The Arabidopsis Mutant *cev1* Links Cell Wall Signaling to Jasmonate and Ethylene Responses

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Biotic and abiotic stresses stimulate the synthesis of jasmonates and ethylene, which, in turn, induce the expression of genes involved in stress response and enhance defense responses. The *cev1* mutant has constitutive expression of stress response genes and has enhanced resistance to fungal pathogens. Here, we show that *cev1* plants have increased production of jasmonate and ethylene and that its phenotype is suppressed by mutations that interrupt jasmonate and ethylene signaling. Genetic mapping, complementation analysis, and sequence analysis revealed that *CEV1* is the cellulose synthase *CeSA3*. *CEV1* was expressed predominantly in root tissues, and *cev1* roots contained less cellulose than wild-type roots. Significantly, the *cev1* mutant phenotype could be reproduced by treating wild-type plants with cellulose biosynthesis inhibitors, and the cellulose synthase mutant *rsw1* also had constitutive expression of *VSP*. We propose that the cell wall can signal stress responses in plants.

INTRODUCTION

Jasmonates (JAs) are a family of cyclopentanone compounds synthesized from linolenic acid via the octadecanoic pathway. They inhibit plant growth generally but also promote diverse processes, including fruit ripening, senescence, tuber formation, tendril coiling, pollen formation, and defense responses against insect pests and pathogens (for review, see Creelman and Mullet, 1997). In plants undergoing normal development, JAs are produced predominantly in germinating seeds and floral tissues (Creelman and Mullet, 1995). The fundamental role of JAs in Arabidopsis fertility and defense was revealed by mutants unable to synthesize or respond to these compounds, which were male sterile and had enhanced susceptibility to pests and pathogens (Staswick et al., 1992; Feys et al., 1994; McConn and Browse, 1996).

JA synthesis is induced by a number of biotic and abiotic stresses, including wounding, water deficit, and pathogen attack (Creelman et al., 1992; Creelman and Mullet, 1995; Penninckx et al., 1998). Elicitors of JA responses include chitin, oligogalacturonides, and cell wall–degrading enzymes (Rojo et al., 1999; Norman-Setterblad et al., 2000). These treatments stimulate the expression of genes such as those encoding vegetative storage proteins (*VSP*s; Benedetti et al., 1995), thionin (*THI2.1*; Epple et al., 1995), and plant defensin (*PDF1.2*; Penninckx et al., 1998), which are expressed characteristically in plants during response to stress and pathogens.

To identify genes involved in JA signaling, we joined the upstream region of the JA-responsive gene *VSP1* to the open reading frame of luciferase (*LUC*) as a reporter and isolated mutants that constitutively expressed LUC, one of which was designated *cev1* (Ellis and Turner, 2001). *cev1* plants have stunted root growth, constitutively express the JA- and ethylene-induced genes *THI2.1* and *PDF1.2* and the basic chitinase gene *CHI-B*, and have enhanced resistance to pests and pathogens (Ellis and Turner, 2001). Here, we further characterize *cev1* and show that the mutation lies within the cellulose synthase gene *CeSA3*, also named *Ath-B* (Arioli et al., 1998) and *IXR1* (Scheible et al., 2001). We present evidence that the inhibition of cellulose synthesis activates JA- and ethylene-dependent stress responses.

RESULTS

cev1 Constitutively Produces JA and Ethylene

cev1 plants have constitutive expression of the JA-responsive genes *VSP* and *THI2.1* and the JA- and ethylene-responsive genes *PDF1.2* and *CHI-B* (Ellis and Turner, 2001). Therefore, we analyzed JA and ethylene production in *cev1* plants. *cev1* plants contained >1500 pmol/g fresh weight, and wild-type plants contained <25 pmol/g fresh weight.

The JA biosynthetic intermediate 12-oxo-phytodienoic acid and the 16:3 fatty acid derivative dinor-12-oxo-phytodienoic acid also were present in higher amounts in *cev1* plants than in wild-type plants (Figure 1). Dark-grown and light-grown *cev1* seedlings produced approximately twice as much ethylene as wild-type seedlings (Table 1).

The Mutant Phenotype of *cev1* Plants Is Partially Suppressed by *etr1* and *coi1*

Previously, we had shown that *VSP* expression and anthocyanin accumulation in *cev1* plants requires *COI1* and that prolific root hair formation requires *ETR1* (Ellis and Turner, 2001). Two other phenotypes that characterize *cev1* plants are shortened hypocotyls in dark-grown seedlings (Figure 2) and shortened roots in light-grown seedlings (Figure 3). In Arabidopsis, ethylene inhibits hypocotyl elongation in dark-grown seedlings, and this response requires *ETR1*, and JA inhibits root growth, which requires *COI1*. Therefore, we examined the contribution of ethylene and JA to these *cev1* mutant phenotypes in the double mutants *cev1 etr1-3* and *cev1 coi1-1* and in the triple mutant *cev1 etr1-3 coi1-1*.

