ROLES OF JASMONATE SIGNALING AND STOMATAL DEFENSE IN THE *ARABIDOPSIS THALIANA-PSEUDOMONAS SYRINGAE* INTERACTION

By

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ABSTRACT

ROLES OF JASMONATE SIGNALING AND STOMATAL DEFENSE IN THE *ARABIDOPSIS THALIANA-PSEUDOMONAS SYRINGAE* INTERACTION

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Plants encounter various biotic and abiotic stresses throughout their life cycles. Accordingly, plants have developed sophisticated defense strategies to cope with stresses. Among these, the plant hormone jasmonate (JA) bestows upon plants the ability to defend against attacks by a wide variety of herbivores as well as necrotrophic pathogens. Perception of pathogen or herbivore attacks promotes synthesis of jasmonoyl-$_{L}$-isoleucine (JA-Ile), the bioactive form of JA, which binds to the COI1-JAZ receptor, triggering degradation of JAZ repressors and induction of transcriptional reprogramming associated with plant defense. Interestingly, some virulent pathogens have evolved strategies to manipulate JA signaling to facilitate their exploitation of plant hosts. For example, strains of the bacterial pathogen *Pseudomonas syringae* produce proteinaceous effectors as well as a JA-mimicking toxin, coronatine (COR), to activate JA signaling and promote disease susceptibility. In the first part of my dissertation research, I explored the possibility that targeted modification of the JA receptor could be a promising new approach to "protect" the disease-vulnerable components of plants. Guided by the crystal structure of the COI-JAZ receptor and evolutionary clues, I succeeded in modifying the JA receptor to allow for sufficient endogenous JA signaling but greatly reduced sensitivity to COR. Transgenic Arabidopsis expressing this modified receptor not only are fertile and maintain a high level of insect defense, but also gain the ability to resist COR-producing pathogens *P. syringae* pv. *tomato* (*Pst*) DC3000 and *P. s.* pv. *maculicola* (*Psm*) ES4326.

The second part of my dissertation research investigates pathogen-induced stomatal closure as an innate immune response. Studies have shown that stomatal closure plays a role in restricting bacterial invasion, whereas highly evolved pathogens produce virulence factors, such as COR in the case of *P. syringae*, to counteract stomatal defense. A previous genetic screen led to identification of six Arabidopsis *scord* (susceptibility to a COR-deficient mutant of *Pst* DC3000) mutants that are defective in bacterium-triggered stomatal closure. I attempted and succeeded in cloning two *SCORD* genes. *SCORD6* encodes a GDP-$_D$-mannose-4,6-dehydratase involved in the de novo synthesis of GDP-$_L$-fucose and *SCORD7* codes for the TRICHOME BIREFRINGENCE (TBR) protein, belonging to TBR-Like protein family, which is proposed to be involved in synthesis and/or modification of pectin or *O*-acetylation of xyloglucan and xylan. Both *scord6* and *scord7* are defective in pathogen-/salicylic acid (SA)-mediated stomatal closure but not in abscisic acid (ABA)-mediated stomatal closure. The identification of *SCORD6* and *SCORD7* genes highlights plant cell-wall-based regulation of stomatal defense and contributes to the general understanding of the multifaceted host defense mechanisms against pathogen infection in plants.

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CHAPTER I

LITERATURE REVIEW: JASMONATE SIGNALING, PATHOGEN/INSECT MANIPULATION OF JA SIGNALING, AND PLANT STOMATAL IMMUNITY

Parts of this chapter are included in a review paper currently under minor revision: Li Zhang, Feng Zhang, Maeli Melotto, Jian Yao, Sheng Yang He (2016) Jasmonate signaling and manipulation by pathogens and insects *J. Exp. Bot.* in revision.

I would like to acknowledge Feng Zhang, Maeli Melotto, and Jian Yao for their contributions to the writing and/or editing of the review article "*Jasmonate signaling and manipulation by pathogens and insects.*"

Abstract

Plants are continuously exposed to various abiotic and biotic stresses throughout their life cycles. Sophisticated signaling networks are developed by plants to defense themselves against pathogen infection, including regulation of jasmonate (JA) signaling and plant stomatal movements. Plants synthesize JA upon developmental cues or environmental stresses to mediate plant development or defense against pathogens and herbivores. Perception of pathogen or herbivore attacks promotes accumulation of jasmonoyl-$_L$-isoleucine (JA-Ile), which binds to the COI1-JAZ receptor, triggering degradation of JAZ repressors and induction of transcriptional reprogramming associated with plant defense. Meanwhile, plant stomata respond to pathogen infection and restrict the entry of pathogens into the apoplastic space via closure of stomata. Recent studies have shown that stomatal closure could be mediated by pathogen-associated

molecular patterns (PAMPs) as well as the plant hormones salicylic acid (SA) and abscisic acid (ABA). Interestingly, some virulent pathogens have evolved various strategies to manipulate JA signaling to facilitate their exploitation of plant hosts and to interfere with PAMP-induced stomatal closure. In this chapter, I will review recent advances in the elucidation of the switch mechanism between transcriptional repression and hormone-dependent transcriptional activation of JA signaling at the protein structural level, various strategies used by pathogens/insects to manipulate JA signaling and the signaling networks that regulate stomatal closure.

Introduction of jasmonate (JA) signaling

Plants encounter various biotic and abiotic stresses throughout their life cycles. The lipid-derived hormone JA bestows upon plants the ability to defend against attacks by a wide variety of herbivores as well as necrotrophic pathogens that kill plant cells for nutrition. However, JA signaling has also been shown to mediate defense against some biotrophic/hemibiotrophic pathogens that obtain nutrients primarily on living plant cells, such as rice resistance to *Meloidogyne graminicola* and *Xanthomonas oryzae* (De Vleesschauwer et al. 2013; Nahar et al. 2011). In addition to its role in regulating defense, JA is also required for plant reproduction and other growth and developmental processes, including lateral and adventitious root formation, seed germination, leaf senescence, as well as formation of glandular trichomes, resin ducts, and nectaries (Campos et al. 2014; Kazan 2015; Wasternack and Hause 2013; Wasternack and Strnad 2016). Interestingly, glandular trichomes, resin ducts, and nectaries can produce diverse compounds that are directly or indirectly involved in plant defense, linking JA's dual roles in development and defense (Campos et al. 2014; Dicke and Baldwin 2010).

A number of recent reviews have discussed topics from JA biosynthesis to molecular genetic dissection of JA signaling (Campos et al. 2014; Goossens et al. 2016; Wasternack and Hause 2013). I refer readers to these excellent reviews. Here I will focus on recent literatures on the elucidation of the structural mechanisms involved in transcriptional repression and activation of JA signaling, the various strategies used by pathogens/insects to manipulate JA signaling, and innovative approaches to interrupt pathogen hijacking of JA signaling for disease control.

Initiation of JA signaling during pathogen/herbivore attacks

It is now widely accepted that pathogen and herbivore attacks are associated with the generation of a variety of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs, such as flagellin), herbivore-associated molecular patterns (HAMPs, such as insect secretions) and/or damage-associated molecular patterns (DAMPs, such as plant cell wall-derived oligogalacturonides and systemin or systemin-like peptides) (Campos et al. 2014; Felton and Tumlinson 2008; Heil and Land 2014; Hogenhout and Bos 2011; Mithofer and Boland 2008; Yamaguchi and Huffaker 2011). These attacker-associated patterns are recognized by plant pattern recognition receptors (PRRs) located at the plant plasma membrane (Brutus et al. 2010; Choi et al. 2014; Mousavi et al. 2013; Qi et al. 2006; Song et al. 2006; Yamaguchi et al. 2010; Yamaguchi et al. 2006). Significant overlaps of gene expression, including genes involved in defense hormone signaling, were observed in PAMP/HAMP/DAMP responses in several genome-wide transcriptome studies (Campos et al. 2014). Studies have also shown rapid accumulation of JA in response to a wide range of MAMPs/HAMPs/DAMPs (Bonaventure et al. 2011; Campos et al. 2014; Doares et al. 1995; Huffaker et al. 2006; Huffaker et al. 2013; Kim et

al. 2014; Lee and Howe 2003; Li et al. 2002; McCloud and Baldwin 1997; Schmelz et al. 2003; Schmelz et al. 2007; Yamaguchi and Huffaker 2011).

The exact mechanism by which MAMP/HAMP/DAMP signaling leads to JA biosynthesis remains elusive. Several intracellular signals, including calcium ions, reactive oxygen species, mitogen-activated protein kinase (MPK) cascades and calcium-dependent protein kinase (CDPKs), have been implicated in the signal transduction from perception of conserved patterns to induction of JA biosynthesis (Ahmad et al. 2016; Arimura and Maffei 2010; Heinrich et al. 2011; Romeis and Herde 2014; Sato et al. 2010; Singh and Jwa 2013; Zebelo and Maffei 2015). Nevertheless, it is not clear whether any of the enzymes involved in the biosynthesis of JA is regulated by CDPK/MPK-mediated phosphorylation, calcium/calmodulin binding or cellular redox changes, although JA-induced phosphorylation of JA signaling components have been observed (Katou et al. 2005; Zhai et al. 2013). JA is synthesized through the oxylipin biosynthesis pathway (Gfeller et al. 2010; Wasternack 2007) starting from the α-linolenic acid that is released from chloroplastic membranes after pathogen/insect attacks. Subsequent catalysis is processed by LIPOXYGENASE (LOX), ALLENE OXIDE SYNTHASE (AOS) and ALLENE OXIDE CYCLASE (AOC) to generate 12-oxo-phytodienoic acid (OPDA) in the chloroplast. OPDA is then transported into the peroxisome, where several cycles of β-oxidation take place and (+)-7-iso-JA is synthesized. After being secreted into the cytosol, (+)-7-iso-JA is conjugated with the amino acid isoleucine (Ile) to generate JA-Ile, the most bioactive form of JA (Wasternack and Hause 2013). Meanwhile, the JA pool in cytosol is converted into JA metabolites to attenuate JA signaling, such as hydroxylation and/or carboxylation (Heitz et al. 2012; Kitaoka et al. 2011; Koo et al. 2011; Koo and Howe 2012). A major area of future

research would be to directly connect MAMP/HAMP/DAMP signaling, which appears to occur mainly in the plasma membrane, cytosol and nucleus, to JA biosynthesis, which occurs mainly in the chloroplast and peroxisome.

The core JA sensing and signaling module

Since the cloning of the *CORONATINE INSENSITIVE1* (*COI1*) gene in 1998 (Xie et al. 1998), which was later found to encode a main component of the JA-Ile receptor complex (Katsir et al. 2008; Melotto et al. 2008a; Sheard et al. 2010; Thines et al. 2007), the core elements of the JA signaling complex have been extensively characterized in the past two decades, leading to a convincing framework for JA perception and initial signal transduction. In "stress-free" plants with low levels of JA, JA-mediated responses are restrained by JASMONATE ZIM DOMAIN (JAZ) proteins (Chini et al. 2007; Fonseca et al. 2009; Thines et al. 2007; Yan et al. 2007). JAZ functions as transcription repressors by binding and repressing the clade IIIe of basic helix-loop-helix (bHLH) transcription factors (TFs) MYCs (Fig. 1A) (Boter et al. 2004; Chini et al. 2007; Heim et al. 2003; Lorenzo et al. 2004). JAZ8 repressor can recruit the TOPLESS (TPL) family of corepressors directly through the TPL-binding ETHYLENE RESPONSE FACTOR (ERF)-ASSOCIATED AMPHIPHILIC REPRESSION (EAR) motif "LxLxL" (Shyu et al. 2012). Besides JAZ8, EAR motifs ("LxLxL" or "DLNxxP") were also identified in JAZ5, JAZ6 and JAZ7 proteins (Kagale et al. 2010) and direct interaction between TPL and JAZ5/6 were detected in interactome experiments (Arabidopsis Interactome Mapping Consortium 2011; Causier et al. 2012). Alternatively or in addition, most other JAZ proteins recruit TPL through binding, *via* the ZIM domain, to the EAR motif-containing adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) (Acosta et al. 2013; Kazan 2006; Pauwels et al. 2010). TPL proteins in turn recruit

histone deacetylases (HDCAs), such as HDA6 and HDA19, resulting in chromatin remodeling and suppression of JA-responsive gene expression (Long et al. 2006; Wu et al. 2008; Zhou et al. 2005). Additionally, JAZ1, JAZ3, and JAZ9 can directly interact with HDA6 leading to chromatin remodeling and repression of JA-responsive genes independently of NINJA and TPL proteins (Zhu et al. 2011).

In response to stresses, plant synthesizes JA-Ile, which directly promotes the interaction between JAZ and COI1, the F-box subunit of the SCF^{COI1} ubiquitin E3 ligase (Fig. 1B) (Katsir et al. 2008; Melotto et al. 2008a; Sheard et al. 2010; Thines et al. 2007; Yan et al. 2009). This hormone-dependent interaction leads to ubiquitination and degradation of JAZ proteins *via* 26S proteasome, and thereby derepression of MYC TFs (Chini et al. 2007; Thines et al. 2007; Yan et al. 2007). JA stimulates extensive transcriptional reprogramming through two branches of transcription activators. In one branch, JA signaling responds to wounding or herbivore attack and induces the production of defense proteins, such as VEGETATIVE STORAGE PROTEIN (VSP), *via* MYCs (Lorenzo et al. 2004). In the other branch, JA acts synergistically with ethylene (ET) upon necrotrophic pathogen attack and induces the production of defense proteins, such as PLANT DEFENSIN1.2 (PDF1.2), *via* the APETALA2/ERF (AP2/ERF) TF family, such as ERF1 and OCTADECANOID-RESPONSIVE ARABIDOPSIS59 (ORA59) (Pieterse et al. 2012; Pre et al. 2008; Wasternack and Hause 2013; Zarei et al. 2011).

Besides the COI1-JAZ-MYC canonical JA-mediated signaling cascade, JAZ repressors also interact with several other TFs, highlighting the multiple roles of JAZ proteins in regulating plant

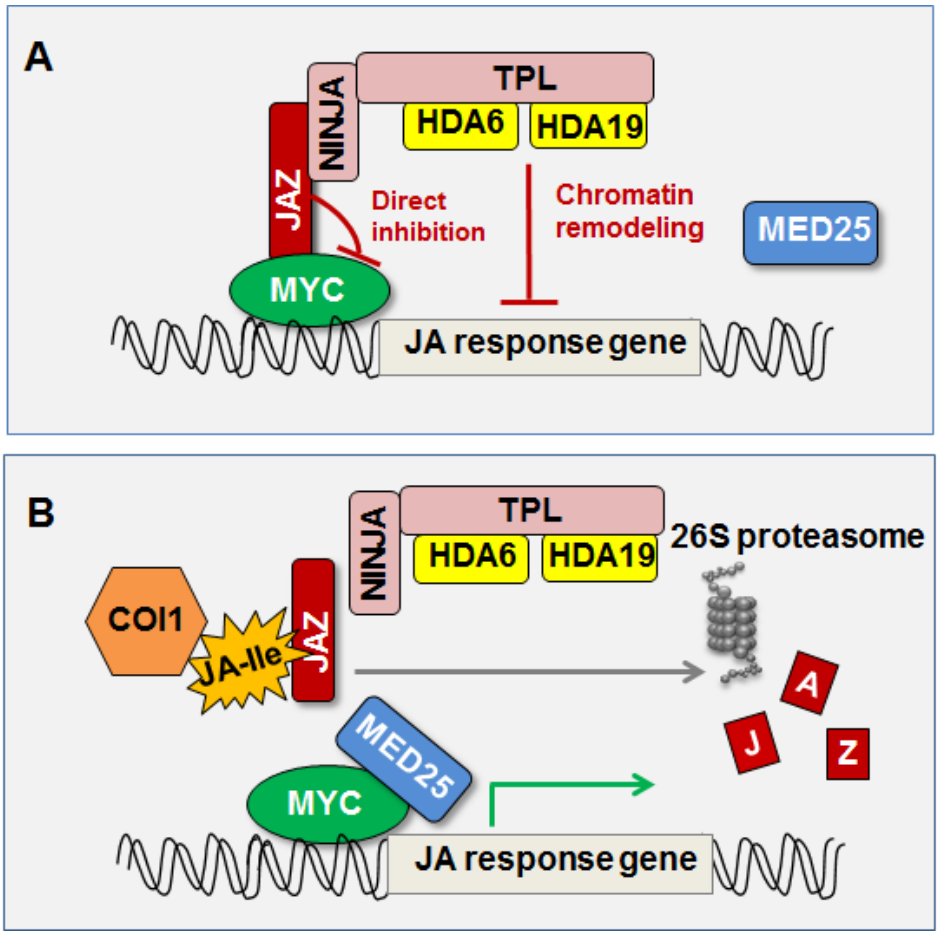

Figure 1: A model of JAZ-mediated transcriptional repression and JA-Ile perception-mediated transcriptional activation of JA signaling. A, In the resting stage, JA-responsive gene expression is suppressed by members of the JAZ protein family, which function as transcription repressors by binding and inhibiting MYC transcription factors through: (1) direct inhibition of the interaction between MYCs and the MED25 subunit of the Mediator co-activator complex (Zhang et al. 2015a); and/or (2) recruiting TOPLESS (TPL) co-repressors either directly (Shyu et al. 2012) or through the NINJA adaptor (Acosta et al. 2013; Kazan 2006; Pauwels et al. 2010). TPL in turn recruits histone deacetylases (HDA6 and HDA19) that repress gene expression through chromatin remodeling (Long et al. 2006; Wu et al. 2008; Zhou et al. 2005). JAZ1/3/9 also directly interact with HDA6 (Zhu et al. 2011). B, JA-Ile facilitates the interaction between JAZ and COI1 to form a co-receptor complex (Katsir et al. 2008; Melotto et al. 2008a; Sheard et al. 2010; Thines et al. 2007; Yan et al. 2009). This co-receptor complex leads to ubiquitination and proteasome-dependent degradation of JAZ repressors by the SCF^{COI1} E3 ubiquitin ligase, resulting in derepression of MYCs (Chini et al. 2007; Thines et al. 2007; Yan et al. 2007). JAZ-free MYCs form homo or heterodimers and bind to the conserved G-box (not shown) in the promoters of JA responsive genes (Fernandez-Calvo et al. 2011). By interacting with MED25 and possibly additional co-activators, MYCs recruit RNA polymerase II and other transcription components (not shown) to transcribe JA-responsive genes (Cevik et al. 2012; Chen et al. 2012).

biology (Chini et al. 2016). The identification of these JAZ-binding TFs shed lights on the mechanism of how JA signaling modulates and integrates plant responses upon developmental or environmental cues. Some examples are: (i) Two members of the R2R3 MYB TF family, MYB21 and MYB24, interact with all JAZ proteins to regulate JA-mediated male fertility (Mandaokar et al. 2006; Song et al. 2011); (ii) multiple JAZ proteins associate with the WD-repeat/bHLH/MYB transcriptional complexes, which comprise the WD-repeat TRANSPARENT TESTA GLABRA1 (TTG1), the clade IIIf bHLH TF TRANSPARENT TESTA8 (TT8), GLABRA (GL3) or ENHANCER OF GLABRA3 (EGL3), and the MYB TF GLABRA1 (GL1) or MYB75, resulting in the repression of the JA-mediated anthocyanin synthesis and trichome initiation (Grebe 2012; Pesch and Hulskamp 2009; Qi et al. 2014; Qi et al. 2011b; Traw and Bergelson 2003). In this case, JAZ proteins directly interact with the bHLH TFs and MYB TFs in the WD-repeat/bHLH/MYB complex and therefore interfere with assembly and function of the WD-repeat/bHLH/MYB complex (Qi et al. 2011b); (iii) Four TFs from the clade IIId bHLH, bHLH3/JA-ASSOCIATED MYC2-LIKE3 (JAM3), bHLH13/JAM2, bHLH14 and bHLH17/JAM1, which are phylogenetically closely related to MYC proteins, were identified to interact with JAZs. These bHLH TFs act as transcription repressors by antagonistically binding to the target sequence of MYC2 or the WD-repeat/bHLH/MYB complex and negatively regulate JA-mediated responses (Fonseca et al. 2014; Nakata et al. 2013; Sasaki-Sekimoto et al. 2013; Song et al. 2013); (iv) Several JAZ proteins interact with the clade IIIb bHLH TFs INDUCER OF CBF EXPRESSION1 (ICE1) and ICE2, resulting in repression of freezing tolerance of Arabidopsis (Hu et al. 2013). Binding specificities of these JAZ-bHLH complexes may be associated with fine-tuning of the JA-mediated responses (Chini et al. 2016); (v) besides the bHLH family, JAZ3 can interact with the YABBY (YAB) family TFs FILAMENTOUS

FLOWER (FIL)/YAB1 and YAB3, repressing JA-mediated anthocyanin accumulation. Moreover, MYB75 is a direct transcriptional target of FIL, regulating anthocyanin accumulation (Boter et al. 2015); (vi) JAZ1, JAZ3, and JAZ9 can bind to ETHYLENE INSENSITIVE3 (EIN3) and EIN3-LIKE1 (EIL1) TFs that positively regulate ET response, thereby suppressing the activity of EIN3 and EIL1 (Zhu et al. 2011); (vii) JAZ1, JAZ3, JAZ4, and JAZ9 proteins interact with the AP2 TFs TARGET OF EAT1 (TOE1) and TOE2. JA-triggered degradation of JAZ proteins release TOE1 and TOE2, both of which repress the transcription of *FLOWERING LOCUS T* (*FT*) and delays the flowering time of Arabidopsis (Zhai et al. 2015). (viii) WRKY57 is a repressor of JA-induced leaf senescence, and it interacts with JAZ4/8 or AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) protein IAA29, regulating the JA-auxin antagonism in leaf senescence (Jiang et al. 2014).

New structural insights into the COI1-JAZ-MYC signaling complex

While molecular, biochemical, and genetic studies support the view that COI1, JAZ, and MYC initiate a JA-dependent signaling cascade, the exact mechanisms of transcription repression or activation were unclear until recently. Two studies elucidated the high-resolution structures of the COI1-JAZ and JAZ-MYC complexes (Sheard et al. 2010; Zhang et al. 2015a). Specifically, the crystal structure of the COI1-JA-Ile-Jas^{JAZ1} complex shed light on how COI1 and JAZ proteins perceive JA-Ile (Sheard et al. 2010). In this structure, the three N-terminal α-helixes of COI1 bind to ASK1, a COI1-interacting subunit within the SCF^{COI1} ubiquitin E3 ligase complex, whereas the 18 tandem leucine-rich-repeats (LRRs) at the C-terminus form a binding pocket for JA-Ile. On the other hand, the Jas^{JAZ1} peptide adopts a bipartite structure in the presence of JA-Ile: (i) the five conserved N-terminal amino acids ("ELPIA") of the Jas^{JAZ1} motif forms a loop

that directly interacts with both JA-Ile and COI1 to trap JA-Ile into the ligand-binding pocket and (ii) the C-terminal region of the Jas^{JAZ1} motif forms an α-helix for docking to the top surface of the COI1 LRR domain (Fig. 2A). Inside the ligand binding pocket, the amide and carboxyl groups of JA-Ile bind to three basic residues of COI1 *via* a salt bridge and hydrogen bond network (Fig. 2C). Overall, the COI1-JAZ crystal structure is consistent with the radio-ligand binding assays showing both COI1 and JAZ proteins are required for high-affinity JA-Ile binding (Sheard et al. 2010). That is, JA-Ile is perceived as a high-affinity ligand by the COI1-JAZ co-receptor complex rather than COI1 or JAZ alone. In addition, the COI1-JA-Ile-JAZ structure provides a convincing explanation for the isomeric specificity of JA-Ile as a preferred ligand described previously (Fonseca et al. 2009; Staswick and Tiryaki 2004). In particular, (3*R*,7*S*)-JA-Ile has a higher binding affinity than (3*R*,7*R*)-JA-Ile to the COI1-JAZ co-receptor, because the aliphatic chain from (3*R*,7*R*)-JA-Ile interferes with binding to COI1 and JAZ1. Analysis of the COI1-JA-Ile-Jas^{JAZ1} crystal structure also led to an unexpected finding of inositol-1,2,4,5,6-pentakisphosphate ($InsP_5$) molecule as a cofactor for the COI1-JAZ interactions. $InsP_5$ molecule was found to interact with both the Jas^{JAZ1} peptide and three arginine residues of COI1 located at the bottom of the JA-Ile binding pocket (Fig. 2D). The InsP binding specificity to the JA receptor is largely determined by the COI1 protein (Laha et al. 2015; Laha et al. 2016).

In addition to binding to COI1 to form the COI1-JAZ co-receptor to perceive JA-Ile, the conserved Jas motif of JAZ proteins is also critical for interaction with MYC for mediating transcriptional repression (Cheng et al. 2011; Fernández-Calvo et al. 2011; Niu et al. 2011). How the same JAZ motif could engage COI1 (for hormone-dependent activation of JA signaling) and MYC (for repression of JA signaling) proteins remained a mystery until a recent report with

detailed MYC3-Jas^{JAZ1} complex structure (Zhang et al. 2015a). The N-terminus of MYC3 contains the JAZ-interacting domain (JID) and the transcription activation domain (TAD), which is required and sufficient for the JAZ-MYC interaction (Cevik et al. 2012; Chen et al. 2012; Fernández-Calvo et al. 2011). Zhang and colleagues (2015a) solved the structures of the Jas^{JAZ1}-MYC3 and Jas^{JAZ9}-MYC3 complexes, revealing that the N-terminus of MYC3 forms a helix-sheet-helix sandwich fold, in which the central five-stranded anti-parallel sheets are surrounded by eight helices (Fig. 2B). The α4 helix of the TAD forms a groove with the JID. In the MYC3-Jas^{JAZ1} and MYC3-Jas^{JAZ9} complex structures, the Jas peptide forms a single, continuous helix to occupy in the groove formed by JID and TAD and becomes an integral part of MYC3 N-terminal fold. Correspondingly, MYC3 undergoes a dramatic conformation change upon engaging the Jas motif sequence (Zhang et al. 2015a).

The JAZ-MYC3 structure provides mechanistic insights into the transcriptional repression and hormone-dependent activation of the JA pathway. First, comparison of the structures of the COI1-JA-Ile-JAZ and JAZ-MYC complexes reveals extensive overlaps in COI1 and MYC binding to the Jas motif. However, the COI1-JAZ interaction involves more amino acid residues in Jas domain compared to the Jas-MYC interaction. Specifically, the N-terminal portion ("ELPIA" in JAZ9) of the Jas motif that is critical for JAZ binding to JA-Ile/COI1 is not essential for MYC-JAZ interaction. Furthermore, in contrast to the continuous helix conformation of the Jas motif in the JAZ-MYC complex, the Jas motif in the COI1-JAZ complex adopts a bipartite conformation (Fig. 2A), involving JA-Ile-dependent unwinding of the N-terminal portion of the Jas helix. It is likely that the more extensive interactions in the COI1-JA-Ile-JAZ complex allow COI1 to compete with MYC for JAZ interaction upon JA-Ile stimulation.

