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Interaction between polygalacturonase-inhibiting protein and jasmonic acid during defense activation in tomato against *Botrytis cinerea*

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Abstract Oligogalacturonic acids (OGAs) generated from in vitro interaction between fungal polygalacturonase (PG) and bean PG-inhibiting protein (PGIP) were shown to activate phytoalexin biosynthesis in soybean. Based on this observation, it was hypothesized that PGIP-dependent generation of OGAs activates plant defence responses in vivo. We tested the hypothesis that PGIP activates jasmonic acid-dependent responses to pathogens. For this purpose, a population of tomato plants segregating for a mutation in the jasmonate receptor CORONATINE INSENSITIVE1 (*coi1*) and for overexpression of pear PGIP (*pPGIP*) was challenged with *Botrytis cinerea*. The *coi1* mutant was hypersensitive to *B. cinerea*, but overexpression of *pPGIP* in the *coi1* mutant background reduced pathogen susceptibility, suggesting that these two genes independently alter defence responses. In addition, pPGIP overexpression suppressed pathogen induction of salicylic acid in the *coi1* mutant and activated expression of acidic ß-1,3-glucanase independently of the *coi1* mutation. However, expression of proteinase inhibitor II (PIN II) in *pPGIP* overexpressing tomato plants was dependent on COI1. Effects of *pPGIP* overexpression on defence are therefore complex and only in the case of PIN II pPGIP acts through *COI1*.

Keywords *Botrytis cinerea* · Jasmonic acid · OGA · PGIP

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Botrytis cinerea causes gray mold and other agronomically important diseases. This necrotrophic pathogen has more than 200 host plants. Endo-polygalacturonase (endo-PG) is an important virulence factor of *B. cinerea*, hydrolyzing polygalacturonic acid of plant cell walls. PG-inhibiting proteins (PGIPs), present in plant cell walls, apparently reduce fungal growth *in planta* because overexpression of pear PGIP (*pPGIP*) in tomato (Powell et al. 2000) and of *AtPGIP1* or

AtPGIP2 in *Arabidopsis thaliana* (Ferrari et al. 2003b) reduces the severity of disease symptoms. Conversely, suppression of *AtPGIP1* in *A. thaliana* enhances susceptibility to *B. cinerea* (Ferrari et al. 2006).

PGIPs are thought to reduce pathogen growth in two ways: 1) PGIPs directly bind to and inhibit pathogen PGs; 2) PGIPs indirectly induce defence responses (Cervone et al. 1989; Stotz et al. 2004). In this report, we explored the latter effect of PGIPs. *Aspergillus niger* PG generates elicitor-active oligogalacturonic acids (OGAs) from polygalacturonic acid in the presence of *P. vulgaris* PGIP (Cervone et al. 1989). Cervone et al hypothesized that interaction between PG and PGIP results in the release of OGAs to activate plant defence responses in vivo.

The jasmonate receptor COI1 is part of an enzyme complex called Skp/Cullin/F-box (SCF^{COI1}), a type of E_3 ubiquitin ligase. The *coi1* and *jar1* mutants of *A. thaliana* (Thomma et al. 1998; Ferrari et al. 2003a) as well as the jasmonate-deficient tomato mutant *def1* (*Defenseless*), prosystemin antisense tomatoes (Diaz et al. 2002), and *jai1* (*coi1* homolog) tomato plants (Vicedo et al. 2009) are hypersusceptible to *B. cinerea*, showing that jasmonic acid (JA) contributes to defence responses against *B. cinerea*.

The observation that OGAs may accumulate when pectic substrates are hydrolysed by endoPGs in the presence of PGIP is supported by in vitro interaction between purified PGs and bean PGIP (Cervone et al. 1989). However, incubation of a more complex PG mixture with pear PGIP did not provide evidence for formation of elicitor active OGAs (Sharrock and Labavitch 1994). Here we tested the hypothesis that increased *pPGIP* expression alters JA-inducible plant defence pathways in vivo. We therefore generated tomato plants that overexpress *pPGIP* in the *coi1* mutant background by crossing respective mutant (Li et al. 2004) and transgenic plants (Powell et al. 2000).

Specifically, tomato plants overexpressing *pPGIP* (Powell et al. 2000) were crossed with a heterozygous *COI1/coi1* tomato (Li et al. 2004). Heterozygous F_1-progeny was selected and selfed. To determine the genotype of the F_2 progeny, genomic DNA was extracted (Miklas et al. 1993); wild-type and *coi1* mutant alleles were amplified according to Li et al. (2004).

