

# The Arabidopsis Mutant *cev1* Has Constitutively Active Jasmonate and Ethylene Signal Pathways and Enhanced Resistance to Pathogens

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**Jasmonates (JAs) inhibit plant growth and induce plant defense responses. To define genes in the Arabidopsis JA signal pathway, we screened for mutants with constitutive expression of a luciferase reporter for the JA-responsive promoter from the vegetative storage protein gene *VSP1*. One mutant, named constitutive expression of *VSP1* (*cev1*), produced plants that were smaller than wild type, had stunted roots with long root hairs, accumulated anthocyanin, had constitutive expression of the defense-related genes *VSP1*, *VSP2*, *Thi2.1*, *PDF1.2*, and *CHI-B*, and had enhanced resistance to powdery mildew diseases. Genetic evidence indicated that the *cev1* phenotype required both *COI1*, an essential component of the JA signal pathway, and *ETR1*, which encodes the ethylene receptor. We conclude that *cev1* stimulates both the JA and the ethylene signal pathways and that *CEV1* regulates an early step in an Arabidopsis defense pathway.**

## INTRODUCTION

Jasmonates (JAs) are a family of cyclopentanone derivatives synthesized from linolenic acid via the octadecanoic pathway that regulate plant growth, plant defense responses, and aspects of development. They inhibit plant growth generally, but in addition, they promote diverse processes including fruit ripening, senescence, tuber formation, tendril coiling, pollen formation, and defense responses against pests and pathogens (reviewed in Creelman and Mullet, 1997). In Arabidopsis, JAs inhibit root elongation (Staswick et al., 1992) and are required for pollen development, anther dehiscence (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000), and defense against insects (McConn et al., 1997) and necrotrophic pathogens (Thomma et al., 1999). JAs also induce many genes, including those for vegetative storage proteins (VSPs; Benedetti et al., 1995), a thionin (*Thi2.1*; Epple et al., 1995; Vignutelli et al., 1998), and a plant defensin (*PDF1.2*; Penninckx et al., 1996).

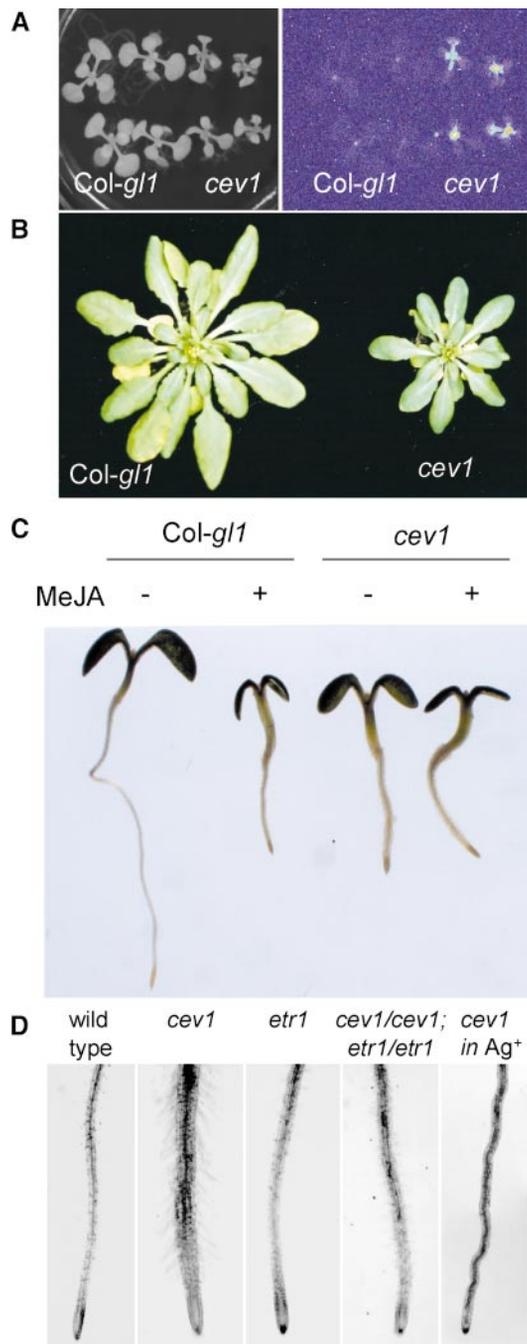
JAs interact with ethylene in the regulation of plant wound and defense responses. Both JA and ethylene are formed when plants are wounded or attacked by pests or pathogens (Creelman et al., 1992; O'Donnell et al., 1996; Kuc, 1997). Mutants defective in either the production or the perception of ethylene or JA have increased susceptibility to several pests and pathogens (McConn et al., 1997; Pieterse et al., 1998; Vijayan et al., 1998; Thomma et al., 1999), indi-

cating that both signal pathways are required for resistance. A possible mechanism for JA- and ethylene-dependent defenses is through their synergistic interaction in the induction of many defense-related genes, including *PR5*, *PDF1.2*, and basic chitinase (*CHI-B*), and of a hevein-like protein (Xu et al., 1994; Penninckx et al., 1998; Norman-Setterblad et al., 2000). However, expression of the JA-responsive *VSP* gene is increased in ethylene-insensitive mutants (Rojo et al., 1999; Norman-Setterblad et al., 2000), suggesting that the ethylene signal pathway represses the induction of *VSP*. JA and ethylene therefore cooperate synergistically in the activation of wound-related defense responses, but some JA responses are antagonized by ethylene.

