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Chapter 5

RSI2 interacts with, and is required for function and stability of tomato resistance protein I-2

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To be submitted

Race-specific disease resistance in plants depends on the presence of Resistance (R) genes. Most R genes encode NB-ARC-LRR proteins carrying a C-terminal Leucine-Rich Repeat (LRR). Few proteins have been identified that interact with this LRR domain, most of which have proposed (co)chaperone activity. Here, we report the identification of RSI2 (Required for Stability of I-2) as an interacting protein of the LRR domain of the tomato R protein I-2. RSI2 belongs to the family of small heat shock proteins (sHSPs). The interaction was found in a yeast two-hybrid screen and confirmed in pull-down assays. VIGS experiments revealed that RSI2 is required for the Hypersensitive Response (HR) induced by autoactive variants of I-2 and Mi-1, a second tomato R protein. Since many sHSPs have chaperone properties, involvement of RSI2 and other R protein (co)chaperones in I-2 and Mi-1 protein stability was examined. RSI2 silencing compromised the accumulation of full-length I-2 accumulation *in planta*, but did not affect Mi-1 levels. HSP90 and SGT1 silencing led to an almost complete loss of full-length I-2 accumulation and a reduction in Mi-1 protein levels. In contrast to RSI2, silencing SGT1 or HSP90 led to an accumulation of I-2 breakdown products. This difference suggests a different molecular mechanism by which RSI2 and HSP90/SGT1 chaperone I-2 protein stability. These data show that R protein function requires RSI2, for stabilizing either the R protein itself or other signalling components involved in initiation of HR.

Introduction

Resistance (R) proteins in plants mediate recognition of specific pathogen-derived factors called Avirulence (Avr) proteins. Upon Avr perception, R proteins initiate defence responses that limit further pathogen ingress. These responses often result in macroscopically visible cell death, referred to as the Hypersensitive Response (HR). The majority of R proteins are NB-ARC-LRR proteins, as they contain a central Nucleotide-Binding and -hydrolysing domain (NB-ARC) and a C-terminal Leucine-Rich Repeat (LRR) domain (Martin et al., 2003; van Ooijen et al., 2007). The LRR forms a potential protein-protein interaction surface and the LRRs of the R proteins RPM1, N, I-2 and Rx have been shown to physically interact with Heat Shock Protein 90 (HSP90) (Hubert et al., 2003; Lu et al., 2003; Liu et al., 2004; de la Fuente van Bentem et al., 2005). HSP90 is a highly conserved molecular chaperone responsible for the stability and function of a large number of proteins (Pearl and Prodromou, 2006). HSP90 consists of an N-terminal domain (NTD) containing an ATP binding and hydrolysis pocket, a central client binding domain, and a C-terminal dimerization domain. The activity of HSP90 is regulated by interactions with a number of co-chaperones that bind HSP90 and in some cases also their clients (Pearl and Prodromou, 2006). For example, the co-chaperone Protein Phosphatase 5 (PP5) interacts both with the C-terminus of HSP90, and the LRR domain of HSP90 client I-2 (de la Fuente van Bentem et al., 2005). Another HSP90-interacting co-chaperone is SGT1 (for suppressor of G2 allele of Skp1). SGT1 consists of three conserved regions: an N-terminal TPR domain, a central HSP90 interacting CS (CHORD-SGT1) domain, and a C-terminal SGT1-specific domain (Azevedo et al., 2002). Several studies have demonstrated physical interactions between HSP90, SGT1, and a third partner; RAR1 (for required for Mla12 resistance) (Hubert et al., 2003; Takahashi et al., 2003; Liu et al., 2004; Azevedo et al., 2006; Botër et al., 2007). RAR1 is composed of two zinc binding domains, CHORD I and CHORD II (Shirasu et al., 1999; Heise et al., 2007). Generally, the combined activities of RAR1, SGT1, and HSP90 are required for R protein stability and accumulation, and thereby for R protein-mediated signalling responses (Azevedo et al., 2006; Botër et al., 2007).

Another class of proteins with chaperone-associated functions are small Heat Shock Proteins (sHSPs), ranging in size from 12-43 kDa, also referred to as the HSP20 family. HSP20s are of variable sequence but are characterised by a conserved region of ~90 amino-acids forming an α-crystallin domain (Caspers et al., 1995). These proteins form large oligomers and perform their ATP independent chaperone function *in vitro* by binding to denatured proteins (Lee et al., 1995; Helm et al., 1997; Kirschner et al., 2000). *In vivo*, sHSPs are believed to confer a protective function by preventing unfolding or disassembly of other proteins (Van Montfort et al., 2001). The only HSP20 linked to disease resistance until now is tobacco HSP17 (Maimbo et al.,

2007). Expression of this gene is induced upon a range of stress treatments. In *hsp17*-silenced *N. benthamiana* plants, expression of defence-related marker genes was compromised, and disease symptoms triggered by *Ralstonia solanacearum* were enhanced, linking HSP17 to disease resistance. However, induction of HR by inoculation with pathogens or by *in planta* expression of the HR-inducing elicitor INF1 was unaffected in these silenced lines (Maimbo et al., 2007). It remains to be investigated whether this HSP17 is involved in R protein-mediated resistance.
Here, we describe identification of RSI2 (Required for Stability of I-2), an HSP20 member that specifically interacts with the tomato I-2 R protein. I-2 confers resistance to *Fusarium oxysporum* (Simons et al., 1998). Besides analysing its physical interaction, we used a virus-induced gene silencing (VIGS) approach to analyse the functional involvement of RSI2 and other known (co)chaperones in HR mediated by autoactive variants of I-2 and a second tomato R protein; Mi-1. Mi-1 belongs to a different subgroup of NB-ARC-LRR proteins (van Ooijen et al., 2007) and confers resistance to root-knot nematode (*Meloidogyne* spec.), potato top aphid and whitefly (Vos et al., 1998). Furthermore, we analysed the effect of silencing RSI2 and other (co)chaperones on I-2 and Mi-1 protein abundance and stability *in planta.*

