Biogenesis and signalling requirements
of plant receptor-like proteins
mediating resistance to fungal pathogens

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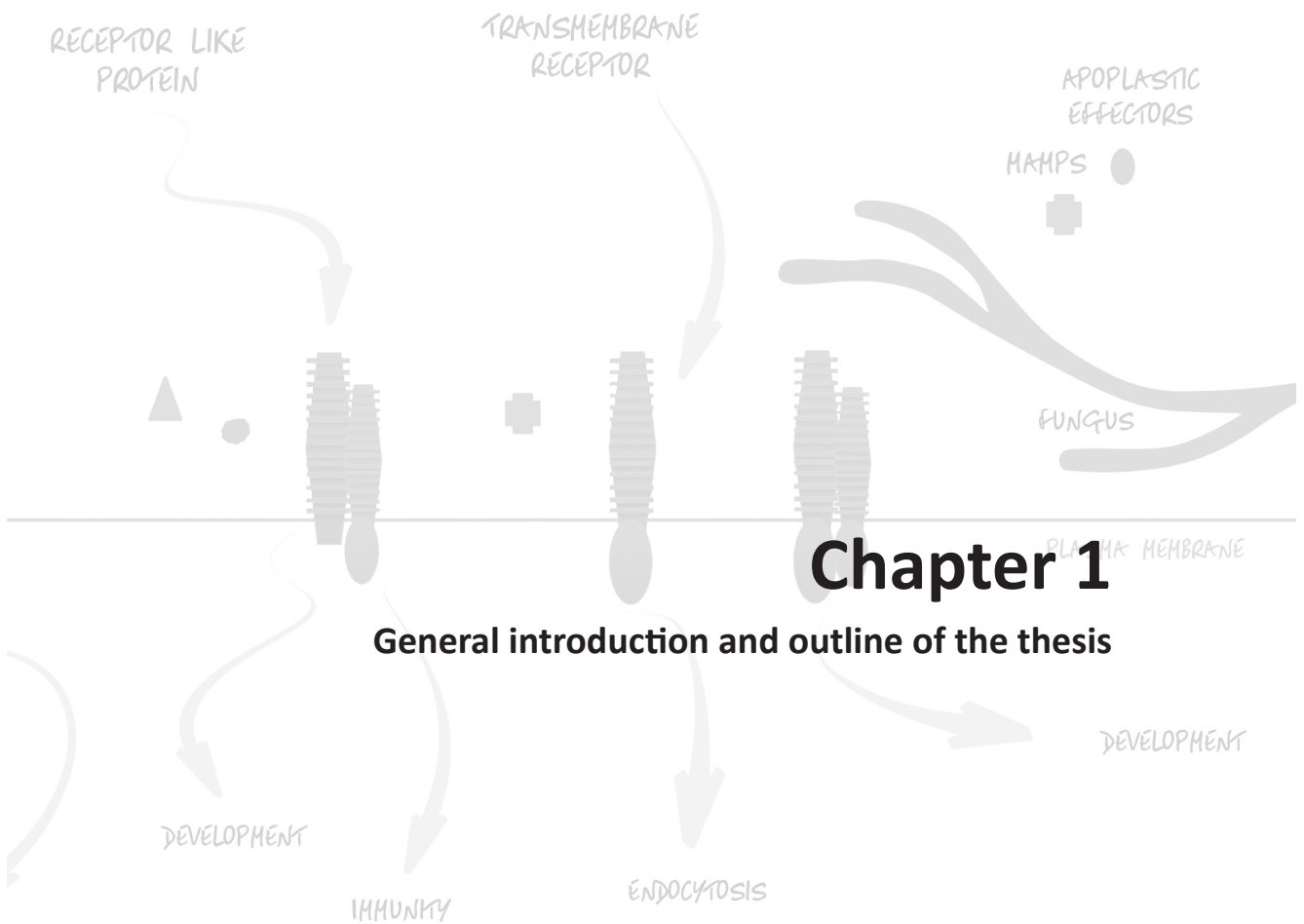
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Chapter 1

General introduction and outline of the thesis

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Abstract

Plants are continuously challenged by pathogens and to defend themselves against pathogenic micro-organisms that breach preformed defences, they possess an innate immune system capable of recognizing invading pathogens and activating a cellular immune response. Conserved molecular patterns of microbes are detected by transmembrane Pattern Recognition Receptors (PRRs), which mediate activation of defence also known as Microbe-Associated Molecular Pattern (MAMP)-triggered immunity (MTI). MTI is overcome by successful pathogens that secrete effector proteins interfering with PRR-mediated immunity. In a co-evolutionary arms race between plants and their attacking pathogens, plants have evolved Resistance (R) proteins capable of recognizing these effectors thereby mounting a strong immune response, known as Effector-Triggered Immunity (ETI), resulting in resistance. Most R proteins are localized in the cytoplasm carry a Nucleotide-Binding (NB) and Leucine-Rich Repeat (LRR) domain and are known as NB-LRRs. In contrast, PRRs are mostly transmembrane Receptor-Like Kinases (RLKs) or Receptor-Like Proteins (RLPs). Both classes of transmembrane receptors consist of proteins with different extracellular domains involved in ligand perception, whereas the cytoplasmic kinase domain of the RLKs is able to initiate a downstream signal-transduction cascade. RLPs lack an obvious cytoplasmic signalling domain and, although the first RLP was identified almost 20 years ago, the mechanism by which this class of receptors mediates immune signalling is still unknown. The studies described in this thesis are aimed at elucidating immune signalling mediated by tomato (*Solanum lycopersicum*) Cf and Ve1 proteins, LRR-RLPs providing resistance against two fungal pathogens with different lifestyles, *Cladosporium fulvum* and *Verticillium dahliae*, respectively. *C. fulvum* thrives in the apoplast of tomato leaf tissue, while *V. dahlia* colonizes the xylem vessels of several crop plants including tomato.

Plant Innate Immunity

In contrast to animals, plants are not mobile and cannot escape from dangerous situations or environmental conditions favouring the chance of microbial infections. Moreover, plants lack an adaptive immune system based on circulating cells [1-6]. Nevertheless, in nature most plants are healthy and disease is the exception. In order to resist attacks by pathogens, plants have pre-formed structural and chemical barriers to protect themselves against pathogenic microbes. For example, leaves are protected by the cuticle, a waxy layer that forms a physical barrier protecting plant cells against invading microbes [7]. However, despite the presence of these barriers, plants are still vulnerable to pathogens that enter through natural openings like stomates and hydathodes or wounds, to pathogens carried by vector organisms and to pathogens that breach preformed defences [8-10]. Upon passing preformed defences, plants rely on the first layer of induced defence responses. This layer of defence is activated upon perception of conserved Microbe-Associated Molecular Patterns (MAMPs), or host-derived damage products that function as Damage-Associated Molecular Patterns (DAMPs), by cell surface Pattern Recognition Receptors (PRRs) [3-5, 11-13] (Fig. 1). PRRs are mostly transmembrane Receptor-Like Kinases (RLKs) or Receptor-Like Proteins (RLPs) [13]. Recognition of MAMPs or DAMPs rapidly activates an array of innate immune responses, such as ion fluxes resulting in alkalization of the apoplast, an influx of Ca^{2+}, production of Reactive Oxygen Species (ROS) and activation of Mitogen-Activated Protein Kinase (MAPK) pathways. These fast responses are followed by activation of defence gene expression, production of secondary metabolites and callose deposition (see [11] and references therein). These combined responses eventually result in MAMP-Triggered Immunity (MTI), which is generally sufficient to halt proliferation of non-adapted microbes [3, 5, 11] (Fig. 1).

However, successful pathogenic microbes are able to overcome MTI and colonise host tissues. In most cases, such pathogens secrete molecules known as effectors that interfere with host immune responses by suppressing MTI, mounting Effector-Triggered Susceptibility (ETS) [14, 15] (Fig. 1). For example, gram-negative bacterial pathogens that colonize the apoplastic space of the leaves inject effectors into host cells by means of the type III secretion system [15, 16]. Many of these bacterial effectors target plant proteins that play crucial roles in the activation of immune responses [15, 16]. Similar to bacteria, numerous fungal pathogens colonize the plant apoplast. Several biotrophic and obligate biotrophic fungal pathogens form specific feeding structures named haustoria, which are in close contact with the plasma membrane of the host cells and enable retrieval of host nutrients. In addition to facilitating the uptake of nutrients, haustoria aid in the delivery of effectors into the host cells [17-19]. However, the translocation mechanisms of fungal effectors are largely unknown [17-19]. There are also effectors secreted by fungi that function in the apoplast and these appear to merely shield MAMPs/DAMPs from plant PRRs or to protect the pathogen against extracellular plant

glucanases, chitinases or proteases [17, 20-24]. Similar to fungi, oomycete pathogens grow extracellularly and often form haustoria from which effectors are secreted that subsequently enter the cells of the host plant [25, 26]. A subset of these effectors, the RxLR effectors, have diverse roles in modulating plant immunity and appear to rely on their N-terminal Arg-x-Leu-Arg amino acid signature for translocation into host cells [25-27]. Crinklers form another group of translocated oomycete effectors and induce host cell death for the benefit of the pathogen [28, 29]. Additional oomycete effectors that are not secreted via haustoria but by intercellular hyphae end up in the apoplast where they interfere with plant cell wall integrity, inhibit glucanases, proteases or induce cell death [25, 30-32]. Importantly, not all effectors of plant-pathogenic microbes function to suppress immune responses. Effectors may also alter the physiology of the host into their own advantage. For example, some fungal and bacterial pathogens increase the expression of a specific group of sugar transporters, presumably to manipulate host sucrose transport in favour of their own metabolism [33].

During co-evolution between plants and pathogens, plants have developed a second layer of defence, enabling them to recognize effectors of pathogens by Resistance (R) proteins, which are also referred to as immune receptors. Perception of an effector by the corresponding R protein results in race-specific resistance to the pathogen and this type of resistance is referred to as Effector-Triggered Immunity (ETI) [3, 5, 34] (Fig. 1). In resistant plants, secreted effectors that end up in the host cytoplasm are mostly recognized by intracellular receptor proteins that comprise the Nucleotide-Binding-Leucine-Rich Repeat proteins (NB-LRRs). Recognition of effectors by NB-LRRs can be either direct or indirect [3, 5, 34-36]. In case of indirect recognition, NB-LRRs monitor host proteins, which are referred to as Virulence Targets (VTs), that are targeted by effectors. VTs are frequently crucial components involved in host immune signalling. The manipulation of the VT by the effector is actually detected by the NB-LRR and the mechanism of effector recognition through monitoring of a VT is referred to as "guarding" [3, 5, 37, 38] (Fig. 1).