Mutants in hormone signal transduction pathways are of two main types: mutants that cause loss of response to the hormone identify genes that normally function as positive regulators, and mutants that cause constitutive activation of the pathway identify negative regulators. Examples of positive regulators include the ethylene receptor *ETR1* (Chang et al., 1993), the *ABI1* and *ABI2* protein phosphatases, which are involved in abscisic acid response (Leung et al., 1994, 1997), and the F-box proteins *TIR1* and *COI1*, which are involved in auxin and JA responses (Ruegger et al., 1998; Xie et al., 1998). Examples of negative regulators include the Raf-like protein kinase *CTR1*, which suppresses ethylene-activated genes (Kieber et al., 1993), and *SPY1*, which suppresses the gibberellin pathway (Jacobsen et al., 1996).

The only reported mutants in the JA pathway have lost JA responsiveness and therefore are likely to define positive regulators (Staswick et al., 1992; Feys et al., 1994; Berger et

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**Figure 1.** Phenotypes of *cev1* Plants.

**(A)** Low-light images of *Col-g1* and *cev1* seedlings containing the *VSP1::luciferase* reporter transgene. Seedlings were grown for 12 days on MS (Murashige and Skoog, 1962) agar. The figure shows the positions of seedlings (left), and a low-light image of the same seedlings sprayed with luciferin solution reveals luciferase activity (right).

**(B)** Four-week-old *Col-g1* (left) and *cev1* (right) plants grown in soil.

**(C)** Seven-day-old *Col-g1* and *cev1* seedlings germinated on MS medium in the presence (+) and absence (-) of 20  $\mu$ M MeJA.

**(D)** Root phenotypes of 7-day-old seedlings of (left to right) *Col-g1*

al., 1996). We have developed a screen for mutations in negative regulators of the JA signal pathway based on constitutive expression of a JA-responsive promoter. For this purpose, we constructed transgenic lines containing the promoter of the JA-responsive gene *VSP1* (Utsugi et al., 1996) fused to the firefly luciferase gene as reporter. The transgenic seed were mutagenized and screened for plants that expressed the reporter construct in the absence of exogenously applied methyl jasmonate (MeJA). Here, we describe a mutant isolated in this screen that we have named constitutive expression of *VSP1* (*cev1*).

## RESULTS

### Isolation of *cev1*

*Arabidopsis* ecotype *Columbia-glabrous* (*Col-g1*) seed homozygous for the *VSP1::luciferase* reporter transgene were mutagenized with ethyl methanesulfonate.  $M_2$  seed were germinated individually in wells of 96-well microtiter plates. After 8 days, seedlings were sprayed with luciferin, and luminescence was measured. Under these conditions, the parental plants did not exhibit luciferase activity. Of the ~20,000 seedlings screened, 359 displayed high luciferase activity, and of these, 240 survived to produce seed. The 240  $M_3$  families were rescreened, and only seven had constitutively high luciferase activity. Test crosses made between these seven mutants gave F1 progeny with wild-type luciferase activity, and F2 populations that segregated for individuals with wild-type luciferase activity and with constitutive luciferase activity. This indicated that the mutants were recessive alleles at different loci that we named *cev1* to *cev7*. *cev1* was chosen for further characterization. It was backcrossed to the parental line, and 56 of 261 F2 progeny tested had constitutive luciferase activity (Figure 1A), indicating that *cev1* segregated as a single recessive allele ( $P > 0.1$ ). *cev1* plants were smaller and were darker green (Figure 1B), and their roots were shorter with longer root hairs than parental plants (Figures 1C and 1D). The *cev1* plants also accumulated purple anthocyanins in their leaves. These morphological traits are characteristic of plants treated with JA (Feys et al., 1994).

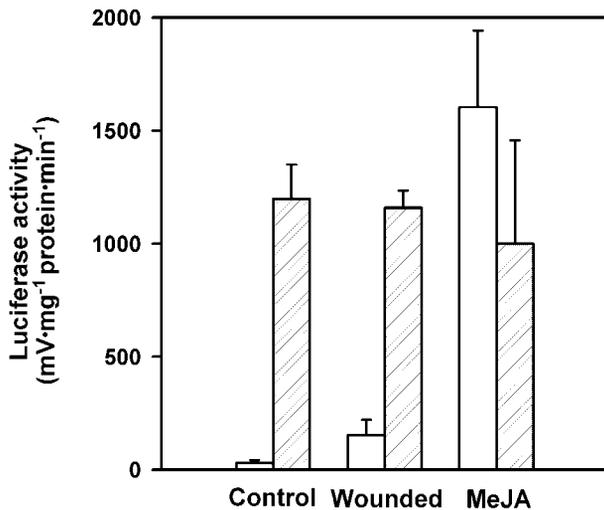
*cev1* was crossed to *Arabidopsis* ecotype *Landsberg erecta*, and the genotypes of *cev1* mutants in the F2 progeny were analyzed with cleaved-amplified polymorphic sequence markers and with simple sequence-linked polymorphic markers (Bell and Ecker, 1994). *cev1* was mapped

