Differences in Cell Death Induction by Phytophthora Elicitins Are Determined by Signal Components Downstream of MAP Kinase Kinase in Different Species of Nicotiana and Cultivars of *Brassica rapa* and *Raphanus sativus*[w]

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Elicitins are small, secreted proteins produced by species of the plant-pathogenic oomycete Phytophthora. They induce hypersensitive cell death in most Nicotiana species and in some cultivars of *Brassica rapa* and *Raphanus sativus*. In this study, two true-breeding Fast Cycling *B. rapa* lines were established that showed severe necrosis (line 7-R) or no visible response (line 18-NR) after treatment with elicitin. Unexpectedly, microscopic examination revealed localized cell death in line 18-NR plants, and expression levels of various defense-marker genes were comparable in both lines. These results suggested that both ''responsive'' and ''nonresponsive'' plants responded to elicitin but differed in the extent of the cell death response. Expression of a constitutively active form of Arabidopsis (*Arabidopsis thaliana*) MAP kinase kinase 4 ($AtMEK4^{DD}$) also induced rapid development of confluent cell death in line 7-R, whereas line 18-NR showed no visible cell death. Similarly, elicitin-responsive Nicotiana species and *R. sativus* cultivars showed significantly stronger cell death responses following expression of $AtMEK4^{DD}$ compared with nonresponsive species/cultivars. Line 7-R also showed higher sensitivity to toxin-containing culture filtrates produced by *Alternaria brassicicola*, and toxin sensitivity cosegregated with elicitin responsiveness, suggesting that the downstream responses induced by elicitin and Alternaria toxin share factors that control the extent of cell death. Interestingly, elicitin responsiveness was shown to correlate with greater susceptibility to *A. brassicicola* (a necrotroph) in *B. rapa* but less susceptibility to *Phytophthora nicotianae* (a hemibiotroph) in Nicotiana, suggesting a more extensive cell death response could cause opposite effects on the outcomes of biotrophic versus necrotrophic plant-pathogen interactions.

Plants have the ability to recognize potential pathogens and resist them by inducing various defense mechanisms. Molecules derived from pathogens are targets for plant recognition and can elicit defense responses even in the absence of the pathogen. These elicitors include nonspecific molecules, such as conserved structural components of the fungal cell wall, the bacterial outer membrane or flagella, and specific molecules produced by particular strains of pathogens such as the avirulence proteins secreted by some fungi (e.g. Avr9 and AvrL567) and type III effectors produced by some bacteria (e.g. AvrB and PopP2; Montesano et al., 2003; Dodds et al., 2004; Lahaye, 2004). Elicitins are small elicitor proteins produced by the pathogenic oomycete genera Phytophthora and Pythium, although not by all species of Pythium. Phytophthora species possess a family of elicitins and elicitin-related proteins divided into three broad classes (Ponchet et al., 1999; Tyler, 2002; Baillieul et al., 2003; Qutob et al., 2003), but the term elicitins generally refers to Class I elicitins, which are secreted abundantly in culture and are well conserved among Phytophthora species. Elicitin treatment of responsive plants induces typical defense responses such as the hypersensitive response (HR, a form of programmed cell death), production of phytoalexins, expression of PR (pathogenesis-related) proteins, and subsequent systemic acquired resistance to various pathogens (Milat et al., 1991; Kamoun et al., 1993; Bonnet et al., 1996; Keller et al., 1996; Cordelier et al., 2003). Induction of HR and systemic acquired resistance by elicitins has been observed in most species of Nicotiana and in some cultivars of *Brassica rapa* (synonym *Brassica campestris*) and *Raphanus sativus*, but not in Solanum species, Capsicum species, *Lycopersicon esculentum*, or Arabidopsis (*Arabidopsis thaliana*; Kamoun et al., 1993; Bonnet et al., 1996; Keizer et al., 1998). Although elicitins from different species show various HR-inducing activities, elicitins from different Phytophthora species usually elicit HR in the same range of plants, indicating that responsive plants recognize elicitins as a conserved feature of Phytophthora species. Thus, elicitins seem to be intermediate between general and specific elicitors.

There have been a number of reports indicating that elicitins function as avirulence factors in Nicotiana-Phytophthora interactions. Most virulent isolates of

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Phytophthora nicotianae (synonym *Phytophthora parasitica*), the black shank pathogen of tobacco, have lost the ability to produce elicitin in culture (Ricci et al., 1992), whereas virulent isolates still able to produce elicitin in culture show down-regulated expression of the elicitin gene *parA1* during growth on the host plant (Colas et al., 2001). These data suggest that the absence or down-regulation of elicitin gene expression in planta constitutes pathogen strategies to avoid or minimize recognition by the host plant. Moreover, elicitin-deficient strains of *Phytophthora infestans* produced by silencing of the *inf1* gene show enhanced virulence on the non-host *Nicotiana benthamiana* (Kamoun et al., 1998).

After elicitin treatment, signaling events characteristic of disease resistance are activated in elicitin-responsive plants. These include the induction of calcium ion influx, transient production of active oxygen species, and activation of mitogen-activated protein kinases (MAPKs; Tavernier et al., 1995; Rustérucci et al., 1996; Zhang et al., 1998, 2000). MAPKs are activated by recognition of various general elicitors (e.g. bacterial flagellin and harpin, and fungal/oomycete cell wall-derived elicitors) and race-specific elicitors (Zhang and Klessig, 2001). In tobacco, elicitin treatment induces activation of the MAPKs salicylic acid-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK; Zhang et al., 1998, 2000). A constitutively active form of NtMEK2, a kinase upstream of SIPK and WIPK, activates both of these MAPKs and induces cell death (Yang et al., 2001), suggesting a MAPK cascade could lead to plant HR in response to elicitin treatment. HSP90 (heat shock protein 90) and SGT1 (suppressor of G2 allele of SKP1), shown by virus-induced gene silencing (VIGS) to be involved in race-specific disease resistance, have also been shown to be essential for HR induction by elicitin in *N. benthamiana* (Peart et al., 2002; Kanzaki et al., 2003; Martin et al., 2003). These data suggest that HR induction by elicitins and race-specific elicitors is controlled by shared or overlapping signal transduction pathways/factors.

In contrast to the knowledge accumulating about plant responses to elicitins, the role of Phytophthora elicitins in plant pathogenesis is less certain. Structural characterization of elicitins reveals a small hydrophilic protein with a hydrophobic pocket, similar to a lipid transfer protein (Boissy et al., 1996; Mikes et al., 1998). Elicitins have been reported to bind phytosterols with 1:1 stoichiometry, to transfer sterol between phospholipidic membranes, and to pick up sterols from plant plasma membranes (Mikes et al., 1998; Vauthrin et al., 1999). Given that Phytophthora and Pythium species are unable to produce sterols, which are essential for their reproduction (Hendrix, 1970), they must take up sterols from their host plants. Therefore, elicitins could be essential factors for the propagation of Phytophthora and Pythium species (Blein et al., 2002), but the importance of elicitins for pathogen viability or pathogenicity has not been confirmed experimentally.

The lipid binding activity of elicitins may also be crucial for their recognition by plants. Elicitins with higher sterol binding efficiency (cryptogein > parasiticein and capsicein) showed stronger elicitor activity (Mikes et al., 1998). Mutated elicitins with less efficient loading of sterols showed lower elicitor activities than wild-type elicitins (Osman et al., 2001). These data suggest that elicitin-sterol complexes may be the targets for plant recognition.

Despite the recent isolation of a number of plant genes responsible for race-specific resistance, limited information is available about plant determinants of responsiveness to elicitins. In this study, we aimed to establish a model system to investigate plant responses to elicitins and focused on determinants of HR induction by elicitins. We established elicitin-responsive *B. rapa* line 7-R, which showed intense leaf necrosis after elicitin treatment or transient expression of an elicitin gene by agroinfiltration, whereas nonresponsive line 18-NR developed no visible response. Microscopic examination of elicitin-treated leaves after lactophenol trypan blue staining revealed that both lines initiate cell death following elicitin treatment, but only responsive line 7-R showed extensive progression or lack of containment of cell death resulting in macroscopic HR. We also investigated the induction of cell death in these two lines by a constitutively active form of Arabidopsis MAP kinase kinase (MAPKK) 4 ($AtMEK4^{DD}$) and Alternaria toxin. Macroscopic symptoms of cell death induced by $AtMEK4^{DD}$ or Alternaria toxin were significantly stronger in line 7-R than line 18-NR. We also tested elicitin-responsive and nonresponsive Nicotiana species and *R. sativus* cultivars and found that differences in visible HR induction in these species/cultivars were also due to differential progression/containment of cell death. These results suggest that the difference between elicitin-responsive and nonresponsive plants could reflect variation in the control of cell death expansion rather than elicitin perception.

