Chaperones of the endoplasmic reticulum are required for Ve1-mediated resistance to *Verticillium*

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SUMMARY

The tomato receptor-like protein (RLP) Ve1 mediates resistance to the vascular fungal pathogen *Verticillium dahliae*. To identify the proteins required for Ve1 function, we transiently expressed and immunopurified functional Ve1-enhanced green fluorescent protein (eGFP) from *Nicotiana benthamiana* leaves, followed by mass spectrometry. This resulted in the identification of peptides originating from the endoplasmic reticulum (ER)-resident chaperones HSP70 binding proteins (BiPs) and a lectin-type calreticulin (CRT). Knock-down of the different BiPs and CRTs in tomato resulted in compromised Ve1-mediated resistance to *V. dahliae* in most cases, showing that these chaperones play an important role in Ve1 functionality. Recently, it has been shown that one particular CRT is required for the biogenesis of the RLP-type *Cladosporium fulvum* resistance protein Cf-4 of tomato, as silencing of *CRT3a* resulted in a reduced pool of complex glycosylated Cf-4 protein. In contrast, knock-down of the various CRTs in *N. benthamiana* or *N. tabacum* did not result in reduced accumulation of mature complex glycosylated Ve1 protein. Together, this study shows that the BiP and CRT ER chaperones differentially contribute to Cf-4- and Ve1-mediated immunity.

INTRODUCTION

Recognition of microbial patterns or damage-associated host molecules by immune receptors results in the activation of immune responses in plants (Boller and Felix, 2009; Jones and Dangl, 2006; Thomma *et al.*, 2011). These immune receptors are localized either in the cytoplasm or at the cell surface (Beck *et al.*, 2012; Jones and Dangl, 2006; Monaghan and Zipfel, 2012). Examples of well-studied cell surface receptors involved in pathogen recognition include the Arabidopsis (*Arabidopsis thaliana*) receptor-like

kinases (RLKs) Flagellin Sensing-2 (FLS2) and the elongation factor-Tu (EF-Tu) receptor (EFR), which are responsible for the recognition of bacterial flagellin and EF-Tu, respectively (Gómez-Gómez and Boller, 2000; Zipfel *et al.*, 2006). Another important class of plant cell surface receptors is the receptor-like proteins (RLPs). Similar to most RLKs, most RLPs carry extracellular leucine-rich repeats (LRRs), but they lack a cytoplasmic kinase domain (Wang *et al.*, 2010). Examples of RLPs that play a role in immunity are the tomato (*Solanum lycopersicum*) Cf proteins (Rivas and Thomas, 2005; Stergiopoulos and de Wit, 2009), Ve1 (Fradin *et al.*, 2009), LeEix2 (Ron and Avni, 2004), Arabidopsis (*Arabidopsis thaliana*) AtRLP30 (Wang *et al.*, 2008), apple (*Malus domestica*) HcrVf2 (Belfanti *et al.*, 2004) and rapeseed (*Brassica napus*) LepR3 (Larkan *et al.*, 2013).

The biogenesis of functional transmembrane receptors requires correct folding and glycosylation. In addition, the transport of non-mature receptors to the plasma membrane should be prevented, as they may be incompetent in ligand binding and subsequent signalling. To ensure that all post-translational modifications required for the maturation of transmembrane receptors take place correctly, endoplasmic reticulum (ER)-resident chaperones regulate ER quality control (ER-QC) (Anelli and Sitia, 2008; Eichmann and Schäfer, 2012; Saijo, 2010). For example, the ER-resident HSP70 binding proteins (BiPs) interact with the HSP40-like stromal-derived factor-2 (SDF2) and J-domain-containing proteins (ERdj3) (Jin *et al.*, 2008; Nekrasov *et al.*, 2009; Schott *et al.*, 2010). The major function of these chaperones in ER-QC is to prevent the accumulation of unfolded proteins by assisting in protein folding and initiating ER stress signalling when unfolded proteins start to accumulate (Eichmann and Schäfer, 2012; Liu and Howell, 2010). Another ER-QC pathway involves N-linked glycosylation and consists of lectin chaperone-assisted folding by the lectin-type calreticulin (CRT) and calnexin (CNX) chaperones. In the ER, a Glc3Man9GlcNAc2 oligosaccharide is first added to asparagine (Asn) (N) residues of nascent glycoproteins (Pattison and Amtmann, 2009). The subsequent action of glucosidases renders the glycoproteins accessible for CRT/CNX-assisted folding (Anelli and Sitia, 2008). A third ER-QC pathway is

