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Conversion of compatible plant–pathogen interactions into incompatible interactions by expression of the *Pseudomonas syringae* pv. *syringae* 61 *hrmA* gene in transgenic tobacco plants

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Summary

The *hrmA* gene from *Pseudomonas syringae* pv. *syringae* has previously been shown to confer avirulence on the virulent bacterium *P. syringae* pv. *tabaci* in all examined tobacco cultivars. We expressed this gene in tobacco plants under the control of the tobacco Δ0.3 TobRB7 promoter, which is induced upon nematode infection in tobacco roots (Opperman *et al.*, 1994, *Science*, 263, 221–223). A basal level of *hrmA* expression in leaves of transgenic plants activated the expression of pathogenesis-related genes, and the transgenic plants exhibited high levels of resistance to multiple pathogens: tobacco vein mottling virus, tobacco etch virus, black shank fungus *Phytophthora parasitica*, and wild fire bacterium *Pseudomonas syringae* pv. *tabaci*. However, the *hrmA* transgenic plants were not significantly more resistant to root-knot nematodes. Our results suggest a potential use of controlled low-level expression of bacterial *avr* genes, such as *hrmA*, in plants to generate broad-spectrum resistance to bacterial, fungal and viral pathogens.

Keywords: disease resistance, *hrp*, *avr*, apoptosis, systemic acquired resistance, hypersensitive response.

Introduction

During host–pathogen co-evolution, many plants have acquired disease resistance genes whose products are involved directly or indirectly in the recognition of pathogen-derived molecules called avirulence factors (Staskawicz *et al.*, 1995). Recognition of pathogen avirulence factors by the plant resistance gene products results in activation of plant disease resistance responses, often including a localized plant cell death response or hypersensitive response (HR) (Dangl *et al.*, 1996; Goodman and Novacky, 1994; Lamb and Dixon, 1997). For example, the *hrmA* gene of *Pseudomonas syringae* pv. *syringae* confers broad-spectrum avirulence on *P. s.* pv. *tabaci* in all examined susceptible host tobacco (Alfano *et al.*, 1997).

The avirulence function of the *hrmA* gene is dependent on hypersensitive response and pathogenicity (*hrp*) genes (Alfano *et al.*, 1997; Heu and Hutcheson, 1993; Leach and White, 1996), many of which are involved in regulation and assembly of a type III protein secretion system (He, 1998). A growing body of indirect evidence suggests that bacterial Avr proteins are delivered directly into the plant cell via the Hrp system (Bonas and Van den Ackerveken, 1997; Collmer, 1998; He, 1998). Purified HrmA protein does not trigger the HR when infiltrated into the apoplast of tobacco leaves; however, transient expression of the *hrmA* gene directly in tobacco cells results in cell death, suggesting recognition of HrmA inside the tobacco cell (Alfano *et al.*, 1997).

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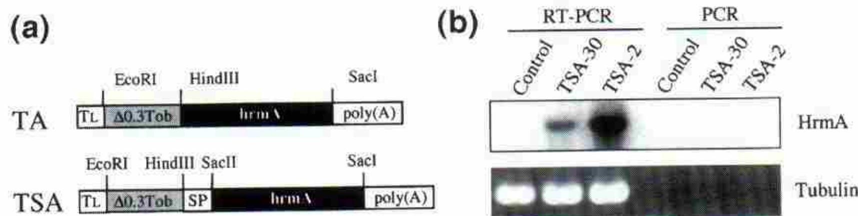

Figure 1. Structures and expression of chimeric *hrmA* genes.
(a) A schematic representation of *hrmA*–T-DNA constructs. The positions of the left T-DNA border (T_L), Δ0.3 TobRB7 promoter (Δ0.3Tob), PR-1b signal peptide sequence (SP), *hrmA* gene and polyadenylation signal (polyA) are illustrated. (b) *hrmA* or β-tubulin transcripts were amplified by RT–PCR as described in Experimental procedures. *hrmA* sequences were detected by Southern blotting using *hrmA*-specific probes. The products of amplification of the β-tubulin gene were visualized by ethidium bromide staining. PCR without prior reverse transcription was used as a control to confirm that the RT–PCR products were attributable to RNA. The source plant (control, TSA-2, or TSA-30) for the RNA is indicated above each lane.

