Expression of a Pathogen-Induced Gene Can Be Mimicked by Auxin Insensitivity

Esther Mayda, Carmen Marqués, Vicente Conejero, and Pablo Vera

Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universidad Politécnica-Consejo Superior de Investigaciones Científicas, Camino de Vera s/n, 46022-Valencia, Spain
Accepted 20 September 1999.

Following perception of a pathogenic attack, plants are able to develop a strong response with the corresponding activation of a plethora of defense-related genes. In this study we have characterized the mode of expression of the *CEVI-1* gene from tomato plants, which encodes an anionic peroxidase. *CEVI-1* expression is induced during the course of compatible viral and subviral infections, like many other defense-related genes, but is induced neither in incompatible interactions nor by signal molecules such as salicylic acid, ethylene, or methyl jasmonate. Additionally, *CEVI-1* is induced in detached leaf tissues following a pathway distinct from that related to the classical wound response. We also describe the characterization of the structural *CEVI-1* gene and compare the mode of expression in different transgenic plant species harboring a *CEVI-1::GUS* construct. Furthermore, we have isolated mutants in *Arabidopsis*, called *dth* mutants, that are deregulated in the control of expression of this gene. From the initial analysis of some of these mutants it seems that activation of *CEVI-1* gene expression correlates with a defect in the perception of auxins by the plant. All these results may suggest that, during systemic infections with viruses, auxin homeostasis is one of the components participating in the regulation of the overall defense response.

Additional keywords: SAR, ToMV.

Higher plants have developed the capacity to integrate an array of defense responses when they are attacked by pathogens. Some of these mechanisms are already established in the plant before arrival of the microbe, whereas others are inducible upon perception of the appropriate pathogenic insult. One common feature of disease resistance is the rapid development of cell death at the infection sites (the so-called hypersensitive response [HR]) (Agrios 1988). The onset of HR in turn activates a signaling process throughout the plant that makes it more refractory to subsequent infections by a broad spectrum of pathogens. This response is called systemic acquired resistance (SAR) (Ross 1961). Ac-

companying these responses, the plant selectively activates an array of active defenses that include the production of antimicrobial secondary metabolites (called phytoalexins), the production of pathogenesis-related (PR) proteins, or even the reinforcement of the cell wall by means of inducing peroxidases that cross-link polymers and proteins to the cell walls. The sufficient duration and efficiency of these defense responses are mandatory for reducing the spread of the pathogen (Ryals et al. 1996). Induction of these defense mechanisms requires the generation of amplifying signal molecules that eventually lead to the activation of the corresponding defense-responsive genes. The earliest signals produced following pathogen recognition are reactive oxygen intermediates (ROIs) (Baker and Orlandi 1995) with superoxide radicals, hydrogen peroxide, and nitric oxide playing pivotal roles in the induction of HR and defense responses (Alvarez et al. 1998; Jabs et al. 1996; Delledonne et al. 1998). Salicylic acid (SA) is a master regulatory molecule that accumulates to considerable levels following pathogen recognition and is implicated in the promotion of HR and SAR by activating defenses systemically (Ryals et al. 1996 and references therein).

Evidence is now accumulating that not all defense-related genes are dependent on SA for their induction. For instance, distinct isoforms of PR proteins are known to be induced by ethylene (Tornero et al. 1997), and the plant defensin gene *PDF1.2* is induced by ethylene or methyl jasmonate but not by SA (Penninckx et al. 1998).

We here show a novel mode of gene regulation that is activated following viral infection. Based on this novel mode of expression, we propose that auxin sensitivity is altered in diseased plants and this mediates the activation of at least part of the plethora of defense-related genes.

RESULTS

Expression pattern of the *CEVI-1* gene from tomato plants.

We previously described the isolation of a group of genes that were induced de novo throughout the plant during the course of the compatible interaction of tomato plants with citrus exocortis viroid (CEV) (Gadea et al. 1996 and references therein). Sequence analysis revealed that most of these genes were classical PR genes whereas others encoded proteins involved in cell wall hardening (e.g., peroxidases) or enzymes related to fatty acid metabolism (e.g., desatu-

Corresponding author: Pablo Vera; Telephone: 34-96-3877864; Fax: 34-96-3877859; E-mail: vera@ibmcp.upv.es

Nucleotide and/or amino acid sequence data have been submitted to the GenBank, EMBL, and DDBJ data bases as accession no. Y19023.

rases, lipoxygenases, etc.). Northern (RNA) blot analysis showed that these genes were coordinately induced in viroid diseased plants, with their level of expression correlating with appearance of viroid-induced disease symptoms in the plant. One of these genes, *CEVI-1* (Vera et al. 1993), encodes a 34-kDa anionic peroxidase that is closely related to other peroxidases from different plant species.