Hypocotyls of dark-grown double mutant *cev1 etr1-3* seedlings were longer than those of *cev1* and not significantly different from those of wild-type and *etr1-3* seedlings (Figure 2). This finding indicates that the shortened hypocotyls of dark-grown *cev1* seedlings can be accounted for by the action of ethylene. Roots of light-grown seedlings of the double mutants *cev1 etr1-3* and *cev1 coi1-1* were signifi-
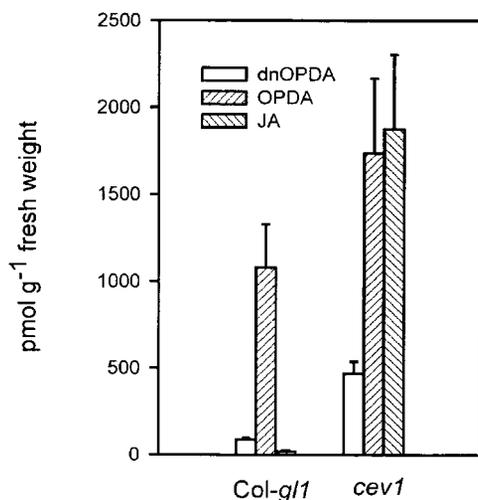
Table 1. *cev1* Plants Overproduce Ethylene

	Col-*gl1*	*cev1*
Dark-grown seedlings	2.1 ± 0.6 nL	4.2 ± 0.3 nL
Light-grown seedlings	22 ± 9 nL g^{-1}	43 ± 6 nL g^{-1}

Ethylene produced by $\sim$500 4-day-old dark-grown seedlings during 3 days of growth or by 200 mg of 10-day-old light-grown seedlings over 24 hours was determined (see also Methods).
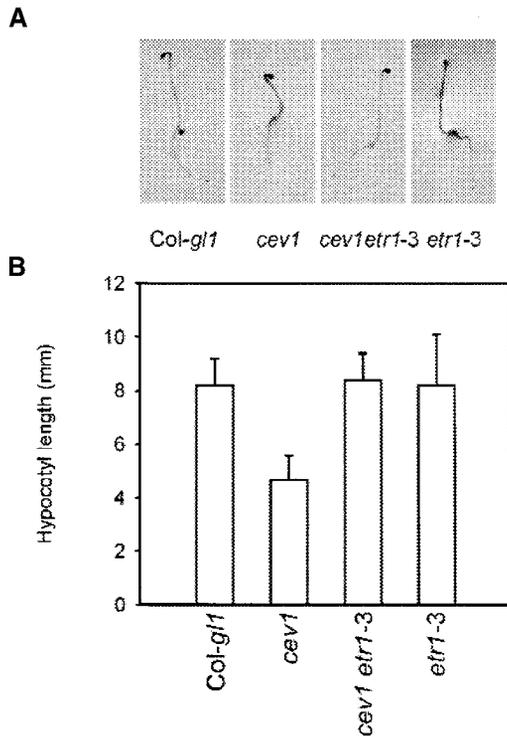
cantly longer than those of the *cev1* mutant, and roots of the triple mutant *cev1 etr1-3 coi1-1* were significantly longer than those of the double mutants but only half the length of those of wild-type seedlings (Figure 3). This finding indicates that the shortened roots of *cev1* plants can be accounted for in part by the action of JA and ethylene.

Positional Cloning of *CEV1*

Cleaved amplified polymorphic sequence and simple sequence length polymorphism markers were used to map the *cev1* mutation to an $\sim$20-kb region on top of chromosome 5 near nga225 and located on the transformation-competent artificial chromosome (TAC) clone K2A11 (Figure 4A). The K2A11 insert was introduced into the *cev1* mutant by *Agrobacterium tumefaciens*–mediated transformation. Plants containing the transgene were selected, and expression of the LUC reporter for *VSP* and root length was measured. Transgenic plants had wild-type activity of the LUC reporter, and root length was not different from that of wild-type seedlings (Table 2). Significantly, the progeny of most self-pollinated primary transformants segregated to give progeny with the *cev1* mutant phenotype and wild-type plants, indicating that the *cev1* mutation had been complemented by sequences in K2A11.

Subclones of K2A11 in the SLJ75515 binary vector were introduced into *cev1* plants by Agrobacterium-mediated transformation. Of these, only clone SB6.6 complemented the *cev1* mutation (Figure 4B). SB6.6 is a 6.6-kb SpeI-BstBI fragment containing one full open reading frame for the putative cellulose synthase, also designated *CeSA3* (Delmer, 1999), and a partial open reading frame from a gene bearing homology with receptor kinase genes. However, clone Xba4 (Figure 4A) containing the entire receptor kinase-like gene failed to complement the *cev1* phenotype. Furthermore, sequencing of *cev1* DNA revealed no mutations in the receptor kinase-like open reading frame.

cev1 plants containing the SB6.6 transgene had wild-type levels of expression of the *VSP1::LUC* reporter gene, their roots were of similar length to those of wild-type seedlings (Table 2), and they produced rosettes similar to those of

Figure 1. The *cev1* Mutant Constitutively Produces JA.

Four-week-old plants were assayed for JA, its intermediate 12-oxo-phytodienoic acid (OPDA), and the 16:3 fatty acid derivative dinor-12-oxo-phytodienoic acid (dnOPDA).

A

Col-*gl1*   *cev1*   *cev1etr1-3 etr1-3*

B

Figure 2. The Ethylene Receptor *ETR1* Is Required for Shortened Hypocotyls in the *cev1* Mutant.

