High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance

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Virus-induced gene silencing was used to assess the function of random *Nicotiana benthamiana* cDNAs in disease resistance. Out of 4992 cDNAs tested from a normalized library, there were 79 that suppressed a hypersensitive response (HR) associated with *Pto*-mediated resistance against *Pseudomonas syringae*. However, only six of these clones blocked the *Pto*-mediated suppression of *P.syringae* growth. The three clones giving the strongest loss of *Pto* resistance had inserts corresponding to *HSP90* and also caused loss of *Rx*-mediated resistance against potato virus X and *N*-mediated tobacco mosaic virus resistance. The role of HSP90 as a cofactor of disease resistance is associated with stabilization of Rx protein levels and could be accounted for in part by SGT1 and other cofactors of disease resistance acting as co-chaperones. This approach illustrates the potential benefits and limitations of RNA silencing in forward screens of gene function in plants.
Keywords: gene silencing/HSP90/plant disease/resistance

Introduction

Plant viruses are activators of RNA silencing through a process that involves 21–25 nucleotide short interfering RNA (siRNA) (Hamilton and Baulcombe, 1999; Waterhouse *et al.*, 2001). By analogy with findings from animals, it seems that the siRNA is processed from a double-stranded RNA (dsRNA) precursor by a DICER RNase III homologue and that it is incorporated as the specificity determinant into an RNA interference specificity complex (RISC) (Hannon, 2002). In virus-induced silencing, the dsRNA either is a viral replication intermediate or is produced by a host-encoded RNA polymerase using a viral RNA template (Mourrain *et al.*, 2000). The viral RNA in this process is thus both an initiator and a target of the silencing mechanism. At the

beginning of the infection cycle, the levels of dsRNA and siRNA would be correspondingly low and the viral RNA replication would be unchecked by silencing. However, at later stages of the cycle when viral dsRNA is more abundant, the higher levels would target RISC to the viral RNA and the rate of viral replication would slow down. Eventually the accumulation of viral RNA would stop or even decline.

Virus-induced RNA silencing, also referred to as virus-induced gene silencing (VIGS), is normally specific for viral rather than host RNA. However, if the viral genome is modified to include host sequences, the corresponding host RNAs are targeted and the symptoms in the infected plant resemble the phenotype of a null or reduced function mutant (Kumagai *et al.*, 1995; Ruiz *et al.*, 1998). This approach to suppression of host gene expression is useful in the assignment of gene function because it is fast (Baulcombe, 1999). The virus vector constructs can be assembled using standard DNA manipulations and, as symptoms develop on the infected plant by 7–21 days post-inoculation (d.p.i.), a loss-of-function phenotype can be produced within a month of having identified a gene of interest.

The use of VIGS for analysis of gene function has been validated by the use of virus vector constructs targeted against genes involved in primary and secondary metabolism, development and disease resistance (Atkinson *et al.*, 1998; Kjemtrup *et al.*, 1998; Jones *et al.*, 1999; Burton *et al.*, 2000; Ratcliff *et al.*, 2001; Jin *et al.*, 2002; Liu *et al.*, 2002a,c; Peart *et al.*, 2002a,b; Slaymaker *et al.*, 2002). Here we describe an application of VIGS in a forward screen of genes required for disease resistance. We produced a normalized library of *Nicotiana benthamiana* cDNA in a vector derived from potato virus X (PVX). This vector was in an *Agrobacterium* Ti plasmid so that *Agrobacterium* inoculation could be used as a high throughput means of initiating virus infection without isolation and *in vitro* transcription of the PVX cDNA (Turpen *et al.*, 1993). Plants were infected with individual PVX constructs from the library, and the resulting symptoms were used as a guide to the function of cloned cDNA. To identify cDNAs of genes involved in disease resistance, the infected plants were assayed for *Pto*-mediated resistance against *Pseudomonas syringae*. We anticipated that silencing would lead to susceptibility to *P.syringae* if the target of VIGS encoded a protein that is, or is similar to, a cofactor of disease resistance.

The primary screen for *Pto* resistance was based on a hypersensitive cell death response that is elicited by the bacterial AvrPto protein in the presence of Pto (Rommens *et al.*, 1995). Out of 4992 cDNAs that were silenced, there were 79 that affected the *Pto*-mediated hypersensitive response (HR), of which there were six that silenced genes required both for the HR and for the *Pto*-mediated

suppression of bacterial growth. Three of these PVX clones with the most pronounced effect on the *Pto* phenotype carried inserts corresponding to HSP90-like chaperonins encoded by a small multigene family. Silencing of these genes also compromised virus resistance mediated by the Rx and N proteins conferring resistance against PVX and tobacco mosaic virus (TMV), respectively. Thus, VIGS is an effective means of screening for novel gene function and is informative about multigene families that may not be readily accessible by mutagenesis because of functional redundancy. The HSP90 proteins may play a role either in stabilization of proteins involved in disease resistance or in assembly of multisubunit complexes required for activation of R proteins and intracellular signalling.

Results

A cDNA library in PVX

A cDNA library for insertion into a PVX agroinfection vector (Figure 1, pGR106) was synthesized using poly-adenylated RNA isolated from fully developed leaves of young *N.benthamiana*. The cDNA was normalized through three cycles of PCR to ensure that cDNAs of abundant mRNAs were not over-represented in the library. The abundance of clones representing rubisco, assessed by colony hybridization of aliquots of the library, was ~100-fold lower (1/600) than in an equivalent library of non-normalized cDNA (1/6). In contrast, rare mRNAs [phytoene desaturase (*PDS*) and *PRF*] were similarly abundant (~1/10 000) before and after normalization (data not shown).

After ligation of the normalized cDNA into the Ti plasmid PVX vectors and transformation into *Agrobacterium*, we confirmed that >95% of the colonies contained inserts of between 400 and 1000 bp in length. Each of 4992 PVX clones in *Agrobacterium* was then inoculated to a single 3 cm high *N.benthamiana* plant at the five-leaf stage. Nearly all (~95%) of the plants developed symptoms of virus infection that, in some instances, were similar to the normal mosaic associated with PVX. However, there were also plants with chlorosis, necrosis, leaf distortion or stunting (Figure 1B). These symptoms were present in many of the plants to some extent but, in ~15% of the plants, they were pronounced. Figure 1B illustrates examples of the most frequently observed symptoms. Presumably these symptoms were due to VIGS of endogenous genes.