and the mutants *cev1*, *etr1*, the double mutant *cev1/cev1; etr1/etr1*, and *cev1* grown in 17  $\mu$ M  $Ag^+$ .

genetically to an 11-cM interval at the top of chromosome 5 between nga225 and nga249. This region contains no experimentally characterized gene for response to JA, ethylene, salicylic acid, or for spontaneous lesions. *cev1* therefore defines a recessive mutation responsible for the complex mutant phenotype described in Figure 1.

**Luciferase Activity Reflects VSP1 Transcript Abundance in Wild-Type and *cev1* Plants Containing the VSP::Luciferase Transgene**

The *VSP::luciferase* transgene faithfully reported *VSP* transcription, as monitored by RNA gel blot analysis. In untreated wild-type seedlings, the transgene was not active (Figure 2), and *VSP* transcripts could not be detected (Figure 3). In seedlings that were wounded or treated with MeJA, however, the transgene was active and *VSP* transcripts were abundant (Figures 2 and 3), in agreement with previous reports (Staswick et al., 1992; Benedetti et al., 1995). Significantly, in untreated *cev1* plants, luciferase activity was high, and *VSP* transcripts were abundant (Figures 2 and 3), which confirmed that the constitutive luciferase activity was not due to a mutation in the transgene. The *VSP1* and *VSP2* genes have 87% DNA sequence identity (Utsugi et al., 1996), and the probe used in this study (Benedetti et al., 1995) likely detects both genes.



**Figure 2.** *VSP1* Promoter Activity in Col-*g/1* and *cev1* Plants.

Col-*g/1* and *cev1* plants containing the *VSP1::luciferase* transgene were harvested and assayed for luciferase activity 12 days after germination. Treated seedlings were wounded with blunt forceps 16 hr before harvest or were transferred to MS plates containing 20 μM MeJA for 48 hr before harvest. Seedlings then were assayed individually for luciferase activity in vitro. Error bars show standard deviations of five replicates for Col-*g/1* (white bars) and *cev1* (gray bars).

**cev1 Seedlings Expressed PDF1.2 and CHI-B Constitutively**

RNA gel blot analysis of Col-*g/1* seedlings indicated that *PDF1.2* was induced by MeJA but not by wounding and that *CHI-B* was induced by wounding but not by MeJA (Figure 3). In mature Arabidopsis plants, however, wounding does not induce *CHI-B* (Samac et al., 1990). Untreated *cev1* seedlings contained substantially more *PDF1.2* mRNA than Col-*g/1* plants treated with MeJA. Surprisingly, wounding or treatment with MeJA reduced the amount of *PDF1.2* mRNA in *cev1* seedlings (Figure 3). Untreated *cev1* seedlings also expressed *CHI-B* constitutively at a level slightly greater than in wild-type plants; however, unlike *PDF1.2*, this gene was not greatly decreased in plants that were wounded or treated with MeJA.

**The VSP1, VSP2, and Thi2.1 Promoters Were Activated Constitutively in the Lamina Tissues of cev1 Plants**

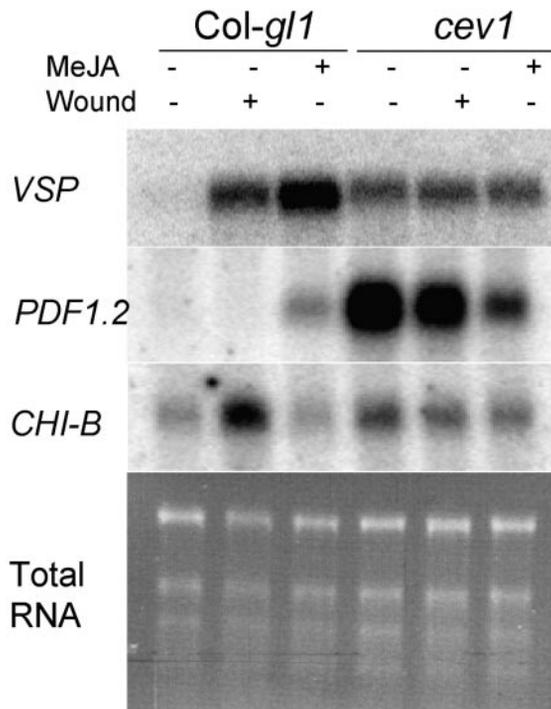
MeJA induces expression of *VSP1*, *VSP2*, and *Thi2.1* in Arabidopsis (Benedetti et al., 1995; Epple et al., 1995; Vignutelli et al., 1998). To investigate the effect of *cev1* on the pattern of expression of these genes, we fused their promoters to β-glucuronidase (*GUS*) as a reporter, introduced the constructs into Col-*g/1* plants by Agrobacterium-mediated transformation, and crossed the transgenes into the *cev1* mutant. In untreated Col-*g/1* plants, the *VSP1::GUS*, *VSP2::GUS*, and *Thi2.1::GUS* transgenes were expressed only in the apical meristem and the midveins, and treatment with MeJA induced expression in the leaf lamina (Figure 4). In *cev1* plants, the *GUS* transgenes were expressed constitutively in the leaf lamina but not obviously so in the midveins, and their expression was not visibly altered by treatment with MeJA (Figure 4).