Results

RSI2 Interacts with the I-2 LRR domain in the yeast two-hybrid system

We reported before the identification of both RSI2 (originally referred to as HSP17) and PP5 as interactors of I-2 bait LRR1-29 in a yeast two-hybrid screen (de la Fuente van Bentem et al., 2005). PP5 has indeed been found using this bait, but RSI2 was identified using a different bait; I-2 LRR12-29, corresponding to amino acid residues 823-1250 (LRR annotation described by (de la Fuente van Bentem et al., 2005)). Screening 6 x 10^6 clones of a tomato cDNA interaction library with bait LRR12-29 revealed two interacting clones. The two cDNA clones carried 733 and 742 basepair inserts (AY150040). The inserts are overlapping and differ only in the length of the 5' regions. Both cDNAs encode a full-length small heat shock protein (sHSP) with a predicted mass of 17.8 kDa. To pinpoint the region of the I-2 protein responsible for the interaction with RSI2, different N- and C-terminal truncations of the I-2 protein were analysed for their interaction in the yeast two-hybrid assay (Figure 1A). Interactions were analysed by the ability to grow on selective plates lacking auxotrophy markers histidine (-HWL) or adenine (-AWL) (Figure 1A). The minimal RSI2-interacting region of the I-2 LRR domain lies within LRRs 15-19, corresponding to amino acids 906-1015 (Figure 1A) Notably, the full-length I-2 protein and the full-length LRR domain (LRR1-29) are expressed (de la Fuente van Bentem et al., 2005), but did not interact with RSI2 in this assay system.

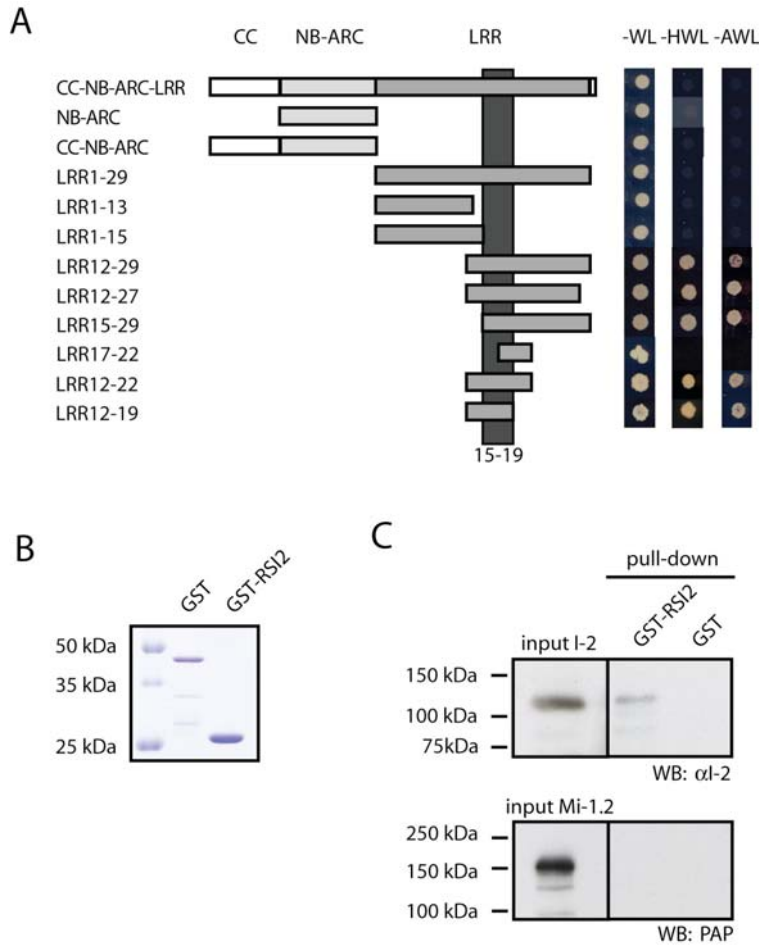

Figure 1

RSI2 interacts with I-2. A) Yeast two-hybrids show interactions between RSI2 and various I-2 baits. The presence of bath and prey plasmids is confirmed by growth on -WL plates whereas the interaction between bait and prey proteins is analyzed on -HWL and –AWL selective plates. The dark-grey area highlights the I-2 region required for RSI2-interaction. B) Coomassie-stained SDS-PAGE gel showing purified GST-RSI2 and GST proteins produced in *Escherichia coli*. C) Western blot (WB) on total protein lysates made from *N. benthamiana leaves* transiently expressing I-2, probed with I-2 antibody (upper panel), or TAP-Mi-1 (lower panel) probed with the PAP antibody. Presence of full-length R proteins in these extracts is shown in the input lanes. GST-RSI2 and GST proteins immobilized on glutathione sepharose beads were incubated with these extracts. Interacting proteins were subjected to SDS-PAGE and Western blot analysis to detect the presence of I-2 or TAP-Mi-1 (right panels).

GST-RSI2 fusion protein interacts with I-2 from plant protein extracts

To verify the interaction identified in the yeast two-hybrid assay between RSI2 and I-2, we next investigated whether RSI2 and I-2 also interact *in planta*. Because the I-2 antibody is not suitable for immunoprecipitations (data not shown), and equipping I-2 with a tag abolishes its activity (van Ooijen et al., 2008), we performed pull-down

experiments of non-tagged I-2 with *Escherichia coli*-produced GST-RSI2 and GST alone. The two GST bait proteins were affinity purified and subsequently analysed on a coomassie-stained SDS-PAGE gel. As shown in Figure 1B, the proteins migrated according to their expected molecular weights (44 and 26 kDa), and little contamination of co-purifying protein was observed.

