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The Cf-9 resistance (R) gene from tomato confers resistance to the fungal pathogen Cladosporium fulvum expressing the corresponding, pathogen-derived avirulence gene product Avr9. To understand how an initial R/Avr recognition event is transmitted and triggers the induction of plant defenses, we investigated early Avr9/Cf-9–dependent activation of protein kinases in transgenic tobacco expressing the Cf-9 gene. We identified two protein kinases of 46 and 48 kD, using myelin basic protein as substrate, that became rapidly activated in a strictly gene-for-gene manner within 2 to 5 min after Avr9 elicitation in both Cf9 tobacco plants and derived cell cultures. Studies with pharmacological inhibitors and effectors revealed that Ca^{2+} influx and a phosphorylation event(s) are required for kinase activation, but neither enzyme is involved in the Avr9-dependent synthesis of active oxygen species. The activation of both kinases is achieved via post-translational mechanisms, and the activation but not inactivation step includes tyrosine phosphorylation. Using specific antibodies, we found that the 46- and 48-kD kinases were similar to WIPK (for wound-induced protein kinase) and SIPK (for salicylic acid–induced protein kinase), two previously characterized mitogen-activated protein (MAP) kinases from tobacco. In addition, Cf9 tobacco plants and cell cultures showed an Avr9-dependent accumulation of the WIPK transcript. Cf9 tobacco suspension cultures are thus a unique system in which to analyze the earliest events in R gene function. These data indicate that (1) the R/Avr-mediated induction of plant defense is accomplished via several parallel signaling mechanisms, and (2) R/Avr–dependent signal transduction pathways are interlinked at MAP kinases with responses of plants not only to non-race-specific elicitors but also to abiotic stimuli, such as wounding and mechanical stress.

INTRODUCTION

The capacity of plants to stop the growth of pathogens and parasites depends on early warning of invasion, followed by the activation of defense mechanisms. Disease resistance (R) genes are part of the plant’s surveillance system and, in so-called gene-for-gene interactions, confer resistance to pathogens that carry the corresponding avirulence (Avr) genes (Flor, 1971). The R gene product is generally considered as a receptor for the matching Avr protein (Staskawicz et al., 1995). Despite the isolation of an increasing number of plant R genes (see below), little is known about the signal transduction chains that follow the R/Avr recognition event. A different picture emerges from the analysis of plant responses after nonspecific elicitation with bacterial or fungal oligosaccharides, proteins, or peptides that are not part of a matching Avr/R gene pair. To date, only one receptor, a 70-kD transmembrane β-glucan elicitor binding protein from soybean, has been characterized, and the corresponding gene has been cloned (Umemoto et al., 1997). In parsley, a 91-kD transmembrane protein, which binds the fungal protein elicitor from Phytophthora sojae with high affinity, has also been analyzed in detail (Nürnberger et al., 1994). Nevertheless, a variety of signaling events have been identified in response to nonspecific elicitation. These include elicitor-induced changes in ion fluxes, an oxidative burst paralleled by an increase in active oxygen species (AOS) production, protein phosphorylation/dephosphorylation, and the generation of fatty acid derivatives (reviewed in Yang et al., 1997; Rushton and Somssich, 1998; Scheel, 1998). However, until recently there has been little direct evidence addressing whether R gene products in gene-for-gene interactions use the same pathways or mechanisms to activate the plant defense response as has been described for nonspecific elicitors.
R gene products, although conferring resistance to different bacteri al, viral, fungal, and nematode pathogens, can be divided into four main classes according to their structural motifs: the nucleotide binding, leucine-rich repeat (NB-LRR) class (e.g., RPS2, L6, N, RPM1, and RPM5), the extracellular membrane-anchored LRR class (e.g., Cf-2, Cf-4, Cf-5, and Cf-9), the protein kinase class (e.g., Pto), and the LRR protein kinase class exemplified by Xa21 (reviewed in Hammond-Kosack and Jones, 1997; Ellis and Jones, 1998). The protein kinase class (e.g., Pto, and the LRR protein kinase class exemplified by Xa21 (reviewed in Hammond-Kosack and Jones, 1997; Ellis and Jones, 1998). The protein kinase domains of Pto and Xa21 suggest a central role for protein phosphorylation in R gene-dependent Avr signal perception and early signal transduction (Martin et al., 1993; Song et al., 1995). However, until recently, an analysis of rapid and transient changes in protein phosphorylation has been available only from studies with nonspecific fungal elicitors in cell cultures (Dietrich et al., 1990; Felix et al., 1991; Viard et al., 1994; Chandra and Low, 1995).

Inhibition experiments revealed that defense-related responses, such as media alkalization, the oxidative burst, defense gene activation, and hypersensitive cell death, are prevented by the protein kinase inhibitors staurosporine and K252a. In contrast, protein phosphatase inhibitors are able to mimic elicitation in the absence of elicitor (Felix et al., 1994; Levine et al., 1994; Mackintosh et al., 1994). In addition, elicitor-induced phosphorylation has also been shown for more downstream events such as the activation of pathogenesis-related transcription factors. For instance, phosphorylation of the nuclear factor PBF-1, probably accomplished via a homolog of the mammalian protein kinase C, is required for the activation of the potato pathogenesis-related PR-10a gene (Després et al., 1995; Subramaniam et al., 1997). Also, phosphorylation of the soybean basic leucine zipper transcription factor G/HBF-1 by a pathogen-induced kinase is a prerequisite for its enhanced binding to the chalcone synthase chs15 promoter (Drège-Laser et al., 1997).

Increasing evidence suggests a role for mitogen-activated protein (MAP) kinase cascades in plant signal transduction (Hirt, 1997; Mizoguchi et al., 1997), and MAP kinases have been suggested to participate in plant defense responses because they become activated after challenge with nonspecific elicitors and pathogen-related signals. These kinases include the fungal elicitor-induced kinases of 44 and 48 kD (Zhang et al., 1998), which were shown to be WIPK (for wound-induced protein kinase; See et al., 1995) and SIPK (for salicylic acid-induced protein kinase; Zhang and Klessig, 1997), a 47-kD elicitor-induced enzyme (Suzuki and Shinshi, 1995), which is likely to be SIPK, and a harpin-induced kinase of 49 kD (Adám et al., 1997), all from tobacco. In addition, Ligterink et al. (1997) showed that when parsley cells are elicited with a peptide derived from a fungal glycoprotein elicitor, a 45-kD MAP kinase becomes activated. Furthermore, the MAP kinase is also transported into the nucleus, suggesting that it participates in the activation of defense-related transcription factors, as has been described in mammalian cells.