Screening of genes required for Pto-mediated resistance

To find out whether genes required for *Pto*-mediated resistance were represented in the PVX library, we used an HR assay. *AvrPto* and *Pto* (Scofield *et al*., 1996; Tang *et al*., 1996) were transiently expressed in leaves of the PVX vector-infected plant in the expectation that an HR would develop only in plants that were fully competent to carry out *Pto*-mediated resistance. Consistent with this expectation, there was an HR in the infiltrated regions of non-infected or PVX:green fluorescent protein (GFP)-infected plants (Figure 2A). Thus PVX did not interfere with the interaction of AvrPto and Pto or with the signalling pathway leading to the HR. In contrast, the
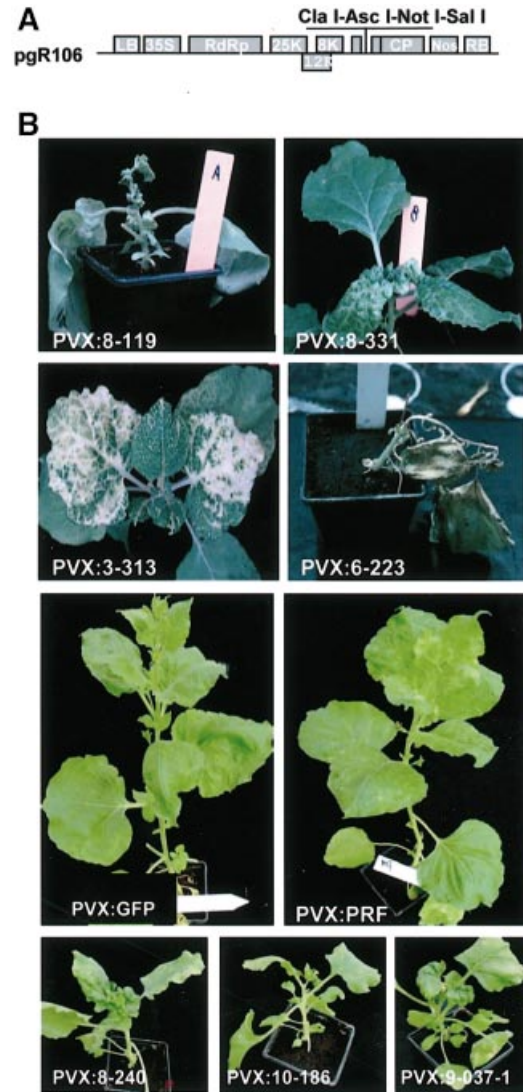

Fig. 1. PVX VIGS vectors. (**A**) Diagrammatic representation of a PVX vector in the pGreen0000 binary plasmid: 35S = 35S promoter of cauliflower mosaic virus; RdRp = PVX RNA-dependent RNA polymerase gene; 25K, 12K and 8K = PVX movement protein genes encoding proteins of the indicated molecular weight; CP = viral coat protein gene; nos = nopaline synthase transcriptional terminator; LB and RB are the left and right T-DNA border sequences. Unique restriction sites for insertion of sequence into the viral vector are indicated. (**B**) Symptoms due to VIGS of histone H4 (PVX:8-119), 16S ribosomal protein L30 (PVX:8-331), chlorophyll a/b-binding protein (PVX:3-213), receptor-like protein kinase (PVX:6-223) and HSP90 (PVX:9-037-1, PVX10-186hsp and PVX:8-240). The PVX:GFP- and PVX:PRF-infected plants did not show symptoms due to VIGS. The plants were photographed 4 weeks after inoculation with the indicated viral constructs.

HR did not develop if the plants were infected with a virus vector construct (PVX:PRF) that would silence the *Prf* cofactor of *Pto*-mediated resistance (Figure 2A) (Peart *et al*., 2002a). Therefore, the loss of the HR could be used as an indicator of genes that are required for *Pto*-mediated resistance.

Out of the 4992 PVX vector clones tested, there were 79 for which the silencing phenotype resulted in compromised AvrPto-induced HR. For each of these 79 clones, the loss of the HR was consistent in at least two replicate tests involving a minimum of three plants each.
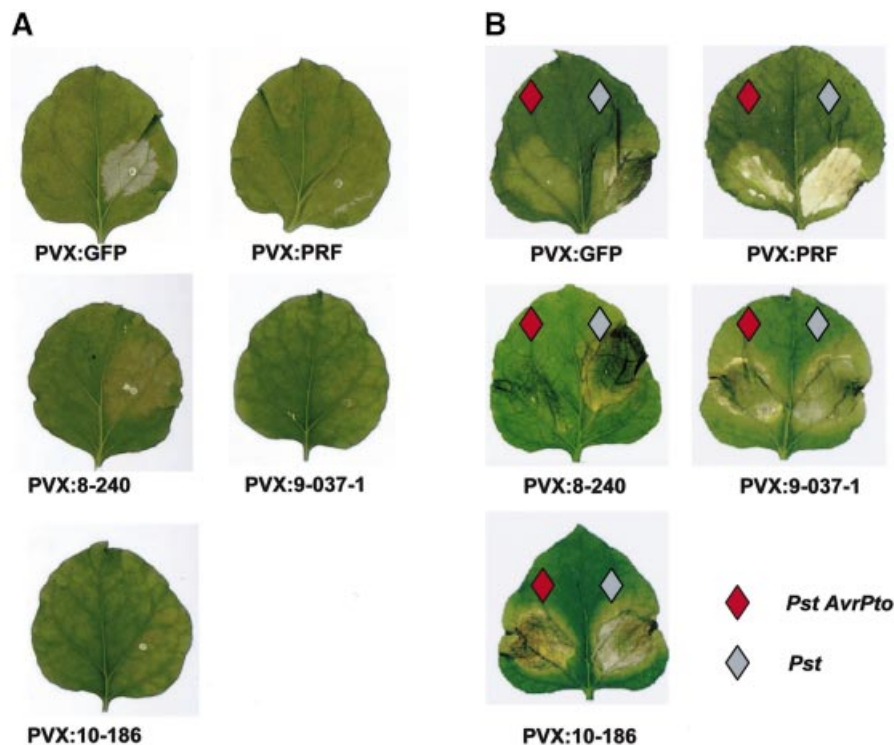
Fig. 2. *Pto* responses are compromised by VIGS of *Prf*. (**A**) *Nicotiana benthamiana* plants were inoculated with the indicated PVX constructs and, after 3 weeks, the upper leaves were infiltrated with a mixture of *Agrobacterium* cultures carrying 35S:Pto and 35S:AvrPto transgenes. In the non-PVX-infected or PVX:GFP-infected leaves, an HR developed after 48 h. This HR was suppressed in plants infected with PVX:PRF or with the indicated clones from the PVX cDNA library. The suppression of HR was weaker with 8-240 than with the other HSP90 constructs. (**B**) *Pto*-transgenic *N.benthamiana* plants (line 38-12) were inoculated with the indicated viral constructs. After 4 weeks, the upper leaves of these plants were challenge-inoculated with dilute cultures of *Pst* that either carried (red symbol) or did not (black symbol) carry *AvrPto*. The *Pst* chlorotic and necrotic symptoms, shown here at 4 days post-challenge inoculation, developed with the virulent *Pst* on all plants or with avirulent *Pst(AvrPto)* inoculated to plants infected with PVX:PRF or with the indicated HSP90 clones from the PVX cDNA library. The symptoms were weaker with PVX:8-240 than with the other HSP90 constructs as with PVX:8-320, PVX:11-9 and PVX:13-21 (not shown).

