4-1-1998

Requirement for the induced expression of a cell wall associated receptor kinase for survival during the pathogen response

Zheng Hui He
Duke University

Deze He
Duke University

Bruce D. Kohorn
Duke University

The Plant Journal (1998) **14**(1), 55–63

Requirement for the induced expression of a cell wall associated receptor kinase for survival during the pathogen response

Zheng-Hui He, Deze He and Bruce D. Kohorn*
Developmental, Cell and Molecular Biology Group, Box 91000, Duke University, Durham, NC 27708, USA

Summary

Pathogen infection of angiosperms must rely on some interaction between the extracellular matrix (ECM) and the invading agent, and may be accompanied by signaling between the ECM and cytoplasm. An *Arabidopsis* cell wall associated receptor kinase (Wak1) has an amino-terminal domain that is tightly associated with the ECM, spans the plasma membrane and has a cytoplasmic protein kinase domain. *Wak1* expression is induced when *Arabidopsis* plants are infected with pathogen, or when the pathogen response is stimulated either by exogenous salicylate (SA) or its analog 2,2-dichloroisonicotinic acid (INA). This *Wak1* induction requires the positive regulator NPR1/NIM1. Thus Wak1 is a pathogen-related (PR) protein. Expression of an antisense and a dominant negative allele of *Wak1* shows that induced expression of *Wak1* is needed for a plant to survive if stimulated by INA. Ectopic expression of the entire Wak1, or the kinase domain alone, can provide resistance to otherwise lethal SA levels. These experiments suggest that Wak1 expression and other PR proteins are protecting plants from detrimental effects incurred during the pathogen response. These results provide a direct link between a protein kinase that could mediate signals from the ECM, to the events that are precipitated by a pathogen infection.

Introduction

The plant cell wall, or extracellular matrix (ECM), is composed of a number of complex carbohydrates and proteins and forms a continuous structure within the tissue of angiosperms (Carpita *et al.*, 1996; Showalter, 1993; Varner and Lin, 1989; Wyatt and Carpita, 1993). The cell wall is dynamic during plant growth and development and mediates interactions with the environment, which in some cases have profound effects on the plant cell (Carpita and Gibeaut, 1993; Roberts, 1994). Pathogen infection of angiosperms can lead to either a hypersensitive response (HR) that is accompanied by local cell death, or to the general induction of systemic acquired resistance (SAR) (Baker *et al.*, 1997). The first contact of a pathogen with a plant must include some form of interaction with the cell wall. Pathogen infection that leads to HR or SAR in *Arabidopsis* and other vascular plants can be associated with a number of cellular changes including cell wall modifications, and the production of reactive oxygen species and phytoelexins (Baker *et al.*, 1997). In SAR, cytoplasmic and systemic levels of salicylate (SA) rise and the expression of pathogen-related (PR) proteins is induced (Hunt *et al.*, 1996). The role of SA in this response remains unknown, although the PR proteins are thought to be involved in directly mediating the plant's response to infection (Chen *et al.*, 1993; Delaney *et al.*, 1994). Much attention has been focussed on those proteins that confer resistance to pathogen infection, and little is known of what communication might occur between the cell wall and plasma membrane when the plant first encounters the pathogen.

Proteins thought to mediate contact between the cell wall and plasma membrane may a priori be expected to be involved in the pathogen response as the first exposure to the pathogen is through the cell wall. A number of genes have been isolated that are predicted to encode proteins that have the potential to mediate cell wall–plasma membrane interactions (Herve *et al.*, 1996), but these predictions remain to be tested. More thoroughly characterized proteins that could mediate cell wall– plasma membrane interactions include the extensin family (Carpita *et al.*, 1996; Miller and Fry, 1993; Wyatt and Carpita, 1993), and the glycine- (Kelley *et al.*, 1988), proline- (Carpita *et al.*, 1996) or arabinogalactan- (Komalavilas *et al.*, 1991) rich proteins, but these may only serve as an adhesive link. A cell wall associated receptor kinase (Wak1) spans the plasma membrane and has a cytoplasmic kinase and an amino-terminal domain tightly bound to the cell wall (He *et al.*, 1996). The extracytoplasmic domain of Wak1 contains several epidermal growth factor repeats (EGF) and limited regions of identity with ECM proteins of metazoans (He *et al.*, 1996; Kohorn *et al.*, 1995). Wak1 could therefore mediate cell wall–cytoplasm signaling. We demonstrate that pathogen induces the expression of *Wak1*, and thus Wak1 is an *Arabidopsis* PR protein. We also demonstrate that this pathogen-induced expression of *Wak1* is required for the plant to survive the cellular response to the patho-

Received 10 October 1997; revised 12 January 1998; accepted 12 January 1998.
*For correspondence (fax +1 919 6138177; e-mail kohorn@acpub.duke.edu).

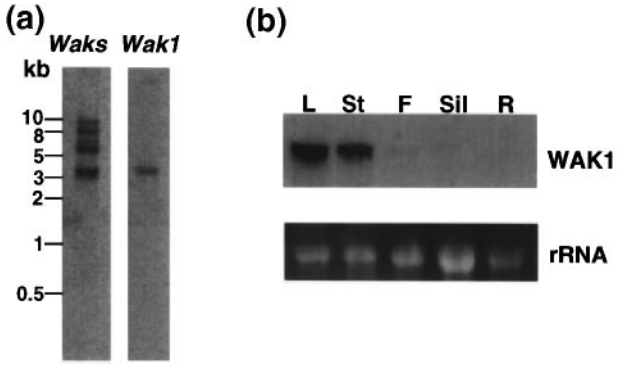

Figure 1. *Wak1*-specific probe.
(a) Autoradiogram of *Hin*dIII-digested *Arabidopsis* genomic DNA probed with either a general Wak probe (Waks) or a Wak1-specific probe (*Wak1*). Size in kb is shown on the left.
(b) Autoradiogram of total *Arabidopsis* RNA from leaves (L), stems (St), flowers (F), siliques (Sil) and roots (R) hybridized to a *Wak1*-specific probe. The ethidium bromide stain of 28S rRNA for each lane is shown below.

gen. Thus the expression of a cell wall-associated receptor kinase is involved in the pathogen response.