Endogenous I-2 is expressed at very low levels in tomato and undetectable in total protein leaf extracts using our affinity purified I-2 antibody (Tameling et al., 2002; van Ooijen et al., 2008). To boost expression, we produced full-length I-2 by transient transformation of *Nicotiana benthamiana* leaves using agroinfiltration. The *N. benthamiana*-produced I-2 (Figure 1C) could readily be detected using the I-2 antibody. Total protein extracts of *N. benthamiana* plants transformed with I-2 were incubated with beads loaded with GST or GST-RSI2. A fraction of the I-2 pool was consistently co-purified with the GST-RSI2 sample, but not with the control sample containing GST alone (Figure 1C). The I-2 protein is stable during the purification procedure under the conditions employed (data not shown). The specific co-precipitation of I-2 with GST-RSI2 confirms the interaction as described above.

Interaction of GST-RSI2 with the R protein Mi-1 was also analysed. A TAP-tagged version of Mi-1 was used, as the Mi-1 antibody cross-reacts with the GST-tag (van Ooijen et al., 2008). The TAP tag does not appear to affect Mi-1 protein function, since the TAP-tagged autoactive mutant Mi-1^{H840A} (van Ooijen et al., 2008) can still induce HR similar to the non-tagged protein (Supplementary Figure 1). After agroinfiltration, the band corresponding to TAP-Mi-1 can be readily detected on western blot using a TAP antibody (Figure 1C). Although TAP-Mi-1 protein is stable throughout the pull-down procedure (data not shown), it did not co-precipitate with the GST-RSI2 fusion protein under the conditions used. Together, these results indicate that RSI2 and I-2 are present in one complex whereas no interaction between RSI2 and Mi-1 was found.

VIGS of RSI2 reveals a role in HR mediated by I-2 and Mi-1

R protein function depends on the activities of a number of chaperones or chaperone-associated proteins (de la Fuente van Bentem et al., 2005; Botër et al., 2007). Here we employed VIGS of RSI2 to analyse whether RSI2 is required for R protein function. For comparison SGT1, RAR1, PP5 and HSP90 were included in this assay. VIGS was induced using the tobacco rattle virus (TRV) silencing system, which was delivered by agroinfiltration of the viral vector constructs into the leaflets of two-week old *N. benthamiana* plants (Ratcliff et al., 2001). To analyse onset and spread of silencing in time, phytoene desaturase (PDS) was used as marker (Demmig-Adams and Adams, 1992). The nearly complete photobleaching, consistently observed three weeks after agroinfiltration, indicates extensive PDS