Presence of *pPGIP* was checked by PCR in the F_2 generation using a forward primer in the 35S promoter (5'GTTCATTTCATTTGGAGAGGACAGGCTT-3') and a reverse primer in the *pPGIP* gene (5'-CTTGCAGCTTGGGAGTG-3'). Segregation of the transgene was tested by kanamycin selection in the F_3 progeny. As the *coi1* mutant does not produce viable seeds (Li et al. 2004), *pPGIP* genotypes in *coi1* mutant seedlings were checked by Southern hybridization and PCR in the F_2 generation.

The *coi1* mutant and *pPGIP*-overexpressing tomato plants are in the genetic backgrounds of cv. Castlemart and cv. T5, respectively. The susceptibilities of both cultivars to *B. cinerea* did not differ significantly (data not shown). Besides, segregation of cv. Castlemart and cv. T5 traits is expected to be random in the F_2 progeny; such traits are thus averaged out when comparisons of the four genotypes are made.

Susceptibilities to *B. cinerea* among segregating F_2 genotypes were analyzed twice without significant experimental effects. Foliar inoculation with *Botrytis cinerea* strain B05.10 was as described (Guimaraes et al. 2004) with the following modifications. A total of ten droplets containing 1,000 conidia in a volume of 2 µl each were placed on detached leaves. Lesion expansion started after 3 days. Lesion diameter and disease frequency were scored. The combined data of two experiments are shown in Fig. 1. Genotype-dependent variation in lesion diameter (Fig. 1a) was relatively larger than genotype-dependent differences in frequency of infection (Fig. 1b). The *coi1* mutant was the most susceptible to *B. cinerea*, both in terms of frequency of infection and lesion diameter. Although there was a trend towards reduction of lesion diameter in *pPGIP*-overexpressing plants when compared to wild-type plants, this difference was not statistically significant. In terms of lesion diameter, the susceptibility of *pPGIP*-overexpressing plants in the *coi1* mutant background was intermediate with respect to *coi1* mutant and *pPGIP*-overexpressing plants. Although *pPGIP* overexpression in the *coi1* mutant background reduced the frequency of infection, this effect was not statistically significant. The approximately additive effects of the *coi1* mutation and *pPGIP* overexpression make it likely that these two genes independently alter susceptibility to *B. cinerea*. Our data support a recent report that OGA-induced resistance in *A. thaliana* to *B. cinerea* is independent of jasmonate, salicylic acid, and ethylene signalling (Ferrari et al. 2007). Although OGAs were reported to activate JA (Doares et al. 1995) and salicylic acid production (Klarzynski et al. 2000), Ferrari et al.

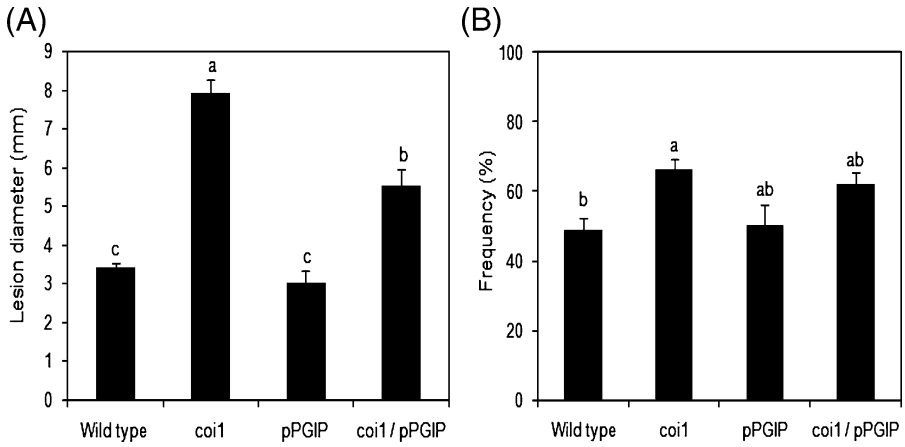

Fig. 1 Effect of pPGIP and JA on defence of tomato against *B. cinerea*. **a** Lesion diameters and **b** frequencies of infection were measured 3 d post inoculation. A total of 41, 32, 10, and 11 replicates were used for wild-type, *coi1* mutant, *pPGIP*-overexpressing, and *pPGIP*-overexpressing plants in the *coi1* mutant background, respectively. A GLM in conjunction with LSM (SAS Institute, Cary, NC) was used to determine significance of genotype effects. Significant differences in lesion diameter as indicated by letters; $P<0.0001$. Differences in frequency of infection between wild-type and *coi1* mutant plants were also significant; $P=0.0003$. Vertical bars show±SE

(2007) have shown that exogenously applied OGAs activate plant defences independently of these compounds.

