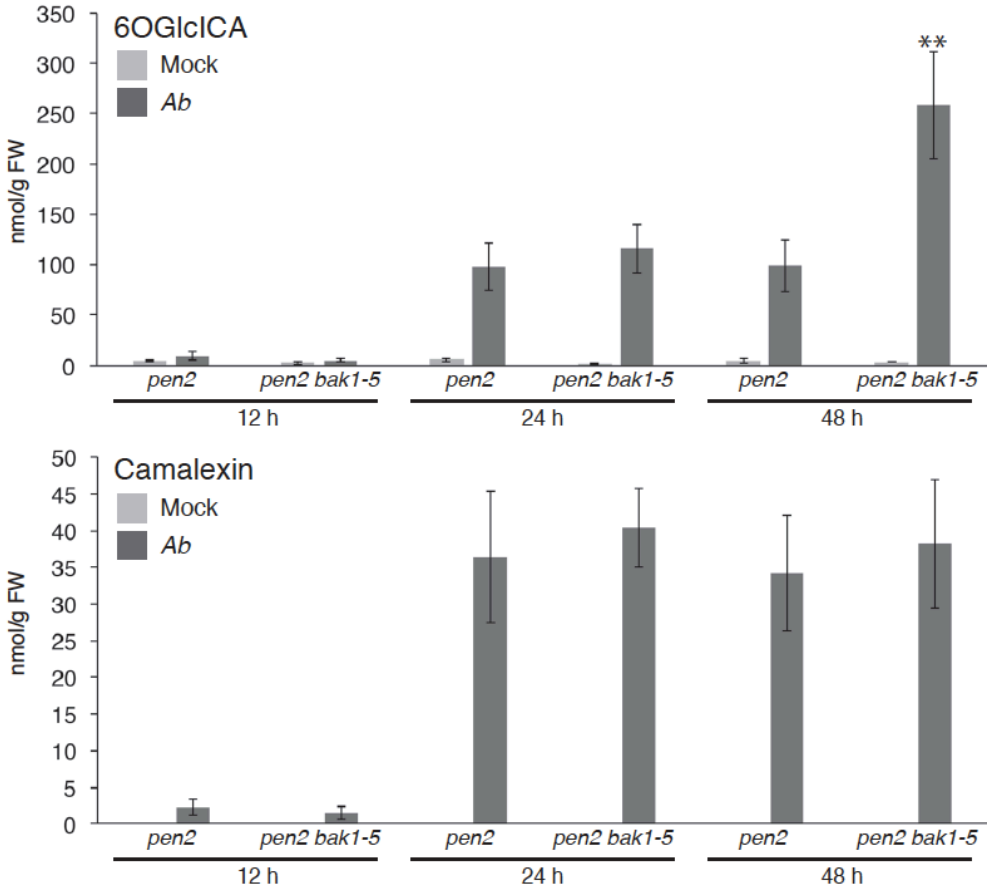

Fig. II-13. The *bak1–5* mutation did not reduce the *Ab*-invasion triggered accumulation of camalexin and ICA.

The *Ab* invasion-triggered accumulations of 6OGlcICA and camalexin were not canceled by the *bak1–5* mutation. Conidial suspensions (5 x 10^5 conidia/mL) of *Ab* were spray-inoculated onto the tested mutant lines. The means of metabolites (nmol/g FW) and SDs from four biological replicates are shown in the graph. The statistical comparison between *pen2* and *pen2 bak1-5* at same timepoint was conducted using two-tailed Student's *t* tests (**$P < 0.01$).

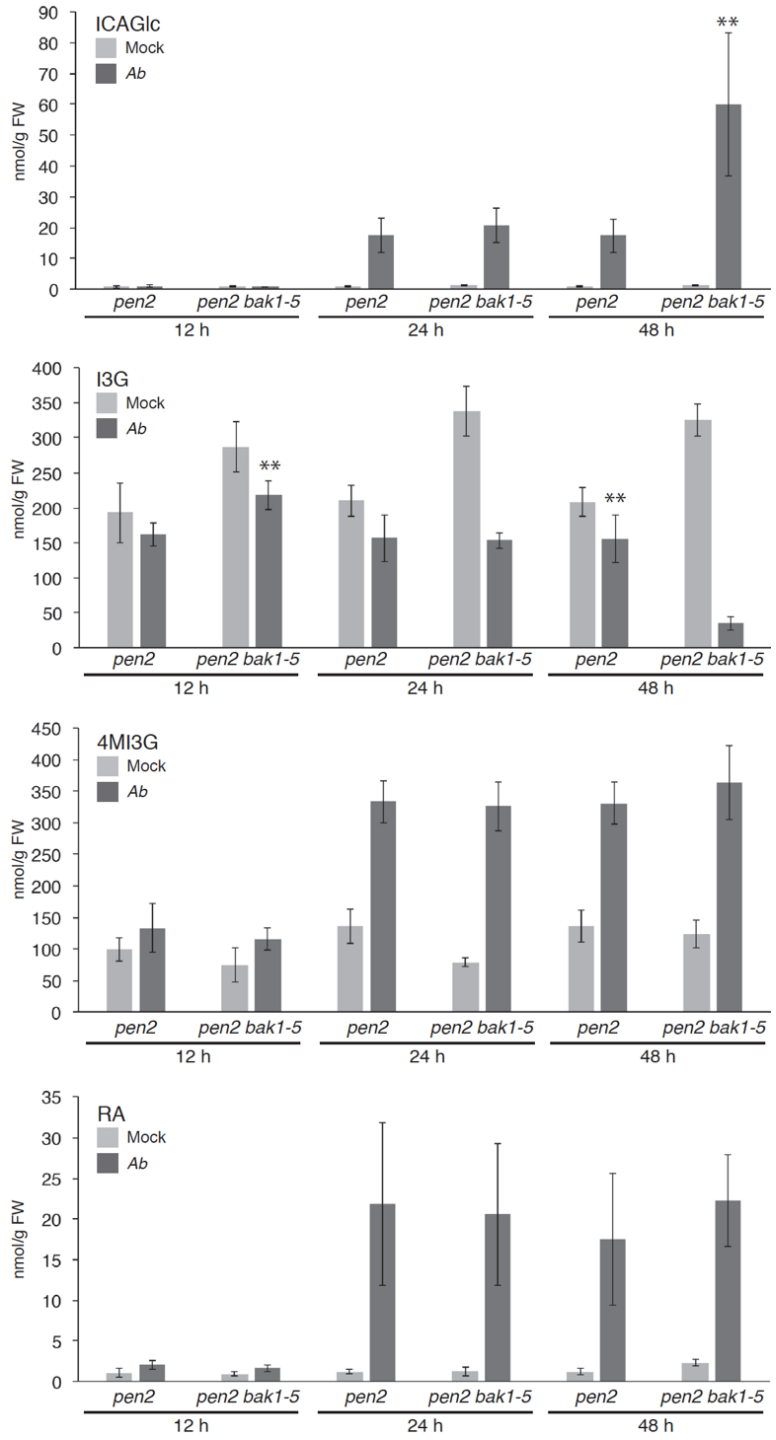

Fig. II-14. The analysis of ICAGlc, I3G, 4MI3G, and RA accumulation in both *pen2* and *pen2 bak1–5* mutants.

Conidial suspensions (5 x 10^5 conidia/mL) of *Ab* were spray-inoculated onto the tested plants. As a control, water was sprayed as a mock treatment. The samples were collected at 12, 24, and 48 hpi. Accumulations of ICAGlc, I3G, 4MI3G, and RA were determined. The means of metabolites (nmol/g FW) and SDs from four biological replicates are shown in the graph. Comparisons between *pen2* and *pen2 bak1–5* at each time point were conducted using two-tailed Student's *t* tests (**$P < 0.01$).