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supported by protein disulphide isomerases and involves the formation of disulphide bridges between free thiol groups present in the client protein (Anelli and Sitia, 2008; Gruber *et al.*, 2006). Eventually, only correctly folded transmembrane proteins are transported to the Golgi apparatus for further maturation and transport to the plasma membrane. Immature and incorrectly folded proteins either re-enter the ER-QC cycle or are degraded by the ER degradation machinery (Anelli and Sitia, 2008; Nakatsukasa and Brodsky, 2008). Consequently, a number of Arabidopsis mutants in ER chaperone components are immunocompromised for bacterial infections. *sdf2* mutants, for example, show reduced immunity mediated by EFR (Nekrasov *et al.*, 2009). Similarly, Arabidopsis mutants in the folding sensor UDP Glc glycoprotein glucosyltransferase (UGGT), the ERD2b HDEL receptor and CRT3 are compromised in EFR-mediated defence responses (Christensen *et al.*, 2010; Li *et al.*, 2009; Nekrasov *et al.*, 2009; Saijo *et al.*, 2009).

The tomato Ve1 receptor is an LRR-RLP that mediates resistance to the fungal vascular wilt pathogens *Verticillium dahliae* and *V. albo-atrum* (Fradin and Thomma, 2006; Fradin *et al.*, 2009; Kawchuk *et al.*, 2001). Ve1 provides resistance to race 1 strains of these pathogens on perception of the secreted Ave1 effector protein (de Jonge *et al.*, 2012; Zhang *et al.*, 2013a). Another group of defence-associated LRR-RLPs is the tomato Cf proteins, involved in resistance to the fungal biotrophic leaf pathogen *Cladosporium fulvum* (Rivas and Thomas, 2005). Recently, it has been discovered that Cf proteins interact with ER-QC chaperones that are required for correct Cf function (Liebrand *et al.*, 2012). Furthermore, it was found that both tomato and *Nicotiana benthamiana* contain four BiP homologues and three CRT-like chaperones, and that Cf-4 especially depends on CRT3a for its biogenesis (Liebrand *et al.*, 2012). CRT3a is an isoform of the plant-specific CRT3 class (Christensen *et al.*, 2010), and silencing of *CRT3a* in *N. benthamiana* results in a reduced pool of mature complex glycosylated Cf-4 protein, whereas the total Cf-4 protein pool is not affected. Silencing of individual BiPs has no effect on Cf-4 function, and silencing of multiple BiPs leads to lethality in *N. benthamiana* (Liebrand *et al.*, 2012).

In this study, we investigated the role of the BiP and CRT chaperones in Ve1-mediated immunity. We immunopurified a functional Ve1-enhanced green fluorescent protein (eGFP) fusion protein from *N. benthamiana* and found that both the CRT- and BiP-type ER chaperones interact with Ve1. Virus-induced gene silencing (VIGS) of the genes encoding these ER-QC chaperones in tomato resulted in reduced Ve1-mediated resistance in most cases. Unexpectedly, we did not detect a reduction in complex glycosylation of Ve1 or a suppression of the Ve1-mediated hypersensitive response (HR) on knock-down of the different CRTs in *N. tabacum* or *N. benthamiana*. Together, our study shows that ER-QC chaperones play an important role in Ve1-mediated immunity. However, clear differences exist between the require-

ment and importance of the various ER-QC chaperones in the maturation of the Cf-4 and Ve1 proteins.

RESULTS

Identification of ER-resident chaperones as Ve1-interacting proteins

Recently, a functional Ve1-eGFP fusion protein has been generated (de Jonge *et al.*, 2012; Zhang *et al.*, 2013a), and we transiently expressed this fusion protein by *Agrobacterium tumefaciens* infiltration in *N. benthamiana* leaves. Ve1-eGFP was subsequently immunopurified using GFP affinity beads, and a tryptic on-bead digestion of the total immunoprecipitate was performed, after which the generated peptides were analysed by mass spectrometry to reveal co-purifying proteins (Liebrand *et al.*, 2012). Two peptides were found to match *N. benthamiana* (Nb) CRT2 (one of the three NbCRTs) and 32 peptides matched one or more of the four NbBiP homologues (Table S1, see Supporting Information). A list of additional co-purifying proteins other than the BiPs and CRTs is presented in Table S2 (Supporting Information). In an alternative approach, Ve1-eGFP was immunopurified from *N. benthamiana* after its transient expression, and the immunoprecipitate was run on sodium dodecylsulphate (SDS) gel and blotted. The blot was incubated with α BiP, detecting endogenous NbBiPs, revealing a clear band at the expected molecular weight of the four NbBiP proteins (Fig. 1A). Collectively, these results show that the NbCRTs and NbBiPs interact with Ve1 on transient expression in *N. benthamiana*.