In some instances, as with PVX:PRF, the loss of HR was complete (e.g. PVX:10-186 and PVX:9-037 shown in Figure 2A). However, there were other clones (e.g. PVX:8-240 shown in Figure 2A) that caused only partial suppression or delay of the HR. The GenBank accession numbers of the insert sequences are presented as Supplementary table 1 available at *The EMBO Journal* Online and on our website (www.sainsbury-laboratory. ac.uk).

To find out whether loss of the AvrPto-induced HR was accompanied by loss of disease resistance, *Pto* transgenic plants (line 38-12) (Rommens *et al.*, 1995) were infiltrated with a dilute suspension (5×10^4 c.f.u./ml) of *P.syringae* pv *tabaci* either without (*Pst*) or with *AvrPto* [*Pst(AvrPto)*]. In plants inoculated with *Pst*, the disease symptoms were manifested at 3 days post-inoculation as a water-soaked lesion in the infiltrated patch of the leaf. After a further 3 days, this lesion became necrotic and surrounded by a chlorotic halo. These symptoms developed irrespective of whether the plants were pre-viously infected with PVX:GFP or PVX:PRF (Figure 2B). However, in the *Pto* transgenic plants infected with PVX:GFP, *Pst(AvrPto)* did not cause necrotic symptoms and the inoculated regions developed only a slight green chlorosis (Figure 2B). Presumably the lack of symptoms in these plants was due to the elicitation of the *Pto*-mediated resistance by *AvrPto*.

In the *Pto* transgenic plants that had been previously infected with PVX:PRF, the water-soaked lesions and subsequent necrosis due to *Pst(AvrPto)* were as severe as with *Pst* (Figure 2B). Similarly, with six out of the 79 PVX clones that suppressed the *Pto-AvrPto*-mediated HR, the *Pto*-mediated resistance was suppressed. With two of these clones (PVX:10-186 and PVX:9-037), the suppres-sion of resistance was as effective as with PVX:PRF. The severe *Pst* symptoms developed within 4 d.p.i. (Figure 2B). However, with the other clones (PVX:8-240, PVX:8-320, PVX:11-9 and PVX:13-21), the *Pst* symptoms did not develop until 6 days or later or were milder than on PVX:PRF-infected plants (Figure 2B). We conclude that these six PVX clones contained inserts representing genes that are required for *Pto*-mediated disease resistance.

Two of the six PVX clones (PVX:10-186 and PVX:9-037) contained chimeric inserts separated by *Asc*I or *Not*I sites used in the original cDNA cloning process. For each of these chimeras, we produced derivative clones (PVX:10-186hsp and PVX:9-037-1) with single inserts that suppressed *Pto*-mediated resistance in exactly the same way as the progenitor. Further studies described below used these single insert clones.

To investigate the effect of these six clones on *Pto* resistance further, we measured bacterial growth in the virus-infected plants. In plants infected with PVX:GFP, the manifestation of *Pto* resistance was differential growth

of *Pst* and *Pst*(*AvrPto*): *Pst*(*AvrPto*) grew more slowly than *Pst* and, by 3 d.p.i., was 30-fold less abundant than *Pst* (Figure 3). As expected, in *Pto* plants infected with PVX:PRF, the loss of *Pto* resistance reduced the differential in growth of these strains. Similarly, following infection with PVX:10-186hsp, PVX:9-037-1 and PVX:8-240, the growth of *Pst*(*AvrPto*) was accelerated and both strains grew at similar rates (Figure 3). However, with PVX:8-320, PVX:11-9 and PVX:13-21, despite the delayed symptoms, there was differential growth of *Pst* and *Pst*(*AvrPto*) as in the PVX:GFP-infected plants (not shown).

To rule out that the loss of *Pto* resistance was an artefact associated with PVX, the 10-186hsp, 9-037-1 and 8-240 cDNA inserts were transferred into vectors based on tobacco rattle virus (TRV). These vectors were inoculated to *N.benthamiana* plants and, 3 weeks later, the plants were tested for the *Pto*-dependent HR and resistance against *Pst* (*AvrPto*). In each instance, the results were the same as with the PVX vector: resistance and HR were compromised (R.Lu, unpublished data).

Characterization of genes required for Pto-mediated resistance

The PVX clones causing pronounced loss of *Pto* disease resistance (10-186hsp, 8-240 and 9-037-1) carried inserts of 549, 499 and 332 bp, respectively, that are similar to an *HSP90* cDNA from tomato (GenBank accession No. M86549) (Figure 4A and C). Correspondingly, the plants infected with PVX:10-186hsp and PVX:9-037-1 contained less HSP90 protein than the equivalent control plants (Figure 4B). From these results, it seems likely that the loss of disease resistance phenotype was due to VIGS of *HSP90*. The other three PVX clones causing loss of Pto disease resistance carried inserts corresponding to snRNA-associated proteins (8-320 and 11-9) or to proteins without known function (13-21) (Supplementary table 1). These clones have not yet been analysed in detail because there was weaker loss of disease resistance than with the *HSP90* clones.

The sequences of the 10-186hsp, 8-240 and 9-037-1 *HSP90* cDNAs were extended by RT–PCR (GenBank accession Nos AY310789, AY310781 and AY310776). These sequences clearly represent a gene family because they were non-identical in overlapping regions. To investigate the number of *HSP90* genes, we carried out RT–PCR of total cDNA with a primer from a conserved region that would have amplified cDNA of other *HSP90* homologues in addition to 10-186, 9-037-1 and 8-240. These RT–PCR cDNAs corresponded to the C-terminal 103 amino acids and the 3′-non-coding region (see arrow in Figure 4A).