To address whether or not *CEVI-1* is induced by other types of pathogens, we inoculated tomato plants with *Tomato mosaic virus* (ToMV), a virus that in the sensitive host used here spreads through all the plant. Figure 1A shows that, at 7 days post inoculation (d.p.i.) with ToMV, *CEVI-1* expression was induced not only in the inoculated leaf but also in upper, noninoculated leaves of the same plant. This induced systemic expression was also observed for the *PR-1* gene used as a control of defenses activation. However,

Fig. 1. Comparative RNA gel blot analysis of *CEVI-1* and *PR-1*. **A**, Induction of *CEVI-1* and *PR-1* mRNA in leaf tissue of tomato plants. Lane 1, mRNA induction in susceptible tomato plants 7 days post inoculation (d.p.i.) with *Tomato mosaic virus* (ToMV) in inoculated leaf (1), immediate upper noninoculated leaf (2), and noninoculated leaves close to apex (3). "Mock": local leaves inoculated with buffer. Lane 2, mRNA induction 7 d.p.i. with ToMV in local leaves from tomato plants carrying (R+) or not carrying (R-) the *Tm-2* disease resistance gene to ToMV. Lane 3, mRNA induction in leaves of tomato plants by citrus exocortis viroid (CEV) infection at 15 d.p.i., and by the incompatible bacteria *Pseudomonas syringae* pv. *syringae* at 2 d.p.i. with an inoculum containing 5×10^7 CFU per ml of bacteria. "Mock": controls inoculated with 10 mM MgCl₂ from samples taken at the same time post inoculation. Lane 4, mRNA induction in intact tomato plants 2 days after infiltration of leaves with buffer ("Mock"), 0.5 mM salicylic acid (SA), or 0.1 mM 1-aminocyclopropane-1-carboxylate (ACC), and in leaves severely wounded with a razorblade or inoculated with 50 mM methyl-jasmonate (MeJA). **B**, Induction of *CEVI-1* and *PR-1* mRNAs in tomato leaf disks floated in buffer alone or buffer containing 0.5 mM SA. Numbers on top: time in hours. **C**, Induction of *CEVI-1* mRNA in leaf disks from wild-type tobacco plants and *NahG* tobacco plants floated in buffer for indicated times. Experiments were conducted twice with similar results.

when resistant tomato cultivars carrying the resistance *Tm-2* gene to ToMV were similarly inoculated with this virus (R+; Fig. 1A), no induced expression of *CEVI-1* was detected at 7 d.p.i., whereas induction of *PR-1* still took place. Analysis of gene expression during this incompatible interaction was also negative at shorter or longer times and did not reveal induction of *CEVI-1*. This suggested that expression of *CEVI-1* is activated in diseased tissue and not during HR. Furthermore, inoculation of tomato plants with the incompatible bacteria *Pseudomonas syringae* pv. *syringae* (P.s.; Fig. 1A) or with the compatible bacteria *P. syringae* pv. *tomato* (data not shown) did not induce expression of *CEVI-1*, while expression of *PR-1* was again evident. All these observations suggested that expression of *CEVI-1* follows a pathogen-inducible mechanism different from that assumed to be common for induction of classical PR genes. To address this possibility in more detail we checked induction of *CEVI-1* by infiltrating leaf tissues in intact plants with signal molecules that mediate induction of defenses, such as SA, 1-aminocyclopropane-1-carboxylate (ACC, the precursor of ethylene), methyl-jasmonate (MeJ), or even by wounding. As shown in Figure 1A, in no one case could we observe induction of *CEVI-1* by any of these molecules, while the corresponding marker genes (e.g., *PR-1* for SA and ACC, or *pin1* for MeJ and wounding [not shown]) were readily induced. However, experiments aimed to reproduce the lack of effect of these molecules on the induction of *CEVI-1* in leaf segments instead of intact plants revealed an interesting event. When detached leaf disks were led to float in buffered solution containing each of the above mentioned chemicals, as described (van der Rhee et al. 1990), we observed that *CEVI-1* expression was induced under these conditions at 6 to 12 h. Remarkably, this induction occurred even in the control leaf disks floated in buffering solution alone (Fig. 1B). Furthermore, the presence of either SA (Fig. 1B) or ACC (data not shown) in the buffer solution did not lead to an over-induction of *CEVI-1* with respect to the levels observed in the control leaf disks floated in buffer. The expected accumulation of *PR-1* mRNAs in SA-treated disks but not in the buffer controls (Fig. 1B) produced a positive control of SA-mediated induction. Inducibility of *CEVI-1* gene expression by detachment was conserved in tobacco plants. When leaf disks of tobacco plants were similarly floated in buffer solution alone and the mRNAs were hybridized with the tomato *CEVI-1* probe a similar pattern of expression for the homologue gene was observed (Fig. 1C). This induction was also reproduced in leaf disks derived from *NahG* tobacco plants (Fig. 1C), thus supporting the conclusion that SA does not mediate the observed mode of gene induction of *CEVI-1*.

Since wounding of intact plants does not induce *CEVI-1* (Fig. 1A, right panel), the observed induction of *CEVI-1* in leaf segments could reflect a kind of osmotic stress imposed on the tissues while floating. However, we did not observe inhibition of this induction when the leaf disks were allowed to stand in MS agar plates or were floated in buffered solutions containing a range of concentrations (10 to 200 mM) of either sucrose, mannitol, NaCl, or KCl (data not shown).

These observations rule out a trivial explanation for the signal and mechanism mediating induction of *CEVI-1* in diseased plants or upon detachment.

Analysis of *CEVI-1* gene expression in transgenic plants containing a promoter-GUS fusion.

CEVI-1 is represented by three copies in the tomato genome (Vera et al. 1993). To avoid any possible cross-reaction between probes of the same family, the structural *CEVI-1* gene was isolated from a genomic library and characterized. Alignment of the genomic and cDNA sequences revealed the presence of three introns in the transcribed region (Fig. 2A). Inspection for conserved motifs in the 5' promoter sequence revealed the presence of two conserved TGTCTC *cis*-acting elements (AuxRE), at positions -958 and -120, which is characteristic of auxin-inducible genes (Guifoyle et al. 1998) (Fig. 2B).

