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LETTER TO THE EDITOR

The *coi1-16* Mutant Harbors a Second Site Mutation Rendering PEN2 Nonfunctional

Coronatine is a phytotoxin produced by several pathovars of *Pseudomonas syringae* that acts as a mimic of methyl jasmonate in plants. Due to the importance of jasmonic acid and its derivatives in responses of plants to biotic and abiotic stress, the *Arabidopsis thaliana* coronatine-insensitive mutant *coi1* is widely used in the plant community. Ellis and Turner (2002) described a conditional fertile *coi1* allele, *coi1-16*, that is being used in a number of laboratories. We have discovered that the *coi1-16* mutant (but not the original *coi1-1*) carries an additional mutation, which might also influence the responses usually studied with *coi1* (i.e., pathogen defense responses). This additional mutation lies in the *PENETRATION2* (*PEN2*) gene, which was identified as a gene required for nonhost resistance of *Arabidopsis* against barley powdery mildew (Lipka et al., 2005).

Nonhost resistance describes the ability of all members of a plant species to successfully prevent colonization by any member of a given pathogen species. Nonadapted pathogens are recognized by the nonhost plant via pathogen-associated molecular patterns, which leads to the activation of defense responses (Nürnberger and Lipka, 2005). Mutant screens have identified genes important for nonhost resistance of *Arabidopsis* against the nonadapted barley powdery mildew fungus *Blumeria graminis* f. sp *hordei* (*Bgh*). *PEN1* encodes a SNARE-syntaxin that is involved in vesicle transport to the site of attempted penetration (Collins et al., 2003; Kwon et al., 2008). A defect in the *PEN2* gene, encoding a glycoside hydrolase, leads to a loss of penetration resistance (Lipka et al., 2005), as does a mutation in *PEN3* encoding an ABC transporter (Stein et al., 2006). The PEN2 protein is a member of the *Arabidopsis* family 1 glycoside hydrolases. Although the catalytic activity of PEN2 has yet to be shown, the substitution of a Glu to Asp at the putative active site renders the protein unable to complement the *pen2* mutant, suggesting that catalytic activity is required for PEN2 function (Lipka et al., 2005).

Apparently, the PEN proteins collectively comprise a set of pre-invasion defense responses activated in nonhost plants, which restrict pathogen growth at the cell periphery. Once this pre-invasion defense is compromised, additional layers of defense responses are able to restrict further spread of the pathogen, since all three *pen* mutants are still resistant to *Bgh*. Post-invasion defense responses against *Bgh* require functional *PAD4*, *EDS1*, and *SAG101* genes (Lipka et al., 2005). On the *pen2 pad4 sag101* triple mutant plant, *Bgh* is able to form conidiospores, suggesting a breakdown of nonhost resistance (Lipka et al., 2005).

The oomycete *Phytophthora infestans*, the causal agent of late blight disease of potato, is not able to infect *Arabidopsis* (Kamoun, 2001). This nonhost–pathogen interaction is characterized by the unsuccessful attempt of the oomycete to penetrate epidermal cells. Cessation of pathogen growth correlates with massive cell wall depositions in epidermal cells (Lipka et al., 2005). Phenotypically, no major symptoms can be detected in *gl1*, a trichomeless mutant that represents the wild type to *pen2-1* (Figure 1A). The *pen2* and *pen3* mutants, but not *pen1*, are compromised in penetration resistance against *P. infestans* (Lipka et al., 2005). *pen2* plants react with visible necrosis formation (Figure 1A), and trypan blue staining shows an increased number of dead cells

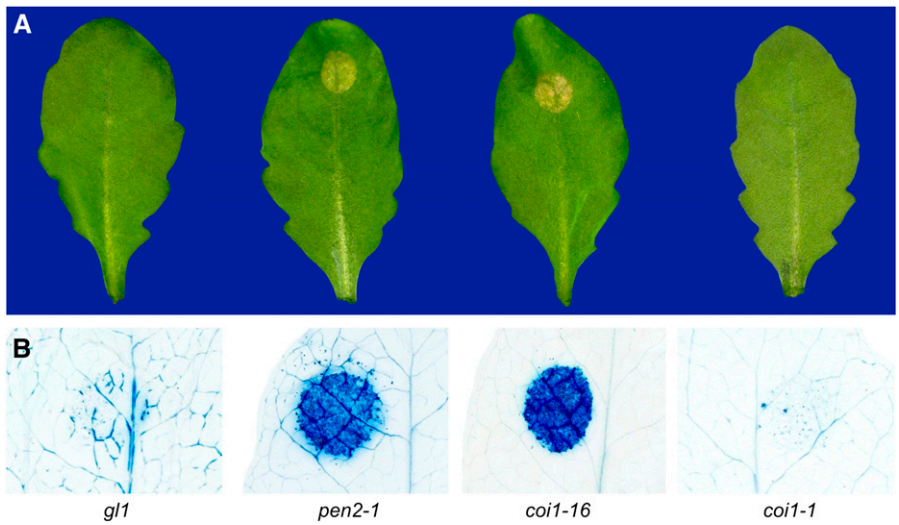

Figure 1. Mutant Phenotypes.