Most of the amplified cDNAs corresponded to the extended sequence of 10-186hsp or 8-240. However, there was an additional sequence (xy8) that is 2.9, 9.7 and 6.8% mismatched with the equivalent region of the 8-240, 9-037-1 and 10-186 cDNAs, respectively (Figure 4D). These four sequences were more similar to the cytoplasmic *HSP90*s (Milioni and Hatzopoulos, 1997; Krishna and Gloor, 2001) of *Arabidopsis* than the organellar forms (Krishna and Gloor, 2001). However, these four *N.benthamiana* genes were more similar to each other than to any of the *Arabidopsis HSP90*s. It is likely,
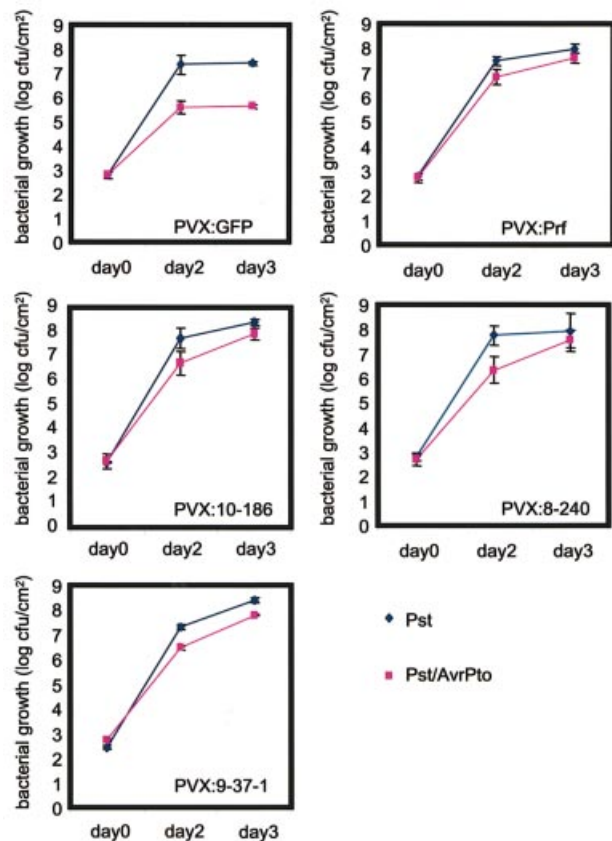

Fig. 3. Growth of *Pst*(*AvrPto*) on *Pto*-transgenic *N.benthamiana* after VIGS of *HSP90* and *PRF*. *Pto*-transgenic *N.benthamiana* (line 38-12) were inoculated with PVX vector constructs as indicated and challenge-inoculated with *Pst* or *Pst*(*AvrPto*) after 4 weeks. The *Pst* inocula were a 10 000-fold dilution of bacterial suspensions (OD_{600} = 1.0 in 10 mM $MgCl_2$), and the *in planta* level of both *Pst* (blue) and *Pst* (*AvrPto*)(pink) was monitored at 0, 48 and 72 h post-bacterial inoculation. The values shown (c.f.u./cm^2) with standard errors were derived from three batches of three plants.

therefore, that the families of genes coding for cytoplasmic HSP90 proteins in these two species evolved from a common gene ancestor through separate gene duplications. The functional difference of cytoplasmic and organellar HSP90 is reinforced by the finding that a PVX clone targeted at an organellar HSP90 (5-328) caused loss of *Pto*-mediated HR but had no effect on the *Pto* disease resistance (Supplementary table 1).

In addition to a role in disease resistance, it is likely that HSP90 proteins have functions in plant growth and development. Consequently, with both the PVX and TRV vectors, there was a VIGS phenotype of stunting and leaf deformation (Figure 1D) that, as with the disease resistance phenotype, was more severe with 10-186 and 9-037-1 than 8-240. In some experiments, these symptoms eventually were so severe that the plants died.

HSP90 is not specific for the Pto disease resistance pathway

From genetic and biochemical analyses, it can be inferred that there are overlapping signal transduction pathways associated with different disease resistance genes (Innes, 1998). It was of interest, therefore, to find out whether

HSP90 silencing affected disease resistance associated with proteins with nucleotide-binding site and leucine-rich repeat domains (NBS-LRRs) encoded by the *N* and *Rx* genes. These proteins confer resistance against PVX (*Rx*) or TMV (*N*), and the experiments were carried out with *N.benthamiana* carrying either *N* or *Rx* transgenes (Bendahmane *et al*., 1999; Peart *et al*., 2002a). These plants were first infected with TRV:HSP90 and later challenge-inoculated with either PVX or TMV constructs that were modified to carry GFP reporter genes.

In *N* genotype plants infected with the TRV empty vector or with TRV:8-240, there was no detectable accumulation of TMV:GFP RNA (Figure 5A). However, following infection with TRV:10-186hsp or TRV:9-037-1, there was marked accumulation of TMV:GFP that could be monitored by northern analysis (Figure 5A). The level
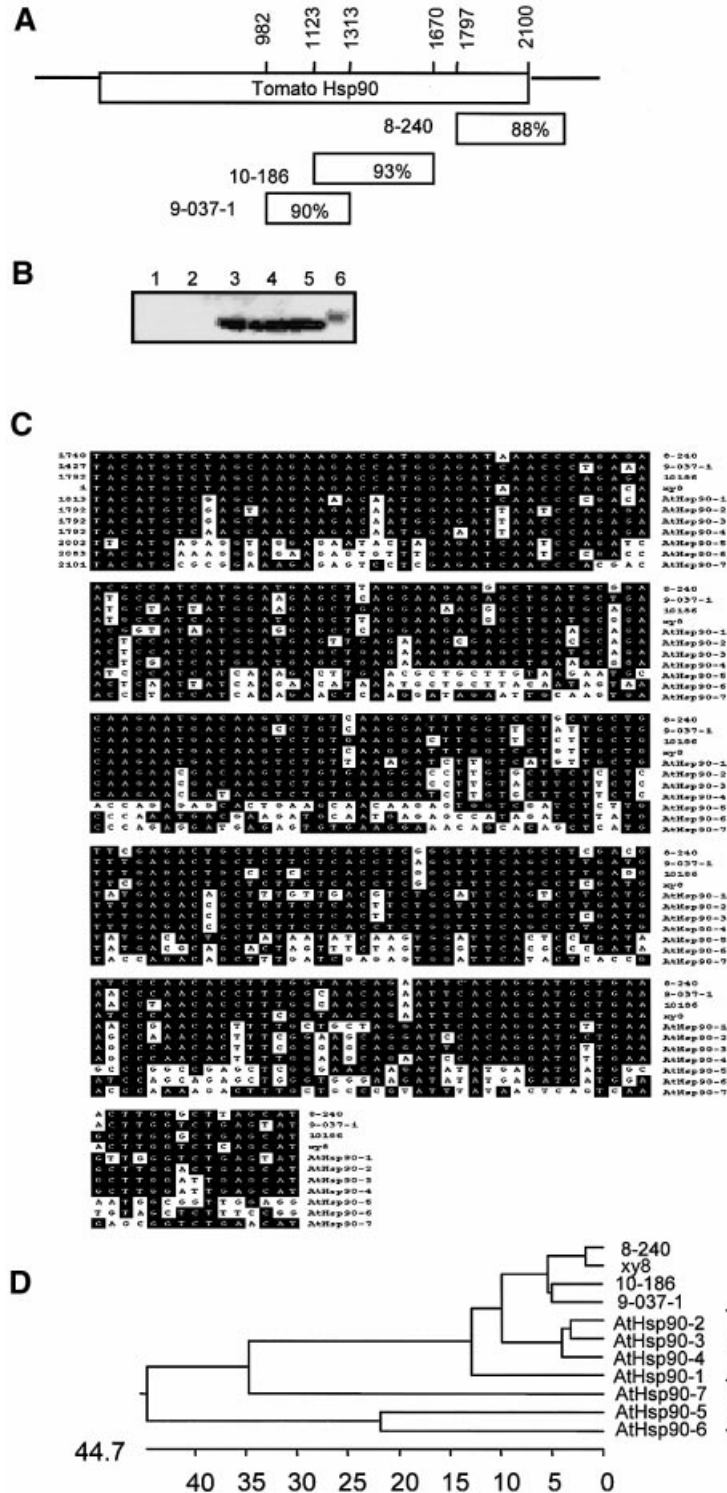
of TMV accumulation was similar to that in plants infected with TRV:NRG1 in which the target of VIGS is required for *N*-mediated resistance (J.Peart and D.C.Baulcombe, unpublished). However, the loss of *N*-mediated resistance in TRV:HSP90-infected plants was incomplete because we could not detect systemic spread of the TMV:GFP. This partial loss of resistance is not intrinsic to the methods used because VIGS of *SGT1* or *N* leads to loss of both local and systemic *N*-mediated resistance against TMV:GFP (Peart *et al.*, 2002a,b).