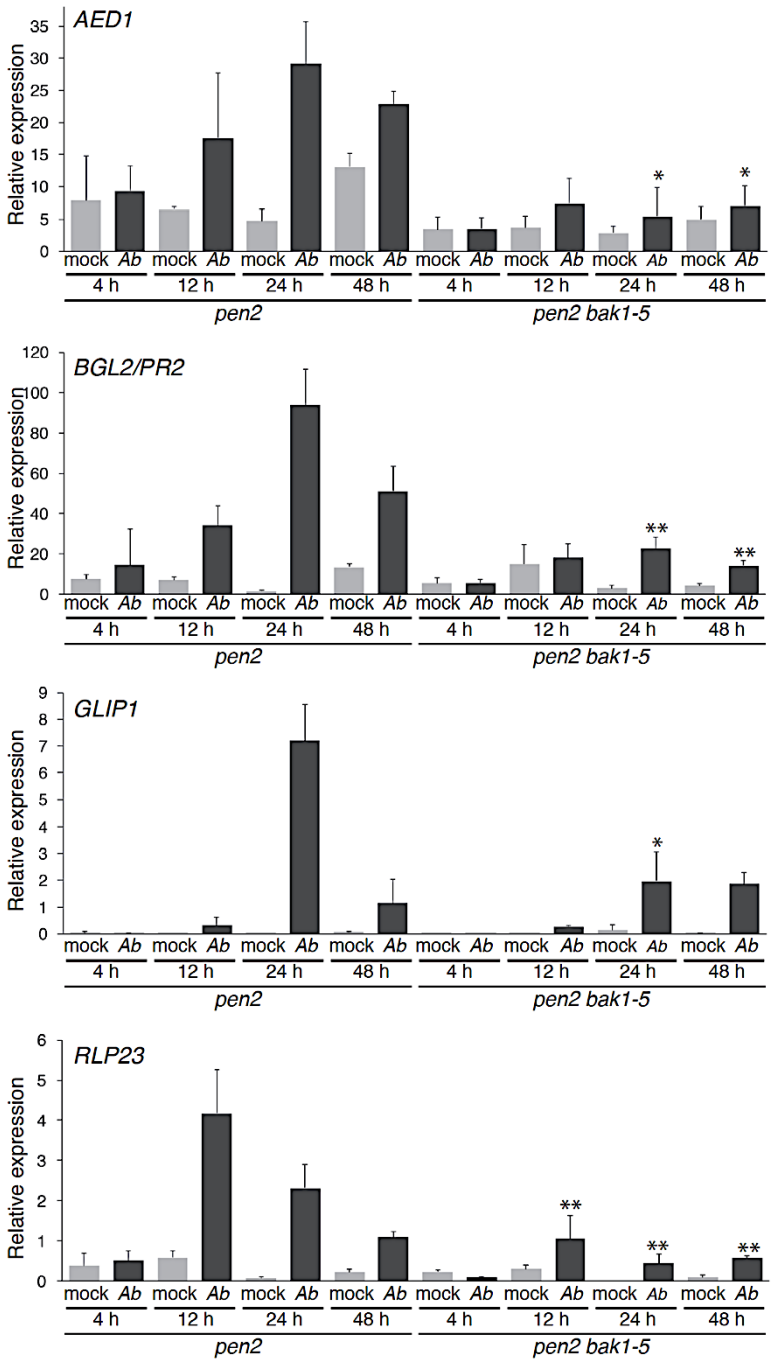

Fig. II-15. The *Ab* invasion-triggered expression levels of *AED1*, *BGL2*, *GLIP1*, and *RLP23* were reduced by the *bak1–5* mutation.

Conidial suspensions (5 x 10^5 conidia/mL) of *Ab* were spray-inoculated onto 4–5-week-old *pen2* and *pen2 bak1–5* plants, and then kept at 100% humidity. The samples were collected at 4, 12, 24, and 48 hpi. Each gene transcript was quantified by RT–qPCR using the gene-specific primers listed in Table II-2. Values were normalized to the expression level of *UBC21*. The means and SDs were calculated from three independent experiments. The statistical analysis was conducted by a two-tailed *t* test. The expression levels of each gene between *pen2* and *pen2 bak1–5* were compared at the same time points and treatment (*$P < 0.05$; **$P < 0.01$).

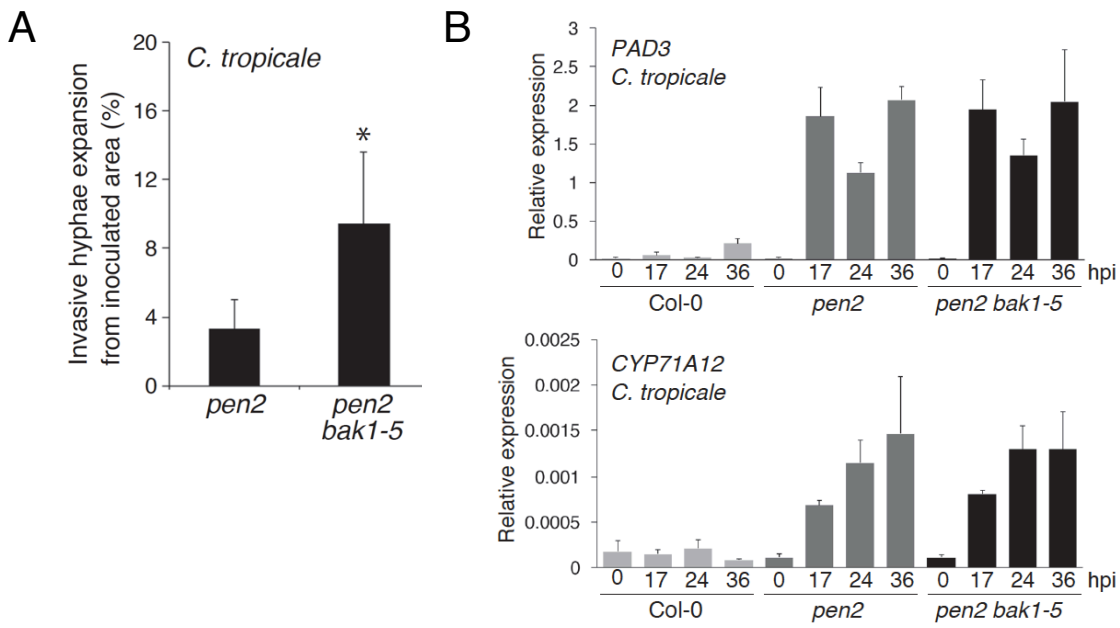

Fig. II-16. The *bak1–5* mutation reduced postinvasive resistance against the nonadapted hemibiotrophic pathogen *Colletotrichum tropicale* (*Ctro*), but did not affect the *Ctro*-invasion-triggered expression levels of *CYP71A12* or *PAD3*.

(A) The *bak1–5* mutation reduced postinvasive resistance against *Ctro*. Aliquots of 5 µL of conidial suspension (2.5 x 10^5 conidia/mL) of *Ctro* were drop-inoculated onto the tested plants. At 4 dpi, the inoculated leaves were collected and stained with Trypan blue. The expansion of invasive hyphae from the inoculated area was investigated using light microscopy, and the percentages of expanded invasive hyphae were determined. The means and SDs were calculated from three independent experiments. The statistical analysis was conducted using two-tailed Student's *t* tests (**$P < 0.01$).

(B) The *Ctro*-invasion-triggered expression of *PAD3* and *CYP71A12* was not canceled by the *bak1–5* mutation. Conidial suspensions (5 x 10^5 conidia/mL) of *Ctro* were spray-inoculated onto the tested plants. Leaf samples were collected at 17, 24, and 36 hpi. Each gene transcript was quantified by RT–qPCR using the gene-specific primers listed in Table II-2. Values were normalized to the expression level of *UBC21*. The means and SDs were calculated from three independent experiments. Statistical analysis using two-tailed Student's *t* tests showed no significant differences between the *pen2* and *pen2 bak1–5* mutants at the same time points.

Table II-1. List of differentially regulated *Arabidopsis* genes in *pen2 bak1-5* plants in comparison with *pen2* plants after *Ab* invasion at 24 hpi.

Locus	Description	*pen2 bak1-5/pen2* [a]
Down-regulated in *pen2 bak1-5*		
At5g52740	copper transport family protein	0.20
At4g04500	cysteine-rich receptor-like protein kinase 37 (*CRK37*)	0.21
At2g26400	acireductone dioxygenase 3 (*ARD3*)	0.21
At4g23150	cysteine-rich receptor-like protein kinase 7 (*CRK7*)	0.29
At3g57260	beta 1,3-glucanase 2 (*BGL2*)	0.30
At5g55450	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein	0.31
At5g10760	aspartyl protease family protein (*AED1*)	0.33
At2g14580	pathogenesis-related protein 1 (*PRB1*)	0.34
At2g32680	receptor like protein 23 (*RLP23*)	0.36
At5g40990	GDSL lipase 1 (*GLIP1*)	0.37
At3g28510	AAA-type ATPase family protein	0.39
Up-regulated in *pen2 bak1-5*		
At1g29660	GDSL esterase/lipase	25,80
At5g44420	ethylene- and jasmonate-responsive plant defensin (*PDF1.2*)	4,39
At1g79400	cation/H(+) antiporter 2 (*CHX2*)	2,58

[a]Up-regulated genes were determined by a greater than 2.5-fold change of normalized signals in their expression ratio (*pen2 bak1-5* vs *pen2*). Down-regulated genes were determined by a smaller than 0.4-fold change of normalized signals in their expression ratio (*pen2 bak1-5* vs *pen2*). The values are the average ratios of three arrays.

Table II-2. List of primers used in this study.