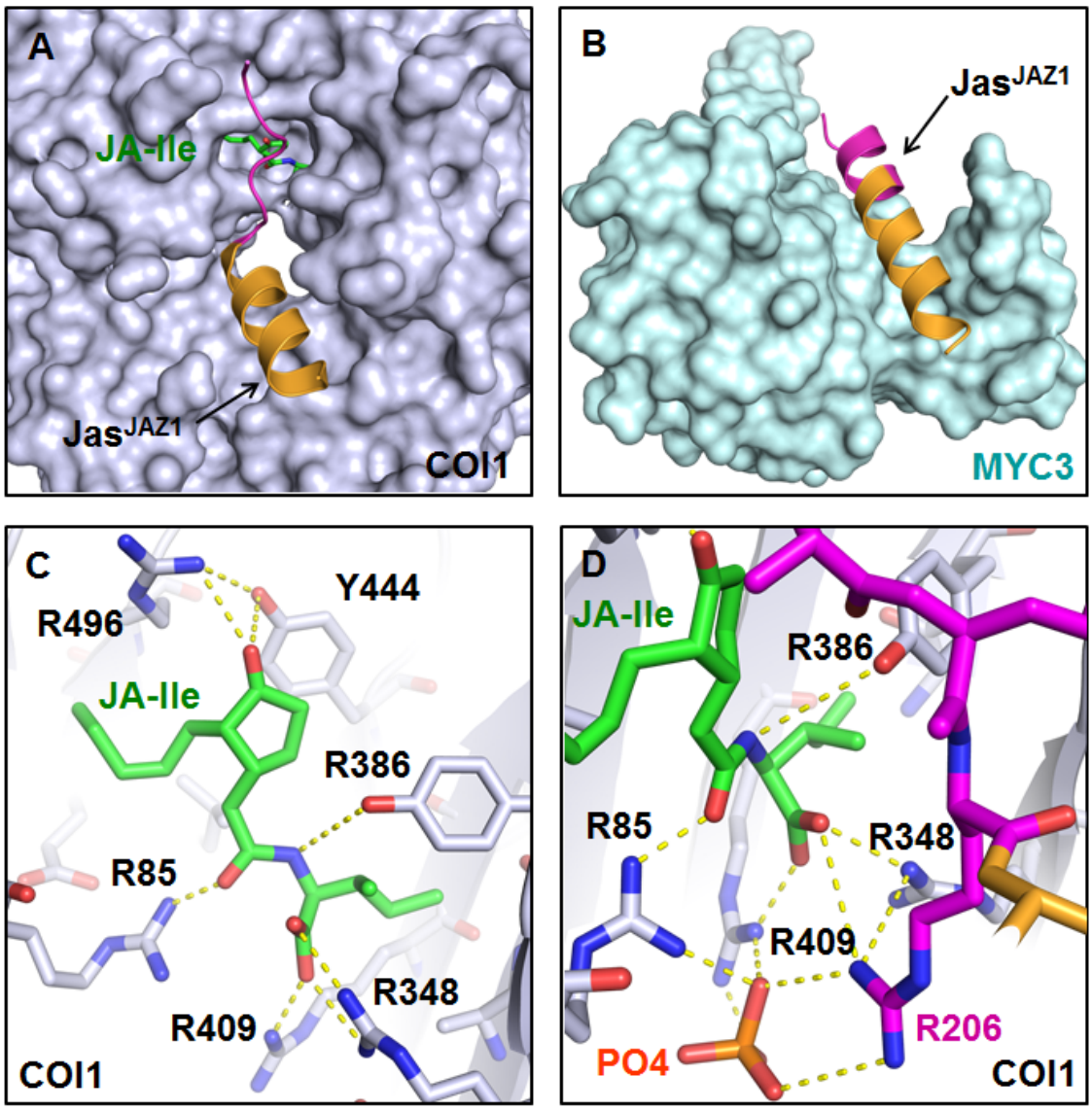

Figure 2: Crystal structures of the COI1-ASK1 complex with JA-Ile and the Jas^{JAZ1} peptide and the MYC3 (N-terminus) complex with the Jas^{JAZ1} peptide. A, In the COI1-Jas^{JAZ1} complex, COI1 forms a binding pocket for JA-Ile. The Jas^{JAZ1} peptide adopts a bipartite structure that contains an N-terminal loop region (magenta) and a C-terminal α-helix (orange). Only parts of the COI1 structure are shown and ASK1 is not shown. B, In the MYC3- Jas^{JAZ1} complex, the Jas^{JAZ1} peptide, adopting a single, continuous helix, occupies the groove formed by JID and TAD in the MYC3 N-terminus. C, JA-Ile interacts with residues in the COI1 ligand-binding pocket. Hydrogen bond and salt bridge networks are shown in yellow dashes. D, The hydrogen bond network in the phosphate (inositol phosphate)-binding site indicates that the $InsP_5$ is a crucial cofactor for JA perception. Images were generated using PyMol software (Schrodinger 2015) and the PDB files 3OGL (A, C, D) (Sheard et al. 2010) and 4YZ6 (B) (Zhang et al. 2015a).

Second, the Jas helix occupies the groove formed by the JID and the TAD in the JAZ-MYC3 structure, and makes direct contacts not only with the JID, but also with the TAD required for transcriptional activation. This unexpected finding suggests possibly a direct competition between the JAZ repressor and transcription coactivators. Indeed, Zhang et al. showed that JAZ could directly inhibit the interaction between MYC3 and MED25 (Zhang et al. 2015a), which is a component of the Mediator co-activator complex required for JA gene expression (Cevik et al. 2012; Chen et al. 2012). Thus, in addition to JAZ-mediated recruitment of TPL/NINJA co-repressors/adaptors for chromatin-based transcriptional repression, JAZ repressors also directly inhibit interactions between MYC proteins and MED25. Such a dual transcriptional repression mechanism may be the key to ensuring tight and dynamic control of JA responses.

Counterattack: Pathogen/insect manipulation of JA biosynthesis and signaling

1. The salicylic acid (SA)-JA antagonism

Plants appear to rely on "cross-talk" between different hormone signaling pathways in order to fine-tune proper immune responses against different types of pathogens. Relevant to this review is the SA-JA antagonistic interaction, which has been extensively studied and reviewed recently (Caarls et al. 2015; Pieterse et al. 2012). In general, JA mediates broad-spectrum resistance against necrotrophic pathogens and herbivorous insects, whereas SA is a major regulator of defense against biotrophic/hemibiotrophic pathogens (Caarls et al. 2015; Campos et al. 2014; Pieterse et al. 2012). Activation of JA signaling has been shown to inhibit SA accumulation through up-regulated expression of NAC TFs (ANAC019, ANAC055, and ANAC072). MYC2 activates the transcription of these NAC TFs *via* direct interaction between MYC2 and the promoter region of these genes. The NAC TFs are reported to inhibit the expression of SA

biosynthesis gene ISOCHORISMATE SYNTHASE1 (ICS1) and activate the expression of an SA methylation gene, BENZOIC ACID/SA CARBOXYL METHYLTRANSFERASE1 (BSMT1) (Zheng et al. 2012). On the other hand, SA-mediated suppression of JA signaling involves several components, including NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), TGA TFs, GLUTAREDOXINS (GRXs), and several WRKY TFs (Caarls et al. 2015; La Camera et al. 2011; Ndamukong et al. 2007; Schmiesing et al. 2016; Spoel et al. 2003; Zander et al. 2012; Zander et al. 2014). For instance, SA induces the expression of GRXs, which block TGA TF-mediated JA response gene expression, including the expression of ORA59 (Ndamukong et al. 2007; Zander et al. 2012). Moreover, SA treatment also reduces the protein level of ORA59 and inhibits the activation of ORA59-regulated gene expression (Van der Does et al. 2013; Zander et al. 2014). Interestingly, egg-extract from *Pieris brassicae* (large white butterfly) induced SA-JA antagonism was recently shown due to reduction of the MYC protein levels, independently of ORA59 (Schmiesing et al. 2016).

In nature, plants encounter attacks by pathogens/insects with different life styles (*e.g.*, necrotrophic vs. biotrophic). It is possible that the SA-JA antagonistic interaction may have evolved as a powerful strategy for plants to fine-tune immune responses based on the type of attackers they encounter at any given time (Pieterse et al. 2012). Conversely, pathogens have developed a plethora of virulence strategies, including evading or manipulating JA-mediated defense, as well as the exploitation of the SA-JA antagonism, to facilitate their survival in the plant. Below we focused our discussion on recent studies that illustrate elegant examples of pathogen/insect manipulation of JA-mediated defense.

2. Activation of JA signaling for pathogenesis

a. Bacterial pathogens

Perhaps the most famous example of pathogen hijacking of JA signaling is mediated by the polyketide toxin coronatine (COR), produced by several pathovars of the hemibiotrophic bacterial pathogen *Pseudomonas syringae*. COR is a structural and functional mimic of JA-Ile (Bender et al. 1999). It contains two moieties: coronafacic acid and coronamic acid, which are conjugated by an amide linkage (Brooks et al. 2004). COR promotes bacterial infection through counteracting PAMP-induced stomatal closure, suppression of plant apoplastic defense, and induction of disease symptoms (Geng et al. 2014). As a remarkable structural mimic of JA-Ile, COR directly binds to the COI1-JAZ receptor with high affinity (Fig. 3) (Katsir et al. 2008; Melotto et al. 2008a; Sheard et al. 2010; Yan et al. 2009; Zhang et al. 2015b; Zheng et al. 2012). COR-mediated activation of the JA signaling pathway leads to suppression of SA-mediated plant defense against *P. syringae* (Brooks et al. 2005; Kloek et al. 2001; Melotto et al. 2006; Zeng and He 2010; Zhang et al. 2015b). It has been reported that COR may also have some virulence functions independently of the SA-JA antagonism, such as regulation of secondary metabolites and suppression of callose deposition (Brooks et al. 2005; Geng et al. 2012; Millet et al. 2010; Uppalapati et al. 2005; Yi et al. 2014).

Although COR is most commonly studied in *P. syringae* (Bender et al. 1999; Geng et al. 2014), production of COR-like compounds has been reported in other bacteria, including *P. cannabina* pv. *alisalensis*, *Streptomyces scabies*, and *Xanthomonas campestris* pv. *phormiicola* (Bender et al. 1999; Fyans et al. 2014; Geng et al. 2014). Moreover, gene clusters involved in COR biosynthesis have been identified in *P. savastanoi* pv. *glycinea*, as well as necrotrophic

Pectobacterium atrosepticum (syn. *Erwinia carotovora* subsp. *atroseptica*), *P. carotovorum* subsp *carotovorum* and *Dickeya* sp. (Bell et al. 2004; Qi et al. 2011a; Slawiak and Lojkowska 2009). Therefore, studies of the actual function of COR/COR-like compounds in both biotrophic and necrotrophic pathogens would help to further elucidate JA function in mediating plant defense. Taken together, these results indicate that biosynthesis of JA-Ile mimics may be a widely utilized strategy by diverse bacterial pathogens to counteract plant defense.

Besides COR, proteinaceous effectors secreted from strains of *P. syringae* have also been shown to activate JA signaling through targeting the COI1-JAZ receptor (Gimenez-Ibanez et al. 2014; Jiang et al. 2013), indicating COI1-JAZ receptor is a common hub for pathogen hijacking. For example, HopZ1a, an acetyl transferase produced by *P. syringae* pv. *syringae* (*Psy*) strain A2, directly interacts with and induces acetylation of JAZ proteins. JAZ acetylation by HopZ1a is associated with its degradation in a COI1-dependent manner, thereby activating JA signaling (Fig. 3) (Jiang et al. 2013). On the other hand, HopX1 produced by *P. syringae* pv. *tabaci* (*Pta*) strain 11528 is a cysteine protease that interacts with and promotes the degradation of JAZ proteins in a COI1-independent manner (Fig. 3) (Gimenez-Ibanez et al. 2014). Interestingly, neither *Psy* A2 nor *Pta* 11528 produces COR/COR-like compounds, indicating that different pathogenic bacteria evolve alternative strategies to target core components of the JA signaling pathway for disease development.

Additionally, the *P. syringae* effector AvrB enhances JA signaling in a COI1-dependent manner in Arabidopsis (He et al. 2004). In this case, the Arabidopsis protein RPM1-INTERACTING PROTEIN4 (RIN4) appears to be involved (Cui et al. 2010; Zhou et al. 2015). AvrB interacts

with RIN4 and activates the plasma membrane-localized H^+-ATPase, AHA1 in a RIN4-dependent manner. Both AHA1 and AvrB enhance the COI1-JAZ interaction and the degradation of JAZs by an unclear mechanism, resulting in stomatal opening and compromised plant defense against *P. syringae* (Fig. 3) (Zhou et al. 2015). Besides targeting the core components of JA signaling, AvrB also interacts with MPK4 and associates with the HEAT SHOCK PROTEIN90 (HSP90) chaperone through RAR1, a co-chaperone for HSP90. Phosphorylation of MPK4 is induced by AvrB in an HSP90-promoted manner, leading to the activation of JA signaling, likely through RIN4 (Cui et al. 2010). Overall, understanding how AvrB activates JA signaling may yield new insights into alternative plant pathways that intercept JA signaling and/or response.

b. Fungal and oomycete pathogens

JA production is a common feature for many plant-interacting fungal pathogens or symbionts (Gimenez-Ibanez et al. 2016; Goossens et al. 2016). For instance, twenty-two JA and related compounds were detected in the culture filtrate of *Fusarium oxysporum* (*Fo*) f. sp. *matthiolae* (Miersch et al. 1999a), and JA biosynthesis has been observed in *Laccaria laccata*, *Pisolithus tinctorius*, *Aspergillus niger* and *Lasiodiplodia theobromae* (Miersch et al. 1999b; Miersch et al. 1999c; Tsukada et al. 2010). Intriguingly, JA production has only been reported in plant-interacting fungi, indicating that these fungi may have evolved the ability to produce JA in order to colonize plants (Goossens et al. 2016). Consistent with this idea, the Arabidopsis pathogens *F. oxysporum* f. sp. *matthioli* (*Fomt*) and *F. oxysporum* f. sp. *conglutinanas* (*Focn*) produce JA, JA-Ile, and JA-Leu (Fig. 3) and exhibit reduced virulence in the *coi1* mutant (Cole et al. 2014), indicating that JA signaling promotes *Fo* infection. Surprisingly, unlike in the case of bacterial

pathogens, COI1-mediated *Fo* pathogenesis is found to be independent of SA signaling and might be due to COI1-mediated lesion development in Arabidopsis (Thatcher et al. 2009). Specifically, Arabidopsis *coi1/NahG* plants, defective both in JA perception and SA accumulation, exhibited a similar level of resistance against *F. oxysporum* 5176 (Fo5176) as the *coi1* plants. Resistance in *coi1* mutant was only detected when leaf necrosis is highly developed in wild-type plants, while no necrosis was observed in *coi1* plants.

Cinnacidin, a structural analog of JA-Ile/COR, has been isolated from the fermentation extract of the fungus *Nectria* sp. DA060097, which closely relates to two woody plant pathogens *N. cinnabarina* and *N. pseudotrichia*. Cinnacidin contains a cyclopentalenone ring and an isoleucine side chain. Comparing to COR, synthetic cinnacidin analog exhibited similar potency in the level of bentgrass seedling growth inhibition, but less effective on Arabidopsis seedling growth inhibition (Irvine et al. 2008). However, whether cinnacidin acts directly on the COI1-JAZ co-receptor or if it is required for fungal virulence is still unknown.

Fungal and oomycete pathogens also produce proteinaceous effectors that activate JA signaling and enhance disease development. For example, several SECRETED IN XYLEM (SIX) effector proteins contribute to the virulence of *Fo* strains (Takken and Rep 2010), including Fo5176 (Thatcher et al. 2012a). One of SIX proteins from Fo5176, Fo5176-SIX4, enhances host JA signaling and Arabidopsis susceptibility (Thatcher et al. 2012a). Similar to the action of fungal-derived JA, no difference in SA-responsive gene expression was detected after inoculation with Δ*six4* mutant or the wild-type Fo5176. This finding reinforces the notion that SA-JA antagonism is not associated with the ability of the soil pathogen *Fo* to colonize the plant host. Additionally,

Fo infection induces the expression of LOB DOMAIN-CONTAINING PROTEIN20 (LBD20), which functions downstream of COI1 and MYC2 to promote pathogenesis. *LBD20* expression is associated with suppression of one branch of JA signaling, marked by the expression of *THIONIN2.1* (*Thi2.1*) and *VSP2*, while no effects were detected on the other branch of JA signaling, marked by unaltered expression of *PDF1.2* (Thatcher et al. 2012b). However, how the Thi2.1/VSP2 branch of JA signaling promotes Fo5176 pathogenesis needs further investigation.

Additionally, an oomycete effector protein from *Hyaloperonospora arabidopsis*, HaRxL44, induces JA/ET signaling, suppresses SA signaling and enhances host disease susceptibility *via* interference with MED19a. MED19a is another member of the Mediator co-activator complex, which regulates SA-triggered immunity (Caillaud et al. 2013). HaRxL44 interacts with and induces degradation of MED19a *via* proteasome to rewire SA-mediated response toward JA/ET-mediated response as a novel strategy of promoting infection (Caillaud et al. 2013).

3. Suppression of JA signaling for pathogenesis and symbiosis

In contrast to biotrophic/hemi-biotrophic pathogens, chewing insects and necrotrophic pathogens suppress JA signaling for their success in host plants. One strategy is to reduce JA accumulation after infection, either by blocking JA biosynthesis or by accelerating JA catabolism. Alternatively, the SA-JA antagonism may be employed for suppression of JA-mediated defense. Emerging studies suggest that both strategies are used by various pathogens.

a. Fungal pathogens and symbionts

Some fungal species have evolved the ability to metabolize JA. For example, the antibiotic biosynthesis monooxygenase (Abm) from the rice blast fungus *Magnaporthe oryzae* coverts both

fungal- and plant-derived JA into 12OH-JA to attenuate JA signaling and facilitate host colonization (Patkar et al. 2015). Loss of Abm in *M. oryzae* leads to the accumulation of methyl-JA (MeJA) in the fungus and induction of plant defense. Therefore, Abm not only attenuates plant JA defense signaling, but also likely converts fungal JA to 12OH-JA to avoid the induction of host defense (Fig. 3) (Patkar et al. 2015). In addition, hydroxylation in the pentenyl side chain of JA was detected in several fungal species, such as *A. niger, P. tinctorius* and *Botryodiplodia theobromae* (Miersch et al. 1993; Miersch et al. 1999b; Miersch et al. 1999c; Miersch et al. 1991). However, whether hydroxylation of JA by these species contributes to pathogenesis remains to be investigated.

Just as biotrophic/hemi-biotrophic pathogenic bacteria activate JA signaling to dampen SA signaling, necrotrophic pathogen can manipulate the SA-JA antagonism for suppression of JA-mediated defense. *Botrytis cinerea* produces β-(1,3)(1,6)-$_{D}$-glucan, an exopolysaccharide that stimulates SA accumulation and antagonistically suppresses JA-response gene expression, including proteinase inhibitors I and II (PI I and PI II) (El Oirdi et al. 2011). Further investigation showed that SA-mediated disease development induced by *B. cinerea* and *Alternaria solani* requires two important regulators of SA signaling: NPR1 and TGA1.a in tomato (El Oirdi et al. 2011; Rahman et al. 2012).

Pathogens/symbionts also secrete proteinaceous effector to suppress JA signaling. For instance, the *Sclerotinia sclerotiorum* integrin-like (SSITL) protein produced by the necrotrophic pathogen *S. sclerotiorum* suppresses JA/ET signaling mediated resistance at the early stage of infection (Zhu et al. 2013). However, the underlying mechanism is not clear. On the other hand,

MYCORRHIZA-induced SMALL SECRECTED PROTEIN7 (MiSSP7) produced by the symbiotic ectomycorrhizal fungus *Laccaria bicolor* is indispensable for the establishment of fungal mutualism in *Populus trichocarpa* (Plett et al. 2011). MiSSP7 expression could be induced by host (poplar) and non-host (Arabidopsis) root excretions (Plett et al. 2011), particularly by the two flavonoids rutin and quercetin (Plett and Martin 2012). Recently, Navarro-Ródenas et al. (2015) found that *L. bicolor* aquaporin LbAQP1 modulates *MiSSP7* expression and the establishment of ectomycorrhizal structures in trembling aspen (*Populus trmuloides*) (Navarro-RoDenas et al. 2015). MiSSP7 enters the plant cell *via* phosphatidylinositol 3-phosphate (PI-3-P)-mediated endocytosis, interacts with PtJAZ6 and inhibits ligand-induced degradation of PtJAZ6 in the host nucleus, thereby blocking the activation of JA signaling and facilitating the establishment of symbiosis (Fig. 3) (Plett et al. 2014; Plett et al. 2011). Contrary to what has been observed for the *P. syringae* effectors HopZ1a and HopX1, the MiSSP7 effector stabilizes the JAZ6 protein, therefore negatively regulating JA signaling. On the other hand, SECRETED PROTEIN7 (SP7) produced by the arbuscular mycorrhizal fungus, *Glomus intraradices* interacts with the plant ERF19 TF in the nucleus, and subsequently suppresses ET-mediated plant defense and enhances mycorrhizal symbiosis of *G. intraradices* in *Medicago truncatula* (Kloppholz et al. 2011).

b. Herbivores and viral pathogens

Insects employ diverse strategies to manipulate the SA-JA antagonism for better accommodation in the host plants, including directly secreting SA or inducing SA signaling. For instance, a high level of SA was found in the locomotion mucus of the slug *Deroceras reticulatum* (Kastner et al. 2014). On the other hand, salivary excretions of the beet armyworm *Spodoptera exigua* have

GLUCOSE OXIDASE (GOX) activity, which could suppress JA-regulated defenses *via* activation of the SA pathway (Diezel et al. 2009; Weech et al. 2008). GOX catalyzes the generation of peroxide from D-glucose (Eichenseer et al. 1999) and is the first insect salivary enzyme identified to suppress wound-induced plant defense against herbivore. GOX from *S. exigua* and *Helicoverpa zea* suppresses terpenoid synthesis in *M. truncatula* and wound-induced production of nicotine and defense against insects in tobacco and tomato plants, respectively (Bede et al. 2006; Musser et al. 2005; Musser et al. 2002). Interestingly, larvae of Colorado potato beetles *Leptinotarsa decemlineata* employ microbial symbionts in their oral secretions to induce SA production, which antagonistically suppresses JA-mediated defense against herbivore in tomato (Chung et al. 2013). Loss of the ability to suppress JA signaling was detected in antibiotic-treated larvae, whereas this ability could be restored with inoculation of the microbial symbionts (Chung et al. 2013).

Oviposition or egg extract triggers SA accumulation and signaling and suppresses JA-regulated plant defense against the generalist herbivore *Spodoptera littoralis* (Bruessow et al. 2010). Remarkably, a recent study showed that *Pieris brassicae* egg extracts not only trigger SA signaling, but also mediate the destabilization of the MYC proteins in an SA-dependent manner (Schmiesing et al. 2016).

As elegant examples of co-evolution, manipulation of the SA-JA antagonism has also been observed in tritrophic interactions to benefit pathogens that are transmitted by insect vectors. In these interactions, insect vectors transmit viruses or phytoplasmas to plants, whereas the microbial pathogens manipulate JA-dependent defense and subsequently affect the performance

of insect vectors. For example, the insect vector *Macrosteles quadrilineatus* transmits Aster yellows phytoplasma strain witches' broom (AY-WB) to the plant host. AY-WB PROTEIN11 (SAP11), secreted by the phytoplasm, binds to and mediates the destabilization of Arabidopsis CINCINNATA (CIN)-related TEOSINTE BRANCHED1, CYCLOIDEA, PROLOFERATING CELL FACTORS (TCP) TFs in the nucleus and this process is regulated by miR319, which guides mRNA cleavage of several *TCP* transcripts (Palatnik et al. 2003; Sugio et al. 2015; Sugio et al. 2011). TCP proteins contain a conserved bHLH DNA-binding domain and regulate various pathways of plant development and defense (Li 2015). Down-regulation of CIN-TCPs reduces the expression of *LOX* genes involved in JA biosynthesis and consequently reduces JA level and signaling in Arabidopsis, which in return benefits the proliferation of the insect vector (Fig. 3) (Sugio et al. 2011). Moreover, down-regulation of CIN-TCPs results in the delayed maturation of vegetative organs, which increases the survival of the biotrophic phytoplasm (Efroni et al. 2008; Li 2015), indicating that both the insect vector and the obligate phytoplasm take advantage of SAP11 suppressed-JA signaling in this tritrophic interaction. Recently, SAP11 was also shown to induce the destabilization of TCP TFs and suppression of JA responses in *Nicotiana benthamiana* (Tan et al. 2016). Suppression of TCP expression and JA-mediated plant resistance are also observed during viral infections (Zhang et al. 2016). For example, *rice ragged stunt virus* (RRSV) infection enhances miR319 accumulation in rice plants. As in the case of AY-WB, miR319 guides mRNA cleavage of several *TCP* genes, and suppresses JA signaling probably also through TCP-mediated *LOX2* expression (Danisman et al. 2012; Schommer et al. 2008; Zhang et al. 2016).

The aphid *Myzus persicae* transmits *cucumber mosaic virus* (CMV) to plants as a strategy to counteract plant defense. The CMV 2b protein is a viral suppressor of RNA silencing (VSR) (Jacquemond 2012), which has roles in symptom induction, virus movement, disruption of SA- or JA-mediated plant defense, in addition to suppression of antiviral RNA silencing (Csorba et al. 2015; Du et al. 2014b). Arabidopsis plants ectopically expressing CMV 2b show mis-regulation of 90% of the JA-responsive genes, whereas 2b protein enhances responses to SA (Lewsey et al. 2010). 2b protein-triggered repression of JA response genes was also detected in *Nicotiana tabacum* and is associated with promoting aphid infection (Ziebell et al. 2011). The negative effect of 2b protein on JA signaling may be partly explained by its interference on the activity of RNA-dependent RNA polymerase 1 (RDR1) (Csorba et al. 2015; Diaz-Pendon et al. 2007). In addition, HC-Pro, another viral VSR protein from *turnip mosaic virus* (TuMV), also affects JA-regulated gene expression in Arabidopsis (Endres et al. 2010). However, further studies with other viruses and their corresponding VSR proteins indicate that VSR-mediated repression of JA response gene expression does not always associate with enhanced aphid performance in *N. benthamiana* (Westwood et al. 2014), indicating JA signaling may play distinct roles in mediating aphid performance in different plant species. Other viral proteins have also been shown to be involved in overcoming JA-mediated host defense, L2 protein from *beet curly top virus* (BCTV) and homologous C2 protein from *tomato yellow leaf curl Sardinia virus* Spain isolate (TYLCSV) or *tomato yellow leaf curl disease* (TYLCD) suppress JA signaling through interacting with COP9 signalosome subunit 5 (CSN5), which affects CSN-mediated deneddylation of SCF-type E3 ubiquitin ligases and their activity (Fig. 3) (Lozano-Duran et al. 2011).

Down-regulation of JA mediated plant immunity is also observed in the tritrophic interaction among the insect vector *Bemisia tabaci*, *tomato yellow leaf curl China virus* (TYLCCNV) and tomato. In this case, the viral satellite gene βC1 is required for the inhibition of JA production and JA-mediated defense against vector infestation (Li et al. 2014b; Salvaudon et al. 2013; Yang et al. 2008; Zhang et al. 2012). βC1 directly binds to ASYMMETRIC LEAVES1 (AS1), which negatively regulates JA response gene expression (Nurmberg et al. 2007; Yang et al. 2008). Moreover, interaction between βC1 and MYC2 has been detected, which reduces MYC2-mediated expression of the terpene synthase genes (Fig. 3) (Li et al. 2014b). Furthermore, manipulated host defense by *B. tabaci* was reported to be beneficial to other insect species. For example, *B. tabaci* suppresses JA-mediated volatile monoterpene (*E*)-*β*-ocimene emission in lima bean and benefits spider mite *Tetranychus urticae* indirectly due to the reduced attraction of predatory mites *Phytoseiulus persimilis* (Zhang et al. 2009).

Some insects even attempt to exploit the intra-pathway antagonism between the ERF branch and the MYC branch of JA signaling for better accommodation (Verhage et al. 2011). Elicitors in the oral secretion of caterpillars of *Pieris rapae* activate the ERF branch of the JA pathway in Arabidopsis, which confers resistance to necrotrophic pathogens (Berrocal-Lobo et al. 2002; Pre et al. 2008). Activation of the ERF branch is associated with suppression of the MYC branch, which mediates resistance to insects (Dombrecht et al. 2007; Fernandez-Calvo et al. 2011; Lorenzo et al. 2004).