(A) Seedlings grown in the dark for 4 days on Murashige and Skoog (1962) (MS) agar.
(B) Lengths of hypocotyls of dark-grown seedlings. T-bars indicate SD values for 10 seedlings.

wild-type plants (Figure 4B). The sequence of the *CeSA3* gene from *cev1* plants revealed a single C-to-T transition in the coding region of the gene that alters the predicted amino acid sequence G617E. Characterization of the cDNA from this gene indicated that the transcript extended from 16,008 to 10,782 of the TAC clone K2A11 and contained 14 introns, including one in the predicted 5′ untranslated region, as noted previously (Scheible et al., 2001). The *cev1* mutation G617E is in a cytoplasmic loop (Figure 4C) in a region conserved within plant cellulose synthases (Delmer, 1999). These results indicate that the G617E mutation in *CEV1*, the predicted cellulose synthase *CeSA3*, is responsible for the *cev1* mutant phenotype.

cev1 Roots Have Reduced Amounts of Cellulose

Analysis of the cellulose content of the *cev1* mutant revealed that its roots contained ∼45% of the cellulose of wild-type roots (12.0% ± 2.4% versus 26.8% ± 7.8% of the ethanol-insoluble cell wall material). However, the cellulose

content of leaf tissues from *cev1* plants was not significantly different from that of wild-type plants.

### *CEV1* Is Expressed in Anthers, Roots, and Midvein Tissue

Expression of the *CEV1* gene was examined by RNA gel blot analysis (Figure 5A) and by histochemical staining of *CEV1*::*β-glucuronidase* (*GUS*) reporter plants (Figures 5B to 5E). *CEV1* mRNA was detected in flower and root tissues and to a lesser extent in leaves and stems (Figure 5A), consistent with previous results (Scheible et al., 2001). Histochemical staining for GUS activity in transgenic plants revealed that expression in roots was confined to the apex of the primary and secondary roots and to root hairs (Figures 5C and 5E). Expression also was observed in the midveins of leaves and in the anthers (Figures 5B and 5D). GUS expression generally was absent from older root tissues and from older leaves and was not observed in inflorescences (data not shown). Wounding or treatment with methyl jasmonate (MeJA) did not increase *CEV1* mRNA by RNA gel blot analysis, nor did it induce GUS expression (data not shown).
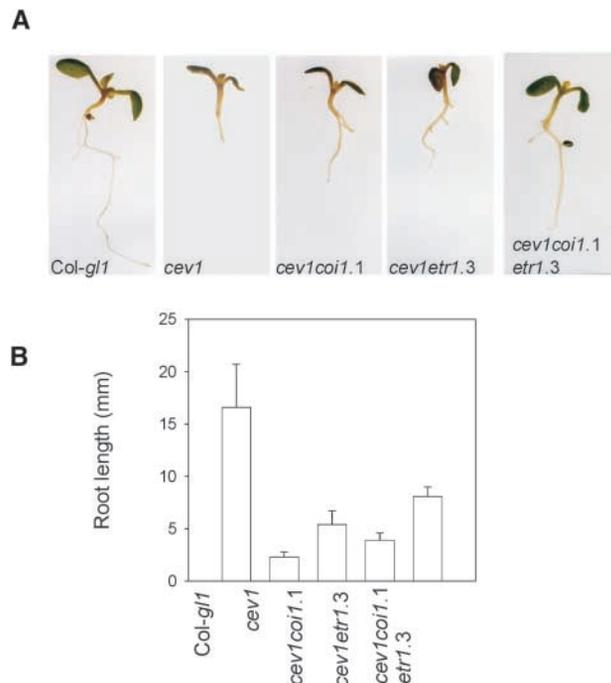
A

Col-*gl1*   *cev1*   *cev1coi1.1*   *cev1etr1.3*   *cev1coi1.1 etr1.3*

B

Figure 3. *ETR1* and *COI1* Are Partially Required for Root Growth Inhibition in the *cev1* Mutant.

(A) Eight-day-old seedlings grown on MS agar.
(B) Root lengths of 12-day-old seedlings grown on MS agar. T-bars indicate SD values for 10 roots.

A

B

C
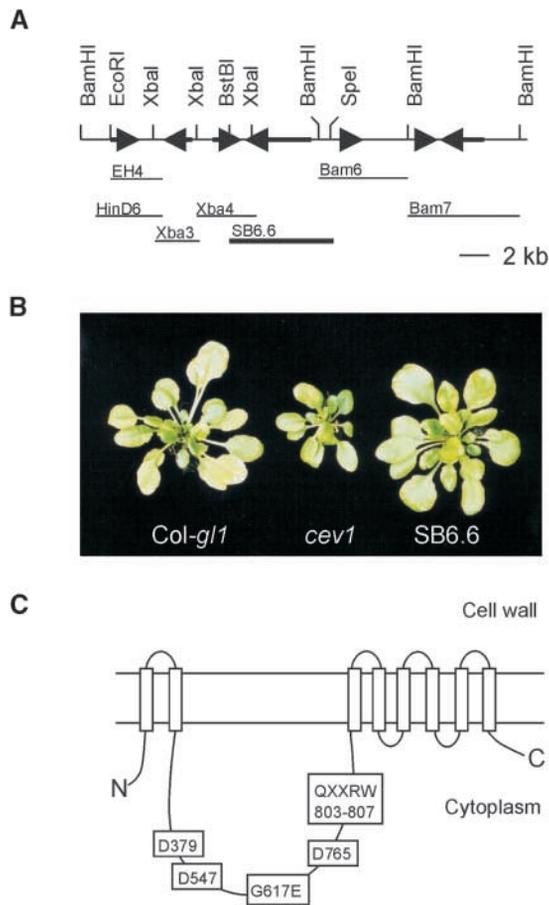
Figure 4. Complementation Analysis of *cev1*.

(A) Partial restriction endonuclease map of the K2A11 TAC clone and DNA fragments used in complementation analysis of *cev1*. Arrows indicate putative open reading frames. The DNA fragment SB6.6 complemented the *cev1* phenotype.

(B) Photographs of 4-week-old wild-type, *cev1*, and SB6.6 plants.

