Regulation of a Hevein-like Gene in *Arabidopsis*

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An *Arabidopsis* cDNA clone was isolated that encodes a protein similar to the antifungal chitin-binding protein hevein from rubber tree latex. This hevein-like (*HEL*) mRNA was inducible by either turnip crinkle virus infection or ethylene treatment. In addition, expression was moderately inducible by treatment with the resistance-inducing compounds salicylic acid and 2,6-dichloroisonicotinic acid. The 786-bp cDNA contains an open reading frame of 212 codons. The deduced amino acid sequence contains a putative signal sequence of 21 amino acids followed by a 43-amino-acid cysteine-rich lectin domain and a 129-amino-acid carboxy-terminal domain. The predicted protein is approximately 70% identical to hevein, to the wound-inducible WIN1 and WIN2 proteins from potato, and to PR-4, a pathogenesis-related protein from tobacco.

Plants respond to pathogen attack by activating a variety of biochemical pathways, some of which include expression of genes encoding antimicrobial proteins (Bowles 1990; Keen 1990). One such response is systemic acquired resistance (SAR), a general resistance to pathogens caused by previous infection with a necrogenic pathogen (Ross 1961; Kuc 1982; Ryals *et al.* 1992). SAR can also be produced by treatment with salicylic acid (SA), a likely endogenous signal (Malamy *et al.* 1990; Métraux *et al.* 1990; Enyedi *et al.* 1992) or 2,6-dichloroisonicotinic acid (INA), a synthetic resistance-activator compound (Métraux *et al.* 1991). In tobacco, SAR correlates with expression of a set of at least nine gene families, which include the five groups of pathogenesis-related (PR) protein-encoding genes (Ward *et al.* 1991b).

Pathogen infection can also result in the evolution of the gaseous plant growth regulator ethylene (Ross and Williamson 1951). One effect of ethylene is to induce genes encoding proteins with *in vitro* antifungal activity, including chitinases and β-1,3-glucanases (Mauch *et al.* 1988; Roberts and Selitrennikoff 1988; Boller 1991). Many of these proteins have been termed "defense proteins," based on their enzymatic activity and regulation by ethylene. In one case, an ethylene-inducible chitinase, when constitutively expressed in transgenic plants, has been shown to increase tolerance to a fungal pathogen (Broglie *et al.* 1991).

To better understand the pathogen response in *Arabidopsis*, we have taken the systematic approach of cloning cDNAs corresponding to genes induced during SAR. Previously, we have shown that three such genes are homologs of tobacco PR-1, PR-2, and PR-5 and are coordinately induced by SA and INA (Uknes *et al.* 1992). Here, we report the isolation and characterization of an *Arabidopsis* cDNA that encodes a protein homologous to PR-4 from tobacco (Friedrich *et al.* 1991) and hevein, an antifungal protein from rubber tree latex (Van Parijs *et al.* 1991). Like hevein, the predicted *Arabidopsis* protein contains an amino-terminal lectin domain that may be involved in chitin binding. Accumulation of this hevein-like (*HEL*) mRNA is strongly induced by ethylene treatment and turnip crinkle virus (TCV) infection and is induced to a lesser extent by SA and INA. Thus, *HEL* may play a role in defense against pathogens in *Arabidopsis*.

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RESULTS AND DISCUSSION

cDNA isolation and characterization.