In addition, the effector calreticulin (Mi-CRT) from the root-knot nematode (RKN) *Meloidogyne incognita* has been shown to suppress JA response gene expression in Arabidopsis

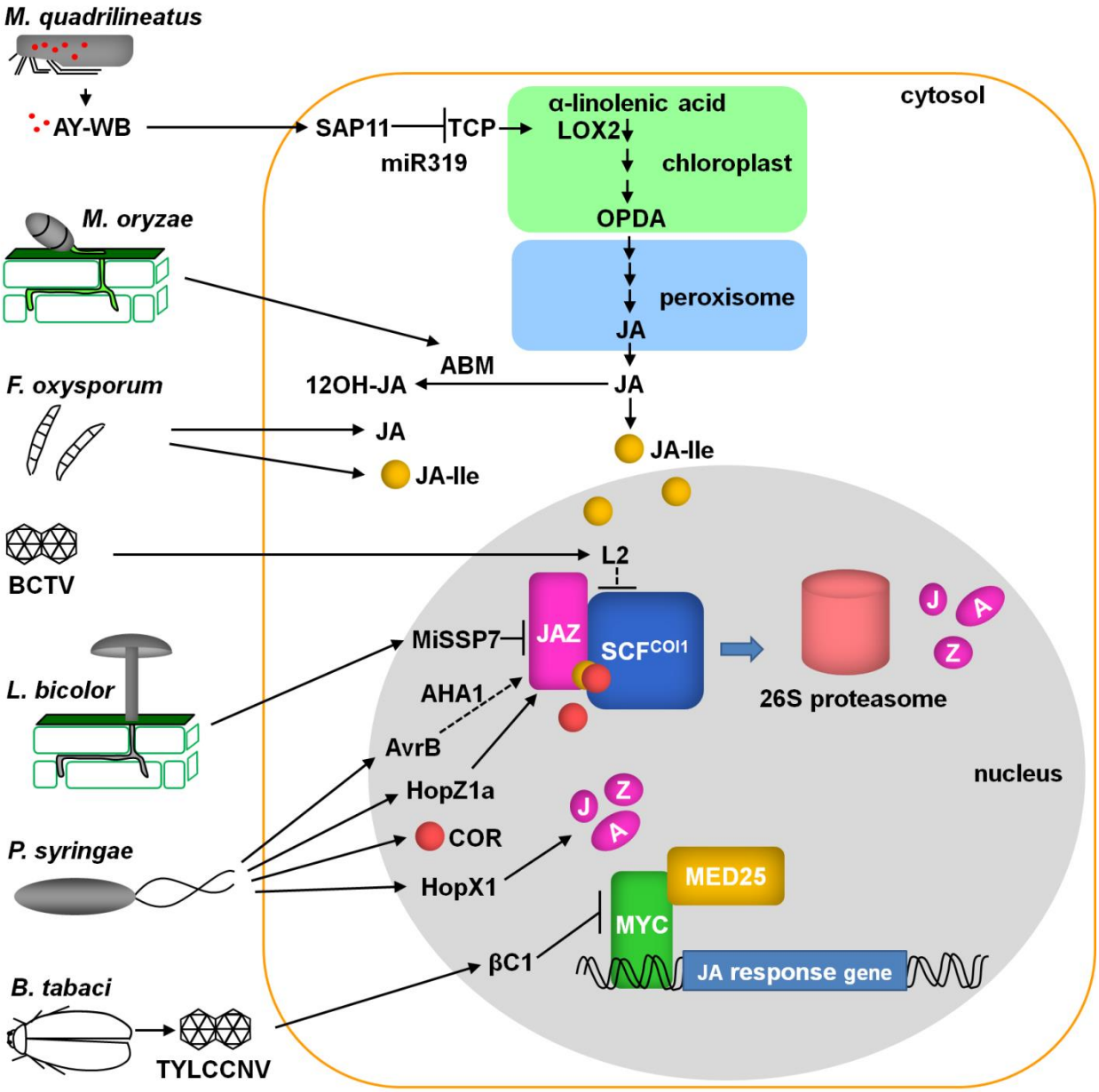

Figure 3: A diagram illustrating plant pathogen hijacking of the core components of JA signaling. Microbial pathogens and insects exploit different strategies to hijack JA signaling. In this diagram, only virulence factors that target the core components of JA biosynthesis or signaling are depicted. The insect vector *M. quadrilineatus* employs phytoplasma AY-WB to suppress JA biosynthesis *via* down-regulation of *LOX2* expression (Sugio et al. 2011). The fungus *M. oryzae* stimulates JA hydroxylation to attenuate JA signaling *via* the ABM effector (Patkar et al. 2015). The mutualist *L. bicolor* suppresses the degradation of JAZ protein by the action of the MiSSP7 effector (Plett et al. 2014). The viral protein BCTV L2 suppresses SCF^{COI1} activity through CSN5 (Lozano-Duran et al. 2011). The insect vector *B. tabaci* employs TYLCCNV to suppress MYC2-mediated gene expression through direct interaction between the βC1 effector and MYC2 (Li et al. 2014b). Conversely, pathogens can also activate JA signaling for pathogenesis. *F. oxysporum* produces JA or JA-Ile and activates JA signaling

Figure 3 (cont'd)
(Cole et al. 2014). The hemibiotrophic bacterium *P. syringae* secretes COR or the AvrB effector to enhance the interaction between COI1 and JAZ co-receptor proteins (Bender et al. 1999; Katsir et al. 2008; Melotto et al. 2008a; Sheard et al. 2010; Yan et al. 2009; Zhang et al. 2015b; Zheng et al. 2012; Zhou et al. 2015). HopZ1a acetylates JAZ proteins and stimulates degradation of JAZ in a COI1-dependent manner (Jiang et al. 2013). HopX1 stimulates JAZ protein degradation in a COI1-independent manner and activates JA signaling (Gimenez-Ibanez et al. 2014).

(Jaouannet et al. 2013). Although the exact function of Mi-CRT in the alteration of JA defense is unknown, Jaouannet and colleagues proposed that Mi-CRT suppresses JA defense likely through chelating calcium in the apoplast and preventing calcium influx (Jaouannet et al. 2013).

Signaling networks that regulate stomatal defense against pathogens

Plants have evolved mechanisms to protect themselves from pathogen attack. The primary immune response in plants is initiated upon recognition of PAMPs by PRRs (Jones and Dangl 2006; Monaghan and Zipfel 2012). For example, flagellin, the main component of the bacterial flagellum, is recognized by FLAGELLIN-SENSITIVE2 (FLS2), a plasma membrane-localized receptor-like kinase (RLK) (Gomez-Gomez and Boller 2000). Bacterial elongation factor Tu (EF-Tu) is recognized by EF-Tu RECEPTOR (EFR) (Zipfel et al. 2006). Mitogen-activated protein kinase (MAPK) signaling, calcium/calmodulin-dependent protein kinase (CDPK) signaling, alkalinization of the extracellular space, production of reactive oxygen species (ROS) and nitric oxide (NO), increased Ca^{2+} influx, transcriptional induction of pathogen-responsive genes, and stomatal closure are all associated with PAMP-triggered immunity (PTI) (Boller and Felix 2009). However, effectors secreted from highly evolved virulent pathogens often suppress or evade PTI and induce effector-triggered susceptibility (ETS), allowing proliferation of pathogens and development of disease symptoms (Jones and Dangl 2006). Each strain of the Gram-negative bacterial pathogen *P. syringae* can secrete approximately 20 to 30 effectors during infection (Chang et al. 2005). To overcome ETS, plants have also evolved a second layer of immunity, effector-triggered immunity (ETI), which is mediated by resistance (R) genes (Jones and Dangl 2006).

Plant stomata play important roles in gas exchange and water transpiration, and stomata aperture changes in response to various environmental signals including relative humidity, CO_2 concentration, light intensity, drought, microbes and plant hormones (Arnaud and Hwang 2015). A complex signaling network is involved in stomatal closure, including kinases/phosphatases, secondary messengers, and ion channel regulation (Arnaud and Hwang 2015; Murata et al. 2015). Stomatal closure in response to abiotic stress is initiated by guard cell membrane depolarization, resulting from inhibition of H^+ extrusion and efflux of Cl^-, malate, and NO^{3-} anions with opening of slow (S)- and rapid (R)-type anion channels. Following membrane depolarization, voltage-regulated out-ward-rectifying K^+ (K^+_{out}) channels are activated leading to K^+ efflux, the consequent massive loss of solutes, decrease of guard cell turgor pressure and induced-stomatal closure (Arnaud and Hwang 2015; Kim et al. 2010; Sirichandra et al. 2009).

The phytohormone ABA is the central regulator of plant resistance against abiotic stress, including stomatal closure under drought condition (Hubbard et al. 2010). Following ABA-induced accumulation of ROS, the non-selective Ca^{2+}-permeable cation (I_{ca}) channels are activated, triggering Ca^{2+} influx and cytosolic calcium $[Ca^{2+}]_{cyt}$ oscillations, which activates the S-type anion channels and suppress the K^+_{in} channels (Kim et al. 2010). NO is also involved in ABA-induced stomatal closure (Arnaud and Hwang 2015).

Moreover stomatal closure has also been shown to be one of the first lines of plant immune response against pathogen infection (Melotto et al. 2006). Foliar pathogens, for example, *P. syringae* live two lifestyles in a successful disease cycle. They initially go through an epiphytic phase on the surface of plants, and subsequently transit into an endophytic phase in the apoplast

of plants (Melotto et al. 2008b). *P. syringae* enters the host tissue through wounds and natural openings, such as stomata (Hirano and Upper 2000). Plant stomata, however, respond to *Pst* DC3000 infection and restrict the entry of *Pst* DC3000 via closure of stomatal aperture (Melotto et al. 2006). In addition, PAMPs including flg22 (a bioactive, 22-amino acid epitope of flagellin), elf26/elf18 (a bioactive, 26/18-amino acid epitopes of EF-Tu), lipopolysaccharides (LPS), oligogalacturonic acid, chitosan and β-1,3 glucans and yeast elicitors (YEL), have been shown to induce stomatal closure in Arabidopsis, tomato, grapevine, barley, Asiatic dayflower, and pea (Allegre et al. 2009; Desikan et al. 2008; Freeman and Beattie 2009; Khokon et al. 2010a; Khokon et al. 2010b; Klusener et al. 2002; Koers et al. 2011; Lee et al. 1999; Liu et al. 2009; Melotto et al. 2006; Srivastava et al. 2009; Zeng and He 2010; Zhang et al. 2008). Specific PRRs are necessary for plants to sense PAMPs in the guard cell and induce stomatal closure (Melotto et al. 2006). For example, FLS2 can recognize flg22 and induce stomatal closure. However, elf18/elf26 induced stomatal closure has not been tested in *efr* mutant yet (Arnaud and Hwang 2015). And whether CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) mediates chitin- or chitosan-induced stomatal closure is unknown (Arnaud and Hwang 2015). Interestingly, BOTRYTIS-INDUCED KINASE1 (BIK1), components involved in different PAMP-induced PTI signaling, is required for flg22-induced stomatal closure (Li et al. 2014a; Zhang et al. 2010).

Plant hormones also play important roles in pathogen-/PAMP-mediated stomatal closure. Studies have shown that PAMP-induced stomatal closure shares a common signaling pathway with ABA-induced stomatal closure, including accumulation of ROS and NO, cytosolic calcium oscillations, activation of S-type anion channels, and inhibition of K^+_{in} channels (Desikan et al. 2008; Klusener et al. 2002; Macho et al. 2012; Melotto et al. 2006; Montillet et al. 2013; Zeng and He 2010; Zhang et al. 2008). In ABA-deficient mutants, stomatal closure is compromised in

response to *Pst* DC3000, flg22 or SA (Melotto et al. 2006; Zeng and He 2010). *ost1-2*, mutant of *OPEN STOMATA1* (*OST1*), acting upstream of ROS production in ABA-induced stomatal closure (Murata et al. 2001; Mustilli et al. 2002), exhibits compromised flg22 and LPS-mediated stomatal closure (Melotto et al. 2006; Zeng and He 2010). Interestingly, the core ABA signaling components, ABSCISIC ACID-INSENSITIVE1 (ABI1), ABI2 and TYPE 2C PROTEIN PHOSPHATASE A (PP2CA) have been shown to be involved in pathogen-mediated stomatal closure (Desclos-Theveniau et al. 2012; Lim et al. 2014; Schellenberg et al. 2010). Other components of ABA signaling have also been reported to be involved in pathogen-induced stomatal closure (Arnaud and Hwang 2015; Desclos-Theveniau et al. 2012; Du et al. 2014a; Lee et al. 2013; Lim et al. 2014; Melotto et al. 2006; Roy et al. 2013; Schellenberg et al. 2010; Zeng and He 2010; Zhang et al. 2008). However, recent studies suggested that ABA functions to potentiate stomatal response capacity other than directly trigger PAMP-mediated stomatal closure (Issak et al. 2013; Montillet et al. 2013).

Another plant stress hormone, SA, also plays important roles in regulating pathogen-induced stomatal closure (Khokon et al. 2011; Melotto et al. 2006; Mori et al. 2001; Zeng et al. 2011; Zeng and He 2010). With a higher concentration compared to ABA, SA induces stomatal closure (Khokon et al. 2011; Mori et al. 2001; Zeng and He 2010). NONEXPRESSOR OF PR GENES1 (NPR1), a master regulator of SA signaling, is required for SA-mediated stomatal closure (Zeng and He 2010). Compromised pathogen-induced stomatal closure has been reported in SA-deficient mutants *enhanced disease susceptibility16-2* (*eds16*-2)/ *SA induction–deficient2-2* (*sid2*-2), *eds5*/*sid1*/*susceptible to COR-deficient Pst DC3000 3* (*scord3*) or transgenic plants *nahG* (Melotto et al. 2006; Zeng et al. 2011; Zeng and He 2010). SA-mediated stomatal closure

is affected in ABA deficient mutant, *aba2-1*, suggesting that ABA signaling is required for SA-mediated stomatal closure and SA might act up-stream of ABA signaling (Zeng and He 2010). However, recent studies show that SA- or ABA-induced stomatal closure is independent at least upstream of ROS accumulation (Khokon et al. 2011). SA-induced ROS production is not affected in *respiratory burst oxidase homologD* (*rbohD*) *rbohF* double mutant and SA does not induce the cytosolic Ca^{2+} oscillations in Arabidopsis guard cells (Khokon et al. 2011). Apoplastic peroxidase (PRX)-mediated extracellular ROS production is required in SA-mediated stomatal closure but is not required in ABA-induced ROS production and stomatal closure (Khokon et al. 2011; Khokon et al. 2010b). With a higher concentration compared to previous study (200 μM vs 20 μM), SA induced stomatal closure was detected in *aba2-2* plants (Issak et al. 2013).

In addition, an ABA-independent oxylipin pathway has been shown to regulate stomatal closure upon pathogen infection (Montillet et al. 2013). Arabidopsis 9-SPECIFIC LIPOXYGENASE (9-LOX) produces reactive electrophile species (RES) oxylipins from fatty acid hydroperoxides (FAHs). Both products and substrates of 9-LOX induce stomatal closure (Montillet et al. 2013). The guard cell-expressed *LOX1* gene is required for flg22- and *Pst* DC3000-induced stomatal closure (Montillet et al. 2013).

The JA signaling regulates plant resistance to insects and necrotropic pathogens (Erb et al. 2012). Unlike ABA or SA, controversial results of JA signaling in modulating stomatal response have been reported. According to Suhita and colleagues (Suhita et al. 2004), exogenous application of MeJA alone caused stomatal closure, with a 50% effect observed at around 5 μM. MeJA-induced

stomatal closure was associated with ROS and NO production and elevation of guard cell Ca^{2+} concentration sharing a common signaling pathway with ABA (Munemasa et al. 2011; Suhita et al. 2004). On the other hand, Melotto and colleagues (Melotto et al. 2008b; Speth et al. 2009) found that high concentrations (>20 μM) of JA and MeJA triggered significant stomatal closure, but lower concentrations (e.g., 2 μM) inhibited ABA-induced stomatal closure. These results suggest that the effect of exogenous JA and MeJA on stomatal response may be dose-dependent (Melotto et al. 2008b). Moreover, a recent study revealed that MeJA inhibits RES oxylipin-mediated stomatal closure, whereas MeJA-mediated inhibition is COI1-independent (Montillet et al. 2013).

Pst DC3000 secreted phytotoxin COR plays important roles in the virulence of *Pst* DC3000 and inhibition of plant stomatal defense. COR is an analog of JA-Ile and can effectively mediate the COI1-JAZ interaction (Brooks et al. 2005). COR-deficient mutant of *Pst* DC3000 exhibited reduced virulence on Arabidopsis and tomato (Brooks et al. 2004; Zeng and He 2010). COR interferes with PAMP-induced stomatal closure and triggers stomatal reopening in a COI1-dependent manner (Melotto et al. 2006; Montillet et al. 2013; Zheng et al. 2012). Importantly, virulence of COR-deficient mutants of *Pst* DC3000 could be restored in SA-deficient plants (Brooks et al. 2005; Melotto et al. 2006; Zeng and He 2010), indicating that COR suppresses stomatal closure through antagonizing the SA signaling. COR induces degradation of JAZ proteins, resulting in release of MYC2 TF and subsequently transcriptional activation of three NAC TFs. These NAC TFs suppress the expression of SA biosynthesis gene *ICS1* and activate the expression of SA metabolism genes *BSMT1* and *SA GLUCOSULTRANSFERASE1* (*SAGT1*) (Zheng et al. 2012). COR also suppresses ABA- and oxylipin-induced stomatal closure (Melotto

et al. 2006; Montillet et al. 2013). Alternatively, recent studies show that activation of ABA signaling could also inhibit COR-induced stomatal opening (Desclos-Theveniau et al. 2012; Lim et al. 2014).

Prospective

Clearly, manipulation of the SA-JA antagonism has emerged as a major theme in plant-pathogen/insect/nematode interactions, and the core components of JA signaling, particularly the COI1 and JAZ co-receptor proteins, appear to be common targets of virulence factors from biotrophic/hemi-biotrophic pathogens. Conventional activation of either the JA or SA signaling pathway, through genetic or chemical manipulation, encounters a risk of improving plant defense against one attacker, but inherently priming plant susceptibility to other attackers, illustrating the complexity and vulnerability of the plant defense network. For example, classical loss-of-function *coi1* mutants exhibit high-level resistance to *P. syringae*, but are greatly compromised in defense against chewing insects and necrotrophic pathogens (Glazebrook 2005; Robert-Seilaniantz et al. 2011; Stintzi et al. 2001; Thaler et al. 2012).

How do we solve this dilemma? One approach would be to modify specific JA signaling components to be insensitive to manipulation of pathogen/insect virulence factors, but preserving the functions of these signaling components in the perception and signal transduction of endogenous JA. Hence, I started my research focusing on constructing amino acid substitutions in the ligand-binding pocket of the Arabidopsis COI1 protein that allows for sufficient signal transduction of endogenous JA-Ile, but has greatly reduced sensitivity to the *P. syringae* toxin COR. I was successful in this effort and generated transgenic Arabidopsis plants expressing an

engineered COI1 receptor that not only maintain a high-level of insect defense, but also gain resistance to the COR-producing pathogens *Pst* DC3000 and *P. s.* pv. *maculicola* (*Psm*) ES4326 (Zhang et al. 2015b). This result provides a proof-of-concept demonstration for modifying the host targets of pathogen virulence factors as a promising new approach to broaden the capacity of host defense against highly evolved pathogens.

I then devoted my research effort to investigate plant stomatal defense in response to pathogen infection and cloned two *SCORD* (*SUSCEPTIBLE TO COR-DEFICIENT Pst* DC3000) genes, which are required for plant stomatal closure upon *Pst* DC3000 infection (Zeng et al. 2011). My identification of *SCORD6* and *SCORD7* genes highlights the importance of plant cell-wall-based regulation of stomatal defense and contributes to the general understanding of the multifaceted host defense mechanisms against pathogen infection in plants.

CHAPTER II

HOST TARGET MODIFICATION AS A STRATEGY TO COUNTER PATHOGEN HIJACKING OF THE JASMONATE HORMONE RECEPTOR

The chapter has been published: Li Zhang, Jian Yao, John Withers, Xiu-Fang Xin, Rahul Banerjee, Qazi Fariduddin, Yoko Nakamura, Kinya Nomura, Gregg A. Howe, Wilhelm Boland, Honggao Yan, and Sheng Yang He (2015) Host target modification as a strategy to counter pathogen hijacking of the jasmonate hormone receptor. *PNAS* 112: 14354-14359.

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Abstract

In the past decade, characterization of the host targets of pathogen virulence factors took a center stage in the study of pathogenesis and disease susceptibility in plants and humans. However, the impressive knowledge of host targets has not been broadly exploited to inhibit pathogen infection. Here, we show that host target modification could be a promising new approach to "protect" the disease-vulnerable components of plants. In particular, recent studies have identified the plant hormone jasmonate (JA) receptor as one of the common targets of virulence factors from highly evolved biotrophic/hemi-biotrophic pathogens. Strains of the bacterial

pathogen *Pseudomonas syringae*, for example, produce proteinaceous effectors as well as a JA-mimicking toxin, coronatine (COR), to activate JA signaling as a mechanism to promote disease susceptibility. Guided by the crystal structure of the JA receptor and evolutionary clues, we succeeded in modifying the JA receptor to allow for sufficient endogenous JA signaling but greatly reduced sensitivity to COR. Transgenic Arabidopsis expressing this modified receptor not only are fertile and maintain a high level of insect defense, but also gain the ability to resist COR-producing pathogens *Pseudomonas syringae* pv. *tomato* and *P. s.* pv. *maculicola*. Our results provide a proof-of-concept demonstration that host target modification can be a promising new approach to prevent the virulence action of highly evolved pathogens.

Introduction

Studies during the past two decades have revealed that plants possess a sophisticated, multi-layered immune signaling network that is regulated by several stress hormones (Robert-Seilaniantz et al. 2011). Most prominently, JA plays a central role in regulating plant defense against a variety of chewing insects and necrotrophic pathogens, whereas salicylic acid (SA) is critical for plant defense against biotrophic or hemi-biotrophic pathogens (Glazebrook 2005; Robert-Seilaniantz et al. 2011; Thaler et al. 2012). During host-pathogen co-evolution, however, many successful plant pathogens developed mechanisms to attack or hijack components of the plant immune signaling network as part of their pathogenesis strategies (Block et al. 2014; Dou and Zhou 2012; Xin and He 2013). As a result, the plant immune system, although powerful, is often fallible in the face of highly evolved pathogens.

The JA signaling cascade has been a subject of intense study, and many important players in this hormone signal transduction system have been identified. Higher plants synthesize different forms of JA, including the most bioactive form jasmonoyl-$_L$-isoleucine (JA-Ile) (Browse 2009; Fonseca et al. 2009; Staswick and Tiryaki 2004; Wasternack 2007; Wasternack and Hause 2013). Perception of JA-Ile occurs through a co-receptor, composed of CORONATINE INSENSITIVE1 (COI1), the F-box subunit of an Skp/Cullin/F-box-type ubiquitin ligase complex, and JASMONATE ZIM DOMAIN (JAZ) proteins, which are transcriptional repressors (Chini et al. 2009; Katsir et al. 2008; Melotto et al. 2008a; Thines et al. 2007; Yan et al. 2007). In the absence of hormone signal, JAZ repressors bind to and repress the transcription factors (TFs; e.g., MYC2) both directly and through the recruitment of the NOVEL INTERACTOR OF JAZ (NINJA) adapter and/or TOPLESS (TPL) co-repressor proteins (Browse 2009; Chini et al. 2009; Chung and Howe 2009; Pauwels et al. 2010; Pauwels and Goossens 2011; Zhang F 2015). In response to developmental or environmental cues, JA-Ile concentration rises, which promotes the interaction between COI1 and JAZs and subsequent degradation of JAZ repressors through the 26S proteasome (Browse 2009; Xu et al. 2002). Activation of MYC and other JAZ-interacting TFs leads to transcriptional reprograming and results in a plethora of JA-mediated physiological responses (Song et al. 2014; Tsuda and Somssich 2015; Vidhyasekaran 2015).

Although activation of the JA signal transduction pathway is essential for plant resistance to chewing insects and necrotrophic pathogens, it also leads to inhibition of SA signaling through hardwired molecular crosstalk between the two pathways (Groen et al. 2013; Pieterse et al. 2012; Robert-Seilaniantz et al. 2011; Tsuda and Somssich 2015; Zheng et al. 2012). Because the SA signaling pathway is critical for plant defense against biotrophic and hemi-biotrophic pathogens,

activation of JA signaling makes plants vulnerable to biotrophic and hemi-biotrophic pathogens. In fact, some strains of the hemibiotrophic bacterial pathogen *P. syringae* have evolved an ability to produce a potent JA-mimicking phytotoxin, COR, to activate JA signaling as an effective means of inhibiting SA defense and promote plant susceptibility (Cui et al. 2005; Geng et al. 2014; Xin and He 2013; Zheng et al. 2012). Furthermore, COR-like compounds are produced by pathogens of other taxa (Bender et al. 1999; Fyans et al. 2014) and proteinaceous effectors from both bacterial and fungal pathogens have been shown to target the COI1-JAZ co-receptor (Gimenez-Ibanez et al. 2014; Jiang et al. 2013; Plett et al. 2014). These recent findings suggest that the COI1-JAZ co-receptor is a common target of manipulation by diverse plant pathogens and represents a prominent vulnerable point of the plant immune network.

COR structurally mimics JA-Ile and directly binds to the COI1-JAZ co-receptor to activate the JA signaling pathway (Fonseca et al. 2009; Katsir et al. 2008; Melotto et al. 2008a). The molecular mimicry of COR is remarkable, as illustrated by its high binding affinity (equal to or higher than JA-Ile) to the COI1-JAZ co-receptor, and by the fact that all previously reported COI1 mutations that affect the action of JA-Ile also affect the action of COR (Browse 2009; Fonseca et al. 2009; Katsir et al. 2008; Melotto et al. 2008a; Sheard et al. 2010; Yan et al. 2009). Interestingly, coronatine-*O*-methyloxime (COR-MO), a potent and highly specific JA-Ile antagonist, was found to inhibit both JA signaling and COR action in Arabidopsis and *Nicotiana benthamiana* (Monte et al. 2014). To date, no COI1 mutations have been shown to differentially affect the action of JA-Ile vs. COR, illustrating the difficulty in uncoupling the molecular actions of these ligands. Nevertheless, a systematic mutagenesis of the COI1-JAZ co-receptor has not been reported.

Guided by the crystal structure of the COI1-JAZ co-receptor and evolutionary clues, we report here the successful generation of a modified JA receptor with a single amino acid substitution in the JA-Ile-binding pocket of the COI1 protein, which allows for sufficient signal transduction of endogenous JA hormone, fertility and plant defense against insects, but confers resistance against COR-producing pathogens, *P. s.* pv. *tomato* (*Pst*) DC3000 and *P. s.* pv. *maculicola* (*Psm*) ES4326. Our results provide a proof-of-concept demonstration that host target modification could be a promising new approach to prevent hijacking of host targets by highly evolved pathogens.

Materials and methods

All experiments reported in this work were performed three or more times with similar results. For computer modeling, coordinates for COR or JA-Ile were obtained from the crystal structures of COI1-JA-Ile/COR-JAZ degron peptide complex (PDB id 3OGL and 3OGK, respectively). In Y2H and *in planta* assays, we standardize the relative potencies of different ligands used (COR, MeJA and JA-Ile) before a new set of experiments. Because of the limited amounts of JA-Ile available for this study, we used other forms of JA if the use of JA-Ile was not absolutely needed. For example, MeJA can be converted to JA-Ile *in planta* and is commonly used in the study of JA signaling (Staswick and Tiryaki 2004). Therefore, we used MeJA, instead of JA-Ile, for *in planta* assays. However, for Y2H experiments we used JA-Ile, because JA or MeJA are not active in yeast (Melotto et al. 2008a; Thines et al. 2007).