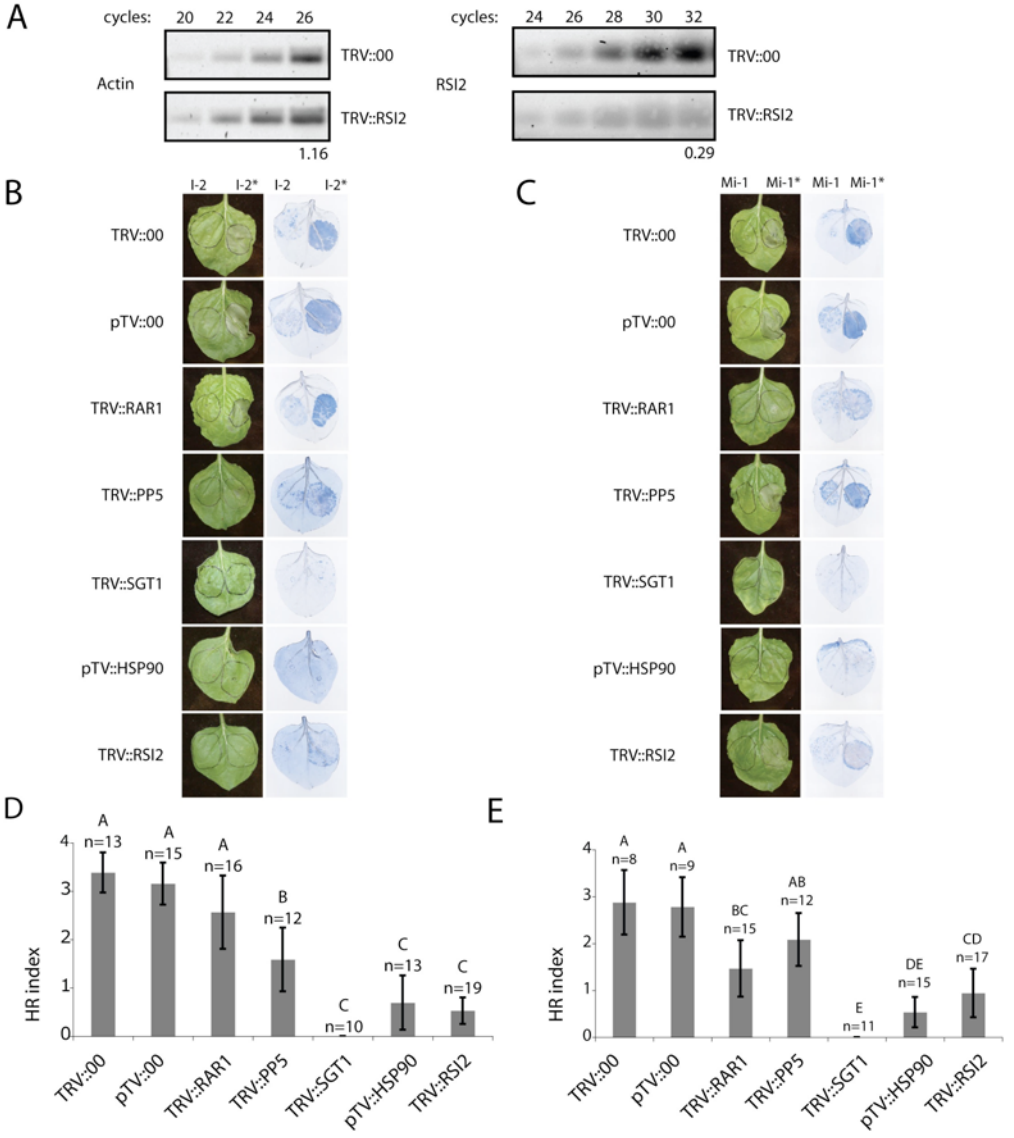

Figure 2

VIGS reveals a role for RSI2 in I-2 and Mi-1-mediated HR signaling. A) Silencing efficiency of RSI2 determined using semi-quantitative RT-PCR (right panel). Actin expression levels were measured as a control for equal cDNA quantity and quality (left panel). Number of PCR cycles are indicated at the top. The relative signal intensity is indicated below (TRV::00 sample indexed at 1). B+C) Agroinfiltration of wild-type (left side of the leaf) or constitutively active mutants (*, right side of the leaf) of I-2 (B) and Mi-1 (C) in *N. benthamiana* three weeks after induction of silencing using the indicated TRV vectors. Pictures were taken 3 days after agroinfiltration and representative leaves were stained using trypan blue to visualize cell death (right panels). D+E) Bar-representation of the severity of HR upon I-2^{D495V} (D) or Mi-1^{D841V} (E) expression in silenced plants. HR was quantified visually on a scale from 0 (no symptoms) to 4 (full necrosis). Significantly different classes can de discerned by a one-way Anova ($P<0.05$). Error bars represent a 95% confidence level.

silencing throughout these plants at this time-point (Supplementary Figure 2). To assess *RSI2* silencing levels, specific primers were designed on the closest *N. benthamiana* homolog available in the TIGR database (GenBank entry DQ275464). Although in the sequenced region, this *N. benthamiana* gene is 80% identical to *RSI2*, it is not predicted to be a silencing target (Xu et al., 2006). However, the relative expression level of this *RSI2* homolog(s) was found to be reduced to 29% in these leaves (Figure 2A). To assess I-2 function in the silenced plants, the upper fully stretched leaves were agroinfiltrated with constructs expressing wild-type I-2 (left side of the leaves) and the constitutively active I-2^{D495V} mutant (right side of the leaves) (Figure 2B). The level of cell death induced by I-2^{D495V} was scored three days after agroinfiltration, on a scale ranging from 0 (absolutely no tissue collapse) to 4 (fully developed HR in the total infiltrated region) as indicated in Supplementary Figure 3. To enhance visibility of HR, the infiltrated leaves were stained for cell death using trypan blue (Figure 2B). Leaves of plants infected with the empty virus control showed clear HR upon expression of I-2^{D495V}, but not upon expression of wild-type I-2 (Figure 2B). This result shows that induction of HR by I-2^{D495V} is not compromised by infection with TRV. In contrast, no (or only minor) tissue collapse induced by I-2^{D495V} was consistently observed in the RSI2 silenced plants (0.5±0.3) compared to the control plants (3.4±0.4), indicating that RSI2 is essential for full I-2 mediated HR. A one-way Anova showed that severity of HR symptoms on RSI2-silenced plants is reduced to similar low levels as on HSP90 and SGT1 silenced plants (Figure 2D). Silencing the established R protein (co)chaperones HSP90 or SGT1 severely suppressed HR triggered by I-2^{D495V}. Silencing of PP5 only partially compromises I-2^{D495} mediated HR, whereas RAR1 silencing did not affect HR significantly.

To analyse whether RSI2 is also involved in HR mediated by Mi-1, we analysed the induction of HR on silenced plants by the autoactivating mutant Mi-1^{D841V} (van Ooijen et al., 2008). In plants infected with the empty viruses, strong HR (2.9±0.7) was induced by this mutant (Figure 2C, right side of the leaves) and not by the wild-type Mi-1 protein (left side of the leaves). In plants silenced for RSI2, HR induced by Mi-1^{D841V} was consistently strongly compromised (Figure 2C and 2E) to 0.9±0.5. These results indicate that RSI2 is not only involved in HR mediated by I-2 but also by Mi-1. Like with I-2^{D495}, Mi-1^{D841V}-mediated HR induction is severely compromised upon SGT1 and HSP90 silencing. PP5 and RAR1 silencing also affect HR induction by Mi-1^{D841V}, although to a lesser extent. These data conclusively show that HR mediated by I-2 and Mi-1 is compromised upon RSI2 silencing.

Silencing RSI2 negatively affects I-2 protein accumulation

The suppressive effect SGT1 silencing has on *N* and *Rx* function is a result of compromised R protein stability due to reduced chaperone activity (Azevedo et al.,

2006; Mestre and Baulcombe, 2006; Botër et al., 2007). HSP20s are also associated with chaperone functions *in vitro* (Nover and Scharf, 1997; Kirschner et al., 2000). To analyse whether the observed effects on HR by silencing RSI2, SGT1, HSP90, PP5 or RAR1 can be attributed to reduced R protein stability, protein accumulation of I-2 and Mi-1 was analysed in the silenced plants. To exclude the possibility that silencing any of these genes adversely influences transgene expression via agroinfiltration *per se*, we agroinfiltrated a construct expressing TAP-tagged GUS protein. Expression levels of this control protein are not altered by silencing the indicated genes (Figure 3C), as shown by a western blot using an antibody against the TAP tag.