Previously, C-terminally-tagged eGFP fusion proteins of the *S. lycopersicum* (Sl) CRTs have been generated (Liebrand *et al.*, 2012), and we investigated here whether Ve1 interacts with the three individual SlCRTs. Therefore, SlCRT2, SlCRT3a and SlCRT3b, all fused to eGFP, were transiently co-expressed with the fusion protein Ve1-haemagglutinin (Ve1-HA) by *Agrobacterium* infiltration in *N. benthamiana*. CRT immunoprecipitation using GFP affinity beads, followed by the detection of Ve1-HA using α HA antibody, revealed that Ve1 interacts with all three SlCRTs, as Ve1-HA is co-immunopurified in all cases (Fig. 1B).

Targeting of tomato BiPs by VIGS compromises Ve1-mediated immunity

To study whether the different BiPs play a role in Ve1-mediated immunity, Tobacco rattle virus (TRV)-based VIGS was employed (Fradin *et al.*, 2009). Tomato cultivar Motelle plants, carrying the Ve1 resistance gene, were *Agrobacterium* inoculated with TRV constructs targeting the expression of the individual BiPs (TRV:Sl-BiP1, TRV:Sl-BiP2, TRV:Sl-BiP3 and TRV:Sl-BiP4) (Liebrand *et al.*, 2012). As a control, plants were inoculated with TRV:GFP. Two weeks after inoculation with the recombinant viruses, plants were

Fig. 1 HSP70 binding proteins (BiPs) of *Nicotiana benthamiana* co-purify with Ve1 and Ve1 co-purifies with tomato lectin-type calreticulins (CRTs). Ve1-enhanced green fluorescent protein (eGFP) was expressed in *N. benthamiana* (A) and Ve1-haemagglutinin (HA) was co-expressed with S/CRT2-eGFP, S/CRT3a-eGFP or S/CRT3b-eGFP in *N. benthamiana* (B). Total protein extracts of the agroinfiltrated leaf tissue were subjected to immunoprecipitation using GFP affinity beads. Total proteins (Input) and immunoprecipitated proteins (IP) were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted. Blots were incubated with α GFP antibody to detect immunoprecipitated Ve1-eGFP (A) or S/CRT-eGFP (B) fusion proteins. Duplicate blots were incubated with α BiP to detect co-immunoprecipitating BiPs (A) or with α HA antibody to detect co-immunoprecipitating Ve1-HA. The Coomassie-stained blot shows the 50-kDa Rubisco band present in the input to confirm equal loading. The experiment was performed twice with similar results and a representative example is shown.

either inoculated with a *V. dahliae* strain expressing *Ave1* or mock inoculated. Subsequently, plants were monitored for a period of 2 weeks for stunted growth, which is a typical disease symptom caused by *V. dahliae* infection. Targeting of *SIBiP1*–*SIBiP3* resulted in stunted growth of Motelle plants on infection with *V. dahliae*, whereas targeting of *SIBiP4* did not compromise Ve1 resistance (Fig. 2). As expected, TRV:GFP inoculation did not result in compromised growth of the plants on inoculation with *V. dahliae*, showing that these plants remained fully resistant to the fungus (Fig. 2). We determined the efficiency of *BiP* silencing by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with specific primers for the individual *BiPs* (Fig. S1A, see Supporting Information). We detected the knock-down of *BiP1* and *BiP2* in lines inoculated with TRV:SI-*BiP1* and TRV:SI-*BiP2*, respectively (Fig. S1A). However, no reduced expression of *SIBiP3* and *SIBiP4* was detected on inoculation with TRV constructs targeting these genes. This is most likely because expression levels of *SIBiP3* and *SIBiP4* are much lower than those of *SIBiP1* and *SIBiP2* (not shown).

