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RAPID COMMUNICATION

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Insight into Types I and II nonhost resistance using expression patterns of defense-related genes in tobacco

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Abstract Plants protect themselves against pathogens using a range of response mechanisms. There are two categories of nonhost resistance: Type I, which does not result in visible cell death; and Type II, which entails localized programmed cell death (or hypersensitive response) in response to nonhost pathogens. The genes responsible for these two systems have not yet been intensively investigated at the molecular level. Using tobacco plants (*Nicotiana tabacum*), we compared expression of 12 defense-related genes between a Type I (*Xanthomonas axonopodis* pv. glycines 8ra) nonhost interaction, and two Type II (*Pseudomonas syringae* pv. syringae 61 and *P. syringae* pv. phaseolicola NPS3121) nonhost interactions, as well as those expressed during *R* gene-mediated resistance to *Tobacco mosaic virus*. In general, expression of most defense-related genes during *R* gene-mediated resistance was activated 48 h after challenge by TMV; the same genes were upregulated as early as 9 h after infiltration by nonhost pathogens. Surprisingly, *X. axonopodis* pv. glycines (Type I) elicited the same set of defense-related genes as did two pathovars of *P. syringae*, despite the absence of visible cell death. In two examples of Type II nonhost interactions, *P. syringae* pv. phaseolicola NPS3121 produced an expression profile more closely resembling that of *X. axonopodis* pv. glycines 8ra, than that of *P. syringae* pv. syringae 61. These results suggest that Type I nonhost resistance may act as a mechanism providing a more specific and active defense response against a broad range of potential pathogens.

Keywords Hypersensitive response-related genes · *Nicotiana tabacum* · Nonhost resistance · Pathogenesis-related genes · Secondary metabolite-associated genes

Abbreviation *Ng-CDM1*: *Nicotiana glutinosa*-cell death marker1 · MAPK: Mitogen-activated protein kinase · *Nt-hsr203J*: *Nicotiana tabacum*-hypersensitive response related203J · *Nt-hin1*: *Nicotiana tabacum*-harpin induced1

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Introduction

Plants need to protect themselves against attack by a wide range of pathogens. A plant species (or its cultivars) susceptible to a given pathogen is referred to as the "host" for that pathogen. Moreover, most pathogens exhibit narrow host specificity, and will not infect "nonhost" species; the resistance of plants to the vast majority of potential pathogens is termed "nonhost resistance" (Dangl et al. 1996; Heath 2000; Kamoun 2001; Thordal-Christensen 2003; Mysore and Ryu 2004). However, neither the mechanisms nor the signaling components have been extensively studied. Since Flor (1971) originally proposed the "gene-for-gene hypothesis", following a study of the interaction between flax and flax rust in 1956, researchers have sought to elucidate the underlying genetic mechanisms of host and nonhost resistance. Many studies have revealed the signal components of these two types of resistance. A number of well-characterized model plants and pathogens are available for the investigation of *R* gene-mediated resistance, since the interaction is highly specific. In contrast, non-specific or general defense mechanisms occur during nonhost resistance, suggesting the involvement of multiple gene/protein interactions (Pedley and Martin 2003; Rivas and Thomas 2005). Recent

advances in molecular biology and biochemistry, such as the development of "omics", have provided researchers with the tools to investigate nonhost resistance in greater detail (Nürnberger and Lipka 2005). For example, loss of function screening (*Arabidopsis* T-DNA mutant and virus-induced gene silencing screening) and microarray analyses have identified several signaling components, including NHO1, SGT1, WIPK, SIPK, EDS, HSP90, and MAPKs (Peart et al. 2002; Yun et al. 2003; Mysore and Ryu 2004; Kang et al. 2003; Nürnberger and Lipka 2005). However, a small direct comparison between host and nonhost resistance has been attempted, and no indicator genes expressed only in nonhost resistance have been found.

To date, plant defenses against pathogens have been divided simply into host and nonhost resistance. Host resistance has been studied intensively in *Arabidopsis-P. syringae*, Tobacco-TMV, and tomato-*P. syringae* model systems, while nonhost resistance remains poorly understood (Tang et al. 1996; Tao et al. 2003; Peart et al. 2005). Mysore and Ryu (2004) proposed two types of nonhost resistance: Type I, which does not result in visible cell death; and Type II, in which a hypersensitive response (HR) occurs, resulting in cell death at the site of infection. However, the gene expression patterns underlying both response types have not yet been compared.