To study the mode of expression of the *CEVI-1* gene, 2,300 bp of the 5' flanking promoter region (from -2300 to the +39 position preceding the initiation ATG codon; Fig. 2B) was fused to the *GUS* (β -glucuronidase) reporter gene. This was introduced into tobacco, tomato, and Arabidopsis plants by transformation with *Agrobacterium tumefaciens*, and the transgenic plants were used to study the transcriptional regulation of *CEVI-1*. Upon infection of *CEVI-1::GUS* transgenic tomato plants with ToMV, GUS expression driven by the *CEVI-1* promoter reproduced the data derived from Northern blot analysis shown in Figure 1A. GUS activity was dramatically induced in the inoculated leaf, and to a lesser extent was also induced in upper, noninoculated leaves of the same plant (Fig. 3A-D). In the latter, the induced expression of GUS is observed in epidermal tissue and is highest in trichomes, which become intensively stained with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-gluc; Fig. 3E). Analysis of GUS expression in transgenic tobacco plants carrying the *N* gene and upon inoculation with *Tobacco mosaic virus* (TMV) did not render induction of GUS activity, not even around the

tissue undergoing HR where expression of a *PRI-GUS* gene, used here as a control, was readily induced (Fig. 3F). Additionally, none of these transgenic plants induced expression of GUS activity driven by the *CEVI-1* promoter in local or systemic leaves after severe wounding (Fig. 3G). However, and consistent with our above observations in Northern blots, leaf disks derived from these transgenic plants showed induced expression of GUS activity at 24 h after detachment. Here, induction took place when disks were floated in buffer alone or in buffer solution containing 0.5 mM SA or 0.1 mM ACC (Fig. 3H). Interestingly, this transcriptional activation was not restricted to the cut edge but extended to the entire leaf disk.

To verify if this mode of gene induction was due to a wound effect, an osmotic stress, or a combination of both, transgenic Arabidopsis plants containing the *CEVI-1::GUS* construct were assayed similarly. Arabidopsis does not have a gene with clear homology to *CEVI-1*, but the pattern of expression of the promoter is similar to that of the other species (data not shown). In Arabidopsis, the flexible and small body of this plant allows performance of floating experiments of leaf segments and their corresponding leaf while the latter still remains attached to the entire plant. These experiments revealed that induction of GUS was observable throughout the detached leaf segments, while the remaining part of the cauline leaf that was similarly floated, while still attached to the plant, did not show any induction of GUS activity, not even in the wounded edges (Fig. 3J). Intact leaves treated under the same floating conditions did not show induction of GUS activity (Fig. 3I). Thus, since induction takes place only in the detached part of the leaf and not in the parts that remain connected to the plant body, we called this enigmatic way of gene activation "detachment response."

The mode of gene regulation described above and the presence of *cis* auxin-like elements in the *CEVI-1* promoter suggested that auxins could be mediating specific aspects of the inducibility of the *CEVI-1* gene. We initially addressed this possibility by testing if this hormone could alter expression of *CEVI-1* under inductive and noninductive conditions. In intact seedlings exogenous application of indole acetic acid (IAA) did not render expression of *CEVI-1* (data not shown), suggesting that auxin is not a positive modulator of *CEVI-1* gene expression. Furthermore, analysis of induced expression of GUS activity in detached leaf tissues from transgenic plants (Fig. 4), or measurement of mRNA levels in wild-type tomato plants (not shown), revealed that the presence of a range of IAA concentrations (from 1 to 100 μ M) had only a minor inhibitory effect (10 to 20% inhibition) on the de novo transcriptional activation of *CEVI-1* by detachment. In these experiments, we could never observe strong suppression of the induction of *CEVI-1*, not even when leaf disks were incubated with supraoptimal concentrations of IAA (100 μ M). This lack of effect was also observed when the auxin analogs naphthyl acetic acid (NAA) or 2,4-D were used in similar experiments (not shown), and suggested that the penetration or transport of IAA was not complete in these experiments. Alternatively, it could be argued that the detached tissues have lost sensitivity to the available IAA supplied exogenously. If this would be the case, one could anticipate that any plant showing constitutive expression of *CEVI-1* could be impaired in auxin perception.

Fig. 2. Characterization of the *CEVI-1* genomic clone. **A**, Schematic organization of the *CEVI-1* gene. Boxes: the four exons. **B**, *Bam*HI; *X*, *Xba*I; *H*, *Hind*III; *E*, *Eco*RI. **B**, Nucleotide sequence of the 5' promoter region of *CEVI-1*. First nucleotide that appeared in the largest cDNA, indicated in bold and used to number (+1) the genomic sequence. Initiation ATG codon is also in bold. TGTCTC motifs are double underlined.

Isolation of Arabidopsis mutants showing constitutive expression of GUS activity driven by the *CEVI-1* promoter.