Rx-mediated resistance against PVX was compromised in plants infected with TRV:9-037-1 and TRV:10-186hsp but not with TRV:8-240 (Figure 5B). This loss of *Rx* resistance was indicated by GFP fluorescence in the leaf inoculated with PVX:GFP or by northern analysis (Figure 5B) on *Rx* genotype *N.benthamiana* inoculated with PVX:GFP. However, the loss of *Rx* resistance was not as strong as in plants infected with TRV:Rx (Figure 5B) and did not allow systemic spread of PVX.

From these results, it seems that HSP90 is required for a process in disease resistance that is common to the three different disease resistance genes *Pto*, *N* and *Rx*. These three examples of disease resistance all involve NBS-LRR proteins: *N* and *Rx* both encode proteins in this class (Whitham *et al.*, 1994; Bendahmane *et al.*, 1999) and *Pto* resistance is dependent on the NBS-LRR product of *Prf* (Salmeron *et al.*, 1996). It was therefore of interest to monitor directly the effect of HSP90 VIGS on an NBS-LRR protein. To carry out this analysis, we used *N.benthamiana* carrying an *Rx-4HA* transgene. The haemagglutinin (HA) epitope-tagged protein conferred resistance against PVX as with the wild-type Rx (P.Moffett, unpublished data) and, despite its low level expression from the endogenous *Rx* promoter, was readily detectable by western blotting (Figure 6). After VIGS with the TRV:HSP90 constructs, the level of Rx-4HA was reduced (Figure 6) in parallel with the effects of these recombinant viruses on *Rx* disease resistance (Figure 5). Thus the 10-186 and 9-037-1 constructs caused a greater reduction of Rx-4HA than did TRV:8-240. In contrast, in plants infected with TRV:SGT1 in which Rx-mediated resistance was also suppressed, there was no detectable effect on the steady-state level of Rx-4HA (Figure 6). It seems, therefore, that the effect of HSP90 in disease resistance could be through direct or indirect effects on the accumulation on the NBS-LRR protein products of disease resistance genes. It also seems that HSP90 and SGT1 influence different stages of the Rx resistance mechanism.

Discussion

VIGS was developed as an alternative to insertional mutagenesis for analysis of gene function. It was proposed as an approach that would be informative about genes that could not be easily investigated by mutagenesis because they have a lethal phenotype (Baulcombe, 1999). In addition, it was intended to be more suitable than transgenic approaches with antisense or inverted repeat constructs for high throughput applications because the viral vector symptoms could be induced without the time-consuming and labour-intensive procedures for plant transformation. Here, by carrying out a survey of nearly 5000 cDNAs, we have validated the suitability of VIGS for high throughput analysis. Moreover, from the high frequency and nature of the extreme symptoms (Figure 1), we have indications that VIGS is informative about genes that are required for growth. Many of the VIGS targets, for example those producing stunted plants, would most probably have a lethal phenotype in a null insertion mutant. However, because the VIGS symptoms are established gradually on a fully developed plant, it is possible to differentiate ways of growth arrest that would not be straightforward in mutants with an embryo lethal phenotype. An additional hypothetical advantage of VIGS over insertional mutagenesis is the possibility of investigating genes in functionally redundant families. The discussion of VIGS and multigene families is included below in the assessment of the *HSP90* gene family in disease resistance.

At present, VIGS has limitations. For example, it may not be a suitable approach for analysis of embryo and seedling characteristics or of genes expressed in fruit and seeds and, for reasons that we do not fully understand, is more effective in *N.benthamiana* and related species than in other hosts. A further limitation of VIGS is the need for large-scale facilities for growing many plants under physical containment. However, it may be possible to miniaturize the procedures so that seedlings are agro-inoculated as they germinate and the VIGS symptoms are assayed in seedlings. Such developments will reduce the