Results

Wak1 *mRNA is pathogen inducible*

Wak1 protein can be detected in most tissues of *Arabidopsis* in most cell types (He *et al.*, 1996). While *Wak* mRNA expression is most abundant in green tissues such as stems and leaves (Kohorn *et al.*, 1992), much lower levels of mRNA can be seen in flowers, siliques and roots (Z.-H. He, I. Cheeseman and B. D. Kohorn, unpublished data). Recently we have identified four other *Wak*-like genes that, along with *Wak1*, are clustered within a 30 kb region. Partial sequence analysis of this locus indicates that the kinase domains are 90% identical and thus it is likely that the Wak1 antiserum directed against the kinase domain would react with all four isoforms, if they are expressed. Moreover, the *Wak1* probe used in our initial studies hybridizes with all of the five coding regions that are separated by *Hin*dIII digestion (Figure 1a; *Wak*s). We have attained sufficient sequence to generate, by PCR, a *Wak1*-specific probe derived from the amino-terminal coding region. The analysis of the other Wak isoforms and the generation of gene-specific probes awaits the completion of the sequence of the 30 kb locus. The *Wak1*-specific probe detects one *Hin*dIII-digested band on a genomic Southern blot (Figure 1a; *Wak1*), and the sequence of this 3.4 kb band shows that it contains only one Wak-coding region (Z.-H. He, I. Cheeseman and B. D. Kohorn, unpublished data). Figure 1(b) shows that this *Wak1*-specific probe detects mRNA in leaves and stems, to a far less extent in siliques and flowers, and not in roots.

To determine if any environmental conditions could induce the production of *Wak1* mRNA we isolated total

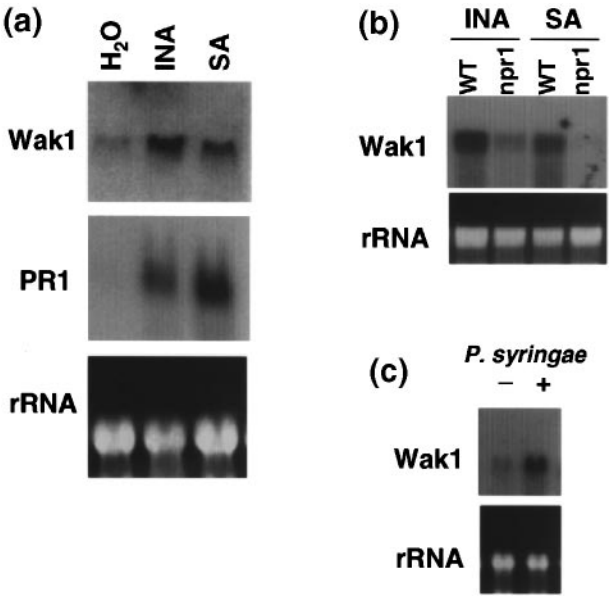

Figure 2. (a) *Wak1* mRNA is induced by INA and SA.
Total RNA was isolated 3 days after plants were sprayed with either H_2O, 1.5 mM INA or SA, analyzed by Northern blotting, and then probed with either ^{32}P-labeled *Wak1* or PR1. The top two panels show autoradiograms, and the bottom panel (rRNA) is the 28S rRNA stained with ethidium bromide.
(b) Total RNA was isolated from wild-type or *npr1* mutant plants that had been sprayed with 1.5 mM INA or SA. The RNA was analyzed by Northern blotting and probed with ^{32}P-labeled *Wak1*.
(c) Total RNA from wild-type plants either mock (–) or infected (+) with *P. syringae* was probed by Northern blot with ^{32}P-labeled *Wak1*.

RNA from whole plants that had been subjected to a variety of stimuli. We then performed Northern blot analysis of this RNA using the *Wak1*-specific probe, and equal loading of RNA was monitored by ethidium bromide staining of the rRNA. Heat and salt stress had no effect on *Wak1* mRNA and wounding produced a decline in the steady state levels (data not shown). Most striking was the induction of *Wak1* message by 2,2-dichloroisonicotinic acid (INA) (Figure 2a). INA is an analog of naturally occuring SA, which is thought to mediate the transduction of cellular signals initiated by pathogen infection. Both exogenous SA and INA can substitute for pathogen to induce a pathogen resistance response in angiosperms. This response includes the induction of three classes of *Arabidopsis* pathogenesis-related (PR) genes, *PR1*, *PR2* and *PR5*, over a period of 3 days. The same Northern blot was also probed for the PR1 mRNA, and Figure 2(a) demonstrates that this mRNA was induced. The *Wak1* message also accumulated upon INA induction, and was reproduced by application of SA (Figure 2a). INA appears to be more effective in inducing *Wak1* mRNA than SA, which is the reverse of that seen for PR1, although both are inductive. These results indicate that *Wak1* is a PR gene in *Arabidopsis*.

NPR1 is thought to be a positive regulator required for the SA-dependent activation of the previously identified PR

genes (Cao *et al.*, 1997). We therefore determined whether *Wak1* expression was affected by the *npr1* mutation that eliminates NPR1 activity. Figure 2(b) shows that in a plant homozygous for the *npr1* mutation the induction of *Wak1* mRNA by INA or SA is greatly reduced. Thus *Wak1* mRNA is probably induced by a similar mechanism as *PR1*, *PR2* and *PR5*, and its expression is coupled to events known to regulate the pathogen response.