Although constitutive high-level expression of an *avr* gene in resistant plants results in a systemic HR and the transgenic plants die (see, for example, Gopalan *et al.*, 1996), it is possible that pathogen-inducible expression of *avr* genes in response to virulent pathogen infection may be used to generate plant disease resistance without the deleterious effects of constitutive *avr* gene expression (as suggested by De Wit, 1992). The tobacco Δ0.3 TobRB7 promoter sequence was previously shown to be induced specifically at the feeding site of root-knot nematodes (Opperman *et al.*, 1994). The reported low level of basal expression and induction by nematode infection make the Δ0.3 TobRB7 promoter an excellent candidate for testing the feasibility of low-level and conditional expression of *avr* genes for generating disease-resistant plants. This report describes such a test. We found that plants that express the *hrmA* gene under the control of the Δ0.3 TobRB7 promoter are not significantly more resistant to nematode infection. However, we also found that extremely low basal expression of the Δ0.3 TobRB7 promoter–*hrmA* gene in plants not infected with nematodes induces general disease resistance responses, resulting in plants that are resistant to a broad range of plant pathogens.

Results

Production and characterization of hrmA*-transgenic tobacco plants*

The *Pseudomonas syringae hrmA* gene was modified for expression in plants as shown in Figure 1(a). Specifically, two constructs were assembled in which the *hrmA* gene was placed under the control of the Δ0.3 TobRB7 promoter (Opperman *et al.*, 1994). One of the Δ0.3 TobRB7 constructs (TA) consisted of just the promoter flanked by the *hrmA* gene, which is expected to express the HrmA protein intracellularly. A second construct (TSA) contained a PR-1b signal peptide at the N-terminus of the *hrmA* coding region, for targeting of the predicted gene product to the endomembrane system (and presumably to the apoplast).

No transgenic plants were obtained with agrobacteria carrying the TA construct, suggesting that the Δ0.3 TobRB7 promoter was sufficiently active in the absence of nematode infection to yield levels of HrmA sufficient to kill transformed plant cells. In contrast, agrobacteria that carried the TSA construct yielded six viable transformed (T1) lines. A comparable number of leaf explants yielded 42 lines when the transformation vector, less the *hrmA* sequences, was used for transformation. All six TSA plant lines were fertile, and Southern blot analysis indicated that kanamycin-resistant progeny maintained the transgene without obvious rearrangement (data not shown). The six TSA lines could be classified into three types: (1) plants that displayed normal growth (three plants), (2) plants with severe stunting and necrosis (one plant), and (3) plants typified by lines 2 and 30. Plants from line 2 were vigorous, with slightly rigid leaves and with some micro-lesions up to 2–3 mm in diameter on older leaves (not shown). Plants from line 30 showed a 10-day retardation of root system development compared to control plants (not shown). However, once roots were established, TSA-30 was indistinguishable from control plants, and lacked the micro-lesions characteristic of plants from line 2. Plants from both lines displayed necrosis in the roots (see Figure 4b) that was not seen in controls.

The altered growth habit of transgenic plants (micro-lesions, slowed root growth, root necrosis) was independent of any challenge by nematodes, suggesting that the Δ0.3 TobRB7 promoter might retain a low level of activity in the absence of nematode infection. To confirm this, the expression level of the *hrmA* gene in T2 (not shown) and homozygous T3 (Figure 1b) progeny from TSA transformants was examined. *hrmA* mRNA could not be detected in TSA transgenic plants by Northern blot analysis (not shown), but was observable in type 3 plants when examined using RT–PCR combined with Southern blot analysis (Figure 1b). The apparent *hrmA* expression level was much higher in the TSA-2 than in the TSA-30 line, which is consistent with the appearance of micro-lesions in TSA-2 but not in TSA-30. Obvious differences (based on

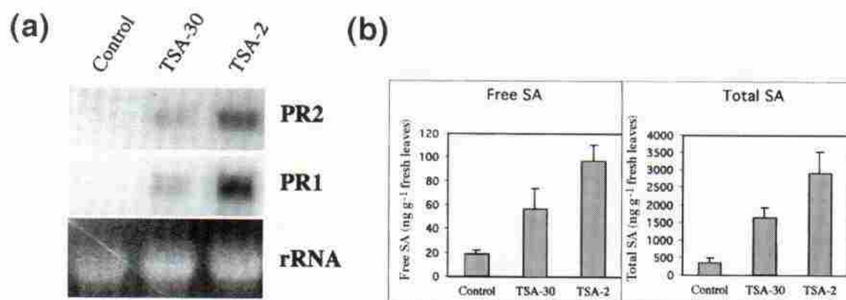

Figure 2. SAR-associated PR gene expression and salicylic acid (SA) accumulation in TSA plants.
(a) Expression of PR genes in TSA plants. Total RNA was isolated from kanamycin-resistant T2 progeny of the TSA-2 and TSA-30 plants or from transformed control plants. Aliquots (20 µg) of total RNA were analysed by Northern blotting, using tobacco *PR1* and *PR2* specific probes as described in Experimental procedures. The ethidium bromide-stained 18S rRNA is shown as a loading control. (b) Salicylic acid levels in control and TSA plants. Levels of free (left) and total (right) salicylic acid in control and *hrmA* transgenic plants. Transformed controls and kanamycin-resistant T2 progeny of the TSA-2 and TSA-30 plants were sampled and salicylic acid levels determined as described in Experimental procedures. Salicylic acid levels for each line were determined in triplicate.