Originally, ETI has been defined as the second layer of plant innate immunity [3, 5] and was considered to be a faster and amplified version of MTI [3, 39]. In addition, ETI was found to frequently culminate in the Hypersensitive Response (HR), which is a form of localized programmed cell death [3, 40]. Despite the distinction between MTI and ETI made in the past, evidence is accumulating that MTI and ETI may actually represent the same process of which the function is to halt ingress of the pathogen [41]. For example, the distinction between MAMPs and effectors does not hold, as not all MAMPs are strictly conserved amongst a class of microbes and certain conserved microbial effectors can equally well be considered as MAMPs [41]. Moreover, a number of MAMPs, including fungal xylanase, actually trigger an ETI-like response, including the HR [42]. Conversely, recognition of effectors does not always trigger a strong ETI response and the amplitude of the defence response may vary for different effector and

receiver pairs [43-45]. Finally, not all immune receptors have an intracellular localization as some are actually cell surface receptors that structurally resemble PRRs [46-48]. Based on these examples, a distinction between immune signalling mediated by cell surface receptors or intracellular NB-LRRs, may actually be a better representation of the different layers of plant innate immunity [4, 41].
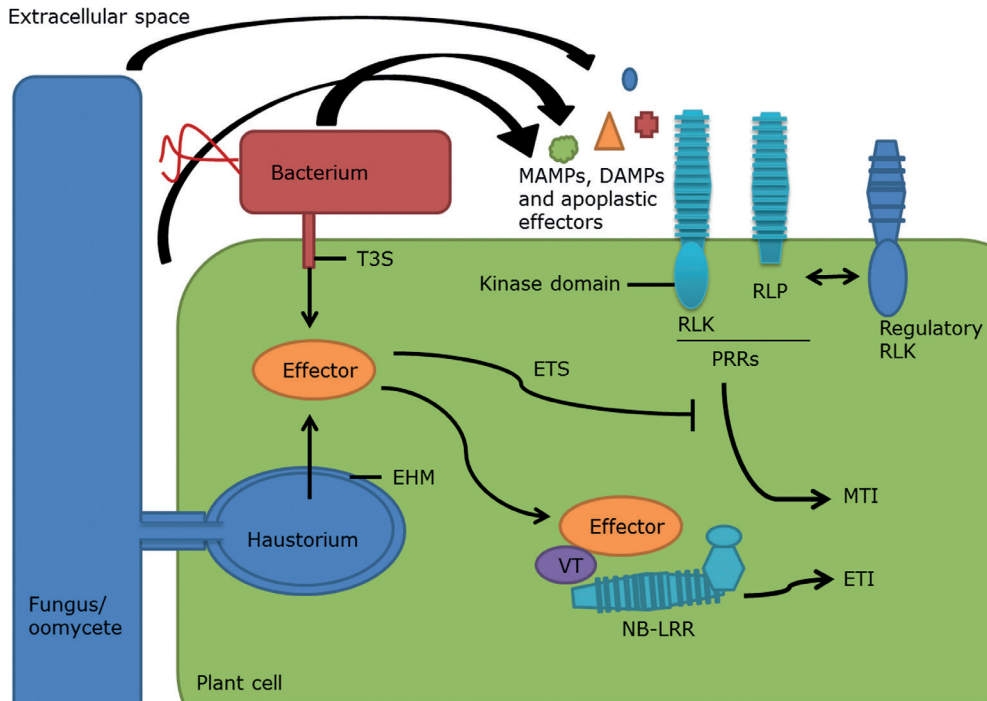
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Figure 1. Schematic overview of the different layers of plant innate immunity and its perturbation by successful pathogens. Bacteria, fungi and oomycetes release Microbe-Associated Molecular Patterns (MAMPs) or actively secrete effectors in the apoplastic space. Furthermore, their invasion may result in the release of host-derived damage products that function as Damage-Associated Molecular Patterns (DAMPs). These are recognized by cell surface-localized, transmembrane Pattern Recognition Receptors (PRRs), which are either Receptor-Like Kinases (RLKs) or Receptor-Like Proteins (RLPs). PRRs may co-operate with additional RLKs that function as so-called "regulatory RLKs". Recognition of ligands by cell-surface PRRs induces MAMP-Triggered Immunity (MTI). By the translocation of effectors into the cytoplasm of plant cells, either via the ExtraHaustorial Matrix (EHM) surrounding fungal or oomycete haustoria or via the Type-III Secretion system (T3S) employed by bacteria, or via secretion of effectors in the apoplast, MTI is effectively suppressed, resulting in Effector-Triggered Susceptibility (ETS). Extracellular and intracellular effectors are detected by cell surface receptors and cytoplasmic R proteins of the Nucleotide-Binding Leucine-Rich Repeat (NB-LRR)-type, respectively, resulting in Effector-Triggered Immunity (ETI). This recognition can take place either directly or indirectly through modification of apoplastic or cytoplasmic Virulence Targets (VTs) by apoplastic or cytoplasmic effectors, respectively. For further details see main text. Figure adapted from [4].

In many cases, plant defence responses are also shaped by plant hormones. In particular, ethylene, jasmonic acid and salicylic acid play important roles in modulating plant defence responses (see [49] and references therein). In addition, an activated local defence response also triggers systemic immunity in distal plant parts, thereby strengthening protection of the plant against future infections [49, 50]. A well-studied example of such a systemic resistance response is the so-called Systemic Acquired Resistance (SAR) response, which is mostly depending on increased levels of salicylic acid [50].

To conclude, plant innate immunity to microbial pathogens relies on the detection of pathogen- or pathogen-induced host-derived molecules that alert the plant of pathogen presence allowing it to mount efficient defence responses against invaders [4, 41]. Both cell-surface and intracellular receptors are involved in pathogen perception and are able to activate defence responses. Here, I will provide an overview of the mechanisms by which cell surface receptors perceive extracellular MAMPs, DAMPs and effectors and how they mediate defence signalling culminating in resistance. Furthermore, I will provide an overview of the cellular mechanisms regulating the biogenesis of functional cell surface receptors.

Cell Surface Receptor-Like Kinase Complexes Involved in Activating Plant Immune Responses
With over 300 members encoded by the genome of the model plant *Arabidopsis thaliana* (*Arabidopsis*), transmembrane RLKs represent a major group of cell surface-localized receptors [51]. All RLKs have the presence of a cytoplasmic kinase domain in common, and in addition they have different extracellular domains such as LRRs, a Lectin- or a Lysin Motif (LysM)-domain [51-55]. RLKs play roles in many different signalling processes including stem cell maintenance, hormone perception, abiotic stress signalling, initiation of symbiosis with microbes and immune signalling [13, 56-59]. In general, the extracellular domain is required for perception of a ligand, whereas the intracellular kinase domain activates the appropriate signalling cascade by phosphorylation of downstream components [13, 56-59]. Moreover, many RLKs function in large protein complexes, frequently comprising additional RLKs and membrane-associated Receptor-Like Cytoplasmic Kinases (RLCKs) [52, 56, 59, 60].

Over the last years, a number of microbial MAMPs and corresponding plant PRRs have been identified [11, 13]. A well-studied bacterial MAMP is flagellin, a protein that forms a structural part of the flagella which are required for their motility [61]. The LRR-RLK Flagellin Sensing-2 (FLS2) of *Arabidopsis* recognizes flagellin, as well as the flagellin-derived peptide flg22 which functions as its elicitor-active epitope, and subsequently triggers a typical MTI response [62]. Very similar to flagellin, the conserved bacterial

protein Elongation Factor-Tu (EF-Tu), involved in translation of RNA, is perceived by the *Arabidopsis* EF-Tu Receptor (EFR), which is also an LRR-RLK [63]. Also in this case, the elicitor-active epitopes derived from EF-Tu, represented by peptides of 18 and 26 amino acids, respectively, can trigger EFR-mediated responses [64]. Functional orthologs of FLS2 are present in many other plants, including tomato (*Solanum lycopersicum*), *Nicotiana benthamiana* and rice (*Oryza sativa*) [11], whereas EFR only appears to be present in members of the Brassicaceae family [11].

Besides LRR-RLKs, the LysM-RLK Chitin Elicitor Receptor Kinase-1 (CERK1) is involved in perception of fungal pathogens by recognizing cell wall derived chitin molecules [65-67]. Recent studies also revealed a role for the LysM-RLK LYK4 (LYsM receptor Kinase-4) in perception of chitin [68]. CERK1 is also involved in perception of the bacterial MAMP peptidoglycan and thereby also contributes to resistance to bacteria [69-72]. CERK1 homologs are conserved and present in different plant species including *Arabidopsis*, rice and tomato [66, 67, 73]. For many other MAMPs the corresponding receptor remains to be identified and it is expected that additional PRRs, which possibly are RLKs, will be identified in the future [11].

RLKs form complexes with additional receptor proteins that are required for the initiation of downstream signalling. In many cases RLKs and RLCKs are recruited that function as regulatory proteins aiding in the activation of cellular signalling [13, 56-59]. In line with this, *Arabidopsis* FLS2 was found to directly interact with the LRR-RLK Somatic Embryogenesis Receptor Kinase-3/BRI1-Associated Kinase-1 (SERK3/BAK1, hereafter referred to as BAK1) upon perception of flagellin [74-77]. BAK1 is a member of the SERK family, consisting of five members in *Arabidopsis* [78]. Interestingly, not only FLS2 but also EFR interacts with BAK1 in a ligand-induced manner [79]. Furthermore, other SERK family members are able to at least partly take over the role of BAK1 in PRR signalling [79]. BAK1 functions as a co-receptor of PRRs as it was found to interact with FLS2 as well as the flg22 ligand [77]. Because BAK1 is required for PRR signalling, it can be considered as a regulatory RLK for multiple PRRs [13]. Next to BAK1, the RLCK Botrytis-Induced Kinase-1 (BIK1) and its close homologs interact with FLS2 and EFR and are required for MTI responses triggered by these PRRs [80-83]. Upon ligand perception, a series of trans-phosphorylation events occur between the PRR, BAK1 and BIK1, after which BIK1 is released from the receptor complex, likely initiating downstream immune signalling [80, 82, 83]. Interestingly, BAK1 and BIK1 are also required for signalling activated by the LRR-RLKs PEP-Receptor-1 and -2 (PEPR1 and PEPR2), which are receptors that perceive endogenous peptides (PEPs) that serve as DAMPS [83-85]. PEPR-mediated signalling also appears to be part of a signal amplification loop involved in augmentation of defence signalling triggered by other PRRs [83, 86]. An additional role of BAK1 is the attenuation of immune responses via phosphorylation of the E3-ubiquitin ligases PUB12 and PUB13 [82, 87]. PUB12 and PUB13 were found to subsequently poly-ubiquitinate FLS2, which tags the receptor to become degraded by the proteasome [87].