To identify a PR-4-like cDNA from *Arabidopsis*, a leaf tissue cDNA library (Uknes *et al.* 1992) was screened at low stringency with a tobacco PR-4 probe (Friedrich *et al.* 1991). Positively hybridizing plaques were obtained at a frequency of about one per 15,000 clones screened. Three lambda clones that hybridized to the probe were purified, and their cDNA inserts were sequenced. All three had identical sequence over their shared regions; the sequence of the longest is shown in Figure 1. The 212-codon open reading frame found in the cDNA is predicted to encode a preprotein having a structure typical of hevein-related polypeptides (Fig. 2). These include hevein itself (Broekaert *et al.* 1990) and the wound-inducible proteins WIN1 and WIN2 from potato (Stanford *et al.* 1989). Each protein contains a signal sequence for targeting to the secretory pathway, an amino-terminal lectin domain rich in cysteine residues, a spacer region, and a carboxy-terminal domain related to PR-4 from tobacco. In HEL, the predicted signal peptide is 21 amino acids in length, based on comparison with the conserved amino termini of the related proteins. The resulting predicted mature protein has a molecular weight of 20,691 and pI of 6.9. The HEL cysteine-rich domain is 43 amino acids long and is separated from a 141-amino-acid carboxy-terminal (PR-4-like) domain by a short seven-amino-acid spacer containing two proline residues. Although the *HEL* cDNA was identified using the tobacco acidic PR-4 as a probe (which lacks a cysteine-rich amino-terminal domain), only cDNAs containing a lectin domain were found when four additional clones were partially sequenced.

The amino-terminal cysteine-rich domain is found in proteins with a variety of biochemical activities, including wheat germ agglutinin (Smith and Raikhel 1989), stinging nettle

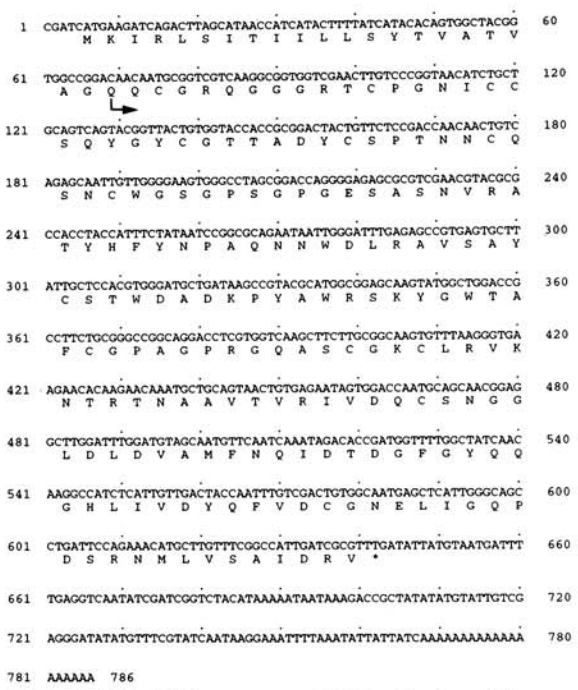

Fig. 1. Hevein-like cDNA sequence and deduced amino acid sequence. The predicted amino terminus of the mature protein, based on comparison with other hevein-like proteins, is indicated by an arrow.

lectin (Broekaert *et al.* 1989), and class I (Shinshi *et al.* 1987) and class IV (Collinge *et al.* 1993) chitinases. This domain is frequently flanked by short, direct repeat sequences and may represent a "module" capable of transposing into diverse genes (Shinshi *et al.* 1990). In prohevein, the 43-amino-acid lectin domain is cleaved from the remainder of the protein and is known to comprise the active antifungal component of the primary translation product. Whether *Arabidopsis* HEL protein is similarly cleaved is an area for further study. The carboxy-terminal domain, related to tobacco PR-4, has no known function.

Overall, the amino acid sequence of the putative mature HEL protein is 66% identical to pro-hevein, 72% identical to WIN1 protein, and 71% identical to WIN2 protein (Fig. 2). In addition, the HEL amino-terminal domain shares eight conserved cysteine residues with the lectin domains of wheat germ agglutinin (Smith and Raikhel 1989) and stinging nettle lectin (Broekaert *et al.* 1989). The carboxy-terminal domain of HEL is 75% identical to tobacco PR-4 and 71% identical to tomato PR-P2 (Linthorst *et al.* 1991).

