

Enhanced Defense Responses in *Arabidopsis* Induced by the Cell Wall Protein Fractions from *Pythium oligandrum* Require SGT1, RAR1, NPR1 and JAR1

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The cell wall protein fraction (CWP) is purified from the non-pathogenic biocontrol agent *Pythium oligandrum* and is composed of two glycoproteins (POD-1 and POD-2), which are structurally similar to class III elicitors. In tomato plants treated with CWP, jasmonic acid (JA)- and ethylene (ET)-dependent signaling pathways are activated, and resistance to *Ralstonia solanacearum* is enhanced. To dissect CWP-induced defense mechanisms, we investigated defense gene expression and resistance to bacterial pathogens in *Arabidopsis thaliana* ecotype Col-0 treated with CWP. When the leaves of Col-0 were infiltrated with CWP, neither visible necrosis nor salicylic acid (SA)-responsive gene (*PR-1* and *PR-5*) expression was induced. In contrast, JA-responsive gene (*PDF1.2* and *JR2*) expression was up-regulated and the resistance to *R. solanacearum* and *Pseudomonas syringae* pv. *tomato* DC3000 was enhanced in response to CWP. Such CWP-induced defense responses were completely compromised in CWP-treated *coi1-1* and *jar1-1* mutants with an impaired JA signaling pathway. The induction of defense-related gene expression after CWP treatment was partially compromised in ET-insensitive *ein2-1* mutants, but not in SA signaling mutants or *nahG* transgenic plants. Global gene expression analysis using cDNA array also suggested that several other JA- and ET-responsive genes, but not SA-responsive genes, were up-regulated in response to CWP. Further analysis of

CWP-induced defense responses using another eight mutants with impaired defense signaling pathways indicated that, interestingly, the induction of JA-responsive gene expression and enhanced resistance to two bacterial pathogens in response to CWP were completely compromised in *rar1-1*, *rar1-21*, *sgt1a-1*, *sgt1b* (*edm1*) and *npr1-1* mutants. Thus, the CWP-induced defense system appears to be regulated by JA-mediated and SGT1-, RAR1- and NPR1-dependent signaling pathways.

Keywords: Elicitor • Ethylene • Induced resistance • Jasmonic acid • *Pseudomonas syringae* • *Ralstonia solanacearum*.

Abbreviations: CC, coiled-coil; CWP, cell wall protein fraction; ET, ethylene; HR, hypersensitive response; ISR, induced systemic resistance; JA, jasmonic acid; LRR, leucine-rich repeat; MAMP, microbe-associated molecular pattern; NB, nucleotide-binding site; POD, D-type of CWP from *P. oligandrum*; PR, pathogenesis related; SA, salicylic acid; SAR, systemic acquired resistance; TIR, Toll and human interleukin-1 receptor-like.

Introduction

Pythium oligandrum is a non-pathogenic soil-inhabiting oomycete and colonizes the rhizosphere of many crop species. It is available as a biocontrol agent (Butt and Copping

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2000, Brozova 2002), because it is responsible for the reduction of soil-borne diseases caused by a number of soil-borne fungal and bacterial pathogens (Martin and Hancock 1987, McQuilken et al. 1990, Al-Rawahi and Hancock 1997, Takenaka et al. 2003, Hase et al. 2006, Hase et al. 2008). Plants recognize a microbe-associated molecular pattern (MAMP), which is the common molecular pattern of microorganisms, and trigger a basal defense system against the pathogens. The disease-suppressive activity of *P. oligandrum* appears to be a result of MAMP-induced resistance against soil-borne pathogens (Picard et al. 2000, Takenaka et al. 2003), in addition to the direct antagonistic effects on soil-borne pathogens: mycoparasitism, competition for nutrients and the production of antimicrobial substances in the rhizosphere (Martin and Hancock 1987, Lewis et al. 1989, Benhamou et al. 1997, Benhamou et al. 1999). Elicitin, a small lipid-binding protein secreted by the oomycetes *Phytophthora* and *Pythium*, shares a MAMP and induces hypersensitive response (HR) cell death in *Nicotiana* species (Ponchet et al. 1999). Recently, Takenaka et al. (2006) reported novel elicitins purified from the cell wall protein fraction (CWP) of *P. oligandrum*, which contain two major glycoproteins, POD-1 and POD-2. The amino acid sequences deduced from the corresponding cDNA sequences of POD-1 and POD-2 (DDBJ accession Nos. AB217820 and AB217821, respectively) suggest that they have elicitin signatures and O-linked glycosylation sites (Takenaka et al. 2006). Furthermore, the CWP of *P. oligandrum* acts as an elicitor that triggers induced resistance against *Ralstonia solanacearum* in tomato and *Rhizoctonia solani* AG2-2 in sugar (Takenaka et al. 2003, Hase et al. 2006, Takenaka et al. 2006, Hase et al. 2008).

Our previous research indicates that the treatment of tomato (*Solanum lycopersicum* cv. Micro-Tom) roots with CWP or a mycelial homogenate of *P. oligandrum* containing CWP induced an increased amount of ethylene (ET) and expression of ET-responsive genes, e.g. ET receptor *ETR4* and ET-responsive transcription factor *ERF2* (Hase et al. 2006, Takahashi et al. 2006). Jasmonic acid (JA)-responsive gene expression, e.g. *PR-6* and *LOX-E*, was also induced in CWP-treated tomato (Takahashi et al. 2006, Hase et al. 2008). Furthermore, CWP-induced resistance to *R. solanacearum* was compromised in *jai1-1* mutant tomato with an impaired JA signaling pathway (Hase et al. 2008). These facts suggested that the activation of the JA- and ET-dependent signaling pathways seems to be associated with the defense response in tomato roots treated with CWP or mycelial homogenate of *P. oligandrum* containing CWP. However, a set of mutant plants with impaired defense signaling pathways should be a useful tool for further investigation of CWP-induced defense systems.