Computational modeling

Coordinates for COR or JA-Ile were obtained from the crystal structures of COI1-JA-Ile/COR-JAZ degron peptide complex (PDB id 3OGK and 3OGL, respectively) and the hydrogen atoms were added using xleap module in the Ambertools. Force field parameters and charges were derived using Antechamber module and GAFF in Ambertools (Wang et al. 2001). The force field ff99SB was used to represent the molecular mechanical potential. The system consisting of COR or JA-Ile along with COI1 and part of the JAZ degron peptide were minimized in two stages using a combination of steepest descent (15000 steps) and conjugated gradient (5000 steps) methods. A strong positional restraint (20 kcals/mole) was applied on all protein and ligand heavy atoms during the first stage of minimization. The protein-ligand complex was minimized again in the second stage, without any positional restraint. *In silico* mutations for A86 and A384 were introduced in COI1 using Pymol (DeLano Scientific LLC, Palo Alto, CA).

Gene cloning, site-direct mutagenesis and plasmid construction

The coding sequences (CDS) of *AtCOI1* and *AtJAZ9* were amplified from total mRNA extract of Arabidopsis Col-0 leaf tissue and cloned into the pCR2.1-TOPO plasmid or pENTR-D TOPO Gateway entry vector (Life Technologies, Grand Island, NY). Specific mutations were introduced into the *AtCOI1* coding sequence directly through the QuickChangeII site directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). For Y2H assays, we first converted the bait and prey vectors pGILDA and pB42AD (Clontech, Mountainview, CA) Gateway cloning-compatible pGILDAattR and pB42ADattR by inserting an attR cassette (Life Technologies, Grand Island, NY) into their multiple cloning site, respectively. Next, the wild-type and mutated *COI1* CDSs in the entry vector were recombined into pGILDAattR using LR ClonaseII (Life

Technologies, Grand Island, NY) to generate C-terminal fusions to the LexA DNA binding domain. The *JAZ9* coding sequences were recombined into pB42ADattR to generate C-terminal fusions to the B42 transcriptional activation domain.

For plant transformation, the *AtCOI1* without stop codon was first cloned into pENTR4A to create pENCOI1C. Next, the native promoter of *AtCOI1* (*pCOI1*; a genomic DNA fragment 1,807 bp upstream of the COI1 start codon) was cloned into pENCOI1C to create a pENpCOI1:COI1 entry vector. $COI1^{A384V}$ was introduced into this vector to create pENpCOI1:$COI1^{A384V}$. Both pCOI1:$COI1^{WT}$ and pCOI1:$COI1^{A384V}$ were recombined into pGWB516 vector (containing a hygromycin resistance gene and a C-terminal 4x c-Myc epitope tag) (Nakagawa and Cuthill 2007) using LR ClonaseII. Confirmed constructs were introduced into *Agrobacterium tumefaciens* (GV3101) by electroporation.

Yeast-two-hybrid for protein-protein interaction

Yeast EGY48 strain carrying the p8Op:LacZ reporter plasmid was co-transformed with pGilda:COI1 (or COI1 mutants) and pB42AD:JAZ9 (or other JAZs) (Clontech, Mountain View, CA). Colonies were selected on SD minimal plates (BD Biosciences, San Jose, CA) supplemented with the -uracil (U)/-tryptophan (W)/-histidine (H) amino acid drop out solution (Clontech, Mountain View, CA). Yeast colonies were cultured overnight in liquid SD -UWH medium, harvested, washed twice in sterile water and adjusted OD_{600} to 0.2 in liquid SD galactose/raffinose -UWH medium (BD Biosciences, San Jose, CA). For Y2H on plates, ten µl culture was spotted onto SD galactose/raffinose-UWH plates with 80 µg/ml X-gal and 10 µM COR (Sigma-aldrich, CA). Blue color indicated protein-protein interactions after 5-7 days 30 °C

incubation. For liquid Y2H assay, cultures were supplemented with designated concentrations of JA-Ile (10 or 30 μM), COR (1 μM) or DMSO. After 16-hour incubation with ligands, the liquid cultures were processed through the Beta-Glo Assay system (Promega, Madison, WI) for detecting the β-galactosidase activity. The JA-Ile stock consists of cis- and trans-isomers, the cis-form being more active. Initial chemical analysis showed 6.8% of the cis-form after synthesis. Protein expression in yeast was detected using anti-LexA antibody (1:5,000, Upstate Biotechnology, Temecula, CA) for detection of COI1 expression from pGilda vector and anti-HA (1:5,000, Roche Life Science, Indianapolis, IN) antibody for detection of JAZ9 expression in pB42AD vector.

Arabidopsis transformation and screening

pCOI1:COI1$^{WT/A384V}$*-4xc-Myc* constructs were transformed into *coi1-30* mutant Arabidopsis plants (Yang et al. 2012). Because homozygous *coi1-30* plants are male sterile, heterozygous plants were identified through genotyping and used for *Agrobacterium tumefaciens*-mediated Arabidopsis transformation (Clough and Bent 1998). Half-strength Murashige and Skoog (MS) medium with 50 μg/ml hygromycin were used to select transgenic T1 seedlings containing *pCOI1:COI1*$^{WT/ A384V}$*-4xc-Myc* transgene. Hygromycin resistant seedlings were transplanted and genotyping was carried out to determine transgenic plants with the homozygous *coi1-30* background. Further screening of T2 or T3 plants were performed for homozygous transgene. Primers used: SALK035548_LP1, CGAATAAATCACACAGCTTATTGG, SALK035548_RP1, GATATGGTTCTTTGTACAACGACG, LBb1.3, ATTTTGCCGATTTCGGAAC, SALK035548_RP, CTGCAGTGTGTAACGATGCTC.

Protein immunoblot analysis

Proteins were extracted from 10- to 12- day old seedlings by protein extraction buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Protein concentrations were measured using the RC/DC protein assay kit (BioRad, Hercules, CA) with bovine serum albumin (BSA) as the standard (BioRad, Hercules, CA). Protein samples with the same total protein concentration were used for immunoblot with rabbit polyclonal anti-c-Myc primary antibodies (1:5,000, Clontech, Mountainview, CA) and goat anti-rabbit secondary antibody (1:20,000).

Co-receptor pulldown assay

Pull-down assays were performed with protein extracts from 4 mg *pCOI1:COI1*$^{WT/A384V}$*-4xc-Myc* plants and 25 μg recombinant MBP-JAZ9-8xHis. Assays were performed in the presence of JA-Ile or COR at various concentrations and incubated for 30 min at 4°C in the binding buffer (Katsir et al. 2008). Eighty μl of Ni resin (Invitrogen, Carlsbad, CA) was added, followed by an additional 15-min incubation period at 4°C. MBP-JAZ9-8xHis-bound Ni resin was washed three times on microcentrifuge spin columns with 0.25 ml binding buffer at 4°C. MBP-JAZ9-8xHis was eluted from the resin with 100 μl of 300 mM imidazole. Proteins bound to MBP-JAZ9-8xHis were analyzed by immunoblotting for the presence of COI1$^{WT/A384V}$-4xc-Myc using anti-c-Myc antibody. MBP-JAZ9-8xHis recovered by the Ni affinity resin was detected by Coomassie Blue staining.

RNA isolation and qPCR assays

Col-0 and transgenic seeds were germinated on half-strength MS medium. Five-day old seedlings were transferred into liquid half-strength MS medium. Segregating *coi1-30* seeds were germinated on half-strength MS medium with 10 μM MeJA and MeJA resistant 5-day old seedlings were transferred into liquid half-strength MS medium. 10 μM MeJA (Sigma, Hercules, CA), 0.2 μM COR or 0.1% DMSO were applied to 12 day old seedlings. Samples were collected after three hours and total RNA was extracted using Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). M-MLV Reverse transcriptase (Life Technologies, Grand Island, NY) and SYBR Green master mix (Life Technologies, Grand Island, NY) were used for reverse transcription and real-time PCR. Primers used: PP2AA3_qRT_F1, GGTTACAAGACAAGGTTCACTC, PP2AA3_qRT_R1, CATTCAGGACCAAACTCTTCAG, JAZ9_qRT_F1, ATGAGGTTAACGATGATGCTG, JAZ9_qRT_R1, CTTAGCCTCCTGGAAATCTG, PR1_qRT_F1, GGCTAACTACAACTACGCTG, PR1_qRT_R1, TCTCGTTCACATAATTCCCAC, SID2_qRT_F2, ACTTACTAACCAGTCCGAAAGACGA, SID2_qRT_R2, ACAACAACTCTGTCACATATACCGT.

Root growth inhibition assays

Col-0, segregating *coi1-30*,transgenic $COI1^{WT}$ and $COI1^{A384V}$ seeds were surface-sterilized, cold stratified, and germinated on half-strength MS agar media containing MeJA, COR or DMSO with indicated concentrations. Seedlings were grown under long day light conditions (16 h light, 100 $\mu E/m^2/s$ and 8 h dark) for 10-12 days before scanning images of roots. Root lengths were measured using ImageJ software (http://rsbweb.nih.gov/ij/).

Bacterial infection assays

The *P. syringae* infection assays in Arabidopsis were performed as described previously (Yao et al. 2013). In brief, 4- to 5-week old (12 h light/12 h dark) Arabidopsis plants were dip-inoculated with bacterial suspension ($1x10^8$ cfu/ml *Pst* DC3000 or *Psm* ES4326 in 0.25 mM $MgCl_2$ solution with 0.025% Silwet-77) or syringe-infiltrated with bacterial suspension ($1x10^6$ cfu/ml *Pst* DC3118 or DB29 in 0.25 mM $MgCl_2$ solution). Bacterial growth was determined by serial dilutions of plant extracts 3 or 4 days after inoculation. Homozygous *coi1-30* plants were selected by genotyping before bacterial infection.

Insect feeding assays

Insect bioassays were performed as described previously (Herde et al. 2013). Briefly, four neonate *S. exigua* larvae were transferred to the center of each six-week old Arabidopsis host plant, grown under 8 h light (100 $\mu E/m^2/s$) and 16 h dark. Eggs of *S. exigua* were obtained from Benzon Research, Inc. (Carlisle, PA, USA). Plants were covered with cup cages. Larval weights were determined after 9 to 12 days of feeding.

Results

A large-scale, targeted alanine substitution mutagenesis of the COI1 protein

We began our study by conducting an expanded mutagenesis of the COI1 protein to identify amino acid residues that might differentially affect the actions of JA-Ile vs. COR. We conducted targeted alanine substitution mutagenesis of the COI1 protein to identify amino acid residues that might differentially affect the actions of JA-Ile vs. COR. At the onset of this study, the crystal structure of the COI1-JAZ co-receptor was not available. However, the crystal structure of the

TIR1-AUX/IAA (for TRANSPORT INHIBITOR RESPONSE 1 [TIR1]-AUXIN/INDOLE 3-ACETIC ACID [AUX/IAA]) co-receptor involved in the perception of the plant hormone auxin was available (Tan et al. 2007). Auxin and JA signaling pathways are highly analogous in hormone sensing and response (Pauwels et al. 2010; Santner and Estelle 2009). In fact, comparative genomic analysis suggested that auxin and JA signaling pathways may have originated from a common ancestor which duplicated and diverged into TIR1 and COI1 for different hormone signaling pathways (Wang et al. 2015). We hypothesized at the time that the ligand-binding surfaces in COI1 would be similarly positioned as those in TIR1, and that key differences in these conserved residues might confer specificity to the differential recognition of the respective ligands (i.e., JA-Ile/COR vs. auxin). Based on this initial criterion, thirty-two amino acids were selected for site-direct mutagenesis to alanine (Table 1). Y2H assays showed that 56% (18 out of 32) of the alanine substitution mutants abolished COR-dependent interaction between COI1 and JAZ9 (Table 1 and Fig. 4A).

When the 18 residues were mapped to the crystal structure of the COI1-JAZ1 co-receptor, which became available later (Sheard et al. 2010), 12 are found to make contacts with ligand, Ins(1,2,4,5,6)P_5 ($InsP_5$) and/or JAZ1 in the ligand-binding pocket. The crystal structure of COI1-JAZ1 co-receptor also shows several additional amino acids in the ligand-binding pocket, which could contribute to the interactions between COI1-ligand, COI1-JAZ and/or COI1-interaction with $InsP_5$ (Sheard et al. 2010). We therefore selected ten additional amino acids for site-directed mutagenesis to alanine (Table 1). Y2H assays showed that three alanine substitutions, Y302A, R326A and Y444A, disrupted the COR-dependent COI1-JAZ9 interaction (Fig. 4B). Thus, a

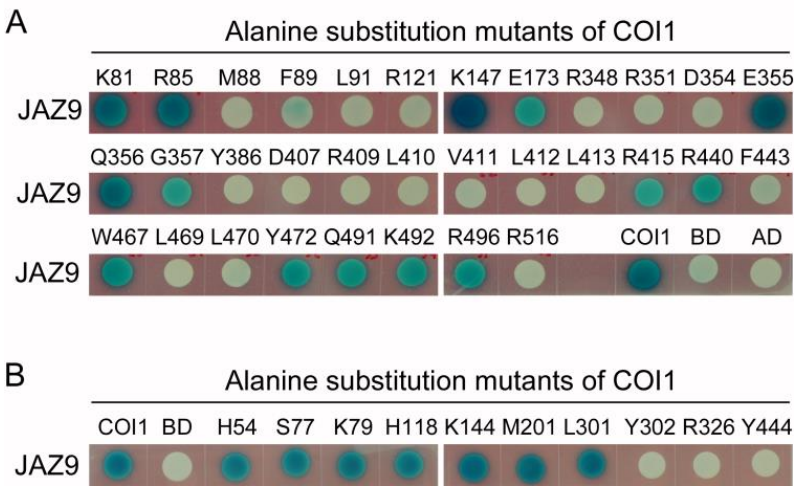

Figure 4: Effect of alanine substitutions on COI1-JAZ9 interactions in Y2H assay in the presence of 10 µM COR. (A) Interactions between JAZ9 and thirty-two alanine substitution COI1 mutants selected based on TIR1 crystal structure. Blue colonies indicate positive interactions. (B) Interactions between JAZ9 and ten additional alanine substitution COI1 mutants selected based on the COI1 crystal structure.

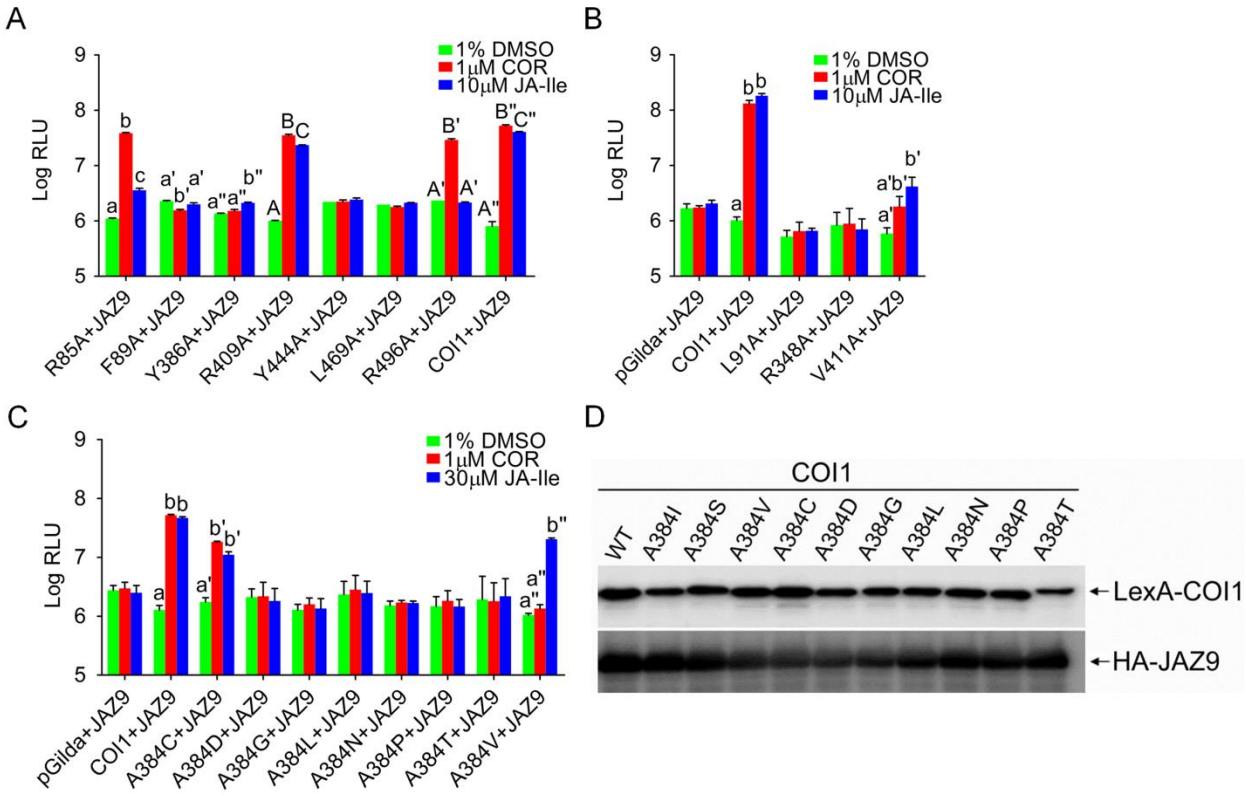

Figure 5: Liquid Y2H results of interactions between COI1 substitution mutants and JAZ9. (A) Liquid Y2H results of JAZ9 interaction with COI1 mutants in which each of seven selected JA-Ile-contacting residues was substituted with alanine. (B) Liquid Y2H results of JAZ9 interaction with COI1 mutants in which each of three additional JA-Ile-interacting residues was substituted with alanine. (C) Liquid Y2H results of JAZ9 and seven additional COI1 mutants with substitutions at the A384 position. (D) Western blot for A384 substitutions expression in yeast, showing that A384V and other A384 substitution mutants are expressed and stable in yeast. Anti-LexA antibody was used for detection of COI1 proteins expressed from pGilda vector and anti-HA antibody was used for detection of JAZ9 expression from pB42AD vector. RLU indicates the degree of interaction between COI1 mutants and JAZ9 in the presence of either 1 μM COR, 10 μM JA-Ile (or 30 μM JA-Ile), or 1% DMSO treatment. Different letters above columns indicate significant differences ($p<0.05$) between different treatments (i.e., DMSO, JA-Ile or COR) for the same set of interacting proteins. For those interacting proteins that do not have letter labels above columns, no significant differences were detected between treatments. Two way ANOVA with Bonferroni posttest was used. Data were presented as mean ± SEM (A: N=2; B, C: N=3).

Table 1: COI1 amino acids selected for alanine substitution.

COI1 Amino Acids	Interaction with JAZ9 [a]	Role in the COI1 ligand-binding pocket[b]	Homologous TIR1 Amino Acid[c]	Role in the TIR1 ligand-binding pocket[c]
Histidine 54[d]	+++	PO_4 contacting	Lysine	Not known
Serine 77[d]	+++	PO_4 contacting	Serine	Not known
Lysine 79[d]	+++	PO_4 contacting	Glutamate	Not known
Lysine 81	+++	PO_4 contacting	Lysine	IP6 coordination
Arginine 85	+++	JA-Ile and PO_4 contacting	Histidine	Auxin binding, IP6 coordination
Methionine 88	No	JAZ1 and PO_4 contacting	Aspartate	Aux/IAA binding
Phenylalanine 89	No	JA-Ile and JAZ1 contacting	Phenylalanine	Auxin and AUX/IAA binding
Leucine 91	No	JA-Ile and JAZ1 contacting	Leucine	Aux/IAA binding
Histidine 118[d]	+++	PO_4 contacting	Arginine	Not known
Arginine 121	No	PO_4 contacting	Arginine	IP6 coordination
Lysine 144[d]	+++	PO_4 contacting	Valine	Not known
Lysine 147	++++	PO_4 contacting	Serine	Aux/IAA binding
Glutamate 173	++	JAZ1 contacting	Glutamate	Aux/IAA binding
Methionine 201[d]	+++	JAZ1 contacting	Cysteine	Not known
Leucine 301[d]	+++	JAZ1 contacting	Serine	Not known
Tyrosine 302[d]	No	JAZ1 contacting	Tyrosine	Not known
Arginine 326[d]	No	JAZ1 contacting	Leucine	Not known
Arginine 348	No	JA-Ile, JAZ1 and PO4 contacting	Arginine	IP6 coordination
Arginine 351	No	JAZ1 contacting	Proline	AUX/IAA binding
Aspartate 354	No	JAZ1 contacting	Glutamate	AUX/IAA binding
Glutamate 355	+++	JAZ1 contacting	Proline	AUX/IAA binding
Glutamine 356	+++	Non	Phenylalanine	AUX/IAA binding

Table 1 (cont'd)

Glycine 357	++	Non	Valine	AUX/IAA binding
Tyrosine 386	No	JA-Ile and JAZ1 contacting	Phenylalanine	Auxin and AUX/IAA binding
Aspartate 407	No	Non	Arginine	IP6 coordination
Arginine 409	No	JA-Ile and PO4 contacting	Arginine	Auxin and AUX/IAA binding
Leucine 410	No	Non	Leucine	Auxin binding
Valine 411	No	JA-Ile and JAZ1 contacting	Cysteine	Auxin and AUX/IAA binding
Leucine 412	No	Non	Isoleucine	Aux/IAA binding
Leucine 413	No	JAZ1 contacting	Isoleucine	Aux/IAA binding
Arginine 415	++	Non	Proline	AUX/IAA binding
Arginine 440	++	Non	Arginine	IP6 coordination
Phenylalanine 443	No	Non	Leucine	Auxin binding
Tyrosine 444[d]	No	JA-Ile and JAZ1 contacting	None	Not known
Tryptophan 467	+++	Non	Methionine	IP6 coordination
Leucine 469	No	JA-Ile contacting	Serine	Auxin binding
Leucine 470	No	Non	Valine	Auxin binding
Tyrosine 472	+++	PO4 contacting	Phenylalanine	AUX/IAA binding
Glutamine 491	+++	Non	Arginine	IP6 coordination
Lysine 492	+++	PO4 contacting	Lysine	IP6 coordination
Arginine 496	+++	JA-Ile and JAZ1 contacting	Arginine	Auxin and AUX/IAA binding
Arginine 516	No	Non	Arginine	IP6 coordination

[a]Y2H assays were conducted with 10 μM COR in the medium. The strength of mutant COI1-JAZ9 interactions was scored relative to COI1-JAZ9 (designated as +++).

[b]COI1 amino acids and their roles in the JAZ1-COI1-JA-Ile interaction as reported (Sheard et al. 2010).

[c]TIR1 amino acids corresponding to those in COI1 and roles in ligand-receptor interaction as reported (Sheard et al. 2010; Tan et al. 2007).

[d]Additional COI1 amino acids selected for mutagenesis based on the crystal structure of the COI1-JAZ1 complex with COR or JA-Ile (Sheard et al. 2010).

total of 21 COI1 residues were identified to be important for COR-induced formation of the COI1-JAZ co-receptor complex in yeast.

To determine whether substitutions that affected COR-dependent COI1-JAZ9 interaction differentially affect JA-Ile-dependent COI1-JAZ9 interaction, we conducted quantitative liquid Y2H assays with 10 alanine substitutions for the amino acids contacting directly with JA-Ile (Sheard et al. 2010) (Fig. 5A, B). We found that (i) seven alanine substitutions disrupted both JA-Ile- and COR- dependent COI1-JAZ9 interaction, (ii) R409A substitution exhibited reduced COI1-JAZ9 interaction in the presence of JA-Ile or COR, and (iii) the R85A and R496A substitutions affect JA-Ile-dependent interaction more than COR-dependent interaction. No substitution was found to disrupt only COR dependent interaction and still maintain JA-Ile-dependent COI1-JAZ9 interaction. Our results therefore strengthen the notion that COR is a remarkable mimic of JA-Ile and that most, if not all, of COI1 residues that are important for the action of JA-Ile are also important for COR action.

Structure-guided modeling of JA-Ile/COR-binding sites in COI1

Our initial mutagenesis was based on alanine substitution, which resulted in a reduction of the side chain size for all the amino acid residues targeted for mutagenesis, except for G357A. Next, we considered increasing the side chain sizes of residues that are in contact with JA-Ile/COR. We noted that, although COR and JA-Ile are highly similar in structure, the flexibilities of COR and JA-Ile in the binding pocket are different. For example, the cyclohexene ring and the ethyl-cyclopropane group of COR appear more rigid than the equivalent parts (the pentenyl side chain and the isoleucine side chain, respectively) of JA-Ile (Sheard et al. 2010). We hypothesized that

the higher rigidity of the cyclohexene ring and the ethyl-cyclopropane group of COR may be more prone than the equivalent parts of JA-Ile to physical hindrance from an increased size of the amino acid side chain with which COR/JA-Ile are in direct contact.

Based on the above hypothesis, residues A86 and A384 attracted our attention for two reasons. First, *in silico* analysis of the putative JA-Ile-binding pockets in diverse plant species for which the COI1 protein sequences are available revealed that, although most residues in the JA-binding site are highly conserved across taxa, residues at positions 86 and 384 exhibit a higher degree of polymorphism (Fig. 6A). In the moss species *Physcomitrella patens*, for example, isoleucine or valine occupy the corresponding position of A384 (Fig. 6A). Positions of A86 and A384 in *Selaginella meollendorffii* are replaced by isoleucine/valine and serine, respectively (Fig. 6A). Previous studies have shown that, although core JA signaling genes are found in *P. patens* (Wang et al. 2015), neither JA nor JA-Ile could be detected in *P. patens* (Stumpe et al. 2010). On the other hand, (9S,13S)-12-oxo-phytodienoic acid (cis-(+)-OPDA), the precursor of JA biosynthesis, is synthesized in *P. patens*, suggesting that *P. patens* may produce an alternative, OPDA-related ligand (Stumpe et al. 2010). We speculated that, during plant evolution, the polymorphism at positions 86 and 384 in the putative COI1 binding pocket may provide a basis for accommodating related ligands of distinct structural features. If so, mutations at these amino acid positions may have a higher chance of producing differential effects on different ligands compared with more highly conserved residues, which are expected to affect different ligands similarly.

Second, we noted that, in the JA-Ile/COR-binding pocket, A86 and A384 make direct contacts with the ligand (Fig. 6A) and are situated close to the cyclohexene ring and the ethyl-cyclopropane group of COR or the equivalent parts of JA-Ile, the pentenyl side chain and the isoleucine side chain, respectively (Fig. 7A, B, Fig. 6B, C). The Cβ atom of A86 is 3.6 Å from the nearest C-atom in the pentenyl side chain of JA-Ile and 3.7 Å from the ethyl group attached to the cyclohexene ring of COR in their respective crystal structures. The Cβ atom of A384 is 4.0 Å from the nearest C-atom of the isoleucine side chain of JA-Ile and 3.6 Å from the ethyl-cyclopropane group of COR. *In silico* mutagenesis followed by energy minimization revealed that the A384V substitution, in particular, would create steric clash with the isoleucine side chain of JA-Ile or the ethyl group attached to the cyclopropane moiety of COR (Fig. 7C, D). However, the flexibility of the isoleucine side chain of JA-Ile would likely allow for its re-adjustment to fit the mutated ligand-binding pocket, whereas the rigidity of the ethyl-cyclopropane group of COR would not (Fig. 7E, F). Taken together, our *in silico* and structural modeling analyses suggest the possibility that mutating alanine to valine at position 384 may result in a ligand-binding pocket that is more unfavorable to the chemical structure of COR than that of JA-Ile.