The Cf-9 gene from tomato confers resistance to races of the leaf mold fungus Cladosporium fulvum expressing the complementary Avr9 gene. Cf-9 has been cloned and encodes an extracytoplasmic membrane-anchored glycoprotein encompassing 27 LRRs (Jones et al., 1994). Avr9 was purified based on its capacity to elicit a Cf-9-dependent necrotic response in tomato leaves (De Wit and Spikman, 1982). The isolation of the corresponding gene revealed that Avr9 encodes a preprotein of 63 amino acids that becomes processed by fungal and plant proteases to a 28-amino acid peptide when secreted (Van den Ackerveken et al., 1992, 1993). Although it is generally proposed that R gene products represent receptors for the products of Avr genes, such an interaction has only been demonstrated for Pto and AvrPto in the yeast two-hybrid system (Scofield et al., 1996; Tang et al., 1996), whereas direct physical contact between Cf-9 and Avr9 has not been proven (De Wit, 1997).

Recently, we showed, in accordance with the gene-for-gene model, that Cf-9 and Avr9 retain their specificity in other plants: functional apoplastically expressed Avr9 peptide isolated from transgenic tobacco plants that harbor the Avr9 transgene under the control of the cauliflower mosaic virus 35S promoter induced necrosis in transgenic Cf9 tobacco and potato (Hammond-Kosack et al., 1994, 1998). Developing this isogenic Cf-9/Avr9 system a step further, Piedras et al. (1998) established Cf9 tobacco cell cultures that respond to Avr9 elicitation with a rapid production of AOS within 5 min.

To understand Cf-9 function, we analyzed early changes in protein phosphorylation that may mediate the Avr/R recognition event. We took advantage of the rapid response of Cf9 tobacco cell cultures to Avr9 and tested whether Avr9 elicitation leads to the activation of protein kinases. In-gel kinase assays with the myelin basic protein (MBP) as a substrate showed that two MBP kinases of 46 and 48 kD become activated in a strict gene-for-gene manner. With the help of specific antibodies, we found that these MBP kinases and previously characterized SIPK and WIPK MAP kinases from tobacco are related (See et al., 1995; Zhang and Klessig, 1997, 1998a, 1998b). Inhibitor studies demonstrated that these rapid and specifically induced MAP kinase pathways are not required for activation of AOS production. We discuss the significance of this intriguing and surprising convergence between the R gene-dependent signaling pathways and those activated by non-race-specific elicitors, wounding, and mechanical stress.

RESULTS

Avr9/Cf-9-Dependent Transient Activation of Protein Kinases

To elucidate the role of protein kinases in the Avr9/Cf-9-dependent defense response, we investigated changes in the protein phosphorylation pattern after elicitation with Avr9 by using an in-gel kinase assay. Tobacco suspension
cultures expressing Cf-9 were challenged with intercellular fluid (IF) obtained from transgenic tobacco plants either containing (IF[Avr9⁺]) or without (IF[Avr9⁻]) the Avr9 peptide. At different time points after elicitation, cells were harvested and protein extracts were prepared. Protein kinase activity was determined by an in-gel kinase assay with MBP as a substrate (Gotoh et al., 1990). Treatment with Avr9 in tobacco cells resulted in a rapid activation of two MBP kinases with estimated molecular masses of 46 and 48 kD (Figure 1A). The enzyme activity became detectable within 5 min of elicitation, peaked at 15 min, and returned to basal levels during the subsequent 2 to 3 hr. Quantitative analysis revealed a six-fold increase in MBP kinase activity at the 15-min time point (Figures 2A and 2B). Although this value varied

![Figure 1. Avr9/Cf-9–Dependent Activation of 46- and 48-kD Kinases in Cell Cultures and Leaves.](image)

(A) and (E) Transgenic tobacco plants carrying the Cf-9 resistance gene ([E] to [H]) or derived suspension culture cells ([A] to [D]) were treated with IF(Avr9⁺) or IF(Avr9⁻), which contain (+) or do not contain (−) the Avr9 peptide. Leaf discs and cell samples were harvested at the indicated times (numbers above the gels given in minutes) after IF challenge, and kinase activity in total cell extracts was analyzed using an in-gel kinase assay with MBP as a substrate. The arrows indicate the 46- and 48-kD MBP kinases.

(B) and (F) Control gels without substrate to test for autophosphorylation. Samples and order of loading are identical to (A) and (E).

(C) and (G) Coomassie Brilliant Blue R 250 staining. Samples and order of loading are identical to (A) and (E).

(D) and (H) Gene-for-gene specificity of the kinase activation. Leaves ([H]) and cell culture samples ([D]) of Cf9 tobacco ([D], lanes 1, 2, and 5; and [H], lanes 1, 2, 3, and 6) or originating from the nontransformed parental line (Petite Havana; [D], lanes 3 and 4; and [H], lanes 4 and 5) were analyzed 15 min after elicitation with IF(Avr9⁻) ([D], lanes 1 and 3; and [H], lanes 2 and 4), with IF(Avr9⁺) ([D], lanes 2 and 4; and [H], lanes 3 and 5), or with chemically synthesized Avr9 peptide ([D], lane 5; and [H], lane 1). In addition, leaves were analyzed 15 min after applying tiny cuts but without injection of IF ([H], lane 6).

Sizes of molecular mass markers in kilodaltons are given by the numbers at right.
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between a five- to 15-fold increase in different experiments, the kinetics remained unchanged. Signals were not due to autophosphorylation, because no phosphorylation occurred after the same samples were separated on gels without substrate (Figure 1B). The addition of IF(Avr9\(^+\)) did not activate the MBP kinases. Equal loading of protein was demonstrated by a Coomassie stain of a parallel gel (Figure 1C).

Control experiments in which the nontransformed parental cell line was elicited with IF(Avr9\(^+\)) and IF(Avr9\(^-\)) revealed that only the Avr9/Cf-9 combination resulted in activated MBP kinases (Figure 1D). This demonstrates that kinase induction occurs as a consequence of an R/Avr gene-for-gene-mediated recognition event. Chemically synthesized Avr9 peptide (see Methods) also caused the activation of the 48- and 46-kD MBP kinases in Cf9 tobacco, indicating that the Avr9 peptide in the IF preparation is responsible for activation.