Arabidopsis thaliana is an excellent model plant for studying plant-pathogen interactions. Several mutants of *A. thaliana* are available, in which salicylic acid (SA), JA or ET

signaling pathways are impaired (Glazebrook 2005). The JA-dependent signaling pathway is impaired in *coi1-1* and *jar1-1* mutants (Staswick et al. 1992, Xie et al. 1998), and the ET-dependent signaling pathway is impaired in *etr1-1* and *ein2-1* mutants (Guzman and Ecker 1990, Chang et al. 1993). In the *eds5-1* mutant (Glazebrook et al. 1996) and *nahG* transgenic plants expressing the bacterial salicylate hydroxylase gene (Delaney et al. 1994), SA-dependent signaling is deficient. The *Arabidopsis npr1-1* mutant was identified by the loss of SA-dependent pathogenesis-related (*PR*) gene expression and disease resistance under systemic acquired resistance (SAR) conditions (Cao et al. 1994, Delaney et al. 1994, Shah et al. 1997). On induction of SAR, NPR1 is translocated to the nucleus and interacts with members of the TGA/OBF subclass of bZIP transcription factors, thereby involving SA-dependent *PR* gene expression (Zhang et al. 1999, Despres et al. 2000, Kinkema et al. 2000, Zhou et al. 2000). Upstream of the SA signaling pathway, *A. thaliana* appears to have at least two mutually exclusive defense signaling pathways employed by two classes of resistance (*R*) genes encoding nucleotide-binding site (NB) and leucine-rich repeat (LRR) domain-containing proteins with coiled-coil (CC) or Toll and human interleukin-1 receptor-like (TIR) motifs at their N-terminal domain (Aarts et al. 1998). *EDS1* and *PAD4*, which encode lipase-like proteins, are required for the function of TIR-NB-LRR class *R* genes (Falk et al. 1999, Jirage et al. 1999, Feys et al. 2001), whereas *NDR1* encoding a plasma membrane-localized protein is essential for the function of most, but not all, CC-NB-LRR class *R* genes (Century et al. 1997, McDowell et al. 2000). Furthermore, it has been demonstrated that REQUIRED FOR Mla12 RESISTANCE 1 (*RAR1*) and SUPPRESSOR OF G-2 ALLELE OF SKIP1 (*SGT1*) are co-chaperones of HEAT SHOCK PROTEIN 90 (*HSP90*) in the folding of NBS-LRR proteins, and are required for efficient defense activation (for reviews, see Shirasu and Schulze-Lefert 2000, Belkadir et al. 2004). Indeed, mutation or silencing of *SGT1*, *RAR1* and *HSP90* compromised some *R* gene-mediated resistance (Peart et al. 2002, Hubert et al. 2003, Lu et al. 2003, Takahashi et al. 2003, Liu et al. 2004).

In this study, to investigate further the CWP-induced defense mechanism, we analyzed the defense response in CWP-treated *nahG* transgenic and 13 mutant [*eds5-1*, *coi1-1*, *jar1-1*, *etr1-1*, *ein2-1*, *npr1-1*, *eds1-1*, *pad4-1*, *ndr1-1*, *sgt1a-1*, *sgt1b* (*edm1*), *rar1-1* and *rar1-21*] plants with impaired defense signaling pathways.

Results and discussion

No necrosis in CWP-treated leaves

As some elicitors of *Pythium* and *Phytophthora* can induce HR-like cell death in *Nicotiana* species (Ponchet et al. 1999), the development of necrosis in CWP-infiltrated leaves of *A. thaliana* was first examined. When the leaves of wild-type

Col-0 were infiltrated with 60 or 300 nmol ml⁻¹ CWP, necrosis was not observed 3 d after infiltration at the macroscopic level (data not shown). In the case of INF1 elicitor belonging to the class I family, only 100 nmol ml⁻¹ INF1 is sufficient to induce HR-like cell death in tobacco (Ponchet et al. 1999). However, even 7 d after infiltration with 300 nmol ml⁻¹ CWP, necrosis was not observed in Arabidopsis leaves at the macroscopic level (data not shown). We have confirmed that CWP did not induce necrosis in tobacco and tomato leaves (Takenaka et al. 2006, Takahashi et al. 2006, Kawamura et al. 2008). Thus, CWP may not be able to induce necrosis in several plant species.

Defense-related gene expression in CWP-treated leaves in Arabidopsis mutants impairing the JA, ET or SA signaling pathway

To investigate whether the defense response is triggered in CWP-treated *A. thaliana* without necrosis, the induction of SA-inducible *PR-1* and *PR-5* genes and JA-inducible *PDF1.2* and *JR2* genes was analyzed in wild-type Col-0, *nahG* transgenic and *eds5-1*, *jar1-1*, *coi1-1*, *ein2-1* and *etr1-1* mutant plants (Supplementary Table S1). *PR-1* and *PR-5* are generally used as markers for the activation of the SA signaling pathway, whereas *PDF1.2* is used as a marker for JA signaling (Takahashi et al. 2004). *JR2* encodes the NAC transcription factor, which accumulates in response to an increased JA level (Titarenko et al. 1997). In our preliminary time-course experiments, *PDF1.2* transcript was detected at 6, 12 and 24 h after CWP treatment by Northern hybridization. The expression of *PDF1.2* in response to CWP reached a maximum level 12 h after CWP treatment (data not shown). Thus, all experiments for defense-related gene expression analysis were conducted 12 h after CWP treatment. JA-inducible *PDF1.2* and *JR2* expression was up-regulated by treatment with 60 nmol ml⁻¹ CWP in wild-type Col-0, but not in *coi1-1* and *jar1-1* mutants with an impaired JA signaling pathway (Fig. 1A). The induction of *PDF1.2* and *JR2* expression according to CWP treatment was also observed in the SA signaling-defective *eds5-1* mutant and *nahG* transgenic plants, but not in the ET signaling-defective *ein2-1* mutant. (Fig. 1B, C). The level of up-regulation of *PDF1.2* and *JR2* expression in response to CWP in the *etr1-1* mutant was not significantly different from that in the wild-type Col-0 (Fig. 1C). On the other hand, SA-inducible *PR-1* and *PR-5* expression was not induced in either the wild-type Col-0, *nahG* transgenic plants or *eds5-1* mutant plants treated with 60 nmol ml⁻¹ CWP, whereas it was induced by exogenous application of SA (Fig. 1). No induction of *PR-1* and *PR-5* expression in wild-type Col-0 was confirmed 24 and 48 h after CWP treatment (data not shown). Therefore, in CWP-treated *A. thaliana*, the JA and ET signaling pathways appear to be activated.