To gain insights into the mechanism of defence against *B. cinerea* in tomato, free salicylic acid concentrations were measured in pathogen and mock-inoculated leaves. Multiple sites of excised tomato leaves were inoculated with *B. cinerea* and placed in a moisture chamber (Guimaraes et al. 2004). Salicylic acid levels were measured 1 and 2 days post inoculation in four replicates of pooled leaf samples as described (Engelberth et al. 2004). Salicylic acid levels were not elevated in leaves of wild-type plants that were inoculated with *B. cinerea* relative to mock-inoculated control samples (Fig. 2). However, a 14-fold increase in salicylic acid was observed in leaves of the *coi1* mutant relative to wild-type leaves 1 day post-inoculation with *B. cinerea*. A *B. cinerea*-induced increase in salicylic acid has, to our knowledge, not been reported previously. Salicylic acid levels in leaves of *pPGIP*-overexpressing plants were reduced relative to the wild type. Most surprisingly, overexpression of *pPGIP* in the *coi1* background abolished the *coi1*-dependent increase in salicylic acid after inoculation with *B. cinerea*.

Similarly to the data on wild type reported here, Govrin and Levine (2002) observed no increase in salicylic acid after *B. cinerea* infection of *Arabidopsis* plants. Interestingly, exposure of the *coi1* mutant to *Pseudomonas syringae* elevated salicylic acid levels, increased *PR1* expression, and enhanced resistance to this bacterial pathogen (Kloek et al. 2001). The effects of salicylic acid on susceptibility to *B. cinerea* are unclear because expression of salicylate hydroxylase (*nahG*) in tomato and *Arabidopsis* increased foliar susceptibility to *B. cinerea* (Audenaert et al. 2002; Govrin and Levine 2002), but leaves of the *npr1 Arabidopsis* mutant are as susceptible to *B. cinerea* as the wild type (Ferrari et al. 2003a).

Proteinase inhibitor II (PIN II) and acidic β-1,3-glucanase are pathogenesis-related proteins that have

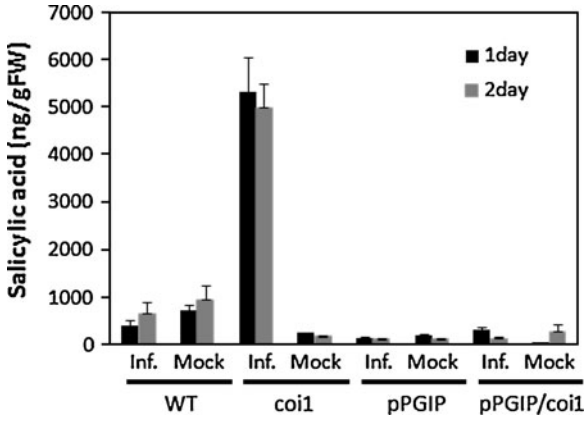

Fig. 2 Salicylic acid levels in wild-type, *coi1* mutant, *pPGIP* overexpressing, and *pPGIP*-overexpressing plants in the *coi1* mutant background 1 and 2 days after mock or *B. cinerea* inoculation (inf.). Vertical bars show±SE of four replicates

frequently been used to monitor host responses to microbial infection. Induction of PIN II is known to be dependent on JA (Li et al. 2004). Polyclonal antibodies against proteinase inhibitor II (PIN II) and against acidic β-1,3-glucanase (PR-N) were obtained from Dr. Clarence Ryan (Washington State University, Pullman, WA) and Dr. Michele Legrand (University of Strasbourg, France), respectively. SDS-PAGE, western blotting, and immunodetection were essentially as described (Stotz et al. 2000). Lyophilized PIN II antiserum was reconstituted in 0.2% Tween-20. Antisera were diluted 1:6,700. Horseradish peroxidase-conjugated antibody was diluted 1:10,000. A chemiluminescent detection system was used to detect the antigen (Pierce ECL Substrate) with a LAS-3000 image reader (Fuji Film, http://fujifilm.jp).