As *Wak1* mRNA expression was now correlated with the PR response, we determined if the message could be induced by infection with pathogen. Wild-type plants were inoculated with *Pseudomonas syringae maculicola* ES4326 (compatible), allowed to incubate for 3 days, and then total RNA was isolated and probed for *Wak1* mRNA by Northern blotting. Figure 2(c) shows that *P. syringae* could also induce *Wak1* mRNA. To see if the mRNA induction was followed by an increase in *Wak1* protein synthesis, total protein extracts were analyzed by Western blotting. There was only a slight change in the steady state levels of Wak1 after stimulation (see below). While this is surprising given the levels of induced mRNA, pre-existing Wak1 protein is extremely abundant (He *et al.*, 1996), and any small, induced change in protein accumulation may be masked. Alternatively the antiserum may be detecting other Wak isoforms; we have recently found at least four other tightly linked, highly similar *Wak* genes and these may provide a basal background that also masks changes in Wak1 protein expression (Z.-H. He, I. Cheeseman and B. D. Kohorn, unpublished data). The Northern blots shown in this work were produced using a probe that is known to be *Wak1* specific (Figure 1a), but we have yet to determine to what extent the other Wak genes contribute to the protein profiles. These results do suggest that since the *Wak1* message is induced by pathogen, INA and SA, it may be required for the response to pathogen.

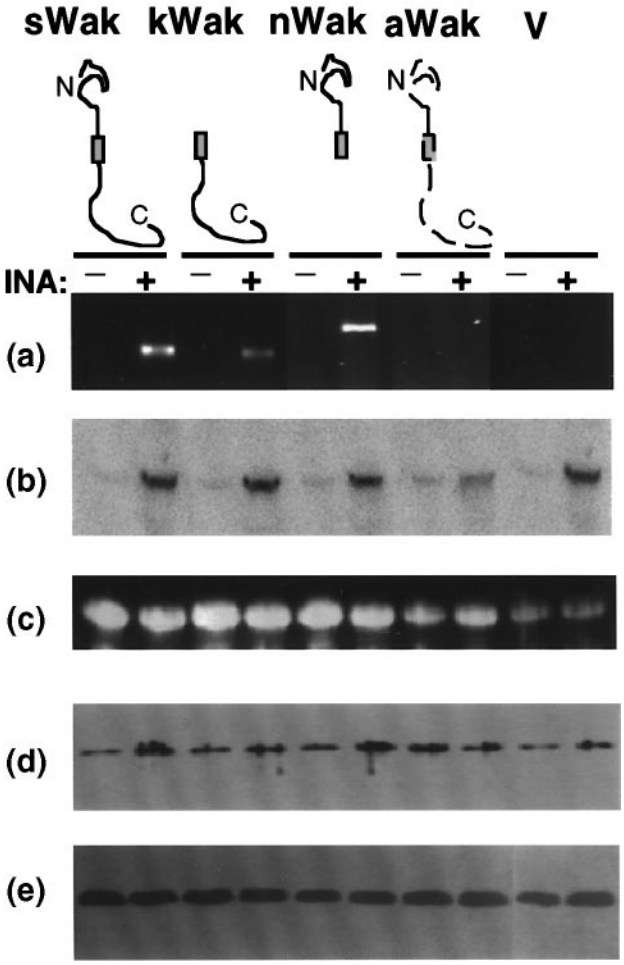

Figure 3. Expression of introduced and endogenous *Wak1* alleles.
Cartoons and names of the *Wak1* alleles transformed into plants are shown above each lane. RNA from each plant [grown in medium containing (+) or not containing (–) inducer] was analyzed by RT-PCR using transgene-specific primers (a), or by Northern blotting and hybridization with a probe specific for the endogenous *Wak1* mRNA (b).
(c) Ethidium bromide staining of 28S rRNA. Total protein was extracted from each plant, Western blotted and probed with Wak1 polyclonal antiserum (d). The same protein samples shown in (d) were probed with cytochrome f antiserum (e) (Smith and Kohorn, 1994).

Wak1 *mRNA induction is required for plant survival during the PR*

The expression of both antisense and dominant negative mutations in transgenic plants can be used to gain an understanding of the function of a given protein. To see if disruption of *Wak1* function had any effect on plant growth and development, we expressed several *Wak1* mutant alleles in transgenic *Arabidopsis*. Our initial experiments used the 35S cauliflower mosaic virus promoter to drive the *Wak1* expression as this is normally constitutively active in most tissues (Holtorf *et al.*, 1995). However, any transformed plants that were recovered failed to express the introduced *Wak1* alleles. We therefore turned to the PR1 promoter that is induced by INA, and remains induced as long as INA is present (Uknes *et al.*, 1993). Plants could first be transformed and selected for a kanamycin marker, and then induced for expression of the various new *Wak1* alleles. These studies were initiated before we knew that endogenous *Wak1* mRNA was INA inducible, although we did establish that INA and SA had only a small effect on wild-type levels of Wak1 protein in wild-type plants. Moreover, to our knowledge the PR1 promoter system continues to be the least intrusive, most available inducible system in *Arabidopsis*.

Four *Wak1* alleles were constructed and introduced into *Arabidopsis* such that their expression could be driven by the PR1 promoter. The 3′ untranslated region (3′UTR) from the *Agrobacterium tml1* gene was used for all of these new alleles to facilitate the detection of the expression above endogenous *Wak1* expression. The four alleles studied are shown in Figure 3, where the Wak1 protein domains expected to be expressed are shown in cartoon