Effects of amino acid substitutions at positions 86 and 384 on JA-Ile/COR-dependent formation of the COI1-JAZ9 co-receptor

To test the hypothesis that mutating A384 or A86 may create a ligand-binding pocket that is more unfavorable to COR than to that of JA-Ile, we first substituted these two alanine residues with the corresponding residues found in lower plant species *P. patens* and *S. moellendorffii* (Fig. 6A). Specifically, the following COI1 mutants were generated: $COI1^{A86I}$, $COI1^{A86V}$, $COI1^{A384I}$, $COI1^{A384S}$ and $COI1^{A384V}$. Quantitative liquid Y2H assays revealed that both $COI1^{A86I}$ and

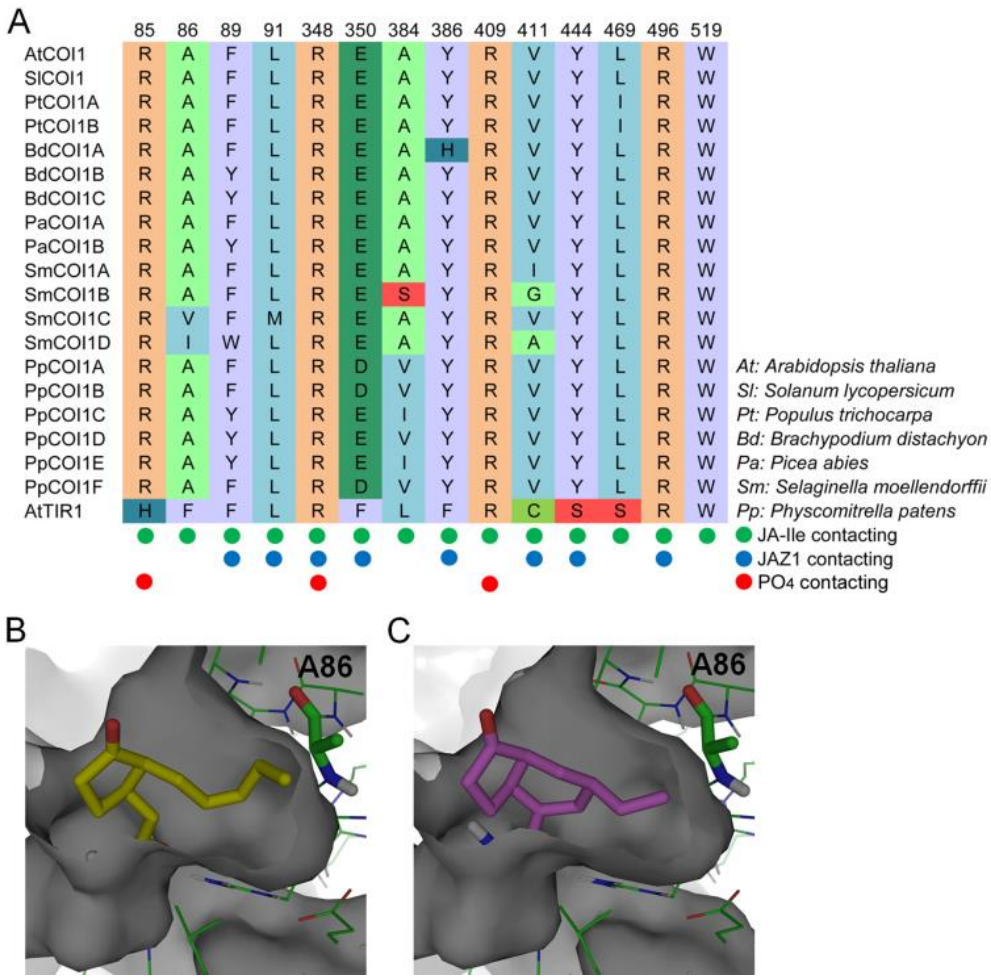

Figure 6: Alignments of amino acids of COI1 orthologs involved in ligand-receptor interaction in the ligand binding pocket and position of A86 with respect to JA-Ile and COR bound in COI1 ligand binding pocket. (A) Fourteen amino acids involved in JA-Ile interaction in the Arabidopsis COI1 protein (Sheard et al. 2010) and the corresponding amino acids in six representative plant species. Green dots indicate amino acids contacting with JA-Ile/COR. Blue dots indicate amino acids that also make contact with JAZ1. Red dots indicate amino acids that also make contact with $InsP_5$. Abbreviations: *Arabidopsis thaliana* (At), *Solanum lycopersicum* (Sl), *Populus trichocarpa* (Pt), *Brachypodium distachyon* (Bd), *Picea abies* (Pa), *Selaginella moellendorffii* (Sm), *Physcomitrella patens* (Pp). (B, C) Binding pose of (B) JA-Ile and (C) COR with respect to A86 in the ligand-binding site of wild type COI1 (PDB id 3OGL and 3OGK, respectively). C-atoms in COI1 are shown in green and those in JA-Ile and coronatine are shown in yellow and magenta, respectively. Ligand-binding site in COI1 is shown in grey-colored surface representation; ligand and A86 residues are shown in stick representation, whereas all other atoms in the protein are shown in line representation.

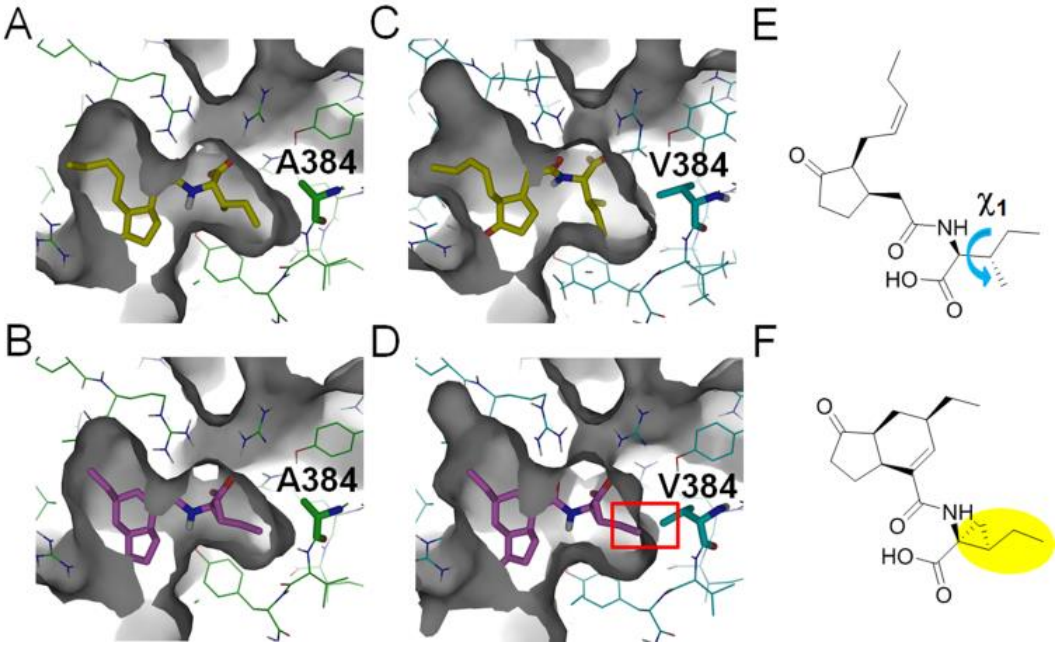

Figure 7: Computer modeling of JA-Ile or COR in the ligand-binding site of COI1 or COI1^{A384V}. (A, B) Binding pose of JA-Ile (A) and COR (B) in the ligand-binding site of COI1 in the crystal structures of the COI1-JAZ1 complex (PDB id 3OGL and 3OGK, respectively). Amino acid contacts in the ligand pocket were described by Sheard *et al*. (Sheard et al. 2010). (C, D) Computer modeling of the A384V substitution showing expected steric clash with the isoleucine sidechain of JA-Ile or the ethyl group attached to the cyclopropane moiety of COR. However, the isoleucine sidechain of JA-Ile can be adjusted in the mutant ligand binding site by rotation of the sidechain dihedral angle, $\chi 1$ of isoleucine (C). In contrast, the steric clash (highlighted in red box) impairs COR binding in the ligand-binding site because the rotatable bond at the equivalent position is absent in COR (D). The ligand-binding site in COI1 is shown in grey-colored surface representation. Ligands and A384/V384 residues are shown in stick representation, whereas all other atoms in the protein are shown in line representation. C-atoms in the WT and mutant COI1 proteins are shown in green and cyan, respectively; those in JA-Ile and COR are shown in yellow and magenta, respectively. In protein and ligand molecules N, O and H-atoms are colored in blue, red and grey, respectively, and, for clarity, non-polar H-atoms are not shown. (E) Molecular structure of JA-Ile with $\chi 1$ torsion angle shown in cyan arrow. (F) Molecular structure of COR in which the cyclopropane moiety restricts the rotational freedom of the terminal ethyl group. The cyclopropane moiety along with the ethyl substitution is highlighted in yellow.

COI1^{A86V} abolished JA-Ile-dependent COI1-JAZ9 interaction, and reduced COR-dependent COI1-JAZ9 interaction (Fig. 8A). This indicated that A86 is critical for the action of both JA-Ile and COR, albeit more critical for JA-Ile than COR.

Substitutions at position 384 exhibited more diverse effects than those at position 86 on JA-Ile/COR-dependent COI1-JAZ9 interaction (Fig. 8A). COI1^{A384I} disrupted both JA-Ile- and COR-dependent interaction, whereas COI1^{A384S} only reduced JA-Ile-dependent interaction. Most interestingly, COI1^{A384V} greatly reduced COR-dependent interaction, but had lesser effect on JA-Ile-dependent COI1-JAZ9 interaction (Fig. 8A). We also found that 10 μM JA-Ile, which contains a mixture of active and inactive isomers of JA-Ile, was equivalent to 0.1 μM pure COR in promoting the COI1-JAZ9 interaction in yeast (Fig. 8B).

We made seven additional substitutions at A384 to determine whether these substitutions would have an effect similar to that of COI1^{A384V}. Of these seven substitutions (representing different types of side chains), A384C reduced, and A384D, A384G, A384L, A384N, A384P and A384T completely disrupted JA-Ile- and COR-dependent interaction (Fig. 5C). In all, no additional substitutions affected COR-dependent COI1-JAZ9 interaction more than JA-Ile-dependent COI1-JAZ9 interaction. Therefore, through extensive mutagenesis efforts we succeeded in identifying a specific amino acid substitution, A384V, in the JA-Ile-binding pocket that preferably affects COR-dependent COI1-JAZ9 interaction, compared with JA-Ile-dependent COI1-JAZ9 interaction in yeast.

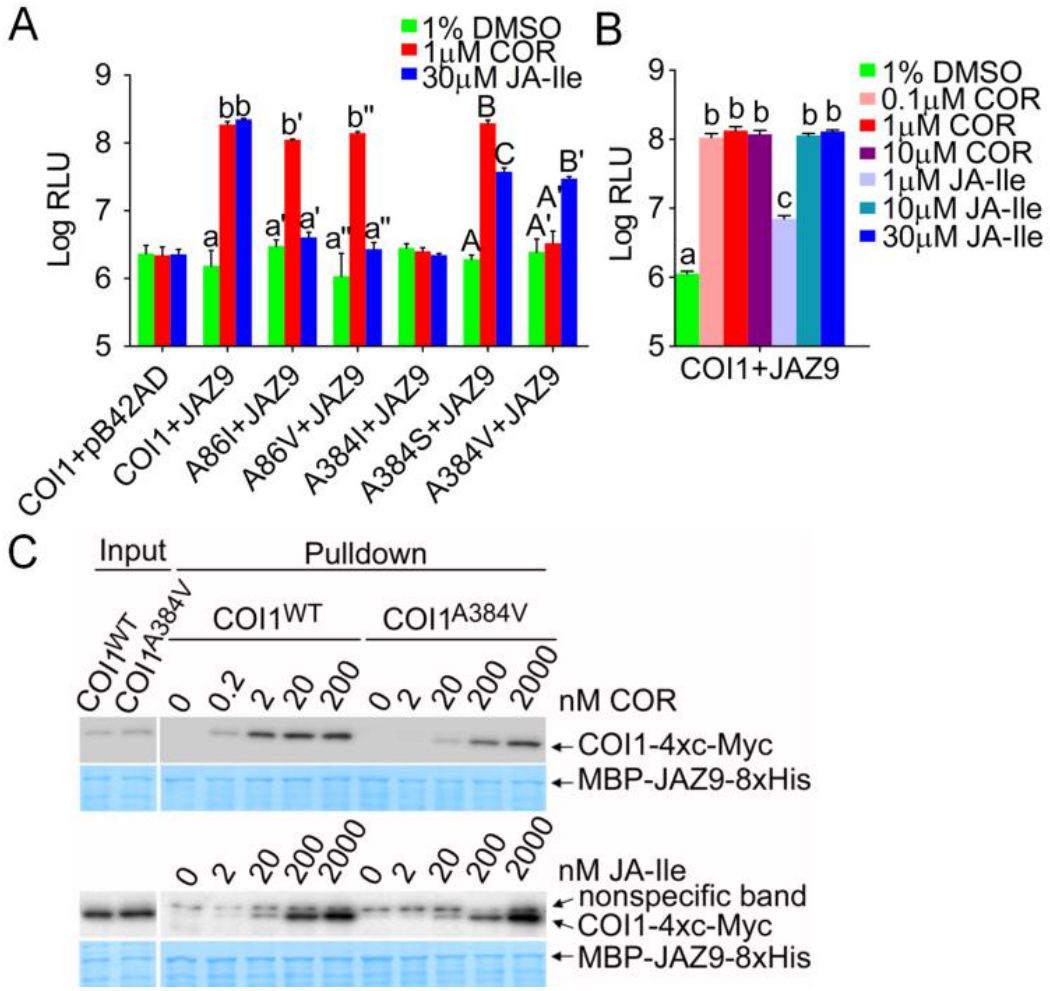

Figure 8: Y2H and pull-down assays for physical interactions between COI1 and JAZ9 proteins. (A) Liquid Y2H results of JAZ9 and mutant COI1 proteins containing amino acid substitutions at position 86 or 384 in the presence of 1 μM COR or 30 μM JA-Ile. (B) Liquid Y2H results of COI1-JAZ9 interaction in the presence of different concentrations of JA-Ile and COR. Relative light units (RLU) indicated the degree of interaction between COI1 mutants and JAZ9. 1% DMSO treatment was used as mock treatment. Different letters of the same type above columns indicated significant differences ($p<0.05$) between different treatments (i.e., DMSO, JA-Ile or COR) for the same set of interacting proteins (N=3, error bars, SEM). For those interacting proteins that do not have letter labels above columns, no significant differences were detected between treatments. Two-way ANOVA with Bonferroni posttest was used for (A). One-way ANOVA with Tukey's multiple comparison test was used for (B). (C) Results of co-receptor pulldown assays. Pull-down assays were performed with protein extracts from *pCOI1:COI1*$^{WT/A384V}$*-4xc-Myc* plants and recombinant *E. coli*-expressed MBP-JAZ9-8xHis in the presence of COR or JA-Ile at indicated concentrations. Proteins bound to MBP-JAZ9-8xHis were analyzed by immunoblotting. Anti-c-Myc antibody was used for detection of COI1$^{WT/A384V}$-4xc-Myc protein. The Coomassie Blue-stained gel shows the amounts of MBP-JAZ9-8xHis pulled down by the Ni affinity resin.

Transgenic Arabidopsis plants expressing COI1^{A384V} are fertile but exhibit differential sensitivities to methyl jasmonate (MeJA) and COR *in vivo*

To determine the physiological relevance of the results from Y2H assays, we produced transgenic Arabidopsis plants (in *coi1-30* null mutant background) that express COI1^{A384V} from the *COI1* native promoter (*pCOI1:COI1^{A384V}-4xc-Myc*; COI1^{A384V} hereinafter). As controls, we also generated transgenic lines that express wild-type COI1 in the *coi1-30* background (*pCOI1:COI1^{WT}-4xc-Myc*; COI1^{WT} hereinafter). First, we determined whether COI1^{A384V} complements the male sterile phenotype in *coi1-30*. JA is essential for male fertility and *coi1* mutants are male sterile (Feys et al. 1994). Consistent with Y2H results showing that COI1^{A384V} maintained substantial JA-Ile interaction, 83% of COI1^{A384V} lines (10 out of 12 lines analyzed) were fertile (Fig. 9A). Four fertile COI1^{A384V} lines were randomly chosen for protein expression analysis and all were found to produce the c-Myc-tagged COI1^{A384V} protein (Fig. 9B). No fertility penalty was detected in COI1^{A384V} plants, as judged by the number of developed siliques and the number of seeds per silique, which are similar to wild-type plants (Table 2).

Next, we performed COI1-JAZ9 pulldown experiments to compare the responsiveness of plant-expressed COI1^{WT} and COI1^{A384V} proteins to serial concentrations of JA-Ile and COR using *E. coli*-expressed JAZ9 protein, following the procedure reported previously (Katsir et al. 2008). These experiments confirmed that a much higher (~100 fold) concentration of COR was required for robust formation of the COI1^{A384V}-JAZ9 co-receptor than for the COI1^{WT}-JAZ9 co-receptor, whereas similar concentrations of JA-Ile were needed to promote the formation of the COI1^{A384V}-JAZ9 and COI1^{WT}-JAZ9 co-receptors (Fig. 8C).

Finally, we conducted further analyses with two representative COI1^{A384V} lines, L1 and L2, to determine their responses to JA- or COR-induced root growth inhibition. Dose-response experiments showed that the effect of 10 μM MeJA, which is converted to the active form JA-Ile *in planta*, was equivalent to that of 0.2 μM COR in wild-type Col-0 plants (Fig. 9C and 10). Unlike wild-type Col-0 plants, the root growth inhibition of COI1^{A384V} plants was significantly less sensitive to 0.2 μM COR than to 10 μM MeJA (Fig. 9C and 10). The potency of 0.2 μM COR in inhibiting root growth in COI1^{A384V} plants was comparable to 0.1 μM MeJA, indicating ~100-fold less effectiveness of 0.2 μM COR in COI1^{A384V} than in Col-0 and COI1^{WT} (Fig. 10). These results were consistent with the differential effects of the A384V substitution on JA-Ile- vs. COR-dependent formation of the COI1-JAZ9 co-receptor observed in both Y2H and COI1-JAZ9 co-receptor pulldown assays, and confirmed that COI1^{A384V} transgenic plants are differentially sensitive to MeJA vs. COR *in vivo*.

Transgenic Arabidopsis plants expressing COI1^{A384V} exhibit differential expression of JA response marker genes in response to MeJA vs. COR

We next examined JA response gene expression in COI1^{A384V} transgenic plants. For this purpose, the expression of the JA-responsive marker gene *JAZ9* was measured by quantitative PCR (qPCR). As expected, *JAZ9* gene expression was induced in Col-0 and COI1^{WT} plants after MeJA or COR application (Fig. 9D). In COI1^{A384V} lines, however, *JAZ9* gene expression in response to COR treatment was significantly reduced compared to Col-0 or COI1^{WT} plants, whereas *JAZ9* expressions in response to MeJA treatment was less affected in this same comparison (Fig. 9D). For example, in COI1^{A384V} L1, MeJA treatment induced the expression of *JAZ9* by 38-fold compared to that in *coi1-30* plants. However, induction of *JAZ9* gene

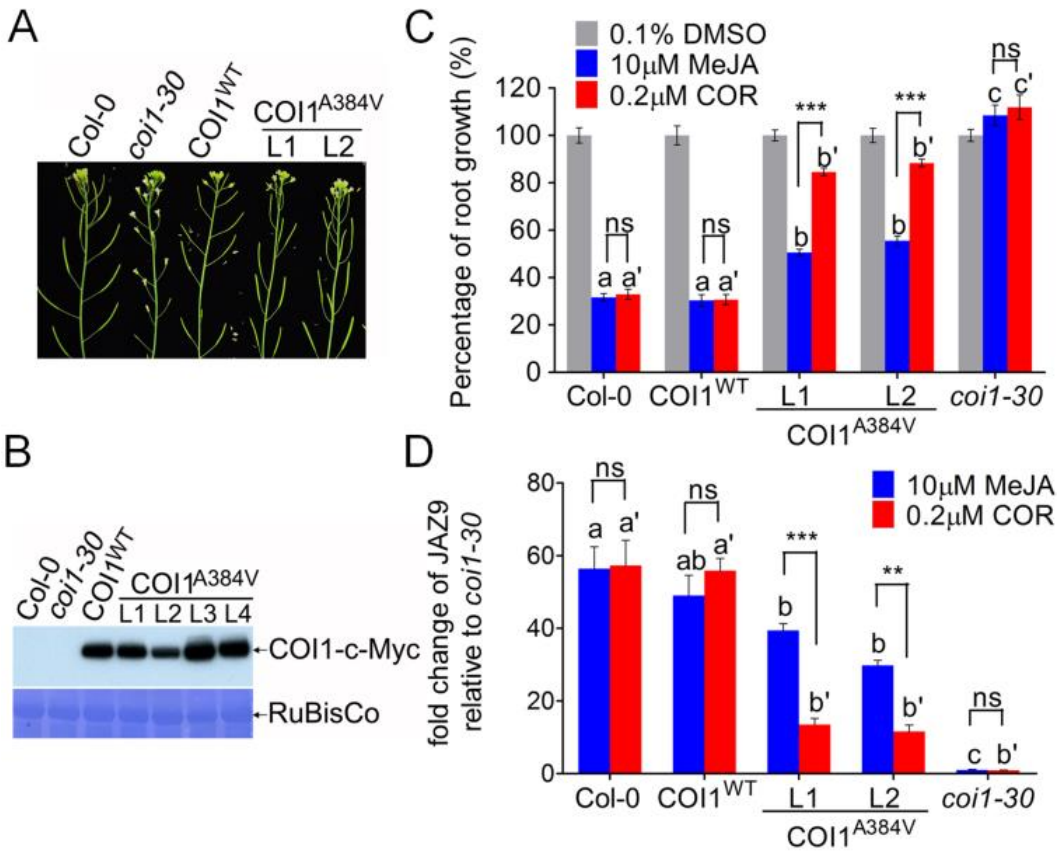

Figure 9: Phenotypes of transgenic COI1^{WT} and COI1^{A384V} plants. (A) A picture showing restoration of male fertility in transgenic *coi1*/*COI1*^{WT} and *coi1*/*COI1*^{A384V} plants. (B) COI1 protein levels in *pCOI1:COI1*^{WT}*-4xc-Myc* and *pCOI1:COI1*^{A384V}*-4xc-Myc* transgenic plants. Coomassie Blue-stained ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) protein was used as loading control. (C) Quantification of root growth assay with 10 μM MeJA or 0.2 μM COR application. Relative root length was compared to mock treatment (0.1% DMSO). Different letters of the same type above columns indicated significant differences ($p<0.05$) between different plant genotypes with the same treatment (MeJA or COR) (N=15, error bars, SEM, except for *coi1-30*, N=7), as determined by two-way ANOVA with Bonferroni posttest. ***$p<0.001$ indicated significant differences between two ligand treatments of the same plant genotype (ns: not significant). (D) Fold changes of *JAZ9* gene expression in Col-0, transgenic COI1^{WT}, COI1^{A384V} and *coi1-30* plants after 10 μM MeJA or 0.2 μM COR induction, relative to those in *coi1-30* plants with 10 μM MeJA. Internal control: PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2AA3, AT1G13320). Different letters of the same type above columns indicated significant differences ($p<0.05$) of gene expression between different plant genotypes with the same ligand treatment (MeJA or COR) (N=4, error bars, SEM), by two-way ANOVA with Bonferroni posttest. **$p<0.01$ and ***$p<0.001$ indicate significant differences between two different ligand treatments of the same plant genotype (ns: not significant).

Table 2: Fertility of Col-0, COI1^{WT}, COI^{A384V} and *coi1-30* plants.

Plant	No. of fertile flowers*	No. of seeds/silique**
Col-0	50/50	62±4
coi1-30/COI1^{WT}	50/50	57±8
coi1-30/COI1^{A384V} L1	50/50	58±6
coi1-30	0/50	0

* Number of flowers that produce siliques/number of total flowers examined. Ten flowers of each plant and five plants of each genotype were examined.
** Five siliques of each plant and a total of five plants were examined.
No statistically significant differences were found by ANOVA ($p = 0.411072$) between Col-0, *coi1-30*/COI1^{WT} and *coi1-30*/COI1^{A384V} L1.

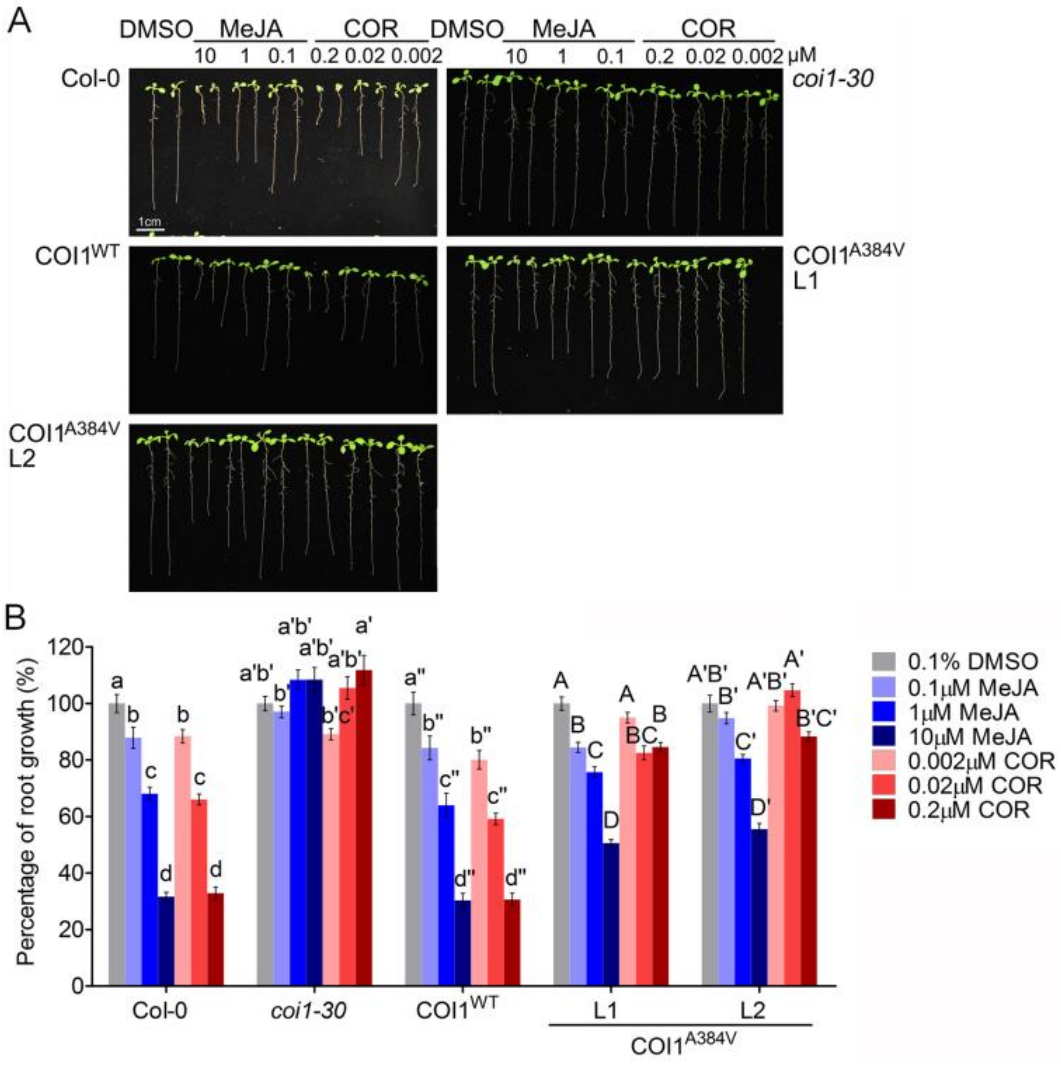

Figure 10: Root growth inhibition assays with a gradient concentration of MeJA or COR. (A) Pictures showing root growth of Col-0, *coi1-30*, COI1^{WT} and COI1^{A384V} seedlings after treatment of a gradient concentration of MeJA or COR. (B) Quantification of root growth assay shown in (A). Relative root length was compared to mock treatment (0.1% DMSO). Error bars represented SEM for 15 seedlings (except for *coi1-30*, 7 seedlings were used) within each treatment. Note that 0.1, 1 or 10 □M MeJA is similar to 0.002, 0.02 or 0.2 □M COR in potency, respectively (i.e., COR is ~50 fold more potent than MeJA). Different letters of the same type above columns indicated significant differences ($p<0.05$) of relative root growth between different treatments (i.e., DMSO, MeJA or COR) within the same plant genotype. Two-way ANOVA with Bonferroni posttest was used.

expression in COI1^{A384V} L1was only 8-fold higher than that in *coi1-30* plants after COR treatment. These results are consistent with the conclusion that the A384V substitution greatly affects the action of COR, while maintaining JA signaling required for substantial JA response gene expression. We also examined the expression of SA-responsive genes *PATHOGENESIS-RELATED GENE 1* (*PR1*) and *SALICYLIC ACID INDUCTION DEFICIENT 2* (*SID2*) in COI1^{A384V} plants and found that *PR1* and *SID2* gene expression were similarly low in Col-0, COI1^{WT} and COI1^{A384V} plants (Fig. 11), indicating that the SA signaling pathway remained quiescent in COI1^{A384V} plants, as in Col-0 and COI1^{WT} plants.