RESULTS

Plant Responses to Elicitin

To establish a model system for the investigation of plant response mechanisms to Phytophthora elicitins, we first tested 53 Arabidopsis accessions (listed in "Materials and Methods") for elicitin responsiveness because only a few Arabidopsis accessions, which were not responsive to elicitins, have been tested and the results reported (Kamoun et al., 1993; Bonnet et al., 1996), despite the fact that Arabidopsis could potentially have been a good model plant for a molecular analysis of elicitin responsiveness. We used an elicitin solution containing approximately 10 to 50 nM elicitin (designated Pc-elicitin) prepared from the culture filtrate of *Phytophthora cinnamomi* (Supplemental Fig. 1A), which induced visible HR in *Nicotiana tabacum* within 2 d of treatment (positive control), but not in *L. esculentum*

(negative control). However, we could find no Arabidopsis accession that showed an HR-like response to Pc-elicitin (Table I). We also tested two additional Nicotiana species, and three *R. sativus* and nine *B. rapa* cultivars. *N. benthamiana*, like *N. tabacum*, showed a visible HR-like response 2 d after Pc-elicitin treatment, whereas there was no obvious response in *Nicotiana amplexicaulis* at the same time point (Table I), although *N. amplexicaulis* has been reported previously to be responsive to elicitins from *Phytophthora sojae* (Qutob et al., 2003). A chlorotic response appeared in *N. amplexicaulis* around 1 week after Pc-elicitin treatment, and the treated area subsequently became desiccated within 2 to 3 weeks of treatment (data not shown). *R. sativus* cv Daikon showed an HR-like response within 2 d of Pc-elicitin treatment, while cultivars White Icicle and Long Scarlet showed no visible response to elicitin, as reported previously (Kamoun et al., 1993; Keizer et al., 1998). All nine cultivars of *B. rapa* tested were nonresponsive to Pc-elicitin (Table I); however, some individuals of Fast Cycling *B. rapa*, which was produced by breeding and selection to obtain plants with shorter life cycles (Williams and Hill, 1986), showed a visible HR-like response to Pc-elicitin (Fig. 1A). Individuals of Fast Cycling *Brassica oleracea* and *Brassica nigra* also showed different degrees of response (Table I; Supplemental Fig. 2), although there were fewer responsive plants in these lines. Fast Cycling *B. rapa* has several advantages as a potential model plant in which to study elicitin responsiveness, such as a short life cycle and a close phylogenetic relationship with Arabidopsis, allowing the possible application of comparative genomics. We therefore decided to further analyze elicitin responsiveness in Fast Cycling *B. rapa*.

Table I. *Induction of cell death by elicitin treatment in different plant species and cultivars*

The responses were scored 2 d after treatment with P. cinnamomi elicitin. −, No visible response; +, nonconfluent cell death; ++, confluent cell death.

Species and Cultivars	*P. cinnamomi* Elicitin
B. rapa	
Fast Plant	−/+/++
cv Sisu	−
cv Valti	−
cv Pak Choy	−
cv Hakurei	−
cv Purple Top White Globe	−
cv Early Purple	−
cv Wong Bok	−
cv Nagaoka 60 Days	−
cv Wong Bok	−
B. oleracea	
Fast Plant	−/+/++
B. nigra	
Fast Plant	−/+
R. sativus	
cv White Icicle	−
cv Daikon	++
cv Long Scarlet	−
Arabidopsis	
53 ecotypes	−
N. tabacum	++
N. benthamiana	++
N. amplexicaulis	−
L. esculentum	−

Selection and Establishment of Elicitin-Responsive and Nonresponsive *B. rapa* Lines from Fast Plants

Individual plants of Fast Cycling *B. rapa* showed different degrees of response to elicitin solution prepared from *P. cinnamomi*, and we categorized them into six classes based on the visible responses of their cotyledons 2 d after Pc-elicitin treatment: (A) complete desiccation, (B) severe cell death in approximately 75% of the treated area, (C) moderate cell death in approximately 50% of the treated area, (D) several spots or small areas of cell death, (E) a few spots of cell death, and (F) no visible cell death (Fig. 1A). The first visible response of plants in classes A and B was a glossy appearance to the undersurface of cotyledons within 6 h of Pc-elicitin treatment, followed by the development of dark necrotic regions within 24 h and complete (class A) or partial (B) desiccation of cotyledons within 48 h. Occasionally, the opposing untreated cotyledons of class A and B seedlings developed a similar degree of cell death to that observed for Pc-elicitin-treated cotyledons of class C and D seedlings. By contrast, plants in classes E and F showed no detectable response within 24 h of treatment, although plants in class E developed a few tiny spots by 48 h after treatment. The strengths of response to Pc-elicitin observed in the cotyledons were reproduced in the true leaves of the same individuals, indicating that response to elicitin is not dependent on the growth stage of the plants and that the different elicitin responsiveness of each individual was probably determined genetically.

To obtain genetically fixed responsive and nonresponsive lines from Fast Cycling *B. rapa*, we initially chose 20 individuals with various degrees of Pc-elicitin responsiveness and commenced the production of genetically fixed lines by self-fertilization using sodium chloride treatment to break the self-incompatibility of *B. rapa* (Monteiro et al., 1988). Self-progenies of these 20 plants showed significant differences in various phenotypes, such as height, branching, shape of leaves, flowering time, pollen productivity, and elicitin responsiveness, indicating considerable genetic diversity within the original Fast Cycling *B. rapa* line. The extent of this genetic diversity was also verified by radiolabeled DNA amplification fingerprinting analysis (Waldron et al., 2002; data not shown). After one more round of self-fertilization, elicitin-responsive line 7-R and nonresponsive line 18-NR were selected, and all progeny of these two lines either showed strong response (line 7-R) or no response (line 18-NR) to Pc-elicitin (Fig. 1B). To further purify the genetic background of these two lines, we repeated the

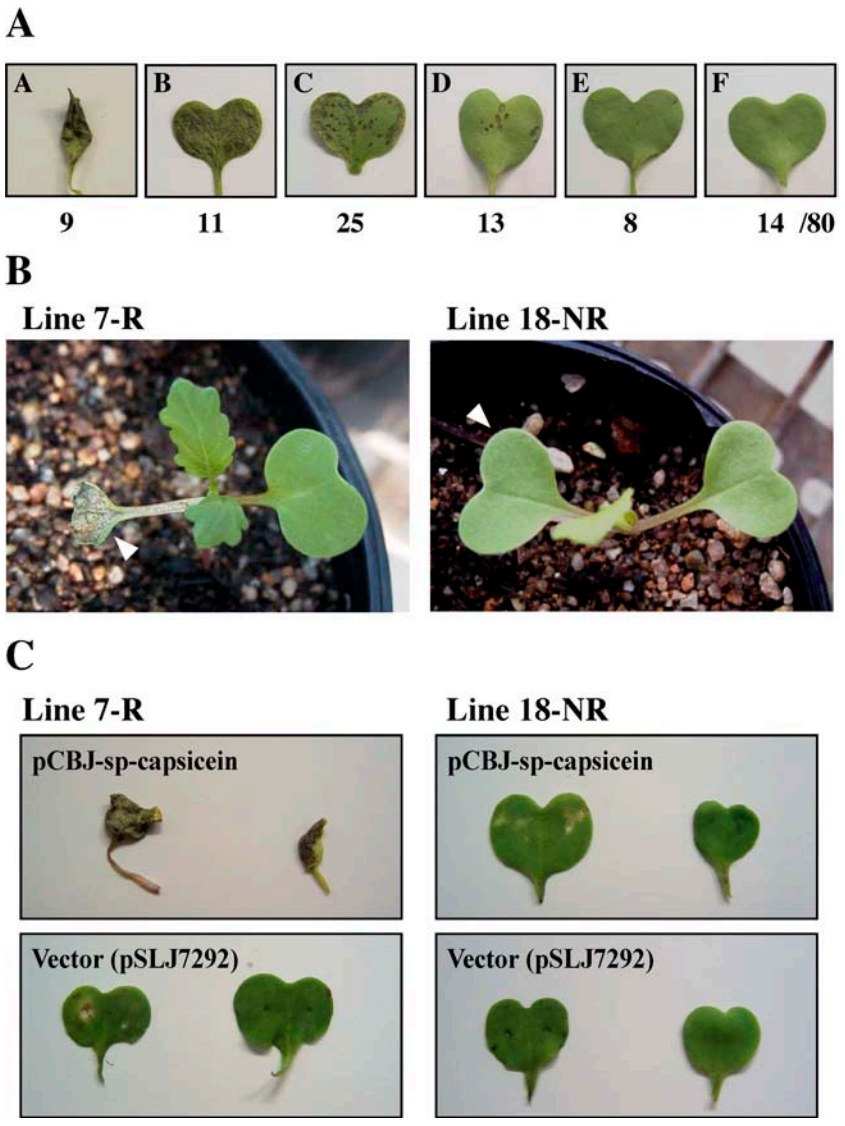

Figure 1. Cell death in Fast Cycling *B. rapa* plants induced by elicitin solution prepared from *P. cinnamomi* culture filtrate or by transient expression of a synthetic elicitin gene. A, Original seed stock of Fast Cycling *B. rapa* contained individuals with different elicitin responsiveness. Cotyledons of 80 Fast Cycling *B. rapa* individuals were treated with elicitin solution. Differential responses to elicitin were categorized into six classes according to the severity of their visible cell death responses. Numbers of plants in each class are given under each section. B, Cotyledons of responsive (line 7-R) and nonresponsive (line 18-NR) lines treated with elicitin solution. Treated cotyledons are indicated by arrowheads. Photographs in A and B were taken 2 d after treatment. C, Cotyledons of responsive (line 7-R) and nonresponsive (line 18-NR) lines infiltrated with *A. tumefaciens* carrying pSLJ7292 (vector) or pCBJ-sp-capsicein (synthetic elicitin gene). The pCBJ-sp-capsicein construct contains a synthetic α-capsicein gene, which is fused in frame with the coding sequence of the PR1 signal peptide of *N. tabacum* and expressed under the control of the CaMV 35S promoter. Photographs were taken 3 d after agroinfiltration.

self-fertilizations four more times before further investigating their elicitin-responsive or nonresponsive characteristics. Responsive line 7-R showed a moderate dwarf phenotype and occasionally produced spots of spontaneous cell death, most probably induced by environmental stresses such as intense light.