form, and antisense (aWak) is shown as a dotted line. Wak1 is normally expressed as a plasma membrane protein with a cytoplasmic kinase domain and an amino-terminal domain tightly bound the cell wall (He *et al.*, 1996). The cDNA sequence (Kohorn *et al.*, 1992) predicts only one hydrophobic region that could function as a signal sequence for translocation, and this sequence serves as the transmembrane domain (He *et al.*, 1996); all introduced alleles contained this sequence. nWak encodes only the transmembrane domain and the extracytoplasmic region, kWak encodes the transmembrane domain and the cytoplasmic kinase, and sWak encodes the entire protein. The control plants (designated V) were transformed with the *Agrobacterium* vector that included the PR1 promoter, the *tml1* 3′UTR, but no *Wak1* sequence. Six independent transformed lines were generated for each construct, and each of these was selfed for two generations to produce a T3. T3 plants homozygous for the new *PR1–Wak1* alleles were identified by PCR and kanamycin segregation analysis. All six representatives of each type of transformation showed the same phenotype (see below) and this phenotype remained linked to the transforming DNA through several generations produced through selfing. One plant population derived from one individual representative of each type of transformation was analyzed by RT-PCR using specific *tml* and *Wak1* probes, and these results are shown in Figure 3(a). RT-PCR was used as Northern blotting with the *tml* probe-provided signal in untransformed wild-type plants. In each transformant, the transgene was not expressed when the plants were grown on MS (normal media, –INA), but addition of 0.1 mM INA (+INA) to the media induced transgene expression within 3 days. The fold increase could not be calculated as there was no mRNA detected on MS medium (– INA), although we assume the message is not abundant as the appropriate size mRNA was barely detected on Northern blots (data not shown). The expression of the endogenous *Wak1* gene was also determined by Northern blot, and this is shown in Figure 3(b). In the nWak, sWak, kWak and V lines, endogenous *Wak1* mRNA was induced by INA. However, in the plants that expressed the antisense *Wak1* allele (aWak, +INA), the endogenous *Wak1* mRNA remained at a similar level to that found in the uninduced plant (aWak, –INA). Equal RNA loading was visualized by rRNA (Figure 3c); loading was equal when compared between the + and –INA of each plant, but was lower for the wild type overall.

We next investigated if the changes in *Wak1* mRNA levels found in the transgenic plants resulted in the alteration of steady state amounts of Wak1 protein. Whole plant extracts were run in polyacrylamide denaturing gels, blotted to nitrocellulose and probed with Wak1 polyclonal antiserum, and the results are shown in Figure 3(d). In no case was the change in Wak1 protein as dramatic as the corresponding mRNA. It may be that the pre-existing levels of Wak1 protein and Wak isoforms are extremely abundant, making any small, induced change in protein accumulation hard to detect. Nevertheless, some changes were found in some of the transformed lines. There was only a small INA-induced increase of endogenous Wak1 in vector-alone transformed plants (V). The sWak-transformed plants had an increased Wak1 level, as predicted from the increase in transgene expression. kWak should be expressed as a smaller protein but this was not detected on the Western blot; the Wak1 serum recognized only the kinase domain, and thus nWak expression could not be detected. In plants expressing the antisense (aWak) allele, Wak1 expression was reduced slightly upon INA induction. Equal loading of total protein was monitored by the detection of cytochrome f (Figure 3e).

Transformants homozygous for the transgenes (six representative lines for each construct) were plated on either MS or MS + INA, and the growth of representative plants is shown in Figure 4. The control vector (V), sWak and kWak alleles had no detectable effect on plant growth. However, while the antisense (aWak) and nWak transformants live on MS (data not shown), they bleached within 7 days on 0.1 mM INA and died after an additional 7 days. Replating onto MS after 7 days of incubation on 0.1 mM INA allowed the aWak and nWak plants to regreen and eventually set seed. Since the antisense plants die on 0.1 mM INA, yet still have levels of *Wak1* mRNA more similar to untreated plants (Figure 3b), it is likely that lethality of the antisense is not simply due to complete elimination of *Wak1*; expression of aWak eliminates the INA induction of the endogenous *Wak1* gene but still allows basal endogenous *Wak1* mRNA expression. This suggests that the induction of *Wak1* may be essential for a plant to survive the PR response. This is consistent with the fact that INA induces the PR response. Figure 4 also shows that the ectopic expression of nWak, like the antisense expression, has a lethal effect on INA, but not MS. As the nWak construct has no effect on the INA induction of endogenous *Wak1* mRNA (Figure 3b), it appears that the lethality is caused by the truncated protein acting through a dominant negative mechanism, although this remains to be shown since the antiserum is not Wak1 specific and we cannot localize protein. These data suggest that the requirement for *Wak1* induction seen on INA may be via a protein product and not only RNA. None of the transformants showed any increased sensitivity or resistance to infection by *P. syringae* (data not shown).

Wak1 expression causes SA resistance

Abnormally high levels of both SA or INA are lethal to plants. If *Wak1* induction is indeed required for a plant to survive a pathogen response then an increase in *Wak1*

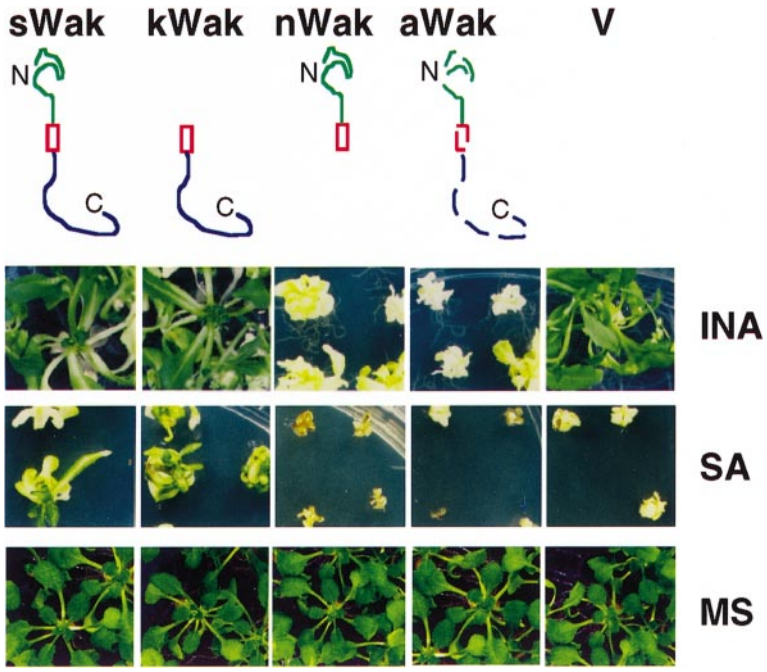

Figure 4. Phenotypes of plants transformed with *Wak1* alleles.
The *Wak1* allele expressed in each plant is shown in cartoon form above each plant. Transformants having *Wak1* alleles nWak, sWak and aWak or vector alone (V) were grown on MS medium containing 0.1 mM INA or 0.5 mM SA for 7 days.

expression may be expected to cause the plant to become more resistant to otherwise lethal doses of SA. The transformants were therefore plated on media that contained 0.5 mM SA, a dose that kills wild type and vector-alone transformed seedlings (Figure 4,V–SA). As the antisense *Wak1* (aWak) and nWak transformed lines die on 0.1 mM INA, it was not surprising that they were also killed by 0.5 mM SA. However, ectopic expression of sWak and kWak allowed these plants to grow and set seed on 0.5 mM SA (Figure 4). Thus the prediction that induced *Wak1* expression causes increased SA resistance was confirmed.