HEL, preprohevein, and WIN2 contain carboxy-terminal extensions, compared to tobacco PR-4 which is extracellular. These short peptides are thought to be involved in targeting to the vacuole (Payne *et al.* 1990; Bednarek and Raikhel 1991; Neuhaus *et al.* 1991), which is consistent with the localization of hevein to the lutoid, a vacuole-derived organelle. Because

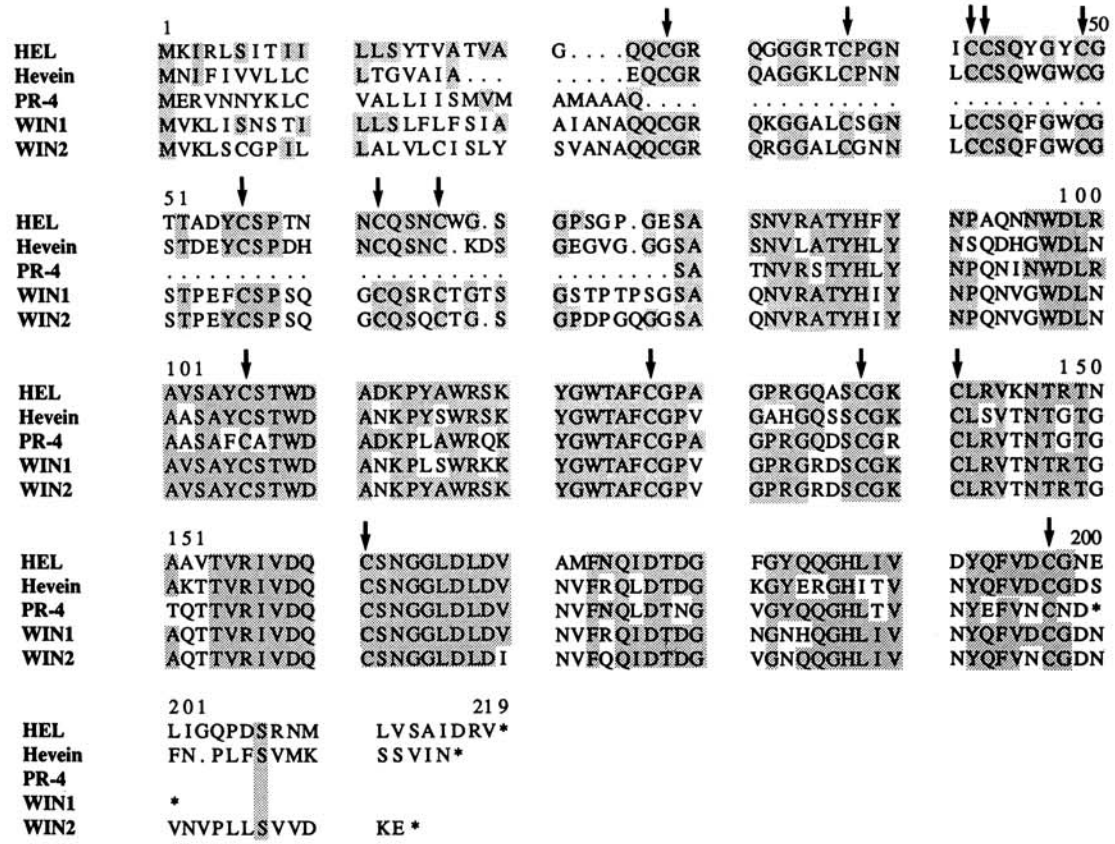

Fig. 2. Alignment of deduced amino acid sequences of hevein-like (HEL) mRNA, hevein (Broekaert *et al.* 1990), tobacco PR-4 acidic protein (Friedrich *et al.* 1991), and WIN1 (Stanford *et al.* 1989) and WIN2 (Stanford *et al.* 1989) proteins from potato. Vertical arrows indicate cysteines. Amino acids identical to HEL are shaded.

HEL, unlike PR-4 from tobacco (Friedrich *et al.* 1991), does not have this extension, it may be secreted to the vacuole.

Expression analysis.