(A) Phenotype of *gl1*, *pen2-1*, *coi1-16*, and *coi1-1* plants after infection with *P. infestans*. Plants were infected by drop inoculation with a zoospore solution of *P. infestans* (5×10^5 spores/mL). Photos were taken 3 d after inoculation.

(B) Visualization of cell death by trypan blue staining. Leaves of *gl1*, *pen2-1*, *coi1-16*, and *coi1-1* plants were infected with a zoospore solution of *P. infestans* (5×10^5 spores/mL) and subjected to trypan blue staining 3 d after infection.

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Table 1. Segregation Analysis of the Cross between *coi1-16* and *pen2-1*

			Phenotype			
			HR		Sterility	
Cross	Progeny	Total	Wild Type	*pen2-1*	Wild Type	*coi1-16*
coi1-16 × *pen2-1*	F1	7	0	7	7	0
coi1-16 × *pen2-1*	F2	461	0	461	354	107

in *pen2* compared with *gl1* (Figure 1B). Postinvasion resistance against *P. infestans* does not require the same components as that against *Bgh*, since *P. infestans* does not show enhanced growth on *pen2 pad4 sag101* triple mutant plants (data not shown).

To identify additional genes or pathways required for nonhost resistance against *P. infestans*, mutants compromised in pathogen responses as well as salicylic acid, jasmonic acid (JA), and ethylene signaling were analyzed. Among these, *coi1-16* shows a clear necrosis phenotype upon drop inoculation of a *P. infestans* zoospore solution (Figure 1A). The phenotype observed for *coi1-16* is similar in extent to that observed on the *pen2* mutant, and the intensity of trypan blue staining in samples from *coi1-16* plants, similar to that of *pen2* plants, is greater than that observed for *gl1* plants (Figure 1B). These results suggested that JA signaling is required for nonhost resistance of *Arabidopsis* against *P. infestans*.

COI1 encodes an F-box protein that is required for the activation of JA-dependent responses. Upon increases in JA levels, COI1 is responsible for the specific degradation of jasmonate ZIM domain proteins, which act as negative regulators of JA-dependent gene expression by binding to the transcriptional activator MYC2/JIN1 (Chini et al., 2007; Thines et al., 2007). The mutant *coi1-1* is male sterile and shows increased susceptibility to pathogens and herbivory (Stintzi et al., 2001). The conditional mutant *coi1-16* was isolated from a mutant screen for methyl jasmonate–insensitive reporter gene expression. *coi1-16* is fertile at temperatures below 20°C; however, root growth inhibition and JA-responsive promoter activity are not restored at lower temperatures (Ellis and Turner, 2002).

To analyze putative additive effects, we crossed the *pen2-1* and *coi1-16* mutants. Surprisingly, no complementation of the *pen2-1* hypersensitive response (HR) phenotype occurred in the F1 generation (Table 1). Moreover, there was no segregation of the HR phenotype in the F2 generation (Table 1). This suggested either that *coi1-16* carries a defective *PEN2* gene, which would not be able to complement the *pen2-1* mutant, or that *pen2-1* contains a mutated *COI1* gene. However, the *pen2-1* mutant is not impaired in male fertility, as would be expected for a plant carrying a defective *COI1* gene (Xie et al., 1998). Moreover, we observed segregation of the *coi1* phenotype (i.e., male sterility) in the F2 generation at nonpermissive temperatures (Table 1). In contrast with *coi1-16*, *coi1-1* does not display the HR phenotype after infection with *P. infestans* (Figure 1).

Therefore, we cloned and sequenced the *PEN2* gene from the *coi1-16* mutant and found that it contains a G-to-A nucleotide exchange corresponding to position 449 of the cDNA. This mutation, subsequently called *pen2-4*, leads to an amino acid exchange from Gly to Asp at position 150. In addition, the G-to-A transition results in the loss of an *Aci*I restriction site (Figures 2A and 2B). To rule out the possibility that the wild type to *coi1-16* already carries this mutation, *gl1* plants were analyzed. The absence of the HR phenotype in *gl1* plants correlated with the presence of the *Aci*I restriction site, suggesting that *gl1* does not contain the *pen2-4* mutant allele (Figure 2B). Moreover, the *PEN2* fragment amplified from genomic DNA of *coi1-1* plants