To verify if auxin sensitivity was involved in the induction of the *CEVI-1* gene promoter, we addressed the search for mutants in transgenic Arabidopsis plants harboring the *CEVI-1::GUS* gene construct. Our screening rationale was that by looking for mutants showing constitutive expression of the reporter gene in intact plants grown under non-inductive conditions, we would identify mutations affecting the regulation of this signal pathway. We mutagenized a homozygous *CEVI-1::GUS* transgenic Arabidopsis line with ethyl methanesulfonate (EMS) as described in Materials and Methods. M₂ plants (from 100 pools) were tested for constitutive expression of GUS. Of 14,000 M₂ plants screened, 63 GUS expressors were identified. These were transplanted to soil, and allowed to set seeds. GUS activity was assayed again in progeny of all these putative mutants to confirm whether the phenotype was heritable. Only 22 lines showed constitutive GUS activity in the M₃ generation.

Two lines (originated from two independent M₁ pools) were found to be homozygous and stable for GUS expression even in the M₅ generation and were chosen for further analysis. We named these mutants *dth2* and *dth23* (for constitutive response to detachment). Both mutants showed no

remarkable differences to wild-type plants in terms of growth and developmental habit under normal growth conditions. Based on genetic analysis (see Table 1), *dth2* and *dth23* are recessive nuclear mutations representing two different complementation groups, and are not linked to the transgene itself.

The pattern of reporter gene expression in the *dth2* and *dth23* mutants is shown in Figure 5A. In the parental wild-type seedlings no GUS activity was detected except in a discrete spot located at the distal end of the cotyledons. Conversely, in *dth2* and *dth23* seedlings (Fig. 5B,C) GUS staining was detected in expanding leaves (predominantly in trichomes) as well as in the cotyledons and the stem, but not in roots. Clockwise, in rosette leaves of these two mutants (Fig. 5E,F) GUS activity was distributed throughout the leaf blade with no expression in the central vein, while leaves from the parental plants did not show detectable GUS expression (Fig. 5D).

Since our hypothesis was that auxin insensitivity could be responsible for the observed expression of *CEVI-1* under inductive conditions, we wondered whether or not these two *dth* mutants would show an auxin-resistant phenotype. To examine the response of these plants to external auxins, seeds from *dth2* and *dth23*, as well as seeds from the parental wild type, were germinated in MS plates containing 0.2 μ M 2,4-D

Fig. 3. *CEVI-1::GUS* gene expression in transgenic plants. Histochemical localization of GUS (β -glucuronidase) activity in the following: **A**, Healthy (noninoculated) tomato leaves; **B**, *Tomato mosaic virus* (ToMV)-infected tomato leaves 5 days post inoculation; **C** and **D**, Upper noninoculated leaves from tomato 5 days post-inoculation of a lower leaf as indicated in **B**; **E**, Magnification of leaf shown in **D**; **F**, TMV-infected transgenic tobacco leaves from transgenic plants containing the *CEVI-1::GUS* construct (upper disk) or the *PR-1::GUS* construct (lower disk); **G**, Local and distal leaf tissues from transgenic tobacco plants containing the *CEVI-1::GUS* gene after severe wounding; **H**, Induced GUS activity in leaf disks from *CEVI-1::GUS* transgenic tobacco plants 24 h after detachment and floated in buffer alone (upper right) or containing either 0.5 mM salicylic acid (SA; lower left) or 0.1 mM 1-aminocyclopropane-1-carboxylate (ACC; lower right). Transgene is not expressed in intact leaves (upper left); **I**, Intact *CEVI-1::GUS* transgenic Arabidopsis plant after floating for 24 h in buffer solution; **J**, Same as in (**I**) except that a portion of cauline leaf was detached and allowed to float similarly to induce GUS activity.

(Maher and Martindale 1980). Figure 5G and H show that, while *dth2* and *dth23* seedlings display significant growth on this medium, the growth of wild-type seedlings is severely inhibited.

Therefore, both the activation of the signaling mechanism leading to transcriptional activation of *CEVI-1* and an auxin-resistant phenotype concurred in these two mutants. In fact, the constitutive expression of GUS activity present in *dth2* and *dth23* plants was not suppressed when the mutant seeds were germinated in IAA-containing plates (data not shown). These observations sustain our hypothesis that auxin might be involved in the activation of the *CEVI-1* gene, and indicate that the mutants here isolated are compromised in the normal plant responses to auxins.

To further validate this hypothesis, the *CEVI-1-GUS* gene construct was used to transform two well-characterized, auxin-resistant mutants, i.e., *axr4* (Hobbie and Estelle 1994b) and *aux1* (Maher and Martindale 1980). Fourteen out of 17 *axr4* kanamycin-resistant transgenic lines for the *CEVI-1-GUS* construct showed constitutive expression of GUS activity (Fig. 5I). Likewise, 24 out of 28 *aux1* ka-

namycin-resistant transgenic lines showed similar constitutive expression of GUS activity (Fig. 5I). Since neither *aux1* nor *axr4* are allelic to *dth2* or *dth23* (E. Mayda and P. Vera, *manuscript in preparation*), these results further sustain the hypothesis that defects in auxin perception are mandatory for the induction of *CEVI-1*.

DISCUSSION

In this study, we have characterized the *CEVI-1* gene from tomato plants encoding an anionic peroxidase. *CEVI-1* is induced in susceptible tomato plants upon infection with viral (ToMV) and subviral (viroid) pathogens concurring with the activation of classical *PR* genes. However, SA, ethylene, or MeJ do not induce *CEVI-1* expression. Thus, although high SA and ethylene levels concur with the observed activation of *CEVI-1* in infected tomato plants (Bellés and Conejero 1989), there is a lack of effect of these two signal molecules on *CEVI-1* expression. This observation suggests that this gene is activated by a different signaling process that runs in parallel with SA- and ethylene-mediated responses in the infected tissues. MeJ has emerged as a novel signal molecule mediating expression of other defense-related genes in an SA-independent fashion (Penninckx et al. 1998). However, *CEVI-1* gene expression responds to neither MeJ nor wounding.