In this study, therefore, we attempted to characterize Type I and Type II nonhost resistance via expression profiles of previously reported defense-related (*PR* genes) and nonhost-related or cell death-related genes (*Ng-CDM1, Nt-hsr203J*, and *Nt-hin1*). In addition, we sought to identify marker genes for Type I or II nonhost resistance, or a unique pattern of expression following infection.

Materials and methods

Plants and pathogen inoculation

Tobacco plants (*Nicotiana tabacum* cv. Xathi nc) were cultivated in a growth chamber under long-day conditions (16 h) at 25 ± 2°C. Ten-week-old tobacco plants were inoculated with sap prepared from TMV-infected tobacco (pH 6.8), following gentle rubbing of the leaf at the infection site with carborundum. The following bacterial strains were used: *Pseudomonas syringae* pv. syringae 61 (Huang et al. 1988), and *P. syringae* pv. phaseolicola NPS3121 (Lindgren et al. 1986), both causal agents of bacterial brown spot on bean; and *Xanthomonas axonopodis* pv. glycines 8ra (Hwang et al. 1992), a causal agent of pustule disease on bean. All bacterial pathogens were cultured in LB medium, supplemented with appropriate antibiotics, at 28°C overnight. Bacterial cells (10^8 cfu/ml) in 10 mM $MgCl_2$ were pressure-infiltrated into the tobacco leaves using a needle-less syringe. Infiltration of 10 mM $MgCl_2$ was used as a control. Inoculated plants were returned to the growth chamber, and leaf tissues were harvested at 1.5, 3, 6, 9, 12, 24, 48, 72 h post-inoculation of each pathogen for isolation of total RNA.

PCR amplification and probes

We used 10 defense-related cDNA clones (Table 2) isolated by differential screening of tobacco (cv Xanthi nc) DNA from resistant, TMV infected samples. Fragments containing homologs of *hin1* (Gopalan et al. 1996) and *hsr203J* (Pontier et al. 1994; 1998) were amplified from 20 ng of chromosomal DNA (*N. tabacum*) by PCR, using oligonucleotide primers (TAGCCACGC ACATGCAAACC and GTGACAATCAAGACGGT AC for *hsr203J*, and GAGCCATGCCGGAATCCAAT and GCTACCAATCAAGATGGCATCTGG for *hin1*), at an annealing temperature, 55°C. The amplified PCR product was separated on a 1% agarose gel, and purified using a gel extraction kit (Qiagen Inc., Valencia, CA, USA). The purified PCR fragment was ligated into the pGEM-T Easy vector (Promega Corp., Madison, WI, USA), and the cloned nucleotide sequences were determined. The cloned *hsr*203J and *hin*1 homologs were named *Nt-hsr203J* and *Nt-hin1*, respectively, and deposited in GenBank under the corresponding accession numbers AF212184 and AF212183 (Suh et al. 2003).

Isolation of total RNA and Northern blot analysis

Total RNA was isolated from inoculated leaf tissues following Choi et al. (1996). Twenty micrograms of total RNA from each sample were separated by formaldehyde-containing agarose gel electrophoresis and transferred onto a Nytran membrane (Amersham, Piscataway, NJ, USA). Loading abundance of total RNA was checked by ethidium bromide staining (0.1 μg/ml). Each cDNA clone was labeled with ^{32}[P]-dCTP using the Prime-a Gene System (Promega Corp.) and subjected to Northern blot hybridization. Both prehybridization and hybridization of the membrane were carried out in 5× SSC, 5× Denhardt's solution, 0.5% SDS and 100 μg/ml salmon sperm DNA, and hybridization was conducted at 60°C overnight. Following hybridization, the membrane was rinsed in 1× SSC and 0.1× SDS at room temperature for 15 min, then washed in 0.5× SSC and 0.1× SDS at 60°C for 2 h. The membrane was exposed to X-ray film (Eastman Kodak Co., Rochester, NY, USA) for 24 h with intensifying screens at −70°C, prior to development. The excrement was repeated three times with similar results.