To determine whether ectopic expression of sWak reduced the endogenous levels of SA and thereby provided resistance, we measured both free and total SA. Measurements were made in tissue exposed for 7 days, just prior to the onset of significant bleaching of the wild type. Table 1 shows the SA levels averaged from three samples of vector-alone (V) or sWak transformants, both grown on either MS or MS + 0.5 mM SA. The SA levels of all plants grown on MS were similar. Surprisingly, the sWak transformants had two- to threefold higher levels of total or free SA than V-transformants when grown on SA. The relative increase may reflect the fact that at the time of harvest the wild-type plants are not yet visibly necrotic, but a portion of the tissue may in fact be dying to SA toxicity; this would underestimate the true levels of SA in these tissues. These results indicate that ectopic expression of *Wak1* does not directly reduce endogenous SA levels.

Table 1. SA levels in transgenic and wild-type plants

	V		sWak	
SA	–	+	–	+
Free SA (ng g^{-1})	549.8 (±13.8)	30 171.8 (±439.5)	432.8 (±118.5)	74 797.2 (±4677.0)
Total SA (ng g^{-1})	1298.8 (±176.4)	434 755.8 (±26 184.1)	744.2 (±31.0)	1 203 074.9 (±212 816.4)

Free (unconjugated O-glucoside) and total SA (ng g^{-1} wet weight tissue) was measured from whole plant tissue [vector-transformed (V) and sWak transformants] grown in the absence (–) or presence (+) of exogenous SA in the medium. Numbers indicate the average of three samples; ± indicates standard deviations.

The resistance seen must therefore result from a different mechanism.

Wak1 affects PR1 expression

The response to pathogen is associated with an increased level of SA and the induced expression of the PR proteins. It is possible that *Wak1* influences the effects of SA by altering PR protein expression. To address this possibility total RNA was isolated from wild-type and transgenic plants expressing the antisense *Wak1* allele (aWak) grown in the presence or absence of INA. The RNA was Northern

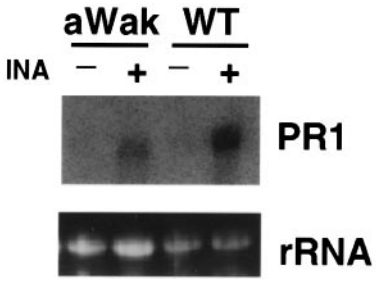

Figure 5. PR1 expression is affected by antisense *Wak1*.
Total RNA was extracted from vector-alone transformed (WT) or antisense *Wak1* (aWak) plants grown on MS or MS plus 0.1 mM INA, Northern blotted and probed with ^{32}P-labeled PR1 DNA. An autoradiogram is shown.

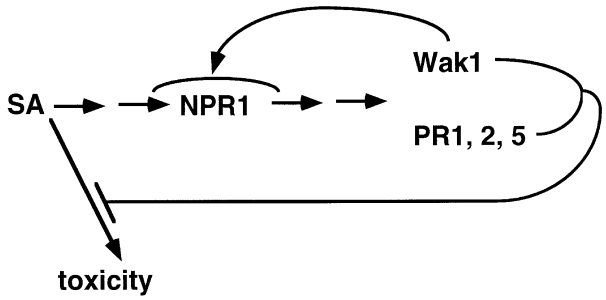

Figure 6. Model for interaction of Wak1 and the pathogen response.
SA is thought to influence the positive regulator NPR1 that is required for *PR1, PR2, PR5* and *Wak1* mRNA induction. PR1, PR2, PR5 and Wak1 may inhibit the toxic affect of SA, and Wak1 may influence NPR1 at some point in the transduction path. See the Discussion for details.

blotted and probed with the PR1 cDNA. Figure 5 shows that, relative to the wild type, PR1 mRNA induction was reduced but not eliminated in plants that expressed aWak.

Discussion

We found that *Wak1* mRNA was induced by INA, SA and *P. syringae* infection. This suggests that *Wak1* mRNA may be needed in some capacity for the response to pathogen. Wak1 is therefore a PR protein, and its induced expression requires the positive regulator NPR1. We have shown that elimination of *Wak1* mRNA induction, via antisense *Wak1* expression, results in plants that cannot survive on otherwise non-toxic levels of INA. Similar results are obtained with non-toxic levels of SA. This implies that *Wak1* mRNA induction is required for a plant to survive a pathogen response. Alternatively these results may suggest that *Wak1* is simply required for plant viability under any conditions. This is less likely an explanation of the antisense phenotype (death on INA) since the levels of *Wak1* mRNA in the INA-induced antisense plants were similar to the levels in the uninduced wild-type, live plants. This does not exclude the possibility that a plant may require a basal level of *Wak1* expression to function normally. It would be best to induce the expression of the *Wak1* alleles by an agent not associated with the resulting phenotype. We used the INA-inducible PR1 promoter since it was the only one available as attempts to employ a constitutive promoter to drive *Wak1* alleles were not successful. Moreover, our initial Western blots indicated only a small influence of INA on Wak1 protein expression.