**cev1 Has Stunted Roots with Reduced Responsiveness to MeJA**

The roots of 10-day-old Col-*g/1* seedlings were 33.6 ± 8.2 mm long and were reduced by 74% to 8.9 ± 1.2 mm by growth on MeJA, confirming previous reports (Staswick et al., 1992; Feys et al., 1994). In contrast, the roots of *cev1* seedlings were 8.5 ± 1.5 mm long and were reduced by 18% to 7.0 ± 1.0 mm by growth on MeJA (Figure 2C). Thus, *cev1* roots were stunted and had reduced sensitivity to inhibition by MeJA.

**Part of the cev1 Mutant Phenotype Is Dependent on COI1**

*COI1* is required for Arabidopsis responses to JAs (Feys et al., 1994). Therefore, we examined whether *COI1* was required for



**Figure 3.** RNA Gel Blot Analysis of Col-*gl1* and *cev1* RNA Extracted from 12-Day-Old Seedlings.

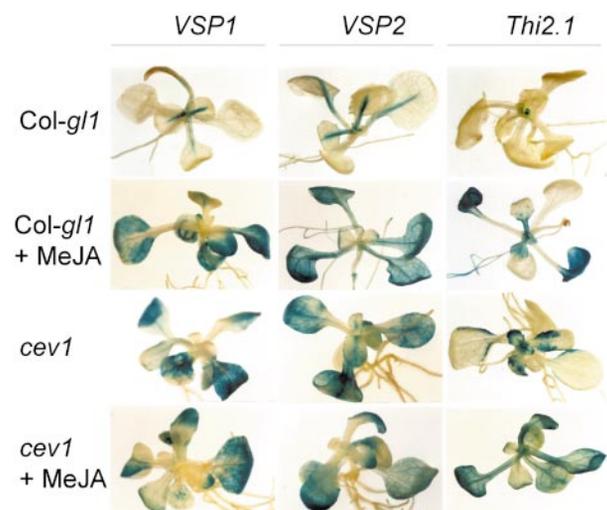
Treated seedlings had been wounded with blunt forceps 16 hr before harvest or were transferred to MS plates containing 20  $\mu$ M MeJA for 48 hr before harvest. Total RNA (0.5  $\mu$ g) was electrophoresed, blotted, and probed with a  $^{32}$ P-labeled DNA fragment of the *VSP* gene, the *PDF1.2* gene, or the *CHI-B* gene.

the *cev1* phenotype by analysis of the double mutant *cev1/cev1;coi1-1/coi1-1*. Significantly, the *cev1/cev1;coi1-1/coi1-1* double mutants did not accumulate anthocyanin as did *cev1* plants (Figure 5A), nor did they express luciferase in the presence or absence of MeJA (Figure 5B). Like *cev1* plants, however, the double mutants had short roots with prolific root hairs. Therefore, *COI1* was required for *cev1*-dependent anthocyanin formation and for *VSP* activity but not for *cev1*-dependent short roots and prolific long root hairs.

#### Part of the *cev1* Mutant Phenotype Is Dependent on *ETR1*

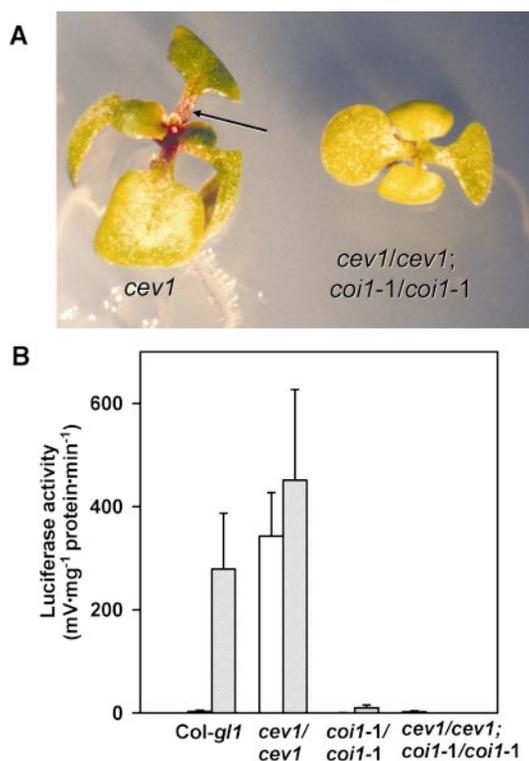
The *cev1* mutant had thickened roots with prolific root hairs and a thickened hypocotyl (Figure 1). Similar features appear in *Arabidopsis* seedlings exposed to ethylene during germination. To determine whether ethylene contributed to this phenotype in *cev1*, we used *etr1/etr1* plants, which are insensitive to ethylene (Chang et al., 1993), to make the