Results and discussion

To elucidate differences between Type I and II nonhost resistance, we selected known tobacco pathogens for this study (Table 1). *P. syringae* pv. syringae 61,

Table 1 TMV and bacterial pathogens used in this study

Pathogens	Characteristics	References
Tobacco mosaic virus	Tobacco, HR-inducing in *N*-gene contained tobacco	Oh et al. (1999)
Pseudomonas syringea pv. syringae 61	Wild-type isolated from wheat, HR-inducing in tobacco	Huang et al. (1988)
P. syringea pv. phaseolicola NPS3121	Bean pathogen, HR-inducing in tobacco	Lindgren et al. (1986)
Xanthomonas axonopodis pv. glycines 8ra	Bean pathogen, non-HR inducing in tobacco	Hwang et al. (1992)

X. axonopodis pv. glycines 8ra, and *P. syringae* pv. phaseolicola NPS3121, have been reported previously as nonhost for tobacco (Huang et al. 1988; Jakobek and Lindgren 1993; Oh et al. 2005). Infiltration of *P. syringae* pv. syringae 61 and *P. syringae* pv. phaseolicola NPS3121 onto the leaves produced HR cell death within 24 h (Fig. 1b). In contrast, *X. axonopodis* pv. glycines 8ra did not induce HR or any visible cell death symptoms, even 4 days after inoculation. These results clearly indicate the presence of two types of nonhost resistance. Since we were unable to locate a suitable candidate bacterium, we instead used the well-characterized TMV–*N* gene interaction (Whitham et al. 1994). The absence of HR following inoculation with a nonhost pathogen raised a question: Does the plant respond differently in Type I and II nonhost resistance at the molecular level? To answer this question, we employed Northern blot analysis to assess the expression patterns of *PR* genes, HR-related genes, and previously known nonhost resistance-related genes. These genes include a reference *PR* gene group (*PR-1, PR-2, PR-3, PR-4, PR-5*, and *SAR8.2*; Table 2). The first group of genes has been reported to be upregulated in plants treated with SA, TMV, or incompatible bacterial pathogens. The second group of genes, encoding HMGR and PAL, or GST, were reported to form second metabolites involved in phytoalexin biosynthesis in Solanaceae, or as oxidative bust-related genes. The third group represents programmed cell death (PCD)-related genes, that are rapidly expressed in response to a nonhost pathogen challenge (Suh et al. 2003, Table 1; Fig. 1b).

The results presented here validate the model of Mysore and Ryu (2004), which incorporates two types of nonhost resistance (type I and II) in plants, and also shed light on the underlying molecular mechanisms. Infiltration with *P. syringae* pv. phaseolicola NPS3121 and *P. syringae* pv. syringae 61 elicited strong HR within 24 h. In contrast, *X. axonopodis* pv. glycines 8ra did not elicit any responses (Fig. 1b). According to an earlier hypothesis (Goodman and Novacky 1994; Agrios 1997), incompatible pathogens must induce the HR phenotype in a nonhost plant. However, more recent data have shown that even pathogenic bacteria, which cause severe symptoms on a host plant, will not induce HR on a nonhost plant (Fink et al. 1990; Jakobek and Lindgren 1993; Kamoun et al. 1998; Klement et al. 1999). These results indicated a requirement for a new model to explain Type I nonhost resistance. In this study, we found that *X. axonopodis* pv. glycines 8ra caused no symptom at the site of infiltration (Table 1; Fig. 1b).

Initially, we confirmed that *X. axonopodis* pv. glycines 8ra elicited Type I nonhost resistance, by comparison with Type II nonhost resistance induced by *P. syringae* pv. syringae 61 and *P. syringae* pv. phaseolicola NPS3121. All three pathogens have been reported as species of pathogen on the legumes. Although all three pathogens produce different lesions in tobacco, our results indicated that each was capable of inducing the expression of defense-related genes. It is interesting to note that there was a greater similarity of expression of *PR-1* and *PR-3* between tissues infiltrated with *P. syringae* pv. phaseolicola NPS3121 and *X. axonopodis* pv.