B. rapa lines 7-R and 18-NR were treated with elicitin solutions prepared from *Phytophthora megasperma*, *P. nicotianae*, *P. sojae*, and *Phytophthora cryptogea*, and severe visible HR to each of the elicitin solutions was found only in line 7-R (Supplemental Fig. 1B), confirming that the response of *B. rapa* line 7-R is not specific to elicitins from particular Phytophthora species but constitutes a broad/general response to Phytophthora elicitins. To confirm that line 7-R was responding to elicitins rather than other components of the elicitin preparations, we synthesized a chimeric transgene for mature α-capsicein, an elicitin produced by *Phytophthora capsici* (Ricci et al., 1989), with the signal peptide (sp) of tobacco PR1a (Cornelissen et al., 1987) fused to the N terminus to enable expression of mature elicitin in the intercellular spaces of transformed plant tissue. The sp-capsicein construct was expressed transiently under the control of the cauliflower mosaic virus (CaMV) 35S promoter by agroinfiltration into cotyledons or leaves of *B. rapa* lines 7-R and 18-NR. Line 7-R showed visible HR-like symptoms induced by sp-capsicein, whereas no visible cell death was induced in line 18-NR (Fig. 1C). There was no induction of HR in either line by agroinfiltration with empty vector (Fig. 1C) or with vector containing a green fluorescent protein (GFP)-hTalin gene under the control of the CaMV 35S promoter (Takemoto et al., 2003; data not shown). This result confirmed that line 7-R responds strongly to elicitins and that line 18-NR does not.

To further examine the cell death response induced by elicitin treatment, we examined Pc-elicitin-treated cotyledons of lines 7-R and 18-NR by light microscopy following staining with lactophenol trypan blue.

Staining of plant cells with trypan blue is indicative of cell death (Keogh et al., 1980). At 6 h after Pc-elicitin treatment, clusters of blue-stained plant cells were observed in line 7-R, and, unexpectedly, line 18-NR also showed some dead cells. Dead cells in line 7-R were usually found in clusters of more than five cells, while dead cells in line 18-NR were usually found as isolated single cells or clusters of only a few cells (Fig. 2A). Within 24 h, cell death in line 18-NR was seen as small clusters of dead cells, whereas cell death in line 7-R plant was almost confluent over the whole treated area of cotyledon (Fig. 2A). In line 7-R, the central part of the petiole of Pc-elicitin-treated cotyledons was often stained by trypan blue, indicating Pc-elicitin may have been transported through the vascular tissues or systemic cell death may have been induced in line 7-R (Fig. 2B). By contrast, treatment with water never induced cell death in lines 7-R and 18-NR. These data raised the possibility that the different magnitudes of the cell death response in lines 7-R and 18-NR could be due to a difference in the progression or containment of cell death rather than the initiation of cell death following elicitin recognition.

Characterization of Progeny from the Cross between Lines 7-R and 18-NR

After genetic purification of lines 7-R and 18-NR by self-fertilization for six generations, a genetic analysis of elicitin responsiveness was initiated by intercrossing the two lines. F1 plants showed moderate responses to Pc-elicitin that varied between classes B to E, suggesting elicitin responsiveness was not dominant (Supplemental Table II). Segregating F2 populations obtained by intercrossing of F1 plants contained plants with phenotypes ranging from classes A to F. Despite the fact that the F1 plants should have had almost identical genotypes, we found a variety of elicitin responses, suggesting environmental effects on elicitin responsiveness. Thus, we further analyzed F3 families derived from individual F2 plants to test their segregation patterns in order to establish F2 genotypes. Sixty-five F2 plants were randomly selected and self-fertilized, and the F3 family from each F2 plant was tested for elicitin responsiveness. Only two F3 families contained progeny showing only class A and B responses to elicitin treatment, while only three F3 families contained progeny showing only class E and F responses (Supplemental Table III). The remaining 60 F3 families gave progeny with phenotypes ranging over classes A to D (13), A to F (28), and C to F (19; Supplemental Table III). Given that line 7-R shows no plants with class E and F phenotypes and line 18-NR shows no plants with class A and B phenotypes but the F1 generates a full range of phenotypes in the F2 (Supplemental Table II), it may be reasonable to pool the F3 families into three classes, i.e. 15 showing no class E or F plants (with corresponding F2 parents presumed to be homozygous responsive), 28 showing a full range of phenotypes (with corresponding F2 parents presumed to be heterozygous), and 22 showing no class A or B phenotypes (with corresponding F2 parents presumed to be homozygous nonresponsive). These data fit a 1:2:1 ratio consistent with a monogenic segregation in the F_2 ($\chi^2_2 = 2.75$, $P>0.2$), suggesting that codominant alleles of a single gene might be involved in the differential elicitin responsiveness of lines 7-R and 18-NR. However, despite a fit to the simplest model possible for the genetic control of elicitin responsiveness in *B. rapa*, these data do not exclude more complex models involving multiple genes.

Transient Expression of a Constitutively Active Form of MAPKK

To investigate the role of MAPKs in the differential elicitin responsiveness of lines 7-R and 18-NR, we employed a constitutively active form of Arabidopsis MEK4 (synonym MKK4), an ortholog of tobacco NtMEK2, which has been reported to mediate HR induction in response to flagellin perception in Arabidopsis (Asai et al., 2002; Ren et al., 2002). Amino acids T224 and S230 of AtMEK4, which are phosphorylated by the upstream MAPK kinase kinase, were each

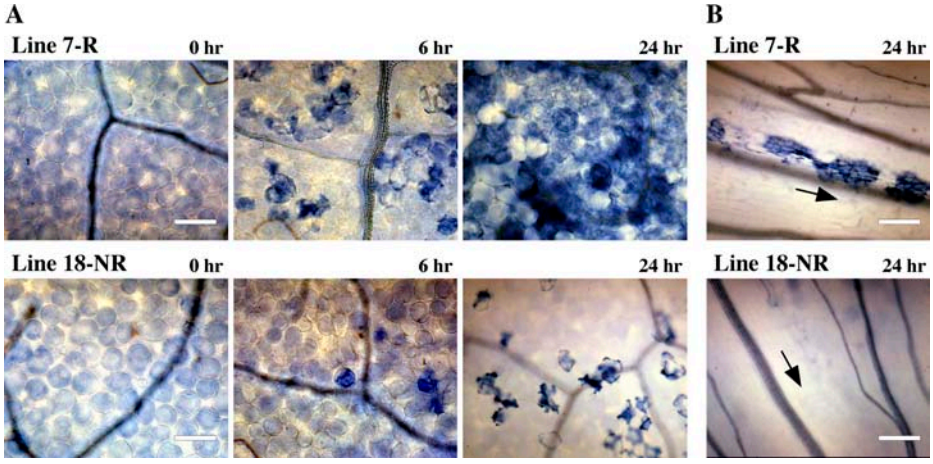

Figure 2. A, Microscopic examination of cell death responses in cotyledons of *B. rapa* lines 7-R and 18-NR. Dead cells were visualized by lactophenol trypan blue staining 0, 6, and 24 h after elicitin treatment. Bar = 100 μm. B, Cell death observed in petiole vascular tissues of cotyledons treated with elicitin. Arrows indicate the direction from the base of the cotyledon toward the hypocotyl. Bar = 200 μm.

replaced by Asp to mimic activation of AtMEK4. The mutant *AtMEK4* gene, designated *AtMEK4*^{DD}, was introduced into a binary plant transformation vector under the control of the CaMV 35S promoter and used in agroinfiltration assays. It was expected that lines 7-R and 18-NR would show similar degrees of HR in response to expression of *AtMEK4*^{DD} if the difference in elicitin response was determined upstream of AtMEK4, e.g. by an elicitin receptor. However, transient expression of *AtMEK4*^{DD} induced significantly stronger HR in line 7-R (Fig. 3). This result suggested that differential strength of HR induction in the two *B. rapa* lines is not determined by their capacity for elicitin recognition but by a component(s) downstream of the MAPK cascade.