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BAK1 was originally identified as an interactor of the LRR-RLK Brassinosteroid-Insensitive-1 (BRI1) [88-90] and, similar as in the case for FLS2, BAK1 functions as a co-receptor for BRI1 by interacting with both the ligand and the receptor [91, 92]. BRI1 perceives plant growth-promoting brassinosteroid hormones (see [93] and references therein) and analogous to what happens during innate immune signalling, reciprocal phosphorylation events between BRI1 and BAK1 occur upon ligand perception and downstream RLCKs are recruited to transduce the cellular signal [93]. In the BRI1 signalling pathway, the RLCKs involved in downstream signalling are BIK1, Brassinosteroid Signalling Kinases (BSKs) and Brassinosteroid-INsensitive-2 (BIN2) [93, 94]. Besides being required for MTI and brassinosteroid signalling, BAK1 also has a role in the containment of cell-death [78, 90, 95], illustrating that this LRR-RLK is involved in a plethora of cellular signalling processes.

In contrast to FLS2, EFR and the PEPRs, *Arabidopsis* CERK1 does not interact with BAK1 and this LRR-RLK is also not required for CERK1-mediated signalling [75, 80]. This suggests that the CERK1 receptor complex consists of markedly different components. Intriguingly, CERK1 signalling in *Arabidopsis* does require functional BIK1 [80]. However, in contrast to treatment with flg22 or elf18, chitin does not induce BIK1 phosphorylation [81], suggesting that upon ligand perception, differences in receptor complex assembly and early phosphorylation events exist between the different PRRs. Indeed, in rice, the small GTPase *Os*Rac1 is a positive regulator of CERK1-mediated immunity [96] and recently, *Os*Rac1GEF (Guanine nucleotide Exchange Factor for *Os*Rac1) was found to interact directly with CERK1 and *Os*Rac1, thereby marking *Os*Rac1GEF and *Os*Rac1 as essential components of the CERK1 receptor complex in rice [97]. The RLCK *Os*RLCK185 was also found to interact with *Os*CERK1 and is required for chitin- and peptidoglycan-induced responses [72], revealing recruitment of yet another component of this receptor complex in rice.

The LRR-RLK Xa21 of rice perceives an unknown molecule secreted by several strains of the bacterium *Xanthomonas* [48]. Several Xa21-interacting proteins have been identified over the years. The ATPase Xb24 directly interacts with Xa21, promotes Xa21 phosphorylation at specific residues and keeps the receptor in an signalling "off" state [98]. Xb25, an ankyrin repeat-containing membrane protein, also interacts with Xa21 and is required for Xa21 accumulation and signalling [99]. Furthermore, the ubiquitin E3-ligase Xb3 interacts with Xa21 and is required for full activation of immune responses [100], whereas the phosphatase Xb15 attenuates the Xa21-mediated immune response [101]. Remarkably, a number of transcriptional regulators were found to interact with the cytoplasmic domain of Xa21 [13]. For example, the WRKY transcription factor *Os*WRKY62 binds to Xa21 and functions as a negative regulator of Xa21-mediated defence [102]. This observation suggests that Xa21 may directly be involved in transcriptional control of defence-associated genes, though it remained puzzling how a plasma-membrane-localized kinase can modulate the transcriptional

activation mediated by nuclear-localized transcription factors. Interestingly, it was recently discovered that upon activation of Xa21, the kinase domain is cleaved off, after which it dissociates and migrates to the nucleus where it regulates immune responses and interacts with *Os*WRKY62 [103]. These data provide a novel mechanism by which RLKs functioning as PRRs activate defence responses.

Biogenesis and ER-QC-Regulated Folding of Cell Surface Receptors
The biosynthesis of functional and mature cell surface receptors takes place in the Endoplasmic Reticulum (ER). The ER is a highly dynamic cell organelle, consisting of tubules and cisternae [104]. Besides playing a role in protein synthesis, the ER is involved in cellular calcium homeostasis, storage of proteins and other compounds, cellular stress signalling, protein glycosylation, chaperone-mediated protein folding and Quality Control (QC) [104-108].

To ensure that only correctly folded and mature receptors reach the plasma membrane, a strict ER-QC mechanism is required. A number of crucial components, involved in different pathways required for ER-QC have been identified in plants over the years [105, 106, 109]. A first ER-QC pathway requires the Heat Shock Protein 70 (HSP70) Binding Proteins (BiPs) and the HSP90 protein GRP94 (Glucose Regulated Protein-94) [109] (Fig. 2). The BiPs form a complex with HSP40-like co-chaperones containing a J domain (the ERdj3 proteins) and Stromal-Derived Factor-2 (SDF2) [110-112]. These chaperone complexes mostly bind to hydrophobic regions of the client protein, thereby assisting in its correct folding and preventing aggregation of unfolded proteins [109].

Frequently, transmembrane receptors carry an extracellular domain that is heavily *N*-linked glycosylated. A second pathway of the ER-QC system is provided by lectin-type chaperones that monitor the *N*-linked glycosylation status of client proteins [109] (Fig. 2). *N*-linked glycosylation is initiated by the addition of a Glucose(Glc)3Mannose(Man)9Glc-N-Acetylglucosamine(NAc)2 oligosaccharide to Asn residues on potential glycosylation sites [107]. Such sites commonly contain the amino acid consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except a Pro [107]. Addition of the oligosaccharide is mediated by the OligoSaccharylTransferase (OST) complex, after which glucosidases-I and -II remove Glc residues, leaving mono-glucosylated glycans on the client protein [107]. These glycans have a high affinity for the ER-QC chaperones CalNeXin (CNX) and CalReTiculin (CRT) [107, 109], of which the CNXs are ER membrane-spanning proteins and the CRTs are proteins of the ER lumen. Both types of chaperones bind unfolded proteins carrying the mono-glucosylated glycans and assist in folding. Upon correct folding, glucosidase-II removes the final Glc residue, after which the client protein is translocated to the Golgi apparatus [109]. Proteins that are not correctly folded after a first round of CNX/CRT-mediated folding are re-glucosylated by UDP-Glc Glycoprotein

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Glucosyltransferase (UGGT) and in this way client proteins can undergo additional rounds of CNX/CRT-assisted folding [109]. Eventually, proteins that do not get properly folded will become target of ER-resident mannosidases which trim Man residues from the glycans, thereby reducing the affinity for CNX and CRT proteins and generating a signal for degradation of the client protein by the ER-Associated Degradation (ERAD) pathway [107, 109]. In fact, Man trimming acts as a sort of molecular timer, in such a way that client proteins that are maintained too long in the ER or that cannot be properly folded, will eventually be degraded.

A third ER-QC pathway involves oxidative folding and the formation of disulfide bridges in client proteins and is based on the action of Protein Disulfide Isomerases (PDIs), which contain thioredoxin domains [109, 113, 114] (Fig. 2).

Recently a number of ER proteins involved in QC and *N*-linked glycosylation were identified to be crucial for functionality and maturation of a number of transmembrane receptors involved in defence and development. It was found that *Arabidopsis*, mutated in a substantial number of ER chaperone genes, is affected in the resistance response against pathogenic bacteria. Mutants in *SDF2* were compromised in immunity mediated by EFR, whereas in rice overexpression of an *OsBiP3*-encoding gene compromised immune responses mediated by Xa21 [111, 115]. Additional *Arabidopsis* mutants were found to be affected in *N*-linked glycosylation and the ER-QC pathway monitoring the glycosylation status of client proteins. For example, mutants in genes coding for the OST complex, such as *OST3/6* and *STT3A* (*STaurosporin and Temperature-sensitive-3A)*, *UGGT*, *Glucosidase II*, the gene encoding the HDEL receptor *ERD2b* and *CRT3* are all compromised in EFR-mediated immune responses [111, 116-121]. Cause of the compromised EFR-mediated immunity was in all cases a reduced accumulation of the receptor itself, whereas in the same mutants FLS2 accumulation and its associated responses were hardly compromised. Additional support for the important role of *N*-linked glycosylation-dependent ER-QC in plant immunity comes from studies on the Induced Receptor Kinase (IRK) involved in resistance to tobacco mosaic virus (TMV), mediated by the NB-LRR protein N [122]. Silencing of two *N. benthamiana CRTs* strongly affected accumulation of the IRK protein and thereby compromised N-mediated resistance [122]. *Arabidopsis* contains three *CRT* homologs and based on their amino acid sequences, CRT3 is markedly different from CRT1 and CRT2 [121, 123, 124]. Based on this observation and on the fact that *crt3* mutant plants, but not *crt1* or *crt2* mutant plants, are compromised in immune responses mediated by EFR, it was speculated that CRT3 has acquired a specific role in the maturation of PRRs [121]. So far, EFR is the only PRR identified to be a client of CRT3, although the observation that *crt3* mutants are more severely compromised in resistance to *Pseudomonas syringae* pv tomato than *efr* mutants, suggests that more PRRs are clients of CRT3 [116].