COI1^{A384V} transgenic plants gained resistance to *Pst* DC3000 and *Psm* ES4326, while maintaining high-level defense against chewing insects

Our analyses so far suggested that we might have succeeded in engineering a modified JA receptor that substantially uncouples endogenous hormone signaling from pathogen hijacking via COR. If so, we expected that the COI1^{A384V} transgenic plants would gain resistance to COR-producing bacterial pathogens, while retaining substantial defense against chewing insects. To test this possibility, we conducted bioassays using *Pst* DC3000 and *Psm* ES4326, two well-known COR-producing hemi-biotrophic pathogens that infect Arabidopsis (Dong et al. 1991; Whalen et al. 1991), and *Spodoptera exigua*, a generalist chewing insect that is susceptible to COI1-dependent defenses in Arabidopsis (Chung et al. 2008). As expected, Col-0 and COI1^{WT} plants were highly susceptible to *Pst* DC3000 (Fig. 12A, B). COI1^{A384V} plants, however, exhibited significantly increased resistance to *Pst* DC3000, as evidenced by greatly reduced bacterial growth and disease symptoms (Fig. 12A, B). Quantitatively, *Pst DC3000* populations in COI1^{A384V} lines were 254- to 42-fold lower than those in Col-0 plants and 189- to 31-fold lower

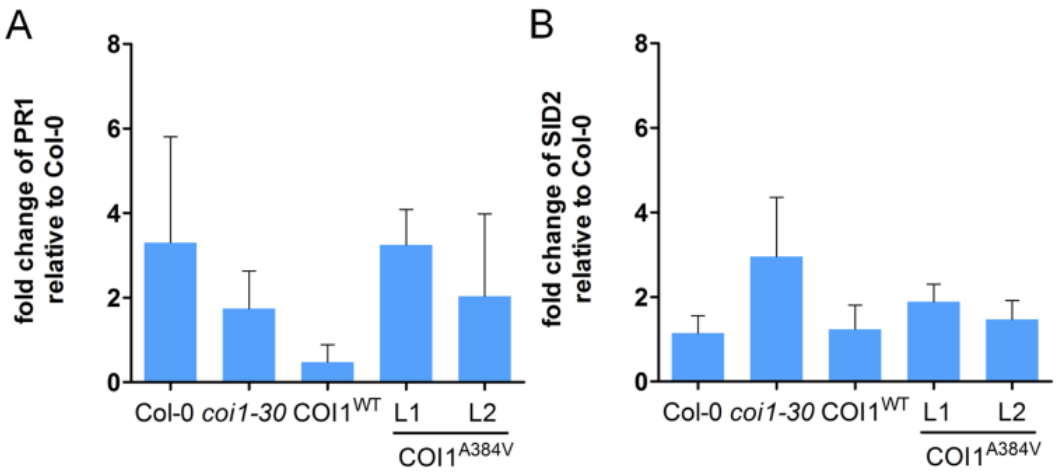

Figure 11: Expression of SA-responsive genes *PR1* and *SID2* in Col-0, COI1^{WT} and COI1^{A384V} plants, relative to that in Col-0 plants. (A) Expression of SA-responsive gene *PR1* in Col-0, COI1^{WT} and COI1^{A384V} plants. **(B)** Expression of SA-responsive gene *SID2* in Col-0, COI1^{WT} and COI1^{A384V} plants. PP2AA3 was used as an internal control gene. One-way ANOVA with Dunnett test was used (N=4, error bars, SEM). No significant difference ($p<0.05$) was detected.

than those in COI1^{WT} transgenic plants (Fig. 12B). Similarly, COI1^{A384V} plants exhibited significantly increased resistance to *Psm* ES4326 compared to wild-type Col-0 or COI1^{WT} plants (Fig. 12C, D). Control experiments showed that *coi1-30* plants were highly resistant to both pathogens in these assays (Fig. 12B, D).

Next, we conducted disease assays using *Pst* DC3118 and DB29, which are mutants of *Pst* DC3000 defective in COR production (Brooks et al. 2004; Zeng et al. 2011). Similar levels of bacterial growth were observed in Col-0, COI1^{WT}, and COI1^{A384V} plants, suggesting that the gained resistance in COI1^{A384V} plants to *Pst DC3000* was largely COR-dependent (Fig. 13).

Finally, we performed insect feeding assays using *S. exigua* neonate larvae. As expected, *S. exigua* grew much more slowly on Col-0 plants than on *coi1-30* mutant plants (Fig. 14), consistent with previous reports (Cipollini et al. 2004; Mewis et al. 2005). The average weight of larvae feeding on *coi1-30* plants was 6-fold higher than larvae reared on COI1^{WT} plants and 5-fold higher than those grown on COI1^{A384V} plants (Fig. 14). Thus, COI1^{A384V} plants maintained an almost wild-type level of defense against *S. exigua*.

Discussion

In the past decade, numerous host targets of bacterial, fungal, oomycete, and nematode virulence factors have been identified, representing major advances in our understanding of plant-microbe interactions. However, this fundamental knowledge has largely not yet been exploited to inhibit disease development. COR was one of the first bacterial virulence factors of which the host target was clearly identified (Fonseca et al. 2009; Katsir et al. 2008; Melotto et al. 2008a) and its

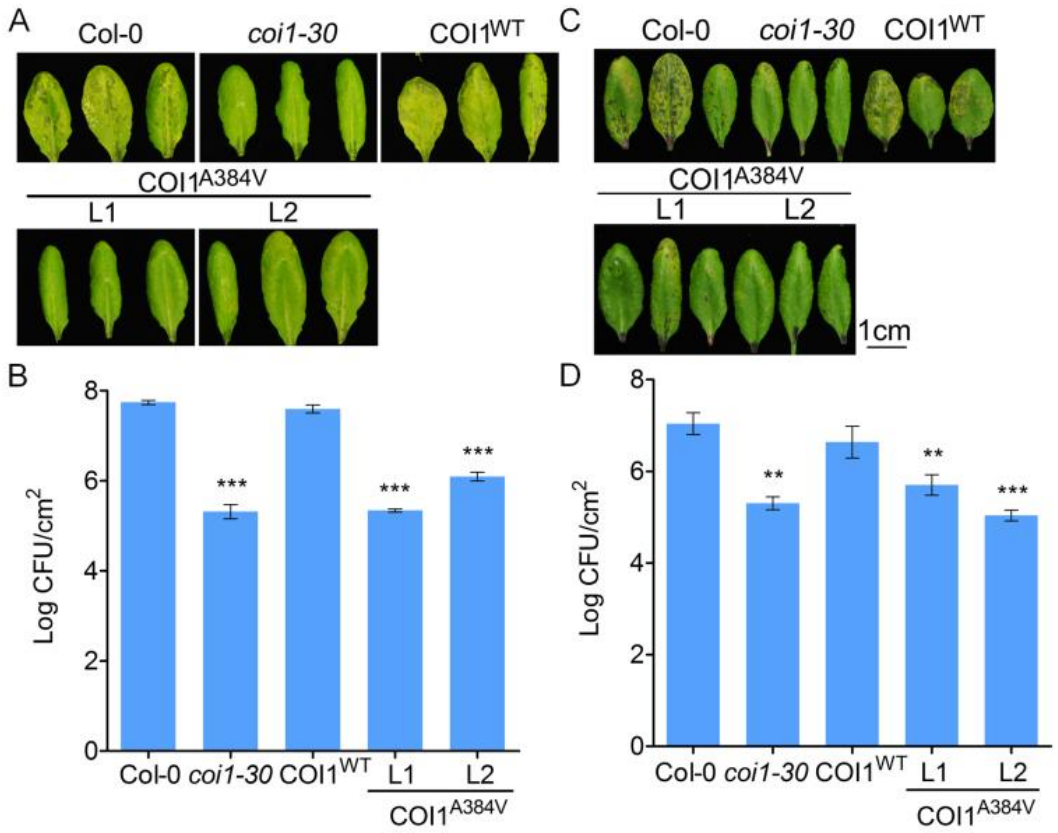

Figure 12: Results of bacterial infection assays with *Pst* DC3000, *Psm* ES4326, and *Pst* DC3118 and *Pst* DB29 (two COR-deficient mutants of *Pst* DC3000). (A, B) Disease symptoms (A) and bacterial populations (B) 3 days after dip-inoculation with $1x10^8$ cfu/ml *Pst* DC3000. ***p <0.001 indicates significant difference between mutant lines and wild-type Col-0 by One way ANOVA with Dunnett test (N=4, error bars, SEM). (C, D) Disease symptoms (C) and bacterial populations (D) 3 days after dip-inoculation with $1x10^8$ cfu/ml *Psm* ES4326. **p<0.01 and ***p <0.001 indicate significant difference between mutant lines and Col-0 wild-type by One way ANOVA with Dunnett test (N=4, error bars, SEM).

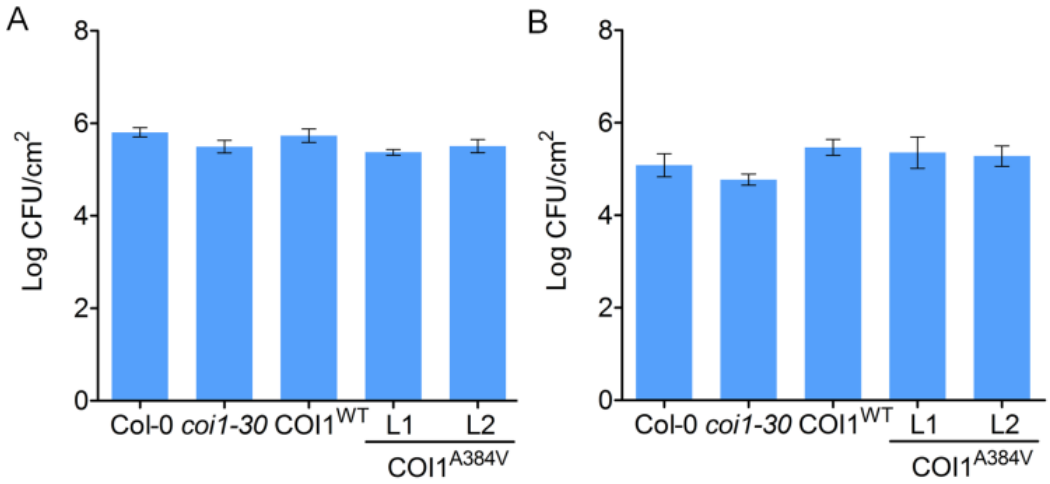

Figure 13: Bacterial populations after infection with COR-deficient *P. syringae* mutants. (A, B) Bacterial populations 4 days after syringe-infilitration with $1x10^6$ cfu/ml *Pst* DC3118 (A) or $1x10^6$ cfu/ml DB29 (B). No significant difference ($p<0.05$) was detected between plant genotypes by One-way ANOVA with Dunnett test (N=4, error bars, SEM).

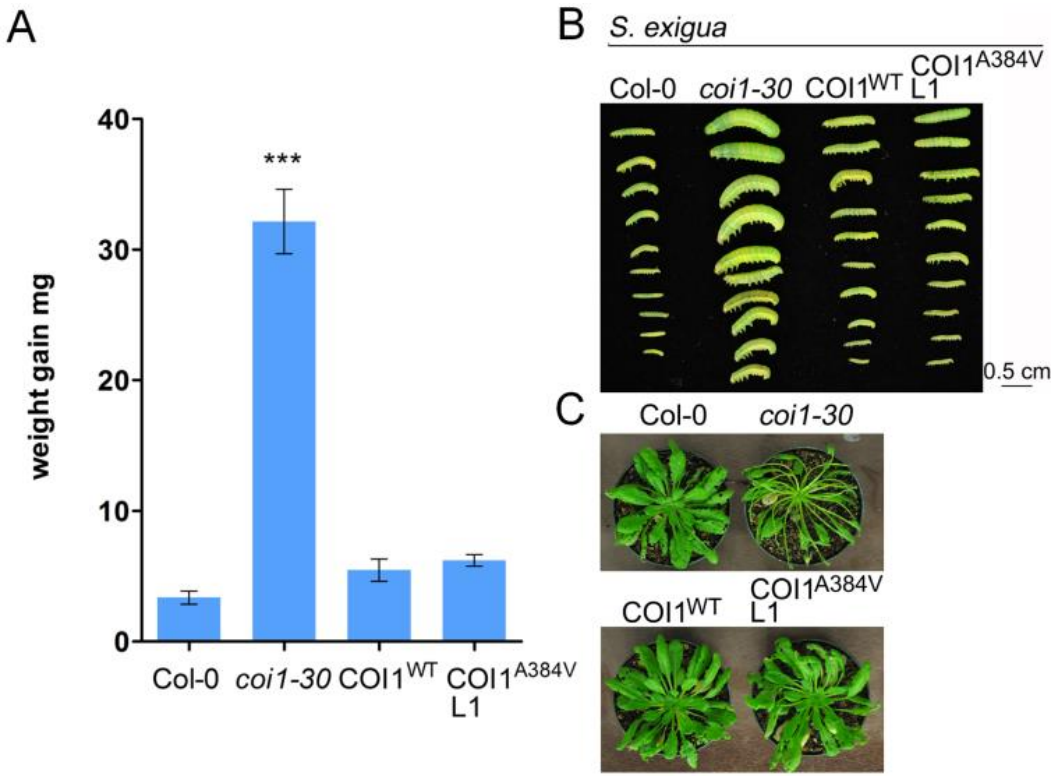

Figure 14: Results of insect feeding assays on COI1^{WT} and COI1^{A384V}. (A) Average weights of 12-day-old *S. exigua* larvae fed on Col-0, *coi1-30*, COI1^{WT} or COI1^{A384V} plants. ***p <0.001 indicates a significant difference in comparisons to Col-0 using One-way ANOVA with Dunnett test (N=10, error bars, SEM). No significant difference was detected in the weight of larvae reared on Col-0, COI1^{WT} and COI1^{A384V} L1 plants. (B) A picture of representative larvae 12 days after feeding. (C) Pictures of Arabidopsis plants after insect challenge.

molecular action on the host target (the JA receptor) was elucidated at the crystal structural level (Sheard et al. 2010). In this study, guided by the crystal structure of the JA receptor, we identified a single amino acid substitution (A384V) in the JA-binding pocket of the COI1 protein that greatly reduces Arabidopsis sensitivity to COR and confers substantial resistance of Arabidopsis to COR-producing *Pst* DC3000 and *Psm* ES4326. Our study provides a proof-of-concept demonstration for the feasibility of making a simple modification to a host target as a promising new approach to counter pathogen virulence, thus expanding the range of pathogens that a plant can defend against.

The COR toxin is produced not only by *P. s.* pv. *tomato* and *P. s.* pv. *maculicola*, but also *P. s.* pvs. *atropurpurea*, *glycinea*, *morsprunorum*, and *porri*, which collectively infect a wide range of plants, including ryegrass, soybean, crucifers, cherry, plum, leeks, and tomato (Bender et al. 1999; Geng et al. 2014). Furthermore, production of COR/COR-like compounds has been reported beyond the *P. syringae* species, including *Pseudomonas cannabina* pv. *alisalensis*, *Streptomyces scabies*, and *Xanthomonas campestris* pv. *phormiicola* (Bender et al. 1999; Fyans et al. 2014; Geng et al. 2014). Finally, gene clusters for COR biosynthesis have been found in *Pseudomonas savastanoi* pv. *glycinea* and *Pectobacterium atrosepticum* (syn. *Erwinia carotovora* subsp. *atroseptica*) (Bell et al. 2004; Qi et al. 2011a). Importantly, transposon insertion mutants of coronafacic acid (CFA)-like polyketide phytotoxin gene clusters in *P. atrosepticum* were shown to have reduced pathogen virulence (Bell et al. 2004). However, it remains to be determined whether these COR-like toxins target the COI1-JAZ co-receptor for their virulence activity. If so, modification of COI1 at A384 or other residues in the JA-binding pocket could represent a broadly applicable approach to improve plant resistance to diverse

pathogens. In addition, because of the simplicity of constructing amino acid substitutions, generation of $COI1^{A384V}$ plants seems particularly amenable using CRISPR-mediated genome editing.

Although our study is focused on uncoupling JA signaling from COR toxin action, recent studies have shown that the JA receptor is also a host target of proteinaceous effectors delivered into the host cell by bacterial pathogens and fungal symbionts (Gimenez-Ibanez et al. 2014; Jiang et al. 2013; Plett et al. 2014). For example, *P. syringae* pv. *syringae*, which is not known to produce COR or COR-like toxins, delivers the effector protein HopZ1a to acetylate and induce JAZ protein degradation, thereby activating JA signaling (Jiang et al. 2013). *P. syringae* pv. *tabaci*, which also does not produce COR or COR-like toxins, delivers the effector protein HopX1 into the host cell to interact with and degrade JAZ via its cysteine protease activity (Gimenez-Ibanez et al. 2014). The *Laccaria bicolor* fungal effector protein MiSSP7 (Mycorrhiza-induced Small Secreted Protein 7) interacts with the host *Populus* PtJAZ6 protein and inhibits JA-induced degradation of PtJAZ6 to promote symbiosis (Plett et al. 2014). Hence, the COI1-JAZ co-receptor has emerged as a common host target for diverse effector proteins of pathogens and symbionts. Further studies to elucidate how these effector proteins modify JAZ proteins could guide future efforts to develop JAZ-based modifications to counter pathogen virulence and enhance beneficial symbiosis. For example, innovative methods may be developed to disrupt the interaction between JAZs and HopZ1a/HopX1 or to block proteolytic degradation of JAZ proteins by HopZ1a/HopX1 as means of protecting plants from pathogen hijacking of the JA receptor.

Together with a recent demonstration of ABA receptor engineering against abiotic stress (Park et al. 2015), our study illustrates that fundamental insights into the plant hormone receptors could indeed lead to innovative methods to manipulate plant hormone receptor signaling with the ultimate goal of improving plant growth and tolerance to abiotic and biotic stresses.

CHAPTER III

IDENTIFICATION OF ARABIDOPSIS *SUSCEPTIBLE TO CORONATINE-DEFICIENT PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000 (*SCORD*) GENES INVOLVED IN STOMATAL DEFENSE

I would like to acknowledge Brad Paasch for his contribution to Figure 19, Dr. Weiqing Zeng for identification of the *scord* mutants and the initial mapping of the corresponding mutations, Dr. Jin Chen for help in genomic sequence analysis, and Dr. Brad Day for providing the elf26 peptide.

Abstract

Recent studies have shown that plants reduce stomatal aperture as an innate immune response to restrict pathogen infection. Highly evolved pathogens produce virulence factors, such as coronatine (COR) from the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, to counteract stomatal defense. COR-deficient mutants of *Pst* DC3000 are compromised in virulence, especially via surface inoculation. The He lab previously isolated eight Arabidopsis (*scord*) mutants that exhibit increased susceptibility to a COR-deficient mutant of *Pst* DC3000. *scord6* and *scord7* were found to be defective in bacterium-triggered stomatal closure. With map-based cloning and next generation sequencing, I identified the *SCORD6* and *SCORD7* genes. *SCORD6* encodes a protein involved in the de novo synthesis of GDP-$_L$-fucose, whereas *SCORD7* encodes for a protein belonging to the TRICHOME BIREFRINGENCE-LIKE (TBL) protein family, of which several members were proposed to be involved in pectin synthesis

and/or modification or *O*-acetylation of xyloglucan and xylan. Both *scord6* and *scord7* are defective only in salicylic acid (SA)-mediated stomatal closure but not in abscisic acid (ABA)-mediated stomatal closure. The identification of *SCORD6* and *SCORD7* genes highlights the importance of plant cell-wall-based regulation of stomatal defense in plants.

Introduction

Plants have evolved mechanisms to protect themselves from pathogen attack. For example, plant stomata, playing important roles in gas exchange and water transpiration, respond to various environmental signals including relative humidity, CO_2 concentration, light intensity, drought, plant hormones, as well as microbes (Arnaud and Hwang 2015). Stomatal closure has also been shown to be one of the first lines of plant immune response against pathogen infection (Melotto et al. 2006). As foliar pathogens, strains of *P. syringae* live two lifestyles in a successful disease cycle. They initially go through an epiphytic phase on the surface of plants, and subsequently transit into an endophytic phase in the apoplast (Melotto et al. 2008b). *P. syringae* enters the host tissue through wounds and natural openings, such as stomata (Hirano and Upper 2000). Stomata respond to *Pst* DC3000 infection and restrict the entry of *Pst* DC3000 via closure of stomatal aperture (Melotto et al. 2006).

In addition to pathogen, pattern-associated molecular patterns (PAMPs), including flg22 (a bioactive, 22-amino acid epitope of flagellin), elf26/elf18 (bioactive, 26/18-amino acid epitopes of elongation factor Tu, EF-Tu), lipopolysaccharides (LPS), oligogalacturonic acid, chitosan and β-1,3 glucans and yeast elicitors (YEL), have been shown to induce stomatal closure in Arabidopsis, tomato, grapevine, barley, *Commelina communis*, and *Pisum sativum* (Allegre et al.

2009; Desikan et al. 2008; Freeman and Beattie 2009; Khokon et al. 2010a; Khokon et al. 2010b; Klusener et al. 2002; Koers et al. 2011; Lee et al. 1999; Liu et al. 2009; Melotto et al. 2006; Srivastava et al. 2009; Zeng and He 2010; Zhang et al. 2008). Specific pattern recognition receptors (PRRs) are necessary for plants to sense PAMPs in the guard cell and induce stomatal closure (Melotto et al. 2006). For example, FLAGELLIN-SENSITIVE2 (FLS2) can recognize flg22 and induce stomatal closure.

ABA is the central regulator of plant resistance against abiotic stress, including stomatal closure under drought condition (Hubbard et al. 2010). Studies have shown that PAMP-induced stomatal closure shares a common signaling pathway with ABA-induced stomatal closure, which includes accumulation of ROS and NO, cytosolic calcium oscillations, activation of S-type anion channels and inhibition of K^+_{in} channels (Desikan et al. 2008; Klusener et al. 2002; Macho et al. 2012; Melotto et al. 2006; Montillet et al. 2013; Zeng and He 2010; Zhang et al. 2008). *ost1-2*, a mutant of *OPEN STOMATA1* (*OST1*), functioning upstream of ROS production in ABA-induced stomatal closure (Murata et al. 2001; Mustilli et al. 2002), exhibits compromised flg22 and LPS-mediated stomatal closure (Melotto et al. 2006; Zeng and He 2010). Several components of ABA signaling have also been reported to be involved in pathogen-induced stomatal closure (Arnaud and Hwang 2015; Desclos-Theveniau et al. 2012; Du et al. 2014a; Lee et al. 2013; Lim et al. 2014; Melotto et al. 2006; Roy et al. 2013; Schellenberg et al. 2010; Zeng and He 2010; Zhang et al. 2008).

SA also plays important roles in regulating pathogen-induced stomatal closure (Khokon et al. 2011; Melotto et al. 2006; Mori et al. 2001; Zeng et al. 2011; Zeng and He 2010). With a higher

concentration compared to ABA, SA induces stomatal closure (Khokon et al. 2011; Mori et al. 2001; Zeng and He 2010). In SA- or ABA-deficient plants, stomatal closure is compromised in response to *Pst* DC3000 (Melotto et al. 2006; Zeng et al. 2011; Zeng and He 2010).

Pst DC3000 secreted phytotoxin COR plays important roles in the pathogen virulence, through activating JA signaling. COR-deficient mutant of *Pst* DC3000 exhibited reduced virulence on Arabidopsis and tomato (Brooks et al. 2004; Zeng and He 2010). Studies showed that COR interferes with PAMP-induced stomatal closure and triggers stomatal reopening in a CORONATINE-INSENSITIVE1 (COI1)-dependent manner (Melotto et al. 2006; Montillet et al. 2013; Zheng et al. 2012). COR induces degradation of JASMONATE ZIM-DOMAIN (JAZ) proteins, resulting in release of MYC2 transcription factor (TF) and subsequently induction of the expression of three NAC (NAM/ATAF/CUC) TFs. These NAC TFs suppress the expression of SA biosynthesis gene *ISOCHORISMATE SYNTHASE 1* (*ICS1*) and activate the expression of SA metabolism genes *BENZOIC ACID/SA CARBOXYL METHYLTRANSFERASE1* (*BSMT1*) and *SA GLUCOSULTRANSFERASE1* (*SAGT1*) (Zheng et al. 2012). Importantly, virulence of COR-deficient mutants of *Pst* DC3000 could be restored in SA-deficient plants (Brooks et al. 2005; Melotto et al. 2006; Zeng and He 2010), indicating that COR suppresses stomatal closure through antagonizing the SA signaling. COR also suppresses ABA- and oxylipin-induced stomatal closure (Melotto et al. 2006; Montillet et al. 2013).

COR suppresses not only stomatal defense, but also apoplastic defense (Zeng et al. 2011). This was shown in a genetic screen for Arabidopsis mutants that could restore the virulence of COR-deficient *Pst* DC3118. In this screen, Zeng and colleagues isolated eight Arabidopsis *scord*

mutants based on increased disease susceptibility to COR-deficient *Pst* DC3118 (Zeng et al. 2011). Further analysis using stomatal assays and infiltration-inoculation assays showed that two *scord* mutations affected only plant stomatal response, two affected only plant apoplastic defense, and the other four affected both. In this study, for the purpose of identifying additional components involved in stomatal defense, I focused on the identification of the *SCORD6* and *SCORD7* genes. SCORD6 is involved in the de novo synthesis of GDP-$_{L}$-fucose and SCORD7 belongs to the TBL protein family. Both of them show defects in cell-wall components and structures. The identification of *SCORD6* and *SCORD7* genes highlights plant cell-wall-based regulation of stomatal defense and contributes to the further elucidation of cell-wall regulation of plant defense upon pathogen infection.

Materials and methods

Plant materials and growth condition

The wild-type Arabidopsis Col-7 and single mutant lines were purchased from the Arabidopsis Biological Resource Center (ABRC), including *tbr-1* (CS3741) (Bischoff et al. 2010; Potikha and Delmer 1995), *mur1-1* (CS6243) (Bonin et al. 1997; Reiter et al. 1993), *mur2-1* (CS8565) (Reiter et al. 1997; Vanzin et al. 2002), *fut4-2* (SALK_125310) (Liang et al. 2013), *fut6-2* (SALK_078357) (Liang et al. 2013), *cgl1-1* (CS6192) (Strasser et al. 2005; von Schaewen et al. 1993; Wenderoth and von Schaewen 2000), and *cgl1-3* (SALK_073650) (Frank et al. 2008). Arabidopsis plants used for induced-stomatal closure assays and bacterial infection assays were grown under 12-h light/12-h dark conditions for 4-5 weeks.

Second generation sequencing and cloning of the mutant genes

Genomic DNA of the *scord6, scord7* mutants and the parent Col-7 plants was extracted using PowerPlant Pro DNA Isolation Kit (Mo-Bio) and sent to the Michigan State University (MSU) Research Technology Support Facility (RTSF) Genomic Core for paired-end sequencing using Illumina's HiSeq 2500 next-generation sequencer.