R protein accumulation in the silenced plants was assessed in *N. benthamiana* leaves agroinfiltrated with I-2 and Mi-1 constructs. Compared to the vector controls, silencing of RAR1 and PP5 was found to reduce I-2 protein accumulation (Figure 3A). No full-length I-2 protein could be detected in plants silenced for SGT1 and HSP90. However, an accumulation of smaller proteins that cross-react with the I-2

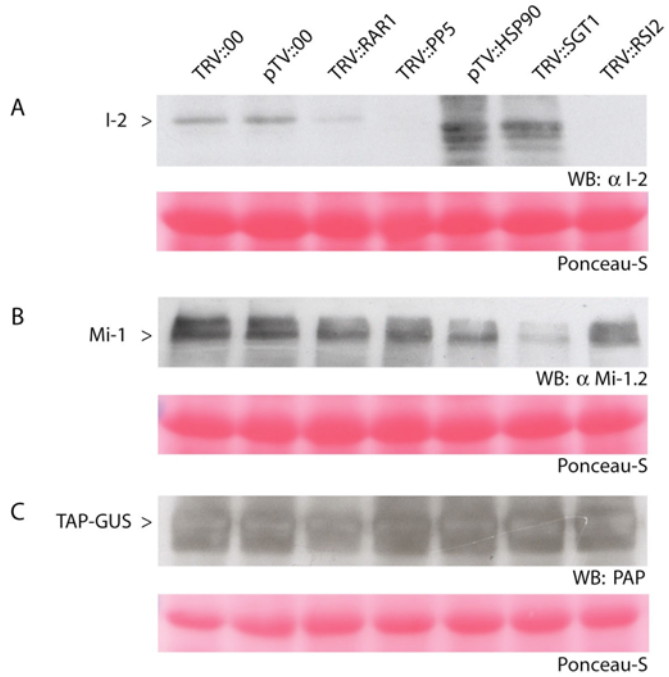

Figure 3

Expression levels of I-2, Mi-1 and GUS protein in silenced *Nicotiana benthamiana* leaves. *N. benthamiana* plants were silenced using the indicated TRV-based silencing construct. Three weeks after induction of silencing, the upper leaves were agroinfiltrated with constructs expressing either I-2, Mi-1 or TAP-tagged GUS. One day after agroinfiltration, leaves were harvested and protein was extracted. 50 µg (I-2 and Mi-1) or 10 µg (TAP-GUS) of total protein was loaded on SDS-page gels. Expression of I-2 (A) Mi-1 (B) or TAP-GUS (C) was analysed on Western blots with the antibody indicated. Equal loading of these blots was confirmed by Ponceau-S staining of Rubisco (lower panels).

antibody was found, suggestive for I-2 derived degradation products. Silencing of RSI2 also led to loss of full-length I-2 accumulation. However, this elimination was not associated with the accumulation of lower molecular weight products.Mi-1 protein abundances in RAR1- or PP5-silenced plants was similar to those observed in the empty virus controls (Figure 3B). Silencing SGT1 led to a clear reduction of Mi-1 protein abundance, whereas HSP90 silencing only slightly affected Mi-1 protein accumulation. In neither case, an increase in lower molecular weight products cross-reacting with the Mi-1 antibody was detected. Hence, although RSI2 is necessary for HR induction by autoactive Mi-1, silencing of RSI2 does not directly affect accumulation of the Mi-1 protein.

To conclude, our data indicate that HSP90 and SGT1 are required for maintaining full-length I-2 and Mi-1 integrity and stability. The functional involvement of RSI2 in I-2 mediated HR is linked to I-2 protein stabilisation, whereas no effect on Mi-1 stability was observed.

Discussion

RSI2 interacts with the I-2 disease resistance protein

In this study we describe the identification of RSI2 as component of the I-2 multiprotein complex. It is unclear whether the RSI2/I-2 interaction is direct, or requires a bridging protein that is functionally conserved in yeast. The RSI2 interacting region was mapped to LRR15-19 of I-2 (Figure 1). This patch differs from that required for the interaction with HSP90 (LRR1-11), but overlaps with the PP5 interacting region (LRR12-22) (de la Fuente van Bentem et al., 2005) suggesting that both proteins might compete for binding. LRR1-29 and full-length I-2 contain the RSI2 interaction patch, however no interaction was observed with these baits in the yeast two-hybrid assay. Likewise a PP5/I-2 interaction in yeast was also found only with truncated versions of I-2 and not with the full-length protein (de la Fuente van Bentem et al., 2005). Possibly folding of extended LRR domains in yeast shields the interaction surface.

The RSI2/I-2 interaction was confirmed in pull-down experiments (Figure 1). Crucial for this experiment was the ability to detect expression of full-length I-2 protein in homogenates of agroinfiltrated *N. benthamiana* leaves. Previous attempts to express I-2 protein using agroinfiltration in a greenhouse or growth chamber did not yield detectable amounts of protein (van Ooijen et al., 2008). Possibly the different conditions used here (lower temperature, light and humidity) enhance the efficiency of *Agrobacterium*-mediated transformations as also reported by (Fu et al., 2006). The enhanced expression under these conditions does not seem to be gene-specific as

enhanced expression was also found with other transgenes tested, such as Mi-1, GUS, PP5 and HSP90 (data not shown).
We do not observe an interaction between RSI2 and Mi-1 in pull-down assays (Figure 1), and accumulation of Mi-1 expression *in planta* was not affected upon RSI2 silencing (Figure 3). However, Mi-1-mediated HR was compromised by silencing RSI2 (Figure 2). Possibly, RSI2 is required for the function of a protein downstream of Mi-1. A possible candidate is the NB-ARC-LRR protein NRC1, that is involved in signalling mediated by many resistance proteins including Mi-1 (Gabriëls et al., 2007). Further studies could reveal whether NRC1 is indeed an RSI2 client.