The *N*-linked glycosylation pathway is also required for the biogenesis of the BRI1 receptor. The mutant receptor BRI1-9 is retained in the ER via CNX/CRT-mediated ER-QC mechanisms in the presence of functional UGGT [123]. CRT3 in particular appears to be involved in retention of BRI-9 in the ER [123] and based on this result it can be concluded that CRT3 does not solely have PRRs as client proteins. Another BRI1 mutant, BRI1-5, is retained in the ER via a BiP- and thiol-mediated pathway [123, 125, 126].
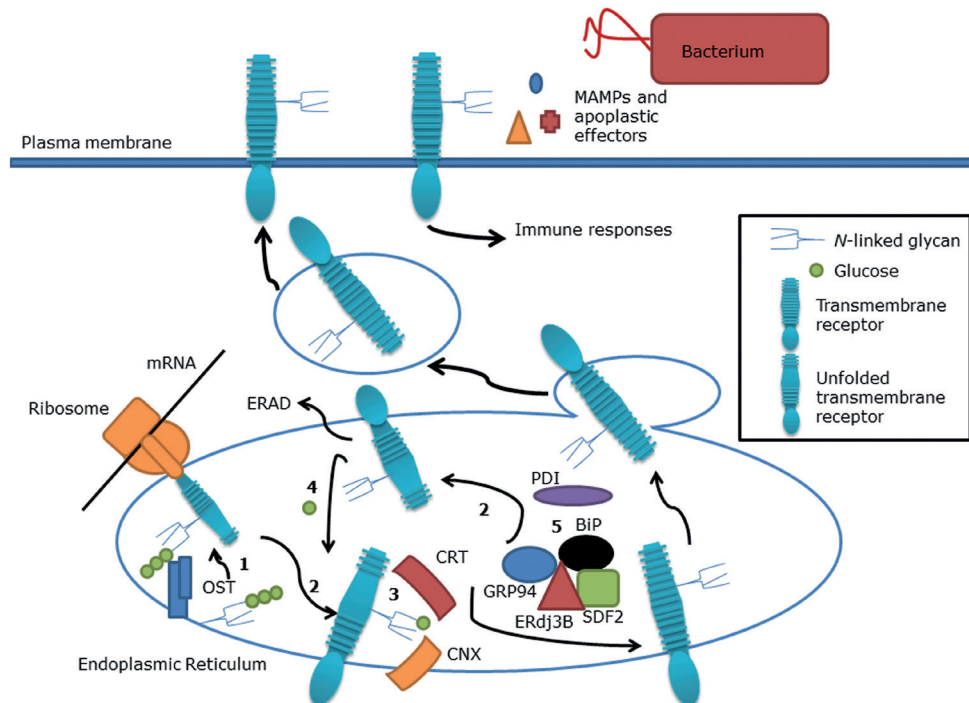

Figure 2. A simplified model of the biogenesis and Endoplasmic Reticulum (ER) Quality Control (QC) requirements of *N*-linked glycosylated transmembrane cell surface-localized receptors. mRNA is translated by ribosomes at the ER and the unfolded receptor, carrying a transmembrane domain, is inserted into the ER membrane. Subsequent crucial steps in ER-QC are indicated. (1) The OligoSaccharylTransferase (OST) complex catalyses the addition of a precursor glycan carrying three glucose moieties to distinct Asn residues (within the Asn-X-Ser/Thr signature) of the extracellular domain of the receptor. (2) Glucosidases remove glucose residues. (3) The mono-glucosylated unfolded receptor is a client for CalNexin/CalReTiculin (CNX/CRT)-mediated folding. (4, 5) In case glucosidase-II has removed the terminal glucose residue, while the client protein is still not correctly folded, additional rounds of re-glucosylation and CNX/CRT-assisted folding may occur. (6) Terminally misfolded proteins will be degraded by ERAD. (7) Besides CNX/CRT-assisted folding, the Binding Protein/Stromal-Derived Factor-2/ HSP40-like co-chaperones containing J domains (BiP/ SDF2/ERdj3B)- and Protein Disulfide Isomerase (PDI)-mediated pathways are also indicated. These ER-QC pathways likely function independently of CNX/CRT-mediated folding. Upon correct folding, the terminal glucose residue of the glycan is removed and the client protein is transported via the Golgi apparatus, where additional glycan modification takes place (not shown), to the plasma membrane. In case the mature receptor is a Pattern Recognition Receptor (PRR), as in this example, it will be capable of recognizing Microbe-Associated Molecular Patterns (MAMPs), Damage-Associated Molecular Patterns (DAMPs) or apoplastic effectors from microbial pathogens and initiate immune signalling. For further details see text. Figure adapted from [105].

The strict requirement by EFR for proteins and chaperones involved in *N*-linked glycosylation and ER-QC for its proper accumulation and functionality, in contrast to FLS2, suggests that EFR is much more vulnerable to under-glycosylation than FLS2. Indeed, by specifically removing putative *N*-linked glycosylated residues from the LRR domains of EFR and FLS2 this was experimentally confirmed [120, 127]. Besides affecting EFR function, under-glycosylation affects EFR accumulation, as a number of EFR glycosylation mutants failed to accumulate to wild-type levels [120, 127].

Eventually, mature receptors are exported from the ER to their next cellular destination, which is the Golgi apparatus. This ER export occurs via Coat Protein Complex-II (COPII)-coated vesicles that are formed at distinct ER exit sites [109, 128]. Little is known about proteins regulating the secretion of transmembrane receptors from the ER. However, recently two Reticulon-like proteins, RTNLB1 (ReTiculoN-Like protein non-metazoan group B-1) and -2, were shown to interact with the cytoplasmic domain of FLS2, thereby modulating ER export of this RLK [109, 128].

Receptor-Like Proteins Represent an Enigmatic Class of Cell-Surface Receptors in Plant Immunity
An enigmatic class of plant cell surface receptors is represented by the RLPs. RLPs have similar extracellular domains as RLKs, but lack a cytoplasmic signalling domain [55, 129, 130]. Most RLPs contain a transmembrane domain, whereas others are attached to the membrane through a Glycosyl Phosphatidyl Inositol (GPI)-anchor [55, 129, 131]. In general, LRR-RLPs have an extracellular LRR domain, carry a transmembrane domain and have a very short cytoplasmic domain lacking obvious signalling signatures [55, 129, 130]. In *Arabidopsis* 57 LRR-RLPs have been identified [129, 130, 132], whereas tomato contains a total of 176 genes that code for LRR-RLPs [133]. LRR-RLPs have been reported to play roles in defence (see below) and in plant development. Examples of the latter are Clavata2 (CLV2) [134], which is involved in meristem stem cell maintenance, and Too Many Mouths (TMM) [135], which is involved in stomatal patterning.

Since LRR-RLPs lack a cytoplasmic signalling domain, it remains enigmatic how these receptors are able to transmit a downstream cellular signal upon ligand detection. For CLV2 and TMM it was found that interaction with LRR-RLKs is required for mediating signal transduction. For example, CLV2 interacts with the LRR-RLK CLV1 and the membrane-associated pseudo kinase Coryne to mediate stem cell signalling [136-138], whereas TMM interacts with the LRR-RLK Erecta to modulate stomatal patterning [139]. Interestingly, the number of LRR-RLPs with assigned functions in plant defence is rapidly growing. However, in contrast to what is known about signalling by defence-associated RLKs, remarkably little is known about the composition of the receptor complexes in which LRR-RLPs are anticipated to be involved and the mechanisms these receptors

activate immune responses. Below, RLPs that play are role in plant defence will be discussed.

In *Arabidopsis* a number of RLPs have been implicated to be involved in defence. The LRR-RLPs RLP18 and RLP30 are both involved in resistance to the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola* [130]. Likewise, loss of function of the LRR-RLP SNC2 (Suppressor of Npr1, Constitutive-2) results in reduced resistance towards *Pseudomonas syringae* pv. *tomato*, whereas a gain of function SNC2 mutant shows a constitutive defence phenotype [140, 141]. SNC2 interacts with the ankyrin-repeat membrane-associated protein Bian DA-1 (BDA-1) and requires this protein for defence signalling [140]. *Arabidopsis RLP52* was reported to be upregulated by chitin and the encoded protein contributes to resistance to the powdery mildew fungus *Erysiphe cichoracearum* [142]. Another *Arabidopsis* LRR-RLP, RFO2 (Resistance to *Fusarium Oxysporum*-2), is required for resistance to the vascular pathogen *Fusarium oxysporum* [143] and recently the bacterial MAMP eMAX (enigmatic MAMP of *Xanthomonas*), which still awaits characterization, was found to be specifically recognized by the *Arabidopsis* LRR-RLP ReMAX (Receptor of eMAX) [144]. The GPI-anchored *Arabidopsis* LysM-RLPs LYM1 (LYsin Motif protein-1) and LYM3 are involved in immune responses against bacteria through mediating recognition of peptidoglycan, possibly in a complex with the LysM-RLK CERK1 [70]. Recently, *Arabidopsis* LYM2 was shown to preferentially localize to plasmodesmata and to limit ion fluxes upon chitin perception, a process independent of CERK1 [145].

RLPs are also known to play a role in defence in other plant species. For example, in apple (*Malus floribunda*) the LRR-RLP HcrVf2 (Homologue of *Cladosporium fulvum* resistance of Vf region-2) mediates resistance to the fungus *Venturia inaequalis* [146]. In rice, the LysM-RLP CeBiP (Chitin elicitor-Binding Protein), which is homologous to the *Arabidopsis* LYMs, interacts with CERK1 and is involved in chitin-triggered immunity [67]. Furthermore, the LYsin motif-containing Proteins (LYPs) LYP4 and LYP6 are involved in peptidoglycan and chitin perception in rice thereby mediating resistance to the bacterial pathogen *Xanthomonas oryzae* and the fungal pathogen *Magnaporthe oryzae* [147].

In tomato, several LRR-RLPs are known to play a role in disease resistance. EIX1 (Ethylene-Inducing Xylanase receptor-1) and EIX2 are required for perception of xylanase of the fungus *Trichoderma viride* [42]. Interestingly, EIX2 was found to act as the genuine xylanase receptor, whereas EIX1 attenuates EIX2-triggered defence responses by interacting with BAK1 [42, 148, 149]. Additional tomato LRR-RLPs are known to play a role in resistance to fungal pathogens. These are the members of the Cf protein family involved in recognizing different effectors *Cladosporium fulvum* and the Ve1 protein, which is involved in resistance to strains of *Verticillium* secreting Ave1. Both the Cf proteins and Ve1 are topic of this thesis and are discussed in more detail below.

Tomato Cf Proteins Are LRR-RLPs Mediating Resistance to Different Strains of *Cladosporium fulvum*

Cladosporium fulvum is a non-obligate biotrophic fungal pathogen of tomato. Asexual *C. fulvum* conidia germinate on the leaf surface and runner hyphae subsequently enter the leaf through stomata [150]. During its lifecycle, *C. fulvum* remains confined to the apoplast from where it obtains its nutrients [47, 151]. Eventually, conidiophores emerge from the stomata and produce conidia that are dispersed and can re-infect tomato [150]. Symptoms caused by *C. fulvum* infection consist of yellowish patches on the upper leaf surface and, in case of a more severe infection, partial wilting and necrosis of the leaf tissue [150]. To promote virulence, *C. fulvum* secretes small cysteine-rich effector proteins into the apoplast [47, 152] and several of these effectors are known to function as Avirulence (Avr) proteins, as they are race-specific and match with particular R proteins of tomato. Additional effector proteins, referred to as Extracellular proteins (Ecps), are secreted by all strains of the fungus and also have matching R proteins in tomato [47, 152-154].