Fig. 4. PVX clones that compromise *Pto*-mediated resistance caused reduced levels of HSP90. (**A**) Sequence alignments. The amino acid sequence corresponding to the ORFs of the 8-240, 9-037-1 and 10-186hsp inserts showed the strongest match to an *HSP90* homologue from several organisms. The diagram illustrates the alignment of these sequences with a tomato *HSP90* (accession No. M96549) (see C) indicating the region and percentage of homology. The arrow corresponds to the RT–PCR-amplified region of *N.benthamiana HSP90* cDNA that generated the sequences shown in (C). (**B**) Western analysis of *N.benthamiana* extracts probed with HSP90-specific antibody. The extracts were taken from plants that had been inoculated 4 weeks previously with TRV constructs carrying the *HSP90*-specific inserts from the PVX clones 9-037-1 (lane 1) and 10-186hsp (lane 2) or from non-inoculated plants (lanes 3–5); a molecular weight marker was loaded on lane 6. Equal loading of the different lanes was confirmed by Ponceau staining of the western blot membrane (not shown). The samples were from *Pto* transgenic (lanes 1–4) or non-transgenic (lane 5) *N.benthamiana*. (**C**) Sequence alignments. The nucleotide sequences of *HSP90* from *N.benthamiana* and *A.thaliana* were aligned using the program Clustal W (Thompson *et al.*, 1994). The aligned sequences correspond to a conserved region in the 3′ end of the coding sequence from the seven different *HSP90*s of *A.thaliana*: four cytosolic type *Athsp90-1*, (AB025606), *Athsp90-2* (AB011476), *AtHsp90-3* (AB011476), *AtHsp90-4* (AB011476); mitochondrial *AtHsp90-5* (AC007167); chloroplastic *AtHsp90-6* (AC009176); and ER-localized *AtHsp90-7* (AL078637) and the extended cDNAs of the *N.benthamiana* 8-240 (2280 nucleotides, accession No. AY310776), 9-037-1 (1893 nucleotides, accession No. AY310781) and 10-186hsp (full length coding sequence of 2301 nucleotides, accession No. AY310789). An additional *N.benthamiana* sequence (xy8) was amplified from a *N.benthamiana* cDNA library with gene-specific primers designed from a conserved region in the cytoplasmic Hsp90 sequences. Numbers on the left side of the sequence correspond to the starting position of the sequence used for the alignment for each gene. (**D**) Dendrogram derived from the sequence alignment described in (C) generated with the program ClustalW (Thompson *et al.*, 1994). The brackets indicate the groups of cytoplasmic (upper) and organellar (lower) forms of *HSP90* (Milioni and Hatzopoulos, 1997; Krishna and Gloor, 2001).
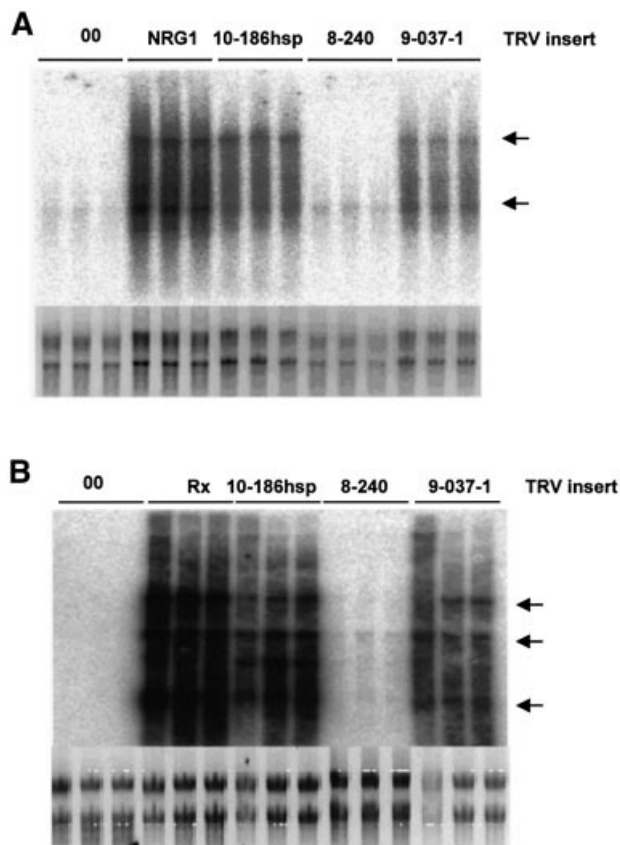
Fig. 5. VIGS of *HSP90*-compromised *N*- and *Rx*-mediated disease resistance. (**A**) *N*-mediated resistance. VIGS was established on *N* transgenic *N.benthamiana* (line 310A) using TRV vectors carrying the inserts from the indicated PVX clones (Peart *et al.*, 2002a). TRV:NRG is a positive control; the insert corresponds to a gene required for *N*-mediated disease resistance that was identified in a separate VIGS screen and will be described in detail elsewhere (J.Peart and D.C.Baulcombe, in preparation). Four weeks post-TRV inoculation, these plants were challenged with TMV:GFP. Accumulation of the TMV:GFP was monitored after a further 7 days by northern analysis. The northern analysis is shown for three independent total RNA preparations for each VIGS construct probed with a GFP-specific probe. The lower panel shows an rRNA loading control. The arrows indicate the major species of viral genomic and subgenomic RNA. (**B**) *Rx*-mediated resistance. VIGS was established on *Rx* transgenic *N.benthamiana* (line Rx18) (Bendahmane *et al.*, 1999) using TRV vectors carrying the inserts from the indicated PVX clones or with an *Rx*-specific insert (Peart *et al.*, 2002a). Four weeks post-TRV inoculation, these plants were challenged with PVX:GFP. Accumulation of the PVX:GFP was monitored after a further 5 days by northern analysis. The northern analysis is shown for three independent total RNA preparations for each VIGS construct probed with a PVX coat protein-specific probe. The lower panel shows an rRNA loading control.

time course, scale and expense of forward screens with VIGS. The use of vectors adapted for other hosts may also extend the applicability of VIGS forward screens (Holzberg *et al.*, 2002; Liu *et al.*, 2002b; Turnage *et al.*, 2002).

A challenge for forward screens of gene function based on VIGS or other silencing approaches will be to ensure complete genome coverage. In the screen described here with 4992 cDNAs, if we conservatively assume 50% redundancy in the library and a genome size equivalent to a typical 25 000 genes, we would have covered ~10% of the transcriptome in our survey. To extend this coverage, we can simply test more clones. However, at some point,
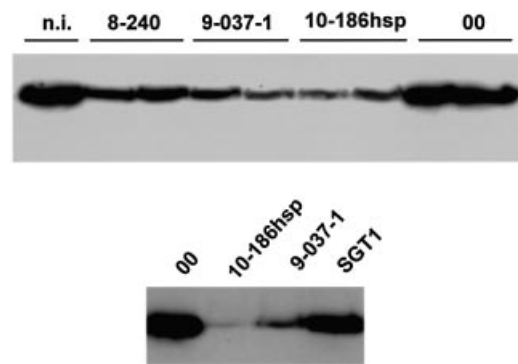

Fig. 6. VIGS of HSP90 caused reduced accumulation of Rx-4HA. Rx-4HA-2 *N.benthamiana* were infected with TRV vectors that were either empty (TRV:00), with an *SGT1* insert (Peart *et al.*, 2002b) or with *HSP90* inserts from the PVX clones 9-037-1, 10-186hsp and 8-240, as indicated. Protein samples were taken at 18 days post-inoculation from the upper leaves. The samples were processed for western blotting and probed with anti-HA antibody. The two panels represent separate experiments. Equal loading was confirmed by Coomassie blue staining of the same samples (not shown). n.i. = non-infected leaf sample.

there would be a diminishing return and it would be necessary to target known expressed sequence tags (ESTs).

The role of the HR in disease resistance

Previous analyses of disease resistance in plants have concluded that the HR is secondary and not necessary for the suppression of the pathogen. For example, in *Rx*-mediated resistance against PVX, the HR was only induced if expression of the elicitor coat protein was uncoupled from virus replication (Bendahmane *et al.*, 1999). It was concluded that the HR is a secondary mechanism that is activated only when the, as yet unidentified, primary mechanism failed to arrest the pathogen completely. The analyses of other resistance systems have come to the same conclusion. These systems involve the *dnd1* mutant in *Arabidopsis* (Yu *et al.*, 1998), the *Sw-5*-mediated resistance against Tospoviruses (Brommonschenkel *et al.*, 2000), *Rsv1*-mediated virus resistance in soybean (Hajimorad and Hill, 2001) and resistance against cauliflower mosaic virus (Cole *et al.*, 2001) in *Nicotiana* species.