Fig. 2 Targeting of individual HSP70 binding protein genes (*BiPs*) by virus-induced gene silencing (VIGS) results in compromised Ve1-mediated resistance to *Verticillium dahliae*. Tomato cultivar Motelle, carrying the *Ve1* resistance gene, was subjected to VIGS by inoculation with the indicated TRV:SI-*BiP* constructs. TRV:GFP served as a control. GFP, green fluorescent protein; TRV, *Tobacco etch virus*. Two weeks after inoculation with the recombinant viruses, plants were either mock inoculated (left plant in each panel) or inoculated with a race 1 strain of *V. dahliae* expressing *Ave1* (right plant in each panel). Photographs were taken at 10 days after *V. dahliae* inoculation. Compromised resistance is reflected by the stunted appearance of the *V. dahliae*-inoculated plants when compared with the mock-inoculated plants. Three independent experiments were performed and a representative example is shown.

Targeting the tomato CRTs by VIGS compromises Ve1-mediated immunity

To determine whether the CRTs are involved in Ve1-mediated immunity, Motelle plants were *Agrobacterium* inoculated with the constructs TRV:SI-CRT2, TRV:SI-CRT3a and TRV:SI-CRT3b (Liebrand *et al.*, 2012), targeting the expression of the different *SICRTs*, and TRV:GFP as a control. Three weeks after TRV inoculation, the plants were inoculated with *V. dahliae* expressing *Ave1* and monitored for stunted growth. Interestingly, targeting of the three individual CRTs in each case resulted in stunting of the plants on inoculation with *V. dahliae* (Fig. 3). This suggests that all three CRTs play a role in Ve1-mediated immunity to *V. dahliae*. Again, the TRV:GFP-inoculated plants remained fully resistant to the fungus (Fig. 3). Successful knock-down of the expression of the different *SICRTs* in the TRV:SI-CRT-inoculated plants was shown by qRT-PCR (Fig. S1B).

Fig. 3 Targeting of individual lectin-type calreticulin genes (*CRTs*) by virus-induced gene silencing (VIGS) results in compromised Ve1-mediated resistance to *Verticillium dahliae*. Tomato cultivar Motelle, carrying the *Ve1* resistance gene, was subjected to VIGS by inoculation with the indicated *Tobacco rattle virus* (TRV) constructs. TRV:*GFP* (GFP, green fluorescent protein) served as a control. Two weeks after inoculation with the recombinant viruses, plants were either mock inoculated (left plant in each panel) or inoculated with a race 1 strain of *V. dahliae* expressing Ave1 (right plant in each panel). Photographs were taken at 10 days after *V. dahliae* inoculation. Compromised resistance is reflected by the stunted appearance of the *V. dahliae*-inoculated plants when compared with the mock-inoculated plants. Three independent experiments were performed and a representative example is shown.

Targeting of the *CRTs* in *N. benthamiana* or *N. tabacum* does not lead to reduced Ve1 accumulation or compromised complex glycosylation of Ve1

Knock-down of *NbCRT3a* by VIGS in *N. benthamiana* compromises the Cf-4-mediated HR and is associated with a reduced accumulation of mature, complex *N*-linked glycosylated Cf-4 protein (Liebrand *et al.*, 2012). To reveal whether the *N*-linked complex glycosylation status of Ve1 changes on targeting of the expression of the different *CRTs*, we inoculated *N. benthamiana* and *N. tabacum* cultivar Samsun with the TRV constructs TRV:SI-*CRT2*, TRV:Nb-*CRT3a* and TRV:SI-*CRT3b* which target the different *N. benthamiana CRTs* (Liebrand *et al.*, 2012). In addition, TRV:*GUS* was used as a control. *Nicotiana tabacum* cultivar Samsun was included because Ve1-mediated recognition of the Ave1 effector results in an HR in this species, whereas this does not occur in *N. benthamiana* (de Jonge *et al.*, 2012; Zhang *et al.*, 2013a). Next, Ve1-eGFP was transiently expressed by *Agrobacterium* infiltration into the leaves of the TRV-inoculated plants. As a control, we also transiently expressed Cf-4-eGFP in the TRV-inoculated *N. benthamiana* plants. Two days later, eGFP-tagged proteins were immunoprecipitated using GFP affinity beads. The total amount of

purified Ve1-eGFP and Cf-4-eGFP proteins was subsequently determined by immunoblotting and detection of the fusion proteins with α GFP antibody, whereas the amounts of complex *N*-linked glycosylated Ve1-eGFP and Cf-4-eGFP were determined using an anti-horseradish peroxidase (HRP) antibody. This polyclonal antibody recognizes the HRP protein by binding to β (1,2)-xylose and α (1,3)-fucose residues that are added to the precursor glycan in the Golgi apparatus (Henquet *et al.*, 2008). Consequently, this antibody cross-reacts with other proteins that are also complex *N*-linked glycosylated (Henquet *et al.*, 2008; Liebrand *et al.*, 2012). Because complex *N*-linked glycosylation takes place in the Golgi, this antibody can be used as a tool to determine the amount of mature, complex glycosylated Ve1-eGFP that has reached the Golgi apparatus.