To test whether the results obtained could be verified using tobacco plants (Figures 1E to 1H), we injected Cf9 tobacco leaves with IF(Avr9\(^+\)) or IF(Avr9\(^-\)), as used for the suspension cultures. Leaf discs were cut at different time points after elicitation, and the protein extracts were analyzed for MBP kinase activity. Again, the two kinases of 46 and 48 kD became rapidly activated after challenge with IF(Avr9\(^+\)) (Figure 1E); however, the 46-kD enzyme is induced to a much lower level than is the 48-kD kinase. The detected
phosphorylation was not due to autophosphorylation (Figure 1F). The activation kinetics, peaking at 15 min after IF treatment, paralleled those observed with elicited suspension cultures (Figures 1C and 2D). In plants as in cell cultures, only the Avr9/Cf-9 combination resulted in full activation (Figure 1H, lanes 2 to 5), and synthetic Avr9 peptide was able to substitute for the IF(Avr9⁺) preparation (Figure 1H, lane 1).

However, in contrast to the elicitation in suspension cultures, the 48-kD kinase was activated 3.3-fold in samples treated with IF(Avr9⁺) compared with the fivefold activation with IF(Avr9⁻) (Figure 2C). A quantification of control experiments (Figure 1H, data not shown) revealed that in the sample in which the leaf received only tiny razor blade cuts (Figure 1H, lane 6), no kinase activation occurred, and it was comparable to an untreated extract (see time point 0 in Figure 1E). Samples that had been flooded with IF exhibited a two- to threefold activation (Figure 1H, lanes 3 to 5), whereas a sixfold activation was observed in the Avr9⁻/Cf-9 gene situation (Figure 1H, lanes 1 and 2). The analyses were independently repeated three times with identical results.

These data clearly demonstrate in accordance with the gene-for-gene hypothesis an Avr9/Cf-9-dependent activation of a 46- and 48-kD MBP kinase in both tobacco plants and suspension cultures.

The 46- and 48-kD MBP Kinases Are Not Required for AOS Production

AOS, synthesized after pathogen attack, have not only been proposed to play a signaling role in plant defense by triggering the hypersensitive response, but AOS might by themselves function as antimicrobial compounds (Lamb and Dixon, 1997). To locate the two MBP protein kinases within a cellular signaling cascade and in particular, to address whether their activation is associated with the Avr9/Cf-9-dependent synthesis of AOS (Piedras et al., 1998), we conducted studies using different inhibitors and effectors related to signaling mediators (Figures 3A and 3B). If not otherwise stated, all samples were analyzed 15 min after elicitation with Avr9. At this time point, the corresponding kinematics showed maximum activity (Figure 2).

Several inhibitors, such as La⁺, an inhibitor of plasma membrane Ca²⁺ channels, staurosporine, an inhibitor of protein kinases, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), an antagonist of calmodulin, and bromophenacylbromide (BPB), an inhibitor of phospholipase A₂ (PLA₂), compromised both the Avr9-induced kinase activation and the Avr9-induced AOS synthesis (Figure 3A, lanes 1, 2, 4, and 5). No change in kinase activation or AOS synthesis occurred when the inhibitors were combined with IF(Avr9⁻) (data not shown). In contrast, diphenyleneiodonium (DPI) did not influence the Avr9-dependent induction of the 46- and 48-kD kinases (Figure 3A, lane 3) but inhibited AOS synthesis. DPI is considered to be an inhibitor of NADPH oxidase, an enzyme that is proposed to be respon-

Figure 3. Influence of Inhibitors and Effectors on the Avr9/Cf-9-Dependent Kinase Activation.

Transgenic Cf9 tobacco suspension culture cells were treated with IF(Avr9⁺) (indicated by Avr9, lanes 1 to 4, 12, and 14) or IF(Avr9⁻) (lane 13) in the presence of inhibitors and effectors of various signaling processes (abbreviations above single lanes are explained below). Cells were harvested at the time points indicated in parentheses.

(A) MBP kinase activity was analyzed by the in-gel kinase assay.
(B) AOS synthesis was determined by the ferricyanide-catalyzed oxidation of luminol.

The inhibitors staurosporine (St; 25 μM; lane and bar 1), LaCl (La; 500 μM; lane and bar 2), and diphenyleneiodonium (DPI; 8 μM; lane and bar 3) were added 5 min before elicitation with IF(Avr9⁺), and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7; 250 μM; lane and bar 4), bromophenacylbromide (BPB; 50 μM; lane and bar 5), and PD98059 (PD; 250 μM; lane 13) were added 10 min before elicitation. The effect of cantharidin (Canth; 5 μM; lanes and bars 9 to 11) and amphetamine B (Ampb; 100 μM; lanes and bars 6 to 8) was investigated in the absence of IF. Data reflecting an elicitor/inhibitor pair or an effector study were repeated in at least three independent experimental series with similar results. Although the absolute amount of H₂O₂ produced might vary slightly in different cell batches, the MBP kinase activation always showed an identical pattern. (Data shown result from two different experiments.) Arrows in (A) indicate the positions of the 46- and 48-kD MBP kinases.
which induces the synthesis of AOS even in the absence of elicitor (Shirasu et al., 1997; Piedras et al., 1998). Remarkably, although cantharidin is able to trigger AOS synthesis during a 3-hr time course after addition (Figure 3B, bars 9 to 11), no activation of the two MBP kinases occurred. In addition, amphotericin B, an antibiotic that provokes elicitor-induced ion fluxes and has been reported to mimic various defense responses (Jabs et al., 1997; Ligterink et al., 1997), failed to induce the MBP kinases 30 min (data not shown) or within 3 hr after addition (Figure 3B, bars 6 to 8). However, as reported (Piedras et al., 1998), and as also shown in Figure 3B, amphotericin B alone was able to induce an oxidative burst peaking at 40 min and 1 hr after the addition of the antibiotic. We conclude from these data that the 46- and 48-kD MBP kinases are independent from the signaling pathway that triggers the Avr9-dependent synthesis of AOS.