Primers used for RT-qPCR				
Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')		
CYP71A12 (At2g30750)	CATTCCCTAAGCCTTCGGTAC	CTTGGAGTTTCTTCATAACA		
CYP82C2 (At4g31970)	CATTTGGTTCGGGAAGAAGA	AGCCAGGGCTCTCAGTCATA		
PAD3 (At3g26830)	TGCTCCCAAGACAGACAATG	GTTTTGGATCACGACCCATC		
UBC (At5g25760)	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC		
AED1 (At5g10760)	GCGTATACAGTATCGTTTACGGCG	GGTAGTGAGAGTTTGCCAGGGCC		
BGL2 (At3g57260)	TGGATCACCGAGAAGGCCAGGG	GCCCACAAGTCTCTAAGGATTAG		
GLIP1 (At5g40990)	GAGCTGATTTGGAGCGGACCTACC	CGAGTGATATATATCGCTCGCG		
RLP23 (At2g32680)	GGAGTGGCTTGTCAAGATAATTGG	CCCAATTTTATCCTCATTTGCCCG		
Primers used for genotyping				
Mutant name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	LBa or LBb primer sequence (5'-3')	Enzyme
pen2-1	TCAGGTAAATCAGTTCGAATCAAG	TGAGGAAACCTGTTGGAGAAAGG		BamHI
bak1-4	ATTTTGCAGTTTTGCCAACAC	CATGACATCATCATCATTCGC	TGGTTCACGTAGTGGGCCATCG	
bak1-5	AAGAGGGCTTGCGTATTTACATGAT	GAGGCGAGCAAGATCAAAAG		RsaI
pepr1	TCGTTTCGGATCACCTAATTG	TTTCACCTGTCAATCCGTTTC	TGGTTCACGTAGTGGGCCATCG	
pepr2	TTGGATCCAACTCATTGAACG	ACGCCAGTCCATGTGAAATC	GCGTGGACCGCTTGCTGCAACT	

Table II-3. List of *Arabidopsis* lines used in this study.

Arabidopsis thaliana line	Reference
Col-0 (WT)	
pen2-1	Lipka *et al.,* 2005
pen2-2	Lipka *et al.,* 2005
pad3-1	Glazebrook and Ausubel, 1994
cyp71A12	Müller *et al.,* 2015
cyp82C2	Rajniak *et al.,* 2015
bak1-4	Chinchilla *et al.,* 2004
bak1-5	Schwessinger *et al,* 2011
pepr1 pepr2	Yamaguchi *et al.,* 2010
cyp71A12 cyp71A13	Müller *et al.,* 2015
cyp79B2 cyp79B3	Zhao *et al.,* 2002
pen2 pad3	Bednarek *et al.,* 2009
pen2 cyp82C2	Pastorczyk *et al.,* 2019
pen2 cyp71A12	Pastorczyk *et al.,* 2019
pen2 bak1-4	In this study
pen2 bak1-5	
pen2 pepr1 pepr2	
myb34 myb51 myb122	Frerigmann and Gigolashvili, 2014
pen2 cyp71A12 cyp71A13	Pastorczyk *et al.,* 2019
cyp71A12 cyp71A13 myb34 myb51 myb122	Pastorczyk *et al.,* 2019

CHAPTER III

EDR1- and ORA59-dependent expression of plant defensin contributes to the postinvasive resistance against fungal pathogen

INTRODUCTION

In Chapters I and II, I revealed that secondary metabolites derived from Trp metabolism, such as indole-3-carboxylic acids (ICAs), camalexin and cyanogenic compound (4-OH-ICN), are involved in postinvasive defense that restricts the expansion of invaded fungal pathogens. It was previously reported that PEN2 and EDR1 are involved in the preinvasive resistance against *Ctro*. PEN2 is a myrosinase, one of the enzymes in Trp-metabolism pathways, that synthetizes indole glucosinolates and restrict the *Ctro* entry (Hiruma *et al*., 2010). *EDR1* (*ENHANCED DISEASE RESISTANCE 1*) encodes a protein kinase homologous to the mitogen-activated protein kinase kinase kinase (MAPKKK) belonging to the Raf family (Frye *et al*., 2011). EDR1 positively regulates the expression of antimicrobial plant defensins (PDFs) in response to pathogens (Hiruma *et al*., 2011). This positive regulation is based on the repression of a transcription factor MYC2 that negatively regulates the expression of the *PDF* genes (Hiruma *et al*., 2011). Notably, the *pen2 edr1* double mutant exhibited more severe defect in preinvasive resistance when compared to the *pen2* and *edr1* single mutants, revealing that PEN2 and EDR1 function independently for the preinvasive resistance against *Ctro* (Hiruma *et al*., 2011; Hiruma and Takano, 2011). However, despite effective invasion by *Ctro* into the *pen2 edr1* double mutant, *Ctro* invasive hyphae did not expand from the inoculated area,

suggesting that both PEN2 and EDR1 are dispensable for postinvasive defense against *Ctro*.

Interestingly, RT-qPCR analyses of the *PDF* genes in *Ab*-inoculated *Arabidopsis* suggested that the expression of the *PDF* genes were induced in postinvasive resistance against *Ab*. In this chapter, I focused on the relation of the *PDF* genes to postinvasive resistance against *Ab*. I revealed that EDR1 is involved in the expression of the *PDF* genes in postinvasive resistance against *Ab*, suggesting the conserved roles of EDR1 for the expression of the *PDF* genes in plant immune responses to fungal pathogens. For further understanding of the mechanism for the induced expression of the *PDF* genes in postinvasive resistance, I also focused on a transcription factor ORA59. It is known that ORA59 is a transcription factor that positively regulates the expression of *PDFs* under jasmonic acid (JA) treatment and inoculation of a necrotrophic pathogen *Botrytis cinerea* (Pré *et al*., 2008). When *Ab* was inoculated, both of the *edr1* mutant and the *ora59* mutant exhibited enhanced lesion development in contrast to the wild-type Col-0, indicating the contribution of EDR1 and ORA59 for the immunity to *Ab*. On the other hand, both EDR1 and ORA59 were dispensable for the preinvasive defense to *Ab*. These results suggested that both EDR1 and ORA59 are involved in the postinvasive resistance against *Ab*. Previous studies indicated that EDR1 is not involved in the regulation of Trp metabolism under pathogen inoculation (Hiruma *et al*., 2011). In Chapter II, I revealed that *Ab* invasion activated the expression of *GLIP1* that is uncoupled from the induced expression of genes involved in Trp metabolism upon *Ab* invasion (Fig. II-15). Then, I also investigated the expression of *GLIP1* in both of the *edr1* mutant and the *ora59* mutant when *Ab* invaded. As a result, the expression pattern of *GLIP1* in these mutants was comparable to the wild-type plants. Thus, the results strongly suggest that a defect of the *edr1* and *ora59* mutant in postinvasive resistance to *Ab* is due to the reduced expression of the *PDF* genes, i.e., EDR1- and ORA59-dependent expression of the *PDF* genes are involved in the postinvasive resistance against *Ab*.

Next, I asked about the recognition mechanism of *Ab* invasion that eventually leads to the expression of the *PDF* genes. In Chapter II, I revealed that BAK1 is dispensable for the activation of the biosynthesis of Trp-derived secondary metabolites when fungal pathogens invade, however, BAK1 is involved in the induced expression of genes encoding AED1, GLIP1, BGL2/PR2 and RLP23 that are known to be related to *Arabidopsis* immunity. Here I investigated the possible contribution of BAK1 to the induced expression of the *PDF* genes upon *Ab* invasion. As a result, the *PDF* gene expression in the *pen2 bak1-5* mutant was not repressed compared with the *pen2* mutant, suggesting that the *bak1-5* mutation has no negative effects on the *Ab* invasion-triggered expression of the *PDF* genes.

RESULTS

Invasion by *Ab* triggered the expression of the *PDF* genes in both EDR1 and ORA59-dependent manner

In Chaper II, I revealed that antimicrobial metabolites derived from Trp metabolism are essential for the postinvasive defense to *Ab*. In order to investigate other antimicrobial compounds involved in this second layered defense against *Ab* in addition to Trp-derived antimicrobial metabolites, I here focused on plant antimicrobial peptides called plant defensins (PDFs). Previous reports revealed that PDFs are strongly induced in response to diverse pathogens such as *Colletotrichum tropicale*, *Fusarium culmorum*, *Botrytis cinerea* and also *Ab* (Penninckx *et al.,* 1998; Sels *et al.,* 2007; Hiruma *et al.,* 2011). Among PDFs, PDF1.2a and PDF1.3 showed antimicrobial activity against *Ab* and *F. culmorum* in *in vitro* assay (Thomma *et al.,* 2002; Sels *et al.,* 2007). To evaluate the potential contribution of the PDFs for the postinvasive defense to *Ab*, *Ab* was inoculated onto WT Col-0, and leaf samples were collected at several time points (4, 12, 24, 48, 72 and 96 hours post inoculation (hpi)) and subsequently the expression of two *PDF* genes, *PDF1.2a* and *PDF1.3*, was investigated by the RT-qPCR experiment. As a result, both *PDFs* with antimicrobial effect was strongly induced at 24 hpi (Fig. III-1). In Chapter II, I revealed that conidia of *Ab* started to germinate at 4 hpi but did not invade plants at this time point. At 12 hpi, germinated conidia of *Ab* started to invade plants, and from 12 hpi to 24 hpi, the fungal entry rate drastically increased from 11 % (at 12 hpi) to 75% (at 24 hpi) (Fig. II-2B). I found that the expression of the *PDF* genes was triggered at 12 hpi and greatly enhanced at 24 hpi, implying that the expression of the *PDF* genes is induced in *Arabidopsis* via invasion by *Ab*. Interestingly, the expression of both *PDF1.2a* and *PDF1.3* were commonly reduced at 48 hpi but increased again at 72 hpi (Fig. III-1).