Interestingly, *CEVI-1* expression is rapidly induced if healthy leaf segments are detached from the plant. This induction is not related to a classical wound response, since expression of *CEVI-1* is not restricted to wounded edges but rather is expressed all along the detached tissues. Thus, it appears as if breaking the proper tissue connections of the leaf and in turn impeding the supply of signals to the detached tissue is the mechanism that sets in motion the rapid transcriptional activation of this gene. This scenario led us to think that, in intact healthy plants, a signaling process might be operating to repress *CEVI-1* expression. If this would be the case, the observed transcriptional activation of *CEVI-1*, either in detached tissues or in diseased plants, could be explained by either the lack of supply of the negative signal or, alternatively, by changing the sensitivity of target cells to this signal. It is noteworthy that the 5' promoter region of *CEVI-1* contains the two AuxREs that are characteristic of auxin-regulated genes (Guilfoyle et al. 1998). Furthermore, a functional analysis of the *CEVI-1* promoter in transgenic plants has revealed that the proximal AuxRE and nearby sequences constitute a *cis*-acting element that is necessary for the inducibility of this gene (E. Mayda and P. Vera, *manuscript in preparation*). However, at variance with what is observed for most genes containing these typical AuxREs, the *CEVI-1* gene is not induced by auxin. Instead, auxin is proposed to repress expression.

To understand in more detail the mechanism underlying the activation of *CEVI-1*, we have isolated mutants of Arabidopsis plants in which the *CEVI-1::GUS* gene was up-regulated in the absence of any external inductive cue, with the rationale that these mutants may be compromised in the negative control operating during normal growth. These were named *dth* (for constitutive response to detachment). Analysis of two of these non-allelic mutants, *dth2* and *dth23*, revealed that they showed a phenotype resistant to the inhibitory growth effect of

Fig. 4. Effect of IAA on the induction of GUS (β -glucuronidase) activity by detachment. Leaf disks from transgenic tobacco plants carrying the *CEVI-1::GUS* transgene were assayed for GUS activity immediately after detachment from the plant (control) or after 36 h of being floating in buffer alone (-IAA) or buffer containing 100 μ M IAA (+IAA). Values represent average of three replicates $\pm$ SE. Similar results were obtained when solutions containing 50, 10, and 1 μ M IAA were used.

Table 1. Genetic segregation of *dth2* and *dth23* mutants

Crosses ^a	Generation	GUS+	GUS-	X ²
Wild type $\times$ <i>dth2</i>	F ₁	0	11	—
	F ₂	42	114	0.214 ^b
Wild type $\times$ <i>dth23</i>	F ₁	0	10	—
	F ₂	38	104	0.150 ^b
<i>dth2</i> $\times$ <i>dth23</i>	F ₁	0	25	—

^a The plant receiving pollen is listed first for each cross.

^b $0.7 > P > 0.5$.

2,4-D, which is characteristic of auxin-insensitive mutants (Maher and Martindale 1980). Furthermore, transgenic plants for bona fide auxin-insensitive mutants, e.g., *axr4* (Hobbie and Estelle 1994b) and *aux1* (Maher and Martindale 1980), transformed with the *CEVI-1::GUS* gene construct revealed that they are also impaired in the negative control that keeps this gene repressed under normal conditions of growth and development.

Thus, from all these results we can speculate that one mechanism mediating the observed up-regulation of *CEVI-1* can be explained by the induction of insensitivity to auxin. This insensitivity must be imposed in the plant during compatible viral infections or provoked in leaf segments when

they are set apart from the plant. Alternatively, the hypothesis of inactivation of normal pools of auxins following these stressing scenarios could be entertained, but this latter possibility seems to be less likely since viroid-infected plants do not show significant alterations in active auxin levels (Rodriguez 1986). In addition, exogenous supply of this hormone to either detached leaf tissue or to the intact *dth* mutants does not restore the repressed status of the gene.

The control of auxin metabolism, including biosynthesis, catabolism, and transport, is a complex and very dynamic process that responds to both developmental and environmental signals (Normanly and Bartel 1999). Also, the molecular basis of auxin sensitivity and response is very com-

Fig. 5. Pattern of GUS (β -glucuronidase) expression in *dth2* and *dth23* plants compared with wild-type Arabidopsis plants containing *CEVI-1::GUS*. **A**, Histochemical staining of GUS activity in a 12-day-old, wild-type transgenic seedling grown aseptically in GM agar medium. **B**, GUS expression of a *dth2* mutant plant grown as in **A**. **C**, GUS expression of a *dth23* mutant plant grown as in **A**. **D**, Full expanded rosette leaf from a wild-type, transgenic plant stained for GUS activity. **E**, Full expanded rosette leaf from a *dth2* plant stained for GUS activity. **F**, Full expanded rosette leaf from a *dth23* plant stained for GUS activity. **G** and **H**, Seeds of Arabidopsis wild type (left) and the *dth2* (center) and *dth23* (right) mutants were germinated on (**G**) control GM plates and (**H**) GM plates containing 0.2 mM 2,4-dichloro-phenoxyacetic acid (2,4-D) for 10 days. **I**, Expression of GUS activity in intact leaves from transgenic *axr4*, *aux1*, and wild-type plants (wt) transformed with the *CEVI-1::GUS* construct.