(C) Scheme of the predicted CEV1 protein showing the cellulose synthase signature motif D,D,D,QXXRW and the position of the *cev1* mutation.

Cellulose Biosynthesis Inhibitors Induce a *cev1* Phenocopy

The cellulose biosynthesis inhibitors 2,6-dichlorobenzonitrile (DCB) and isoxaben (IXB) were found to reproduce the *cev1* phenotype. Twelve-day-old wild-type Arabidopsis seedlings placed on 5 μM DCB or 5 μM IXB for 3 days became stunted, leaves displayed epinasty, and there was visible accumulation of anthocyanin in the midveins (Figure 6). These effects also were caused by MeJA and are characteristic of the *cev1* phenotype. Significantly, growth of the aerial parts of the *coi1* mutant was not affected visibly by any of these treatments (Figure 6A). However, root growth of

Table 2. Complementation of the *cev1* Phenotype

	VSP1::luciferase Activity (mV mg^{-1} min^{-1})	Root Length (mm)
Col-*gl1*	6.4 ± 2.8	18.5 ± 2.5
cev1	98.1 ± 6.8	5.4 ± 0.8
K2A11;*cev1*	9.3 ± 2.7	18.5 ± 2.9
SB6.6;*cev1*	4.6 ± 2.7	17.1 ± 2.2

All plants contained the *VSP1*::*LUC* transgene. Seedlings were grown for 12 days on Murashige and Skoog agar. Luciferase activity and the length of the primary roots were determined for at least 10 seedlings.

coi1 plants was inhibited by DCB and IXB but not by MeJA. Treatment with DCB or IXB induced the production of both *PDF1.2* and *VSP* mRNA in wild-type plants, whereas treatment with 20 μM MeJA induced the production of *VSP* mRNA but little *PDF1.2* mRNA (Figure 6B).

We compared the time course for induction of the LUC reporter for *VSP* after exposure to MeJA and the cellulose biosynthesis inhibitors IXB and DCB (Figure 7). MeJA induced LUC activity after 3 h, but IXB and DCB increased LUC activity only after 12 to 24 h.

rsw1 Expresses Increased Levels of a JA-Responsive Gene

The cellulose synthase mutants *rsw1* and *prc1* were examined for the expression of JA-responsive genes. The *rsw1-1* mutant appears normal at 22°C, but when it is incubated at 30°C, it has defective cellulose synthesis and its growth is stunted (Arioli et al., 1998). Dark-grown *prc1* plants are stunted, although light-grown plants appear normal (Fagard et al., 2000). When grown at 30°C, *rsw1* seedlings had considerably higher levels of *VSP* mRNA than wild-type plants (Figure 6C), and at 22°C, no differences in *VSP* expression were observed (data not shown). At 30°C, neither *rsw1* nor *cev1* plants expressed *PDF1.2* mRNA (data not shown), indicating that the higher temperature may adversely affect the expression of this gene. Dark-grown *prc1*, *cev1*, and wild-type plants were examined for the expression of *VSP* and *PDF1.2* mRNA, but these transcripts were not detected in any of the seedlings. However, light is required for *VSP* expression (Berger et al., 1995) and apparently for *PDF1.2* as well.

DISCUSSION

The *cev1* mutant was isolated in a screen for mutants with constitutive expression of the LUC reporter for the *VSP1*
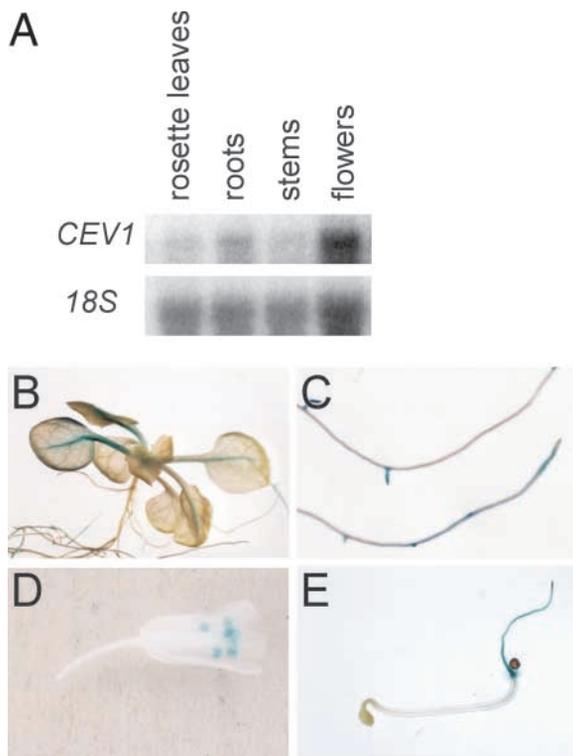
Figure 5. Expression Pattern of the *CEV1* Gene.

(A) Total RNA was extracted, and 1 μg was electrophoresed, blotted, and probed for *CEV1* or *18S* mRNA.
(B) to **(E)** Histochemical assays of GUS activity in *CEV1*::*GUS* transgenic plants.
(B) Rosette of a 2-week-old seedling.
(C) Roots of 2-week-old seedlings.
(D) Flower.
(E) Three-day-old dark-grown seedling.

promoter, and it was found subsequently to have constitutive expression of other JA- and ethylene-responsive genes, including *VSP2*, *PDF1.2*, *CHI-B*, and *THI2.1* (Ellis and Turner, 2001), and enhanced resistance to pests and pathogens (C. Ellis, I. Karafyllidis, and J.G. Turner, unpublished results). Here, we show that *cev1* plants overproduce JA and ethylene, and this is largely responsible for the altered morphology and pattern of gene expression in this mutant. Plants that are wounded or treated with the fungal pathogen *Alternaria brassisicola* also have increased synthesis of both JA and ethylene and increased expression of genes regulated by JA and ethylene (Creelman et al., 1992; Penninckx et al., 1998; Rojo et al., 1999). Therefore, *cev1* plants reproduce many of the physiological alterations that characterize wounded and infected plants.