The blots incubated with α GFP revealed that similar amounts of Ve1-eGFP were purified from *N. benthamiana* and *N. tabacum* inoculated with the different TRV constructs that target the *CRTs*, or with TRV:*GUS* (Fig. 4). Replicate blots incubated with α HRP revealed that the pool of complex *N*-linked glycosylated Ve1-eGFP is equal, regardless of which TRV construct is used for the inoculation of the plants, both for *N. benthamiana* and *N. tabacum* (Fig. 4A,B). As expected, *N. benthamiana* plants inoculated with TRV:Nb-*CRT3a* showed a strongly reduced complex glycosylation on Cf-4-eGFP (Fig. 4B) (Liebrand *et al.*, 2012). To a lesser extent, this was also observed for plants inoculated with TRV:SI-*CRT3b* (Fig. 4B). These results show that the Ve1 protein is indeed complex *N*-linked glycosylated, but targeting of the individual *CRTs* does not reduce the amount of complex glycosylated Ve1 protein (Fig. 4A,B). This is in contrast with Cf-4, for which the amount of complex glycosylated protein is strongly reduced when *SlCRT3a* is targeted. Hence, the compromised resistance to *V. dahliae* on targeting of the different *CRTs* is probably not a result of reduced accumulation of complex mature *N*-linked glycosylated Ve1 protein.

Targeting the BiPs and *CRTs* in *N. tabacum* does not compromise the Ve1-mediated HR

To determine whether the knock-down of *CRTs* and *BiPs* compromises the Ve1-triggered HR, *N. tabacum* (cv. Samsun) (*Nt*) was inoculated with TRV:SI-*BiP1*, TRV:SI-*BiP2*, TRV:SI-*BiP3* and TRV:SI-*BiP4* targeting the different *N. benthamiana BiPs* (Liebrand *et al.*, 2012), as well as with TRV:SI-*CRT2*, TRV:Nb-*CRT3a* and TRV:SI-*CRT3b* targeting the *CRTs*. Although its genome has not yet been sequenced, we anticipated that, based on the very high genome sequence homology between *N. benthamiana* and *N. tabacum*, these constructs would also target the corresponding close homologues of *N. tabacum*. Three weeks post-viral inoculation, expanded leaf sections were *Agrobacterium* infiltrated to transiently co-express Ve1 and the matching effector Ave1. As controls, we included TRV:*GUS* and TRV:Nb-*SOBIR1*/Nb-*SOBIR1*-like.

TRV:*GUS* does not have a plant target, whereas TRV:*Nb-SOBIR1*/*Nb-SOBIR1-like* targets the homologues of the RLK *SOBIR1*, required for the Ve1-mediated HR in *N. tabacum* (Liebrand *et al.*, 2013).

Fig. 4 Ve1-enhanced green fluorescent protein (eGFP) contains complex *N*-linked glycans and virus-induced gene silencing (VIGS) of the different lectin-type calreticulin genes (*CRTs*) does not affect Ve1 glycosylation. Ve1-eGFP was transiently expressed in *Nicotiana tabacum* (cultivar Samsun) (A) or *N. benthamiana* (B), silenced for the different *CRTs*. As a control in (B), Cf-4-eGFP was also transiently expressed in *N. benthamiana* silenced for the different *CRTs*. The fusion proteins were subsequently immunopurified using GFP affinity beads, run on sodium dodecylsulphate (SDS) gel and blotted. Blots were incubated with α GFP antibody to reveal the total amount of immunopurified Ve1 and Cf-4 proteins, and duplicate blots were incubated with a horseradish peroxidase antibody (α HRP) to reveal the pool of mature complex glycosylated purified protein. The experiment was performed twice with similar results and a representative example is shown. TRV, *Tobacco rattle virus*.

Remarkably, targeting of the different *BiP* and *CRT* homologues in *N. tabacum* did not significantly compromise the Ve1-mediated HR (Fig. 5). As expected, inoculation with the TRV construct targeting *NtSOBIR1* resulted in a compromised Ve1-mediated HR (Fig. 5). These results are in contrast with the reduced Ve1-mediated resistance in tomato (Figs 2 and 3), but in agreement with the presence of *N*-linked glycosylated Ve1 receptor protein in the plants inoculated with the TRV:*CRT* constructs (Fig. 4).