To exclude the possibility that these results might be due to higher transformation efficiency of line 7-R compared to line 18-NR, a β-glucuronidase (GUS) gene was introduced by agroinfiltration and the transient expression of the gene was evaluated by GUS staining. No significant difference in GUS staining was observed between 7-R and 18-NR lines, although slightly less GUS staining was seen occasionally in line 7-R (data not shown).

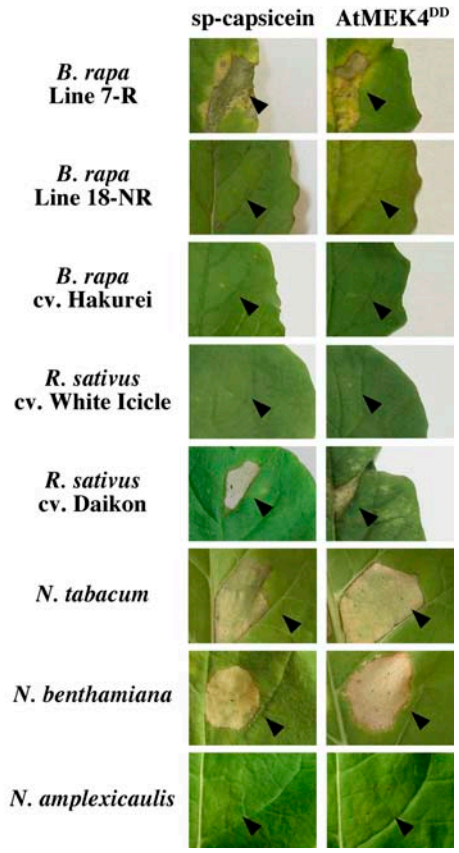

Figure 3. Hypersensitive cell death induced by transient expression of elicitin or *AtMEK4*^{DD} genes in *B. rapa* cultivars, *R. sativus* cultivars, and Nicotiana species. *A. tumefaciens* cultures carrying pCBJ-sp-capsicein or pCBJ-AtMEK4^{DD} were infiltrated into intercellular space of plant leaves. Photographs were taken 3 d after agroinfiltration. Results shown are representative of three separate experiments.

Elicitin- and MEK^{DD}-Induced Cell Death in Different Plant Species

To test whether the correlation between elicitin- and MEK^{DD}-induced cell death extended to other *B. rapa* cultivars and other plant species showing variation in elicitin responsiveness, we tested four *B. rapa* cultivars, two *R. sativus* cultivars, and three Nicotiana species plus Arabidopsis and *L. esculentum*. Transient expression of the sp-capsicein gene caused visible necrosis in *R. sativus* cv Daikon, *N. tabacum*, and *N. benthamiana*, whereas the four *B. rapa* cultivars (cvs Sisu, Valti, Pak Choy, and Hakurei), *R. sativus* cv White Icicle, *N. amplexicaulis*, Arabidopsis, and *L. esculentum* showed no visible response within 3 d of agroinfiltration (Fig. 3; Table II; data not shown). However, *N. amplexicaulis* often developed a weak chlorotic response followed by desiccation of the treated area within 2 weeks. These results are consistent with the responses observed with elicitin solutions prepared from *P. cinnamomi* culture filtrates. Interestingly, plant cultivars or species responsive to elicitin always developed confluent HR in response to the transient expression of *AtMEK4*^{DD}, whereas those nonresponsive to elicitin did not, although some, including Arabidopsis and *L. esculentum*, showed a weak response more than a week after agroinfiltration (Fig. 3; data not shown). Transient expression of wild-type *AtMEK4* or *AtMEK4*^{R}, an inactive mutant of *AtMEK4*, caused no response in elicitin-responsive *B. rapa*, *R. sativus*, or *N. tabacum* (Supplemental Fig. 3). Equivalent agroinfiltration transformation efficiencies were also verified for these plants by GUS gene expression, and no significant difference was observed between elicitin-responsive and nonresponsive species/cultivars (data not shown).

Lactophenol trypan blue staining and microscopic analysis revealed that *N. amplexicaulis* and elicitin nonresponsive cultivars of *B. rapa* and *R. sativus*, like *B. rapa* line 18-NR, developed only small clusters of dead cells 24 h after Pc-elicitin treatment (Supplemental Fig. 4), while *N. tabacum*, *N. benthamiana*, and *R. sativus* cv Daikon, like *B. rapa* line 7-R, showed confluent cell death (Supplemental Fig. 4). These data suggest that all of the *B. rapa* and *R. sativus* cultivars and Nicotiana species tested have the ability to recognize and respond to elicitin, and in all of these plants the determinant of cell death severity is located downstream of the MAPK cascade.

Expression of Defense Genes in Responsive and Nonresponsive *B. rapa*

Since it was shown that both *B. rapa* lines 7-R and 18-NR could initiate cell death in response to elicitin, we investigated the expression patterns of defense-marker genes in both lines after Pc-elicitin treatment. These marker genes include the *Pathogenesis-Related 1* (*PR-1*) gene as a marker of salicylic acid (SA)-mediated induction of defense genes, *Plant Defensin 1.2* (*PDF1.2*) gene as a marker for ethylene- and jasmonic acid-

Table II. *Summary of plant responses to treatment with P. cinnamomi elicitin or A. brassicicola toxin and agroinfiltration with capsicein, constitutively active MAPKK, or control gene expression constructs*

−, No visible response; +, nonconfluent cell death; ++, confluent cell death; +++, rapid/confluent cell death; nd, not determined.

Species and Cultivars (Lines)	*P. cinnamomi* Elicitin	*A. brassicicola* Toxin	Agroinfiltration		
			sp-capsicein	AtMEK4^{DD}	GFP-hTalin
B. rapa					
Fast plant line 7-R	++	+++	++	++	−
Fast plant line 18-NR	−[a]	+	−	−	−
cv Sisu	−[a]	+	−	−	−
cv Valti	−[a]	+	−	−	−
cv Pak Choy	−[a]	+	−	−	−
cv Hakurei	−[a]	+	−	−	−
R. sativus					
cv White Icicle	−[a]	nd	−	−	−
cv Daikon	++	nd	++	++	−
N. tabacum	++	nd	++	++	−
N. benthamiana	++	nd	++	++	−
N. amplexicaulis	−[a]	nd	−	−	−

[a]Localized cell death was detected by microscopic observation after lactophenol trypan blue staining.

mediated induction of defense genes, and *Copper Zinc Superoxide Dismutase 1* (*CSD1*) gene, which is reported to be induced coincident with HR and by the SA analog isonicotinic acid (Uknes et al., 1992; Penninckx et al., 1998; Kliebenstein et al., 1999). We also investigated *Lesion Simulating Disease 1* (*LSD1*), *LSD-One-Like 1* (*LOL1*), and *Bax Inhibitor 1* (*BI-1*) genes, which are negative (*LSD1*) and positive regulators (*LOL1* and *BI-1*) of hypersensitive cell death (Dietrich et al., 1997; Epple et al., 2003; Kawai-Yamada et al., 2004). Primers for these marker genes were designed using Arabidopsis sequences and corresponding Brassica sequences if available (Supplemental Table I).

In both *B. rapa* lines 7-R and 18-NR, expression of *PR-1* and *PDF1.2* was clearly induced within 24 h after Pc-elicitin treatment (Fig. 4). Unexpectedly, expression of *PR-1* was significantly greater in line 18-NR than in line 7-R, whereas the expression of *PDF1.2* was the same in both lines.

In Arabidopsis, LSD1/LOL1 are thought to be positive/negative regulators of the expression of *CSD1*, which encodes superoxide dismutase, a potential suppresser of cell death through detoxification of O_2^- (Kliebenstein et al., 1999; Epple et al., 2003). Expression of *LOL1* was very weak in both lines and there was no detectable induction after Pc-elicitin treatment (data not shown). Expression of *CSD1* was found to be induced in both lines 7-R and 18-NR soon after Pc-elicitin treatment (Fig. 4). *LSD1* was induced slightly in both lines 18 to 24 h after Pc-elicitin treatment. Expression of *LSD1* and *CSD1* was often slightly stronger in line 7-R than in line 18-NR (Fig. 4), counter to a possible role in limiting the cell death response in line 18-NR. Arabidopsis *CSD1* has been reported to be induced by the SA analog isonicotinic acid, but is also induced strongly in plant cells undergoing cell death (Kliebenstein et al., 1999). Given CSD1 is expected to be a suppresser of HR, the higher expression of CSD1 in line 7-R may reflect attempted negative feedback of cell death as a result of the extensive cell death that has already occurred. From these results, it would appear that the differential elicitin responsiveness of lines 7-R and 18-NR cannot

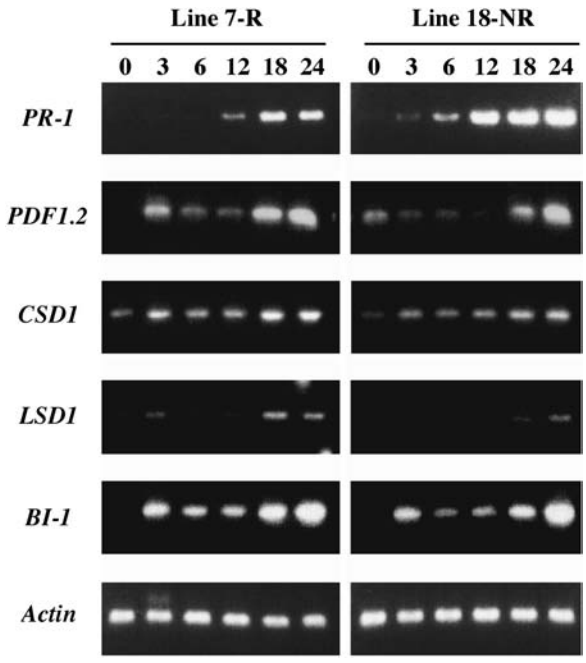

Figure 4. Expression profiles of marker genes for defense responses in *B. rapa* line 7-R and line 18-NR at various times after treatment with elicitin solution. Accumulation of mRNA for *PR-1*, *PDF1.2*, *CSD1*, *LSD1*, and *BI-1* was determined by RT-PCR with specific primers. RT-PCR analysis of actin gene expression is shown as an internal control. Results shown are representative of three separate experiments.

be explained by differential expression of the *CSD1*, *LSD1*, or *LOL1* genes.