double mutant *cev1/cev1;etr1/etr1*. The double mutants were similar to *cev1* plants in that they were slightly stunted with small dark-green leaves, but they differed from *cev1* plants in that they lacked prolific root hairs, had thinner roots (Figure 1D) and thinner hypocotyls, and had two to five times greater constitutive luciferase activity from the *VSP1::luciferase* transgene. *cev1* seedlings germinated on 17  $\mu$ M  $Ag^+$ , which antagonizes the perception of ethylene (Chang et al., 1993), had thin roots, and root hairs were absent (Figure 1D). To test the effect of perturbations to the ethylene signal pathway alone on expression of the *VSP::luciferase* transgene, we crossed this construct into the *etr1/etr1* and *ctr1/ctr1* (which has constitutive ethylene responses; Kieber et al., 1993) mutant backgrounds. Ten-day-old transgenic seedlings were transferred to medium containing 20  $\mu$ M MeJA for 2 days, and luciferase activity was measured. In the wild type, *etr1/etr1*, and *ctr1/ctr1* backgrounds, luciferase activity was  $90 \pm 24$ ,  $1097 \pm 210$ , and  $5 \pm 3$  mV  $\cdot$  mg $^{-1}$  protein  $\cdot$  min $^{-1}$ , respectively. This indicated that activation of the ethylene signal pathway suppressed expression of the *VSP::luciferase* transgene. *Arabidopsis* seedlings germinated in the dark in the presence of ethylene have stunted roots with prolific root hairs, a thickened hypocotyl, and an exaggerated apical hook. *cev1* seedlings germinated in the dark had stunted roots with prolific root hairs and a thickened hypocotyl, but they lacked an apical hook. We observed that 20  $\mu$ M JA in the growth medium (Murashige and Skoog [MS] agar) suppressed formation of the exaggerated apical hook in dark-grown *ctr1* seedlings, and in dark-grown wild-type seedlings germinated in the presence of 5



**Figure 4.** GUS Expression Driven by the *VSP1*, *VSP2*, and *Thi2.1* Promoters in Col-*gl1* and *cev1* Seedlings.

Seedlings were grown for 12 days on MS medium or for 10 days on MS medium and transferred to medium containing 20  $\mu$ M MeJA for 2 days and then stained for GUS activity.



**Figure 5.** Effect of the *coi1-1* and *etr1* Mutations on the *cev1* Phenotype.

**(A)** Ten-day-old seedlings of *cev1/cev1* (left) showing anthocyanin in petioles (arrow) and the *cev1/cev1; coi1-1/coi1-1* (right) double mutant.

**(B)** *VSP1* promoter activity in wild-type seedlings, the *cev1/cev1* and *coi1-1/coi1-1* mutants, and the *cev1/cev1; coi1-1/coi1-1* double mutant. Ten-day-old seedlings containing the *VSP1::luciferase* transgene in these mutant backgrounds were transferred to MS medium or MS supplemented with 20 μM MeJA for 2 days. Seedlings were extracted individually, and luciferase activity was measured. Error bars show standard deviations of 10 replicates without MeJA (white bars) and with MeJA (gray bars).

parts per million (ppm) ethylene, but not in dark-grown *coi1-1* seedlings germinated in the presence of 5 ppm ethylene. Apparently, JA suppressed the ethylene-induced apical hook in a *COI1*-dependent manner.

**cev1 Plants Exhibit Increased Resistance to Three Powdery Mildew Pathogens**

*Col-g/1* plants are susceptible to several species of powdery mildew, including *Erysiphe cichoracearum* UCSC1 (Xiao et al., 1997), *Erysiphe orontii* MGH, and *Oidium lycopersicum* Oxford. We examined whether the *cev1* mutation affected colonization by these pathogens. Seven days after inoculation, hyphal growth and conidiophore development on *Col-*

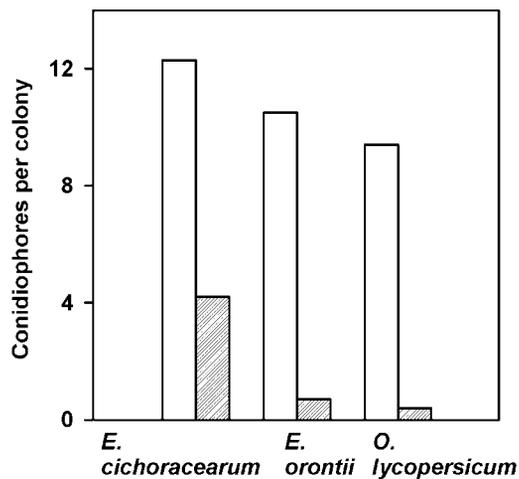
*gl1* plants were significantly greater than on *cev1* plants (Figure 6). Fungal colonies on the *cev1* plants were smaller and fewer in number than those on *Col-g/1* plants.