So far, all identified R proteins mediating resistance to *C. fulvum* are transmembrane LRR-RLPs that are predicted to localize to the plasma membrane [47, 152]. These so-called Cf proteins each mediate recognition of a specific *C. fulvum* Avr protein and thereby the *Cf* genes and their matching *Avrs* are a perfect example of the gene-for-gene interaction between plants and their pathogens [47, 152]. Over the years, a number of matching Cf/Avr pairs has been identified, which are Cf-2/Avr2, Cf-4/Avr4, Cf-4E/Avr4E and Cf-9/Avr9 [23, 47, 150, 152, 155-162]. Cf-9 was the first LRR-RLP that was identified in plants [163]. Not much later, Cf-2, Cf-4, Cf-4E and Cf-5, as well as Cf homologs from wild-tomato varieties, were discovered [23, 47, 150, 152, 155-162]. Interestingly, all *Cf* genes are located in large genomic clusters where cross-overs may rapidly occur to generate new Cf variants (see [152] and references therein).

For a number of *C. fulvum* effectors the virulence function has been elucidated. Avr4 functions as a chitin-binding protein, effectively protecting fungal hyphae against plant chitinases [23, 164]. Ecp6 is a secreted protein that sequesters small chitin fragments that function as MAMPs [21, 165]. Avr2 inhibits the plant cysteine protease Rcr3 (Required for *Cladosporium* resistance-3) and binding of Avr2 to Rcr3 triggers Cf-2-mediated resistance [22, 160]. Hence, Cf-2 is believed to have evolved to guard the VT Rcr3 [37, 160]. The intrinsic functions of the additional Avrs and Ecps that have been identified remain to be elucidated.

Transient expression of Cf proteins, in combination with their corresponding Avr ligands in *Nicotiana tabacum* or *N. benthamiana* triggers a specific HR, showing that Cf proteins are functional in these plants and that the required downstream signalling partners are present [166]. Remarkably, Cf-4 also recognizes Avr4 homologs from *Mycosphaerella fijiensis*, a fungal pathogen on banana [153] and *Dothistroma septosporum*, a pine

pathogen [167], and based on these observations Avr4 can be considered as a conserved MAMP and Cf proteins can be considered to function as MAMP receptors, instead of acting as R proteins [41]. The extracellular LRR domain of Cf proteins is important for ligand recognition and several studies have tried to identify the crucial LRRs that determine ligand specificity by domain swapping [168-172]. LRRs 11-12 are for example crucial for Avr9 recognition by Cf-9 and replacement of Cf-9-specific residues in additional LRRs by Cf-4-specific residues results in loss of Cf-9 function [168, 169]. For Cf-4, the LRR B-domain confers specificity and specific residues in LRRs 13-16 are required for its functionality [168, 169]. Later, fine mapping experiments in which the LRRs of Cf-9 and Cf-9B were shuffled, revealed that LRRs 5-15 of Cf-9 and the complete N-terminal part of Cf-9B, up to LRR 15, are required for functionality [170, 171]. Regarding Cf-2 and Cf-5, domain swap experiments revealed that the LRR C1-domain is crucial for recognition specificity of both proteins [172]. Furthermore, a screen using Cf-9 mutants revealed numerous solvent-exposed Trp and Cys residues to be required for activity [173]. Cf proteins are heavily *N*-linked glycosylated on their LRRs and most of the extracellular Asn residues in Cf-9 (which are part of the Asn-X-Ser/Thr signature) are decorated with *N*-linked glycan moieties [173, 174]. Mutation of most of these Asn residues was found to compromise Cf-9 function [173].

Experiments using tobacco cell cultures revealed that within minutes upon perception of the matching ligand, Cf proteins mediate a ROS burst [175, 176]. In addition, ion channels are activated resulting in increased ion fluxes [177] and a K^+ outward ion channel was found to participate in this ion flux [177]. H^+-ATPases also play a role in the Cf-triggered response and at least one H^+-ATPase is known to be dephosphorylated upon Avr perception [178]. Furthermore, a rapid production of Phosphatidic Acid (PA), which is a second messenger molecule produced by Phospholipase C (PLC) enzymes, was observed [179]. In line with this observation, tomato PLC4 and PLC6 were found to be required for Cf-4-mediated defence responses [180]. MAPK pathway activation, as well as activation of Calcium-Dependent Protein Kinases (CDPKs), are also associated with Cf-mediated resistance responses [181-184]. Before the onset of the HR, a massive transcriptional reprogramming occurs, which is indicative of defence gene expression [185-188]

A number of Cf-interacting proteins, as well as several downstream components, have been identified as members of the defence signalling pathway activated by these LRR-RLPs. The thioredoxin CITRX (Cf-9-Interacting ThioRedoXin) was identified by a yeast two-hybrid screen using the cytoplasmic domain of Cf-9 as a bait [189]. CITRX negatively regulates Cf-9-mediated immune responses and may act as an adaptor protein connecting Cf-9 to the cytoplasmic kinase ACIK1 (Avr9/Cf-9-Induced Kinase-1), which is a positive regulator of Cf-9-mediated defence. CITRX and ACIK1 were found to interact by yeast two-hybrid and co-ImmunoPrecipitation (co-IP) assays [189-191]. ACIK1 was originally found to be upregulated during the Cf-9-mediated HR and silencing

of the encoding gene compromises Cf-9-mediated resistance [191]. Silencing of *CITRX* in tomato caused a strong yellowing of the leaves [189], suggesting an additional role of the encoded protein in chloroplasts. In line with the latter observation, the closest homolog of tomato CITRX in *Arabidopsis*, Trx*p*, is shown to be localized in the chloroplast [192]. These results suggest that tomato CITRX may actually not reside in the cytosol but in chloroplasts. Further studies are required to confirm that CITRX actually localizes to the cytosol or alternatively is released from the chloroplasts to interact with the cytoplasmic part of Cf-9. Another Cf-interactor found by yeast two-hybrid screens is VAP27 (Vesicle-Associated Protein-27), which is a SNARE (Soluble N-ethylmaleimide–sensitive factor Attachment protein REceptor) protein that may be required for cellular trafficking of Cf receptor complexes [193]. The gene encoding the NB-LRR NRC1 (NB-LRR protein Required for HR-associated Cell death-1), was shown to be differentially regulated upon Cf-4 activation and NRC1 is required for full functionality of Cf-4, Cf-9 and additional immune receptors [186, 194]. In addition, HSP90 is required for Cf-4-mediated responses, possibly by stabilizing the receptor complex downstream of NRC1 [186, 194]. Also important for Cf-9-mediated HR is the F-box protein, ACIF1 (Avr9/Cf-9–Induced F-Box-1). ACIF1 transcripts rapidly accumulate during Avr9-triggered, Cf-9-mediated HR and silencing of the encoding gene compromises this HR [195].

All Cf-interacting proteins described at the start of my thesis work were identified by yeast two-hybrid analyses and biochemical evidence of these interactions remains lacking. Therefore, our understanding of the Cf-receptor complex is still very limited. Interestingly, it was recently found that silencing of tomato *SERK1* compromises Cf-4-mediated defence in tomato [196]. This suggests that the regulatory SERK family of RLKs may take part in a receptor complex together with the Cf proteins and that Cf proteins may interact with RLKs to activate downstream signal transduction pathways.

Tomato Ve1 Is an RLP Mediating Resistance to Strains of *Verticillium* Secreting Ave1
Verticillium dahliae and *Verticillium albo-atrum* are a non-obligate vascular pathogens that have a broad host range and, in addition to tomato, also infect the model plants *N. benthamiana* and *Arabidopsis* [197]. *Verticillium* is a soil-borne pathogen that enters the roots, colonizes the vascular tissue and causes symptoms including chlorosis, wilting and stunting [197]. To promote virulence, *Verticillium* secretes effector proteins [198]. During its lifecycle, *Verticillium* conidia spread through the plant via the xylem and via micro-sclerotia and melanised mycelium *Verticillium* can survive for long periods in the soil. A locus in tomato conferring resistance to race 1 strains of *Verticillium* has been identified. This locus contains two genes that code for the transmembrane LRR-RLPs Ve1 and Ve2 [197, 199] and it was found that Ve1 is the functional receptor mediating resistance to race 1 strains of *Verticillium* [46], whereas Ve2 appeared to be a non-functional receptor [46, 200]. Strikingly, transfer of *Ve1* from tomato to *Arabidopsis*

resulted in a gain of resistance to race 1 strains of *V. dahliae* [196]. Sequencing of a race 1 and race 2 strain of *V. dahliae* and subsequent comparative genomics revealed a secreted protein, named Ave1 (Avirulence on Ve1 tomato), as the effector being recognized by Ve1 [198]. Intriguingly, Ve1 also mediates resistance to a *Fusarium oxysporum* strain expressing an Ave1 homolog [198].

Important plant components required for Ve1-mediated resistance have been identified using mutants in *Ve1*-transgenic *Arabidopsis* and gene silencing experiments in *Ve1*-expressing tomato. Required components were found to be involved in hormone signalling, such as ETR1 (EThylene Response-1) and JAR1 (Jasmonic Acid Resistant-1) [46, 196], but also in R protein-mediated resistance like NDR1 (Non-race specific Disease Resistance-1), EDS1 (Enhanced Disease Susceptibility-1), NRC1, MEK2 (Mitogen-activated Extracellular signal-regulated Kinase kinase-2), NPR1 (Non-expressor of PR1) and the F-box protein ACIF1 [46, 196]. Interestingly, the RLKs SERK1 and BAK1, are also required for Ve1-mediated resistance [46, 196], suggesting that the LRR-RLP Ve1, similar to other transmembrane receptors involved in defence and development, requires SERK family members for its functionality. Recently it was found that transient co-expression of Ve1 with its matching Ave1 ligand in *N. tabacum* triggers an HR and that Virus-Induced Gene Silencing (VIGS) is possible in different *N. tabacum* cultivars [201]. This opens possibilities for quickly testing components putatively required for Ve1-responses in a transient expression system.