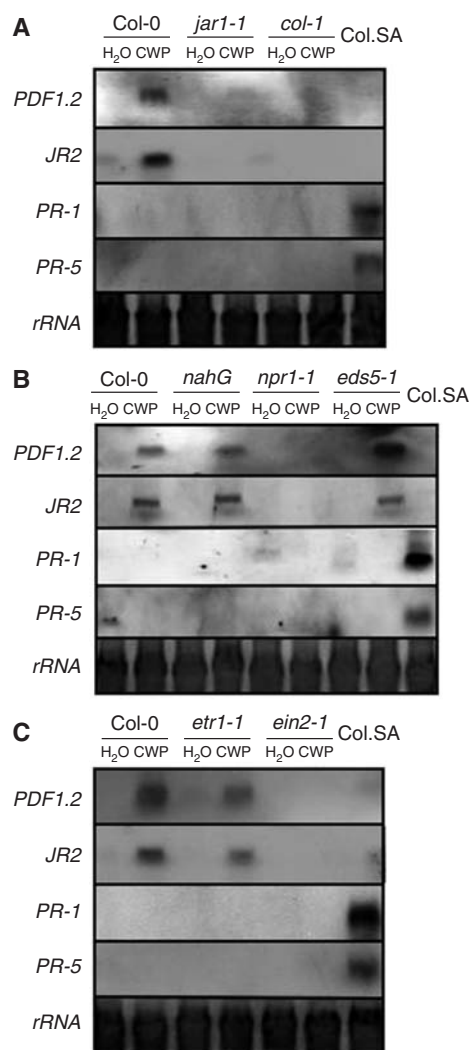


Fig. 1 Expression of defense-related genes in CWP-treated leaves of *Arabidopsis thaliana* wild-type Col-0, the *nahG* transformant and *npr1-1*, *eds5-1*, *jar1-1*, *coi1-1*, *etr1-1* and *ein2-1* mutants. Expression of JA-responsive *PDF1.2* and *JR2* genes and SA-responsive *PR-1* and *PR-5* genes was analyzed in wild-type Col-0, the *nahG* transformant and the six mutants by Northern hybridization 12 h after CWP treatment. Gene expression was also analyzed in distilled water-treated wild-type Col-0, the *nahG* transformant and the six mutants as negative controls. (A) Defense-related gene expression in CWP-treated leaves of wild-type Col-0, and *jar1-1* and *coi1-1* mutants in which the JA signaling pathway was impaired. (B) Defense-related gene expression in CWP-treated leaves of wild-type Col-0, *nahG* transgenic Col-0, and *npr1-1* and *eds5-1* mutants in which the SA signaling pathway was impaired. (C) Defense-related gene expression in CWP-treated leaves of wild-type Col-0, and *etr1-1* and *ein2-1* mutants in which the ET signaling pathway was impaired. Expression of these defense-related genes in the leaves of wild-type Col-0 with 1.0 mM of exogenously applied SA (Col.SA) for 12 h was analyzed by Northern hybridization as a positive control of the induction of SA-responsive gene expression. rRNA was detected as an internal control. Each set of experiments was repeated three times and representative data are presented.

To investigate further the altered gene expression at the genomic level, global gene expression in CWP-treated leaves of *A. thaliana* was analyzed using cDNA array composed of about 24,000 non-redundant clones. Expression of 169 clones was up-regulated by >3-fold following CWP treatment. JA-inducible genes encoding PDF1.2b, tomato protease inhibitor I homolog and basic chitinase, and ET-inducible genes encoding ET-responsive transcription factors and ACC synthase for ET biosynthesis were included among the up-regulated genes (Supplementary Table S2). However, SA-inducible genes were not listed among them. On the other hand, the expression of 24 genes was down-regulated by one-third following CWP treatment (Supplementary Table S3). Thus, the activation of JA- and ET-dependent signaling pathways was also confirmed by cDNA array analysis.

Generally, the SA-dependent defense response is associated with the induction of HR and SAR against a wide range of biotrophic pathogens, but it is not effective against necrotrophic pathogens (Durrant and Dong 2004, Glazebrook 2005). The JA-dependent defense response, which is characterized by activation of the gene encoding PDF1.2 in Arabidopsis, is not associated with HR and SAR, but is considered to provide an alternative defense against necrotrophic pathogens (McDowell and Dangl 2000, Glazebrook 2005). JA also plays an important role in the induced resistance triggered by beneficial microorganisms such as non-pathogenic fluorescent *Pseudomonas* spp., which is referred to as rhizobacteria-mediated induced systemic resistance (ISR) (Pieterse et al. 2000). ET signaling is often co-activated with JA signaling for defense responses to pathogens, and induces PR gene expression similar to that found in JA-responsive genes (Xu et al. 1994). ISR requires the JA-dependent signaling pathway in combination with the ET-dependent signaling pathway (Pieterse et al. 1998). The JA signaling pathway was clearly activated in CWP-treated Arabidopsis. The ET signaling pathway was also induced in response to CWP with the activation of the JA signaling pathway. On the other hand, it has been reported that when the leaves of *A. thaliana* were inoculated with the oomycete pathogen *Phytophthora infestans*, significant induction of PDF1.2 was observed (Huitema et al. 2003). Thus, the signaling pathway for the CWP-induced defense system may be partially associated with that for rhizobacteria-mediated ISR or non-host resistance to oomycetes in *A. thaliana*.