Arabidopsis BI-1 is an inhibitor of plant cell death induced by H_2O_2 or by expression of mammalian Bax (Kawai-Yamada et al., 2004). Expression of the *B. rapa* homolog of *AtBI-1* was induced to similar extents in both lines 7-R and 18-NR within 3 h of Pc-elicitin treatment (Fig. 4), suggesting that induction of *BI-1* gene expression was not sufficient to stop the progressive cell death induced in line 7-R. Overall, these results clearly show that line 18-NR is as "responsive" to elicitin as line 7-R with respect to defense gene induction.

Characterization of *LOL1* and *LSD1* Genes from Responsive and Nonresponsive *B. rapa*

Line 7-R allows extensive progression of the elicitin-induced cell death response and occasionally develops sectors of spontaneous cell death. These characteristics reminded us of the progressive lesion-mimic mutants such as *lsd1* and *acd1* of Arabidopsis (Lorrain et al., 2003). LSD1 and the recently characterized LOL1 are zinc-finger proteins that are antagonistic negative and positive regulators of cell death progression, respectively (Dietrich et al., 1997; Epple et al., 2003). Our preliminary experiments to map genes controlling the extensive cell death phenotype in line 7-R indicated weak linkage to some Brassica markers corresponding to a region of Arabidopsis chromosome 1 near the *LOL1* gene (data not shown). Thus, we analyzed the *LOL1* genes in lines 7-R and 18-NR. Using degenerate primers (Supplemental Table I), we isolated and sequenced *B. rapa LOL1* homologs from both *B. rapa* lines. The nucleic acid sequences of the *LOL1* genes were identical in the two lines and they encoded a protein showing 98% amino acid identity with Arabidopsis LOL1 (Supplemental Fig. 5). We also isolated partial sequences of *LSD1* homologs. Each line had at least two *LSD1* homologs with minor sequence variations between the two lines (data not shown). We investigated the distribution of these variants in progenies of the two lines but found no correlation between elicitin responsiveness and distribution of *LSD1* homologs in F_2 progeny of the cross between lines 7-R and 18-NR (data not shown). From these results, it would appear that the differential elicitin responsiveness of lines 7-R and 18-NR cannot be explained by variation in the coding sequences of the *LSD1* or *LOL1* genes.

Induction of Cell Death by Alternaria Toxin in Responsive and Nonresponsive *B. rapa*

Alternaria brassicicola is a necrotrophic pathogen of Brassica that causes black leaf spot disease. *A. brassicicola* produces a toxin in culture media that causes necrosis on Brassica and other plant species (MacDonald and Ingram, 1986), and the Brassica-specific AB-toxin is produced specifically upon germination of *A. brassicicola* (Otani et al., 1998). Since plants often show typical "resistance" reactions in response to toxin treatment (Wang et al., 1996; Navarre and Wolpert, 1999; Stone et al., 2000), we decided to test the sensitivities of *B. rapa* lines 7-R and 18-NR to Alternaria toxin. Culture filtrate (Ab-cf) was collected from a 2-week-old culture of *A. brassicicola* and used as a crude toxin solution. Interestingly, Ab-cf caused strong cell death on line 7-R, while line 18-NR showed only a very weak response within 2 d of treatment (Fig. 5A). Furthermore, >50 randomly selected plants from different F3 families obtained from the cross between lines 7-R and 18-NR were tested simultaneously for responsiveness to Ab-cf and Pc-elicitin. Interestingly, the cell death response induced by Ab-cf was always comparable to that caused by Pc-elicitin (Fig. 5B), indicating that the determinant of cell death severity that differentiates line 7-R from line 18-NR is common to both elicitin- and Alternaria toxin-induced cell death pathways. Inoculation tests with *A. brassicicola* were carried out to evaluate the effect on disease severity of the differential responsiveness of lines 7-R and 18-NR to Alternaria toxin. Agarose blocks with growing cultures of *A. brassicicola* were placed on detached leaves of lines 7-R and 18-NR. Severe disease symptoms, such as brown necrotic lesions surrounded by areas of chlorosis, were evident within 2 d after inoculation of line 7-R. Slower development of disease symptoms was observed in line 18-NR, but no difference in disease severity was evident between the two lines by 1 week after inoculation (Fig. 6A). This result suggests that the degree of toxin sensitivity can affect the rate at which the disease progresses but does not affect the ultimate outcome of the interaction.

Infection of *N. tabacum* and *N. amplexicaulis* by *P. nicotianae*

Cotyledons of elicitin-responsive *N. tabacum* and nonresponsive (or weakly responsive) *N. amplexicaulis* were inoculated with *P. nicotianae*, the casual agent of black shank of tobacco. In *N. tabacum*, infection with *P. nicotianae* induced limited plant cell death within 24 h, which appeared to limit the rate and extent of pathogen infection but did not contain it completely (Fig. 6B). *P. nicotianae* that escaped from containment by clusters of dead plant cells showed no further induction of plant cell death in surrounding cells (Fig. 6B). This result is consistent with a previous report showing that expression of the elicitin gene in virulent *P. nicotianae* is down-regulated on the host plant (Colas et al., 2001). By contrast, infection of *N. amplexicaulis* with *P. nicotianae* caused no cell death but resulted in extensive growth of the pathogen within 24 h (Fig. 6B). By 48 h after inoculation, extensive hyphal growth was evident in cotyledons of both *N. tabacum* and *N. amplexicaulis*; however, massive production of sporangia was obvious only in *N. amplexicaulis* (Fig. 6B). This result suggests that the higher elicitin responsiveness of *N. tabacum* affects the

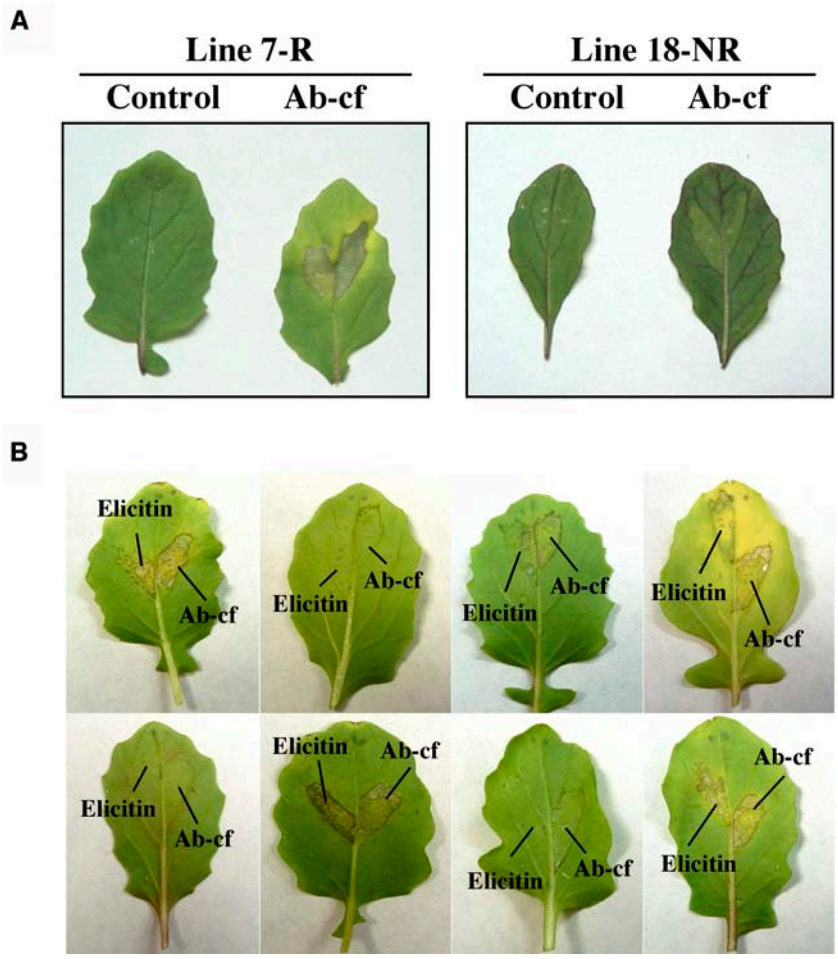

Figure 5. Cell death in *B. rapa* induced by Alternaria toxin and elicitin solution. A, *B. rapa* leaves were treated with potato dextrose broth media (Control) or culture filtrate of *A. brassicicola* isolate MBH3-1 (Ab-cf). B, Leaves of F3 plants from the *B. rapa* cross line 7-R × line 18-NR were treated with elicitin solution (Elicitin) or culture filtrate of *A. brassicicola* (Ab-cf) and photographed 2 d after treatment.

rate of *P. nicotianae* infection, which could reduce disease severity and the production of pathogen propagules.