Correlation between the Avr9-Dependent Activation of MBP Kinases and the Phosphorylation of Tyrosine Residues

The molecular masses of 46 and 48 kD and the utilization of MBP as a substrate suggest that the Avr9-induced protein kinases are MAP kinases. The activation of MAP kinases involves phosphorylation at both serine/threonine and tyrosine residues (Hirt, 1997; Mizoguchi et al., 1997). To investigate the involvement of tyrosine phosphorylation, we analyzed Cf-9-expressing tobacco leaves and suspension cultures, challenged with either IF(Avr9<sup>1</sup>) or IF(Avr9<sup>2</sup>), by immunoblotting and immunoprecipitation using a phosphotyrosine-specific monoclonal antibody (Figure 4). In the immunoblot, two signals at estimated molecular masses of 46 and 48 kD could be detected after treatment with IF(Avr9<sup>1</sup>) within 5 min of elicitation, reaching a maximum signal intensity at 15 min that then remained constant (Figure 4A). No such signals could be detected in samples treated with IF(Avr9<sup>2</sup>) (data not shown). We next combined immunoprecipitation with the antiphosphotyrosine antibody with the in-gel kinase assay (Figure 4B). MBP kinase activity at 48 kD could be immunoprecipitated from leaf and suspension culture samples, which had been elicited with IF(Avr9<sup>1</sup>), but not after treatment with IF(Avr9<sup>2</sup>) or in controls in which a monoclonal antihemagglutinin (HA) antibody. To confirm the specificity of the phosphotyrosine antibody, extracts were precipitated in the presence of 5 mM phosphoserine (P-Ser) or 5 mM phosphotyrosine (P-Tyr). The MBP kinase activities in the immunocomplexes were analyzed with the in-gel kinase assay.

48-kD proteins, whereas the 48-kD kinase phosphorylation signal is clearly more pronounced in the in-gel kinase assay (Figure 1A). This suggests that the 46-kD MBP kinase is less active and robust under the experimental in-gel conditions used and could also explain why no 46-kD signal was detected after the immunoprecipitation in Figure 4B.

The Avr9-Induced 46- and 48-kD Kinases Are Related to Stress-Induced MAP Kinases from Tobacco

Several plant MAP kinases recently have been reported to be involved in signaling processes after various environmental stimuli such as wounding, mechanical stress, drought,
but also pathogens, derived elicitors, and defense-related signals (reviewed in Hirt, 1997; Somssich, 1997; Yang et al., 1997; Scheel, 1998). Our data with elicited tobacco leaves (Figure 1H) suggested that the extent of MBP phosphorylation in the in-gel kinase assay depends on the nature and strength of the inducing stimuli. Full activation of the 48-kD MBP kinase in leaves seems to be derived from a specific elicitation component and a nonspecific flooding component. Furthermore, we could demonstrate that MBP kinase activity is transiently induced after wounding of tobacco leaves or vigorous stirring of tobacco cell cultures (data not shown).

We next studied whether the Avr9-induced 46- and 48-kD MBP kinases are related or even identical to protein kinases activated after wounding or stirring and investigated directly a potential identity between the Avr9-induced kinases and two previously characterized MAP kinases from tobacco that are induced by environmental stimuli: (1) WIPK, a MAP kinase of ~46 kD, whose gene was isolated from a differential screen for genes induced in an N-dependent manner after tobacco mosaic virus (TMV) infection (Seo et al., 1995); and (2) SIPK, an enzyme of ~48 kD, which has recently also been shown to become activated by nonspecific fungal elicitors and during N-dependent recognition of TMV (Zhang and Klessig, 1997, 1998a; Zhang et al., 1998).

Cf9 tobacco suspension cultures were challenged with IF(Avr9\textsuperscript{+}) or IF(Avr9\textsuperscript{−}), and protein extracts were subjected to immunoblot and immunoprecipitation combined with in-gel kinase assays. The antibodies applied were raised against N-terminal peptides originating from SIPK (p48N; Zhang et al., 1998) or WIPK (p44N; Zhang and Klessig, 1998b), or against the C-terminal six amino acids of the alfalfa MAP kinase MMK4 (M24; Jonak et al., 1996; H. Hirt, unpublished results) (see Figure 5A). The sequence comparison revealed that WIPK shares with MMK4 at the C terminus six out of six amino acids; thus, the M24 antisera was also expected to recognize WIPK from tobacco.

The immunoblot (Figure 5B) revealed that the p48N antibody detected a polypeptide of 46 kD, whereas p44N and M24 recognized a protein of 46 kD. All signals were constitutively present during the time course, irrespective of whether the protein extracts originated from cells elicited with IF(Avr9\textsuperscript{+}) or IF(Avr9\textsuperscript{−}). Any differences in intensity were due to unequal protein loading. The immunoprecipitations combined with subsequent analysis of the precipitates by the in-gel kinase assay revealed that the Avr9-induced 48-kD MBP kinase could be precipitated with the p48N (SIPK) antibody, whereas the 46-kD enzyme is complexed by the p44N (WIPK) antibody (Figure 5C, lane 2). In the presence of an excess of the corresponding SIPK and WIPK peptides, against which the antibodies had been raised, these immunoprecipitations were compromised (data not shown). Furthermore, SIPK activity was also precipitated after mechanical stress stimuli such as wounding in leaves (data not shown) and mechanical stirring in cell cultures (Figure 5C, lane 4) and after salicylic acid treatment (Figure 5C, lane 5). The 46-kD signal is weak (Figure 5C, lower panel, lane 6) because repeated freezing of the total extract used as marker leads to a less active WIPK enzyme. Immunoprecipitations with the M24 antibody also recognized the 46-kD kinase after Avr9 challenge (data not shown). The difference in the estimated molecular mass of WIPK—46 kD in this study compared with 44 kD reported by Zhang and Klessig (1998b)—is likely to be due to the migration behavior of different prestained molecular mass markers.

To further differentiate the 48-kD Avr9-induced MBP kinase from the 46-kD enzyme, we repeated the in-gel assay of total extract and p48N immunocomplexes in the presence of 5 \( \mu \)M staurosporine during the final gel incubation step (Figure 5D). Only the 48-kD MBP kinase activity was inhibited, whereas the 46-kD kinase remained active.

**Avr9/Cf-9-Dependent Induction of WIPK Transcript Accumulation**

Besides a rapid and transient increase in MAP kinase activation, members of the WIPK class of MAP kinases in plants also respond to various biotic and abiotic environmental stimuli with increases in the corresponding MAP kinase mRNA levels (Seo et al., 1995; Jonak et al., 1996; Bögre et al., 1997; Ligterink et al., 1997; Zhang and Klessig, 1998a, 1998b). To further investigate a potential link between specific elicitation and responses after wounding and mechanical stress, we conducted RNA gel blot analyses by using WIPK as a probe. Cf9-expressing tobacco plants and suspension cultures were elicited with IF(Avr9\textsuperscript{+}) and IF(Avr9\textsuperscript{−}), and RNA was isolated at different time points after elicitation. As controls, nontransformed tobacco plants wounded with carborundum or suspension cultures that had been stimulated by mechanical stirring were used.