the yield of RT–PCR product) in the levels of expression of the *hrmA* gene in younger and older leaves from TSA-2 and TSA-30 plants were not apparent (data not shown). Expression of the *hrmA* gene in type 1 plants could not be detected by RT–PCR (data not shown). Owing to the severely restricted growth habit, the type 2 plant was not assessed for *hrmA* expression.

Expression of defence-associated characteristics in TSA plants

The appearance of micro-lesions on old leaves of TSA-2 and delayed root development in TSA-30 suggest that low-level constitutive expression of the *hrmA* gene in these plants may have led to induction of hypersensitive and systemic defence responses. This possibility was tested by measuring the levels of expression of two pathogenesis-inducible genes. When RNA from leaves of TSA-2 and TSA-30 plants was hybridized with probes specific for the *PR-1* and *PR-2* genes (encoding β-1,3-glucanase), constitutive expression of these genes was observed in the TSA lines, but not in the control plants (Figure 2a). Moreover, there was a positive correlation between the levels of expression of these two genes in the TSA lines (Figure 2a) and the quantities of *hrmA* RT–PCR product that were observed (Figure 1b).

In wild-type plants, induction of the *PR1* and *PR2* genes by incompatible pathogens is mediated by salicylic acid, the synthesis of which is dramatically increased as a consequence of activation of defence responses. To better understand the means by which *hrmA* expression induces PR1 and PR2 expression, the levels of salicylic acid in the TSA-2 and TSA-30 plants were compared with the levels in control plants. As shown in Figure 2(b), the levels of free and total salicylic acids in the TSA plants were greater than those seen in controls. Interestingly, the TSA-2 plants, which had the highest levels of PR gene (Figure 2a) and *hrmA* (Figure 1b) expression, also possessed the highest salicylic acid levels.

Resistance to pathogens

The results shown in Figure 2 are consistent with the hypothesis that low-level *hrmA* gene expression may suffice for the induction of defence responses. This was further explored by studying the responses of T2 (shown in Figures 3 and 4) and T3 (not shown) progeny of the TSA-2 and TSA-30 lines to two viruses (tobacco vein mottling virus [TVMV] and tobacco etch virus [TEV]), a bacterial pathogen (*Pseudomonas syringae* pv. *tabaci*), and a fungal pathogen (*Phytophthora parasitica* var. *nicotianae* isolate 62).

Two differences between the TSA lines and control plants were observed when plants of each line were inoculated with either of the two viruses. Within 1 day of inoculation, the TSA plants exhibited HR-like necrosis on the leaves that had been inoculated with TVMV or TEV (a typical response for the TSA-2 plants is shown in Figure 3a). This response was restricted to the inoculated leaf tissue. In this study, the necrosis was only seen in the TSA plants. Moreover, such effects have never been observed in numerous previous inoculation studies with TVMV and TEV with other tobacco lines, transgenic or otherwise (Fellers *et al.*, 1998; Maiti *et al.*, 1993; Xu *et al.*, 1997; Xu *et al.*, 1998).

Beginning about 5 days post-inoculation, typical vein mottling or leaf etch symptom was invariably detected on the uninoculated upper leaves of control plants that had been infected with TVMV or TEV, and considerable coat protein (indicative of the accumulation of progeny virus) could be detected in the upper leaves of infected control plants (Figure 3b). In contrast, the uninoculated upper leaves of infected TSA plants remained free of disease symptoms, and virus accumulation could not be