plex (Leyser 1997). During the past few years, different auxin-response mutants have been identified (Hobbie and Estelle 1994a). These genetic studies indicate that the response to auxin depends on the function of the ubiquitin-proteasome pathway, as exemplified by studies of the *axr1* and *tir1* mutants. The *AUXIN RESISTANT 1* (*AXR1*) gene encodes a protein that participates in the activation of the ubiquitin-related protein RUB1 (del Pozo et al. 1998). On the other hand, the *TRANSPORT INHIBITOR RESISTANT 1* (*TIR1*) gene encodes a protein of the F-box family that is part of a ubiquitin-protein-ligase complex (E3) known as SKP1 CDC53 FboxTIR1 (Ruegger et al. 1998). Apparently, *AXR1* and *TIR1* mediate auxin response by regulating the degradation of specific proteins, presumably those involved in auxin-regulated gene expression (del Pozo and Estelle 1999 and references therein). Interestingly, there is some parallelism between auxin perception and perception of the wound response mediated by jasmonate. The MeJ-insensitive *coi1* mutant is compromised in wound responses and resistance to insect attack, and *COI1* encodes a protein of the F-box family (like *TIR1*) (Xie et al. 1998). This suggests that *COI1* protein may serve to mark repressor proteins for proteolytic removal and in turn reveal a jasmonate-dependent response.

It has also been shown that a specific plant MAPKKK kinase (NPK1) activates an MAPK cascade that leads to the suppression of normal auxin responses and that can be reversed by an MAPK phosphatase (Kovtun et al. 1998). This could be explained by antagonistic signaling pathways that use a distinct MAPK cascade pathway, balancing auxin sensitivity. Again, there are parallelisms between auxin perception and perception of wound and pathogenic signals. A 48-kDa MAP kinase is activated in tobacco by SA (Zhang and Klessig 1997), suggesting the implication of an MAPK cascade in mediating plant defense signaling. Also, another MAP kinase (ERMK) is involved in a receptor-mediated activation of plant defense reactions toward pathogens (Ligterink et al. 1997). Likewise, an MAPK pathway has been described during the wound response as mandatory to induce accumulation of JA (Seo et al. 1995).

Relevant to the above discussion is the observation that elicitor-induced expression of some defense-related genes in tobacco protoplasts (Jouanneau et al. 1991), and transcription of *PAL* and *CHS* genes in carrot cell cultures (Ozeki et al. 1990) are negatively controlled by auxins.

Furthermore, injection of *A. tumefaciens* (bearing functional *tms* genes in the Ti plasmid) into tobacco leaves inhibits the HR induced by injection of the incompatible bacterial pathogen *Pseudomonas syringae* (Robinette and Matthyse 1990). All these examples indicate that auxin, wound, and pathogen perception may share conserved mechanisms of signal transduction pathways that may cross-talk at some point. The observation that in *dth* mutants the activation of the *CEVI-1* gene and auxin insensitivity concurs further suggests that there is an interactive cross-talk system between pathogen elicitation and auxin homeostasis. Such an interaction in the *dth* mutants could provide an original system to investigate the effect of auxin responsiveness in attempts to understand how plants mount and coordinate an array of defense signaling mechanisms to counter-act pathogen invasion.

MATERIALS AND METHODS

Plant material, growth conditions, and treatments.

Three-week-old tomato and tobacco plants were inoculated with ToMV or TMV as described (Tornero et al. 1997). *P. syringae* inoculations were as described (Jordá et al. 1999). In the case of *Arabidopsis thaliana* Col-O, surface-sterilized seeds were germinated on GM agar plates (1% Bacto agar, 0.5× Murashige and Skoog (MS) salts [Gibco BRL, Gaithersburg, MD], 1% sucrose, 1× B5 vitamins and 0.5 g of MES [morpholineethanesulfonic acid] per liter, pH 5.8). For synchronous seedling germination, the plates were kept in the dark for 2 days at 4°C and then transferred to 23°C with continuous light. For induction upon detachment, 1.5-cm disks were punched out from leaves and floated in a petri dish on buffer (30 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 6.9), containing or not any of the chemicals at the indicated concentrations for 24 to 48 h at 22°C (14 h light, 8 h dark cycles). After incubation, leaf disks were directly analyzed for GUS expression or were frozen and stored at -70°C.

Library screening and DNA sequence analysis.

Library screening with radiolabeled cDNA was performed as previously described (Tornero et al. 1997). A tomato genomic DNA library constructed in λ-EMBL3 (Clontech, San Diego, CA) was screened with a radiolabeled *CEVI-1* cDNA probe (Vera et al. 1993), and the positive clones were isolated, purified, and characterized as described (Tornero et al. 1997).

RNA blot analysis.

RNA was purified from different tomato and tobacco plant tissues as described (Jordá et al. 1999), and 15 µg of total RNA was electrophoresed on 1% agarose gels containing formaldehyde and blotted onto Nytran membranes (Schleicher and Schuell, Dassel, Germany). Equal loading of RNA was verified by ethidium bromide staining of the gel before transfer to the membrane. Radiolabeled probes were prepared by random priming with T7 polymerase (Pharmacia, Uppsala, Sweden). Hybridization and washing conditions of filters were done as described (Jordá et al. 1999).