A total of ~3 Gbp genome sequences for each sample were obtained and the coverage was 20- to 25-fold. For quality control, the sequence quality was considered satisfactory according to FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Because there was no assembled Col-7 genome sequence, I used the TAIR10 version of the Col-0 genome sequence as the reference genome for assembling. In order to identify the location of T-DNA insertion, I combined the Col-0 TAIR10 genome and the T-DNA insertion sequence (pSKI015) (Weigel et al. 2000) to generate the new reference genome. Using Bowtie2 (Langmead and Salzberg 2012), the reads of the *scord6*, *scord7* and Col-7 genome were aligned to the combined genome. Using SAMtools (Li et al. 2009a), three types of reads were detected: reads aligned to only Arabidopsis genome, only T-DNA insertion sequences, or both Arabidopsis and T-DNA insertion sequences. Reads aligned to both Arabidopsis genome and T-DNA insertion sequence were used to determine the location of T-DNA insertion in the genome of *scord6* or *scord7*.

Meanwhile SAMtools and VCFtools were used to identify sequence variations including single-nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs) (Danecek et al. 2011; Li et al. 2009a). Three output files were generated, including SNPs and INDELs between Col-0 and Col-7 genome, between Col-0 and *scord6* genome and between Col-0 and *scord7* genome.

SNPs/INDELs with the QUAL value >= 30 were selected. Homozygous variations detected only in the *scord6* or *scord7* genome, but not in the Col-7 genome were selected and compared to the candidate regions predicted by prior physical mapping (Zeng et al. 2011). Only nonsynonymous SNPs or INDELs locating in the open-reading frame of the genes in the candidate regions of the *scord6* and *scord7* genomes were selected. Primers used in targeted PCR for confirming the presence of deletions in *scord6* and *scord7* mutants includes:

SCORD6_FP CACCATGGCGTCAGAGAACAACGGAT;

SCORD6_RP AGGTTGCTGCTTAGCATCCATGTAT;

SCORD7_FP CACCATGGCGTCAGACGCCGTTAAGTAT;

SCORD7_RP AGTTTTTCGTTGTGTTTGGCTGAGCTGA

Generation of transgenic Arabidopsis

Full-length coding DNA sequence (CDS) of *SCORD6*/*MUR1* (AT3G51160) and *SCORD7*/*TBR* (AT5G06700) genes were cloned from Col-0 and transferred into the donor vector pDONR207 using BP Clonase II (Invitrogen) to generate the entry clone. The entry clone was then recombined into the destination vector pDest-35S-X-YFP-6xHis (Reumann et al. 2009) to generate constructs that express C-terminal YFP-tagged proteins using LR Clonase II (Invitrogen). Constructs were used for *Agrobacterium tumefaciens*-mediated Arabidopsis transformation of *scord6* or *scord7* plants (Clough and Bent 1998). Half-strength Murashige and Skoog (MS) medium with 50 μg/mL kanamycin was used to select transgenic seedlings containing 35S:*SCORD6/SCORD7-YFP* transgenes.

Bacterial Infection Assays

P. syringae infection assays in Arabidopsis were performed as described previously (Yao et al. 2013). In brief, 4- to 5-wk-old Arabidopsis plants were dip-inoculated with bacterial suspension (1×10^8 cfu/mL *Pst* DC3118 in 0.25 mM $MgCl_2$ solution containing 0.025% Silwet-77) or syringe-infiltrated with bacterial suspension (1×10^6 cfu/mL *Pst* DC3118 in 0.25 mM $MgCl_2$). Bacterial population was determined by serial dilutions of plant extracts 3 d after inoculation. For PAMP-induced protection, plant leaves were syringae-infiltrated with 0.5 μM flg22 (EZBiolab), 1 μM elf26 (EZBiolab), or 0.1% DMSO for 22 hours following by infiltration of bacterial suspension (5×10^7 cfu/mL *Pst* DC3000 in 0.25 mM $MgCl_2$ solution). Bacterial growth was determined by serial dilutions of plant extracts 1 d after inoculation. For benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH)-induced protection assays, 100 μM BTH (Chem Service) or 0.1% DMSO containing 0.005% Silwet was sprayed 24 hours before infiltration of bacterial suspension (5×10^7 cfu/mL *Pst* DC3000 in 0.25 mM $MgCl_2$). Bacterial population was determined by serial dilutions of plant extracts 1 d after inoculation.

Stomatal closure assay

Leaf discs (~3 mm × 3 mm) were collected one hour after lights were turned on in the growth chamber and were submerged in MES buffer (25mM MES-KOH pH6.15, 10mM KCl) with 100 μM SA (Sigma-Aldrich), 10 μM ABA (Sigma-Aldrich) or 0.1% DMSO as mock treatment for one hour. For measuring pathogen-induced stomatal closure, leaf discs were submerged in water or bacterial suspension ($1x10^8$ cfu/mL *Pst* DC3118 in water) for one hour. Stomatal apertures were captured using Olympus FluoView 1000 Spectral-based Laser Scanning Confocal Microscope with the blue diode laser (405 nm) (Olympus) for detecting auto-fluorescence of the

cell wall of the pore aperture. The length and width of the pore aperture were measured using ImageJ (https://imagej.nih.gov/ij/). At least 30 stomata were measured for each treatment and the ratio of width/length was graphed as a measure of stomatal closure.

Scanning electron microscopy (SEM)

SEM samples were prepared and scanned at the Center for Advanced Microscopy, MSU. In brief, samples were fixed at 4 ºC for one and a half hours in 4% glutaraldehyde, and then buffered with 0.1 M sodium phosphate at pH 7.4 for 40 min. Following a brief rinse in the buffer, samples were dehydrated in an ethanol series (25%, 50%, 75%, 95%) for 50-60 min in each ethanol concentration, and then in 100% ethanol one hour each for three times. Samples were critical point dried in a Leica Microsystems model EM CPD300 critical point dryer (Leica Microsystems) using liquid carbon dioxide as the transitional fluid. Samples were then mounted on aluminum stubs using high vacuum carbon tabs (SPI Supplies, West Chester, PA) and coated with osmium (~ 10 nm thickness) in an NEOC-AT osmium coater (Meiwafosis Co., Ltd.). Finally, samples were examined in a JEOL JSM-6610LV scanning electron microscope at 12 kV, WD 15 (JEOL Ltd.).

Results

Initial identification of the *SCORD6* and *SCORD7* genes

Although all *scord* mutants were isolated from an activation tagging population generated by Weigel et al. (Weigel et al. 2000), only two of the *SCORD* genes, *SCORD3* and *SCORD5*, were identified in a previous study through plasmid rescue (Zeng et al. 2011). The T-DNA insertion sites in the six other *scord* mutants could not be recovered using plasmid rescue or iPCR cloning

(Zeng et al. 2011). The inability to recover T-DNA insertions by these methods might result from complicated rearrangements or loss of T-DNA sequences in these Arabidopsis mutants (Laufs et al. 1999). Next-generation sequencing has been shown to be successfully used to identify transposon and T-DNA insertion sites in plants (Lepage et al. 2013; Williams-Carrier et al. 2010). Notably, a recent study used Illumina paired-end sequencing to locate T-DNA insertion sites in four distinct Arabidopsis leaf-shape mutants (Polko et al. 2012), which were segregated from the same activation tagging population as *scord* mutants (Weigel et al. 2000). Hence, I took a similar approach to identify T-DNA insertions in *scord6* and *scord7* mutants (See Materials and methods). The T-DNA insertion in the *scord7* genome is located in Chr1 between AT1G28080 (a RING finger protein) and AT1G28090 (a polynucleotide adenylyltransferase family protein). However, in a previous study, the *SCORD7* gene was mapped to Chr5 in a region between 1.97Mbp and 2.18Mbp (Zeng et al. 2011). This indicated that T-DNA insertion in the *scord7* mutant was not responsible for the mutant phenotype. The *scord6* mutation was previously mapped to the 18.84 Mb-19.03 Mb interval of Chr3. However, T-DNA sequences were not found in the *scord6* mutant. Furthermore, no pSKI015-associated herbicide glufosinate resistance could be detected in the *scord6* mutant, indicating that this mutant has lost T-DNA insertion.

Because of a lack of association between the *scord6* and *scord7* mutant phenotypes and the location of T-DNA insertion, I took an alternative approach to identifying the *SCORD6* and *SCORD7* genes. Specifically, variations including SNPs and INDELs were selected and located to the candidate regions of the *scord6* and *scord7* mutants, respectively (See Materials and methods). Finally, a 25bp deletion locating near the 3' end of AT3G51160 (*MUR1*) was detected

in the *scord6* genome within the candidate region (18.84 Mb-19.03 Mb/III) (Fig. 15A). A 58bp deletion, covering part of the 5' UTR region and AT5G06700 (*TBR*) gene, was found in the *scord7* genome within the candidate region (1.9 Mb-2.14 Mb/V) (Fig. 15A). No other nonsynonymous SNPs or INDELs in the coding regions of the candidate regions of the *scord6* or *scord7* genomes were found. The presence of the *scord6*/*scord7*-associated deletion was confirmed by targeted PCR and sequencing using the *scord6*/*scord7* genomic DNA.

Mutations in *MUR1* and *TBR* genes affect Arabidopsis resistance to the COR-deficient mutant of *Pst* DC3000

SCORD6/*MUR1*/*GMD2* gene encodes an isoform of GDP-$_D$-mannose-4,6-dehydratase, which catalyzes the first step of the de novo synthesis of GDP-$_L$-fucose from GDP-$_D$-mannose. *mur1* mutants were first identified by Reiter et al. from an ethyl methanesulfonate-mutagenized population (Reiter et al. 1993). With a point mutation S162F in the *MUR1* gene (Fig. 15A), *mur1-1* exhibits almost a complete loss of $_L$-fucose in shoot derived cell wall material (Bonin et al. 1997; Reiter et al. 1993). *SCORD7*/*TBR*, on the other hand, belongs to the *TBL* gene family, of which several members were proposed to be involved in synthesis and/or modification of polysaccharides, including pectin, xyloglucan and xylan (Bischoff et al. 2010; Gille et al. 2011; Urbanowicz et al. 2014; Vogel et al. 2004; Xiong et al. 2013; Yuan et al. 2016a; Yuan et al. 2013; 2016b; c). The *tbr-1* mutant contains a point mutation G427E in the *TBR* gene (Fig. 15A) and exhibits significant loss of trichome birefringence (Bischoff et al. 2010; Potikha and Delmer 1995).

I ordered *mur1-1* and *tbr-1* mutant alleles and transformed the *scord6* and *scord7* mutants with

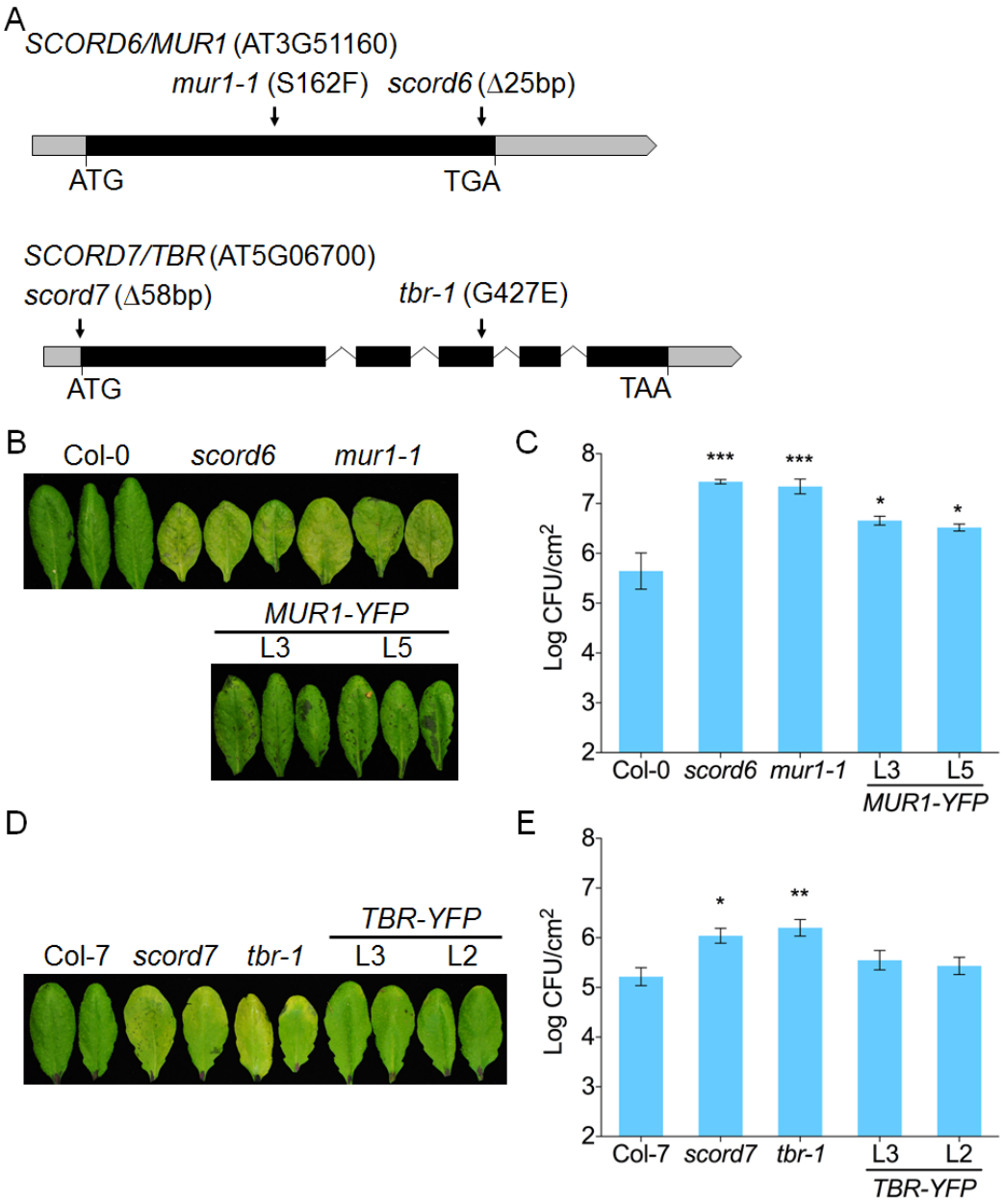

Figure 15: Identification of the *SCORD6* and *SCORD7* genes. (A) Schematic depiction of the Arabidopsis *MUR1* (AT3G51160) and *TBR* (AT5G06700) loci. Exons are depicted as black boxes, and untranslated regions (5' UTR and 3' UTR) are shown as gray boxes. Arrows indicate the positions of deletions or SNPs for different allelic mutant lines. Disease symptoms (B, D) and bacterial populations (C, E) 3 d after dip-inoculation with 1×10^8 cfu/mL *Pst* DC3118. *(0.01 < P < 0.05), **(0.001 < P < 0.01), and ***(P < 0.001) indicate significant differences between mutant lines and wild-type Arabidopsis by one-way ANOVA with Dunnett's test (n = 4, error bars, SEM).

the corresponding *MUR1*/*SCORD6* or *TBR*/*SCORD7* genes under the control of CaMV 35S promoter. Two independent lines of the second generation of 35S:*MUR1/SCORD6-YFP* and 35S:*TBR/SCORD7-YFP* were used for further study. The *mur1-1* and *tbr-1* mutants and the transgenic plants were examined for their immunity responses to *Pst* DC3118 infection by dip-inoculation. Like *scord6* plants, *mur1-1* plants show compromised defense against *Pst* DC3118 (Fig. 15B, C). In contrast, transgenic Arabidopsis plants, expressing 35S:*MUR1-YFP* in the *scord6* background, were partially restored in resistance to *Pst* DC3118 (Fig. 15C). The *tbr-1* mutant also exhibits compromised defense against *Pst* DC3118, similar to the *scord7* mutant and transgenic plants expressing 35S:*TBR-YFP* in the *scord7* background were recovered in plant defense against *Pst* DC3118 (Fig. 15D, E).

Mutations in *MUR1* and *TBR* genes affect pathogen/SA-induced stomatal movements

Next, I tested whether *mur1-1* and *tbr-1* mutants, like the *scord6* and *scord7* mutants, are affected in stomatal defense. Compared to the control water treatment, the stomatal apertures of wild-type Col-7 exhibit significantly reduced ratio of width/length in response to *Pst* DC3118 inoculation, as previously described (Zeng et al. 2011). In contrast, stomatal apertures of the *scord6*, *mur1-1*, *scord7* and *tbr-1* mutants do not response to *Pst* DC3118 inoculation (Fig. 16A, B). These results suggest compromised stomatal defense after pathogen infection in these four mutants. I then used different stomatal closure-stimulating chemicals ABA and SA (Melotto et al. 2006; Zeng and He 2010) to examine the levels of stomatal closure in these four mutants. With SA treatment, wild-type Col-7 show significant stomatal closure, whereas all four mutants exhibit no reduction of stomatal aperture compared to mock treatment, indicating compromised SA-mediated stomatal closure in these mutants (Fig. 16C, D). With ABA treatment, however

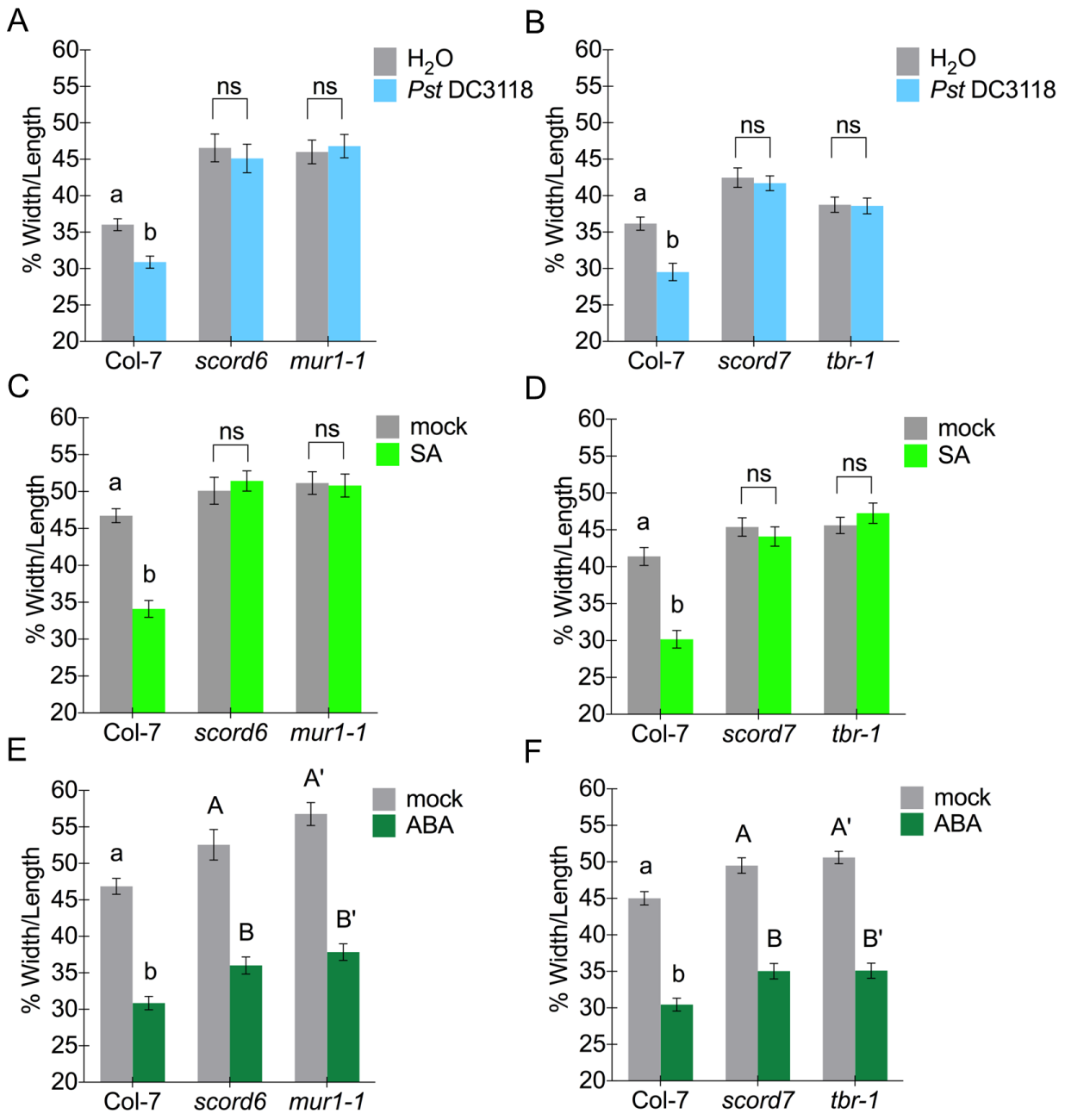

Figure 16: Results of bacterium or chemical-induced stomatal closure in wild-type and mutant plants. (A, B) Stomatal aperture 1 h after inoculation with 1×10^8 cfu/mL *Pst* DC3118 or water. Different letters above columns indicated significant differences ($P < 0.05$) of stomatal sizes between water and bacteria treatment with the same plant genotype ($n > 30$, error bars, SEM), according to two-way ANOVA analysis with Bonferroni posttest, (ns: not significant). (C-F) Stomatal aperture after 100 µM SA (C, D) or 10 µM ABA (E, F) treatments for 1 hour. Different letters of the same type indicated significant differences ($P < 0.05$) of stomatal size between mock (0.1% DMSO in MES buffer) and chemical treatment (SA or ABA) with the same plant genotype ($n > 30$, error bars, SEM), according to two-way ANOVA analysis with Bonferroni posttest, (ns: not significant).

wild-type Col-7 and the mutants show significantly induced stomatal closure (Fig. 16E, F), indicating mutations in *MUR1* and *TBR* genes affect only pathogen/SA-induced stomatal closure, while maintaining responsiveness to ABA.

As indicated previously, both *MUR1* and *TBR* genes are involved in the synthesis/modification of cell wall components. The maintained stomatal response suggests that the altered guard cell wall components in *mur1-1* and *tbr-1* mutants are not associated with ABA-induced stomatal closure, indicating that the basic mechanics of *mur1/scord6* and *tbr-1/scord7* mutant guard cells are intact. Therefore, the compromised stomatal closure after pathogen/SA treatment might be a result from changes in steps other than the basal ability of guard cell movement.

Taken together, my functional examination of *mur1-1* and *tbr1* mutant alleles and transgenic complementation of *scord6* and *scord7* mutations by *MUR1* and *TBR* genes, respectively, suggested that I have identified the *SCORD6* and *SCORD7* genes.

Both *scord6* and *scord7* mutants show abnormal cell walls in guard cells and trichomes

$_{L}$-Fucose is a component of several cell wall polymers, including pectin, xyloglucan, as well as glycoproteins. *mur1* mutant plants show a dwarf phenotype under continuous light and compromised mechanical properties of the primary cell wall, likely resulting from a lack of fucose-containing side chains in pectin or xyloglucan (Reiter et al. 1993). Consistent with this possibility, Arabidopsis *cgl* mutants, lacking fucosylated N-glycans, are morphologically indistinguishable from wild-type Arabidopsis, indicating the dwarfism of the *mur1-1* mutant is not due to a deficiency of $_{L}$-fucose in N-glycosylated proteins (Reiter et al. 1993; von Schaewen

et al. 1993). The *tbr-1* mutant exhibits a loss of trichome birefringence, which is visible under polarized lights, due to severely reduced crystalline cellulose in *tbr-1* trichomes (Bischoff et al. 2010; Potikha and Delmer 1995). Reduced amount of esterified pectins, increased activity of pectin methylesterase, and altered patterns of wound-induced callose deposition in trichomes and surrounding cells were also detected in the *tbr-1* mutant (Bischoff et al. 2010; Potikha and Delmer 1995). As both mutations in *MUR1* and *TBR* genes affect plant cell wall properties, I paid a close look at guard cell walls, in these mutants. With SEM, I observed morphological differences in the central cell walls between the two guard cells that form each stoma (Fig. 17A). Compared to wild-type stomata, both *scord6* and *mur1-1* mutants show absence of raised central ridges of stomatal aperture. Moreover, I observed differences in the surface of trichomes as well (Fig. 17B). The collapsed appearance of trichome papillae, the cuticular wax droplets, was found in the *scord6* and *mur1-1* mutants, indicating the cell wall defects are not restricted to guard cells. Although the mechanisms of papillae formation or function remain elusive, trichomes with strong papillae defects transmitted more lights, suggesting possible function of papillae in light scattering (Suo et al. 2013). Morphological differences were also detected in guard cell walls and trichomes in the *scord7* and *tbr-1* mutants (Fig. 17A, B). Like *scord6*/*mur1-1* stomata, *scord7* and *tbr-1* stomata have greatly reduced central ridges between the guard cells. In addition, the trichome papillae were lost almost completely in the *scord7* and *tbr-1* mutants, indicating different degrees of effects of MUR1 and TBR proteins on cell wall architecture.

The *scord6* mutant shows compromised apoplastic defense

In addition to compromised stomatal defense, the *scord6* mutant was previously found to be compromised in apoplastic defense, whereas the *scord7* mutant is affected only in stomatal

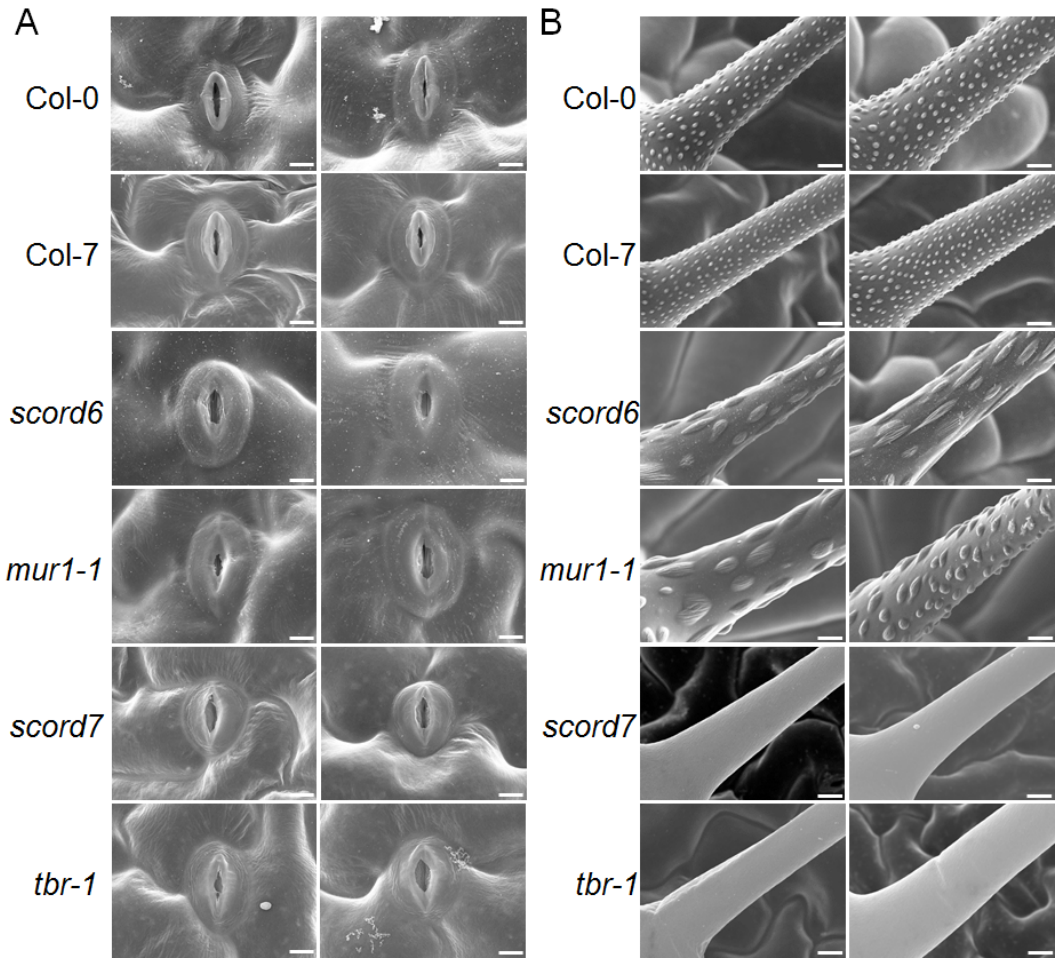

Figure 17: SEM images of the cell wall of guard cells (A) and surface of trichomes (B).
Scale bar = 5 µm (A), Scale bar = 10 µm (B)

defense (Zeng et al. 2011). To determine whether the *mur1-1* and *tbr1* mutants have a similar phenotype to the *scord6* and *scord7* mutants, respectively, I infiltrated *Pst* DC3118 directly into the leaf apoplast of Col-7, *scord6*, *mur1-1*, s*cord7* and *tbr-1* plants and bacterial populations was counted 3 day after infection. Only *scord6* and *mur1-1* mutants show increased bacterial growth (Fig. 18A, B), indicating compromised apoplastic defense in the *scord6* and *mur1-1* mutants.