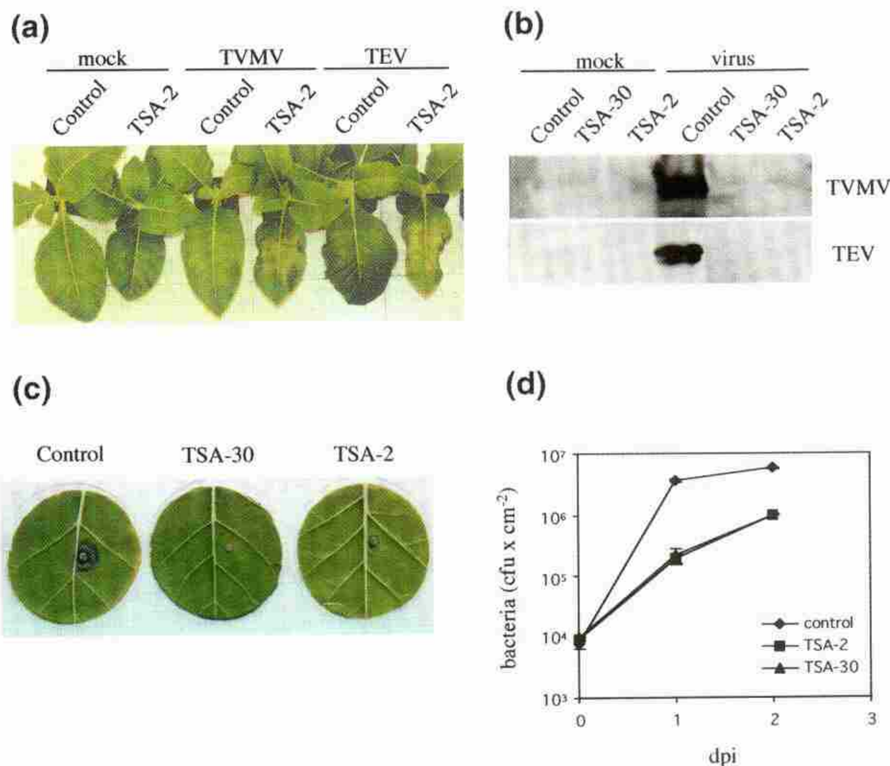

Figure 3. Resistance of TSA plants to different pathogens.
(a) Appearance of TVMV or TEV inoculated leaves of controls and representative TSA-2 plants, showing the HR-like necrosis that is characteristic of the TSA plants. The photograph was taken 1 day after inoculation with the indicated virus. (b) Immunoblot determination of TVMV and TEV coat protein levels in plants inoculated with TVMV and TEV. Samples were taken 2 weeks after inoculation and analysed by immunoblotting using anti-TVMV or anti-TEV antisera as appropriate (Fellers *et al.*, 1998). (c) Resistance of *hrmA* transgenic plants to *Phytophthora parasitica* var. *nicotianae* isolate 62. TSA plants were compared with a transformed control, as described in Experimental procedures. Leaf samples are from the plant lines indicated above the sample. (d) Growth of *P. s.* pv. *tabaci* on inoculated TSA or control plants. The data represent the mean of three replicate experiments. Note that standard deviations were plotted for all points, but that these were too small to be easily seen in the plot. Also note that the lines for the TSA-2 and TSA-30 plant lines are largely coincident.

detected in these plants (Figure 3b). These results indicate that, after the initial hypersensitive reaction to infection that is seen in the inoculated leaf, the TSA plants are wholly resistant to further systemic spread of TVMV and TEV.

Four days after inoculation of a control plant leaf with *Phytophthora parasitica* var. *nicotianae* isolate 62, a necrotic area (20 mm diameter) was apparent, indicative of successful infection by this pathogen (Figure 3c). In contrast, cell death was restricted to the leaf tissue that was touched by the pathogen plug in leaves from TSA-2 and TSA-30 plants, indicative of a local HR (Figure 3c). Moreover, these leaves were devoid of the fungus (not shown).

After inoculation of control plants with *Pseudomonas syringe* pv. *tabaci*, the bacterial population at the inoculation site was found to increase approximately 700-fold over the course of 2 days (Figure 3d), and characteristic symptoms (spreading necrosis and chlorosis) were apparent at these sites (not shown). In inoculated TSA-2 and TSA-30 plants, the rate of bacterial growth was much lower than was apparent in inoculated control plants, such that bacterial populations were about 5% of that seen on controls after 1 day post-inoculation, and 17% of that seen in controls after 2 days post-inoculation (Figure 3d). In addition, symptoms indicative of disease were conspicuously absent on inoculated TSA plants (not shown).

These results shown in Figure 3 were obtained with kanamycin-resistant T2 progeny of the primary TSA-2 and TSA-30 transformants. Similar results were also obtained with homozygous T3 progeny of this line, indicating a similar level of resistance in homozygous and hemizygous plants. T2 progeny from type 1 *hrmA* transgenic plants (which showed completely normal growth, see above) were also tested for resistance to the pathogens listed above, to explore the possibility that these plants might also possess a degree of disease resistance. However, these plants showed no indications of resistance to these pathogens (not shown).