Surprisingly, the constitutive defenses of the *cev1* mutant result from a recessive mutation in the cellulose synthase gene *CeSA3*. This raises the significant question of whether

JA and ethylene signaling in the *cev1* mutant results from an effect of the altered enzymatic activity of the mutant protein CEV1/G617E, possibly induced by reduced wall synthesis or by increased substrate level, or from a signaling property of the CEV1/G617E protein.

To address this question, we examined other cellulose synthase mutants and the effects of inhibitors of cellulose biosynthesis. Plants treated with the cellulose biosynthesis inhibitors IXB and DCB were stunted and epinastic, accumulated anthocyanin, and had enhanced expression of *VSP* and the JA- and ethylene-responsive gene *PDF1.2*. These phenotypes, except for the stunted roots, were suppressed in *coi1* plants, indicating that JA signaling was required for many of the effects of IXB and DCB.

We also found that plants containing the *rsw1* mutation in the cellulose synthase gene *CeSA1* constitutively expressed the JA-responsive gene for *VSP*. In addition, dark-grown *cev1* plants, such as *prc1* and *rsw1* plants (Fagard et al., 2000), have stunted hypocotyls. We have demonstrated that in *cev1* plants, these are effects of increased JA and ethylene signaling.

However, not all cellulose synthase mutants induce stress signaling. For example, *ixr1* and *ixr2* in *CeSA3* and *CeSA6*, respectively, are gain-of-function mutations that confer resistance to the cellulose biosynthesis inhibitor IXB (Scheible et al., 2001). These alleles do not cause stunted growth, anthocyanin accumulation, or epinasty, unlike the loss-of-function *cev1* and *prc1* alleles. Also, with the exception of irregular xylem and a slight decrease in growth, mutations in the *IRX1* and *IRX3* genes, which are required for cellulose synthesis in secondary cell walls, do not have morphological abnormalities (Turner and Somerville, 1997).

The time course for induction of the JA-responsive *VSP*::*LUC* transgenes by IXB and DCB indicated that LUC increased only 24 h after treatment, whereas LUC activity increased 3 h after treatment with MeJA, which is approximately the same time for the wound induction of *VSP* expression (Creelman et al., 1992; I. Karafyllidis and J.G. Turner, unpublished data). This finding indicates that IXB- and DCB-induced JA signaling is likely a secondary effect of these inhibitors. IXB and DCB inhibit the incorporation of radiolabeled Glc into cellulose within 1 h of treatment (Heim et al., 1990).

Hence, alterations in metabolite levels or in turgor pressure resulting from less Glc being used for cell wall synthesis should occur much more rapidly than the observed induction of stress responses. Therefore, we suggest that the delay is attributable to the time required for the growth and production of cells with altered cell walls and that JA and ethylene signaling in *cev1*, *rsw1*, and in plants treated with IXB and DCB is a consequence of altered wall composition or structure.

Relatively little is known about the function of the plant cell wall in signaling. However, in yeast, signaling in response to several stimuli, including temperature, osmotic stress, and pheromones, may involve the cell wall (Davenport et al.,

A

B

C
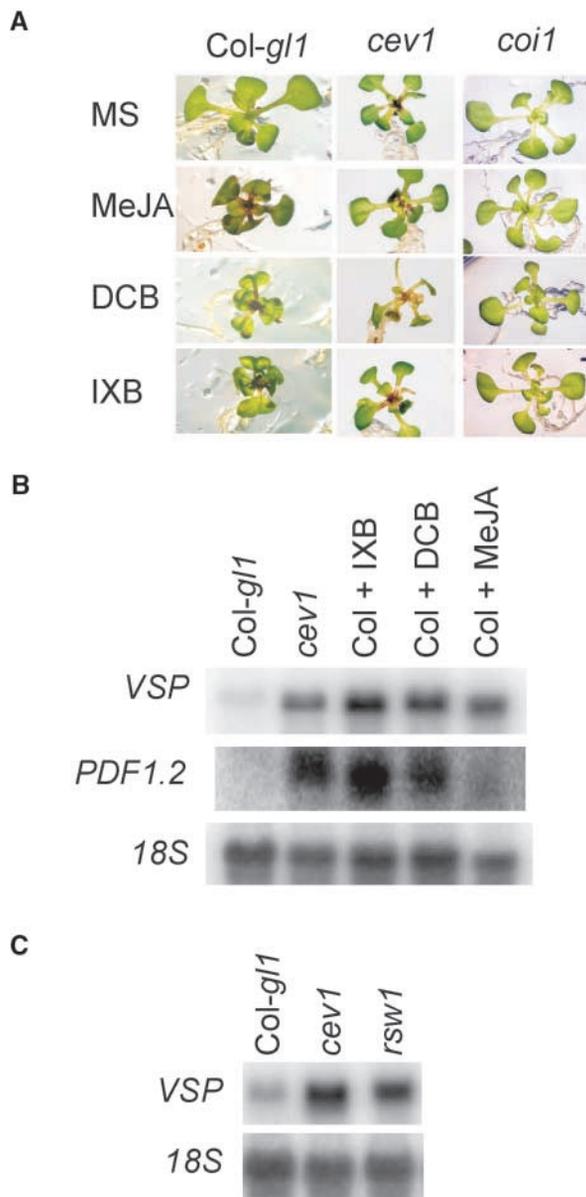
Figure 6. Cellulose Biosynthesis Inhibitors Induce *COI1*-Dependent JA Responses.