In Chapter II, I revealed that Trp-derived metabolites contribute to the postinvasive defense to *Ab*. Therefore, I decided to compare the expression pattern of the *PDF* genes

with genes involved in Trp metabolism when *Ab* was inoculated. For this, *Arabidopsis* WT Col-0 was inoculated with *Ab*, and the expression pattern of Trp metabolism-related genes (*CYP71A12*, *PAD3*, *CYP79B2*, *CYP81F2*, *CYP82C2* and *CYP79B3*) was investigated by RT-qPCR analysis (Fig. III-2). The analysis revealed that the expression of *PAD3*, *CYP71A12*, *CYP82C2*, and *CYP79B2* was not detectable at 4 hpi and started to be detected at 12 hpi (Fig. III-2). As for *CYP81F2* and *CYP79B3*, their expression was detected at 4 hpi but increased at 12 hpi in common with *PAD3*, *CYP71A12*, *CYP82C2*, and *CYP79B2*. Importantly, the subsequent expression patterns of the all tested genes were not consistent with that of the *PDF* genes (Fig. III-2). In contrast to the *PDF* genes, the drastic reduction in the expression at 48 hpi was not observed in the tested Trp metabolism-related genes. Also, the clear recovery of the expression observed in the *PDF* genes was not detected in the case of the Trp metabolism-related genes. These results strongly suggest that a regulatory manner for expression of the *PDF* genes is distinct from that for the Trp metabolism-related genes in the case of *Ab* inoculation. Related to this, I additionally investigated the expression of *Pathogenesis related protein 1* (*PR1*) that is known to be induced under pathogen infection and SA treatment (van Loon *et al*., 2006). *PR1* is also frequently used as a marker for SA signaling. The *PR1* expression was detected at 12 hpi of *Ab* and increased at 24 hpi (Fig. III-3), suggesting a possibility that *Ab* invasion activates the SA pathway.

I next investigated the factors required for the induced expression of the *PDF* genes in *Ab* inoculation. As mentioned, the *edr1* mutant inoculated with *Ctro* showed the reduction of the *PDF* expression, suggesting the involvement of EDR1 in the induction of the *PDF* gene expression (Hiruma *et al*., 2011). Therefore, it was investigated whether EDR1 is involved in the expression of the *PDF* genes (*PDF1.2a* and *PDF1.3*) when *Ab* was inoculated. Notably, loss of EDR1 caused the severe reduction of the *PDF* gene expression in the inoculation of *Ab*, suggesting that EDR1 is also important for the induced expression of the *PDF* genes in *Ab* inoculation (Fig. III-1). ORA59 is a transcription factor that belongs to *A*PETALA2/ETHYLENE RESPONSE FACTOR

(AP2/ET) family and binds to the GCC-box motif in the promoter region of *PDF1.2a* (Van der Does *et al*., 2013). *Arabidopsis* defective in *ORA59* decreased the *PDF1.2a* and *ORA59* overexpressing plants increased the expression of *PDF1.2a* in response to a necrotrophic pathogen *Botrytis cinerea* (Pré *et al*., 2008). Here I investigated whether ORA59 contributes to the expression of the *PDF* genes in response to *Ab* by inoculating *Ab* on the *ora59* mutant plants. As a result, in the *ora59* mutant, the expression of *PDF1.2a* and *PDF1.3* was almost completely canceled (Fig. III-1), suggesting the essential roles of ORA59 for the *PDF* expression in response to *Ab*.

EDR1 and ORA59 contribute to the postinvasive resistance to *Ab* via activation of the *PDF* gene expression

Next, I investigated a possible contribution of EDR1 and ORA59 to *Arabidopsis* immunity to *Ab*. For this propose, *Ab*-triggered lesion development was measured in the *edr1* and *ora59* mutant plants. The assay suggested that the *edr1* and *ora59* plants showed enhanced lesion development in contrast to Col-0 (Fig. III-4A, 4B), although a statistical test failed to detect a significant difference between Col-0 and *edr1*. Also, lesion development was more severe in the *ora59* mutant than in the *edr1* mutant. The finding suggested that both EDR1 and ORA59 are involved in *Arabidopsis* immunity to *Ab*. Then the contribution of EDR1 and ORA59 for the entry control of *Ab* was investigated. *Ab* was inoculated onto Col-0, the *pen2* mutant, the *edr1* mutant and the *ora59* mutant, and the invasion ratio was determined. There were no significant differences in the invasion ratio between the examined mutants and Col-0, suggesting that EDR1 and ORA59 are dispensable for the preinvasive defense to *Ab* (Fig. III-5). Collectively, these results demonstrate that EDR1 and ORA59 are involved in the postinvasive resistance to *Ab*.

I also here revealed that EDR1 and ORA59 are commonly required for the expression of the *PDF* genes in *Ab* inoculation. Then it was investigated whether EDR1 and ORA59 control the expression of other genes involved in the postinvasive resistance to *Ab*. Previous studies indicated that EDR1 is not involved in the regulation of Trp

metabolism (Hiruma *et al*., 2011). In Chapter II, I showed that the *pen2 bak1-5* mutant exhibited enhanced susceptibility than the *pen2* mutant to *Ab*. Microarray analysis narrowed down *bak1-5* sensitive genes such as *AED1*, *GLIP1*, *BGL2* and *RLP23* that are known to be related to *Arabidopsis* immunity (Table II-1). Remarkably, GLIP1 (GDSL LIPASE1) is a secreted protein that exhibits antimicrobial activity against *Ab* hyphae and conidia (Oh *et al*., 2005), thus GLIP1 is likely involved in the postinvasive resistance to *Ab*. I investigated the *GLIP1* expression in both *edr1* and *ora59* mutant plants when *Ab* was challenged. As a result, I found that the *GLIP1* expression in these mutants was comparable to that in the WT Col-0 (Fig. III-6), thus, EDR1 and ORA59 are not involved in the *GLIP1* expression triggered by the *Ab* invasion, whereas the *Ab*-triggered *GLIP1* expression was sensitive to *bak1-5*. Consistent with this, the expression of the *PDF* genes, controlled by EDR1 and ORA59, was not reduced in the *pen2 bak1-5* mutant compared with the *pen2* mutant when *Ab* was inoculated, indicating that the *Ab*-triggered expression of the *PDF* genes was not repressed by *bak1-5* (Fig. III-7). These results supported the idea that a defect of the *edr1* and *ora59* mutants in the postinvasive resistance against *Ab* is due to the reduced expression of the *PDF* genes. Therefore, EDR1- and ORA59-dependent expression of the *PDF* genes is involved in this second-layered defense to *Ab*.

DISCUSSION

It is known that signaling pathways by plant hormones such as jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) are important for plant immunity. Plant defensins (PDFs) is a well-known small cysteine-rich antimicrobial polypeptide that are induced in response to pathogen infection and JA/ET treatment (Penninckx *et al.,* 1996; 1998; Epple *et al.,* 1997; Hiruma *et al.,* 2011). Among 17 PDFs family of *Arabidopsis*, PDF1.2a and PDF1.3 were reported to be involved in immunity to multiple pathogens such as *C. tropicale* (*Ctro*), *C. higginsianum* (*Ch*), *Botrytis cinerea* and *Ab* (Penninckx *et al.,* 1996; 1998; Hiruma *et al.,* 2011). In this study, the RT-qPCR experiment suggested that the expression of the *PDF* genes started to be induced in response to the initiation of *Ab* invasion at 12 hpi (Fig. III-1). Interestingly, *Ab*-triggered expression of the *PDF* genes showed clear reduction at 48 hpi and also clear recovery at 72 hpi (Fig. III-1). This expression pattern was not observed in the case of the Trp metabolism-related genes (Fig. III-2), i.e., the expression of these genes is generally sustained after *Ab* invasion. How the *PDF* genes exhibit this dynamic expression patterns in *Ab* inoculation? I now hypothesize that the expression of the *PDF* genes is induced by *Ab* invasion but then is quickly repressed, whereas the expression of Trp metabolism-related genes is induced by *Ab* invasion and is sustained in contrast to the *PDF* genes. The reason why the expression of the *PDF* genes is recovered at 72 hpi is probably that *Ab* starts to invade neighboring cells around this time point, which activates the transient expression of the *PDF* genes in these new invaded cells. To assess this hypothesis, it is important to perform microscopic observation of *Ab* infection, for example, time course observation of *Ab* infection behavior on *Arabidopsis* transgenic plants expressing GFP under the control of *PDF* promoter or the *PAD3* promoter.