Resistance of the T2 progeny of the TSA-2 and TSA-30 plants to three different nematode species was also evaluated. Two of these (*Meloidogyne javanica* and

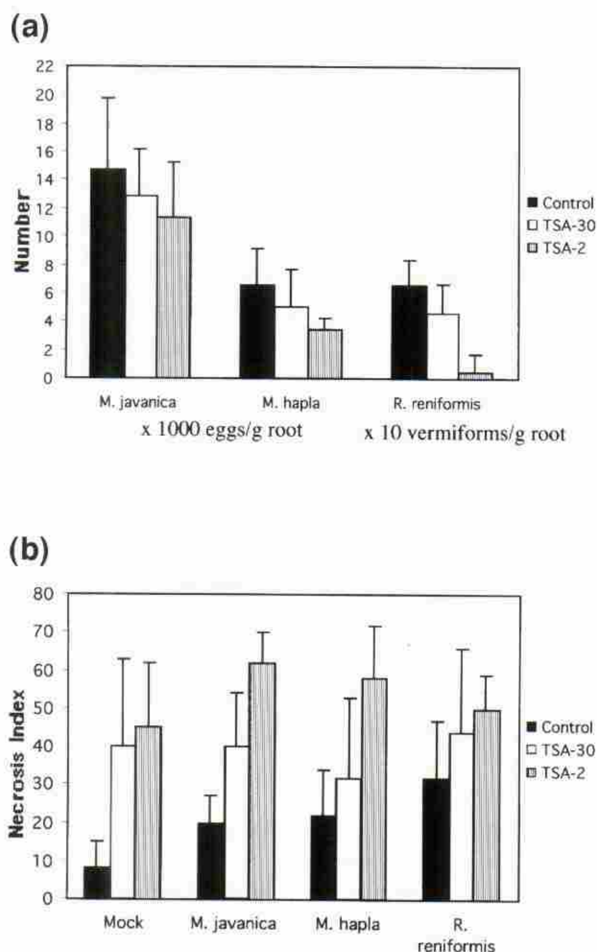

Figure 4. Responses of *hrmA* transgenic plants to three nematode species.
(a) Egg number per gram root is given for *Meloidogyne* species and vermiform number for *Rotylenchulus reniformis*. (b) Root necrosis index of TSA plants is given with and without nematode inoculation, with a minimum of 0 and maximum of 100. The data bars show the mean of six replicate experiments. Error bars represent the standard error.

Meliodogyne hapla) induce multi-nucleate giant cells as feeding structures, and thus should produce structures in which the TSA gene is highly expressed. The third (*Rotylenchulus reniformis*) induces multi-cellular synctia, rather than multi-cellular giant cells. In transgenic tobacco plants, the Δ0.3 TobRB7 promoter was observed to be inactive in similar feeding structures (Opperman *et al.*, 1994), and we expected that the TSA gene would have little effect against the latter nematode. Plants were infected with the different nematodes, and the numbers of root knots, eggs or vermiforms, and degrees of root necrosis after four or five life cycles (12 weeks) were compared. Interestingly, for the two *Meloidogyne* species, there were no large or statistically significant differences between control and the TSA plants in the numbers of root knots (not shown) or eggs (Figure 4a). A greater degree of root necrosis was observed in the TSA plants compared with the control plants (Figure 4b). The observed root necrosis consisted of browning or blackening of the surface layer of root cells. However, this necrosis was apparently not caused by the pathogens, as mock-inoculated plants also displayed a similar level of necrosis (Figure 4b). Interestingly, the TSA-2 plants were more resistant to *R. reniformis* than the control plants, judging from vermiform number (Figure 4a).

Discussion

The results presented here indicate that low-level constitutive expression of the *Pseudomonas syringae* pv. *syringae hrmA* gene can yield plants that possess high levels of resistance against a broad range of microbial plant pathogens (Figure 3). Thus, as has been found for the plant-encoded receptors of Avr proteins (Oldroyd and Staskawicz, 1998; Tang *et al.*, 1999), expression of bacterial genes whose products are involved in specific defence responses in plants can condition plants for increased resistance to a broad range of pathogens. The mechanism by which enhanced disease resistance is attained remains unclear. One possibility is suggested by the work of Alfano *et al.* (1997), who showed that intracellular expression of HrmA can induce cell death. It is possible that the TSA-2 and TSA-30 plants possess a level of HrmA that is insufficient to trigger a full-scale HR response, and that pathogen infection increases the expression of the *hrmA* gene at the inoculation site. The increased *hrmA* expression would then activate a higher level of defence responses, including the appearance of a macroscopic HR. Alternatively, a low level of *hrmA* gene expression in the transgenic plants may partially activate defence mechanisms, including the HR cell death programme. Consistent with this prediction, we found that *hrmA* plants constitutively express PR genes, and, in TSA-2 plants, possess microscopic HR-like lesions in older leaves.