Because structural homologs of HEL in other species can be induced by pathogen infection, we analyzed the response of the *HEL* gene to infection by TCV. *Arabidopsis thaliana* ecotype Di-0 exhibits a necrotic response to TCV infection (Simon *et al.* 1992) similar to the hypersensitive response of tobacco (Xanthi nc) to tobacco mosaic virus. Figure 3A shows gel blot hybridization of RNA isolated from TCV-infected (primary) tissue and noninfected (secondary) tissue from TCV-infected plants. Four days after TCV infection, *HEL* mRNA was induced 14- and 6-fold in the primary and secondary tissues, respectively. Infection of ecotype Col-0 with the compatible pathogen *Pseudomonas syringae* pv. *tomato* DC3000 induces *HEL* gene expression less than three-fold at 6 days after inoculation (data not shown).

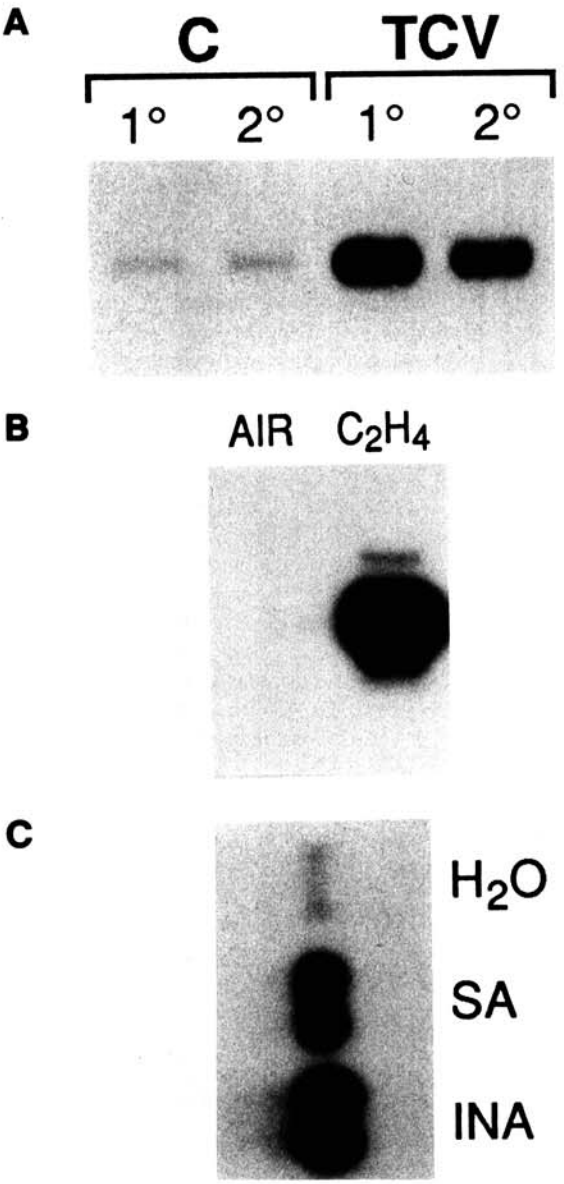

Fig. 3. Expression analysis of hevein-like (*HEL*) mRNA. **A,** Turnip crinkle virus (TCV) induction of *HEL* mRNA. Total RNA was isolated from mock-inoculated leaves (C) or from TCV-infected (1°) and noninfected (2°) leaves of TCV-infected plants 4 days after treatment. **B,** Ethylene induction of *HEL*. Total RNA was isolated from ethylene (C_2H_4)- or air-treated leaves 24 hr after treatment. **C,** Resistance activator induction of *HEL* mRNA. Total RNA was isolated from salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), and dH_2O-treated leaves 1 day after treatment. Blots were probed with the *HEL* cDNA shown in Figure 1.