WIPK transcripts accumulate in suspension cultures after elicitation with Avr9 (Figure 6A, first to sixth lanes). The increase in RNA level became evident within 30 min, and maximal accumulation (~15-fold) was reached at 1 to 2 hr before returning to the basal level. No induction of the WIPK transcript occurred in cells challenged with IF(Avr9\textsuperscript{−}) (Figure 6A, seventh to 12th lanes).

Tobacco plants wounded with carborundum exhibited a rapid and transient twofold accumulation of the WIPK transcript (Figure 6B), first described by Seo et al. (1995). Addition of IF(Avr9\textsuperscript{+}) also resulted in increased WIPK mRNA levels, and the induction kinetics paralleled those of the corresponding cell culture experiment. Surprisingly, in contrast to the elicitation in tobacco cells, WIPK transcript accumulation could also be detected during the first 60 min after treatment of the plants with IF(Avr9\textsuperscript{−}). No WIPK transcript accumulation was observed in leaves that received only small cuts. This indicates that injection of IF into leaves causes a flooding response that overlays the specific Avr9 elicitation response, whereas addition of IF to suspension...
cultures does not represent a stress stimulus to the system. Equal loading of RNA was demonstrated by an ethidium bromide stain of the corresponding agarose gels (Figures 6A and 6B, gels at bottom). The RNA blot analysis was repeated twice, resulting in identical kinetics of Avr9-induced WIPK transcript accumulation.

These data demonstrating an Avr9/Cf-9–dependent induction of the WIPK transcript further support the idea that the specific defense response and general wound/stress response pathways converge.

**DISCUSSION**

**Function of SIPK and WIPK in the Plant Defense Response**

To elucidate Cf-9 function and subsequent downstream signaling events, we investigated Avr9/Cf-9–dependent changes in protein phosphorylation by using an in-gel kinase assay. We chose transgenic tobacco expressing the Cf-9 gene...
cause, compared with Cf9 tomato, tobacco not only revealed a stronger and faster response to the Avr9 elicitor (Hammond-Kosack et al., 1998), but the derived Cf9 cell cultures represent a more amenable experimental system (Piedras et al., 1998).

In this study, we demonstrate that two tobacco MBP kinases of 46 and 48 kD become activated in an Avr9/Cf-9–dependent manner and that the kinase activation conforms with the gene-for-gene hypothesis. Immunoprecipitation analysis revealed that the 46- and 48-kD kinases are WIPK and SIPK, respectively, and thus represent members of the stress-responsive class of MAP kinases in plants. Although less total WIPK protein seems to be present in tobacco leaves compared with tobacco cell cultures, explaining the weaker in-gel kinase signal, SIPK and WIPK activation kinetics, peaking at 15 min after challenge and returning to basal levels within the subsequent 2 to 3 hr, are identical in both tobacco cell cultures and leaves.

MBP kinase activation obtained after IF(Avr91) injection into Cf9 tomato cotyledons shows a comparable kinetic to that from transgenic tobacco (data not shown). Thus, identical signaling components and pathways appear to be involved in the Avr9/Cf-9–dependent hypersensitive response induction in tomato and tobacco. This observation validates the investigation of R gene function in a heterologous plant system, carrying the R gene of interest as a transgene. Moreover, it proves that these responses are R gene dependent rather than due to another gene cointrogressed with the R gene by plant breeders.

The rapid Avr9/Cf-9–dependent activation of SIPK and WIPK suggests that both kinases directly participate in the transmission of an R gene–mediated signal to activate the plant defense response. However, the biological role of these enzymes is not defined, and there is no experimental proof that they are required for pathogen resistance. One could also envisage that a MAP kinase is not involved in the transduction of the primary (elicitation) signal but rather becomes activated in response to some physiological changes and participates in resetting the system.

A genetic approach to establish a function of SIPK and WIPK in the plant defense response would be to analyze mutant lines compromised in the corresponding kinase. No such data are available at this time. On the other hand, our results with Cf9 tobacco also validate an investigation of R gene function in, for example, Arabidopsis, for which mutant collections are available, and a search for transposon insertions in AtMPK3 and AtMPK6, the orthologous WIPK and SIPK genes from Arabidopsis (Mizoguchi et al., 1995, 1996), is already in progress.

However, due to genetic redundancy, the MAP kinase knock-out mutant might display no or only a subtle phenotype. For example, in yeast, the two MAP kinases Fus3p and Kss1p are responsible for mating and invasive growth, respectively. In the deletion mutant of Fus3p, however, mating still occurs, whereas invasive growth is induced inappropriately. Thus, in the case of the Fus3p deletion, the MAP kinase Kss1p, although catalyzing a different biochemical function in the wild type, is recruited (Madhani et al., 1997). Interestingly, WIPK sense-suppressed lines from tobacco respond to wounding with an increase in the mRNA level of acidic PR-1 and PR-2 protein and an increase in salicylic acid that cannot be observed in wounded wild-type leaves (Seo et al., 1995). These responses previously have only been reported after pathogen attack but not after

**Figure 6.** Avr9/Cf-9–Dependent Induction of the WIPK Transcript. RNA was extracted from tobacco leaves or suspension culture cells at the indicated time after elicitation with IF(Avr91) or IF(Avr92), or after mechanical stress stimuli. Total RNA (20 μg per lane) was separated on denaturing formaldehyde gels and blotted onto nylon membranes. The blots were hybridized with a radiolabeled DNA fragment containing the complete coding region of the WIPK gene. Gel loading is shown by ethidium bromide staining (lower panels). Numbers at right indicate positions of RNA length markers.

(A) Suspension culture cells containing the Cf-9 gene were challenged with IF(Avr91) or IF(Avr92) or were mechanically stressed by vigorous stirring for 1 min.

(B) Cf9 tobacco plants were induced with IF (see above); nontransformed tobacco plants (Petite Havana) were wounded with wet carborundum.
mechanical wounding. Thus, in the transgenic lines, suppressed WIPK might be functionally replaced by another MAPK (maybe SIPK) that activates that inappropriate response.