If the HR is a secondary feature of disease resistance, it would be expected, as we found, that VIGS of some targets would not affect the mechanisms leading to suppression of the pathogen but would interfere with the secondary cell death response. However, there was an unexpected high proportion of genes (73/79) with an HR-specific VIGS phenotype and no effect on pathogen growth. An explanation for this finding could be related to the number of components in the signal transduction pathways. If the part of the pathway affecting the growth of the pathogen involves fewer proteins than the HR branch, there would be correspondingly fewer VIGS targets. Alternatively, the HR part of the pathway may be affected by diverse VIGS targets that, perhaps including those that affect the general metabolic status of the cell, are not directly involved in disease resistance.

However, whatever the explanation, the discrepant results with the HR and disease resistance assays emphasize how these are very different tests. The HR

test involves expression of the elicitor at a high level from the 35S promoter, whereas in the pathogen it would normally accumulate only at very low levels. The lesson for future VIGS screens is that direct tests of disease resistance are more useful than assays based on an HR.

The role of HSP90 proteins in disease resistance

HSP90 proteins facilitate protein folding, and it could be that NBS-LRR proteins or other components of the disease resistance pathways are dependent on HSP90 for correct folding, as in other systems (Nathan *et al*., 1997). The misfolded proteins could be either degraded or not biologically active. We cannot rule out this explanation. However, there are indications that HSP90 may be more specifically involved in disease resistance than is implied by a general role in protein folding. For example, there are mutations in *HSP90* genes of *Arabidopsis* resulting in specific loss of disease resistance that do not lead to severe morphological phenotypes. One *HSP90* mutation affects *RPS2* (Takahashi *et al*., 2003), whereas another affects *RPM1* (J.Dangl, personal communication). The findings that HSP90 interacts directly with proteins involved in disease resistance including SGT1 (Takahashi *et al*., 2003; J.Dangl, personal communication) are also an indication that this chaperonin has a specific role in disease resistance.

The VIGS phenotype of *HSP90 is* similar to that of *SGT1* in that there is partial or complete loss of many disease resistance responses including those associated with Rx, N and Pto (Figures 3 and 6) (Peart *et al*., 2002b). Silencings of *SGT1* and *HSP90* were also similar in causing loss of non-host resistance against *P.syringae* pv. *maculicola* and in having no effect on non-host resistance against *Xanthomonas campestris* pv *campestris* (I.Malcuit, unpublished data). These similarities may be significant because there are structural motifs that are common to SGT1 and HSP90-associated proteins known as co-chaperones. One co-chaperone of HSP90, p23, has sequence characteristics of the CS domain of SGT1 (Garcia-Ranea *et al*., 2002) that binds directly to the RAR1 disease resistance signalling protein. Other HSP90 co-chaperones including Hop have a tandem arrangement of three degenerate 34 amino acid repeats referred to as the tetratricopeptide repeats (TPRs) that are also present in Sgt1 (Azevedo *et al*., 2002). TPR domains bind specifically to the conserved C-terminal motif MEEVD of HSP90 (Chen *et al*., 1998; Young *et al*., 1998).

A simple prediction, by extrapolation from these results, is that HSP90 would bind directly to SGT1 via the TPR domain. The bound SGT1 would then serve as a co-chaperone that could influence downstream signalling proteins through interactions of the CS domain. However, from the Rx-4HA accumulation data (Figure 6), we infer that HSP90 cannot only act in association with SGT1. HSP90 silencing results in reduced levels of Rx-4HA, whereas SGT1 silencing has no effect. Thus, there must also be an HSP90 effect at a level that is SGT1 independent and that affects the stability of Rx. This process may influence stability of other similar proteins because an *HSP90* mutation in *Arabidopsis* causes destabilization of a disease resistance protein that, like Rx, has a nucleotide-binding site and leucine-rich repeat (J.Dangl, personal communication).

Does VIGS silence one or more members of the HSP90 multigene family?

VIGS can be effective with as few as 28 nucleotides of sequence identity between the insert in the virus vector and the target RNA (Thomas *et al*., 2001). It is therefore quite likely that the 9-037-1 and 10-186hsp inserts from the highly conserved coding sequence region would have silenced all *HSP90* mRNAs. Consistent with this expectation, the western analysis showed almost complete absence of HSP90 in the tissues infected with the PVX vectors carrying these inserts (Figure 4B).

In contrast, the VIGS clones targeted at less divergent regions of the *HSP90* mRNA had either weak or no suppression of disease resistance. For example, the 8-240 clone that corresponds to the 3′ region coding sequence and the untranslated region had only small and inconsistent effects on HSP90 protein levels and weaker effects than 9-037-1 or 10-186 on the various types of disease resistance (Figures 3 and 5) or on Rx levels (Figure 6). Similarly, PVX constructs with inserts from the 3′-untranslated regions of *HSP90* mRNAs did not cause loss of disease resistance (9-295; Supplementary table 1, and R.Lu, unpublished information). One interpretation of these results is that there is functional redundancy in the *HSP90* gene family. However, we could not assess how efficiently these 3′-specific constructs had silenced the corresponding mRNA and formally we cannot rule out the possibility that there is functional differentiation of HSP90 isoforms.

HSP90 VIGS affected plant growth (Figure 1B) as well as disease resistance. The growth effects correlated with the effects on disease resistance in that 9-037-1 and 10-186hsp had more pronounced effects than 8-240. From this result, it could be concluded that HSP90 is involved in similar signalling pathways affecting normal plant growth and disease resistance. However, it could also be that HSP90 in plants, as in other eukaryotes, is required to chaperone correct folding of a subset of essential proteins (Nathan *et al*., 1997). Alternatively, as suggested recently, it could be that HSP90 is required to buffer cells against the epigenetic effects of misfolded proteins (Queitsch *et al*., 2002). If that is the case, the growth phenotypes observed here (Figure 1D) could reflect the damage caused by the accumulation of misfolded proteins.

Materials and methods

Plant material

The plants were either non-transgenic *N.benthamiana* or transgenic derivatives carrying *Rx* (line Rx18) and *N* (line 310A) and *Pto* (lines 38-12) (Rommens *et al*., 1995; Bendahmane *et al*., 1999; Peart *et al*., 2002a) as indicated in the text. The line Rx4HA-2 carrying an *Rx-4HA* construct was assembled by transformation with a modified version of pB1-RxHA (Bendahmane *et al*., 1999). The modification was the addition of three HA epitope tags (data not shown) to the 5′ end of the *Rx* opening reading frame in pB1-RxHA (Bendahmane *et al*., 1999). The resulting construct was used for *Agrobacterium*-mediated transformation of *N.benthamiana* and stable transformants were selected on kanamycin. The experiments shown were carried out in plants of line Rx4HA-2 that are homozygous for the *Rx-4HA* transgene and in which the PVX resistance was as strong as in lines transformed with wild-type Rx (P.Moffett unpublished data).