Ethylene is associated with the plant response to pathogen infection (Ross and Williamson 1951). Figure 3B shows that ethylene was able to induce *HEL* mRNA abundance up to 108-fold after 24 hr of treatment. In contrast, ethylene does not induce PR-1, PR-2, or PR-5 mRNA abundance in *Arabidopsis* (K. Lawton, S. Dotter, S. Uknes, and J. Ryals, unpublished). Therefore, *HEL* appears to be regulated in a manner similar to that of the ethephon-inducible class I chitinase of *Arabidopsis* (Samac *et al.* 1990) and the basic isoforms of the tobacco PR proteins, which are induced by ethylene and also induced locally by pathogen infection (Abeles and Forrence 1970; Memelink *et al.* 1990; Boller 1991). Due to the compact rosette form of *Arabidopsis*, ethylene evolved from primary TCV lesions may cause the induction of *HEL* in secondary uninfected tissue, as has been observed in the response of tobacco to tobacco mosaic virus (De Laat and Van Loon 1983).

We also tested chemical agents known to induce SAR for their ability to induce expression of *HEL*. In *Arabidopsis*, SA and the synthetic resistance activator INA induce PR-1, PR-2, and PR-5 mRNAs more than 20-fold (Uknes *et al.* 1992). As seen in Figure 3C, INA was able to induce *HEL* mRNA abundance more than 10-fold at 1 day after treatment. SA induced

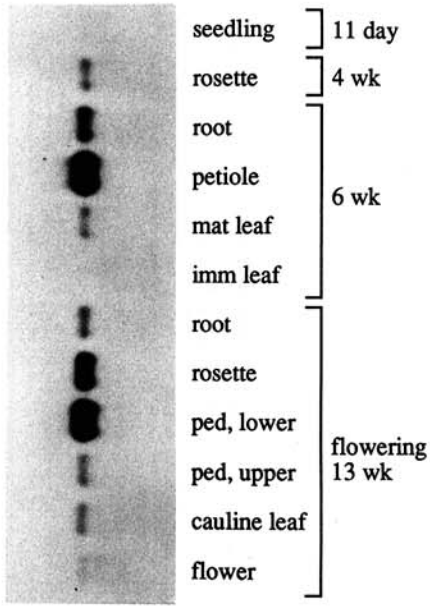

Fig. 4. Developmental expression of *HEL* mRNA. Total RNA isolated from vegetative and flower tissues at various stages during development was hybridized with the *HEL* cDNA shown in Figure 1. Four- and six-week-old plants had not initiated flowering under our growth conditions. Mat leaf = mature, fully expanded leaves without petioles; imm leaf = immature leaves with smaller rosettes; ped, lower = peduncles, lower portion; ped, upper = peduncles, upper portion and pedicels.

HEL mRNA more than fivefold at 1 day after treatment, with some variation resulting from changes in background expression levels of *HEL*. Thus, induction of *HEL* by INA and SA is substantially lower than has been observed for various SAR genes in tobacco (Ward *et al.* 1991a) and *Arabidopsis* (Uknes *et al.* 1992).

Hevein has been shown to be slightly induced by abscisic acid (Broekaert *et al.* 1990), and some PR genes have been suggested to be regulated by auxin and cytokinin (Antoniw *et al.* 1980; Shinshi *et al.* 1987; Memelink *et al.* 1990). However, when applied exogenously to entire plants, none of these growth regulators significantly increased *HEL* mRNA abundance (data not shown).

The PR proteins of tobacco are known to accumulate in leaves of healthy flowering plants (Fraser 1981). To determine whether *HEL* expression was developmentally regulated, we performed gel blot hybridization on RNA isolated from various tissues during different stages of plant development. Figure 4 shows that *HEL* mRNA is abundant in petioles and lower peduncles of flowering plants. Both tissue types contain a relatively high proportion of vascular tissue; whether this anatomy is relevant to *HEL* expression is unknown. *HEL* mRNA also appears at low levels in mature leaves of nonflowering healthy plants and, like *WIN2*, is weakly expressed in intact roots (Stanford *et al.* 1989). The expression of *HEL* in older leaves may be the result of increased ethylene production in senescing tissue (Yang and Hoffman 1984).