Silencing experiments reveal a role for RSI2 in HR signalling
RSI2 is, like SGT1, HSP90 and PP5, required for HR triggered by autoactivating mutants of I-2 and Mi-1 (Figure 2). The relative expression of the closest *N. benthamiana* RSI2 homolog is reduced to 29% upon VIGS using the tomato *RSI2* sequence. The presence of other, possibly even closer related, unknown *RSI2* genes in the *N. benthamiana* genome can not be excluded, and silencing might therefore also target other RSI2 homologs. Nevertheless, silencing using the heterologous tomato RSI2 sequence is apparently sufficient to suppress I-2 and Mi-1 mediated HR signalling.
That silencing of SGT1, HSP90 or RAR1 reduces I-2 mediated HR is in line with an earlier study (de la Fuente van Bentem et al., 2005). In that study PP5 was shown to interact with the I-2 LRR domain, however, no effect on I-2 mediated HR was observed upon VIGS of PP5 (de la Fuente van Bentem et al., 2005). Here, we find a relatively small but statistically significant effect on I-2 mediated HR. The difference between these studies might be due to the enhanced efficiency of the *Agrobacterium*-mediated transformation and hence silencing levels (Fu et al., 2006). Support for enhanced silencing efficiency is the more extensive bleaching observed upon PDS silencing in our study as compared to (de la Fuente van Bentem et al., 2005).
We found that HR mediated by a constitutively active variant of Mi-1 is dependent on SGT1 and HSP90, consistent with the involvement of these genes in Mi-1 mediated resistance towards whitefly in tomato (Bhattarai *et al.*, 2007). RAR1 was found not to be involved in Mi-1 mediated resistance to whitefly (Bhattarai *et al.*, 2007), whereas we show that this protein is required for full HR induction by Mi-1. This difference might suggest that HR is not involved or not required for whitefly resistance. The observation that PP5 silencing reduces Mi-1^{D841V}-mediated HR is consistent with the reported yeast two-hybrid interaction between the two (de la Fuente van Bentem et al., 2005).

Chaperones involved in R protein stability

We describe that silencing of RSI2 severely reduces I-2 protein accumulation *in planta* (Figure 3), pointing out a role for RSI2 in chaperoning I-2. Silencing of HSP90 and SGT1 also leads to a reduction in the abundance of full-length I-2 and Mi-1. The interaction between SGT1 and HSP90 has been shown to be necessary for SGT1 to fulfil its functions in resistance mediated by the R protein Rx (Botër et al., 2007). Interestingly, SGT1 is also linked to protein degradation by the ubiquitin/26S proteasome pathway, as it is important for the function of several SCF (for SKP1/CULLIN1/F-box protein) complexes (Kitagawa et al., 1999; Azevedo et al., 2002; Gray et al., 2003). Since SGT1 is also involved in maintaining protein stability, it is thought to be an important regulator determining the fate of a client protein. Silencing SGT1 or interaction partner HSP90 might thus destabilise the full-length I-2 protein by reduced chaperoning activity, but at the same time, the cell might not be able to degrade the resulting unstable, incorrectly folded I-2 products because the link to the proteasome is broken by SGT1 silencing. This link could explain the accumulation of degradation products that cross-react with the I-2 antibody (Figure 3). Upon RSI2 silencing this accumulation is not observed, indicating that this protein is involved in stabilizing I-2, but not in its elimination by the 26S proteasome.

HSP20s have been shown to prevent aggregation of client proteins in an HSP90 and ATP independent manner, RSI2 might function in a similar fashion. Alternatively, RSI2 might function in conjunction with HSP90. HSP20s are characterised by a HSP20/α-crystalline fold, forming a beta-sandwich of two parallel β-sheets. The crystal structure of wheat HSP20 (van Montfort et al., 2001) reveals that this fold closely resembles that of the CS domain (Finn et al., 2006), as found in SGT1 and in p23, where it is required for their binding to HSP90 (Boter et al., 2007). P23 regulates human HSP90 activity by a direct interaction with the ATP-bound active form of HSP90 (Johnson et al., 1994). *In vitro*, p23 has been shown to interact with partially folded proteins and to prevent these from aggregation (Bose et al., 1996). The related fold of HSP20s and p23, together with the experimentally verified chaperoning activities of both, could indicate a similar biochemical function as chaperones and co-regulators of HSP90 activity. The absence of the I-2 breakdown products in RSI2 silenced plants (that was observed in SGT1 and HSP90 silenced plants), might indicate that RSI2 is mainly involved in the chaperoning functions (possibly together with the HSP90/SGT1 machinery) and is not involved (or redundant) in the process of targeted protein degradation via SGT1. Efforts to test this hypothesis and show an interaction between RSI2 and HSP90 in yeast two-hybrid where not successful (results not shown). Likewise, we where unable to establish the presence of a ternary complex of I-2, RSI2 and HSP90 in our RSI2 pull-downs (Figure 2) after probing the blot with an HSP90 antibody (data not shown).

Future studies should reveal whether RSI2 functions as a p23-like regulator of the HSP90/SGT1 machinery, or that it performs it chaperone functions independent of HSP90.

To summarize, we report an HSP20 that engages in the I-2 protein complex and positively contributes towards a full HR mediated by autoactive I-2 and Mi-1 mutants. We observe a significant effect of RSI2, SGT1 and HSP90 silencing on I-2 protein accumulation, showing that these proteins are required for I-2 accumulation. Mi-1 protein abundance was also reduced upon silencing of HSP90 and SGT1 but not by RSI2. Possibly another RSI2 client protein required for Mi-1 mediated HR is affected in these plants. These results add RSI2 to the list of chaperones that are required for R protein function.

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Materials and methods

Yeast two-hybrid

The PJ69-4a host strain was grown on Minimal Medium (MM: 2% glucose, 0.17% yeast nitrogen base, 0,5% $(NH_4)_2SO_4$) supplemented with 0.002% Uracil, 0.004% D/L-Methionine, 0.01% L-Leucine (L), 0.002% L-Tryptophane (W), 0.002% Adeninesulfate (A) and 0.002% L-Histidine (H). PJ69-4a harbors three reporter genes that can be transcriptionally activated upon reconstitution of the Gal4 transcription factor. These genes are the *E. coli LacZ* gene, whose expression can be detected by X-gal staining of the yeast colony, and the yeast *ADE2* and *HIS3* genes, which can be selected for by adenine and histidine prototrophy, respectively. If selection for an auxotrophy marker was desired, one or more of the components was omitted from the medium (MM-W, L, A, H). PJ69-4a was transformed with plasmids that contain bait (pAS2-1) and prey (pACT2) constructs (Clontech). Tryptophane and leucine prototrophic transformants were selected on MM-WL plates. Droplets of a cell dilution corresponding to 10^4 cells were spotted on MM-WL, MM-HWL and MM-AWL plates. The I-2 baits used in this study have been described before (de la Fuente van Bentem *et al.*, 2005).