Defense-related gene expression in CWP-treated leaves in Arabidopsis mutants impairing SAR and R gene-mediated resistance

Arabidopsis mutants with SAR or R gene-mediated disease resistance were used to investigate further the molecular mechanism of the CWP-induced defense system. In the CWP-treated *npr1-1* mutant, up-regulation of PDF1.2 and

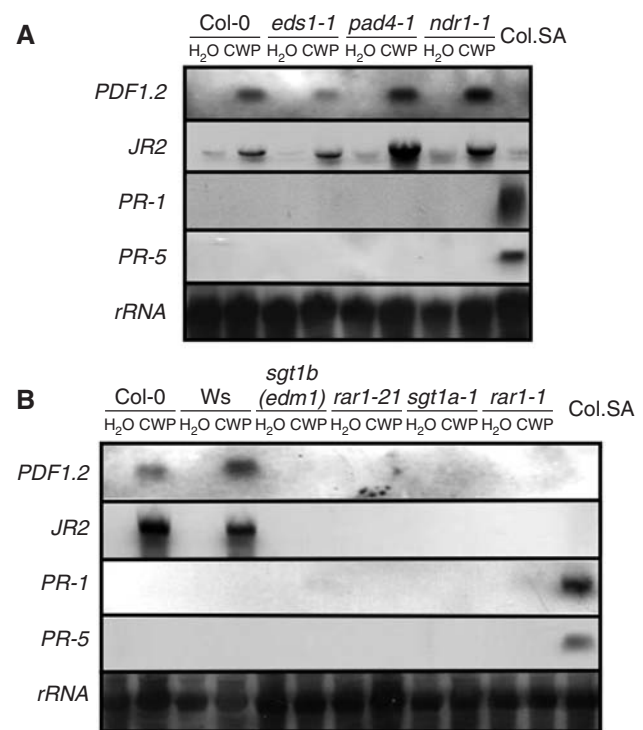


Fig. 2 Expression of defense-related genes in CWP-treated leaves of *Arabidopsis thaliana* wild-type Col-0 and Wassilewskija (Ws), and *eds1-1*, *pad4-1*, *eds5-1*, *sgt1b* (*edm1*), *rar1-21*, *sgt1a-1* and *rar1-1* mutants. Expression of JA-responsive PDF1.2 and JR2 genes and SA-responsive PR-1 and PR-5 genes was analyzed in wild-type Col-0 and the seven mutants by Northern hybridization 12 h after CWP treatment. Gene expression was also analyzed in distilled water-treated wild-type Col-0 and the seven mutants as negative controls. (A) Defense-related gene expression in CWP-treated leaves of wild-type Col-0, and *eds1-1*, *pad4-1* and *eds5-1* mutants. (B) Defense-related gene expression in CWP-treated leaves of wild-type Col-0 and WS, and *sgt1b* (*edm1*), *rar1-21*, *sgt1a-1* and *rar1-1* mutants. Expression of these defense-related genes in the leaves of wild-type Col-0 with 1.0 mM of exogenously applied SA (Col.SA) for 12 h was analyzed by Northern hybridization as a positive control of the induction of SA-responsive gene expression. rRNA was detected as an internal control. Each set of experiments was repeated three times, and representative data are presented.

JR2 expression was compromised (Fig. 1B), indicating that the activation of the JA and ET signaling pathways in response to CWP appeared to require NPR1, which is partially required for SAR. However, in CWP-treated *eds1-1*, *pad4-1* and *ndr1-1* mutants with R-mediated resistance in *A. thaliana*, PDF1.2 and JR2 expression was induced as well as in CWP-treated wild-type Col-0 (Fig. 2A).

To study further the dependence upon other defense signaling-related genes, the expression of PDF1.2 and JR2 in *sgt1a-1*, *sgt1b* (*edm1*), *rar1-1* and *rar1-21* mutants was analyzed. The *sgt1a-1* and *rar1-1* mutants had the Wassilewskija