The resistance mechanisms activated in transgenic *hrmA* plants do not appear to be effective in controlling *M. javanica* and *M. hapla* (root-knot nematodes). We had hypothesized that induced expression of *hrmA* in nematode feeding cells (giant cells) would stop nematode infection because giant cells act as nutrient sinks (Bird and Loveys, 1975) and serve as a permanent feeding site for the nematode (Hussey, 1985; Wyss and Grunder, 1992; Wyss *et al.*, 1992). The failure to observe such an effect suggests that the *hrmA*-triggered constitutive defence responses in the TSA plants are not effective against nematode infection, perhaps because giant cells are not able to respond to signals that activate the HR/cell-death pathway. In nematode-resistant tomato plants that carry the R gene *Mi*, there is no development of the feeding site upon infection. Instead, a localized region of necrotic cells,

characteristic of a hypersensitive response, develops near the site where the giant cell would normally form (summarized in Williamson, 1998). This suggests that localized HR may be effective in limiting nematode infection, and that cells at the sites of nematode infection are able to mount an HR response. The reasons why TSA plants do not respond in a like manner are not clear. The root necrosis that is observed in the TSA-2 and TSA-30 plants might condition root cells so that they are no longer able to mount a large-scale cell-death response. More interestingly, root cells may be inherently unable to respond to HrmA as do cells in other parts of a plant; the observed (limited) necrosis may be the full extent of the response, and this may be insufficient to limit nematode ingress. It is also possible that the Δ0.3 TobRB7 promoter behaves differently in the root cells of the TSA plants than has been reported in other studies, owing to the necrosis that is seen in unchallenged plants. The present study does not allow a distinction between these possibilities.

The TSA-2 and TSA-30 plants possess characteristics indicative of the establishment of systemic acquired resistance (SAR) in these plants (Figure 2). Others have reported that systemic acquired resistance to compatible root-knot nematodes in tomato can be conditioned by prior inoculation with incompatible nematodes (Ogallo and McClure, 1996), and that inoculation of cotton plants with incompatible nematodes results in an increase in phytoalexin levels (Veech and McClure, 1977). These observations suggest that systemic defences are effective against root knot nematodes. In this light, the lack of resistance to the root knot nematodes in the TSA-2 and TSA-30 plants is puzzling. Again, the root necrosis may limit the effectiveness (or even induction) of SAR in roots, thereby permitting infection by the root knot nematodes.

Alfano *et al.* (1997) showed that intracellular expression of the *hrmA* gene triggers HR cell death in tobacco, whereas infiltration of purified HrmA protein to the apoplast of tobacco leaves does not. It was therefore suggested that HrmA acts inside the plant cell to trigger the HR. In this study, we failed to generate transgenic tobacco plants that produce the HrmA protein intracellularly. We did obtain a number of transgenic plants that presumably express the HrmA protein extracellularly, but the number was small. The difficulty of producing transgenic plants that express HrmA extracellularly was unexpected, given that HrmA does not appear to elicit HR from outside of the tobacco cell. A likely explanation is that the transport of the pre-HrmA polypeptide may be somewhat less than 100% efficient; this, along with a low level of constitutive activity of the Δ0.3 TobRB7 promoter, would result in levels of intracellular HrmA high enough to kill many transformed cells. It is also possible that the predicted product of the TSA gene, the signal peptide–HrmA polypeptide, might fold in a way that interferes with transport and intracellular signalling by HrmA.

An additional surprising finding of our study was the low-level activity of the Δ0.3 TobRB7 promoter in *hrmA* plants. A previous study (Opperman *et al.*, 1994) indicated that a Δ0.3 TobRB7 promoter–GUS gene was active only in giant cells, with undetectable levels of expression (based on histochemical staining for GUS activity) in other cells. The present study shows that the Δ0.3 TobRB7 promoter is active at levels below the sensitivity of the GUS reporter system, but detectable by more sensitive methods (such as RT–PCR). This constitutive expression is apparently the reason why plants designed to express intracellular HrmA could not be produced.

The two plant lines examined in this study (TSA-2 and TSA-30) were similar in their responses to all of the pathogens tested, even though the apparent levels of expression of SAR-related characteristics (PR gene expression, salicylic acid accumulation) were rather different in these two lines (Figure 2). This indicates that relatively modest induction of SAR-associated properties may suffice for effective protection against a range of pathogens, and suggests that the resistant properties we observed in this limited number of lines will probably be general features of plants that express the *hrmA* gene in a low-level, constitutive manner.

In summary, we suggest that expression of pathogen *avr* genes at low levels and/or under the control of pathogen-inducible promoters is a viable strategy for genetic engineering of broad-spectrum pathogen resistance in plants. To effectively use this strategy, one needs to consider two factors.