Promoter-GUS fusion, plant transformation, and analysis of transgenic plants.

The oligonucleotide o35Nprom (5'-GGGATCCTTGATGA GTGATAGACAAC-3') served as primer for the incorporation of synthetic *Bam*HI restriction sites in the *CEVI-1* promoter. This and a T3 primer were used to polymerase chain reaction (PCR) amplify a 2.3-kb DNA fragment from the genomic clone inserted in pBluescript. The synthetic *Bam*HI site was introduced into the promoter upstream regions at positions -1 relative to the translation initiation site. The 2.3-kb *Xba*I-*Bam*HI fragment was cloned upstream of the β-glucuronidase gene in pBI101.1 (Jefferson 1987). The resulting fusion was verified by nucleotide sequence analysis with primers specific for the promoter region. The resulting plasmid was then mobilized into *A. tumefaciens* LBA4044 or C58, as described (Bevan 1984). Tobacco (*Nicotiana tabacum* cv. Xanthi nc) plants were transformed as described (Horsch et al. 1984). Tomato (*Lycopersicon esculentum*, cv. Rutgers) plants were

transformed as described (McCormick et al. 1986). *Arabidopsis thaliana* Col-0 plants were transformed as described (Bechtold et al. 1993). Primary transformants were allowed to self-fertilize and F₁ seeds were collected and germinated on MS agar medium containing kanamycin as selection. Transgenic seedlings were then transferred to soil. Transformants were assayed for GUS activity by a fluorimetric assay or by an in situ assay with the colorogenic substrate X-gluc (Jefferson 1987).

Arabidopsis mutants isolation.

A homozygous *Arabidopsis* transgenic line carrying the *CEVI-1::GUS* construct was mutagenized by soaking approximately 20,000 seeds in 120 ml of 0.3% ethyl methane-sulfonate for 8 h with gentle shaking. Seeds were then washed with water for 1 h and sown in soil. The mutagenized seeds were sown onto 100 separate flats and were allowed to grow, self-fertilize, and set seed. A representative population of each lot was germinated on GM agar plates with 50 µg of kanamycin per ml. Twelve days after planting, seedlings were numbered and a single leaf was removed from each and assayed for GUS activity by a fluorimetric assay or alternatively by an in situ assay with the colorogenic substrate X-gluc (Jefferson 1987). The M₂ seedlings constitutively expressing GUS activity were transplanted to soil for seed setting. This procedure was repeated with the M₃, M₄, and M₅ progeny of these putative mutants to ensure that the phenotype was heritable and to identify the homozygous mutants.

Genetic analysis.

Crosses were performed by dissecting and emasculating unopened buds and then using the pistils as recipients for pollen from five opened flowers. Backcrosses with the parental *CEVI-1::GUS* transgenic line were performed with *CEVI-1::GUS* plants as the pollen donor. The reciprocal crosses were also performed. F₁ and F₂ plants were grown and tested for GUS activity as described above. Segregation of constitutive GUS activity in the F₂ generation was analyzed with chi-square test for goodness of fit.

Test of auxin insensitivity.

To determine the response of *dth2*, *dth23*, and the Col-0 parental line seedlings to 2,4-dichloro-phenoxyacetic acid (2,4-D), surface-sterilized seeds were germinated on GM plates supplemented with 0.2 µM 2,4-D and the effect followed over time as described (Maher and Martindale 1980).

ACKNOWLEDGMENTS

We thank R. T. Chetelat for providing tomato seeds, J. Ryals for providing tobacco *NahG* seeds, I. García for providing viruses, G. Martin and C. Castresana for providing the bacteria used in these experiments, and P. Tornero for helpful discussions. We also thank M. D. Comín for help in the generation of transgenic plants and for taking care of plants. We acknowledge the Spanish Ministry of Science and Education for financial support to P. V., and C. M. thanks the Spanish Ministry of Science and Education for a fellowship.

LITERATURE CITED

Agrios, G. N. 1988. Plant Pathology. Academic Press, London.
 Alvarez, M. E., Pennell, R. I., Meijer, P.-J., Ishikawa, A., Dixon, R. A., and Lamb, C. 1998. Reactive oxygen intermediates mediate a sys-