DISCUSSION

Ve1 is a transmembrane RLP carrying extracellular LRRs which mediates resistance to race 1 strains of the vascular wilt fungi *V. dahliae* and *V. albo-atrum* (Fradin and Thomma, 2006; Fradin *et al.*, 2009; Kawchuk *et al.*, 2001). Hence, Ve1 requires post-translational modifications and ER-QC-assisted folding before being guided to the plasma membrane (Anelli and Sitia, 2008). Indeed, we observed that Ve1 is associated with complex *N*-linked glycans (Fig. 4). Furthermore, the protein interacts with BiP and CRT ER-QC chaperones (Fig. 1). Here, we have shown that targeting of *BiP1*, *BiP2* and *BiP3* genes by VIGS in tomato compromises Ve1-mediated immunity to *V. dahliae*, whereas targeting of *BiP4* does not affect resistance to the pathogen (Fig. 2). Targeting of the *CRT* chaperones by VIGS in tomato also compromises Ve1-mediated resistance to *V. dahliae* (Fig. 3), revealing that multiple BiPs and CRTs play a role in Ve1-mediated immunity. Remarkably, targeting of the *CRTs* in *N. tabacum* does not affect the Ve1-mediated HR (Fig. 5). This suggests that the Ve1-activated pathway triggering the HR is still intact, and that the HR may thus not be required for Ve1-mediated resistance against *Verticillium*. In support of this, it has been shown recently that Ve1-mediated resistance in Arabidopsis does not require the HR (Zhang *et al.*, 2013b).

Recently, it has been shown that tomato Cf proteins carry complex *N*-linked glycans and interact with various ER-QC chaperones (Liebrand *et al.*, 2012). For example, Cf-4 has been found to interact with BiP and CRTs in co-immunopurification assays. In addition, silencing of the *N. benthamiana* and tomato *CRT3a*

Fig. 5 Targeting of the individual HSP70 binding protein (*BiP*) and lectin-type calreticulin (*CRT*) genes in *Nicotiana tabacum* does not lead to a compromised Ve1-mediated hypersensitive response (HR). *Nicotiana tabacum* cv. Samsun was subjected to virus-induced gene silencing (VIGS) by inoculation with the indicated constructs. Three weeks later, Ve1 was transiently co-expressed with the matching ligand Ave1. Photographs of infiltrated spots were taken another 4 days later. TRV:*GUS* and TRV:Nb-*SOBIR1*/Nb-*SOBIR1-like* were included as controls. Three independent experiments were performed and representative examples are shown. TRV, Tobacco rattle virus.

homologues strongly compromises Cf-4-mediated immune responses (Liebrand *et al.*, 2012). In another study on Cf-4-mediated responses, it was found that *BiPs* are differentially regulated during mounting of the Cf-4/Avr4-triggered HR (Xu *et al.*, 2012).

Ve1 function does not seem to depend on one particular CRT. Indeed, targeting the three *SiCRTs* individually by VIGS resulted in a compromised Ve1-mediated resistance. This is in contrast with the findings for Cf-4, which specifically requires CRT3a for its functionality. This chaperone was found to be particularly required for the accumulation of complex, *N*-linked glycosylated Cf-4, but not for the stability of the total Cf-4 protein pool in *N. benthamiana* (Fig. 4) (Liebrand *et al.*, 2012). Interestingly, Ve1 is still complex glycosylated on targeting *CRT3a* and the Ve1 protein accumulates to similar levels as in control plants (Fig. 4). In addition, the Ve1-triggered HR in TRV:Nb-*CRT3a*-inoculated *N. tabacum* plants was still intact. Thus, a reduced accumulation of complex glycosylated Ve1 probably does not explain the compromised Ve1-mediated resistance.