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Aims and Outline of the Thesis

Over the last years much progress has been made on the identification and characterization of *C. fulvum* and *Verticillium* effectors. Furthermore, a number of downstream components of the signalling pathways triggered by effectors and mediated by matching Cf and Ve1 proteins have been identified. However, still relatively little is known regarding the biogenesis of these two LRR-RLPs and the composition of the receptor complex that initiates defence signalling upon ligand perception by these extracellular receptors. For example, although proposed more than ten years ago, biochemical evidence for an interacting RLK or cytoplasmic kinase required for the functionality of Cf and Ve1 proteins remains lacking [47, 163].

The aims of this thesis were to identify and characterize novel proteins interacting with Cf proteins and Ve1, which mediate resistance to two fungal pathogens with different life-styles. The rationale was that these interacting proteins are required for the function of these LRR-RLPs and that their characterisation would shed light on their role in LRR-RLP biogenesis and signalling. To identify novel interacting proteins, a biochemical approach was followed in which the LRR-RLPs were fused to enhanced Green Fluorescent Protein (eGFP) and subsequently subjected to affinity purification. The RLP-eGFP fusion proteins were immunopurified after their expression *in planta* and the immunoprecipitates were analysed by mass-spectrometry. Proteins co-purifying with the LRR-RLPs would subsequently be functionally tested for their requirement in resistance mediated by the Cf proteins and Ve1, and characterised in more detail.

Chapter 2 describes the first successful immunoprecipitations of functional LRR-RLPs and the identification of the BiP and CRT ER-chaperones as interactors of the Cf-4 protein. Both types of chaperones play a role in ER-QC and we studied their role in Cf-4 biogenesis by Virus-Induced Gene Silencing (VIGS). Silencing of individual *BiPs* did not compromise Cf-4-mediated resistance, indicating there is gene redundancy and the biogenesis of functional Cf-4 protein was not hampered, whereas silencing of multiple *BiPs* appeared lethal for *N. benthamiana*. Interestingly, silencing of a specific *CRT3* homolog (*CRT3a*) in tomato severely compromised Cf-4-mediated resistance to an Avr4-secreting strain of *C. fulvum*. Similarly, it was found that silencing of the *N. benthamiana CRT3a* homolog compromises the Cf-4/Avr4-triggered HR. The molecular mechanism behind this observation was uncovered by determining the glycosylation status of the Cf-4 protein upon silencing of the individual *CRTs*. Strikingly, silencing of *CRT3a* severely compromised complex *N*-linked glycosylation of Cf-4, indicating that this decoration is essential for Cf protein function.

In **chapter 3** the requirement of the BiP and CRT ER-QC chaperones for Ve1-mediated resistance to *V. dahliae* is investigated. In contrast to the specific requirement of CRT3a for Cf-4 function, silencing of individual *BiPs* and *CRTs* in almost all cases resulted in

compromised Ve1-mediated resistance of tomato to *V. dahliae*. Remarkably, the Ve1/Ave1-triggered HR in tobacco was not compromised upon silencing of the individual ER-QC chaperones and likewise, Ve1 was still complex *N*-linked glycosylated upon silencing of the *CRTs*. Together, this chapter shows that the BiP and CRT chaperones differentially contribute to resistance mediated by Cf-4 and Ve1.

Chapter 4 describes the identification of the LRR-RLK Suppressor Of BIR1-1 (SOBIR1), and its close homolog SOBIR1-like, as interactors of the LRR-RLPs Cf-4 and Ve1. SOBIR1 was found to constitutively interact with Cf proteins and Ve1, as well as with a number of additional LRR-RLPs involved in defence or in development. Remarkably, SOBIR1 did not interact in co-IPs with the LRR-RLKs FLS2 and BAK1 and neither with two additional LRR-RLKs that were tested. Hence, SOBIR1 appears to specifically interact with LRR-RLPs. Silencing of the genes encoding the SOBIR1 homologs resulted in a reduced Cf-4- and Ve1-triggered HR in *Nicotiana* species and compromised resistance to *C. fulvum* and *V. dahlia*e, respectively, in tomato. Additionally, in contrast to wild-type *Arabidopsis*, an *Arabidopsis sobir1* knock-out mutant expressing Ve1 is not resistant to *V. dahliae* expressing Ave1. It was also observed that knock-down of *SOBIR1* expression in *N. benthamiana* leads to reduced accumulation of the Cf-4 and Ve1 proteins. Hence, SOBIR1 is required for accumulation of these defence-associated LRR-RLPs. Together, the identification of SOBIR1 provides the first evidence that the LRR-RLPs Cf-4 and Ve1 form a complex with an RLK.

Chapter 5 is the general discussion, focussing on the role ER-QC chaperones in plant immunity and in more detail on the role of SOBIR1 as a regulatory LRR-RLK of plant LRR-RLPs. SOBIR1 was found to interact with multiple LRR-RLPs associated with defence or development. These results, combined with the fact that SOBIR1 has known roles in promoting plant defence responses as well as in developmental responses, like floral organ shedding, suggests that this LRR-RLK plays a conserved role in many different signalling processes mediated by LRR-RLPs. These results resemble the observations made for members of the well-studied SERK family of regulatory LRR-RLKs. For example, BAK1 (SERK3) is involved in functioning of receptor complexes mediating MAMP-triggered responses, as well as in receptor complexes that perceive plant brassinosteroid hormones. An overview of plasma membrane-localized receptor complexes in which SERKs and/or SOBIR1 are known to be involved is provided. Together, our data support the conclusion that these two LRR-RLKs both have essential, conserved and distinctive functions in both plant development and innate immune responses.

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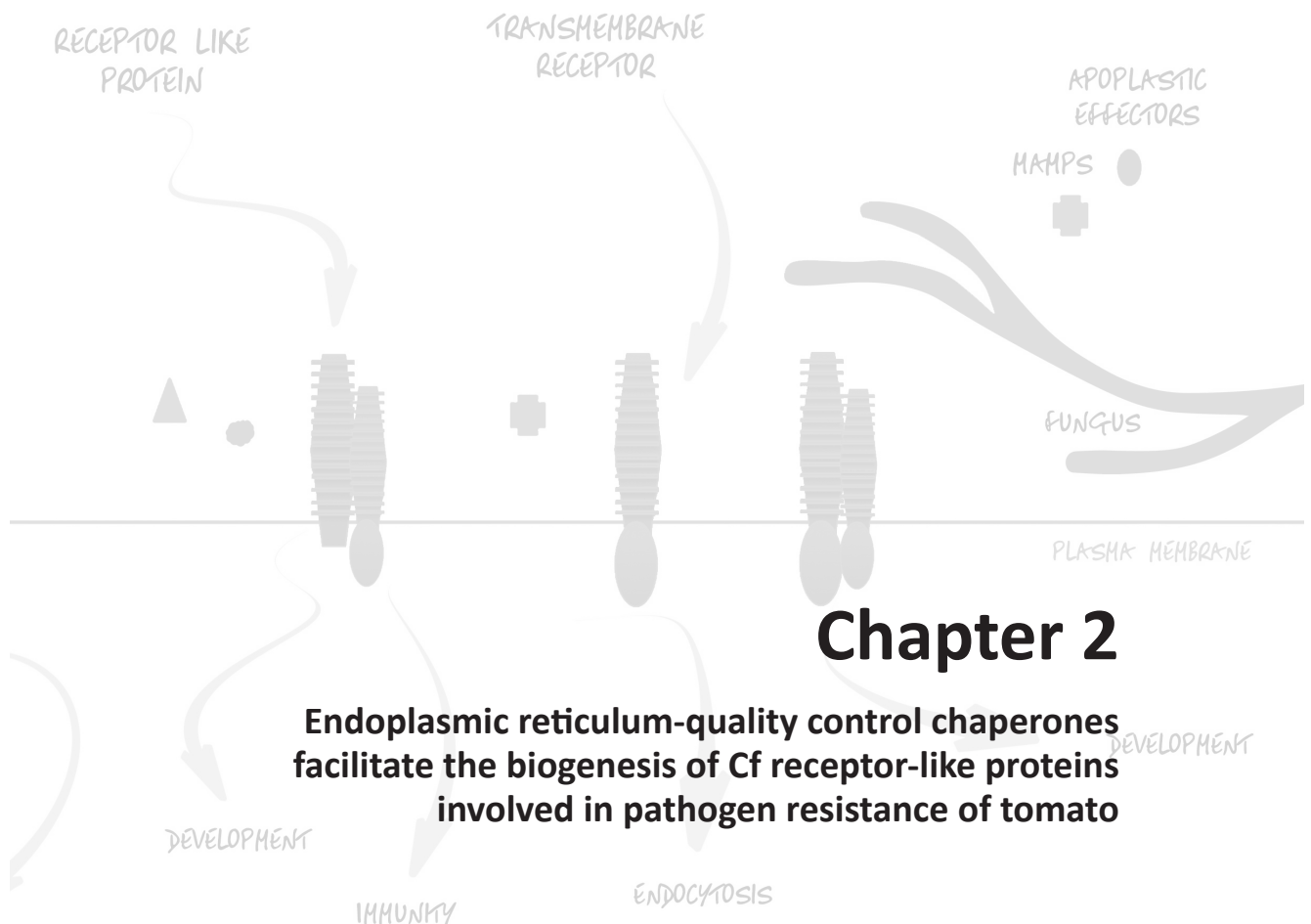
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Chapter 2

Endoplasmic reticulum-quality control chaperones facilitate the biogenesis of Cf receptor-like proteins involved in pathogen resistance of tomato

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Abstract

Cf proteins are Receptor-Like Proteins (RLPs) that mediate resistance of tomato (*Solanum lycopersicum*) to the foliar pathogen *Cladosporium fulvum*. These transmembrane immune receptors, which carry extracellular Leucine-Rich Repeats (LRRs) that are subjected to post-translational glycosylation, perceive effectors of the pathogen and trigger a defence response that results in plant resistance. To identify proteins required for the functionality of these RLPs, we performed immunopurification of a functional Cf-4-eGFP fusion protein transiently expressed in *Nicotiana benthamiana*, followed by mass spectrometry. The Endoplasmic Reticulum (ER) HSP70 Binding Proteins (BiPs) and lectin-type CalReTiculins (CRTs), which are chaperones involved in ER-Quality Control (ER-QC), were co-purifying with Cf-4-eGFP. The tomato and *N. benthamiana* genomes encode four BiP homologs and silencing experiments revealed that these BiPs are important for overall plant viability. For the three tomato CRTs, VIGS targeting the plant-specific *CRT3a* gene resulted in a significantly compromised Cf-4-mediated defence response and loss of full resistance to *C. fulvum*. We show that upon knock-down of *CRT3a* the Cf-4 protein accumulated, but the pool of Cf-4 protein carrying complex-type *N*-linked glycans was largely reduced. Together, our study on proteins required for Cf function reveals an important role for the calreticulin ER chaperone CRT3a in the biogenesis and functionality of this type of RLPs involved in plant defence.