**DISCUSSION**

JAs regulate both plant development and defense. Signaling pathways for other plant hormones, including ethylene, auxin, and abscisic acid, have been defined through the characterization of mutants, some of which reveal negative regulators. However, only four JA response mutants have been isolated, on the basis of JA-insensitive root growth, and these are presumed to define positive regulators (Creelman and Mullet, 1997). We sought mutants with constitutive JA responses that, we reasoned, would define negative regulators of the JA pathway, and isolated *cev1*.

The *cev1* mutant was identified by the constitutive expression of a reporter gene controlled by the JA-responsive *VSP1* promoter. It also exhibited other phenotypes: the plants were stunted, had short roots with an excess of root hairs, accumulated anthocyanins, and constitutively expressed the JA-regulated genes *VSP1*, *VSP2*, and *Thi2.1* (Feys et al., 1994; Bohlmann et al., 1998) and the ethylene- and JA-regulated genes *PDF1.2* (Penninckx et al., 1998) and *CHI-B* (Norman-Setterblad et al., 2000). *cev1* also altered the tissue-specific pattern of expression of *VSP1*, *VSP2*, and *Thi2.1* in Arabidopsis. In soybean, *VSPs* are localized in the vacuoles of paraveinal mesophyll and bundle sheath cells of leaves (Franceschi et al., 1983). Similarly, *Col-g/1* plants expressed *GUS* gene fusions to the *VSP1*, *VSP2*, and *Thi2.1* promoters only in the leaf midveins and the apical meristem region. However, in *Col-g/1* plants treated with MeJA, these promoters were expressed at high levels in the leaf lamina. In untreated *cev1* plants, the same *GUS* transgenes also were expressed at high levels in the leaf lamina, and their activity and pattern of expression were not altered detectably by treatment with MeJA. Therefore, the activity of the *VSP1*, *VSP2*, and *Thi2.1* promoters in the *cev1* mutant resembles their activity in *Col-g/1* plants treated with MeJA. This finding indicated that the *cev1* mutant had a constitutively active JA signal pathway, and we investigated whether this could account for its other phenotypes.

*COI1* is required for Arabidopsis responses to JA, including increased expression of *VSP*, accumulation of anthocyanin, and inhibition of root growth (Feys et al., 1994; Xie et al., 1998). We tested whether these characteristics in *cev1* also required *COI1*. The *cev1/cev1; coi1-1/coi1-1* double mutant had low activity of the *VSP1* promoter and reduced anthocyanin formation (Figure 5), indicating that *COI1* was required for the constitutive expression of these phenotypes. *cev1* therefore regulates a step before *COI1* in the JA pathway. However, the double mutant had short roots, indicating that *COI1* was not required for this phenotype in *cev1*. We conclude that there is a constitutively active JA signal



**Figure 6.** Resistance of *cev1* to Powdery Mildews.

Four-week-old Col-*gl1* and *cev1* plants were inoculated with *E. cichoracearum* UCSC1, *E. orontii* MGH, or *O. lycopersicum* Oxford. After 7 days (for *E. cichoracearum*) or 5 days (for *E. orontii* and *O. lycopersicum*), leaves were fixed and cleared in a lactophenol solution and stained with trypan blue, and the number of conidiophores per colony was recorded. Numbers are the means from 50 colonies for Col-*gl1* (white bars) and *cev1* (gray bars). An unpaired *t* test analysis indicated that for each fungus, differences between the means for the Col-*gl1* and *cev1* plants were statistically significant ( $P < 0.001$ ).

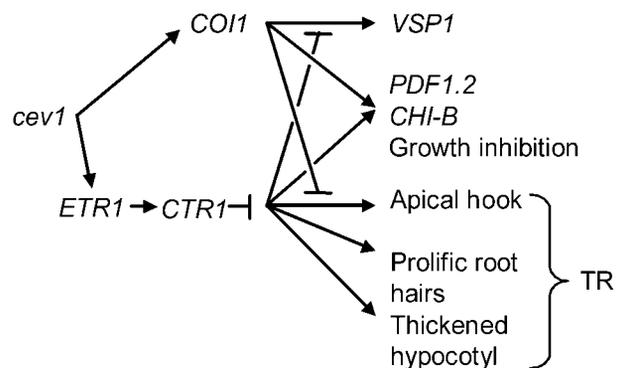
pathway in *cev1* that causes the enhanced formation of anthocyanin and the constitutive expression of *VSP*. However, JA alone cannot account for the inhibited root growth in this mutant.