If the hypothesis is correct, this would raise a question why the expression of the *PDF* genes must be transient in invaded cells? One possibility is that the sustained expression of the *PDF* genes might have negative effects on the regular development of

Arabidopsis. However, the constitutive expression of *PDF1.2a* in *Arabidopsis* showed enhanced immunity but did not cause alteration in plant phenotype in normal growth conditions (Stotz *et al*., 2009; Hiruma *et al*., 2011). Thus, the transient expression of the *PDF* genes is unlikely due to its negative effects on *Arabidopsis*. I here also revealed that the expression of *PR1* was induced after *Ab* invasion, suggesting the activation of SA signaling pathway in invaded sites. It is known that the SA signaling works antagonistically with the JA signaling (Niki *et al*., 1998). Thus, the activation of the SA signaling via *Ab* invasion might result in the repression of the JA-related responses including the expression of the *PDF* genes.

EDR1 is not essential for preinvasive resistance to *Ab* (Fig. III-5), whereas *EDR1* is involved in preinvasive resistance to *Ctro*. Similarly, *PEN2* is not essential for preinvasive resistance to *Ab* (Fig. II-2D), whereas *PEN2* is involved in preinvasive resistance to *Ctro* (Hiruma *et al*., 2010). These results further support that plants take diverse strategies to control entry of fungal pathogens with different invasion strategies. In contrast to the case in preinvasive resistance, *EDR1* is involved in postinvasive resistance against *Ab*. Importantly, I here revealed that EDR1 is critical for the induced expression of the *PDF* genes upon *Ab* invasion. Thus, EDR1 is required for the induced expression of the *PDF* genes in response to different pathogenic fungi, *Ctro* and *Ab*, suggesting a conserved role of EDR1 for the pathogen-induced *PDF* expression. Also, previous studies indicated that EDR1 is not involved in the pathogen-mediated activation of Trp metabolism (Hiruma *et al*., 2011). Furthermore, I also revealed that EDR1 is not involved in the *Ab*-induced expression of *GLIP1* (Fig. III-6). These results suggested that EDR1-dependent expression of the *PDF* genes contributes to postinvasive resistance to *Ab*.

It is also known that the expression of the *PDF* genes is activated through transcription factor ORA59 in several conditions (Pré *et al*., 2008). In response to JA/ET signals, ORA59 binds to the GCC-box motif in the promoter region of *PDF1.2a* (Does *et al*., 2013) and activates its expression. I also revealed that *ORA59* is required for the

induced expression of the *PDF* genes in *Ab* inoculation, further supporting the critical roles of ORA59 for the expression of the *PDF* genes. Notably, *ORA59* is involved in postinvasive resistance against *Ab*. Thus, these results suggest the relation of EDR1- and ORA59-dependent *PDF* expression to *Arabidopsis* postinvasive resistance against *Ab*. In the case of *Ab* inoculation, lesion development in the *ora59* mutant was more severe than that in the *edr1* mutant (Fig. III-4A, 4B). Importantly, the expression of the *PDF* genes was mostly canceled in the *ora59* mutant in the *Ab* inoculation, whereas the *PDF* genes were slightly expressed in the *edr1* mutant (Fig. III-1). This finding further strengthens the contribution of EDR1- and ORA59-dependent *PDF* expression to the postinvasive resistance against *Ab*. Because EDR1 is a protein kinase, I also hypothesize that EDR1 might target ORA59, i.e., *Ab* invasion activates EDR1 via some unknown mechanism, and then EDR1 might activate ORA59 via direct interaction and phosphorylation of ORA59. In order to clarify this hypothesis, it is necessary to investigate (i) the phenotypes of the *edr1 ora59* double mutant to *Ab* inoculation, (ii) a possible interaction of EDR1 with ORA59, and (iii) a possible ability of EDR1 to phosphorylate ORA59.

It remains unclear about the recognition machinery of *Ab* invasion that triggers the expression of the *PDF* genes. The *Ab*-induced expression of the *PDF* genes was not canceled in the presence of the *bak1-5* mutation (Fig. III-7), indicating that *bak1-5* gives no negative effects on this immune response, in contrast to the *Ab*-induced expression of several secreted protein genes including *GLIP1*. Thus, EDR1-ORA59-PDFs pathway is not regulated by PRR system that is canceled by *bak1-5*. As I mentioned, the *Ab*-induced expression of genes involved in Trp-related secondary metabolism is not sensitive to *bak1-5*. Thus, a common upstream machinery related to recognition of *Ab* invasion might regulate the expression of both *PDF* genes and Trp-related metabolism genes. However, the expression pattern of the *PDF* genes in *Ab* infection is distinct from that of Trp-related metabolism genes (Fig. III-1 and III-2), thus, alternatively, distinct pathogen recognition machineries might independently regulate Trp-related metabolism pathway and EDR1-ORA59-PDFs pathway at gene expression level. Further studies on recognition

machineries for pathogen invasion is prerequisite for understanding of molecular background of postinvasive resistance in higher plants.

MATERIALS AND METHODS

Fungal materials

The fungal cultures used in this chapter was described in Chapter II.

A. thaliana lines and growth conditions

The *A. thaliana* accession Col-0 was used as the WT plant. The mutants *pen2-1*, *pen2-2* (Lipka *et al*., 2005), *edr1-1* (Frye *et al*., 2011), *ora59* (Pré *et al*., 2003), *pen2 edr1* (Hiruma *et al*., 2011), *pen2 bak1-5* (in Chapter II) were used in this study. *Arabidopsis* seeds were sown on rockwool (Grodan; http://www.grodan.com) and kept at 4˚C in the dark for 2 days, and later grown at 25˚C with a cycle of 16 h light and 8 h dark in Hoagland medium. *Arabidopsis* lines used in this study was listed on Table III-2.

Pathogen inoculation, lesion development and fungal invasion assays.

Pathogen inoculation, lesion development and fungal invasion assays performed in this chapter was described in Chapter II.

RT–qPCR analysis

RT–qPCR analysis was performed in according to the methods described in Chapter I. The primers used in this study are listed in Table III-1.

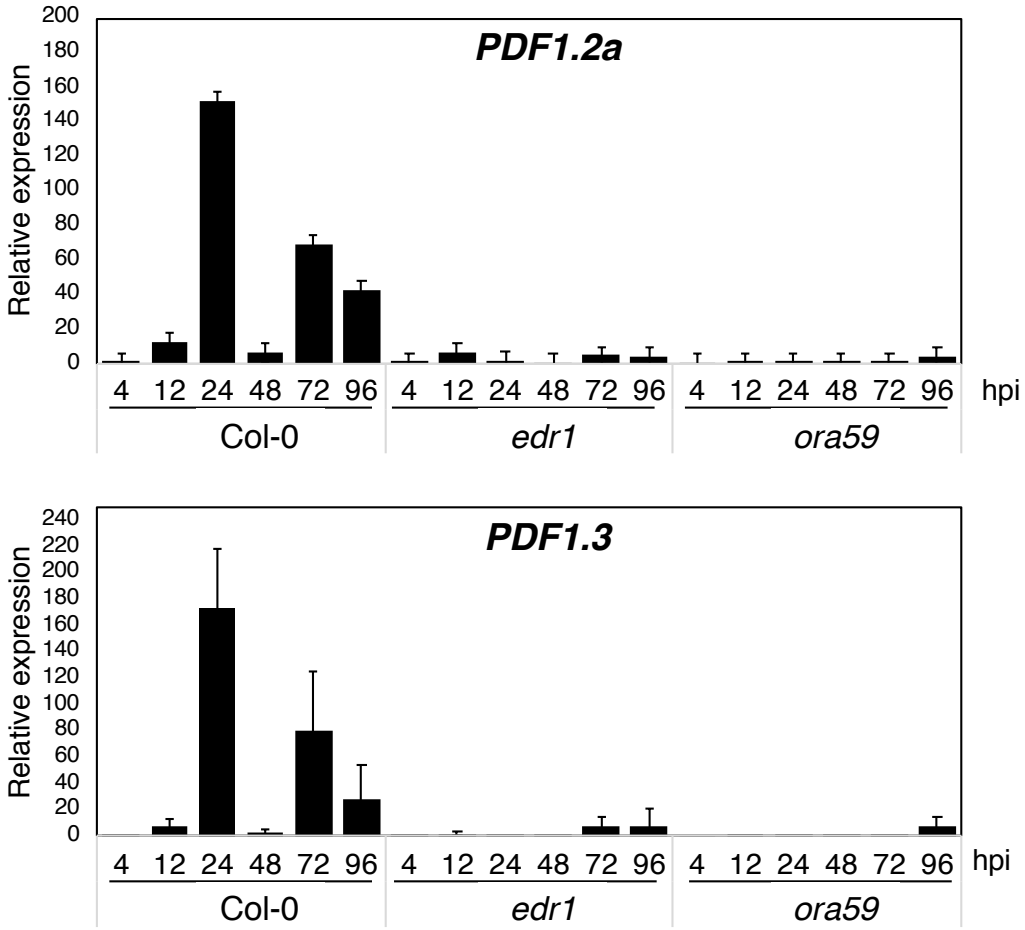

Fig. III-1. *Ab* invasion triggered the activation of antimicrobial peptide genes *PDF1.2a* and *PDF1.3*.