DISCUSSION

Phytophthora species are some of the most destructive pathogens of crops and natural plant communities. They produce highly conserved elicitor proteins known as elicitins, which are cultivar-specific elicitors in Brassica species and *R. sativus* (Kamoun et al., 1993; Ponchet et al., 1999). Investigations of plant responses to elicitins could contribute to the protection of a wide range of plants against Phytophthora diseases. In this study, we established two *B. rapa* lines that showed a clear macroscopic difference in elicitin responsiveness (Fig. 1; Supplemental Fig. 1); however, microscopic examination revealed that both lines showed localized cell death soon after elicitin treatment (Fig. 2). Similarly, all *B. rapa* and *R. sativus* cultivars and Nicotiana species tested showed microscopic cell death following Pc-elicitin treatment (Supplemental Fig. 2), indicating that elicitin perception is not cultivar/species specific. Since elicitins from different Phytophthora species showed comparable necrosis-inducing activity in the two *B. rapa* lines and *N. tabacum* (Supplemental Fig. 1; data not shown), elicitin perception would appear to be a pathogen genus (Phytophthora)/plant genus-specific phenomenon.

Although we tested 53 Arabidopsis accessions, we did not find any elicitin-sensitive plants. A similar trial with 50 accessions, mentioned briefly in a review article by Yu (1995), also failed to identify an elicitin-responsive line of Arabidopsis. Arabidopsis leaves (accessions Col-0 and Ws-0) showed no microscopic evidence of cell death after elicitin treatment (data not shown) in contrast to all elicitin-nonresponsive Nicotiana, Brassica, and Raphanus species/cultivars, which showed microscopic evidence of cell death (Fig. 2; Supplemental Fig. 4). These results confirm that Arabidopsis is not responsive to elicitin and indicate fundamental differences between actual nonresponsive plant species and so-called ''nonresponsive'' species/cultivars of the genera Nicotiana, Brassica, and Raphanus. This suggests that Arabidopsis, unlike Nicotiana, Brassica, and Raphanus, may not possess an elicitin receptor.

A specific 193-kD plasma membrane component of *N. tabacum* cells has been identified as an elicitin binding site (Bourque et al., 1999). Mutated elicitins with a lower efficiency of sterol loading showed

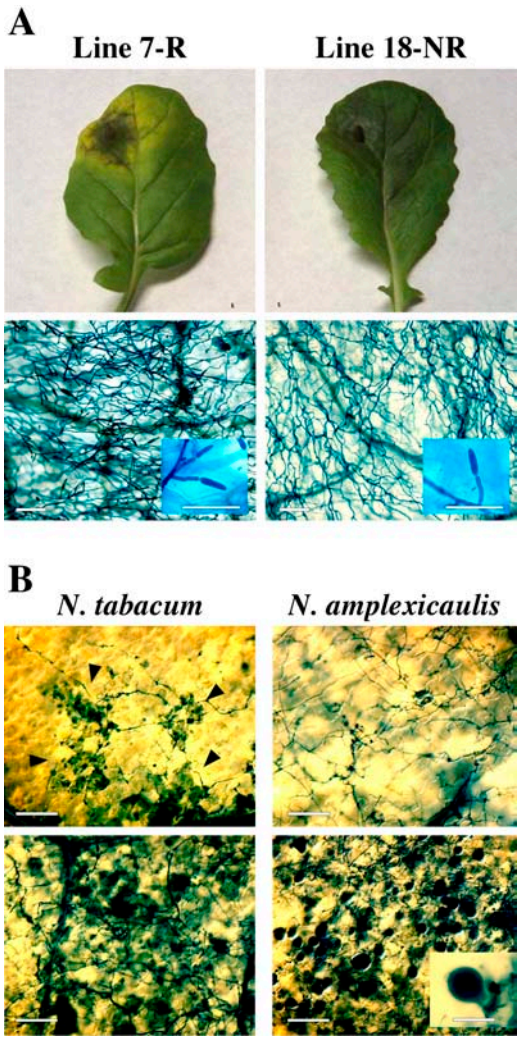

Figure 6. A, Disease symptoms on *B. rapa* lines 7-R and 18-NR after inoculation with *A. brassicicola*. Line 7-R showed more severe symptoms compared to line 18-NR at the same time point (top). Extensive fungal growth and sporulation (inset) of *A. brassicicola* were observed in both lines 7-R and 18-NR (bottom). All photographs were taken 60 h after inoculation. Bars = 50 μm. B, Infection of *N. tabacum* and *N. amplexicaulis* with *P. nicotianae* 24 (top) and 48 (bottom) h after inoculation. Incomplete containment of pathogen growth by dead plant cells was often observed in *N. tabacum* 24 h after inoculation (indicated by arrowheads) in contrast with extensive growth of pathogen in *N. amplexicaulis* (top). Extensive growth of *P. nicotianae* was observed in both *N. tabacum* and *N. amplexicaulis* 48 h after inoculation, but production of numerous sporangia (inset) is obvious only in *N. amplexicaulis* (bottom). Bars = 100 μm (main panels) and 50 μm (inset).

reduced association with this binding site and lower HR induction (Osman et al., 2001), indicating that the sterol binding capacity of the elicitin is biologically important to induce HR. However, Arabidopsis and *L. esculentum*, which showed no microscopic evidence of cell death (data not shown), also possess elicitin binding sites (Bourque et al., 1999; Ponchet et al., 1999). It is possible that Arabidopsis and *L. esculentum* have a defective elicitin receptor that is able to bind elicitin, but is unable to transduce a signal or is missing a signaling component downstream of the receptor. Alternatively, elicitin binding could reflect a phenomenon that is unrelated or indirectly related to defense activation, e.g. it could be binding to a pathogenicity target that is not under surveillance by a resistance mechanism in Arabidopsis and *L. esculentum* but is under surveillance in Brassica. Because all the *B. rapa* and *R. sativus* cultivars we tested were able to respond to elicitin, investigating the differences between responsive and ''nonresponsive'' cultivars of these species would not lead to the identification of an elicitin receptor. However, the application of heterologous complementation systems, e.g. introduction of a *Brassica* gene into Arabidopsis, could be a possible strategy to isolate an elicitin receptor.

It has been reported that orthologous MAPKs are activated by race-specific disease resistance interactions and by treatment with general elicitors in different plant species (Zhang and Klessig, 2001). Thus, the MAPK cascade may be considered a convergence point for signal transduction pathways leading to host-specific and non-host resistance. Elicitin treatment induced the activation of SIPK and WIPK in *N. tabacum* and *N. benthamiana* (Zhang et al., 1998, 2000), suggesting that induction of HR by elicitin also employs the MAPK cascade. Overexpression of *AtMEK4*^{DD}, orthologous to the upstream MAPKK of SIPK and WIPK, induced stronger cell death in elicitin-responsive *B. rapa* line 7-R, Nicotiana species, and *R. sativus* compared with that in line 18-NR and other nonresponsive plants (Fig. 3), suggesting that extensive cell death induced in elicitin-responsive plants was determined downstream of MAPKK activation in these species (Fig. 7). Recently, it has been reported that VIGS of *NbrbohA* or *B* (*N. benthamiana* respiratory burst oxidase homologs) reduced/delayed HR by the *P. infestans* elicitin INF1 in *N. benthamiana* (Yoshioka et al., 2003). By contrast, StMEK^{DD}-induced death was compromised only by VIGS of *NbrbohB*, suggesting that cell death induced by elicitin and MEK^{DD} are not entirely equivalent in *N. benthamiana*. Moreover, simultaneous VIGS of both *N. benthamiana SIPK* and *WIPK* didn't compromise cell death induced by INF1 (Sharma et al., 2003). These results indicate that the MAPK cascade activated by MEK^{DD}-SIPK/WIPK is not the sole signal transduction pathway for the induction of cell death by elicitin or is not involved in elicitin-induced cell death in *N. benthamiana*. Therefore, it is possible that the cell death we observed after expression of *AtMEK4*^{DD} was not related to the cell death induced by elicitin treatment. However, this is unlikely because SIPK and WIPK are the two major MAPKs activated by elicitin treatment (Zhang et al., 2000).