Genetic evidence indicated that the ethylene signal pathway was constitutively expressed in *cev1*. *cev1*-dependent prolific root hairs and thickened roots and hypocotyls were suppressed by *etr1* in the *cev1/cev1;etr1/etr1* double mutant, revealing that the ethylene signal pathway was activated by the *cev1* mutation. Roots of the double mutant were shorter than those of wild-type plants, however, indicating that ethylene alone cannot account for this phenotype of *cev1*. It is possible that JA and ethylene act independently to cause short roots in *cev1*. The enhanced luciferase activity from the *VSP1::luciferase* transgene in the *cev1/cev1;etr1/etr1* double mutant compared with that of the *cev1* mutant indicated that an ethylene-dependent signal suppressed the induction of the *VSP* promoter by JA. This finding was confirmed by our observation that MeJA-induced activity of the *VSP::luciferase* transgene in wild-type plants was greater in the *etr1* mutant background and suppressed in the *ctr1* background, and it supports a previous report that ethylene antagonizes the JA induction of *VSP* (Rojo et al., 1999). Seedlings germinated in the dark in the presence of ethylene display the triple-response that includes an exaggerated apical hook (Kieber et al., 1993). We

observed that *cev1* displayed part of the triple response but lacked the apical hook, and that JA suppressed the ethylene-induced apical hook in a *COI1*-dependent manner. This indicated that the JA signal pathway also suppresses an ethylene-induced response. Previous studies have shown that JA and ethylene jointly regulate transcription of 36 of 2375 genes tested (Schenk et al., 2000). Our results indicate that these signal pathways also have some mutually antagonistic effects.

Expression of defense-related genes was consistent with the activation of the ethylene and JA pathways in *cev1*. JA and ethylene interact synergistically to induce *PDF1.2* (Penninckx et al., 1998) and *CHI-B* (Norman-Setterblad et al., 2000), which could account for the observed constitutive expression of *PDF1.2* and *CHI-B* in untreated *cev1* plants (Figure 3). We conclude that constitutive activation of the JA and ethylene signal pathways in *cev1* plants is sufficient to account for the phenotype of these plants, and for the observed pattern of gene induction, and we summarize our findings in a model in Figure 7.

JAs are required, alone or in combination with ethylene, for defense against insects and necrotrophic pathogens (McConn et al., 1997; Vijayan et al., 1998; Thomma et al., 2000). We demonstrate that *cev1* plants have enhanced resistance to the biotrophic, obligately pathogenic powdery mildew pathogens *E. cichoracearum* UCSC1, *E. orontii* MGH, and *O. lycopersicum* Oxford, to which Col-*gl1* plants are normally susceptible (Xiao et al., 1997). Many antifungal agents are synthesized in response to JA, alone or in combination with ethylene, including thionins (Epple et al., 1995)



**Figure 7.** Model for the *cev1* Mutant Phenotype.

The *cev1* mutation activates the JA and ethylene signal pathways, possibly by inducing the synthesis of JA and ethylene, or by regulating *COI1* and *ETR1* directly. The model accounts for the observed *cev1* mutant phenotype through independent effects of the activated JA and ethylene signal pathways, and through cross-talk between these pathways causing activation and suppression of different responses. TR indicates triple response, arrows indicate activation, and bars indicate suppression.

and defensins (Penninckx et al., 1998). Although genes for these proteins are not induced by powdery mildew infection (Reuber et al., 1998), they are constitutively expressed in *cev1* plants and therefore could contribute to the observed resistance.

The *cev1* mutation causes constitutive activation of the JA and ethylene signal pathways and *CEV1*, therefore, may function at an early step in these pathways, either as regulator of the levels of JA and ethylene or as regulator of the flux through the JA and ethylene signaling pathways. Its characterization should increase our understanding of early steps in plant wound and defense responses.

## METHODS

### Construction of Transgenic Lines

The *VSP1* promoter was isolated from a  $\lambda$ FIX library of *Arabidopsis thaliana* Landsberg genomic DNA. Polymerase chain reaction (PCR) was used to amplify a 1.5-kb DNA fragment from the 5' untranslated region of *VSP1* using the primers 5'-CTCTCTAGAGGGCGAACTCGAGCTCC-3' and 5'-AGGATTTTCATAAGCTTTTGTATGGT-3'. The luciferase gene from pGEMluc (Promega) was inserted into a pBI101 vector (Clontech, Palo Alto, CA) in place of the  $\beta$ -glucuronidase (*GUS*) gene using the BamHI and SacI restriction endonuclease sites. The *VSP1* upstream region was digested with HindIII and inserted 5' to the luciferase gene. This construct was introduced via electroporation into *Agrobacterium tumefaciens* GV3101 pMP90, which in turn was used to transform Columbia-*glabrous* (*Col-g1*) plants (Bechtold et al., 1993). A 1.5-kb DNA fragment containing the 5' untranslated region of *VSP2* was isolated via PCR using the primers 5'-CTTCTTAATTAAGCTTATCTTC-3' and 5'-GAGGATTTT-CATGGATCCTAATGG-3'. The 5' untranslated regions of *VSP1* and *VSP2* also were cloned into the HindIII and the HindIII and BamHI sites of pBI101, respectively. These constructs were introduced into *Col-g1* by *Agrobacterium*-mediated transformation to create the *VSP1::GUS* and *VSP2::GUS* lines. The *Thi2.1::GUS* line has been described previously (Xie et al., 1998).