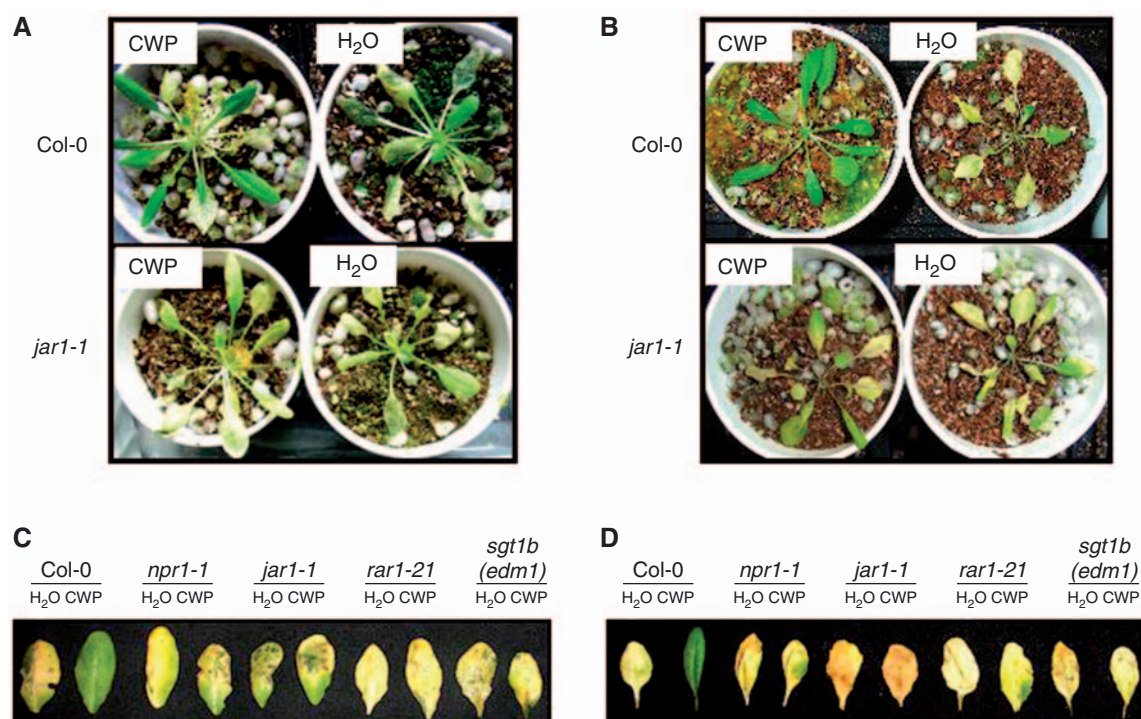


Fig. 3 Response to two bacterial pathogens in CWP-treated *Arabidopsis thaliana* wild-type Col-0 and four mutants. Systemic symptoms in CWP-treated wild-type Col-0 and *jar1-1* mutants 5 d after inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 (A) and *Ralstonia solanacearum* (B). Chlorosis on the leaves of wild-type Col-0 and *npr1-1*, *jar1-1*, *rar1-21* and *sgt1b (edm1)* mutants 5 d after inoculation with *P. syringae* pv. *tomato* DC3000 (C) and *R. solanacearum* (D). Systemic and local symptoms were also observed in distilled water-treated wild-type Col-0 and the four mutants as controls. Each set of experiments was repeated three times, and representative data are presented.

(Ws) background, while the *rar1-21* and *sgt1b (edm1)* mutants had the Col-0 background (**Supplementary Table S1**). The enhanced downy mildew susceptibility (*edm1*) mutant, in which a 35 kb deletion containing the *sgt1b* locus occurs, was used as the *sgt1b* mutant in this study. Up-regulation of *PDF1.2* and *JR2* expression in response to CWP was compromised in *sgt1a-1*, *sgt1b (edm1)*, *rar1-1* and *rar1-21* mutants, but induced in CWP-treated wild-type Col-0 and Ws (**Fig. 2B**). These results suggest that CWP-induced activation of the JA signaling pathway appears to be independent of *EDS1*, *PAD4* and *NDR1* but dependent upon *SGT1a*, *SGT1b* and *RAR1*, which are generally required for R gene-mediated resistance in plants.

Response to bacterial pathogens in CWP-treated *Arabidopsis*

Arabidopsis thaliana is a host plant for *Pseudomonas syringae* and *R. solanacearum*. In order to examine CWP-induced resistance to *P. syringae* and *R. solanacearum* in *A. thaliana*, bacterial growth and symptoms were analyzed in wild-type Col-0 and *jar1-1*, *npr1-1*, *rar1-21* and *sgt1b (edm1)* mutants in which up-regulation of JA-inducible *PDF1.2* and *JR2* expression was compromised (**Figs. 3, 4**). Chlorotic symptoms

developed in wild-type Col-0 infected with virulent *P. syringae* pv. *tomato* DC3000 at 5 d after inoculation, but they were suppressed in CWP-treated Col-0 (**Fig. 3A, C**). Growth of *P. syringae* pv. *tomato* DC3000 decreased in CWP-treated Col-0 compared with control Col-0 treated with distilled water (**Fig. 4A, B**). The suppression of chlorotic and wilt symptoms and bacterial growth was also observed in CWP-treated Col-0 infected with virulent *R. solanacearum* (**Figs. 3B, D; 4C, D**). These results suggest that enhanced resistance to two bacterial pathogens in response to CWP was induced in wild-type Col-0. On the other hand, severe symptoms developed in CWP-treated *jar1-1* mutants infected with *P. syringae* pv. *tomato* DC3000 or *R. solanacearum* (**Fig. 3A, B**). The symptoms also developed in *npr1-1*, *rar1-21* and *sgt1b (edm1)* mutants treated with CWP, as well as in distilled water-treated wild-type Col-0 (**Fig. 3C, D**). Decreased bacterial growth caused by CWP treatment was also compromised in *jar1-1*, *npr1-1*, *rar1-21* and *sgt1b* mutants infected with *P. syringae* pv. *tomato* DC3000 (**Fig. 4A, B**) or *R. solanacearum* (**Fig. 4C, D**). These results indicate that enhanced resistance to two bacterial pathogens was induced in *A. thaliana* treated with CWP and was dependent upon *JAR1*, *NPR1*, *RAR1* and *SGT1b*.