***Agrobacterium*-mediated transformation and silencing**

Agrobacterium tumefaciens strain GV3101(pMP90) (Koncz and Schell, 1986) was transformed with the indicated vectors and grown to OD_{600}=0.8 in LB-Mannitol (10 g/l tryptone, 5 g/l yeast extract, 2.5 g/l NaCl, 10 g/l mannitol) medium supplemented with 20 µM acetosyringone and 10 mM MES pH 5.6. Cells were pelleted and resuspended in infiltration medium (1x MS salts, 10 mM MES pH 5.6, 2% w/v sucrose, 200 µM acetosyringone) and infiltrated at OD_{600}=1. Importantly, all *Agrobacterium* infiltrations were performed in a laboratory (no direct sunlight, 20º C) rather than a growthchamber or a greenhouse. Two week-old *N. benthamiana* leaves were used for infiltration with TRV-based silencing constructs. Subsequently, the plants were kept in the laboratory for 3 days and then transferred to the greenhouse to allow spread of the TRV virus. *Agrobacterium*-mediated expression of the R proteins was performed in the laboratory 2.5 weeks after silencing. Trypan blue staining was performed as described before (van Ooijen *et al.*, 2008).
RSI2 silencing was performed with the tobacco rattle virus (TRV) system by cloning an *Eco*RI/*Xho*I fragment from the cDNA pACTII library clone (de la Fuente van Bentem *et al.*, 2005) into pYL156 (Liu *et al.*, 2002). An *Eco*RI/*Sal*I fragment from the tomato PP5 TPR domain pAS2-1 bait vector (de la Fuente van Bentem *et al.*, 2005) was ligated into pYL156 digested with *Eco*RI/*Xho*I. An *Bam*HI/*Sal*I fragment from the SGT1 silencing vector used by (Peart *et al.*, 2002) is ligated into pYL156 digested with *Bam*HI/*Xho*I. RAR1 was silenced using the silencing vector described by (Liu *et al.*, 2002). HSP90 silencing is performed using the TRV silencing vector described by (Ratcliff *et al.*, 2001) and the RNA2 clone used in (de la Fuente van Bentem *et al.*, 2005). Silencing was performed with the corresponding RNA1 construct (Ratcliff et al., 2001; Liu et al., 2002). Empty RNA2 vectors were used as negative controls.

RT-PCR

RNA was extracted with Trizol. cDNA was amplified from 1 ug RNA using SupersrciptIII (invitrogen, according to manufacterer). RSI2 fragments were amplified using the primer set (ctgaagcacatgtgtttaaggcc and cttgacatcaggcttcttcac). Agarose gel was stained for 12 hrs using SYBR Green (Invitrogen) and scanned using a STORM phosphoimager (Amersham Bioscience). The signal intensities were measured using ImageQuant and corrected for background levels.

Binary vector construction

The binary vectors for wild-type I-2, I-2 D495V, wild-type Mi-1 and Mi-1 D841V have been described by (van Ooijen *et al.*, 2008). The TAP tagged wild-type Mi-1 construct was generated by PCR on pSE23 (Gabriëls *et al.*, 2007) using (aaaaagcaggctctatggaaaaacgaaaagatatt) and agaaagctgggttcttaaataaggggatattcttctg). Gateway *att*B sequences were added by adapter PCR using the primer set (ggggacaagtttgtacaaaaaagcaggct) and (ggggaccactttgtacaagaaagctgggt). The PCR products were transferred to binary vector CTAPi (Rohila *et al.*, 2004) by the Gateway one-tube protocol for cloning *att*B-PCR products directly into destination vectors (Invitrogen). To obtain a TAP-tagged autoactive mutant, a *Bsp*119I/*Bcu*I fragment was exchanged between the non-tagged Mi-1 H840A construct described in (van Ooijen *et al.*, 2008) and the wild-type clone. This removes the stop codon in the original mutant construct and allows a translational fusion to the tag. The TAP-GUS control was generated by a Gateway LR reaction from the GUS control plasmid included in the LR kit (Invitrogen) to the binary vector NTAPi (Rohila *et al.*, 2004). Clones were sequenced to verify the correct insert sequence.

One-way Anova

For the statistical analysis of HR induction by autoactive R protein mutants on silenced plants, HR was visually scored three (I-2) or four (Mi-1) days after agroinfiltration. Data sets from three independent silencing experiments on 5 to 8 plants per silenced gene were scored, adding up to 30 (SGT1) to 47 (RSI2) independent scores. On these data, a one-way Anova was performed with StatView using a significance interval of 95%. Error bars in charts represent a 95% confidence level calculated using Microsoft Excel.