PIN II was expressed in mock and pathogen-inoculated leaves of wild-type plants (Fig. 3). PIN II expression in excised leaves is not surprising because this protein is known to be wound-responsive (Green and Ryan 1972; Gustafson and Ryan 1976). PINII was also strongly expressed in mock-inoculated leaves of *pPGIP*-overexpressing plants. However, PIN II expression was suppressed in infected leaves of *pPGIP*-overexpressing plants. PIN II was not expressed in the *coi1* mutant background in the presence or absence of *pPGIP* overexpression (Fig. 3). Thus, overexpression of *pPGIP* does not activate PIN II expression in the *coi1* mutant background. We therefore conclude that, in the case of PIN II expression, *pPGIP* acts through the jasmonate pathway.

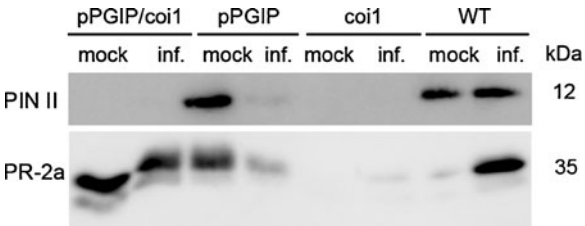

Fig. 3 Protein expression of PIN II and PR-2a (acidic glucanase) in wild-type, *coi1* mutant, *pPGIP* overexpressing, and *pPGIP*-overexpressing plants in the *coi1* mutant background 1 and 2 days after mock or *B. cinerea* inoculation. PR-N antiserum was used for detection of PR-2a in tomato. Note that the small induction of PR-2a expression in response to infection (inf.) is supportive of COI1-independent induction, probably by SA (van Kan et al. 1995). However, most of the induction is COI1-dependent; PR-2a is also known to respond to JA (Ding et al. 2002)

Inoculation with *B. cinerea* induced acidic β-1,3-glucanase, also referred to as PR-2a (Ding et al. 2002), in wild-type leaves, but PR-2a induction was negligible in leaves of the *coi1* mutant (Fig. 3). As with PIN II, PR-2a was expressed in mock-inoculated leaves of pPGIP-overexpressing plants, but less so in leaves that were pathogen challenged. Unexpectedly, PR-2a expression was high in mock and pathogen-inoculated leaves of *coi1* mutant plants that overexpress *pPGIP*. These data show that PR-2a regulation by *pPGIP* is independent of *COI1*.

Our analysis of *pPGIP* expression in the background of the *coi1* mutant is not entirely compatible with the hypothesis that OGAs generated by fungal endo-PGs in the presence of PGIP activate defence responses (Cervone et al. 1989; Bishop et al. 1981). Our data showed an additive effect of pPGIP expression in the background of the *coi1* mutant, suggesting that, with respect to the resistance phenotype, JA signalling is not

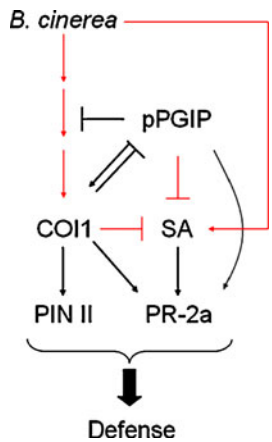

Fig. 4 Proposed model of PGIP- and JA-induced defence responses against *B. cinerea* in tomato leaves. *Lines* in *red* are based on pathogen-induced changes in free SA levels; an pathogen-induced SA increase is only observed in the *coi1* mutant in the absence of *pPGIP* transgene expression. *Black lines* are based on protein expression data. PIN II is induced by the pathogen in a COI1-dependent fashion. The pPGIP transgene activates PIN II expression in the absence of the stimulus, but induced PIN II expression is low in transgenic plants. This suggests that pPGIP interferes with the JA pathway. Moreover, the wild-type *COI1* allele appears to negatively regulate the pPGIP-mediated up-regulation of PIN II. PR-2a is positively regulated by COI1, SA, and pPGIP. Down-regulation of induced PR-2a levels does not occur in *coi1* mutant plants overexpressing pPGIP. This also supports the negative effect of the wild-type *COI1* allele on PR-2a and PIN II expression. No change in PR-2a expression is seen in the *coi1* mutant because pPGIP-dependent expression is independent of COI1. Note that PGIP and JA independently activate the defense responses

related to pPGIP action (Fig. 4). Overexpression of *pPGIP* apparently alters defence signalling or perturbs cell wall metabolism such that induced responses to *B. cinerea* infection are altered. In the case of PIN II expression, pPGIP acts through COI1. The effects of *pPGIP* overexpression on SA and PR-2a production are independent of the jasmonate pathway.

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