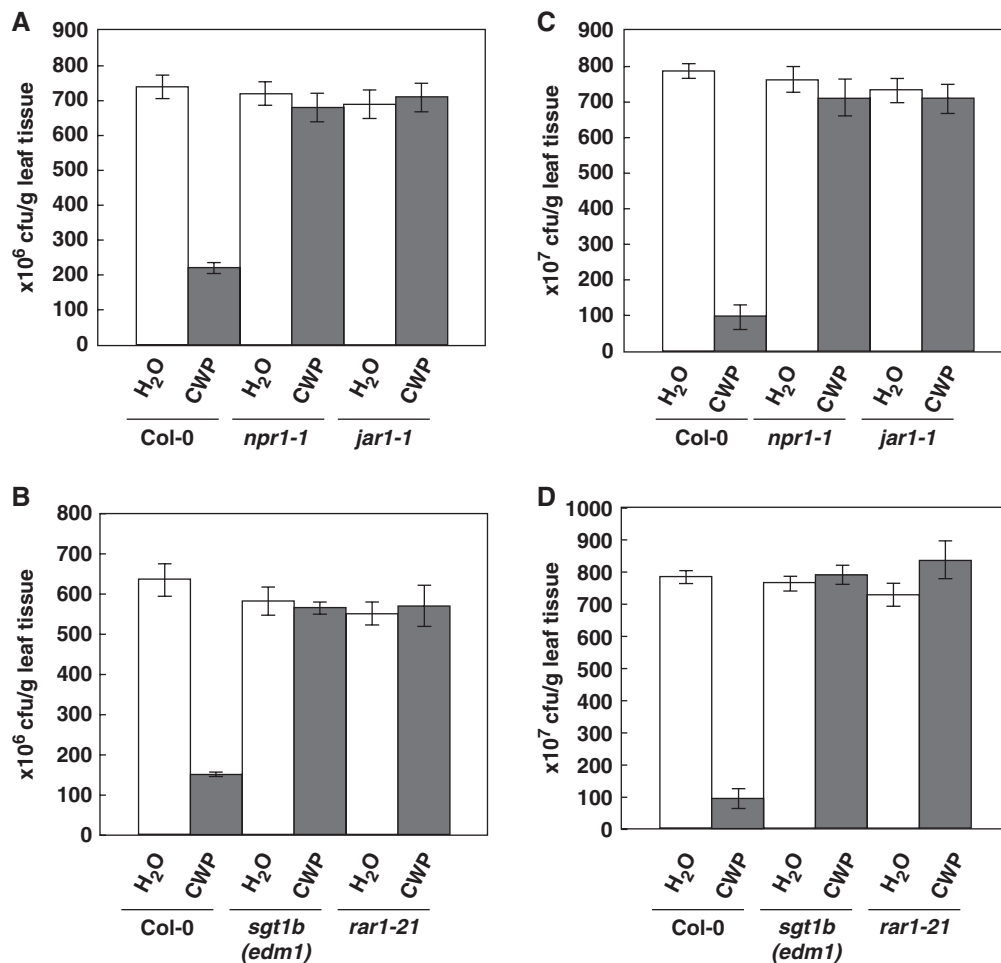


Fig. 4 Bacterial growth in CWP-treated *Arabidopsis thaliana* wild-type Col-0 and four mutants. Bacterial growth in CWP-treated wild-type Col-0 and *npr1-1*, *jar1-1*, *rar1-21* and *sgt1b (edm1)* mutants 5 d after inoculation with *Pseudomonas syringae* pv. tomato DC3000 (A, B) and *Ralstonia solanacearum* (C, D). Growth of these two bacteria was also measured in distilled water-treated wild-type Col-0 and the four mutants as controls. Each set of experiments was repeated three times, and representative data are presented. The error bar indicates standard error.

The compromise of CWP-induced resistance in the *jar1-1* mutant impairing the JA signaling pathway is consistent with the phenotype of CWP-treated *jai1-1* mutant tomato against bacterial pathogens (Hase et al. 2008). Since the virulence compound coronatine of *P. syringae* is a JA analog (Xie et al. 1998), there is still a possibility that this phenotype of the *jar1-1* mutant against *P. syringae* may be caused by the insensitivity of the *jar1-1* mutant against coronatine. However, studies using the *jar1-1* mutant demonstrated that *jar1-1* mutation has no detectable impact on plant susceptibility to virulent *P. syringae* (Pieterse et al. 1998, Clarke et al. 2000, Laurie-Berry et al. 2006). Thus, the JA signaling pathway is likely to be a key signaling pathway for CWP-induced resistance to bacterial pathogens in *Arabidopsis* and tomato.

In the defense response, NPR1 plays an important role in the activation of the SA signaling pathway and SAR, where it

functions in the nucleus and acts as a positive regulator of SA signaling (Kinkema et al. 2000, Dong 2004, Durrant and Dong 2004). On the other hand, NPR1 is also required for ISR induced by non-pathogenic rhizobacteria and confers resistance to pathogens that are dependent on JA and ET signaling pathways (Pieterse et al. 1998). In the ISR, NPR1 appears to act in the cytosol (Spoel et al. 2003). Furthermore, NPR1 plays a crucial role in the cross-talk between SA and JA/ET signaling (Spoel et al. 2003). JA-responsive defense gene expression and induced resistance to two bacterial pathogens in CWP-treated *A. thaliana* also required NPR1. The role of NPR1 in the CWP-induced defense system should be clarified to provide better understanding of the signaling pathways regulating CWP-induced defense systems.