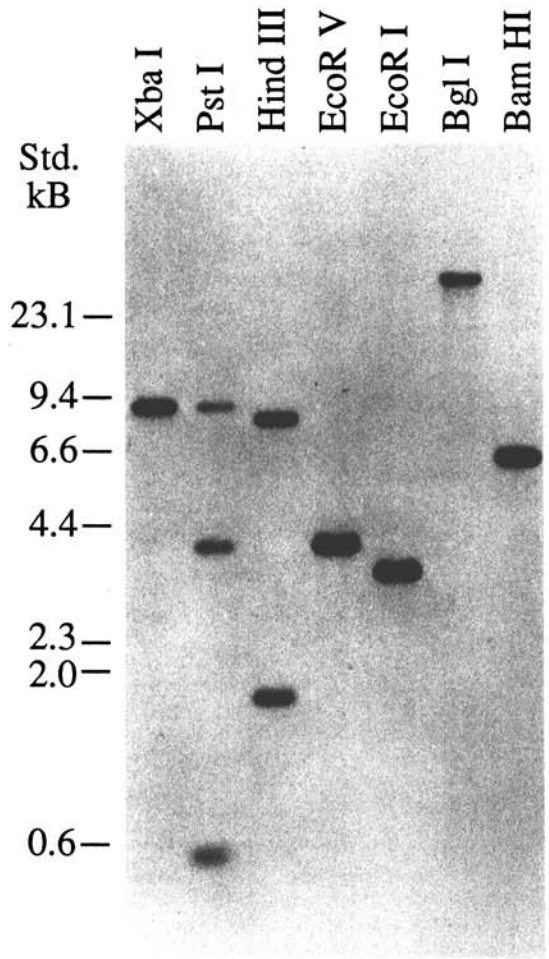

Fig. 5. Complexity of the *Arabidopsis HEL* gene family. DNA (1 μg) of *Arabidopsis thaliana* ecotype Col-0 was digested with the indicated restriction enzymes. The gel blot was probed with the *HEL* cDNA in Figure 1.

Complexity of the *HEL* gene family.

To analyze the genetic complexity of *HEL*, Southern blot analysis was performed on *Arabidopsis,* using various regions of the gene as probes at both high and low stringencies. The high-stringency hybridization profile using the entire cDNA clone to probe *A. thaliana* ecotype Col-0 DNA is shown in Figure 5. The *HEL* cDNA contains two *Pst*I sites and a single *Hin*dIII site; thus, all the bands present can be predicted from the cDNA sequence and are consistent with a single *HEL* gene. Hybridization of a similar blot at low stringency revealed several additional bands (data not shown). Using 5′ and 3′ fragments of the cDNA as probes at low stringency, we determined that the new bands are homologous to the 5′ lectin portion of the *HEL* gene only (data not shown). A lectin domain-containing *Arabidopsis* class I chitinase gene has been reported in the literature (Samac *et al.* 1990). However, when an identical blot was probed with the lectin domain of the *Arabidopsis* class I chitinase, the hybridization pattern did not correspond to the unidentifed bands detected by the *HEL* lectin domain at low stringency, indicating that the amino-terminal cysteine-rich domain of the chitinase was not sufficiently similar to that of *HEL* to be detected by cross-hybridization.

Genes similar to *HEL* in other plants are members of multigene families. The *WIN1* and *WIN2* family from potato contains at least five members per haploid genome (Stanford *et al.* 1989), and tobacco PR-4 belongs to a gene family of three to four members (Friedrich *et al.* 1991). Other *HEL* homologs may exist in *Arabidopsis*, but our data indicate that none are closely related. Because *HEL* appears to be a single gene, it represents an interesting prospect for investigating the regulation of expression of this class of genes.

Possible role for HEL in defense against pathogens.