Therefore, the question remains on how *CRT* silencing affects Ve1 function. One explanation could be that CRTs play a role in

Ve1-mediated immunity of tomato, which is independent of their role in *N*-linked glycosylation. Arabidopsis CRT2 has been shown to be involved in the salicylic acid-dependent expression of defence genes (Qiu *et al.*, 2012). Silencing of different *CRTs* may thus hamper an adequate response after ligand perception by Ve1. Alternatively, other downstream components involved in Ve1-mediated immunity may rely on CRT-mediated ER-QC. Indeed, several proteins required for Ve1 signalling have been identified, such as the RLKs *SOBIR1*, *SERK1* and *SERK3/BAK1* (Fradin *et al.*, 2009, 2011; Liebrand *et al.*, 2013), and targeting the *CRTs* may affect the folding and glycosylation of these RLKs. Thus, Ve1-mediated immunity to *V. dahliae* may be affected indirectly when the *CRTs* are targeted. These results reveal a differential requirement of CRTs in Ve1- and Cf-4-mediated immunity. Although Cf-4 relies strongly on *SiCRT3a* but not on *SiCRT2* or *SiCRT3b* (Liebrand *et al.*, 2012), Ve1 requires all three CRTs for the RLP to mediate immunity.

ER-QC chaperones play a role in the biogenesis and functionality of a number of additional plant transmembrane receptors. In *N. tabacum*, CRT2 and CRT3a have been shown to be required for the accumulation of the plasma membrane-localized induced

receptor kinase (IRK), which is involved in N-mediated resistance to *Tobacco mosaic virus* (Caplan *et al.*, 2009). In rice (*Oryza sativa*), OsBiP3 interacts with the plasma membrane-localized RLK Xa21 involved in resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*. In this case, overexpression of OsBiP3 results in enhanced resistance to the bacterium (Park *et al.*, 2010). In Arabidopsis, mutant forms of the brassinosteroid receptor BRI1 are retained in the ER by CRT3 (Hong *et al.*, 2008; Jin *et al.*, 2007, 2009). Furthermore, mutations in a number of ER-QC chaperones strongly compromise the functionality of the immune receptor EFR in Arabidopsis (Li *et al.*, 2009; Lu *et al.*, 2009; Nekrasov *et al.*, 2009; Saijo *et al.*, 2009). In particular, *crt3* mutants accumulate EFR to reduced amounts. In contrast, FLS2 accumulation and downstream responses are hardly affected in these ER-QC chaperone mutants. Perturbation of the N-linked glycosylation status of EFR and FLS2 reveals that underglycosylation strongly affects EFR function, whereas this is not the case for FLS2 (Häweker *et al.*, 2010; Saijo, 2010; Sun *et al.*, 2012). Interestingly, weakly defective *crt3* Arabidopsis mutants accumulate EFR to wild-type levels, but display compromised EFR-triggered responses, showing that abundance control and QC of the EFR receptor can be uncoupled (Saijo *et al.*, 2009). This may also explain why Ve1-mediated defence is reduced on silencing of the individual CRTs, whereas Ve1 protein accumulation remains unaltered. Together, our results show that Ve1-mediated immunity to *V. dahliae* depends on ER-QC-assisted folding mediated by the BiP and CRT families.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Nicotiana benthamiana, *N. tabacum* cultivar Samsun and tomato (*Solanum lycopersicum*) cultivar Motelle plants, carrying the *Ve1* gene, were grown in the glasshouse under 16 h of light at 21 °C and 8 h of darkness at 19 °C. Supplemental light of 100 W/m² was applied when the light intensity dropped below 150 W/m². The relative humidity was approximately 75%.

Binary vectors for *Agrobacterium* infiltrations and VIGS

The constructs pBIN-KS-Ve1-eGFP (pSOL2095Ve1::GFP) and pBIN-KS-Cf-4-eGFP have been described previously (Liebrand *et al.*, 2012; Zhang *et al.*, 2013a). pB7K40-Ve1-HA, p35S:Ve1 and pSOL1292-Ave1 have been described elsewhere (Fradin *et al.*, 2009; Fradin *et al.*, unpublished data; Zhang *et al.*, 2013a). Plasmids directing the expression of the *SICRT*-eGFP fusions and all TRV constructs for VIGS experiments have been described previously (Liebrand *et al.*, 2012; Zhang *et al.*, 2013a). All binary plasmids were transformed to *A. tumefaciens* strain C58C1, carrying helper plasmid pCH32, for *Agrobacterium* infiltration assays. *Agrobacterium* infiltrations were performed as described previously (van der Hoorn *et al.*, 2000; Zhang *et al.*, 2013a).