EDS1 and PAD4 positively regulate SA accumulation in the EDS1/PAD4-dependent R gene-mediated response (Wiermer et al. 2005). NDR1 also plays an indispensable role

in some *R* gene-mediated resistance responses. However, in *ndr1-1*, *eds1-1* and *pad4-1* mutants treated with CWP, defense gene expression and resistance to bacterial pathogens were not compromised, indicating that CWP-induced defense responses may be conferred by signaling pathway(s) distinct from *R* gene-mediated responses. However, the CWP-induced defense response was compromised in *rar1-1*, *rar1-21*, *sgt1a-1* and *sgt1b* (*edm1*) mutants. RAR1 and two SGT1s have interactive functions in disease resistance for regulating the accumulation and folding of particular NB-LRR proteins, the major class R proteins, by interacting transiently with HSP90 (Hubert et al. 2003, Takahashi et al. 2003, Liu et al. 2004). Silencing of *SGT1* in *Nicotiana benthamiana* suggested a requirement of SGT1 not only for NB-LRR protein and other R proteins, but also for some non-host resistance (Peart et al. 2002). In Arabidopsis, it has also been demonstrated that two *SGT1* genes, *SGT1a* and *SGT1b*, are expressed and involved in host disease resistance by NB-LRR proteins and basal resistance (Austin et al. 2002, Peart et al. 2002, Tör et al. 2002, Tornero et al. 2002, Azevedo et al. 2006, Zhou et al. 2008). In our study, induced resistance to bacterial pathogens in CWP-treated Arabidopsis was dependent on RAR1 and SGT1. There is no evidence for the involvement of the *R* gene in the recognition of CWP or downstream signaling for CWP-induced resistance; a recent investigation on NB-LRR proteins suggested that the NB-LRR proteins confer resistance to a wide variety of microbes and do have alternative functions that are different from the immune receptor function (Tameling and Joosten 2007). Thus, an interaction of RAR1 and SGT1 with uncharacterized NB-LRR proteins may play a role in the defense responses activated by CWP.

On the other hand, SGT1b is also required for the activities of the SCF^{TIR}-mediated auxin response and the SCF^{COL}-mediated JA response (Gray et al. 2003, Lorenzo and Solano 2005). The Arabidopsis *COI1* gene is required for the JA-mediated defense response against pathogens (Xie et al. 1998). COI1 protein contains an F-box motif and associates physically with AtCUL1, AtRbx1 and the Skp1-like proteins ASK1 and ASK2 to assemble SCF^{COI1} ubiquitin-ligase complexes (Skp1-Cdc53-F-box protein) (Xu et al. 2002). SCF^{COI1} is thought to target key regulators of the JA signaling pathway for ubiquitination and subsequent degradation by the 26S proteasome. The JA signaling pathway was clearly activated in CWP-treated Arabidopsis. Thus, because the SCF^{COI1}-mediated JA signaling pathway was impaired, CWP-induced resistance to bacterial pathogens and activation of JA signaling might be compromised in an *sgt1* mutant background. To the best of our knowledge, the role of *SGT1* and *RAR1* in disease resistance induced by non-pathogenic microorganisms is still poorly understood. Further study on the function of SGT1 and RAR1 in CWP-induced JA signaling and enhanced resistance to bacterial pathogens in Arabidopsis should provide deeper understanding of disease resistance

induced by colonization in the rhizosphere by non-pathogenic *P. oligandrum*.

Recently, some receptors recognizing MAMPs at the cell surface were isolated (Nurnberger and Kemmerling 2006, Bittel and Robatzek 2007). Furthermore, a component that is associated with the receptor has been identified (Schwessinger and Zipfel 2008). For example, Arabidopsis BAK1 physically interacts with the flagellin-derived 22 amino acid peptide (flg22) recognition receptor FLAGELLIN SENSING 2 (FLS2) and is required for the flg22-induced defense response (Chinchilla et al. 2007). Thus, identification of the CWP recognition receptor or receptor complex and its associated components will clarify the earliest event of the CWP-induced disease resistance mechanism and activation of downstream defense signaling at the molecular level.

Materials and Methods

Plants and growth condition

Arabidopsis thaliana ecotypes Col-0 and Ws, *nahG* transgenic Col-0 (Delaney et al. 1994) and 13 mutants comprising *eds5-1* and *pad4-1* (Glazebrook et al. 1996), *npr1-1* (Cao et al. 1994), *coi1-1* (Xie et al. 1998), *jar1-1* (Staswick et al. 1992), *etr1-1* (Chang et al. 1993), *ein2-1* (Guzman and Ecker 1990), *eds1-1* (Parker et al. 1996), *ndr1-1* (Century et al. 1995), *sgt1a-1* and *rar1-1* (Azevedo et al. 2006), *sgt1b* (Tör et al. 2002) and *rar1-21* (Tornero et al. 2002) were grown at 25°C under continuous illumination (7,000 lux) in a mixture of vermiculite and perlite (1:1 mixture), and irrigated with a 1,000-fold-diluted Hyponex solution at 3 d intervals. The detailed information about the 13 mutants used in this study is presented in **Supplementary Table S1**.

Preparation of CWP elicitor

Preparation of CWP from the cell wall fraction of *P. oligandrum* isolate MMR2 was conducted by the method of Takenaka et al. (2006). To analyze defense-related gene expression, 60 nmol ml⁻¹ CWP was infiltrated into the leaves of 4-week-old *A. thaliana* using a disposable 1 ml plastic syringe, and total RNA was isolated 12 h after infiltration for gene expression analysis. To examine the induction of necrosis, 300 nmol ml⁻¹ CWP was infiltrated into the leaves and the progression of necrosis at the infiltration site was observed every 24 h until 1 week after infiltration.