Table 2 Defense-related genes used in this study

Classification (gene name)	Organisms	GenBank accession no.	Reference
First group: pathogenesis-related protein genes			
PR-1a (*PR protein-1a*)	*Nicotiana glutinosa*	U49241	Yun et al. (1996, 1999)
PR-2 (*acidic β-1,3-glucanase*)	*N. glutinosa*	U49242	
PR-3 (*acidic chitinase*)	*N. tabacum*	M29868	Park et al. (1999)
PR-4b (*PR protein-4b or hevein like protein*)	*N. tabacum*	M60282	
PR-5 (*Osmotin-like protein*)	*N. tabacum*	X95308	
SAR8.2	*N. tabacum*	M97361	
Second group: second metabolites—and oxidative burst—associated genes			
PAL (*Phenylalanin amonia lyase*)	*N. tabacum*	X78269	Kang et al. (1998)
HMGR (*3-hydroxy-methylglutaryl CoA reductase*)	*N. tabacum*	AF004232	
GST (*Glutathion-S-transferase*)	*N. tabacum*	AF443177	
Third group: cell-death-related genes			
Ng-CDM1 (*N. glutinosa*-cell death marker1)	*N. glutinosa*	AF208022	Suh et al. (2003)
Nt-hin1 (*N. tabacum*-harpin induced1)	*N. tabacum*	AF212183	
Nt-hsr203J (*Nt*-hypersensitive response related203J)	*N. tabacum*	AF212184	

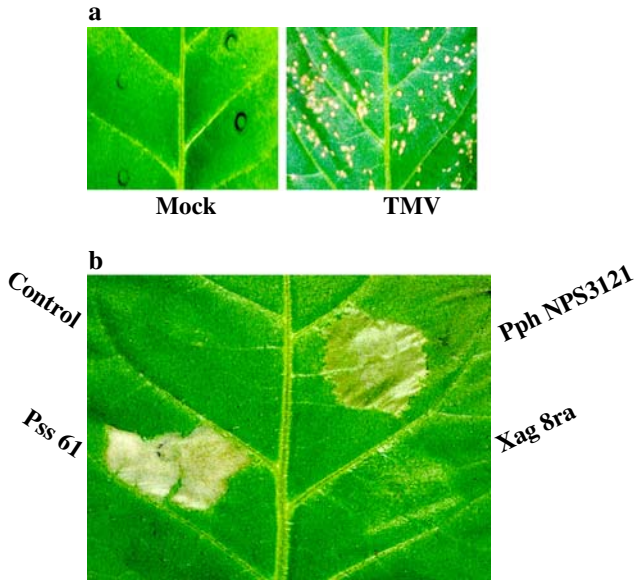

Fig. 1 HR cell death and null responses of tobacco leaves following host and nonhost pathogen inoculations. **a** HR cell death caused by TMV inoculation. HR cell death symptoms occurred 48 h following infection (*right*). Ten-week-old tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were inoculated with sap prepared from TMV-infected tissues. Control plants were asymptomatic (**left**). **b** HR cell death in tobacco leaves 20 h after infiltration with two HR-inducing (*P. syringae* pv. syringae 61 and *P. syringae* pv. phaseolicola NPS3121) strains, or one non-HR inducing bacterium (*X. axonopodis* pv. glycines *8ra*). Bacterial suspensions were infiltrated at a concentration of $\sim 10^8$ cfu/ml in 10 mM $MgCl_2$. Controls were infiltrated with $MgCl_2$ and were asymptomatic. *Pss* 61 *Pseudomonas syringae* pv. syringae 61; *Pph* NPS3121 *P. syringae* pv. *phaseolicola* NPS3121; *Xag* 8ra *Xanthomonas axonopodis* pv. *glycines* 8ra