(1) It would be desirable to express a broad-spectrum *avr* gene that can elicit resistance response in many cultivars and plant species so that the same *avr* expression construct can be used to generate resistance in multiple plants and cultivars. The *hrmA* gene appears to be such a broad-spectrum *avr* gene; it triggers an HR in all examined tobacco cultivars (Alfano *et al.*, 1997) and transformed *Arabidopsis thaliana* (Q. Li and S. Shen, unpublished observations). Whether it triggers an HR in other plant species needs to be examined. Many *avr* genes are identified initially based on their ability to trigger HR and resistance in one or a few cultivars of a given plant species (Leach and White, 1996). However, further examination of the effects of these *avr* genes in other plant species often uncovers additional plant species and cultivars that react with an HR to these *avr* genes.

(2) A low basal level of expression is critical for this strategy to work, owing to the potential for proteins such as HrmA to elicit defence responses, including cell death, in the absence of pathogen challenge. This potential is manifest in the present report as a severe growth defect in

some plant lines, and modest but significant differences (micro-lesions, root necrosis) in lines with very low expression levels. These observations indicate the need for a delicate balance in plants that express the *hrmA* gene, with expression levels high enough to trigger and maintain systemic defences, but low enough to avoid large-scale cell death. Delineation and attainment of an expression level that optimizes the disease resistance but minimizes growth aberrations will be essential for the full realization of the potential application of genes such as *hrmA* for disease resistance in crops.

Experimental procedures

Recombinant DNA manipulations

Recombinant DNA manipulations were performed basically as described by Sambrook *et al.* (1989). Tobacco (*Nicotiana tabacum* cv. Wisconsin 38) genomic DNA was used as template for amplification of the Δ0.3 TobRB7 promoter (Opperman *et al.*, 1994; Yamamoto *et al.*, 1991). Oligonucleotides Δ0.3 TobRB7-5′ (5′-GGAATTCAGCTTATCTAAACAAAGTTTTAAATTC-3′) and Δ0.3 TobRB7-3′ (5′-GTAAGCTTCTGAGCGATCCTTCTCACTAGAAAAA-TGCCC-3′) were used for PCR. The PCR product was digested with *Eco*RI and *Hin*dIII (sites underlined in the primer sequences), and cloned into pBluescript KS+ (Stratagene), and the recombinant product confirmed by DNA sequencing.

Sequences encoding the signal peptide of the pathogenesis-related protein 1b (PR-1b) were amplified by PCR using *N. tabacum* cv. Samsun NN genomic DNA as template and the oligonucleotides PR1b-5′ (5′-CACGAAGCTTACCATGGGATTTTTT-CTCTTTTCAC-3′, *Hin*dIII site underlined) and PR1b-3′ (5′-TCCCGCGGGAGTTTTGGGCAT GAGAAG-3′, *Sac*II site underlined) as primers (Cornelissen *et al.*, 1986). The amplified sequence was cloned into pBluescript KS+ and confirmed by DNA sequencing.

The *hrmA* gene was amplified by PCR using *Pseudomonas syringae* pv. *syringae* strain 61 genomic DNA as template and PCR primers designed according to the *hrmA* sequence (Heu and Hutcheson, 1993). Two 5′ primers were made with different restriction sites for cloning with or without the *PR1-b* signal peptide sequence (Figure 1) (*hrmA*-5′-1, 5′-CACGAAGCTTACC-ATGGACCCTATCCATGC-3′; *hrmA*-5′-2, 5′-TCCGCCGCGGGGTG-AACCCTATCCATGC-3′, *Hin*dIII and *Sac*II sites underlined). A common 3′ primer (*hrmA*-3′: 5′-GGTGGAGCTCAGTTTCGCGC-CCTGAG-3′, *Sac*I site underlined) was used for both constructs. The full-length *hrmA* DNA was first cloned into pBluescript KS+ and the insert was verified by DNA sequencing. Various combinations of promoters, signal peptide sequences and the *hrmA* gene were then made in the T-DNA binary vector pKYLX71:35S^2 (Maiti *et al.*, 1993; Mogen *et al.*, 1992). Figure 1 shows various expression cassettes constructed in this study.

Tobacco transformation and plant growth conditions

The resulting pKYLX71:35S^2 derivatives were mobilized into *Agrobacterium tumefaciens* (pGV3850) and the transconjugants used to transform tobacco (*Nicotiana tabacum* L. cv. KY14) as described in detail elsewhere (Li and Hunt, 1995). All leaf pathogen inoculation experiments were carried out in a growth chamber with 16 h light and 8 h dark at 22–25°C. The nematode inoculation tests were performed in a greenhouse. KY14 plants transformed with the vector pKYLX71:35S^2 was used as control in all experiments.