2

INTRODUCTION

Recognition of non-self molecules by immune receptors, of which a class is represented by resistance proteins, initiates plant immune responses leading to resistance to invading pathogens [1, 2]. In tomato (*Solanum lycopersicum*), the extracellular biotrophic fungal pathogen *Cladosporium fulvum* causes leaf mold disease and by the secretion of effector proteins its virulence is promoted [3-7]. Recognition of *C. fulvum* race-specific effector proteins (also referred to as Avirulence (Avr) proteins) by resistant tomato plants is mediated by Cf proteins. Cf proteins are transmembrane proteins that are classified as Receptor-Like Proteins (RLPs), carrying an extracellular Leucine-Rich Repeat (LRR)-domain but lacking an obvious intracellular signalling domain, which is in contrast to Receptor-Like Kinases (RLKs) that possess an intracellular kinase domain [8]. Recognition of an effector of *C. fulvum* by the matching Cf protein triggers the activation of defence responses that eventually results in resistance to *C. fulvum*, a mechanism commonly referred to as effector-triggered immunity [1]. In this interaction, resistance is associated with the Hypersensitive Response (HR), which is a form of localized programmed cell death [1, 8, 9].

The biogenesis of functional transmembrane receptors, which involves proper folding and post-translational modifications such as glycosylation, takes place in the Endoplasmic Reticulum (ER). To prevent secretion of immature immune receptors not competent of ligand binding, to the plasma membrane a strict control mechanism of the different steps in protein maturation is required. ER-Quality Control (ER-QC) is a process that involves several pathways, all ensuring that eventually only mature, correctly folded proteins are transported to their final cellular destination [10]. One important pathway requires the abundant ER Heat Shock Protein (HSP) 70 chaperones, referred to as Binding Proteins (BiPs). These BiPs form a complex with the HSP40-like co-chaperones containing J domains (ERdj3) and Stromal-Derived Factor-2 (SDF2) [11-13]. The BiP complex aids in client protein folding, thereby preventing protein aggregation, and is involved in ER-stress signalling [10]. A second ER-QC pathway involves *N*-linked glycosylation and lectin chaperone-assisted folding of nascent proteins. *N*-linked glycosylation is initiated by addition of a $Glc_3Man_9GlcNAc_2$ oligosaccharide to asparagine (N) residues of *N*-linked glycosylation sites with the consensus sequence (NxS/T), by the OligoSaccharylTransferase (OST) complex. Glucosidase I and II cleave glucose residues from this glycan to leave mono-glucosylated glycans on the client protein. Subsequently, the lectin chaperones CalNeXin (CNX) and CalReTiculin (CRT) are able to fold the client protein [14]. When correctly folded, glucosidase II removes the final glucose residue and the client protein is then translocated to the Golgi apparatus. Proteins that are still not correctly folded are re-glucosylated by the folding sensor UDP-Glucose Glycoprotein glucosylTransferase (UGGT) and are subjected to another round of CNX/CRT-assisted folding. Glycoproteins that are still not properly folded are recognized by specific lectins with mannosidase activity, causing mannose trimming. The loss of mannose moieties

reduces the affinity for CNX/CRT, which is a signal for ER-Associated protein Degradation (ERAD) [10, 15]. Eventually, the last step in ER-QC involves the formation of disulfide bonds between free thiol groups present in the client protein, a modification which is mediated by protein disulfide isomerases [16, 17].

Following ER-QC-assisted folding and glycosylation, proteins are transferred to the Golgi apparatus where the *N*-linked glycans are further modified into complex-type *N*-linked glycans. In plants, in the Golgi a β(1,2)-xylose and α(1,3)-fucose residue are linked to the precursor core glycans of glycoproteins [18]. Eventually, functional RLPs are anticipated to carry complex-type *N*-linked glycans as numerous putative *N*-linked glycosylation sites are for example present in the LRR domains of different Cf proteins [19]. Although the complexity of the *N*-linked glycans remains to be elucidated, mutational analysis of the 22 putative *N*-linked glycosylation sites present in the Cf-9 protein revealed that most of these sites are indeed glycosylated and contribute to Cf-9 activity. Four glycosylation sites are even essential for Cf-9 function and removing all glycans by PNGaseF treatment caused a strong mobility shift, showing that Cf-9 is heavily glycosylated [19, 20]. The observation that functional Cf proteins are abundantly glycosylated indicates that this type of RLPs requires stringent ER-QC for their maturation to biologically active proteins.

A number of studies have addressed the identification of proteins that are required for Cf function. To date, all Cf-interacting proteins were found by yeast-two hybrid analyses, using the C-terminus of Cf-9 as a bait [21, 22]. Attempts to isolate and characterize Cf complexes *in vivo* following a biochemical approach, have failed up till now. To identify factors interacting with Cf proteins, we C-terminally tagged Cf proteins with enhanced Green Fluorescent Protein (eGFP). By transient *Agrobacterium*-mediated expression in *Nicotiana benthamiana*, a Solanaceous plant in which Cf proteins are functional [23], we produced the tagged Cf proteins, allowing immunopurification of Cf-eGFP-containing protein complexes using GFP-affinity beads. We found BiP and CRT ER chaperones as co-purifying proteins, pointing to an important role of ER-QC in the biogenesis of functional Cf proteins. We discovered that, when compared to *Arabidopsis thaliana*, tomato and *N. benthamiana* contain one additional *BiP* gene. Furthermore, the latter two plants carry two *CRT3-like* genes, instead of only one for *Arabidopsis*. Silencing of *CRT3a* resulted in severely compromised Cf-4 functionality in *N. benthamiana* and tomato and we found a strong reduction in complex-type *N*-linked glycosylation of the Cf protein is responsible for this phenotype. Altogether, we show *in vivo* interaction between Cf proteins and ER-QC chaperones upon transient expression of the Cf proteins in *N. benthamiana* and reveal an important role for CRT3a in the biogenesis of a tomato RLP that is involved in resistance to a fungal pathogen.

RESULTS

Cf-eGFP Fusion Proteins are Functional and Cf-4-eGFP is Efficiently Immunopurified from Transiently Transformed *Nicotiana benthamiana* Leaves

We chose eGFP to C-terminally tag various Cf proteins for which the matching effector from *C. fulvum* has been identified (Fig. 1A). We also eGFP-tagged the Cf-like protein Peru2, which is a Cf homolog from *Solanum peruvianum* that shows constitutive activity when expressed in *N. benthamiana* and *N. tabacum* [24].
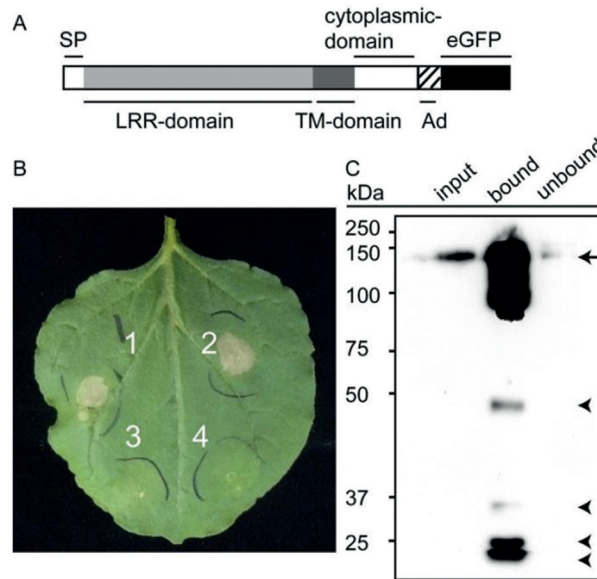
Figure 1. Tomato Cf proteins with a C-terminal eGFP tag are functional and are efficiently immunopurified using GFP-affinity beads. (A) Schematic representation of recombinant Cf proteins C-terminally tagged with eGFP. SP, signal peptide; LRR, leucine-rich repeat; TM, trans membrane; Ad, cloning adapter. (B) Co-expression of Cf-4-eGFP with the *C. fulvum* Avr4 effector by *Agrobacterium*-mediated expression in *N. benthamiana* results in a Cf-4/Avr4-specific HR (1). This HR developed equally fast and had the same intensity as the HR obtained upon co-expression of untagged Cf-4 with Avr4 (2). Cf-4-eGFP and untagged Cf-4 did not trigger an HR when co-expressed with the *C. fulvum* effector Avr9 (3 and 4, respectively). Leaves were photographed three days after *Agrobacterium* infiltration. (C) Immunopurification of Cf-4-eGFP, transiently expressed in combination with the viral silencing suppressor P19 in *N. benthamiana*. A total protein extract of the infiltrated leaf tissue was subjected to immunopurification using GFP-affinity beads. Total proteins (input), immunopurified proteins bound to the beads and eluted by boiling the beads in SDS sample buffer (bound), and proteins remaining in the supernatant (unbound) were separated by 8% SDS-PAGE and subjected to immunoblot analysis using αGFP antibody. The strong band at 140 kDa has the size of Cf-4-eGFP (arrow). Putative Cf-4-eGFP degradation products are observed at 45 kDa, 37 kDa and 20 kDa. A band of 25 kDa is observed at the expected size of free eGFP (arrowheads).

Agrobacterium tumefaciens-mediated transient transformation of the Cf-eGFP proteins, in combination with the matching or a non-matching Avr effector of *C. fulvum* in *N. tabacum*, revealed functionality of the tagged Cfs as an HR occurred when a matching

Cf-eGFP/Avr pair was expressed (Supplemental Fig. S1). An HR was not observed when non-matching Cf/Avr pairs were expressed, whereas the Peru2-eGFP fusion caused a constitutive HR (Supplemental Fig. S1). We also expressed Cf-4-eGFP in combination with Avr4 in *N. benthamiana* and similar results were obtained (Fig. 1B). The time period over which the HR developed and its intensity were similar to the HR caused by non-tagged Cf-4.