Inhibitor Studies Demonstrate Independence of the MAP Kinase Pathway from That Leading to AOS Production

By investigating the responses of Cf9 tobacco to the Avr9 elicitor in the presence of various pharmacological inhibitors, we could show that the MAP kinase pathway and the pathway to AOS production are parallel pathways, both activated in a Ca^{2+}-dependent and possibly a PLA_2-dependent manner.

SIPK/WIPK still become activated in the presence of DPI, which inhibits AOS production (Jabs et al., 1997; Piedras et al., 1998), whereas PD98059, a highly specific inhibitor of MAP kinase kinases, abolished SIPK and WIPK activation without affecting the Avr9-dependent AOS synthesis. PD98059 has not previously been reported to be useful for the study of plant MAP kinase pathways.

Lanthanum chloride inhibited MBP kinase activation and AOS synthesis, indicating that both responses are located downstream of a Ca^{2+} influx channel, and a role of Ca^{2+} was further confirmed by the inhibition of both responses in the presence of the calmodulin antagonist W7. An Avr9-dependent influx of Ca^{2+} ions has indeed been demonstrated in Cf9 tobacco leaf guard cells (M.R. Blatt, A. Grabov, J. Brearley, K.E. Hammond-Kosack, and J.D.G. Jones, unpublished results).

The protein kinase inhibitor staurosporine compromised Avr9-dependent AOS synthesis and SIPK/WIPK activation, indicating that both responses require an upstream phosphorylation event. Staurosporine- or K252a-dependent inhibition of the MBP kinase activation in response to non-race-specific elicitation has been described earlier (Grosskopf et al., 1990; Suzuki and Shinshi, 1995; Adám et al., 1997; Zhang et al., 1998). By applying the kinase inhibitor during the in-gel assay, we could in addition show that staurosporine compromises SIPK and WIPK activation as well as SIPK activity, whereas WIPK activation is not affected. This observation allows us to distinguish between both kinase activities and might provide a useful tool for classifying the biotic/abiotic stress-induced tobacco MBP kinases as well as their corresponding orthologs from other plant species that become activated after challenge with various external stimuli (see below).

Finally, both responses were inhibited in the presence of the PLA_2 inhibitor BMB. Thus, it is unlikely that SIPK/WIPK activate a PLA_2 upstream of the AOS synthesis, as described for mammalian cells (Lin et al., 1993; Hazan et al., 1997). However, a potential SIPK- and/or WIPK-mediated PLA_2 activation in an independent pathway, for example, triggering jasmonic acid synthesis, cannot be ruled out.

The Avr9-Induced 46- and 48-kD MBP Protein Kinases Are Regulated Post-Transcriptionally

The Avr9-dependent induction of kinase activity is neither paralleled by an increase in SIPK and WIPK protein amount nor SIPK mRNA, whereas a delayed increase in WIPK transcript was observed. This indicates that SIPK and WIPK activation is achieved solely by post-translational modifications. The properties of WIPK are reminiscent of two MAP kinases from alfalfa and parsley, whose elicitor and wound-induced transient activation kinetics were also followed by an increase in mRNA level, whereas the amount of protein remained unchanged (Jonak et al., 1996; Bögre et al., 1997; Ligterink et al., 1997). In contrast, an N-gene-dependent increase in WIPK mRNA and WIPK protein preceding WIPK activation occurred in tobacco plants after infection with TMV (Zhang and Klessig, 1998a). This deviation might be due to different experimental systems. In Cf9 tobacco and parsley, the corresponding Avr9 or oligopeptide elicitor is applied extracellularly, and the response is mediated via a corresponding transmembrane determinant, either the R gene product (Cf-9) or a 91-kD binding protein (Nürnberger et al., 1994). In the N experiment, the analysis was performed with TMV-infected tobacco after a shift from high temperature (no resistance) to lower temperature, which allows rapid activation of defense responses and development of resistance.

To investigate the post-translational modification of SIPK/WIPK, we used an antiphosphotyrosine antibody and could show that activated SIPK from Avr9-challenged samples but not from the control was immunoprecipitated. A similar correlation between tyrosine phosphorylation and the activation of MAP kinases in plants was shown previously in the contexts of wounding and nonspecific elicitation (Usami et al., 1995; Adám et al., 1997; Stratman and Ryan, 1997; Zhang et al., 1998; Zhang and Klessig, 1998a, 1998b). In addition, we analyzed the Avr9/Cf-9-dependent changes in tyrosine phosphorylation during the time course. In tobacco leaves and cell cultures, 46- and 48-kD MBP kinase activation is paralleled by an increase in phosphorylated tyrosine residues. Surprisingly, in contrast to the transient mode of kinase activation, the phosphotyrosine signal remained unchanged for at least 2 to 3 hr after elicitation. These data suggest that the inactivation of both enzymes is not initially accomplished by dephosphorylation at the tyrosine residue.

Signaling Pathways Originating from Different Extracellular Stimuli Are Interlinked at the Level of Specific MAP Kinases

By analyzing Avr9/Cf-9-induced protein kinases in tobacco, we were able to identify the induced 46- and 48-kD MBP kinases as WIPK and SIPK. Recently, both enzymes were also shown to be activated in tobacco leaves in an N gene-dependent manner after TMV infection. Both kinases be-
came activated within 4 hr after a shift to the permissive temperature, and the activation of both enzymes remained high during the subsequent 4 hr (Zhang and Klessig, 1998a). Furthermore, SIPK activation is also obtained after nonspecific elicitation, salicylic acid treatment, and mechanical stress stimuli such as wounding or stirring (our data; reviewed in Scheel, 1998). WIPK, originally discussed in the context of wounding (Seo et al., 1995), is in addition activated by nonspecific elicitation (Zhang et al., 1998), and WIPK mRNA accumulates after specific and nonspecific elicitation as well as mechanical stress.

We conclude from these data that not only signal pathways derived from different R genes share components but also that signaling triggered by such diverse biotic and abiotic stimuli as wounding, mechanical stress (stirring), non-specific elicitation, and salicylic acid treatment is interlinked at certain MAP kinases. This raises two questions: (1) At which step do the distinct pathways merge, that is, at the MAP kinase or upstream? (2) How can various stimuli that are transmitted via identical signaling components result in different responses?