Ab conidial suspension ($5x10^5$ conidia/mL) was spray-inoculated onto 4 to 5-week-old *Arabidopsis* plants, and inoculated leaves were collected at 4, 12, 24, 48, 72 and 96 hours post inoculation (hpi). *PDF1.2a* and *PDF1.3* transcripts were quantified by RT-qPCR using the gene specific primers listed in Table III-1. Values were normalized to the expression level of *UBC21*. The means and SDs were calculated from three independent experiments.

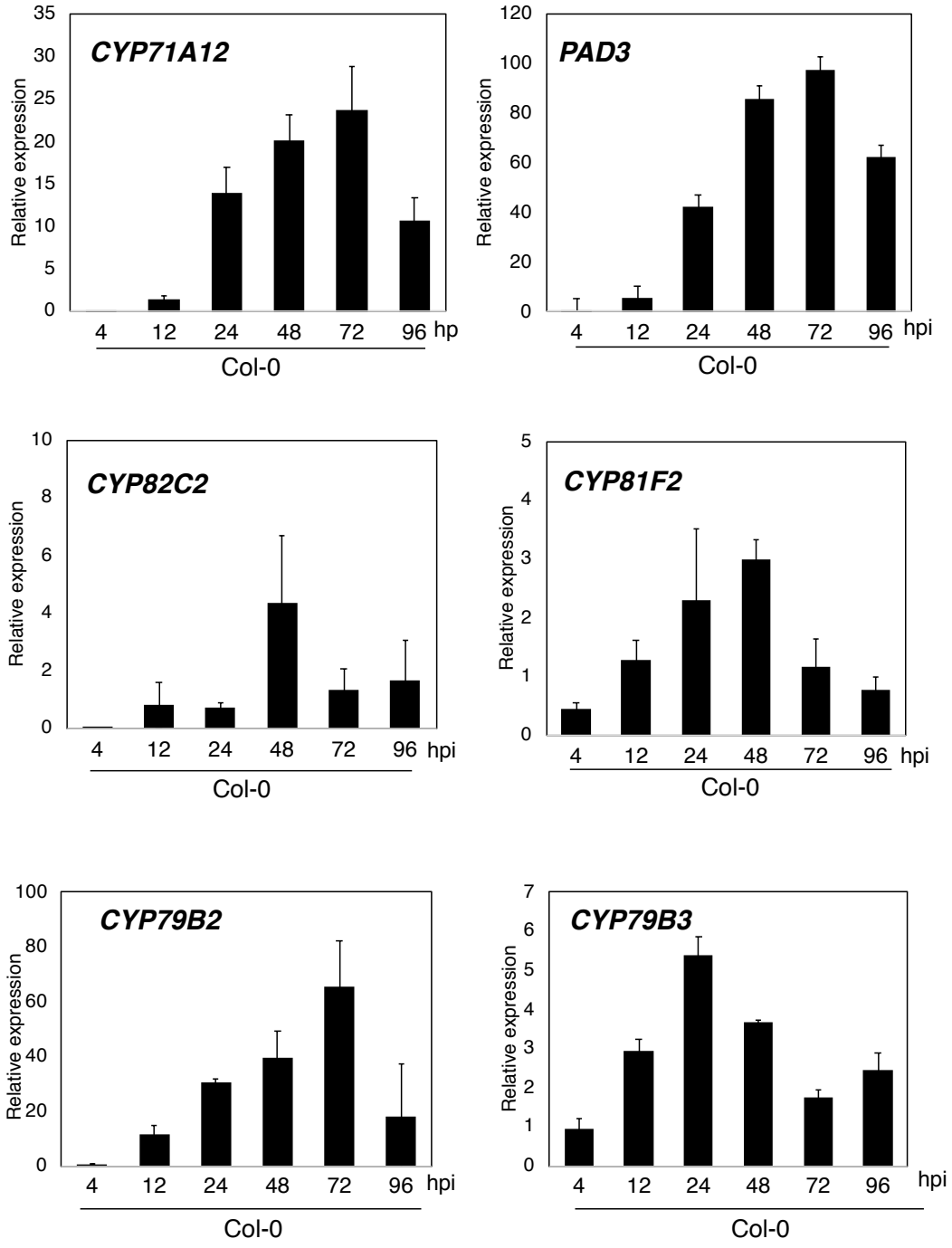

Fig. III-2. Expression pattern of Trp-pathway related genes in *Ab* inoculation.
Ab conidial suspension ($5x10^5$ conidia/mL) was spray-inoculated onto 4 to 5-week-old *Arabidopsis* WT Col-0 plant, and inoculated leaves were collected at 4, 12, 24, 48, 72 and 96 hours post inoculation (hpi). *CYP71A12*, *PAD3*, *CYP82C2*, *CYP81F2*, *CYP79B2* and *CYP79B3* transcripts were quantified by RT-qPCR using the gene specific primers listed in Table III-1. Values were normalized to the expression level of *UBC21*. The means and SDs were calculated from three independent experiments.

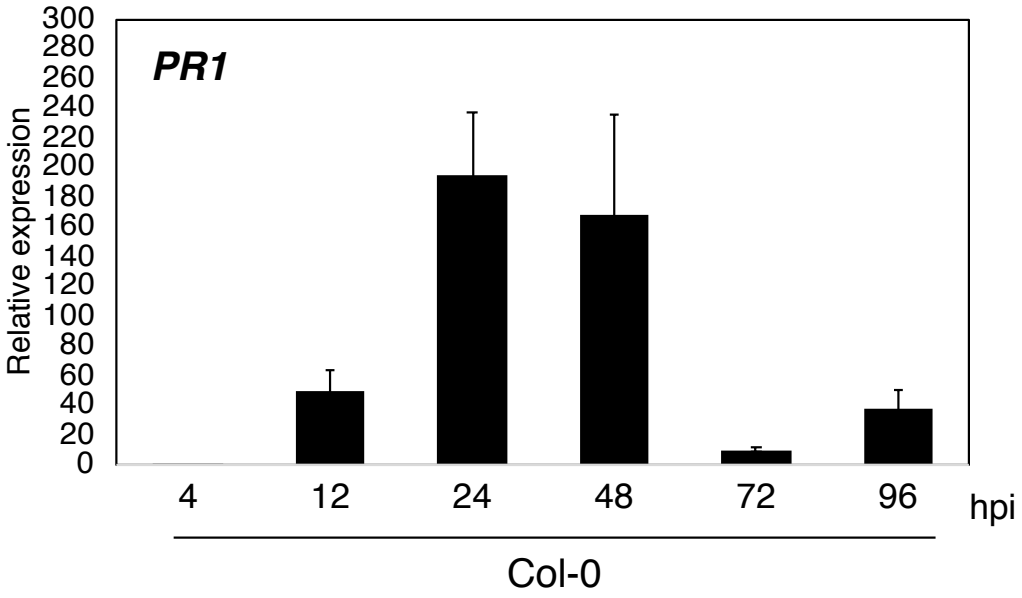

Fig. III-3. *Ab* invasion triggered the expression of a SA-signaling marker *PR-1*.
Ab conidia suspension ($5x10^5$ conidia/mL) was spray-inoculated onto 4 to 5-week-old *Arabidopsis* WT Col-0 plant, and inoculated leaves were collected at 4, 12, 24, 48, 72 and 96 hours post inoculation (hpi). *PR1* transcript was quantified by RT-qPCR using the gene specific primers listed in Table III-1. Values were normalized to the expression level of *UBC21*. The means and SDs were calculated from three independent experiments.