VIGS experiments and *V. dahliae* disease assays

VIGS in *N. benthamiana*, *N. tabacum* cultivar Samsun and tomato cultivar Motelle was essentially performed as described previously (Fradin *et al.*, 2009; Liebrand *et al.*, 2012; Zhang *et al.*, 2013a). We used *Agrobacterium* inoculation to express both pTRV:RNA1 and pTRV:RNA2 (Liu *et al.*, 2002). *Verticillium dahliae* race 1 strain JR2 was used for the inoculation of tomato. Fungal inoculations and disease scoring were performed as described previously (Fradin *et al.*, 2009, 2011). *Agrobacterium*-mediated co-expression of p35S:Ve1 and pSOL1292-Ave1 in TRV-inoculated *N. tabacum* cv. Samsun was performed at a final optical density at 600 nm (OD₆₀₀) = 1 for each construct (Zhang *et al.*, 2013a).

RNA extraction and qRT-PCR

For qRT-PCR, leaves of four plants inoculated with a TRV-silencing construct were ground and subjected to RNA extraction plus first-strand cDNA synthesis, as described previously (Liebrand *et al.*, 2012). Primers matching the individual *SIBIPs*, *SICRTs* and *SIActin* are given in Table S3 (see Supporting Information). qRT-PCR was performed as described previously (Liebrand *et al.*, 2012).

Protein identification by mass spectrometry

Immunopurification of the Ve1-eGFP fusion protein and GFP-HA control from transiently transformed *N. benthamiana* leaves was performed as described previously for Cf-4-eGFP (Liebrand *et al.*, 2012). In brief, proteins were extracted using extraction buffer containing 150 mM NaCl, 1% IGEPAL CA-630 (NP40), 50 mM Tris, pH 8, and one tablet of protease inhibitor cocktail (Roche, Almere, the Netherlands) per 50 mL. One gram of leaf material (fresh weight) per 2 mL of extraction buffer was used and 10 mL of total extract was subjected to immunopurification using 60 µL (50% slurry) of GFP_TrapA affinity beads (Chromotek, Planegg, Germany). The beads were incubated with the protein extract for 1 h, after which they were washed five times with extraction buffer. Subsequently, a trypsin on-bead digestion was performed and the peptide mixture was analysed by mass spectrometry using an Orbitrap XL mass spectrometer (Thermo Scientific, Etten-Leur, the Netherlands), as described previously (Liebrand *et al.*, 2012).

Co-immunopurifications and immunoblotting

Co-immunopurifications were performed as described previously (Liebrand *et al.*, 2012). We used 15 µL (50% slurry) of GFP_TrapA affinity beads for the immunopurifications. The beads were washed five times with the extraction buffer before eluting with SDS loading buffer. After blotting to poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad, Veenendaal, the Netherlands), the membranes were incubated with αGFP (anti-GFP-HRP, 130-091-833, MACS antibodies, Leiden, the Netherlands), αHA (anti-HA-peroxidase clone 3F10, 12013819001, Roche) or αHRP (rabbit anti-HRP, 323-005-021, Jackson ImmunoResearch, Suffolk, UK) as a primary antibody, and goat anti-rabbit-HRP (anti-rabbit IgG-HRP, A9169, Sigma, Zwijndrecht, the Netherlands) as a secondary antibody. All antibodies were used at the dilutions recommended by the manufacturer.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Determination of the expression levels of *Solanum lycopersicum* HSP70 binding protein (*SIBiP*) (A) and lectin-type calreticulin (*SICRT*) (B) genes in *Tobacco rattle virus* (TRV)-inoculated tomato plants. Tomato plants were inoculated with the indicated TRV-silencing constructs and relative transcript levels of *SIBiP1* and *SIBiP2* (A) as well as of *SICRT2*, *SICRT3a* and *SICRT3b* (B) were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Expression levels were standardized to those in the TRV:*GUS*-inoculated plants and normalized to endogenous *SlActin*. RNA used as a template for qRT-PCR was extracted from a mixture of four individually silenced tomato plants. The standard deviation shows the variation between three technical repeats.

Table S1 Sequences and Mascot ion scores of peptides matching co-purifying endoplasmic reticulum (ER)-resident chaperones, identified by mass spectrometry of a tryptic digest of immunopurified Ve1-enhanced green fluorescent protein (eGFP), transiently expressed in *Nicotiana benthamiana*.

Table S2 Additional Ve1-enhanced green fluorescent protein (eGFP) co-purifying proteins identified by mass spectrometry. Shown are the number of unique mass spectra divided by the number of unique peptides identified for each protein in the Ve1-eGFP and GFP-haemagglutinin (HA) control immunopurification.

Table S3 List of oligonucleotide primers used.