(A) and **(B)** Seedlings were grown on MS agar for 12 days, transplanted onto MS agar supplemented with 20 μM MeJA, 5 μM IXB, or 5 μM DCB, and incubated for another 3 days.
(A) Seedlings of wild-type, *cev1*, and *coi1-1* plants.
(B) Total RNA was extracted, and 1 μg was electrophoresed, blotted, and probed for *VSP* or *PDF1.2* mRNA.
(C) Seedlings were germinated for 5 days on MS agar at 22°C and transferred to 30°C for another 7 days. Total RNA was extracted, and 1 μg was electrophoresed, blotted, and probed for *VSP* mRNA.

1995; Kamada et al., 1995; Buehrer and Errede, 1997). These signals are mediated by the RHO1 GTPase, which is a regulatory subunit of the β-1,3-glucan synthase complex that synthesizes one of the major cell wall components in yeast (Qadota et al., 1996). RHO1 normally is activated by TOR2, a protein that has homology with phosphatidylinositol kinases (Schmidt et al., 1997).

However, mutations in genes for cell wall biosynthesis, and chemically induced cell wall defects, activate RHO1 GTPase-dependent signaling directly and independently of *TOR2* (Bickle et al., 1998). This finding agrees with our finding that in Arabidopsis, mutations in genes for cell wall biosynthesis activate JA and ethylene signaling. In addition, mutations in an Arabidopsis chitinase-like gene, which is presumed to be involved in cell wall modification, also activate the production of ethylene (Zhong et al., 2002). It appears that cell wall integrity in Arabidopsis, like that in yeast, can mediate signaling events.

The cell wall of plants has some functional similarities to the extracellular matrix (ECM) of mammalian cells, which plays a key role in cell signaling (Reuzeau and Pont-Lezica, 1995). In mammalian cells, integrin receptors interact with the ECM and, in response to stimuli, including mechanical stress, activate RhoA, a homolog of RHO1 (Qadota et al., 1994; Clark and Brugge, 1995). In mammals, peptides containing the amino acid sequence RGD disrupt interactions between integrin and protein ligands within the ECM (Ruoslahti, 1996).

RGD peptides also disrupt the formation of Hechtian strands in plasmolyzed Arabidopsis cells, indicating that integrin-like proteins occur in Arabidopsis even though integrin genes have not been identified in its genome (Canut et al., 1998). RGD peptides also decrease plant defense responses at sites of fungal infection, including the production of hydrogen peroxide and callose (Mellersh and Heath, 2001). Evidently, adhesion between the cell wall and the plasmalemma occurs through integrin-like linkages and can signal plant defense responses.

Oligosaccharides and cell wall material have long been known to have signaling properties in plants (Darvill and Albersheim, 1984). Oligosaccharides and fungus-derived cell wall fragments have been demonstrated to elicit JA synthesis (Gundlach et al., 1992; Doares et al., 1995). However, in Arabidopsis, oligosaccharides promote ethylene production and inhibit the expression of JA-regulated genes (Rojo et al., 1999), and in tomato, JA induces polygalacturonase expression, suggesting that the oligogalacturonides produced in planta likely act downstream of JA synthesis (Ryan, 2000). Although clarification of their roles is required, the evidence indicates a connection between oligosaccharides and wound response.

Inhibitors of cellulose synthesis, and mutations in cellulose synthase genes, may cause the release of oligosaccharides and cell wall–derived compounds that subsequently activate JA synthesis and stress signaling. Mutations in cellulose synthase genes cause alterations in wall composition.
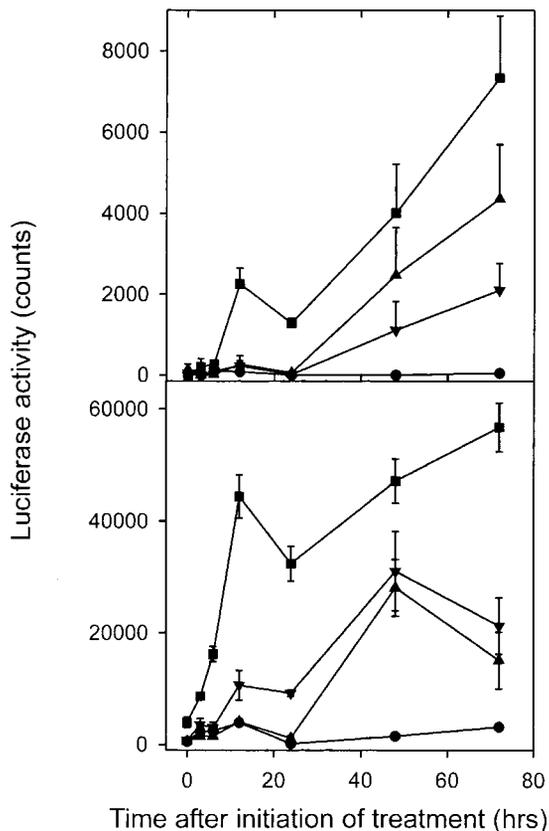
Figure 7. Time Course of the Induction of *VSP* Expression by Cellulose Biosynthesis Inhibitors.