glycines 8ra, than was found between the former and *P. syringae* pv. syringae 61. This suggests that the plants are responding to the pathogens without necessarily producing HR. It is also possible that plants facilitate their resistance mechanisms by activating a PCD-independent pathway. These findings parallel the identification of the *defense no death 1 (dnd1)* mutant in *Arabidopsis* (Clough et al. 2000). As a result, we propose that Type I nonhost resistance is an evolutionarily older defense mechanism than Type II nonhost resistance, or gene-for-gene resistance. A *dnd1* mutant, which carried a mutation in the DND1 locus of *Arabidopsis*, lacked HR but retained expression of resistance mechanisms against avirulent *P. syringae*, including the induction of *PR* gene expression and the suppression of bacterial growth (Clough et al. 2000). In the case of Type II nonhost resistance, plants require a more coordinated suite of proteins for recognition of specific bacterial effectors such as cell wall materials or flagella (Nürnberger et al. 2004). Upon recognizing a pathogen, the plant initiates several downstream signaling pathways, resulting in a HR phenotype. When a suspension of *X. axonopodis* pv. glycines 8ra was infiltrated at $10^{3\text{-}4}$ cfu/ml onto the leaf, the bacterial population increased to 10^7 cfu/ml, without producing any symptoms. This suggests that *X. axonopodis* pv. glycines 8ra may possess certain effectors to evade the plant's defenses (Oh et al. 2005), a finding in agreement with previous studies on other bacteria (Espinosa and Alfano 2004; Abramovitch and Martin 2004). In contrast, the growth of Type II nonhost pathogens was quickly contained.

Rub-inoculation of TMV on the tobacco leaves induced expression of all 12 genes from 48 h post-inoculation. Particularly, *GST* and *Nt-hin1* genes were instantly expressed even at 1.5 h, and at 48 and 72 h post-inoculation. Infiltration of *P. syringae* pv. syringae 61 was elicited significantly in *PR-1, PR-3*, and *Nt-hsr203j* transcription 6 h after challenge, and *PR-4, PR-5, SAR8.2, PAL*, and *Nt-hin1* 9 h after inoculation. Expression of *HMGR* mRNA by *P. syringae* pv. syringae 61 was initiated as early as 3 h after challenge. Expression of *GST* and *Ng-CDM1* genes was activated only temporally at 9 and 12 h, and at 12 and 24 h, respectively, after inoculation. *PR-2, PR-4, PR-5*, and *GST* mRNA began to be induced 6 h after infiltration of *P. syringae* pv. phaseolicola NPS3121. *SAR 8.2, HMGR, Nt-hin1*, and *Nt-hsr203J* transcription was activated from 3 h after inoculation. Low levels of *PR-3* and *Ng-CDM1* expression were detected 12 h after infiltration. However, the expression of *PAL* mRNA was varied at different time points. Infection of *X. axonopodis* pv. glycines 8ra in the tobacco leaves was induced in 10 out of 12 genes tested in this experiment 6 h post-inoculation. We did not detect any transcriptions of *Ng-CDM1* following inoculation of *X. axonopodis* pv. glycines 8ra. The mRNA level of *PAL* was significantly declined right after inoculation and increased to 12 h. We obtained similar results from three independent experiments.

The expression of *Nt-hsr203J* (*Nicotiana tabacum*-hypersensitive response related) and *Nt-hin1* (*Nicotiana tabacum*-harpin induced) might be considered to be reliable indicators for differentiating between host and nonhost resistance. Following inoculation with TMV, these genes were strongly induced at a later stage of infection, from 48 to 72 h. In contrast, inoculation with *P. syringae* pv. syringae 61, *P. syringae* pv. phaseolicola NPS3121, or *X. axonopodis* pv. glycines 8ra initiated expression within 3–6 h (Fig. 2). Interestingly, the *Nt-hsr203J* transcript was rapidly and specifically lowered by12 h following inoculation with both *P. syringae* pv. syringae 61 and *X. axonopodis* pv. glycines 8ra, but remained at significantly elevated levels 24–48 h post-inoculation with *P. syringae* pv. phaseolicola NPS3121. The expression pattern of *Ng-CDM1* (*Nicotiana glutinosa*-cell death marker1) was different for the two pathogen types. *Ng-CDM1* was originally identified as an important indicator of HR-induced cell death, and the Type II nonhost resistance (*P. syringae* pv. syringae 61 and *P. syringae* pv. phaseolicola NPS3121) pathogens induced its expression at 12 h; however, expression disappeared by 48 or 72 h. In contrast, the expression of *Ng-CDM1* was not detected in plants challenged with *X. axonopodis* pv. glycines 8ra. In agreement with this