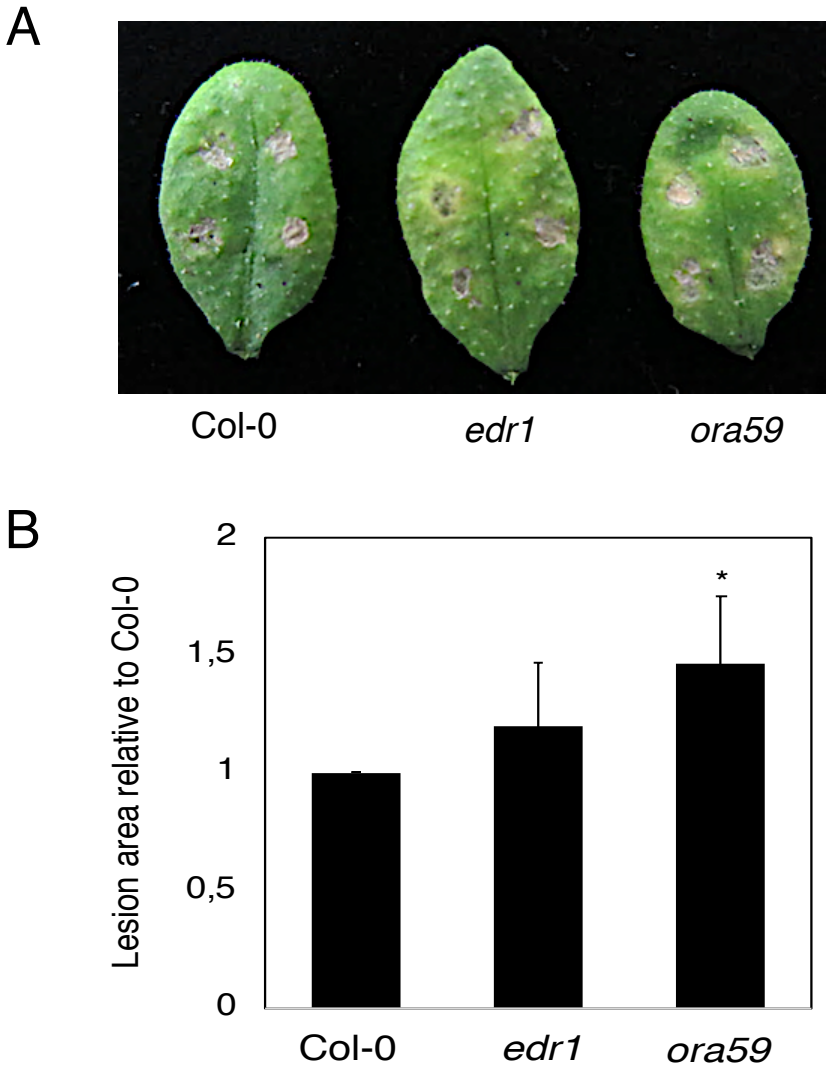

Fig. III-4. EDR1 and ORA59 contribute to the postinvasive defense against *Ab*.
(A) Lesion development in the case of *Ab* inoculation. Conidial suspension of *Ab* (1x 10^5 conidia/mL) was drop-inoculated onto mature leaves of 4 to 5-week-old plants of the tested *Arabidopsis* lines. The photogpah was taken at 4 dpi.
(B) Quantitative analysis of lesion development caused by *Ab*. Conidial suspension of *Ab* (1x 10^5 conidia/mL) was drop-inoculated onto mature leaves of 4–5-week-old plants of the tested *Arabidopsis* lines. At 4 dpi, lesion development was measured. The means and SDs were calculated from three independent experiments. For the statistical analysis, lesion development of *edr1* and *ora59* was compared with WT Col-0 using two-tailed Student's *t* tests (*$P < 0.05$).

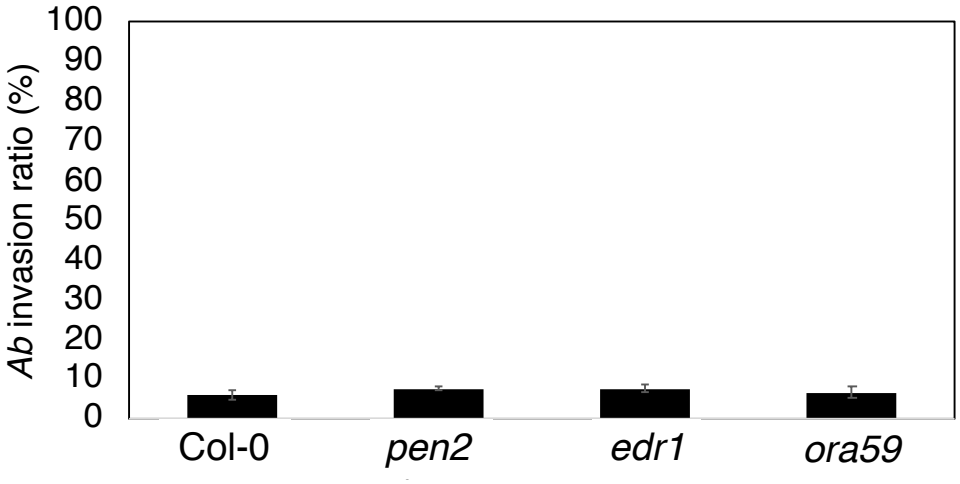

Fig. III-5. EDR1 and ORA59 are not essential for preinvasive defense to *Ab*.
Conidial suspension (1 x 10^5 conidia/mL) of *Ab* was drop-inoculated onto 4 to 5-week-old plants and kept at 100% humidity. The inoculated leaves were collected at 12 hpi, and then subjected to trypan blue viability staining assay. The presence or absence of invasive hyphae from at least 50 germinating conidia were counted in each experiment. The means and SDs were calculated from three independent experiments. Statistical comparisons of the *Ab* invasion ratios on WT Col-0 and tested mutants were conducted using two-tailed Student's *t* tests. The invasion ratios of mutants were not significantly different from WT Col-0.

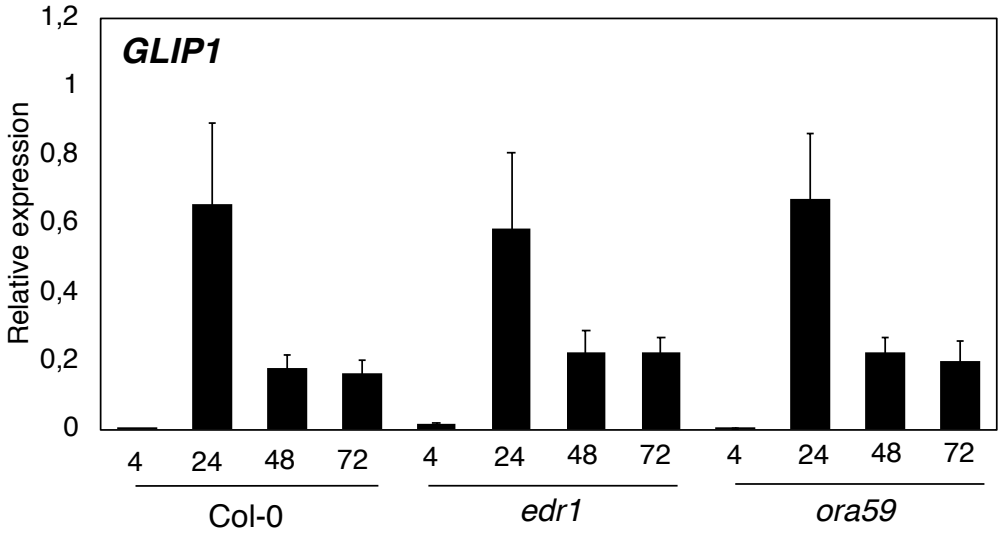

Fig. III-6. The expression of *GLIP1* was not affected in *edr1* and *ora59*.
Ab conidial suspension (5 x 10^5 conidia/mL) was spray-inoculated onto 4 to 5-week-old *Arabidopsis* WT Col-0, *edr1* mutant and *ora59* mutant plant. The inoculated leaves were collected at 4, 24, 48 and 72 hours post inoculation (hpi). *GLIP1* transcript was quantified by RT-qPCR using the gene specific primers listed in Table III-1. Values were normalized to the expression level of *UBC21*. The means and SDs were calculated from three independent experiments.