Gene expression analysis by Northern hybridization

The expression of two SA-inducible *PR* genes (*PR-1* and *PR-5*) and two JA-inducible genes (*PDF1.2* and *JR2*) of *A. thaliana* was analyzed. Total RNA was isolated from the leaves of *A. thaliana* by the acid guanidium-phenol-chloroform method (Chomczynski and Sacchi 1987). A 15 µg aliquot of total RNA was loaded in each lane of a 1.2% denaturing agarose gel. Northern hybridization was performed according

to Sambrook and Russell (2001). To detect the expression of *PR-1*, *PR-5* and *PDF1.2* genes, ~500 bp fragments of cDNA of each gene described previously were used as probes for Northern hybridization analysis (Takahashi et al. 2004). The DNA fragments for detecting transcripts from *JR2* by Northern hybridization were amplified by reverse PCR with primers 5'-AAGCTCCCCTCAAAGACGTC-3' and 5'-ACGACA CGACAAGTCCAAAG-3' (Titarenko et al. 1997). First-strand cDNA as a template for reverse PCR was reverse-transcribed from total RNA of the leaves of *A. thaliana* ecotype Col-0 using a ThermoScript RT-PCR kit (Life Technologies, Rockville, MD, USA). A 1 µg aliquot of first-strand cDNA was added to 50 µl of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 2 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.2 µM of each primer and 5 U of Go-Taq DNA polymerase (Promega, Madison, WI, USA) for PCR. The reaction ran with the program 30 cycles at 94°C for 1 min, 55°C for 2 min and 73°C for 1 min. The PCR product was purified and cloned into the *EcoRV* site of pBluescript SK+ (Stratagene, La Jolla, CA, USA). To confirm that the expected DNA was cloned, the nucleotide sequence of each insert was determined by the Sanger method using an ABI model 310A automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The amplified *PR-1*, *PR-5*, *PDF1.2* and *JR2* DNA fragments were labeled with digoxigenin using a DNA labeling kit (Roche, Penzberg, Germany) and detected using the CDP-Star reagent (New England Biolabs, Beverly, MA, USA) following the instruction manuals. Each set of experiments was repeated three times, and representative data are presented in Figs. 1 and 2.

cDNA array analysis

To investigate the alteration in the gene expression pattern of CWP-treated leaves of *A. thaliana* ecotype Col-0, global gene expression was analyzed using Agilent Arabidopsis cDNA array (Agilent Technologies, Santa Clara, CA, USA). A total of 24,000 non-redundant genes comprised the array. Total RNA was isolated 12 h after treatment from Arabidopsis leaves infiltrated with 60 nmol ml⁻¹ CWP, and distilled water was used as a control using an RNeasy plant mini kit (Qiagen, Hilden, Germany). For biological repetition, total RNA was prepared from two independent plants treated with distilled water or CWP. To avoid a bias as a consequence of differences in efficiency of the labeling, two pairs of cRNA samples (Cy3-labeled cRNA of distilled water-treated Col-0 and Cy5-labeled cRNA of CWP-treated Col-0, or Cy5-labeled cRNA of distilled water-treated Col-0 and Cy3-labeled cRNA of CWP-treated Col-0) were used for hybridization on Arabidopsis ver. 3 Oligo Microarray (Agilent Technologies, Santa Clara, CA, USA). The procedures for labeling the cRNA probe, hybridization and data analysis were described previously (Sekine et al. 2008). Signal intensities were calculated and normalized using the global normalization method.

Data points whose intensities in both channels were equal to or greater than the background plus 2.6 times the standard deviation were further analyzed. *P*-values for the difference between gene expression in CWP-treated Col-0 and that in distilled water-treated control Col-0 were calculated using a *t*-test with multiple tests correction. Only genes that were significantly different (*P* < 0.01) in both of the two dye-swap experiments were used for further analysis. The ratio of the gene expression value in CWP-treated Col-0 to that in distilled water-treated control Col-0 for each data point was averaged from the two dye-swap experiments. Genes up-regulated by >3-fold in comparison with the control, or down-regulated by less than a third in the leaves treated with CWP were listed.

Pathogenic bacteria and disease assessment

To examine resistance to *R. solanacearum*, 4-week-old *A. thaliana* roots were soaked with mycelial homogenate containing about 30 nmol ml⁻¹ CWP or distilled water as a control for 24 h. The mycelial homogenate was prepared from 5 g fresh weight of the washed mycelial mats homogenized with 10 ml of distilled water, and adjusted to 5 × 10⁵ oospores ml⁻¹ using distilled water. The plants were cut so that each retained 2 cm roots 24 h after soaking, and the roots were soaked with 1 × 10⁸ c.f.u. ml⁻¹ of virulent *R. solanacearum* isolate 8242, race 1, biovar 4 for 3 min, and then grown in a mixture of vermiculite and perlite (1:1 mixture) as described above for an additional 5 d. The bacterial solution was prepared by the method described previously (Nakaho et al. 2004, Hase et al. 2008). Plants were coded and inspected daily for wilting symptoms. Each assay was repeated in three successive trials and three plants were inoculated in each experiment. For measurement of bacterial growth, three plants were harvested 5 d after inoculation. The plants were homogenized with 100-fold distilled water. The homogenate was diluted with distilled water at 10- to 10⁵-fold for counting bacterial colonies. Appropriate dilutions were placed on CPG agar medium containing 0.1% of yeast extract and 0.001% of tetrazolium chloride (Hendrick and Sequeira 1984). Plates were incubated at 28°C for 2 d, and then the bacterial colonies were counted. Bacterial counts were expressed as colony-forming units per mg of leaf tissue.

To examine resistance to *P. syringae* pv. *tomato* DC3000, 60 nmol ml⁻¹ CWP was infiltrated into the middle region of a fully expanded leaf of a 4-week-old *A. thaliana* plant and incubated at 25°C under 7,000 lux for 12 h. At 5 d after spraying with virulent *P. syringae* pv. *tomato* DC3000 at a concentration of 7 × 10⁷ c.f.u. ml⁻¹ in 0.05% silwet 77, disease assessment via symptom development and measurement of bacterial growth were performed according to the method described previously (Sekine et al. 2008). Three independent plants were used for each measurement of the growth of *P. syringae*, and the levels of bacterial growth were expressed

as the average number \pm standard error of bacteria. Each set of experiments was repeated three times, and representative data are presented in Figs. 3 and 4.

Supplementary data

Supplementary data are available at PCP online.

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