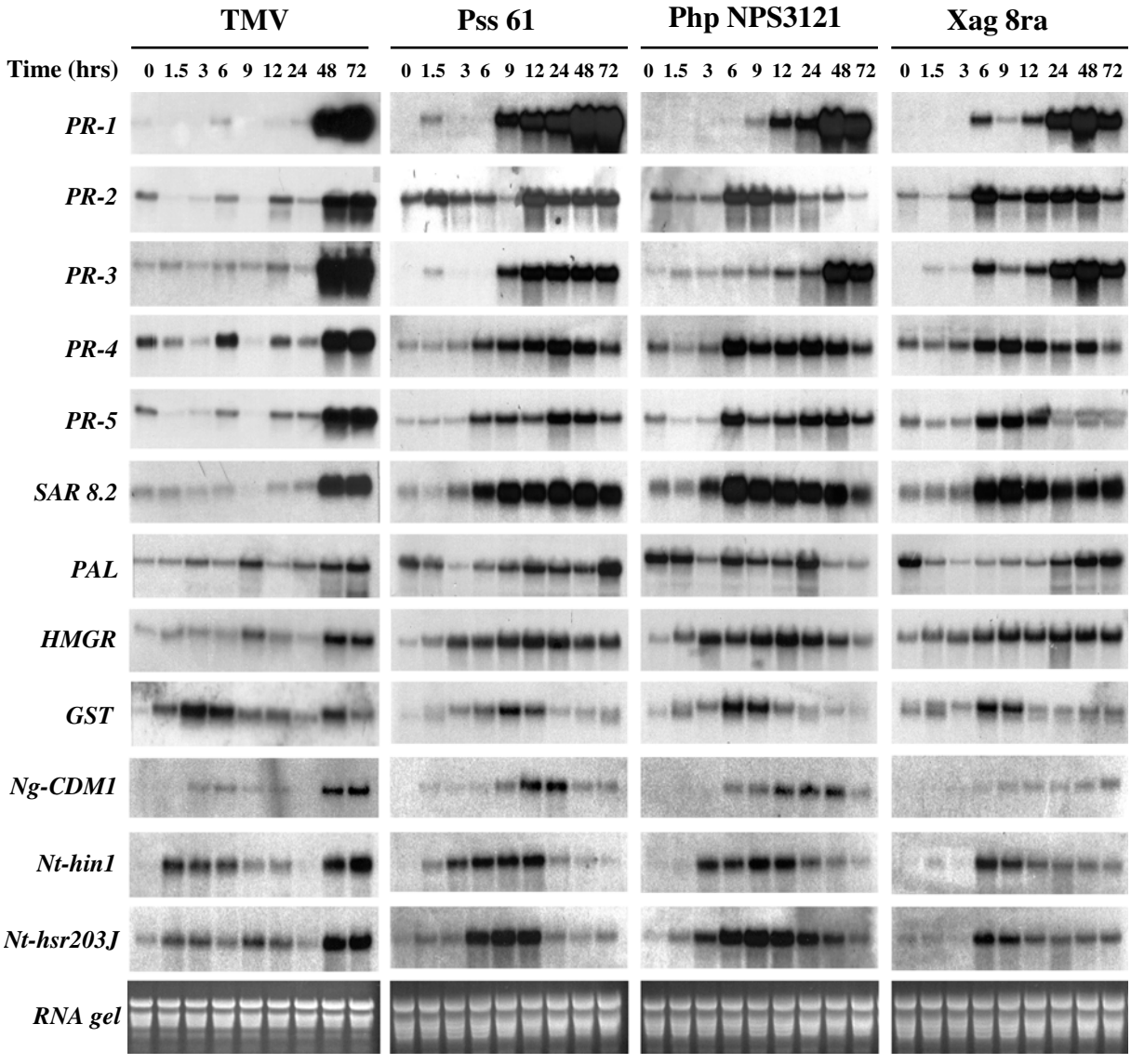

Fig. 2 Expression of defense-related genes during host and nonhost resistance in tobacco. Time course for expression of *PR* genes, secondary metabolites, oxidative burst, and cell death-related genes during HR cell death in tobacco following infection by TMV (host response), *P. syringae* pv. *syringae* 61, *P. syringae* pv. phaseolicola NPS3121, and *X. axonopodis* pv. *glycines* 8ra. Ten-week-old tobacco plants were inoculated and leaf tissues were harvested for total RNA isolation at the times indicated. Samples containing 20 μg of total RNA were blotted onto Nytran membranes and hybridized with the ^{32}P-labeled cDNA probes indicated in the figure. *PR-1* pathogenesis-related protein 1; *PR-2* β-1,3-glucanase; *PR-3* chitinase; *PR-4* hevein-like protein; *PR-5* osmotin-like protein; *PAL* phenylalanine ammonia lyase; *HMGR* 3-hydroxy-3-methylglutaryl CoA reductase; *GST* glutathione-S-transferase; *hin*1 harpin induced; *hsr203J* hypersensitive reaction related; and *Ng-CDM1 N. glutinosa* cell death marker1