In animals, perception of extracellular and intracellular stimuli is also transduced into the cell by shared MAP kinase cascade components. There, two members of different MAPK subgroups, SAPK (for stress-activated protein kinase) or JNK (for J N-terminal kinase) and the p38 kinase, become activated by a variety of environmental stresses (Nishida and Gotoh, 1993; Herskowitz, 1995; Raingeaud et al., 1996). Different stimuli elicit distinct signaling pathways merging at MAP kinase activation (Nishina et al., 1997), and the output of SAPK/J NK or p38 kinase has been shown to be regulated via the duration and the activation level of the respective MAP kinase. SIPK and WIPK activation might be regulated by similar mechanisms in plants. We showed in this study that treatment of tobacco leaves with tiny cuts, flooding with control IF, or elicitation with Avr9 resulted in different SIPK activation levels. On the other hand, kinase activation kinetics after mechanical stress stimuli are more transient and shifted toward shorter time points compared with Avr9 elicitation. This holds not only for tobacco (see also Seo et al., 1995; Suzuki and Shinshi, 1995; Usami et al., 1995; Zhang and Klessig, 1998b; Zhang et al., 1998), but transient kinase activation was also seen after mechanical stress in tomato (Stratman and Ryan, 1997) and alfalfa (Bögre et al., 1996; Jonak et al., 1996).

However, another model to explain how, despite shared MAP kinase cascade components, different responses can be achieved is based on studies in yeast. There, two distinct developmental pathways for filament formation and mating share the MAP kinase kinase kinase (Ste11p) and MAP kinase kinase (Ste7p), whereas a pathway-specific MAP kinase (either Fus3p or Kss1p) is used. It is hypothesized that multiprotein complexes (e.g., Ste11p/Ste7p/Fus3p) are formed that sequester the corresponding MAP kinase (reviewed in Madhani and Fink, 1998). In plants, the most detailed description of gene families constituting MAP kinase cascades is available from Arabidopsis. To date, nine MAP kinase members classified into four subgroups, five MAP kinase kinases, and eight MAP kinase kinase kinases have been identified (Mizoguchi et al., 1997). Furthermore, interactions between AtMPK4 (MAP kinase) and AtMEK (a MAP kinase kinase from Arabidopsis) as well as between AtMEK and AtMEKK1 (MAP kinase kinase kinase) have been suggested (Mizoguchi et al., 1997). Assuming that in tobacco also multiple gene family members of each MAP kinase cascade component exist, it is tempting to speculate that distinct responses are accomplished by creating analogous stimulus/pathway-specific multikinase complexes.

By investigating Avr9/Cf-9–dependent changes in protein phosphorylation, we identified two MAP kinases, SIPK and WIPK, that are located in signaling pathways independent from the induction of AOS synthesis. On the other hand, both kinases are integrated in more than one stress response pathway. This indicates that signal transduction triggering plant defense against pathogens cannot be considered as a linear chain of events but reflects a complex network with interconnections and cross-talk with other stress response pathways. Thus, only an integrated approach, combining the analysis of lines mutated in distinct signaling components with a careful physiological and biochemical characterization, will allow dissection of underlying processes and unravel R gene function.

METHODS

Plant Culture Conditions and Treatments

Plants (Nicotiana tabacum cv Petite Havana) or the transgenic Cf9 line generated by transforming Petite Havana with cosmid 9-34.8 carrying Cf-9 (Hammond-Kosack et al., 1998) were grown in Levington’s M3 compost in a growth cabinet at 28°C with a 16-hr-light/8-hr-dark cycle (300 to 650 µE m⁻² sec⁻¹ photon flux density; 70 to 80% relative humidity). For elicitor treatment, leaves of plants in the sixth to eighth leaf stage, when the flower bolt had just started to rise, were challenged with intercellular fluid (IF) originating from transgenic tobacco that produces the Avr9 peptide apoplastically (IF[Avr9⁺]) or with control IF lacking Avr9 (IF[Avr9⁻]) (De Wit and Spikman, 1982; Hammond-Kosack et al., 1994, 1998). To inject, we set tiny razor blade cuts at the lower leaf side in which the IF was flooded with a syringe. At a given time point, the leaf was detached and two leaf discs (15-mm circles) of each injected panel were cut with a cork borer, immediately frozen in liquid nitrogen, and stored at −70°C until analysis. Mature tobacco leaves (Petite Havana) were wounded by a gentle rubbing of wet carborundum on the upper leaf epidermis. Suspension cultures of tobacco cells Petite Havana and the derived Cf9 line (Piedras et al., 1998) were subcultured at 2-week intervals in Murashige and Skoog (1962) medium supplemented with 3% sucrose, B5 vitamins, 2,4-dichlorophenoxyacetic acid (1 mg/mL), and kinetin (0.1 mg/mL), pH 5.7. Log phase cells were used 4 days after a 1:10 dilution. Before an experiment, the cells were washed three times with assay buffer (5 mM Mes, pH 6, 175 mM mannitol, 0.5 mM CaCl₂, and 0.3 mM K₂SO₄) with 30-min intervals of shaking.
between the single steps, distributed into single flasks (1 g of cells per 20 mL), and allowed to equilibrate in the shaker for 3 hr at 22°C and 180 rpm (Piedras et al., 1998). For elicitation, 75 μL of IF(Avr9+) or IF(Avr9−) was added. At the time indicated, the cells were harvested by filtration, immediately frozen in liquid nitrogen, and stored at −70°C. To apply mechanical stress, we vigorously stirred equilibrated with a magnetic stirrer for 1 min before the flask was incubated on the shaker again.

To study the effect of various effectors and inhibitors, we added the compounds 5 min before elicitation with Avr9 (0.5 mM lanthanum chloride, 0.8 μM diphenyleneiodonium [DPI], and 25 μM staurosporine), 10 min before elicitation (250 μM N-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide [W7], 250 μM 2'-amino-3'-methoxylavone [PD98059], [Calbiochem], and 50 μM 4-bromophenacyl bromide [BPP]), or at time point 0 without further addition of IF (5 μM cantharidin, 100 μM amphotericin B, and 500 μM salicylic acid). If not otherwise mentioned, all compounds were purchased from Sigma (Poole, UK). The synthetic Avr9 peptide was used at a concentration of 10 mM (Piedras et al., 1998).

**Determination of Active Oxygen Species**

Aliquots (0.2 mL) of all cell samples analyzed for protein kinase activity were tested in parallel for active oxygen species (AOS) production by using the ferricyanide-catalyzed oxidation of luminol, as described previously (Schwacke and Hager, 1992; Piedras et al., 1998).