(A) *VSP1::LUC* activity.
(B) *VSP2::LUC* activity.
Seedlings were grown on MS agar for 12 days and transplanted onto MS agar supplemented with 20 μM MeJA, 5 μM IXB, or 5 μM DCB. *LUC* activity was measured at the times indicated using low-light images of plants taken with a liquid nitrogen–cooled charge-coupled device imaging system. T-bars indicate SD values for eight seedlings. Circles, control; squares, MeJA; triangles, IXB; inverted triangles, DCB.

For example, *rsw1* root cell walls contain approximately half the amount of cellulose and twice the amount of starch of wild-type plants, along with a modest increase in pectin (Peng et al., 2000). *prc1* cell walls contain 50% more hemicellulose and slightly less pectin than wild-type plants (Fagard et al., 2000), and *cev1* cell walls have reduced cellulose. These altered walls may contain high levels of elicitors of JA and ethylene signaling, or the altered wall architecture may allow the untimely release of such molecules. We were not able to "wash out" elicitors from *cev1* plants, although this may be the result of the limited solubility or mobility of potential elicitors.

We show here that the *cev1* phenotype is caused by a mutation in the putative cellulose synthase gene *CeSA3*,

that *cev1* roots have reduced cellulose content, and that the most striking phenotypes of this mutant are caused by its overproduction of JA and ethylene. Furthermore, other genetic or chemical disruptions of cellulose synthesis result in the activation of these signaling pathways. Therefore, we propose that the cell wall can mediate JA- and ethylene-dependent stress and defense responses.

METHODS

Nomenclature

Analysis of the *Arabidopsis thaliana* genome has identified at least 10 cellulose synthase genes, which have been designated *CeSA1* to *CeSA10* (Delmer, 1999; Richmond and Somerville, 2000). The cellulose synthase gene responsible for the *cev1* phenotype, *CeSA3*, also has been designated *Ath-B* (Arioli et al., 1998) and *IXR1* (Scheible et al., 2001). Other mutants discussed in this article include *rsw1*, also designated *CeSA1* (Arioli et al., 1998), and *prc1*, also designated *ixr2* and *CeSA6* (Fagard et al., 2000).

Growth Conditions

prc1-1 and *rsw1-1* seeds were a gift from M. McCann (John Innes Centre, Norwich, UK) and R.E. Williamson (Australian National University, Canberra, Australia). Unless stated otherwise, plants were grown as described previously (Ellis and Turner, 2001) at 22°C in a 16-h photoperiod on 0.5 × Murashige and Skoog (1962) (MS) agar (seedlings) or soil (mature plants). Seedlings for hypocotyl measurements were grown on MS medium for 4 days in the dark. Seedlings for root measurements were grown for 12 days under a 16-h photoperiod. Construction and selection of the *cev1 coi1-1* and *cev1 etr1-3* double mutant lines were as described previously (Ellis and Turner, 2001). The *cev1 coi1-1 etr1-3* triple mutant line was constructed from a cross between the *cev1 coi1-1* and *cev1 etr1-3* double mutant lines. The triple mutants were selected on the basis of their lack of anthocyanin accumulation and their lack of prolific root hairs.

For treatment with cellulose biosynthesis inhibitors, 12-day-old seedlings grown on MS agar were transferred to MS medium supplemented with 5 μM 2,6-dichlorobenzonitrile (Aldrich) or isoxaben (RdH Laborchemikalian, Seelze, Germany) and grown for another 1 to 3 days. To test *VSP* levels in the *rsw1-1* mutant, plants were germinated on MS medium for 5 days and transferred to 30°C for 7 days before harvest and extraction of RNA.

Cloning of the *CEV1* Gene

The *cev1* mutant, in a Columbia (Col)-*gl* background, was crossed with Landsberg *erecta* plants, and 416 F2 individuals exhibiting the short-root phenotype of *cev1* were selected for analysis. DNA from these plants was used to map the *CEV1* gene to an ~20-kb region on chromosome 5 using a combination of cleaved amplified polymorphic sequence and simple sequence length polymorphism markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994) with data obtained from TAIR (http://www.arabidopsis.org). This region was located on the transformation-competent artificial

chromosome clone K2A11, which was obtained from the ABRC (Columbus, OH).

The clone was introduced into *cev1* plants by *Agrobacterium tumefaciens*–mediated transformation (Clough and Bent, 1998), and it complemented the mutation. Restriction endonuclease fragments of the K2A11 clone were ligated into the binary vector pSLJ75515, introduced into *cev1* plants by Agrobacterium-mediated transformation, and tested for complementation of the mutant phenotype. Clone SB6.6 was found to complement the mutant phenotype.

Overlapping fragments of the *CEV1* gene and the *cev1* mutant were amplified by PCR using the following pairs of primers: 5′-CCATCCCAAGATTCTCCTCTTCGTCTTC-3′ and 5′-AAGTGAGGG-TGACTAGATGAC-3′, 5′-GTACAGGAATGACGAAGCGAG-3′ and 5′-CCCTCTTCTATGTCATCGAG-3′, 5′-CATTTGTCAAAGATCGTAGAG-C-3′ and 5′-AGAGGAACAATAGAACCAGCTG-3′, and 5′-CTG-GAGAAGCGATTTGGACAG-3′ and 5′-GAACGAACAGACAGATAC-ACAC-3′. These fragments were sequenced with the aid of BigDye dideoxy terminator mix (Applied Biosystems, Foster City, CA).