Viral constructs, virus inoculation and cDNA library construction

The TRV vector construct for agroinoculation (pTV00) and inoculation procedures have been described previously (Ratcliff *et al*., 2001). The

PVX vector construct (pgR106) is a derivative of previously described clones (Chapman *et al.*, 1992) (GenBank accession No. AY297843). A similar vector with *Cla*I, *Sma*I and *Sal*I sites for insertion of foreign sequence is also available (pgR107: GenBank accession No. AY297842). Inoculation procedures for the TRV vector were as described (Ratcliff *et al.*, 2001). With PVX constructs, we used a toothpick that was touched to an *Agrobacterium* colony and then used to pierce the leaf or petiole of a 2–3 cm high seedling. Systemic PVX infection developed after 5–10 days presumably because wounded cells were transformed with the Ti plasmid and were a source of infection for the rest of the plant.

Construction of the cDNA library in the PVX vector is described below. The *PRF* insert in PVX:PRF and TRV:PRF was generated by PCR with primers Prf2a (5′-GTTGGCATGCCAGGATTGGGC-3′) and Prf1s (5′-ACAAGGCTTAAG ATAGTGTGG-3′); the PCR products correspond to nucleotides 3361–4035 in the ORF of the tomato *Prf* gene (Peart *et al.*, 2002a) (accession No. AF479624).

Total RNA for cDNA library construction was treated with RNase-free DNase (Promega), and recovered by ethanol precipitation. Reverse transcription used standard procedures, and cDNA normalization has been described previously (Ko, 1990; Kohchi *et al.*, 1995). To facilitate cDNA amplification and subsequent cloning, a 'lone' linker, which is an annealing product of two primers AscI A (5′-GAGATATTA-GGCGCGCCTACTC) and AscI B (5′-GAGTAGGCGCGCCTAATAT), was added to both ends of all random cDNAs. Three rounds of normalization were performed consecutively. The normalized cDNAs were digested with *Asc*I and *Not*I, and cDNA fragments of 400 bp to 1 kb were purified, ligated into the PVX vector construct pGR106 and transformed into *Agrobacterium* GV3101 competent cells. To assess the efficiency of normalization, an aliquot of the ligation reaction was transformed into *Escherichia coli* and the transformants were assayed by colony hybridization with probes specific for *rubisco*, *PDS* and *Prf*.

PCR of HSP 90 sequences
Hsp90 cDNA sequences from *N.benthamiana* were amplified using the SMART™ cDNA RACE amplification kit (BD Bioscience-Clontech, Palo Alto, CA) with *HSP90* gene-specific primer X (5′-GATATTTACTA-CATTACTGGTGAGAGCAAGAAGGC-3′) and the library primer AP1. Nested PCR was performed with an *HSP*-specific nested primer Y (5′-TCTAGCATGGCTGGATACATGTCTAGCAAGA-3′) and nested library primer AP2 according to the manufacturer's instructions (BD Bioscience-Clontech). Both X and Y primers were designed from a region in the *HSP90* sequence that is conserved in *N.benthamiana*, tomato and *Arabidopsis* cytosolic *Hsp90* sequences.

Monitoring of HR and bacterial and viral disease on plants
The HR test was carried out by infiltration of *N.benthamiana* upper leaves with a mixture of *Agrobacterium* cultures carrying *35S:Pto* and *35S:AvrPto* transgenes (Scofield *et al.*, 1996). *Pseudomonas syringae* pv *tabaci* (*Pst*) used for *Pto*-mediated resistance assay was *Pst* 11528 which is virulent on resistant tomato cultivars expressing *Pto* on *Pto*-transgenic *N.benthamiana* plants (Rommens *et al.*, 1995). *Pst* 11528 (AvrPto) is a AvrPto-expressing *Pst* transconjugant strain which is avirulent on resistant tomato cultivars expressing *Pto* and on *Pto*-transgenic *N.benthamiana* plants (Rommens *et al.*, 1995). All *Pst* strains were grown in L medium only supplemented with 25 µg/ml kanamycin to maintain the transconjugation vector pDSK519 and 100 µg/ml rifampicin for the selection of *Pst* strains. Bacterial suspensions were inoculated onto leaves using a needleless syringe. Inoculation of diluted inocula [10 000-fold dilution from original cell suspension (OD$_{600}$ ± 1.0)] was used for disease symptom and bacterial growth assays. To measure *in planta* growth of *Pst* strains, three replicate samples, each containing leaf discs from three plants, were collected from inoculated leaves. Leaf discs were ground in 10 mM MgCl$_2$, serially diluted and plated onto L medium containing kanamycin (25 µg/ml) and rifampicin (100 µg/ml). Colonies were counted after 24 h growth at 28°C.

PVX:GFP and TMV:GFP derivatives were inoculated by infiltration or toothpick of *Agrobacterium* strains carrying the viral cDNA in Ti plasmid vectors, and RNA analysis was as described previously (Peart *et al.*, 2002a). The probe used to detect TMV is the full-length GFP sequence. The full-length PVX coat protein sequence was used to make a probe for detection of PVX:GFP in the resistance assay test.

Protein analysis
For the analysis of the HSP90 protein, leaf samples were extracted in 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM dithiothreitol (DTT), 1% Triton X-100, 1.5% polyvinylpyrrolidone supplemented with protease inhibitors (protease inhibitor cocktail for plant and tissue extracts, Sigma). A 50 µg aliquot of total protein was loaded onto a 6% SDS–polyacrylamide gel and, after electrophoresis, the gels were electroblotted onto PVDF membrane. Immunological reactions were carried out with a primary antibody HSP90α (aE-17), (Santa Cruz Biotechnology Inc., Santa Cruz, CA) which is an affinity-purified goat polyclonal antibody against a conserved peptide mapping near the N-terminus of HSP90α of *Arabidopsis thaliana*. For analysis of Rx-4HA levels, the western blots were prepared and probed with anti-HA antibody (3F10, Roche) as described (Bendahmane *et al.*, 2002).

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

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Note added in proof

A recent paper [Kanzaki,H., Saitoh,H., Ito,A., Fujisawa,S., Kamoun,S., Katou,S., Yoshioka,H. and Terauchi,R. (2003) Cytosolic HSP90 and HSP70 are essential components of IFN1-mediated hypersensitive response and non-host resistance to *pseudomonas cichorii* in *Nicotiana benthamiana. Mol. Plant Pathol.*, **1**, 383–391] also describes the use of VIGS to reveal a role of HSP90 in disease resistance.