result, we found that in plants challenged with *X. axonopodis* pv. glycines, the expression of *Nt-hsr203J* and *Nt-hin1* 8ra occurred earlier, and was weaker than expression induced by inoculation with either *P. syringae* pv. syringae 61 or *P. syringae* pv. phaseolicola NPS3121 (Fig. 2). Expression of *Nt-hsr203J* and *Nt-hin1* is reported to be rapidly induced by bacterial effectors, such as hairpins or avrPto, during HR-mediated cell death in tobacco plants (Gopalan et al. 1996). Taken together, these results suggest that the reason why *X. axonopodis* pv. glycines 8ra did not show any visible HR phenotype may be related to the expression pattern of *Ng-CDM1, Nt-hsr203J*, and *Nt-hin1*. Once the plant has recognized the presence of a pathogen, via identification of pathogen effectors and PAMPs (pathogen-associated molecular patterns, e.g. lipopolysaccharides and flagellin), these three genes can be tightly regulated downstream of the HR signaling pathway (Alfano and Collmer 1996; Nürnberger et al. 2004; Zeidler et al. 2004; Zipfel et al. 2004). Support for this hypothesis is provided by the observation that when these three genes have been silenced, using virus-induced gene silencing (VIGS), inoculation of tobacco or *Nicotiana benthamiana* with *P. syringae* pv. syringae 61 or *P. syringae* pv. phaseolicola NPS3121 results in the HR (Ryu et al. 2004).

Differences between host (TMV) and nonhost (*P. syringae* pv. syringae 61, *P. syringae* pv. phaseolicola NPS3121, and *X. axonopodis* pv. glycines 8ra) resistance were observed at the molecular level, and expression of some defense-related genes, including *PR-1, PR-2, PR-3, SAR 8.2*, and *Ng-CDM1*, was more delayed during *R* gene-mediated host resistance than in nonhost resistance. We could not conclude that expression of these genes was delayed by inoculation with TMV, since inoculated tobacco plants that contained the *N* gene showed a typical HR phenotype within 48 h, when rub-inoculated with viral RNA (Fig. 1a). However, HR was observed within 24 h of infiltration with *P. syringae* pv. syringae 61 or *P. syringae* pv. phaseolicola NPS3121. Recently, many "omic" technologies have become available to study gene expression profiles to find the main components that determine host or nonhost resistance. A microarray experiment with the *Arabidopsis* GeneChip®, which included over 8,000 genes, was used to compare the host pathogen, *P. syringae* pv. tomato (*AvrRpt2*) and nonhost pathogen, *P. syringae* pv. *phaseolicola* in *Arabidopsis* containing *RPS2* (Tao et al. 2003). The results did not indicate a significant difference between the genes expressed during host and nonhost resistance; however, gene expression was delayed following infection by a nonhost pathogen, compared to a host resistance response. To compare host and nonhost resistance in tobacco, bacterial pathogens containing defined avirulent proteins will need to be tested against *P. syringae* pv. syringae 61 and *P. syringae* pv. phaseolicola NPS3121. In the *Arabidopsis* system, infiltration of *P. syringae* pv. phaseolicola NPS3121 did not result in any visible HR, thus indicating Type I nonhost resistance. This supports our finding that even though a pathogen does not induce HR, it nonetheless elicits expression of defense-related genes.

Our finding that three defense-related gene sets exhibited different expression patterns may help elucidate nonhost resistance. In particular, the expression patterns of *Ng-CDM1, Nt-hsr203J*, and *Nt-hin1* may allow discrimination between host and Type I or Type II nonhost resistance responses. A greater understanding of Type I nonhost resistance may provide a novel strategy for developing PCD-free resistance against pathogens.

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