Reporter Gene Construction and Assays

To construct the *CEV1*::*β-glucuronidase* (*GUS*) reporter gene, the region corresponding to −3 to −970 upstream of the *CEV1* gene was amplified using primers 5′-GTCATCTAGATGCTTCCAACACCG-3′ and 5′-CACATCTAGACATGCACCACCAG-3′. This fragment was confirmed by DNA sequencing, cloned into the XbaI site of pBI101 (Clontech, Palo Alto, CA), and introduced into Col-*gl1* plants by Agrobacterium-mediated transformation (Clough and Bent, 1998). The *VSP1*::*luciferase* (*LUC*) transgenic line has been described previously (Ellis and Turner, 2001). To create the *VSP2*::*LUC* transgenic line, the *LUC* gene from pGEMluc (Promega) was inserted into a pBI101 vector (Clontech) in place of the *GUS* gene using the BamHI and SacI restriction endonuclease sites.

A 1.5-kb DNA fragment containing the 5′ untranslated region of *VSP2* was isolated via PCR of genomic DNA using primers 5′-CTT-CTTAATTAAGCTTATATCTTC-3′ and 5′-GAGGATTTTCATGGATCC-TAATGG-3′. This fragment was cloned into the HindIII and BamHI sites of the pBI101/LUC vector and introduced into Col-*gl1* plants by Agrobacterium-mediated transformation (Clough and Bent, 1998). Histochemical staining for GUS activity and in vitro and in vivo LUC activities were assayed as described previously (Ellis and Turner, 2001).

Jasmonate and Ethylene Determinations

Jasmonate was determined as described previously (Hause et al., 2000). Ethylene was determined according to Guzman and Ecker (1990) with slight modifications. To determine ethylene in etiolated seedlings, ~500 seeds were placed in a vial containing MS medium that was closed with a SUBA-seal, leaving a headspace volume of ~5 mL. Seeds were kept at 4°C for 3 days and then incubated in the dark for 3 days at 22°C before samples were removed for analysis. To determine ethylene produced by light-grown seedlings, ~200 mg fresh weight of 10-day-old light-grown seedlings was placed into vials containing MS medium. The vials were sealed and incubated in the light for 24 h before samples were removed for analysis. Headspace samples (10 μL) were analyzed using a 610 series gas chromatograph (ATI Unicam, Thermo DNIX, Houston, TX). Ethylene standards were from Aldrich. All experiments were performed in triplicate.

Cellulose Measurements

Seedlings were grown on vertical surfaces of MS agar for 3 weeks, at which time samples were harvested, frozen in liquid nitrogen, ground with a mortar and pestle, and homogenized in 50 mM Tris, pH 7.2, and 1% SDS. Samples were filtered through a fine mesh (5 μm), washed with ethanol and acetone, and resuspended in water. An aliquot was reserved for determination of total cell wall sugar content. The cell wall material was digested with 2 M trifluoroacetic acid for 90 min at 120°C, cooled, and centrifuged (4000*g* for 5 min). The resulting cellulose pellet was washed with water and quantified colorimetrically using the phenol/sulfuric acid method (Dubois et al., 1956).

Root and Hypocotyl Measurements

Roots and hypocotyls were measured with a caliper. At least 10 plants of each genotype were measured for each data set.

RNA Gel Blot Analysis

RNA gel blot analysis was performed as described previously (Ellis and Turner, 2001).

Accession Number

The accession number for *CeSA3* is AF027174.1. The accession number for clone K2A11 is AB018111.

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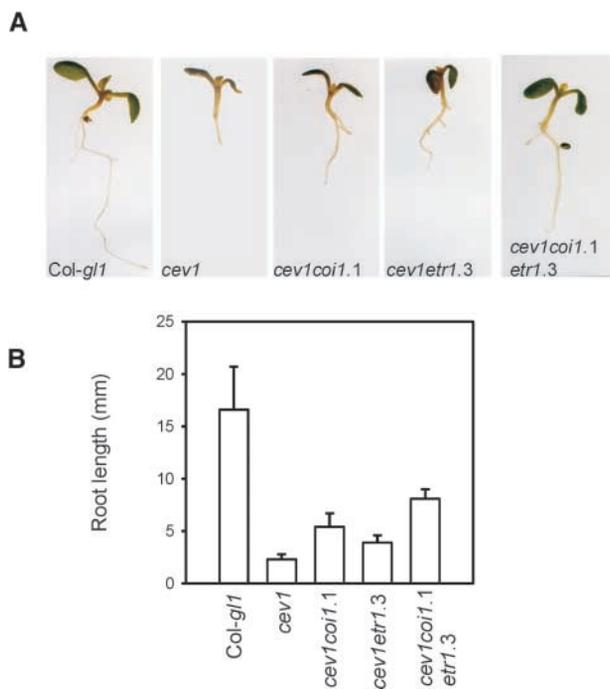**Berger, S., Bell, E., Sadka, A., and Mullet, J.E.** (1995). *Arabidopsis*

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Correction

Christine Ellis, Ioannis Karafyllidis, Claus Wasternack, and John G. Turner. (2002). The Arabidopsis Mutant *cev1* Links Cell Wall Signaling to Jasmonate and Ethylene Responses. Plant Cell **14,** 1557–1566.

The labels on the *x* axis of Figure 3B were misaligned in the original publication. The corrected figure is shown below.

Figure 3. *ETR1* and *COI1* Are Partially Required for Root Growth Inhibition in the *cev1* Mutant.

(A) Eight-day-old seedlings grown on MS agar.
(B) Root lengths of 12-day-old seedlings grown on MS agar. T-bars indicate SD values for 10 roots.

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