Transgenic lines were analyzed by DNA gel blotting to determine the number of copies of the transgenes and by measurement of luciferase activity to determine reporter gene activity. Lines that contained a single copy of the transgene and exhibited a high level of reporter gene expression were chosen for use in this study.

### Luciferase and GUS Assays

*A. thaliana Col-g1* seed homozygous for the *VSP1::luciferase* transgene were mutagenized by immersion in 0.3% ethyl methane-sulfonate for 15 hr,  $M_1$  plants were raised, and  $M_2$  seed were germinated individually in the wells of 96-well microtiter plates containing 150  $\mu$ L of Murashige and Skoog (1962) (MS) agar. Eight-day-old seedlings were sprayed with 1 mM luciferin in 0.01% Triton X-100, and luminescence was measured in an EG&G Wallac Victor Multilabel Counter (Perkin-Elmer, Boston, MA). In vitro luciferase assays were performed with an LKB Wallac 1251 Luminometer (LKB, Stockholm, Sweden) using the method of Mudge et al. (1996) and lu-

ciferase assay reagent (Promega). Low-light images of plants were taken with a liquid nitrogen-cooled charge-coupled device camera (Princeton Instruments, Trenton, NJ) using Metamorph imaging software (Universal Imaging, West Chester, PA). Protein concentration was measured by the Bradford (1976) method. GUS staining was according to Xie et al. (1998).

### Isolation of Double Mutants

To isolate double mutants, we crossed *cev1* plants homozygous for the *VSP1::luciferase* transgene to *coi1-1* plants also homozygous for the *VSP1::luciferase* transgene, and F2 progeny plants were scored for male sterility (a marker for *coi1-1*) and for short roots (a marker for *cev1*). Of 787 F2 plants examined, 455 had long roots (>15 mm) and were fertile, 153 had long roots and were male sterile, 132 had short roots (<12 mm) and were fertile, and 47 had short roots and were male sterile, conforming to an expected ratio of 9:3:3:1 ( $P > 0.5$ ) for two independently segregating genes. The presumed double mutants (male sterile and short roots) were confirmed to be *coi1-1/coi1-1* by a cleaved-amplified polymorphic sequence marker for the *coi1-1* allele (Xie et al., 1998) and were confirmed to be *cev1/cev1* by backcrossing to *cev1* plants, which gave F1 progeny that were fertile and had short roots.

Approximately one-quarter of the F2 population from the cross *cev1/cev1* to *etr1/etr1* had small dark-green leaves (a marker for *cev1*), and the roots of approximately three-quarters of these lacked prolific root hairs (a marker for *etr1*). This indicated that *etr1* abolished the root hair phenotype of *cev1* plants and was consistent with *etr1* segregating as a dominant mutation. Test backcrosses of these candidate double mutants to their *cev1* and *etr1* parents identified lines with the double mutation *cev1/cev1;etr1/etr1*.

### RNA Gel Blot Analysis

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Crawley, UK) from 12-day-old seedlings that had been wounded or treated with methyl jasmonate (MeJA), and *VSP* mRNA was determined by RNA gel blot analysis. RNA gel blotting was performed as described previously (Benedetti et al., 1995). Blots were probed with random primed  $^{32}$ P-labeled DNA fragments (MegaPrime kit; Amersham). The *VSP* probe has been described previously (Benedetti et al., 1995). The *PDF1.2* probe was amplified from genomic DNA by PCR using the primers 5'-GCATGTCGATAGTCCATTACGT-3' and 5'-ACATGGGACGTAACAGATACAC-3' (Penninckx et al., 1998). The *CHI-B* probe was amplified from genomic DNA by PCR using the primers 5'-GATGGGCTACAGCACCAGAC-3' and 5'-GTAACAATC-AAGATTACCACCAGG-3' (Samac et al., 1990).

### Conidiophore Determinations

Plants were grown for 4 weeks under short-day conditions, and then ~10 plants were inoculated with powdery mildew as described previously (Xiao et al., 1997). After 7 days (for *Erysiphe cichoracearum*) or 5 days (for *Erysiphe orontii* and *Oidium lycopersicum*), leaves were fixed and cleared in a lactophenol solution and stained with trypan blue (Reuber et al., 1998). The number of conidiophores per colony then was recorded.

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**The Arabidopsis Mutant *cev1* Has Constitutively Active Jasmonate and Ethylene Signal Pathways and Enhanced Resistance to Pathogens**

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