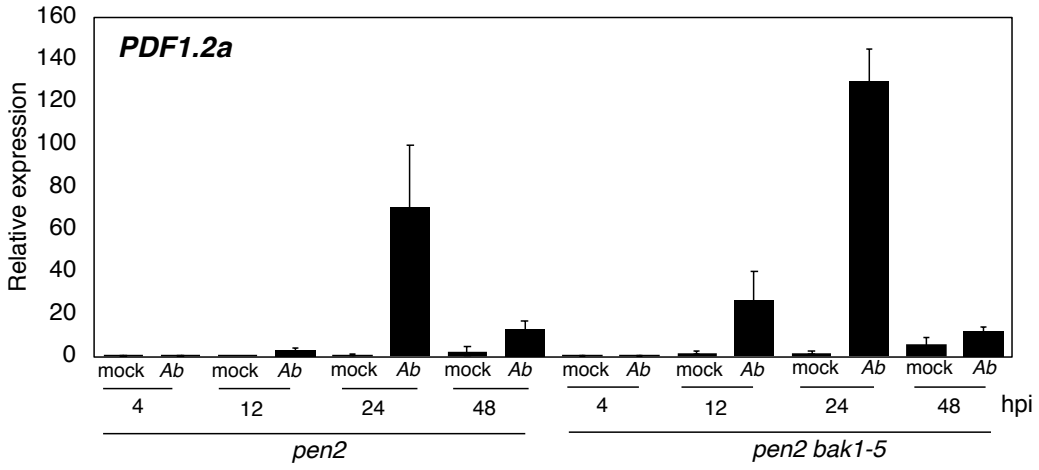

Fig. III-7. The *Ab*-triggered expression of *PDF1.2a* is not reduced by the *bak1-5* mutation.

Ab conidial suspension (5 x 10^5 conidia/mL) was spray-inoculated onto 4 to 5-week-old *Arabidopsis pen2* and *pen2 bak1-5* mutant plant, and the inoculated leaves were collected at 4, 12, 24 and 48 hours post inoculation (hpi). *PDF1.2a* transcript was quantified by RT-qPCR using the gene specific primers listed in Table III-1. Values were normalized to the expression level of *UBC21*. The means and SDs were calculated from three independent experiments.

Table III-1. List of primers used in this study

Primers used for RT-qPCR			
Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	
PDF1.2a (At5g44420)	TTTGCTGCTTTCGACGCAC	CGCAAACCCCTGACCATG	
PDF1.3 (At2g26010)	AAGCACCGATAATGGTGGAAGCAC	GTATAATTGGTAGTCATTGGTAGC	
CYP71A12 (At2g30750)	CATTCCCTAAGCCTTCGGTAC	CTTGGAGTTTCTTCATAACA	
CYP82C2 (At4g31970)	CATTTGGTTCGGGAAGAAGA	AGCCAGGGCTCTCAGTCATA	
PAD3 (At3g26830)	TGCTCCCAAGACAGACAATG	GTTTTGGATCACGACCCATC	
UBC (At5g25760)	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC	
CYP79B2 (At4g39950)	GTAACTTCGGAGCATTCGT	TCGCCGGATATCACATCC	
CYP79B3 (At2g22330)	CAGCCTTTGCTTACCGCTGAT	GGCGTTTGATGGGTTGTCTG	
CYP81F2 (At5g57220)	ATTGTCCGCATGGTCACAGGGAG	GTAGCCGTGTCCGAACACTTTAAG	
PR1 (At2g14610)	AAGAGGCAACTGCAGACTCA	TCTCGCTAACCCACATGTTC	
GLIP1 (At5g40990)	GAGCTGATTTGGAGCGGACCTACC	CGAGTGATATATATCGCTCGCG	
Primers used for genotyping			
Mutant name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Enzyme
pen2-1	TCAGGTAAATCAGTTCGAATCAAGAAC	TGAGGAAACCTGTTGGAGAAAGGATC	BamHI
bak1-5	AAGAGGGCTTGCGTATTTACATGATCAGT	GAGGCGAGCAAGATCAAAAG	RsaI

Table III-2. List of *Arabidopsis* lines used in this study

Arabidopsis thaliana line	Reference
Col-0 (WT)	
pen2-1	Lipka *et al.,* 2005
edr1	Frye *et al.,* 2001
ora59	Pré *et al.,* 2008
pen2 bak1-5	In this study

SUMMARY

CHAPTER I. CYP71A12-dependent biosynthesis of indole-3-carboxylic acids is involved in the postinvasive resistance against *Colletotrichum tropicale*

Arabidopsis thaliana mounts nonhost resistance response against nonadapted hemibiotrophic fungus *Colletotrichum tropicale* (*Ctro*), which requires PEN2-dependent synthesis of antifungal metabolites derived from tryptophan (Trp). When *C. tropicale* invades *Arabidopsis* defective in this entry control, postinvasive resistance is newly activated as a second layered defense, which blocks further expansion of invasive hyphae. CYP79B2 and CYP79B3 are key enzyme for the biosynthesis of Trp-derived secondary metabolites. *cyp79B2 cyp79B3* mutant is fully susceptible to *Ctro*, suggesting the importance of Trp-pathway derived secondary metabolites for the immunity. This mutant is also defective in both pre- and postinvasive resistance against *Ctro*, indicating the importance of Trp-pathway derived secondary metabolites. Analysis of series of *Arabidopsis* mutants defective in Trp-metabolism pathway revealed that indole-3-carboxylic acids derivatives (ICAs) as well as camalexin are indispensable for the postinvasive resistance, whereas these secondary metabolites were dispensable for the preinvasive resistance to *Ctro*. Metabolites profiling of *Arabidopsis* mutants treated with *Ctro* showed that CYP71A12 has an important contribution to the accumulation of ICAs whereas CYP71A13 is critical for camalexin accumulation upon pathogen infection. These findings suggest distinct roles of these two homologous P450 monooxygenases in the immune responses. The postinvasive resistance to *Ctro* was associated with plant cell death similar to HR cell death. To assess whether plant cell death observed in postinvasive resistance to *Ctro* is mediated by NLRs, I utilized mutations in *RAR1* and *SGT1*. Analysis of fungal hyphal expansion showed that *RAR1* and *SGT1* have no clear contribution to postinvasive resistance and HR-like cell death in response to the invasion by *Ctro*.

CHAPTER II. *bak1–5* mutation uncouples tryptophan-dependent and independent postinvasive immune pathways triggered in *Arabidopsis thaliana* by multiple fungal pathogens

I found that both CYP71A12 and CYP71A13 are critical for *Arabidopsis*' postinvasive resistance toward both the necrotrophic *Alternaria brassicicola* and the adapted hemibiotrophic *C. higginsianum* in addition to *Ctro*. Metabolite analyses suggest that the production of ICAs and camalexin is induced upon pathogen invasion, while phenotypic comparison of *cyp79B2 cyp79B3* and *pen2 cyp71A12 cyp71A13* plants indicates that the contribution of ICAs to postinvasive resistance is dose-dependent. I also found that the *bak1–5* mutation significantly reduced postinvasive resistance against *C. tropicale* and *A. brassicicola*, indicating that a pattern recognition receptor complex commonly contributes to this second defense-layer against pathogens with distinct infection strategies. Unexpectedly, I revealed that the *bak1–5* mutation had no detectable effects on Trp-metabolite accumulation triggered by pathogen invasion. Further comparative gene expression analyses suggested that pathogen invasion in *Arabidopsis* activates (i) *bak1–5* insensitive Trp-metabolism that leads to antimicrobial small molecules and (ii) a *bak1–5* sensitive immune pathway that activates the expression of antimicrobial protein genes such as *AED1*, *BGL2*/*PR2*, *GLIP1* and *RLP23*.

CHAPTER III. EDR1- and ORA59-dependent expression of plant defensin contributes to the postinvasive resistance against fungal pathogen

Arabidopsis exhibits durable resistance that is composed by two layers of defense: preinvasive resistance and postinvasive resistance. *PEN2* and *EDR1* is involved in the preinvasive resistance against *Ctro*. In preinvasive resistance to *Ctro*, both PEN2-dependent synthesis of Trp-related antifungal metabolites and EDR1-regulated expression of plant defensins (PDFs) are necessary. However, it remains to be elucidated

(i) whether EDR1 also regulates the expression of PDFs during postinvasive defense and (ii) whether the expression of PDFs is involved in postinvasive defense against fungal pathogens. I found that the invasion by *A. brassicicola* resulted in the transient expression of *PDFs* where it resulted in the sustained expression of Trp-metabolism pathway genes. I also revealed that *EDR1* is required for the induced expression of the *PDF* genes upon the pathogen invasion. ORA59 is a transcription factor that regulates the expression of *PDFs*. Here, I also found that *ORA59* is critical for the induced expression of the *PDF* genes upon the pathogen invasion. The pathogen-induced expression of *PDFs* is not canceled by the *bak1-5* mutation. Importantly, the inoculation assay of *A. brassicicola* indicated that *EDR1* and *ORA59* are involved in the postinvasive resistance against *A. brassicicola*, suggesting the involvement of PDFs in the *Arabidopsis* postinvasive resistance in addition to the Trp-related secondary metabolites.

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