**Preparation of Protein Extracts**

Leaf and cell samples were ground in liquid nitrogen, thawed in 2 volumes of extraction buffer (50 mM Heps, pH 7.4, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 10 mM Na3VO4, 50 mM β-glycerophosphate; 1 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride [AEBSF]; Pefabloc), 2 μg/mL antipain, 2 μg/mL aprotinin, and 2 μg/mL leupeptin), and centrifuged at 21,000 rpm for 20 min at 4°C in a microcentrifuge. The supernatant was desalted on an NAP-5 column (Pharmacia), which was equilibrated with elution buffer (20 mM Heps, pH 7.4, 1 mM MgCl2, 1 mM NaF, 1 mM Na3VO4, 5 mM β-glycerophosphate, 1 μg/mL antipain, 1 μg/mL aprotinin, and 1 μg/mL leupeptin). The crude extracts were stored at −70°C. The protein concentration was determined using the BCA protein assay kit (Pierce, Chester, UK) with BSA as a standard.

**In-Gel Kinase Assay**

Initially, the in-gel kinase assay was performed as described previously (Gotoh et al., 1990; Suzuki and Shinshi, 1995; Usami et al., 1995). Briefly, 20 μg of total protein per lane was separated on an 11.25% SDS–polyacrylamide gel embedded with 0.25 mg/mL myelin basic protein (MBP; Sigma) as a kinase substrate. After electrophoresis, the SDS was removed by two washings (30 min each) in buffer A (50 mM Tris-HCl, pH 8.0, and 20% isopropanol) followed by two washings in buffer B (50 mM Tris-HCl, pH 8.0, and 5 mM β-mercaptoethanol). The proteins in the gel were denatured in buffer C (50 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, and 6 M guanidinium chloride) for 1 hr at room temperature and were renatured overnight at 4°C in buffer D (50 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, and 0.04% Tween 20). After equilibration in buffer E (40 mM Heps, pH 7.4, 2 mM DTT, 15 mM MgCl2, and 0.1 mM EGTA) for 30 min at room temperature, the gel was incubated in 10 mL of buffer E including 40 μM ATP plus 1.85 MBq (50 μCi) γ-32P-ATP (3000 Ci/mmol; Amersham, Little Chalfont, UK) for 90 min at room temperature. The reaction was stopped by transferring the gel into 5% trichloroacetic acid (w/v) and 1% sodium phosphate (w/v). Unincorporated γ-32P-ATP was removed by an intensive washing for 3 hr with at least five changes of the same solution. The gels were dried onto Whatman (Maidstone, UK) 3MM paper and exposed to Kodak X-OMAT film (Sigma) or analyzed using a PhosphorImager (Stratagene, La Jolla, CA). The size of protein kinases was estimated by using different prestained molecular mass markers.

For the correlation of Avr9-induced MBP kinases with known stress-induced MAP kinase from tobacco, a different protocol for the in-gel kinase assay was applied (Zhang and Klessig, 1997). Briefly, after electrophoresis, the gel was washed three times in buffer F (25 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 0.1 mM Na3VO4, 5 mM NaF, 0.5 mg/mL BSA, and 0.1% Triton X-100) for 30 min at room temperature, followed by three washes with buffer G (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM Na3VO4, and 5 mM NaF) at 4°C, including the overnight incubation. Finally, the gel was washed once with buffer H (25 mM Tris-HCl, pH 7.5, 2 mM EGTA, 12 mM MgCl2, 1 mM DTT, and 0.1 mM Na3VO4) for 30 min and then incubated in 10 mL of buffer H with 200 nM ATP plus 1.85 MBq γ-32P-ATP for 90 min; both steps occurred at room temperature. The reaction was stopped and the gel further processed as described above. The latter protocol, lacking a guanidinium denaturation step and performing the kinase reaction in a 200-fold lower concentration of nonlabeled ATP, appears to be more sensitive than the protocol described previously (Gotoh et al., 1990).

**Immunoblotting**

Crude extracts (20 μg total protein per lane) were separated on an 11.25% SDS gel, and proteins were transferred onto nitrocellulose (Amersham) by wet electrophoretic transfer (Mini-Protean II system; Bio–Rad). For analysis with the antiphosphotyrosine antibody, the blots were blocked overnight in TBS buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) with 10% BSA at 4°C and then incubated with the monoclonal antibody PT-66 (Sigma) in a 1:2000 dilution for 1 hr at room temperature in TBS buffer including 1% BSA. After three washes in TBS, the blot was incubated with horseradish peroxidase–conjugated anti-mouse IgG (Promega) (1:10,000 dilution) for 1 hr at room temperature and washed again. The signals were visualized using an enhanced chemiluminescence system (ECL kit; Amersham) following the manufacturer’s instructions.

For immunoblot analysis with kinase-specific antibodies, the blots were blocked in TBS buffer with 5% fat-free milk for 1 hr at room temperature and then probed with the following antibodies: M24 (1:10,000; H. Hirt, unpublished data), p44N (1:5000; Zhang and Klessig, 1998b), and p48N (1:10,000; Zhang et al., 1998). Alkaline phosphatase-conjugated goat anti-rabbit IgG (1:4000 dilution; Sigma) was used as a secondary antibody, and the reaction was visualized by hydrolysis of tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (Sigma) as substrate.

**Immunoprecipitation**

Leaf and cell culture crude extracts in elution buffer (100 μg; see above) were incubated in a volume of 300 μL in IP buffer (25 mM
RNA Gel Blot Analysis

RNA was extracted from elicited, wounded, and stressed leaves (four discs of 1.5 cm) or cell culture material (2 g of cells), as described previously (Dean et al., 1985). Twenty micrograms of total RNA (per lane) was separated on a denaturing formaldehyde–agarose gel. After blotting to nylon membranes, the blot was hybridized with a radiolabeled 1.2-kb BamHI-SacI cDNA fragment that encompasses the complete coding region of the tobacco oxidase gene (Seo et al., 1995), following the protocol of Church and Gilbert (1984). Briefly, filters were hybridized with the probe in 0.2 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA (w/v), and 7% SDS (w/v) at 65°C overnight, followed by washings with (1) 100 mM sodium phosphate, 1 mM EDTA, and 1% SDS, and (2) 50 mM sodium phosphate, 1 mM EDTA, and 0.5% SDS at the same temperature. The filters were exposed to Kodak X-OMAT film and analyzed using a PhosphorImager.

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