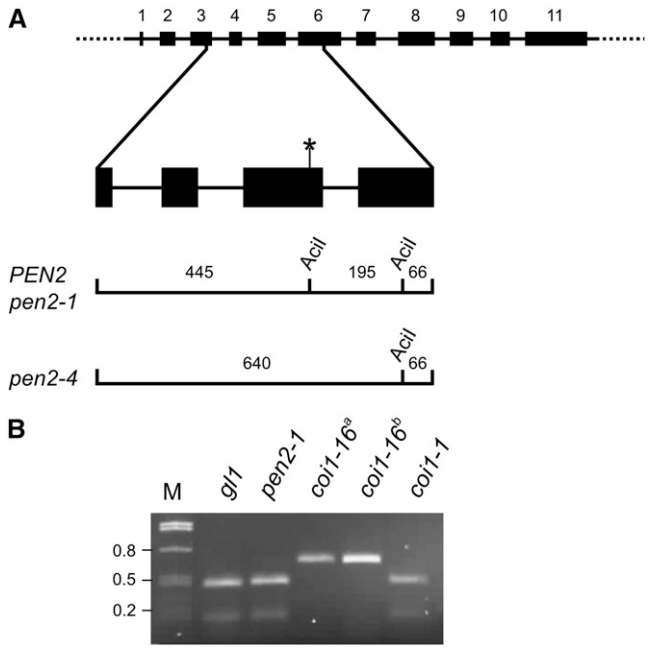

Figure 2. Structure of *PEN2* and *PEN2-4* Genes.

(A) Structure of the *PEN2* gene and location of the mutation in exon 5 in the *pen2-4* gene from *coi1-16* (marked by an asterisk). The G-to-A transition results in the elimination of an *Aci*I site. Below are shown the positions of the *Aci*I restriction sites in a 706-bp fragment amplified from *PEN2*, *pen2-1*, and *pen2-4*.
(B) Restriction of the 706-bp fragment from *PEN2* (amplified using the primers 5′-AAACGTTGCCGTTGATTTCT-3′ and 5′-CAGCAACACTAGCGCCATTA-3′) from *gl1*, *pen2-1*, *coi1-16*, and *coi1-1* plants with *Aci*I. The letters ''a'' and ''b'' indicate two different lines of *coi1-16*.

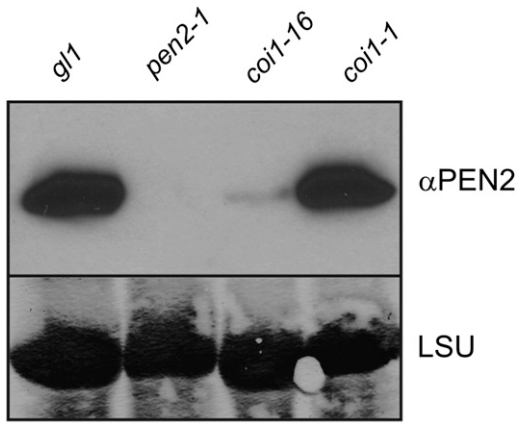

Figure 3. Determination of PEN2 Protein Levels in *gl1*, *pen2-1*, *coi1-16*, and *coi1-1* Plants.

Proteins were extracted from leaves of untreated plants and subjected to immunoblot analyses using PEN2 antiserum (αPEN2; Lipka et al., 2005). Filters were stained with amido black to visualize equal loading. LSU, large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase.

carried the *Aci*I restriction site and yielded restriction fragments of the same size as did *gl1* and *pen2-1* (Figure 2B).

To address possible causes of the *pen2* phenotype of *coi1-16*, PEN2-4 protein levels in unchallenged *coi1-16* plants were analyzed using PEN2 antiserum (Lipka et al., 2005). PEN2 protein is not detectable in *pen2-1* plants compared with significant levels of PEN2 in *gl1* plants (Figure 3). We found highly reduced amounts of PEN2-4 protein in *coi1-16* plants, whereas *coi1-1* contains PEN2 to similar levels as *gl1* (Figure 3). These results suggest that the protein encoded by the *pen2-4* gene of *coi1-16* is unstable, and the inability of PEN2-4 to accumulate to high levels would explain the *pen2* phenotype of *coi1-16*.

These analyses show that *coi1-16* carries a mutant allele of *PEN2*, which we have named *pen2-4*. In contrast with the knockout *pen2* alleles *pen2-1*, *pen2-2*, and *pen2-3* (Lipka et al., 2005), the *pen2-4* allele encodes a protein with highly reduced stability. PEN2 is required for penetration resistance against nonadapted pathogens and, importantly, is also involved in defense against host pathogens, such as *Plectosphaerella cucumerina* and *Pythium irregulare* (Lipka et al., 2005; Adie et al., 2007). Therefore, studies with pathogens using *coi1-16* should be evaluated carefully because the mutation in *pen2*, rather than the one in *coi1*, might be responsible for alterations in pathogen-related phenotypes.

Our findings emphasize the importance of performing multiple backcrosses to minimize the risk of phenotypic misinterpretation due to a second site mutation. However, for closely linked mutations, even repeated backcrossing cannot ensure successful separation. Therefore, complementation experiments or resequencing of ethyl methanesulfonate mutants are required to conclusively ascribe a mutant phenotype to the loss of a specific gene function.

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