Three lines of evidence suggest that HEL protein is involved in the response of *Arabidopsis* to pathogen infection. First, the predicted translation product shares extensive homology to known antifungal lectins, in particular, hevein. Second, pathogen infection induces *HEL* mRNA accumulation both locally and systemically. Third, ethylene, an inducer of antifungal activities in other species (Boller 1988, 1991), strongly induces *HEL* expression. Thus, the *HEL* gene product may comprise an antimicrobial activity that is inducible by physiological stimuli related to pathogen infection.

MATERIALS AND METHODS

cDNA isolation.

The *HEL* cDNA was isolated by screening a leaf tissue cDNA library (Uknes *et al.* 1992) at low stringency with a tobacco PR-4 acidic cDNA probe (Friedrich *et al.* 1991). Duplicate plaque lifts were taken with nitrocellulose filters (Schleicher & Schuell, Keene, NH) (Ausubel *et al.* 1987). Probes were labeled by random priming (Feinberg and Vogel-

stein 1983) with the Prime It kit (Stratagene, La Jolla, CA). Hybridization and washing were according to Church and Gilbert (1984) at 50° C. Positive plaques were purified, and plasmids containing the cDNA inserts were *in vivo* excised and sequenced (Hattori and Sakaki 1986). Sequence comparisons were performed using the GAP (Deveraux *et al.* 1984) and PILEUP programs (Genetics Computer Group, Madison, WI).

Expression analysis.

Total RNA was purified from frozen tissue samples by phenol-chloroform extraction followed by lithium chloride precipitation (Lagrimini *et al.* 1987). Samples (5 μg) were separated by electrophoresis through formaldehyde agarose gels and blotted to nylon membrane (GeneScreen Plus, NEN, Boston, MA) as described (Ausubel *et al.* 1987). Equal loading of lanes and equal transfer of RNA were confirmed by ethidium bromide staining. Hybridization and washing were at 65° C according to Church and Gilbert (1984). Fold-induction was determined using a Betascope blot analyzer (Betagen, Waltham, MA).

Southern analysis.

One microgram of *Arabidopsis* DNA was digested with each of seven restriction enzymes, two of which cut within the *HEL* cDNA. Digests were separated on an 0.8% agarose gel and blotted to nylon membrane (GeneScreen Plus, NEN) as described (Southern 1975). The *HEL* cDNA probe was labeled with the random-priming DNA labeling system (GIBCO BRL, Gaithersburg, MD). Hybridization conditions were the same as those used for the high-stringency RNA blots. Identical blots were probed at low stringency (50° C) with the lectin domain only, the PR-4 domain only, or the entire *HEL* cDNA. An additional blot was probed with the polymerase chain reaction-amplified lectin domain of *Arabidopsis* class I chitinase (Samac *et al.* 1990) at high stringency.

Plant growth and treatment.

Plant growth conditions were as described (Uknes *et al.* 1992). Best results were obtained when plants were not watered immediately before chemical treatment. For chemical induction experiments, 4- to 6-wk-old plants were sprayed to imminent runoff with water, 0.65 mM INA (25% a.i. in wettable powder), or 5 mM SA. Leaves were harvested 24 hr after treatment. Ethylene induction was performed by placing plants in 60-L chambers containing air only or 100 ppm of ethylene for 24 hr. *A. thaliana* ecotype Col-0 (Columbia) was used for all experiments except pathogen induction. TCV-M infection of *Arabidopsis* ecotype Di-0 (Dijon) has been described (Simon *et al.* 1992; Uknes *et al.* 1993). Primary and secondary leaves of bentonite- or TCV-treated plants were harvested 4 days after infection. For the developmental time course, aerial portions of seedlings that had developed two true leaves were harvested at 11 days after planting. Other developmental samples were collected from various organs at 4 and 6 wk and during flowering at 13 wk. Four- and six-week-old plants had not initiated flowering under our growth conditions. Thirteen-week-old plants had multiple inflorescences and siliques and had initiated leaf senescence.

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