temic signal network in the establishment of plant immunity. *Cell* 92: 773-784.
 Baker, C. J., and Orlandi, E. W. 1995. Active oxygen species in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:299-321.
 Bechtold, N., Ellis, J., and Pelletier, G. 1993. In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C. R. Acad. Sci. Paris Life Sci.* 316:1194-1199.
 Bellés, J. M., and Conejero, V. 1989. Evolution of ethylene production, ACC and conjugates ACC levels accompanying symptom development in tomato leaves infected with citrus exocortis viroid CEVd. *J. Phytopathol.* 127:81-85.
 Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.* 12:8711-8721.
 del Pozo, J. C., and Estelle, M. 1999. Function of the ubiquitin-proteasome pathway in auxin response. *Trends Plant Sci.* 4:107-112.
 del Pozo, J. C., Timppe, C., Callis, J., and Estelle, M. 1998. The ubiquitin-related RUB1 and auxin response in *Arabidopsis*. *Science* 280: 1760-1763.
 Delledonne, M., Xia, Y., Dixon, R. A., and Lamb, C. J. 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature* 394:585-588.
 Gadea, J., Mayda, M. E., Conejero, V., and Vera, P. 1996. Characterization of defense-related genes ectopically expressed in viroid-infected tomato plants. *Mol. Plant-Microbe Interact.* 9:409-415.
 Guifoyle, T., Hagen, G., Ulmasov, T., and Murfett, J. 1998. How does auxin turn on genes? *Plant Physiol.* 118:341-347.
 Hobbie, L., and Estelle, M. 1994a. Genetic approaches to auxin action. *Plant Cell Environ.* 17:525-540.
 Hobbie, L., and Estelle, M. 1994b. The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. *Plant J.* 7:211-220.
 Horsch, R. B., Fraley, R. T., Rogers, S. G., Sanders, P. R., Lloyd, A., and Hoffmann, N. 1984. Inheritance of functional foreign genes in plants. *Science* 223:496-498.
 Jabs, T., Dietrich, R. A., and Dangel, J. L. 1996. Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* 273:1853-1856.
 Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep.* 5:387-405.
 Jordá, L., Coego, A., Conejero, V., and Vera, P. 1999. A genomic cluster containing four differentially regulated subtilisin-like processing protease genes is in tomato plants. *J. Biol. Chem.* 274:2360-2365.
 Jouanneau, J.-P., Lapous, D., and Guern, J. 1991. In plant protoplasts, the spontaneous expression of defense reactions and the responsiveness to exogenous elicitors are under auxin control. *Plant Physiol.* 96: 459-466.
 Kovtun, Y., Chiu, W.-L., Zeng, W., and Sheen, J. 1998. Suppression of auxin signal transduction by a MAPK cascade in higher plants. *Nature* 395:716-720.
 Leyser, O. 1997. Auxin: Lessons from a mutant weed. *Physiol. Plant.* 100:407-414.
 Ligerink, W., Kroj, T., zur Nieden, V., Hirt, H., and Scheel, D. 1997. Receptor-mediated activation of a MAP kinase in pathogen defense of plant. *Science* 276:2054-2057.
 Maher, E. P., and Martindale, S. J. B. 1980. Mutants of *Arabidopsis thaliana* with altered responses to auxin and gravity. *Biochem. Genet.* 18:1041-1053.
 McCormick, S., Niedermeyer, J., Fry, J., Barnason, A., Horsch, R., and Fraley, R. 1986. Leaf disc transformation of cultivated tomato *L. Esculentum* using *Agrobacterium tumefaciens*. *Plant Cell Rep.* 5:81-84.
 Normanly, J., and Bartel, B. 1999. Redundancy as a way of life - IAA metabolism. *Curr. Opin. Plant Biol.* 2:207-213.
 Ozeki, Y., Komamine, A., and Tanaka, Y. 1990. Induction and repression of phenylalanine ammonia-lyase and chalcone synthase enzyme proteins and mRNAs in carrot cell suspension cultures regulated by 2,4-D. *Physiol. Plant.* 78:400-408.
 Pennincx, Y. A. M. A., Thomma, B. P. H. J., Buchala, A., Métraux, J.-P., and Broekaert, W. F. 1998. Concomitant activation of jasmonate and ethylene response pathway is required for induction of a plant defense gene in *Arabidopsis*. *Plant Cell* 10: 2103-2113.
 Robinette, D., and Matthysse, A. G. 1990. Inhibition by *Agrobacterium tumefaciens* and *Pseudomonas savastoni* of development of the hypersensitive response elicited by *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 172:5742-5749.

- Rodriguez, J. L. 1986. Estudio de las alteraciones fisiológicas y bioquímicas causadas por el viroide de la exocortis de los cítricos CEV. Ph.D. thesis. University of Valencia, Valencia, Spain.
- Ross, A. F. 1961. Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14:340-358.
- Ruegger, M., Dewey, E., Gray, W. M., Hobbie, L., Turner, J., and Estelle, M. 1998. The TIR1 protein from *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast Grr1p. *Genes Dev.* 12:198-207.
- Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H.-Y., and Hunt, M. D. 1996. Systemic acquired resistance. *Plant Cell* 8:1809-1819.
- Seo, S., Okamoto, H., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y. 1995. Tobacco MAP kinase: A possible mediator in wound signal transduction pathway. *Science* 270:1988-1992.
- Tornero, P., Gadea, J., Conejero, V., and Vera, P. 1997. Two *PR-I* genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. *Mol. Plant-Microbe Interact.* 10:624-634.
- van der Rhee, M. D., van Kan, J. A. L., Gonzalez-Jaen, M. T., and Bol, J. 1990. Analysis of regulatory elements involved in the induction of two tobacco genes by salicylate treatment and virus infection. *Plant Cell* 2:357-366.
- Vera, P., Tornero, P., and Conejero, V. 1993. Cloning and expression analysis of a viroid-induced peroxidase from tomato plants. *Mol. Plant-Microbe Interact.* 6:790-794.
- Xie, D.-X., Feys, B. F., James, S., Nieto-Rostro, M., and Turner, J. G. 1998. *COI1*: An *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science* 280:1091-1094.
- Zhang, S., and Klessig, D. F. 1997. Salicylic acid activates a 48-kD MAP kinase in tobacco. *Plant Cell* 9:809-824.