SA is a major regulator of plant defense against bacterial pathogens. Using LC-MS, I investigated the SA levels of the *scord6* and *mur1-1* mutants. Both the free SA and the conjugated SAG levels in *scord6* and *mur1-1* mutants are lower after *Pst* DC3118 infection compared to wild-type Arabidopsis (Fig. 19A, B), indicating that, in the context of bacterial infection, mutations in the *MUR1* gene not only affect the ability of exogenous SA to induce guard cell movement, but also SA accumulation. On the other hand, the *scord7* mutant accumulates a comparable level of *Pst* DC3000-induced SA comparing to wild-type plants (Zeng et al. 2011), indicating that, besides stomatal defense, mutations in *MUR1* and *TBR* genes affect other aspects of plant defense differently.

The *scord6* mutant is compromised in elf26-induced protection

EF-Tu receptor (EFR) is a well-characterized Arabidopsis PRR, which perceives bacterial EF-Tu to trigger PTI (Zipfel et al. 2006). Previous studies showed that EFR is highly glycosylated and this glycosylation is necessary for the stability and function of EFR (Haweker et al. 2010; Li et al. 2009b; Nekrasov et al. 2009; Saijo et al. 2009). On the other hand, FLS2, the receptor for flagellin (Gomez-Gomez and Boller 2000), was less glycosylated. The abundance of FLS2 protein and plant resistance were affected to a less extent or not affected in N-glycan mutants or

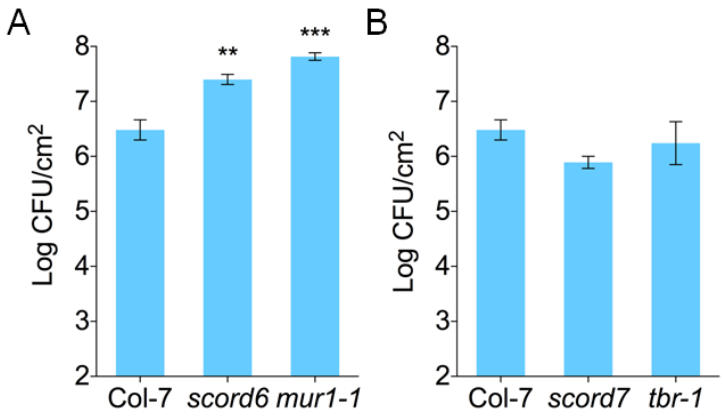

Figure 18: Arabidopsis apoplastic defense in wild-type and mutant plants. (A, B) Bacterial populations 3 d after infiltration-inoculation with 1×10^6 cfu/mL *Pst* DC3118. **0.001 < P < 0.01 and ***P < 0.001 indicate significant differences between mutant lines and wild-type Col-7, analyzed by one-way ANOVA with Dunnett's test (n = 4, error bars, SEM).

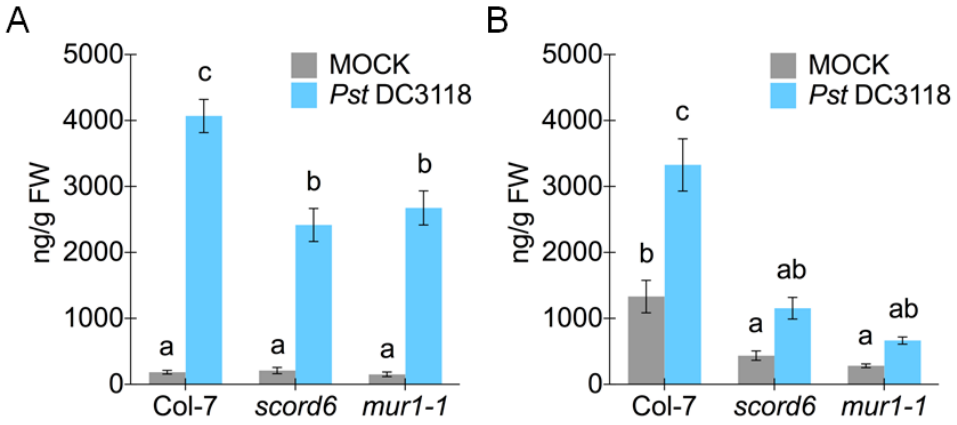

Figure 19: SA levels after pathogen infection. (A) Free SA and (B) SAG levels 24 hour after 1×10^6 cfu/mL *Pst* DC3118 infection. Different letters above columns indicated significant differences (P < 0.05) of SA or SAG levels (n = 6, error bars, SEM), analyzed by two-way ANOVA with Tukey's test.

mutants for ER quality control (Haweker et al. 2010; Li et al. 2009b; Nekrasov et al. 2009; Saijo et al. 2009). Because $_{L}$-Fucose is an important component of the carbohydrate chains of glycoproteins (Strasser 2016) and the *mur1-1* mutation has been shown to affect the glycoprotein profiles in Arabidopsis (Rayon et al. 1999), I tested the hypothesis that EFR-mediated PTI may be compromised in the *scord6* and *mur1-1* mutants. I used flg22 (a bioactive peptide from bacterial flagellin; Monaghan and Zipfel 2012) or elf26 (a bioactive peptide of bacterial EF-Tu; Monaghan and Zipfel 2012) to induced FLS2- and EFR-mediated PTI, which is then assessed by *Pst* DC3000 infection via infiltration-inoculation. Like wild-type Col-7 plants, both *scord6* and *scord7* mutants show increased resistance to *Pst* DC3000 after flg22 pre-treatment, comparing to mock treatment, which indicates that flg22-induced PTI is not affected in *scord6* and *scord7* mutants (Fig. 20A). In contrast, elf26-induced PTI was greatly compromised in the *scord6* and *mur1-1* mutants (Fig. 20B). Bacterial population in *scord6* and *mur1-1* mutants after elf26 pre-treatment was significantly higher than those in wild-type plants, implying the loss of ability of these two mutants to induce PTI with elf26 treatment (Fig. 20B). The *scord7* and *tbr-1* mutants, on the other hand, exhibit normal elf26-induced PTI (Fig. 20B).

Next, I also test whether BTH, the synthetic analog of SA, can induce protection against *Pst* DC3000 in these mutants. Both *scord6* and *mur1-1* mutants show effective BTH-induced resistance against *Pst* DC3000 (Fig. 20C), indicating that, BTH-induced protection remains intact in these two mutants. Interestingly, with infiltration-inoculation of the secretion-defective mutant *Pst* Δ*hrcC*, increased bacteria growth were detected in *scord6* and *mur1*-1 mutants compared to wild-type plants (Fig. 20D), indicating that the *scord6* and *mur1-1* mutants might confer defects in basal defense.

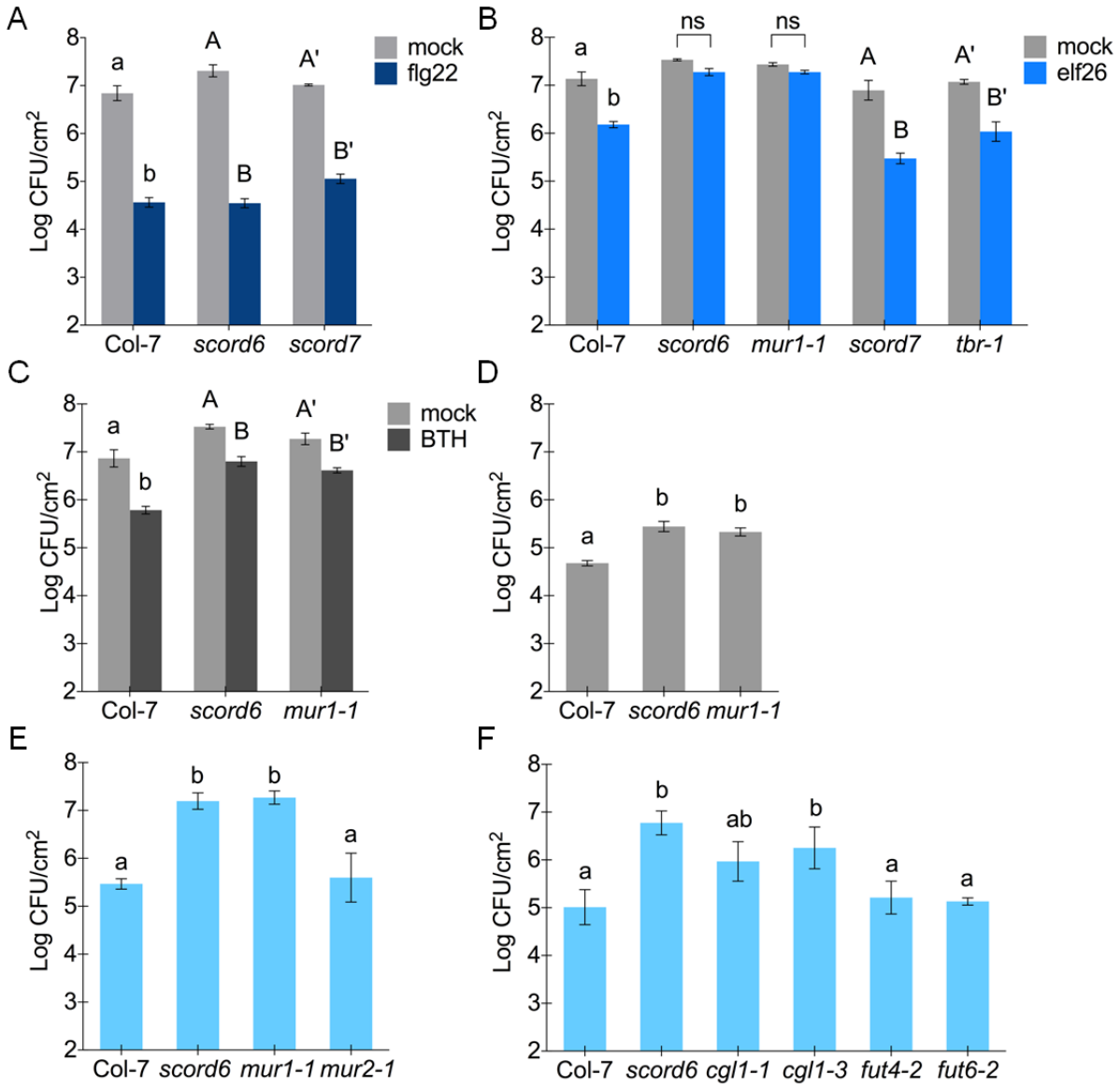

Figure 20: flg22, elf26 and BTH-induced protection in wild-type and mutant plants and plant defense of different mutants against *Pst* Δ*hrcC* or *Pst* DC3118. (A, B) Bacterial populations 1 d after infiltration-inoculation (5×10^7 cfu/mL *Pst* DC3000) with 22 h pre-induction of 0.5 μM flg22 (A), 1 μM elf26 (B) or 100 μM BTH. Different letters of the same type above columns indicated significant differences ($P < 0.05$) of bacterial population between mock and flg22/elf26/BTH with the same plant genotype (n = 4, error bars, SEM); analyzed by two-way ANOVA with Bonferroni posttest. (D) Bacterial populations 1 day after infiltration-inoculation with 5×10^7 cfu/mL *Pst* Δ*hrcC* mutant. (E, F) Bacterial populations 3 d after infiltration-inoculation with 1×10^6 cfu/mL *Pst* DC3118. Different letters above columns indicate significant difference ($P < 0.05$) of bacterial population among different plant genotypes after *Pst* Δ*hrcC* (D) or *Pst* DC3118 (E, F) inoculation; analyzed by one-way ANOVA with Tukey's test (n = 4, error bars, SEM).

Fucosylation in xyloglucan and O-glycans is not associated with Arabidopsis defense against *Pst* DC3118

A previous study showed that *mur1* mutants exhibit a deficiency of $_{L}$-fucose biosynthesis and a lack of fucose-containing side chains in pectin, xyloglucan and glycoproteins (Reiter et al. 1993). However, *cgl* mutants lack fucosylated N-glycans are morphologically indistinguishable from wild-type Arabidopsis, indicating the dwarfism of *mur1-1* is not due to deficiency of $_{L}$-fucose in N-glycoproteins (Reiter et al. 1993; von Schaewen et al. 1993). To determine whether defects in fucosylation in xyloglucan and/or O-glycans play a role in the disease phenotypes of *scord6*/*mur1-1* mutants, I examined Arabidopsis mutants that are defective in fucosylation of xyloglucan (*mur2-1*; a xylogylcan fucose transferase, Vanzin et al. 2002) or O-glycan (*fut4-2* and *fut6-2*; O-glycan fucose transferases, Liang et al. 2013) for their response to *Pst* DC3118 infection. These mutants show normal resistance to *Pst* DC3118 compared to wild-type plants (Fig. 20E, F), indicating that defects in fucosylation of xyloglucan or O-glycan do not affect plant defense against *Pst* DC3118. Interestingly, similar trichome phenotype was observed in *mur2-1* mutant (Vanzin et al. 2002), indicating that the collapsed trichome papillae is not associated with the susceptibility of *scord6* and *mur1-1* mutants against *Pst* DC3118. Moreover, consistent with the function of N-glycan in regulating EFR abundance and function, *cgl1-1* and *cgl1-3* mutants show increased bacteria growth after *Pst* DC3118 infection, compared to wild-type plants (Fig. 20F).

Future plans and discussion

In this study, I have identified two *SCORD* genes, both of which show possible association between cell wall defects and compromised plant defense. SCORD6/MUR1 is an isoform of

GDP-$_{D}$-mannose-4,6-dehydratase which functions in $_{L}$-fucose de novo biosynthesis and affects fucosylation in xyloglucan, pectin and glycoproteins (Bonin et al. 1997; Reiter et al. 1993). SCORD7/TBR belongs to the TBL family and *tbr-1* mutant exhibits altered pectin esterification and cell wall patterns (Bischoff et al. 2010; Potikha and Delmer 1995). While both SCORD6 and SCORD7 proteins regulate certain aspects of plant cell wall, they function similarly in stomatal defense, but act differently in apoplastic defense and elf26-induced PTI. With the examination of different ligand-induced stomatal defense or apoplastic defense in these two cell-wall related mutants, my study could help to further elucidate plant cell-wall-based regulation of plant immunity and contributes to the general understanding of the multifaceted host defense mechanisms against pathogen infection in plants.

I proposed in results that $_{L}$-fucose deficiency in *scord6* and *mur1-1* mutants might affect the abundance of EFR protein due to lack of mature N-glycan. In order to test this hypothesis, anti-EFR antibody could be used to detect the EFR protein levels with or without pathogen infection. To further confirm the defects in elf26-induced PTI in *scord6* and *mur1-1* mutants, qPCR for elf26-induced downstream signaling components could be carried out after elf26 induction in wild-type, *scord6*, *mur1-1* and *efr* plants. For example, *CYP81F2* and *WRKY33* are genes significantly induced after elf18 treatment within one hour in wild-type plants while lack of induction was detected in *efr* mutant (Li et al. 2009b; Roux et al. 2011). With Col-7 and *efr* mutant as control, I am able to test to what extent *scord6* and *mur1-1* mutants reduce the elf26-triggered protection. Meanwhile, *CYP81F2* and *WRKY33* genes could also be induced by flg22 treatment (Gravino et al. 2016; Li et al. 2009b; Roux et al. 2011) and could be used to confirm unaffected protection after flg22 treatment in *scord6* and *mur1-1* mutants. Moreover, mutants of

N-glycan fucosyltransferases *fut11*, *fut12* and *fut13* (Kaulfurst-Soboll et al. 2011; Villalobos et al. 2015) will be used in *Pst* DC3118 dip-inoculation to confirm that fucosylation in N-glycan affects plant defense against *Pst* DC3118.

In this study, altered cell wall architecture is associated with defects in plant response to pathogen and SA treatments. The abnormal appearance of the central ridges might due to the altered pattern of the outer cuticular ledges of guard cells, which are primarily composed of the nonpolymerized cuticular waxes and the cutin polyesters. The outer cuticular ledges of guard cells have long been reported to function to prevent water loss (Lu et al. 2012; Schonherr and Ziegler 1975). Although the mechanisms of formation and function of the cuticular ledges remains elusive, *ECERIFERUM9* (*CER9*), a cuticle biosynthesis gene, was show to be a possible cuticle-associated drought tolerance determinant (Lu et al. 2012). Recently, Pautov and colleagues proposed that the outer ledges prevent wide opening of the stomatal pore and stomatal rising above the surface of leaf epidermis in woody plants using modeling (Pautov et al. 2016), indicating possible relationship between outer cuticular ledges and plant stomatal closure. Notably, the cutin deficient glycerol-3-phosphate acyltransferase *gpat4 gpat8* mutant, exhibiting absent cuticular ledges similar to that of *scord6* and *mur1-1* mutants, can still respond to ABA to a similar extent of wild-type stomata (Li et al. 2007), which is consistent with the responsiveness of *scord6* and *mur1-1* mutants to ABA. Together with our results that *scord6* and *scord7* show compromised pathogen-/SA-induced stomatal closure but not ABA-induced stomatal closure, the absent or altered outer cuticular ledges in guard cells might associate with pathogen- /SA-mediated the guard cell movement. Alternatively, there might be signaling component that were affected with altered cell wall polymers in *scord6* and *scord7* mutants, which function in SA-

mediated stomatal closure independent or upstream of ABA-induced stomatal closure. Hence further identification of this signaling component would help to elucidate the signaling pathway in plant stomatal defense.

In a previous study, Zeng and colleagues found that *scord7* mutant was unable to close stomata after ABA treatment (Zeng et al. 2011), which is not consistent with our result that *scord7* mutant maintains ABA-induced stomatal closure. The reason might due to the different strategies of stomatal assay, as in previous studies stomatal movement is detected with leaf peels while I used whole leaf discs in stomatal assays. The difference in these two experiments is the absence or presence of certain cells or structures, such as mesophyll cells or sub-stomatal cavity, which might facilitate the stomata movement in *scord7* mutant during ABA-induced stomatal closure.

CHAPTER IV

CONCLUSION

Host target modification represents a promising strategy to engineer host-vulnerable competent for enhanced host immunity

I have constructed the A384V amino acid substitution in the ligand-binding pocket of the Arabidopsis CORONATINE INSENSITIVE1 (COI1) protein that allows for sufficient signal transduction of endogenous jasmonate (JA)-Ile, but has greatly reduced sensitivity to the *Pseudomonas syringae* toxin coronatine (COR). Moreover, transgenic Arabidopsis plants expressing the engineered $COI1^{A384V}$ receptor not only remain fertile and maintain a high-level of insect defense, but also gain resistance to the COR-producing pathogens. Therefore the generation of $COI1^{A384V}$ mutant helps to solve the dilemma of salicylic acid (SA)-JA antagonism in conventional activation of either pathway through genetic or chemical manipulation. Hence, my study strengthens the notion that hormone receptor engineering holds promise to improve crop yield and abiotic and biotic stress tolerance in plants.

Interestingly, an engineered abscisic acid (ABA) receptor PYRABACTIN RESISTANCE 1 (PYR1) was shown to acquire a new recognition capacity toward an existing agrochemical mandipropamid (Park et al. 2015). Application of mandipropamid could efficiently activate ABA responses and improve drought tolerance in transgenic plants (Park et al. 2015). Together with my results, it is shown that fundamental insights into the plant hormone receptors have begun to

yield innovative methods to manipulate plant hormone receptor signaling with the ultimate goal of improving plant tolerance to abiotic and biotic stresses.

Moreover, recent studies have shown that the COI1-JAZ co-receptor is a common host target for diverse proteinaceous effectors of pathogens and symbionts (Gimenez-Ibanez et al. 2014; Jiang et al. 2013; Plett et al. 2014). For example, *P. syringae* pv. *syringae* secret the effector protein HopZ1a to induce JAZ protein degradation in an acetylation-dependent manner, thereby activating JA signaling (Jiang et al. 2013). *P. syringae* pv. *tabaci* deliver the effector protein HopX1 into the host cell to interact with and degrade JAZ via its cysteine protease activity (Gimenez-Ibanez et al. 2014). Additionally, the *Laccaria bicolor* fungal effector protein MiSSP7 (Mycorrhiza-induced Small Secreted Protein 7) interacts with host *Populus* PtJAZ6 protein and inhibits JA-induced degradation of PtJAZ6 to promote symbiosis (Plett et al. 2014). Further study to elucidate how these effector proteins modify JAZ proteins could guide future efforts to develop JAZ-based modifications to counter pathogen virulence and enhance beneficial symbiosis.

Cell-wall regulation of plant stomatal immunity potentially stimulates studies of plant immunity to a new direction

I have identified the *SCORD6* and *SCORD7* genes. I believe that I provided unambiguous evidence that *SCORD6* is *MUR1* and that *SCORD7* is *TBR*. Both *MUR1* and *TBR* genes were previously known only for their involvement in the biosynthesis or modification of pectin, xyloglucan and/or xylan. My results show that these two genes are required for pathogen-triggered stomatal closure, thereby implicating a role of cell-wall synthesis or modification in

pathogen-mediated stomatal closure. To my knowledge, this may be the first time that specific cell-wall regulation is connected to stomatal defense.

Although the exact roles of cell wall components remain to be investigated, it is generally believed that guard cell walls need to be both strong and elastic in order to sustain the high internal pressure and reversible movements (Jones et al. 2005). Cell wall components, cellulose, hemicellulose, and pectin, have all been shown to be involved in basic guard cell movement in response to abiotic stress or chemical treatments. For example, dynamic reorganization of cellulose microfibrils takes place during stomatal movement in response to light, dark, ABA and fusicoccin, the stomata opening toxin from *Fusicoccum amygdale* (Rui and Anderson 2016). The cellulose synthesis mutant *cesa3*^{je5} and hemicellulose xyloglucan deficient *xxt1xxt2* mutant exhibit significant difference of stomatal movement compared to wild-type plants during fusicoccin-induced stomatal opening and ABA-induced stomatal closure (Rui and Anderson 2016). In addition, high proportion of stomatal closures was detected in tomato plants overexpressing a hot pepper XYLOGLUCAN ENDOTRANSGLUCOSYLASE/ HYDROLASE (XTH) (Choi et al. 2011). Meanwhile, guard cell walls are enriched in phenolic esters of pectin (Jones et al. 2005), which play a role in the flexibility of guard cell walls (Jones et al. 2003). Changes in pectin composition/modification during development or enzymatic treatments also affect stomatal movement in response to light, abiotic stress or chemical treatments, such as fusicoccin, ABA, CO_2 concentration or mannitol-containing osmoticum (Amsbury et al. 2016; Jones et al. 2005; Jones et al. 2003; Merced and Renzaglia 2014). Other factors in cell wall regulation have also been shown to affect guard cell movement in response to light, drought or pH, such as guard cell specific expansin (AtEXPA1), β-AMYLASE1 (BAM1), as well as

tubuliln (Prasch et al. 2015; Swamy et al. 2015; Wei et al. 2011; Zhang et al. 2011). Although examples of defects in cell wall composition or modification associated with abnormal guard cell movement have been reported, how exactly the structure and dynamics of guard cell walls mediate stomatal movement and function remains elusive (Rui and Anderson 2016). Comprehensive studies of cell wall biosynthesis and modification mutants in various ligand/chemical-induced stomatal closure or opening would help to clarify both common and unique effects of wall components on stomatal movements triggered by various external and internal signals.

Besides cell-wall-regulated plant stomatal defense, plant cell walls also function to protect host against pathogen or parasite attacks, either as the first layer of barrier or as reservoir of defense elicitors (Malinovsky et al. 2014; Nuhse 2012). Generally, the tightly packed crystalline microfibrils make plant cell wall hard to access or penetrate (Nuhse 2012). Several examples of wall stiffness and plant resistance have been reported. For example, soil borne fungi can only invade the elongation zone of roots after first colonization in root tips. The reason might be that the cell walls in the elongation zone are temporarily weakened and thinned (Gunawardena and Hawes 2002), which increase the accessibility to fungi. Moreover, enhanced resistance to *P. syringae* was detected in Arabidopsis plants over-expressing extensin which increases wall stiffness (Wei and Shirsat 2006). I found that the *scord7* and *tbr-1* mutants exhibit loss of trichome birefringence, indicating reduced crystalline cellulose in trichome (Bischoff et al. 2010). Hence, these mutants might be good candidates for further study of a potential association between wall stiffness and plant resistance against different pathogens including fungi species.

In contrast to cellulose microfibrils, pectin and xyloglucan are easier to access and break down by cell wall degrading enzymes (CWDEs), such as endo-polygalacturonases (EPGs), pectate lyases (PLs), pectin methyl-esterases (PMEs) and endo-xylanses (Lionetti et al. 2012; Nuhse 2012), which are used by pathogens to breach plant cell wall integrity and facilitate pathogen infection. On the other hand, oligosaccharide fragments released from wall polysaccharides during pathogen infection could function as damage-associated molecular patterns (DAMPs) to mediate plant resistance. For example, degradation of homogalacturonan (HGA), one type of pectin, releases oligogalacturonides (OGs), which are potent defense response elicitors mediating rapid and strong defense responses (Doares et al. 1995; Galletti et al. 2009). Wall-associated protein kinases (WAKs) have been shown to be receptors of OGs (Brutus et al. 2010), which confer higher binding affinity to OGs with degree of polymerization (DP) 9-15 comparing to highly polymerized OGs (Kohorn and Kohorn 2012).

The level of methyl esterification of HGA, mediated by PMEs, seems critical to EPGs and PLs, which preferentially cleave non-esterified HGA (Kars et al. 2005; Lionetti et al. 2012; Micheli 2001). Whereas Pectin lyases act preferentially on highly methyl esterified HGA. Altered expression of PMEs in response to *B. cinerea* (AbuQamar et al. 2006; Raiola et al. 2011) has been reported and increased activity of PMEs after treatment with PAMPs or infection with necrotrophic fungus *Alternaria brassicicola*, or *P. syringae* pv. *maculicola* (*Psm*) ES4326 leads to decreased methyl esterification of HGA, enhanced production of OGs and enhanced DAMP signaling (Bethke et al. 2014). Several *pme* mutants exhibit increased susceptibility to *Psm* ES4326 than wild-type plants, indicating a role of PMEs in plant immunity. In the case of PME-mediated resistance against *A. brassicicola* and *Psm* ES4326, it appears that plants have evolved

to compensate wall integrity for OGs generation. In other cases, however, plants preferentially maintain the wall integrity. For example, increased levels of intact pectin and enhanced resistance to powdery mildew *Erysiphe cichoracearum* and *E. orontii* were detected in *powdery mildew resistant6* (*pmr6*) mutant (Vogel et al. 2002). It seems that in this case inaccessible HGA may provide more protection compared to OG-mediated plant response.

In all, many aspects of the plant cell wall-based plant immunity are not well understood. I believe that study of cell-wall-mediated plant immunity and plant stomatal defense would be an intriguing field for future study and could spark new understanding of the multi-layered plant defense. The role of pectin in plant immunity may be more complicated than simply polysaccharide integrity versus enzymatic breakdown and further studies are needed. Both *scord6* and *scord7* mutations affect the modification of pectin which might affect the generation or composition of OGs, therefore these two mutants could be used for the study of pectin composition and OGs-mediated plant defense, as well as the interplay between OGs generation and wall integrity in plants.

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