Protein extraction and Western blotting

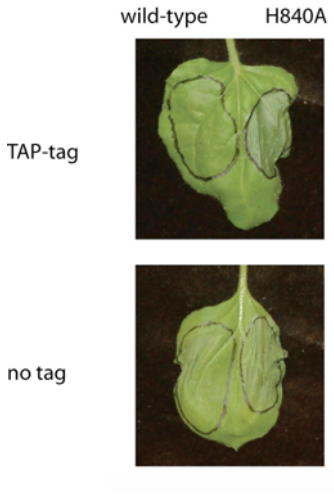
For the analysis of R protein expression level in silenced plants, 3 leaves from 3 independent silenced plants (9 total) were harvested and pooled 24 hours after agroinfiltration with GUS, I-2 or Mi-1 and frozen in liquid nitrogen. After grinding the tissue, it was allowed to thaw in 1 ml protein extraction buffer per 500 mg of tissue (25 mM Tris pH 8, 1 mM EDTA, 150 mM NaCl, 5 mM DTT, 0,1% NP-40, 1 x Roche Complete protease inhibitor cocktail and 2% poly-(vinyl-poly-pyrolidone) (PVPP)). Extracts were cleared by centrifugation at 12,000 *g* for 10 minutes at 4º C and the supernatant was passed over 4 layers of Miracloth. Protein concentrations were measured using a Bradford assay (Bradford, 1976) and standardised with extraction buffer without PVPP. The extract was mixed with Laemmli sample buffer. 50µg of total plant protein was separated on 8% SDS-PAGE gels and blotted on PVDF membranes using the semi-dry blotting system TE77 (GE Healthcare). Equal loading was verified by staining the membrane using Ponceau S. 5% skimmed milk powder was used as a blocking agent. As a primary antibody, a 1:4000 dilution of the I-2 or Mi-1 antibody (van Ooijen *et al.*, 2008) was used and as a secondary antibody a 1:4000 dilution of goat-anti-rabbit linked to horseradish peroxidase. For TAP-GUS detection, a 1:10,000 dilution was used of the PAP antibody (Sigma). Luminescence was visualised with ECL Plus (GE healthcare) and BioMax MR film (Kodak).

GST-RSI2 production and pull-down

E. coli strain BL21 (DE3) was transformed with empty pGEX-KG and with pGEX-KG containing full-length RSI2. The latter was generated by cloning the full-length RSI2 sequence from the pACTII interaction clone into pGEX-KG digested with *Nco*I/*Xho*I. 5 ml LB overnight culture supplemented with 50 µg/ml carbenicillin was diluted 10 times. After 2 hours of incubation at 37º C, protein expression was induced by incubation at room-temperature for 5 hours in the presence of 1.5 mM IPTG. Pelleted cells were frozen at -20º C and thawed by resuspending in 10 ml ice-cold PBS pH 7.4, supplemented with 1x protein inhibitor cocktail (Roche). Lysozyme was added to a final concentration of 1mg/ml and the suspension was rotated gently at 4º C for 30 min. After adding Triton X-100 (0.5%) the mixture was rotated for an additional 30 minutes at 4º C. The cell mixture was sonicated on ice 2 times for 2 minutes. Cell debris was removed by two centrifugation steps of 20 minutes at 18000 *g* at 4º C. The supernatant was 1x diluted in PBS pH 7.4 and aliquots were frozen. To 1 ml of extract, 200 µl 50% GST beads slurry (GE Healthcare) was added and the capture was performed rotating at 4º C for 1.5 hour. Pelleted beads were washed 4 times with 0.5 ml ice-cold PBS pH 7.4 supplemented with Roche Complete protease inhibitors. I-2 or Mi-1-TAP protein was extracted from *N. benthamiana* tissue in which I-2 or Mi-1-TAP was expressed by *Agrobacterium* transformation. 10 mg of total protein lysate was supplemented with an additional 0.1% NP40 to obtain the interaction buffer and added to 10 µg of immobilised GST-RSI2 or GST protein. The final interaction buffer conditions were 25 mM Tris pH 8, 1 mM EDTA, 150 mM NaCl, 5 mM DTT, 0.2% NP-40, 1 x Roche Complete protease inhibitor cocktail. This mixture was incubated overnight at 4º C to allow the interaction to take place. The beads were pelleted and washed for 5 times with interaction buffer. Proteins were eluted by addition of Laemmli sample buffer to the pelleted beads and samples were run on an 8% SDS-PAGE gel. Blotting and detection procedures as described above.

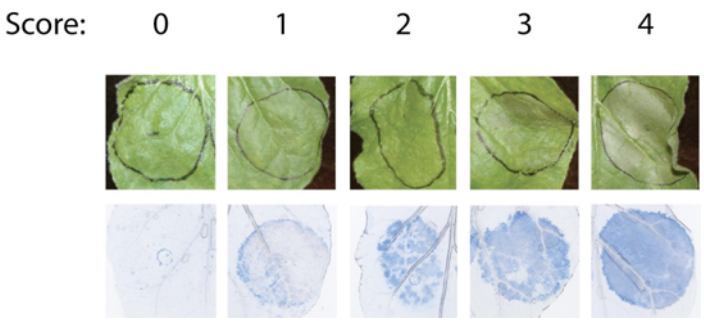
The sequence of RSI2 was deposited in genbank under accession number AY150040.

Supplementary data



Supplementary Figure 1
C-terminally TAP tagged Mi-1 H840 induces HR. Agroinfiltrated *N. benthamiana* leaves expressing wild-type Mi-1 or constitutively active mutant Mi-1 H840A, either C-terminally TAP-tagged (top leaf) or non-tagged (bottom leaf).

Supplementary Figure 2
Nicotiana benthamiana plants were silenced for phytoene desaturase to visualise the efficiency of silencing. Due to a disturbed carotenoid biosynthesis, the plants become susceptible to photobleaching. Pictures were taken three weeks after agroinfiltration with TRV::PDS.



Supplementary Figure 3
The visual range of HR intensity scores. HR initiated by agroinfiltration of the autoactive mutants I-2^{D495V} and Mi-1^{D841V} on silenced plants was scored on a visual range from 0 to 4. Representative leafs of all 5 classes are depicted (top panels) and cell death is visualised by Trypan blue staining (lower panels). 0: no visible cell death, 1: first symptoms (a grey shade) of tissue collapse in the infiltrated region on the lower side of the leaf, but no symptoms on the top side of the leaf, 2: clearly developing tissue collapse on the lower side of the leaf and first symptoms of HR on the upper side of the leaf, 3: clear HR visible on the upper side of the leaf, but not in the total infiltrated zone, 4: total infiltrated region shows cell death.

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