Defense genes are often induced in compatible plant-microbe interactions, but their expression is usually slower and weaker compared to that in incompatible interactions (Alfano and Collmer, 1996; Tao et al., 2003). By contrast, the timing and amount of

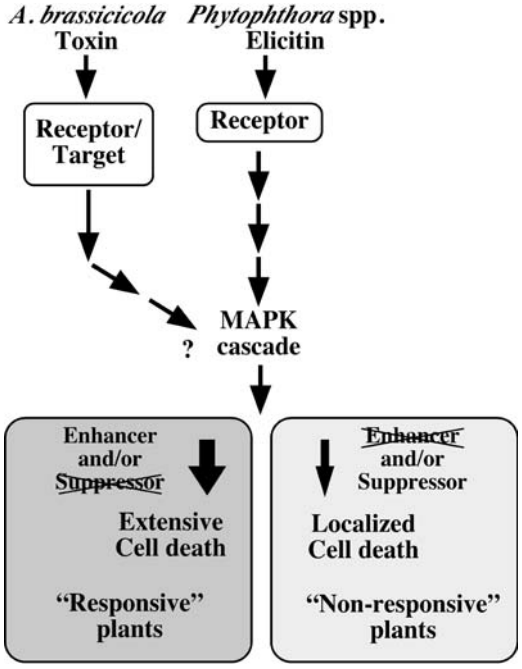

Figure 7. Schematic diagram summarizing a model for plant signal transduction leading to the induction of hypersensitive cell death in response to elicitin. Both ''responsive'' and ''nonresponsive'' plants possesses an elicitin receptor and can initiate signal transduction leading to hypersensitive cell death and expression of defense genes. ''Responsive'' plants can show visible cell death due to the presence of an enhancer or the absence of a suppressor of cell death progression, while nonresponsive plants only induce localized cell death, normally invisible to the naked eye. The enhancer or suppressor of cell death acts downstream of the MAPK cascade. Severity of cell death induced by Alternaria toxin is also determined by the same enhancer/suppressor of cell death.

defense gene expression after elicitin treatment was comparable in lines 7-R and 18-NR (Fig. 4), suggesting that line 18-NR is able to recognize and respond to elicitin just as well as line 7-R. Unexpectedly, the expression of *PR-1* after elicitin treatment was significantly stronger in line 18-NR than in line 7-R, whereas *PDF1.2* was expressed at almost the same level in both lines (Fig. 4). This result suggests that the SA level might be higher in line 18-NR after elicitin treatment. SA has been reported to provide negative feedback controlling the extent of cell death (Shapiro and Zhang, 2001; Devadas and Raina, 2002; Zhang et al., 2004). Differential SA accumulation could be responsible for the differences in cell death extension between *B. rapa* lines 7-R and 18-NR.

Various reports indicate that toxins produced by fungal pathogens, such as the nonspecific toxin fumonisin B1 from *Fusarium moniliforme* and the host-selective toxin victorin from *Cochliobolus victoriae*, induce typical ''resistance'' responses in sensitive plants, such as the production of active oxygen species, expression of PR-proteins, and accumulation of phytoalexins as well as the induction of plant cell death (Mayama et al., 1986; Stone et al., 2000). Cell death induced by toxins often shows features typical of programmed cell death, e.g. cleavage of DNA into nucleosomal fragments (DNA laddering), chromatin condensation, and alteration of mitochondrial permeability (Wang et al., 1996; Navarre and Wolpert, 1999; Yao et al., 2001; Curtis and Wolpert, 2004). Alternaria toxin caused stronger cell death in line 7-R than that in line 18-NR or elicitin nonresponsive *B. rapa* cultivars (Fig. 5; Table II), and elicitin responsiveness and toxin sensitivity cosegregated in F3 plants from the cross between lines 7-R and 18-NR (Fig. 5). These results suggest that Alternaria toxin-induced cell death acts through the same signaling pathways as elicitin-induced cell death. The higher toxin sensitivity found in line 7-R affected the rate of disease progression in *B. rapa* lines inoculated with *A. brassicicola* (Fig. 6A). Given that elicitins are considered avirulence factors for responsive plants, the higher sensitivity to Phytophthora elicitin and Alternaria toxin of *B. rapa* line 7-R could cause opposite effects on the outcomes of these two plant-pathogen interactions, reflecting the different pathogenic strategies of these pathogens; Alternaria species are necrotrophic pathogens causing severe disruption of host cells early in infection, while Phytophthora species are usually biotrophic or hemibiotrophic pathogens that keep host cells alive (at least initially) to enable nutrient uptake from plant cells. In support of this hypothesis, we found that infection of elicitin-responsive *N. tabacum* with *P. nicotianae* induced host cell necrosis at the site of pathogen attack and delayed pathogen development compared to nonresponsive (or weakly responsive) *N. amplexicaulis*, which showed no host cell necrosis at the site of pathogen attack but instead showed rapid pathogen development and sporulation (Fig. 6B). These data suggest an interesting variation in the control of pathogen-induced cell death, with the more extensive cell death allowed in responsive plants perhaps contributing to basal resistance against biotrophic or hemibiotrophic pathogens but greater susceptibility to necrotrophic pathogens and vice versa. Although the reduction in the growth rate of the necrotrophic pathogen on the less-responsive plant or the hemibiotrophic pathogen on the more-responsive plant may be a relatively subtle effect, it could slow the rate of disease development and have a cumulative effect over multiple pathogen growth cycles leading to a decrease in pathogen load, a delay in the onset of a disease epidemic, and a reduction in its severity.

This study was initiated with the expectation that the naturally occurring variation in elicitin response within responsive genera or species might be due to variation in elicitin perception. We have clearly shown that this is not the case and that the variation exists downstream of a convergence point in elicitin-induced, MAPK-induced, and toxin-induced cell death pathways (Fig. 7). Although variation in elicitin perception is not involved, these findings are important for several reasons: first, natural variation in elicitin response within elicitin responsive genera or species does not

reflect variation in recognition and cannot be used as a basis for isolation of an elicitin receptor; second, they reveal a convergence point in the downstream effector phase of presumably distinct signaling pathways leading to elicitin-induced and Alternaria toxin-induced cell death; third, a general regulator of programmed cell death other than LSD1, LOL1, or BI-1 appears to be involved; and, finally, they reveal a potentially important naturally occurring variation in the regulation of the extent of pathogen-induced cell death (of which one manifestation is elicitin responsiveness versus elicitin nonresponsiveness) that affects the growth of necrotrophs and hemi/biotrophs in opposite ways. Near-isogenic lines are being established from lines 7-R and 18-NR for further genetic analysis aiming to isolate the gene(s) responsible for the more extensive cell death response in Brassica. The outcome of these experiments will provide new insight into the regulation of programmed cell death induced by general elicitors and toxins in plant-microbe interactions.

MATERIALS AND METHODS

Plant Growth Conditions

Seed of fast cycling *Brassica rapa*, *Brassica oleracea*, and *Brassica nigra* plants were obtained from the Crucifer Genetics Cooperative (University of Wisconsin, Madison, WI). Seed of *B. rapa* cultivars Wong Bok, Hakurei, and Pak Choy and *Raphanus sativus* cultivars White Icicle, Long Scarlet, and Daikon were obtained from Arthur Yates (Milperra, Australia). Seed of *B. rapa* cultivars Nagaoka 60 Days F1 and Early Purple were obtained from Mr. Fothergill's Seeds (Seven Hills, Australia). Seed of *B. rapa* cultivars Sisu and Valti were provided by Dr. Saara Lang (University of Helsinki, Finland). *Nicotiana amplexicaulis* was obtained from Dr. Peter Lawrence (Queensland Department of Primary Industries, Biloela, Australia). Plants were grown under standard glasshouse conditions at 25°C by day and 18°C by night. For reverse transcription (RT)-PCR analysis, *B. rapa* lines were grown in a growth cabinet at 21°C with 16 h of light (100 μmol photons m^{-2} s^{-1}) per day. Arabidopsis (*Arabidopsis thaliana*) accessions were obtained from Dr. Nobuharu Goto (SENDAI Arabidopsis Seed Stock Center, Miyagi University of Education, Sendai, Japan). Arabidopsis accessions used in this work were Ak-1, Ang-1, Ba-1, Bl-1, Bla-1, Blh-1, Bor-0, Bs-1, Bur-0, Bus-1, Cal-0, Can-0, Cen-0, Co-1, Col-0, Di-2, Edi-0, Es-0, Est-1, Gr-1, Gre-0, Hau-0, Hi-0, Hir-1, Ita-0, Kas-1, Kin-0, Kl-1, Kn-0, Ko-5, Lapal, Ler-er, Lip-0, Lu-1, Mh-0, Ms-0, Mt-0, Mv-0, Nd-0, Nok-3, Np-0, Oy-0, Pa-1, Pak-1, Pak-2, Pak-3, Pog-0, Ri-0, RIB1, Sap-2, Sendai-4, Tsu-0, Tul-0, Van-0, Ws-0, and Yo-0. They were grown in a growth cabinet under the same conditions as *B. rapa* used for RT-PCR analysis.