Our results suggest that the induction of *Wak 1* mRNA is protecting the plant from the effects of a pathogen response. We predicted that increased *Wak1* expression may protect a plant from otherwise lethal doses of SA or INA, and this can indeed be achieved through the ectopic expression of either the entire Wak1 protein, or the kinase domain alone. The mechanism for this protection is at present unknown, although since the expression of the Wak1 cytoplasmic kinase is effective in SA resistance it suggests that this domain may be involved in activating cytoplasmic events. One simple explanation is that induced *Wak1* expression leads to decreased SA levels, thereby reducing toxicity. However, the SA levels appeared two- to threefold higher and thus alternative mechanisms need not be invoked. The two- to threefold higher levels may also be misleading as tissue was necessarily harvested at times before SA became visibly lethal, but cell death may have occured in the wild-type plants resulting in apparent different levels with respect to the sWak plants. As these initial studies failed to provide any evidence of a reduction in SA levels by sWak expression, and since an apparent increase was contrary to the proposed mechanism, additional measurements on the other transgenic plants were not done. Measurements of reactive oxygen species and oxidized cellular components may give clues as to how *Wak1* is able to influence SA affects, although this is unlikely given the observation that H_2O_2 is not a second messenger of SA (Hunt *et al.*, 1996). Rather than hunt for cellular changes we plan to seek interactions between the *Wak1* alleles and known mutations in the SAR response pathway (Baker *et al.*, 1997; Hunt *et al.*, 1996). Mutations in *NPR1* or its allele *NIM1* that result in the reduction of PR1, PR2 and PR5 induction are also sensitive to normally non-toxic amounts of SA (Cao *et al.*, 1994; Delaney *et al.*, 1994). Since *npr1* causes a decrease in *Wak1* mRNA, this sensitivity may be due to the additional absence of *Wak1* induction. Alternatively, some or all of the PR proteins may be required to protect the plant from the toxicity effected by increased SA levels, and this is diagrammed in Figure 6. This function of the PR proteins, including that of Wak1, may not be an exclusive role.

Antisense (aWak) expression not only leads to death during INA induction, but also to the reduction of the expression of PR1. As NPR1 is a positive regulator of PR1 expression, it suggests that Wak1 may influence this activation. This feedback effect, shown in Figure 6, may act either upstream, downstream or at NPR1 directly. In aWak transgenic plants PR1 induction is not reduced to the

same extent as Wak1, suggesting that the Wak1 feedback on the NPR1 pathway may be synergistic but not essential for PR expression.

The induced expression of the *Wak1* alleles in plants leads to the induction of their mRNA, but we have not differentiated the transgenic protein from the endogenous Wak1. The polyclonal Wak1 serum is likely to recognize all of the multiple Wak isoforms, as they have a high degree of identity in the kinase domain (Z.-H. He and B. D. Kohorn, unpublished data), and until isoform-specific antiserum is generated we cannot determine the respective contribution of each of the endogenous Wak genes. A specific epitope tag is needed to determine where the nWak is localized in the cell, as the current Wak1 antiserum is directed toward epitopes within the kinase domain. nWak should contain sufficient targeting information as the signal sequence is predicted to lie in the transmembrane domain (He *et al*., 1996; Kohorn *et al*., 1992). Expression of the smaller kWak (the kinase domain alone) has a phenotypic effect, but we have not detected this smaller protein in whole cell extracts. Pulse chase and immunoprecipitation may be required to detect this labile species, although the limited solubility of Wak1 renders these experiments extremely difficult (He *et al*., 1996). In plants expressing the sense *Wak1* (sWak) there is a slight increase in total Wak1 protein after INA induction, and we assume that this increase comes from the contribution of the transgene as this rise is greater than seen in wild-type plants. Fusion proteins that include the GFP should help to distinguish introduced protein from the endogenous, and this may help to determine if the genetic effect of ectopic expression of the *Wak1* alleles is indeed due to alteration of protein localization or function. The levels of total Wak1 protein declined in the antisense (aWak) plant, and this is consistent with the reduction in mRNA levels. We could not detect the antisense message and this may be due to lability that occurs when the antisense mRNA is associated with its target message. It is highly unlikely that any of the affects we observed are due to co-suppression or transcription/translation factor dilution, as then each of the different transgenes would have provided similar phenotypes and this was not the case. Our results are represented by six independent transformation events for each construct, and thus represent a transgene effect and not one peculiar to individual transformation events.

Expression of any of the *Wak1* alleles had no affect on the resistance or susceptibility of *Arabidopsis* to *P. syringae* infection. We have not determined if other pathogens are affected, but we note that there are at present no known resistance genes that are tightly linked to the *Wak1* locus, which maps to the upper arm of chromosome 1 (Z.-H. He and B. D. Kohorn, unpublished data). The lack of *Wak1*-related resistance is not surprising, however, as our results suggests that Wak1 is itself not interacting with the pathogen, but rather is required by the plant during the response to the pathogen.

We have suggested that since Wak1 has both a tight association with the cell wall and is a cytoplasmic kinase, it could serve to mediate signals between the two compartments. The findings reported here also demonstrate that *Wak1* has an essential role in maintaining plant function during a pathogen response. Why *Wak1* induction is needed during the pathogen response is not clear, although we have noticed that PR1 and PR5 are secreted cell wall proteins that localize in the same cellular compartment as the amino-terminus of Wak1 (He *et al*., 1996; Hunt *et al*., 1996). The pathogen response is coupled to a number of changes in the cytoplasm including an oxidative burst. If a pathogen were to activate pathways that alone provide resistance, but are also subsequently damaging to the cell, activation of the Wak1 pathway may either prevent such damage or activate a radical scavenging system. Alternatively the synergistic activation of a Wak1 pathway may be required to abate the activation of certain events stimulated by pathogen or SA. The involvement of a receptor-like protein kinase that is induced by pathogen also remains to be established (Wang *et al*., 1996). All modes of action predict that Wak1-associated activity may well play a role in normal development in the absence of the pathogen response, in a capacity where changes in the cell wall can be transmitted to the cytoplasm. Changes in the cell wall have indeed been linked to oxidative bursts and cellular physiologies that can have similarities to those observed for pathogen interactions (Baker *et al*., 1997). This reasoning is in agreement with the emerging concept that other pathogen-related proteins, even in organisms as diverse as *Drosophila* (Lemaitre *et al*., 1996), have normal cellular functions (Wilson *et al*., 1997).