Expression of the hrmA *and PR genes*

RT–PCR was used for *hrmA* transcript detection. Total RNA was isolated from the transgenic plants using the TRIzol Reagent kit according to manufacturer's instruction (Gibco BRL). A 5 μg aliquot of purified total RNA was added to a 30 μl reverse transcription mixture, according to the manufacturer's recommendations (Ambion Inc.). Oligo(dT) was used as the first-strand primer. The resultant first strand was used as a template for subsequent PCR reactions (Sambrook *et al.*, 1989), using the primers PR1b-5′ and *hrmA*-3′. PCR products were separated in a 1% agarose gel, blotted to Nytran Plus membranes (Schleicher & Schuell Inc.), and the filter probed with ^{32}P-labelled *hrmA* DNA (prepared by random-primed synthesis). As an internal equal loading control, the tobacco β-tubulin DNA was also amplified from the same first-strand mixture using suitable primers (tubulin forward: 5′-CTTGCATTGGTACACAGG-3′; tubulin reverse: 5′-ACTTGAAA-CCCACGCTCCTC-3′). Controls were also performed without reverse transcription.

Northern blot analysis (Sambrook *et al.*, 1989) was used for detection of PR gene expression. Plasmids carrying tobacco *PR1* and *PR2* sequences (kindly provided by Dr Yinong Yang, Department of Plant Pathology, University of Arkansas, and Dr Santanu Dasgupta, Department of Agronomy, University of Kentucky, respectively) were labelled with [α-^{32}P]-dCTP using a random priming kit (Stratagene). Aliquots (20 μg) of total RNA from each treatment were fractionated in a 1.0% agarose/formaldehyde gel and subsequently blotted to Nytran Plus membranes. Hybridizations were performed with the appropriate probes in Northern MAX Prehyb/Hyb Buffer (Ambion Inc.) according to the manufacturer's instructions.

Pathogen resistance assays

All pathogen resistance assays were carried out with both T2 and T3 progenies of the transgenic plants in independent experiments. Tobacco vein mottling virus (TVMV) and tobacco etch virus (TEV) inoculations were performed as described elsewhere (Fellers *et al.*, 1998). Two weeks after inoculation, disease symptoms were scored and two leaf discs (about 100 mg) were taken from top leaf and ground in 100 μl SDS–PAGE loading buffer. A 20 μl aliquot of the supernatant was analysed by immunoblot analysis as described in detail elsewhere (Fellers *et al.*, 1998).

Phytophthora parasitica var. *nicotianae* isolate 62 (kindly provided by B. Kennedy, University of Kentucky) was grown on an oatmeal medium plate (5% Gerber oatmeal and 2% agar, w/v). After the fungal mycelia had spread throughout the plate (approximately 7 days), a plug of medium containing the fungal mycelia was taken by excision with a 6 mm cork borer. A piece of tobacco leaf (the 5th leaf from top) was cut to the size of a 9 cm Petri dish, and placed upside down on top of a piece of water-soaked Whatman paper. The mycelium–agar plugs were set on the leaf disc with the mycelium side contacting the leaf surface. The dish was sealed and set at 28°C with 16 h light and 8 h dark. Leaves were photographed 4 days after inoculation.

Inoculations with *Pseudomonas syringae* pv. *tabaci* WF4 were performed as described by Li *et al.* (2000). Samples of inoculated tissue were taken daily by excision with a 6 mm cork borer.

Bacteria inside the leaf discs were released by grinding the tissue in a microfuge tube in sterile water and plated on LB medium. The bacterial population was determined based on the numbers of colonies formed in LB plates, as described by Bertoni and Mills (1987). The mean values from three plates for each of three independent inoculations are presented.

The plants used for the nematode resistance test were grown in a 1:1 mixture of sand and soil. *Meloidogyne* spp. eggs were used as inocula prepared by a NaOCl extraction protocol (Barker, 1985) from nematode-infected tomato plants. Each pot (one plant) was inoculated with 10 000 eggs. After 12 weeks, root gall and root necrosis indices were evaluated using a scheme of 0–100 (100 = gall or necrosis covering the entire root). Eggs were extracted from roots with the NaOCl protocol, stained with acid fuchsin and counted (Barker, 1985). For the *R. reniformis* resistance test, the vermiforms were prepared from tomato plants by the flotation–centrifugation–sieving method (Barker, 1985) using nested 35-mesh and 400-mesh sieves. Approximately 10 000 vermiforms were used to inoculate each pot (one plant). After 3 months of development, plant resistance was evaluated by counting the vermiforms extracted from the soil and roots using the same extraction method.

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