Fungal and Oomycete Pathogens

Phytophthora cinnamomi isolate H1069, *Phytophthora megasperma* H1053, *Phytophthora nicotianae* H1033, *Phytophthora sojae* H1188, and *Phytophthora cryptogea* H1121 (Gabor et al., 1993) were grown on V8 medium (10% [v/v] cleared V8 juice, 20 μg mL^{-1} β-sitosterol, 0.1 mg mL^{-1} $CaCO_3$, 1.7% [w/v] Bacto agar, pH 6.0 to 6.5) in the dark at 25°C. To collect culture filtrates, *Phytophthora* spp. were cultured for 14 d in the dark at 25°C in a liquid Glc/Asn medium optimized for elicitin production (25 mg mL^{-1} Glc, 1 mg mL^{-1} Asn, 0.5 mg mL^{-1} KH_2PO_4, 0.5 mg mL^{-1} yeast extract, 0.25 mg mL^{-1} $MgSO_4 7H_2O$, 1 μg mL^{-1} thiamine; Kamoun et al., 1993). Culture medium was harvested by filtration through a 0.20-μm filter (Minisart; Sartorius AG, Goettingen, Germany) and dialyzed against water using SnakeSkin dialysis tubing (7 kD molecular mass cutoff; Pierce Biotechnology, Rockford, IL). Concentration and purity of elicitins was verified by silver staining of SDS-PAGE gels (Supplemental Fig. 1). *Alternaria brassicicola* isolate MBH3-1 was provided by Dr. Jeremy Burdon (CSIRO Plant Industry, Canberra, Australia) and grown and stored in the dark at 25°C on potato dextrose agar (PDA) media. To collect culture filtrate, *A. brassicicola* was cultured in potato dextrose broth media in the dark at 25°C for 14 d. The culture medium was harvested after filtration through a 0.20-μm filter (Minisart; Sartorius AG). Detached leaves of *B. rapa* were inoculated with *A. brassicicola* by placing agarose blocks (2 mm × 2 mm) excised from the edges of *A. brassicicola* colonies growing on potato dextrose agar media onto the detached leaves. For *P. nicotianae* inoculation, 5-μL drops of a suspension of the elicitin-producing *P. nicotianae* isolate H1111 at 1×10^4 spores mL^{-1} were placed on detached cotyledons of 2-week-old seedlings of *Nicotiana tabacum* and *N. amplexicaulis*. Inoculated cotyledons were incubated on moist filter paper in petri dishes kept at 25°C.

Construction of Vectors and Agrobacterium-Mediated Transient Gene Expression

Primer sequences used in this study are listed in Supplemental Table I. A gene for mature elicitin fused at the N terminus to a PR-1 signal peptide was synthesized using 24 primers, SyE/F01-12, and /R01-12. Amino acid sequences of the mature elicitin and PR-1 signal peptide were derived from *Phytophthora capsici* α-capsicein (Ricci et al., 1989) and tobacco PR-1a (Cornelissen et al., 1987), respectively. The 5′ ends of primers were phosphorylated with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and ligated with Taq DNA ligase (New England Biolabs). PCR was performed with the ligation mix and primers C-Eli-F and SyE/R12 to generate a *Cla*I-*Eco*RI cassette designated sp-capsicein. The Arabidopsis MAPKK gene *AtMEK4* (At1g51660; Ren et al., 2002) was amplified from genomic DNA of Arabidopsis ecotype Columbia using primers Cl-AtMEK4-F and AtMEK4-EI-R. Using the PCR product as a template, the 5′ and 3′ ends of *AtMEK4* were amplified separately by PCR with primers Cl-AtMEK4-F and -BI-MR or BI-AtMEK4-MF and -R to introduce two amino acid substitution mutations (T224D and S230D) causing constitutive activation of the MAPKK (Ren et al., 2002). *Cla*I-*Bam*HI and *Bam*HI-*Eco*RI cassettes for the 5′ and 3′ ends of $AtMEK4^{DD}$ were cloned into the *Cla*I and *Eco*RI sites of pBluescript SK^- (Stratagene, La Jolla, CA) to generate a *Cla*I-*Eco*RI cassette designated $AtMEK4^{DD}$. Primers Cl-AtMEK4-F and Bg-AtMEK4(R)-MR were used to amplify and mutagenize the 5′ end of AtMEK4 to generate $AtMEK4^R$, in which the essential catalytic Lys at position 108 was replaced by an Arg (Yang et al., 2001). The *Cla*I and *Bgl*II cassette containing the mutagenized 5′ end of AtMEK4 and the *Bgl*II-*Eco*RI cassette containing the 3′ end of wild-type AtMEK4 were assembled in pBluescript SK^- to make a *Cla*I-*Eco*RI cassette of full-length $AtMEK4^R$.

The *Cla*I-*Eco*RI cassettes of sp-capsicein, AtMEK4, $AtMEK4^{DD}$, or $AtMEK4^R$ were cloned into the *Cla*I-*Sac*I site of pBluescript SK^- together with the *Eco*RI-*Sac*I cassette containing the CaMV 35S terminator sequence excised from pCBJ-GFP-hTalin (Takemoto et al., 2003) to generate sp-capsicein-3′/pBS, AtMEK4-3′/pBS, $AtMEK4^{DD}$-3′/pBS, and $AtMEK4^R$-3′/pBS, respectively. Sp-capsicein-3′, AtMEK4-3′, $AtMEK4^{DD}$-3′, and $AtMEK4^R$-3′were excised with *Cla*I and *Sac*I and cloned into the *Hin*dIII-*Sac*I sites of pSLJ7292 (described in http://www.jic.bbsrc.ac.uk/sainsbury-lab/jonathan-jones/plasmid-list/plasmid.htm) together with the *Hin*dIII-*Cla*I cassette containing the 35S promoter and tobacco mosaic virus omega leader sequence from pCBJ-GFP-hTalin to generate pCBJ-sp-capsicein, pCBJ-AtMEK4, pCBJ-$AtMEK4^{DD}$, and pCBJ-$AtMEK4^R$, respectively.

Agrobacterium-mediated transient gene expression, also known as agroinfiltration, was performed as described previously with minor modification (Kapila et al., 1997). *Agrobacterium tumefaciens* strain AGL-1 (Lazo et al., 1991) containing pSLJ7292, pCBJ-GFP-hTalin, pCBJ-sp-capsicein, pCBJ-AtMEK4, pCBJ-$AtMEK4^{DD}$, or pCBJ-$AtMEK4^R$ was cultured at 28°C until stationary phase, washed, and resuspended in infiltration medium (1× Murashige and Skoog salts, 10 mM MES, pH 5.6, 3% [w/v] Suc, and 200 μM acetosyringone). The bacterial suspension was injected through small holes made by a needle into the intercellular spaces of plant leaves using a plastic syringe without a needle.

To verify that different cultivars of *B. rapa*, *R. sativus*, or different species of Nicotiana were all receptive to Agrobacterium-mediated transient gene expression, *A. tumefaciens* strain GV3101 containing pCAMBIA1305.2 (AF354046; CAMBIA, Canberra, Australia) was used to express the GUS gene. GUS staining was carried out 2 d after agroinfiltration according to the protocol described by Jefferson et al. (1987).

RT-PCR

First-strand cDNA was synthesized from 1 μg of total RNA of *B. rapa* in a reaction volume of 20 μL with oligo dT primer and 1× first-strand buffer (Invitrogen, Carlsbad, CA), 10 mM dithiothreitol, 5 mM dNTPs, and 20 ng μL^{-1}

oligo dT primer (12−18). The reaction was incubated at 65°C for 5 min, then at 37°C for 10 min before adding 200 units M-MLV reverse transcriptase (Invitrogen) and 8.725 units RNAguard porcine RNase inhibitor (Amersham Biosciences, Buckinghamshire, UK). The reaction mix was then incubated at 37°C for 60 min and at 95°C for 5 min. cDNA solutions were diluted 20 to 40 times based on the level of actin gene expression determined by PCR with primers ACT2-F and -R (Supplemental Table I). The absence of contamination by genomic DNA was verified by the different sizes of actin gene PCR products obtained from genomic DNA and cDNA. PCR was performed with 0.5 units μL^{-1} REDTaq DNA polymerase (Sigma, St. Louis) in 1× REDTaq PCR reaction buffer (Sigma), 200 μM dNTPs, 0.66 μM specific primers for the genes under investigation, and 2 μL of diluted cDNA solution in a 15-μL reaction volume with 30 thermal cycles. Sequences of specific primers are provided as supplemental data (Supplemental Table I).

Lactophenol Trypan Blue Staining and Light Microscopy

To monitor plant cell death and fungal growth, plant leaves were stained as described previously (Takemoto et al., 2003). Leaves treated with elicitin or inoculated with *A. brassicicola* were cleared in methanol for more than 24 h and boiled for 3 min in lactophenol trypan blue stain (10 mL of water, 10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, 10 mg of trypan blue). After the leaves had cooled to room temperature for 1 h, the stain was replaced with 1 g mL^{-1} chloral hydrate. Stained leaves were decolorized overnight and viewed using an Axioplan universal microscope (Zeiss, Jena, Germany).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Sequence data from this article have been deposited with the GenBank data library under accession number AB193295.

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