Experimental procudures

Plant growth conditions

Arabidopsis thaliana ecotype Columbia was either grown in pots on Metro-Mix 220 growing medium (Grace Sierra Horticultural Products Company, Milpitas, CA) or on Petri plates with MS (Murashige and Skoog, 1962) under a cycle of 14 h of light and 10 h of darkness at 22°C. All seeds were cold-treated at 4°C for more than 48 h before placement in the growth environment. INA 0.1 mM or SA 0.5 mM was included in the agar MS medium where indicated. Alternatively, plants were sprayed with 1.5 mM INA or SA as indicated.

Construction of transgenic lines containing different Wak1 *alleles*

The transformation vector (pWCT) that contains the *Arabidopsis* PR1 gene promoter between the T-DNA borders was kindly provided by Dr John Ryals of CIBA-GEIGY Corp. This binary vector has the 4.4 kb PR1 promoter and accepts downstream 5′ *Xho*I–*Eco*RI

3′ fragments. The *Eco*RI site is flanked on its 3′ border by the 3′ untranslated region (UTR) of the *tml1* gene of *Agrobacterium*. pWCT also contains the eukaryotic kanamycin resistance marker within the T-DNA borders. Four different constructs were generated where each used the PR1 5′UTR and the *tml* 3′UTR and differed only in the amount of *Wak1* coding sequence. The initiating methionine was provided by the PR1 sequence. sWak: full-length *Wak1* coding sequence, amino acids (aa) 1–602. kWak: transmembrane domain and the kinase domain, aa 209–602. nWak: extracellular domain and the transmembrane domain, aa 1–246. aWak: antisense copy of *Wak1* including the entire *Wak1* cDNA in reverse orientation. All constructs were initially cloned using PCR-generated Wakl fragments (with Pfu polymerase; Boehringer Mannheim) in *Escherichia coli* XL-1-Blue, and confirmed by sequencing. *Agrobacterium* (strain GV3101) carrying each construct was used to transform 4-week-old *A. thaliana* by vacuum infiltration (Bechtold *et al.*, 1993). Transformants were identified by growing plants on MS plates containing kanamycin (50 μg ml^{-1}). For each construct six independent lines were selected. Homozygous transformants (T3) were confirmed by both segregation and PCR analysis and were analyzed for phenotypes and gene expression.

RNA extraction and Northern blot analysis

Tissues were frozen in liquid N_2 and total RNA was isolated by an 80°C phenol extraction method (Verwoerd *et al.*, 1989). Twenty micrograms of total RNA was fractionated through a formaldehyde agarose gel and blotted to a hybridization membrane (Hybond N^+; Amersham) (He *et al.*, 1996). Ethidium bromide (40 μg ml^{-1}) was added to each sample to allow visualization of RNA under UV light for confirmation of equal loading. ^{32}P-labeled DNA probes: a *Wak1*-specific probe was prepared using primers to the amino-terminal coding region and PCR with Taq polymerase according to the manufacturer's suggestions (Boehringer Mannheim). A general Wak probe that hybridized to all five Wak coding regions was generated through random-primed synthesis from a *Wak1* cDNA according to the manufacturer's suggestions (Boehringer Mannheim). Radioactivity was analyzed using a Phospholmager and ImageQuant analysis software (Molecular Dynamics, Sunnyvale).

Analysis of transgene expression

The detection of transgene expression was carried out by RT-PCR. Total RNA was LiCl precipitated and treated with DNase-free RNase A, phenol/chloroform extracted, and ethanol precipitated. Primer R86 (5′-GCCTCCCCTTCATCGTCC-3′) homologous to the *tml* 3′UTR was used for first-strand and subsequent PCR synthesis from nWak, sWak and kWak. 5′ primers are as follows: 1840F (5′-ATGGAACTGCTCTCAGGTCA AAAGG-3′) for both sWak and kWak, where PCR produced a 460 bp fragment: 388F (5′-GAAACTACAT-CAGGTCGC ATCAAGCACAT-3′) for nWak where PCR produced a 650 bp fragment. Reverse primer anti-800 (5′-CGCTTACATGGG-AACACCGTCTGAAGATAGC) and forward primer anti-40 (5′-CT-CGAGCATTATTGATGAAGCAACA-3′) were use to provide a 660 bp PCR fragment from aWak. All primers were successfully tested first on plasmid templates. The RT-PCR reaction was carried out in a total volume of 20 μl using the GeneAmp® EZ r*Tth* RT PCR kit (Perkin Elmer) according to the manufacturer's protocol, including 0.75 μM primers, 250 ng RNA, 200 μM dNTP, 1.0 mM $MnCl_2$, and five units of r*Tth* DNA polymerase. The RT-PCR was carried out at 72°C for 2 min followed by 60°C for 30 min. For PCR amplification 4 μl of the RT mix was placed into 16 μl of PCR mixture: 1.6 μl 10× chelating buffer, 1 μl 20 mM $MgCl_2$, 1 μl primer (2.5 μM) and 12.4 μl water. PCR reactions were performed with an initial denaturation at 95°C for 2 min followed by 40 cycles of [95°C 1 min, 60°C 1.5 min] and a final cycle of 60°C for 7 min. The RT-PCR products were analyzed in a 2.5% agarose gel and imaged by an Eagle-Eye system (Stratagene).

Protein analysis

Wak1 protein extraction and Western blot analysis was performed as described elsewhere (He *et al.*, 1996).

Infection with P. syringae

Plants were grown on soil in 2.5 inch square pots covered with fine mesh veil. Four-week-old plants were thoroughly wetted by dipping into a solution containing *P. s. maculicola* ES4326. The growth of the pathogen bacteria and the preparation of dipping solutions were as previously described by Cao *et al.* (1994).

Determination of SA levels

Ten-day-old seedlings of both vector-transformed (V) and sWak plants grown on MS plates or MS plates containing 0.5 mM SA, were frozen in liquid nitrogen, and 1 g of each tissue was extracted and quantified for free (unconjugated O-glucoside) SA and total SA (Delaney *et al.*, 1995). This service was kindly provided by Mike Willits of Ciba Geigy Corp.

Acknowledgments

We would like to thank Karen Bernd, Mireille Perret, Iain Cheeseman and Xinnian Dong for wonderful discussions. We are grateful to John Ryles and Mike Willits for pWCT and the SA analysis, and to Xinnian Dong for the *npr1* RNA. This work was supported financially by NSF MCB 9218931.

References

Baker, B., Zambryski, P., Staskawicz, B. and Dinesh-Kumar, S.P. (1997) Signaling in plant–microbe interactions. *Science*, **276**, 726–733.

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*, **316**, 1194–1199.

Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X. (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell*, **6**, 1583–1592.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S. and Dong, X. (1997) The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell*, **88**, 57–63.

Carpita, N. and Gibeaut, D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with physical properties of the walls during growth. *Plant J.* **3**, 1–30.

Carpita, N., McCann, M. and Griffing, L.R. (1996) The plant extracellular matrix: news from the cell's frontier. *Plant Cell*, **8**, 1451–1463.

Chen, Z., Silva, H. and Klessig, D.F. (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science*, **262**, 1883–1886.

Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl Acad. Sci. USA*, **81**, 1991–1995.

Delaney, T., Friedrich, L. and Ryals, J. (1995) *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc. Natl Acad. Sci. USA*, **92**, 6602–6606.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. and Ryals, J. (1994) A central role of salicylic acid in plant disease resistance. *Science*, **266**, 1274–1250.

He, Z.-H., Fujiki, M. and Kohorn, B.D. (1996) A cell wall-associated, receptor-like protein kinase. *J. Biol. Chem.* **271**, 19789–19793.

Herve, C., Dabos, P., Galaud, J.P., Rouge, P. and Lescure, B. (1996) Characterization of an *Arabidopsis thaliana* gene that defines a new class of putative plant receptor kinases with an extracellular lectin-like domain. *J. Mol. Biol.* **258**, 778–788.

Holtorf, S., Apel, K. and Bohlmann, H. (1995) Comparison of different constitutive and inducible promoters for the overexpression of transgenes in *Arabidopsis thaliana*. *Plant. Mol. Biol.* **29**, 637–646.

Hunt, M.D., Neuenschwander, U.H., Delaney, T.P., Weymann, K.B., Friedrich, L.B., Lawton, K.A., Steiner, H.Y. and Ryals, J.A. (1996) Recent advances in systemic acquired resistance research – a review. *Gene*, **179**, 89–95.

Kelley, B., Sauer, N. and Lamb, C.J. (1988) Glycine-rich cell wall proteins in bean: gene structure and association of the protein with the vascular system. *EMBO J.* **7**, 3625–3633.

Kohorn, B.D., He, Z.-H. and Fujiki, M. (1995) A receptor-like kinase with an EGF domain in the cell wall. In *Protein Phosphorylation in Plants*, Vol. 39 (Shewry, P.R., Halford, N.G. and Hooley, R., eds. Oxford: Oxford University Press, pp. 297–304.

Kohorn, B.D., Lane, S. and Smith, T.A. (1992) An *Arabidopsis* serine/threonine kinase homologue selected in yeast for its specificity for a thylakoid membrane protein. *Proc Natl. Acad. Sci. USA*, **89**, 10989–10992.

Komalavilas, P., Zhu, J.K. and Nothnagel, E.A. (1991) Arabinogalactan-proteins from the suspension culture medium and plasma membrane of rose cells. *J. Biol. Chem.* **266**, 15956–15965.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M. and Hoffmann, J.A. (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell*, **86**, 973–983.

Miller, J.G. and Fry, S.C. (1993) Spinach extensin exhibits characteristics of an adhesive polymer. *Acta Bot. Neerl.* **42**, 221–231.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Roberts, K. (1994) The plant extracellular matrix: in a new expansive mood. Structures at the plant cell surface. *Curr. Opin. Cell Biol.* **6**, 688–694.

Showalter, A.M. (1993) Structure and function of plant cell wall proteins. *Plant Cell*, **5**, 9–23.

Smith, T.A. and Kohorn, B.D. (1994) Mutations in a signal sequence for the thylakoid membrane identify multiple protein transport pathways and nuclear suppressors. *J. Cell Biol.* **126**, 365–374.

Uknes, S., Dincher, S., Friedrich, L., Negrotto, D., Williams, S., Thompson-Taylor, H., Potter, S., Ward, E. and Ryals, J. (1993) Regulation of pathogenesis-related protein-1a gene expression in tobacco. *Plant Cell*, **5**, 159–169.

Varner, J.E. and Lin, L.-S. (1989) Plant cell wall architecture. *Cell*, **56**, 231–239.

Verwoerd, T.C., Dekker, B.M. and Hoekema, A. (1989) A small-scale procedure for the rapid isolation of plant RNAs. *Nucl Acids Res.* **17**, 2362.

Wang, X., Zafian, P., Choudhary, M. and Lawton, M. (1996) The PR5K receptor protein kinase from *Arabidopsis thaliana* is structurally related to a family of plant defense proteins. *Proc. Natl Acad. Sci. USA*, **93**, 2598–2602.

Wilson, I., Vogel, J. and Somerville, S. (1997) A common theme in plants and animals? *Curr. Biol.* **7**, 175–178.

Wyatt, S.E. and Carpita, N.C. (1993) The plant cytoskeleton–cell wall continuum. *Trends Cell Biol.* **3**, 413–417.