We subsequently investigated whether we could immunopurify Cf-eGFP fusion proteins. Therefore, we transiently co-expressed Cf-4-eGFP with the P19 silencing suppressor in *N. benthamiana* and incubated a total protein extract of the infiltrated leaves with GFP-affinity beads. Immunoblot analysis using an αGFP antibody revealed a specific band at about 140 kDa (the molecular weight of Cf-4-eGFP) in the total protein extract (Fig. 1C, input) and a very strong signal in the eluate of the beads (Fig. 1C, bound), indicating successful immunopurification of the Cf-4 fusion protein.

The ER-Resident BiP and CRT Chaperones Co-Purify with Cf-4-eGFP

To identify Cf-4-eGFP co-purifying proteins, *N. benthamiana* was transiently transformed with Cf-4-eGFP and the fusion protein was subjected to immunopurification with GFP-affinity beads. Immunopurified proteins were separated by a short SDS-PAGE run and Coomassie-stained. Two abundant proteins, one of ~140 kDa and one of ~70 kDa, were detected (Fig. 2A) and the bands were excised, subjected to in gel tryptic digestion and the generated peptides were analyzed by mass spectrometry. The identification of peptide sequences originating from Cf-4-eGFP in the 140 kDa band confirmed immunopurification of the Cf protein (Fig. 2B), whereas analysis of the 70 kDa protein revealed several peptides originating from ER luminal Binding Proteins (BiPs), which are highly conserved members of the HSP70 family of chaperones. We identified several peptides originating from different *Nb*BiP isoforms and projected them on the *Sl*BiP1 sequence (Fig. 2C and Supplemental Table S1).

Following an alternative approach, we performed direct on-bead tryptic digestion upon immunopurification of Cf-4-eGFP, revealing that, besides BiPs, the ER lectin-like CalReTiculin (CRT) chaperones co-purified with the Cf-4 protein. Using a database consisting of Solanaceous plant protein sequences, we identified various peptides matching CRT proteins. We projected the identified peptides on the full-length tomato CRT protein sequences and found that a total of six unique peptides matched to the sequence of a tomato homolog of the plant-specific CRT3 class, which we named *Sl*CRT3a (Fig. 2D and Supplemental Table S1). Furthermore, we found one peptide matching another tomato CRT3 homolog, here referred to as *Sl*CRT3b (Fig. 2E and Supplemental Table S1) and three unique peptides matching a tomato CRT2 homolog, *Sl*CRT2 (Fig. 2F and Supplemental Table S1). When we performed the same protocol on leaf tissue of *N.*

benthamiana transiently expressing GUS in the presence of the P19 silencing suppressor, peptides matching to the BiP and CRT chaperones were not identified.
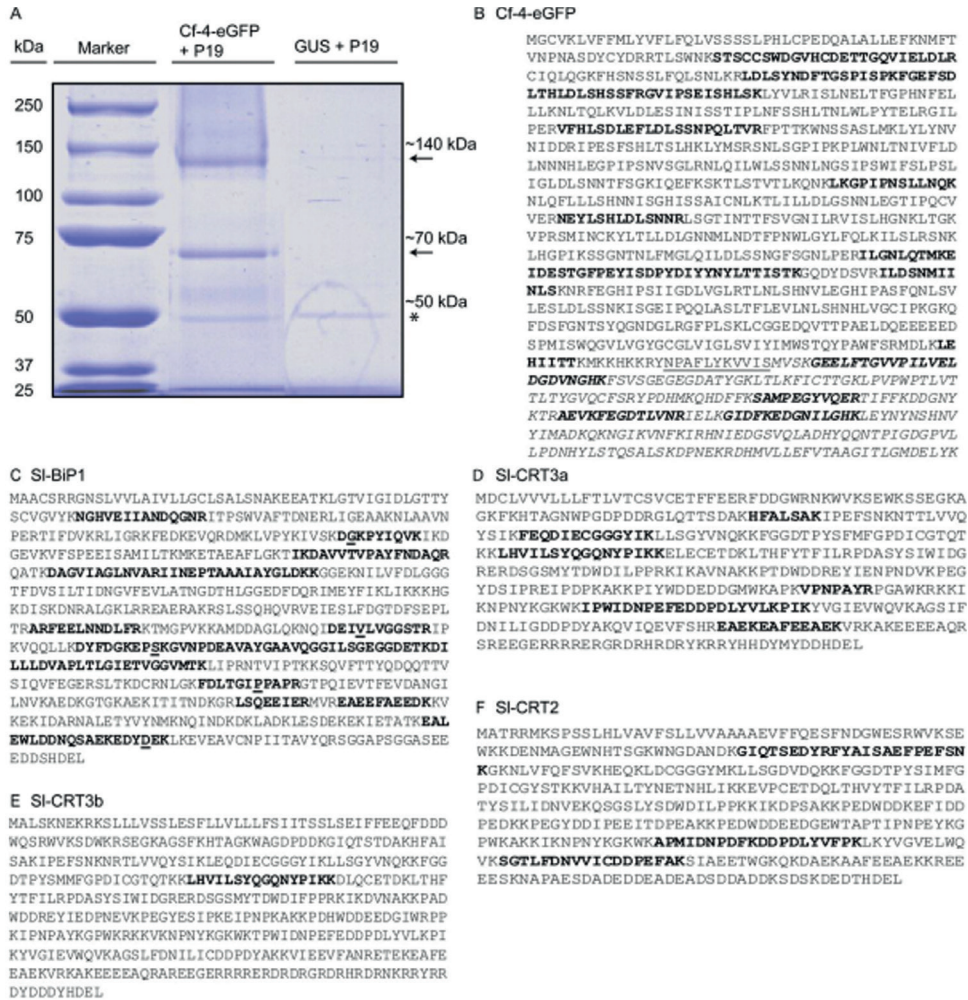
A

Marker / Cf-4-eGFP + P19 / GUS + P19

kDa: 250, 150, 100, 75, 50, 37, 25

~140 kDa, ~70 kDa, ~50 kDa *

B Cf-4-eGFP

MGCVKLVFFMLYVFLFQLVSSSSLPHLCPEDQALALLEFKNMFT
VNPNASDYCYDRRTLSWNK**STSCCSWDGVHCDETTGQVIELDLR**
CIQLQGKFHSNSSLFQLSNLKR**LDLSYNDFTGSPISPKFGEFSD
LTHLDLSHSSFRGVIPSEISHLSK**LYVLRISLNELTFGPHNFEL
LLKNLTQLKVLDLESINISSTIPLNFSSHLTNLWLPYTELRGIL
PER**VFHLSDLEFLDLSSNPQLTVR**FPTTKWNSSASLMKLYLYNV
NIDDRIPESFSHLTSLHKLYMSRSNLSGPIPKPLWNLTNIVFLD
LNNNHLEGPIPSNVSGLRNLQILWLSSNNLNGSIPSWIFSLPSL
IGLDLSNNTFSGKIQEFKSKTLSTVTLKQNK**LKGPIPNSLLNQK**
NLQFLLLSHNNISGHISSAICNLKTLILLDLGSNNLEGTIPQCV
VER**NEYLSHLDLSNNR**LSGTINTTFSVGNILRVISLHGNKLTGK
VPRSMINCKYLTLLDLGNNMLNDTFPNWLGYLFQLKILSLRSNK
LHGPIKSSGNTNLFMGLQILDLSSNGFSGNLPER**ILGNLQTMKE
IDESTGFPEYISDPYDIYYNYLTTISTK**GQDYDSVR**ILDSNMII
NLS**KNRFEGHIPSIIGDLVGLRTLNLSHNVLEGHIPASFQNLSV
LESLDLSSNKISGEIPQQLASLTFLEVLNLSHNHLVGCIPKGKQ
FDSFGNTSYQGNDGLRGFPLSKLCGGEDQVTTPAELDQEEEEED
SPMISWQGVLVGYGCGLVIGLSVIYIMWSTQYPAWFSRMDLK**LE
HIITT**KMKKHKKRYN*PAFLYKVVISMVSK***GEELFTGVVPILVEL
DGDVNGHK**FSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVT
TLTYGVQCFSRYPDHMKQHDFFK**SAMPEGYVQER**TIFFKDDGNY
KTR***AEVKFEGDTLVNR**IELKG***IDFKEDGNILGHK**LEYNYNSHNV
YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVL
LPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK*

C Sl-BiP1

MAACSRRGNSLVVLAIVLLGCLSALSNAKEEATKLGTVIGIDLGTTY
SCVGVYK**NGHVEIIANDQGNR**ITPSWVAFTDNERLIGEAAKNLAAVN
PERTIFDVKRLIGRKFEDKEVQRDMKLVPYKIVSK**DGKPYIQVK**IKD
GEVKVFSPEEISAMILTKMKETAEAFLGKT**IKDAVVTVPAYFNDAQR**
QATK**DAGVIAGLNVARIINEPTAAAIAYGLDKK**GGEKNILVFDLGGG
TFDVSILTIDNGVFEVLATNGDTHLGGEDFDQRIMEYFIKLIKKHG
KDISKDNRALGKLRREAERAKRSLSSQHQVRVEIESLFDGTDFSEPL
TR**ARFEELNNDLFR**KTMGPVKKAMDDAGLQKNQ**IDEIVLVGGSTR**IP
KVQQLLK**DYFDGKEP**SKGVNPDEAVAYGAAVQGGILSGEGGDETKDI
LLLDVAPLTLGIETVGGVMTKLIPRNTVIPTKKSQVFTTYQDQQTTV
SIQVFEGERSLTKDCRNLGK**FDLTGIPPAPR**GTPQIEVTFEVDANGI
LNVKAEDKGTGKAEKITITNDKGR**LSQEEIER**MVR**EAEEFAEEDK**KV
KEKIDARNALETYVYNMKNQINDKDKLADKLESDEKEKIETATK**EAL
EWLDDNQSAEKEDYDEK**LKEVEAVCNPIITAVYQRSGGAPSGGASEE
EDDSHDEL

D Sl-CRT3a

MDCLVVVLLLFTLVTCSVCETFFEEERFDDGWRNKWVKSEWKSSEGKA
GKFKHTAGNWPGDPDDRGLQTTSDAK**HFALSAK**IPEFSNKNTTLVVQ
YSIK**FEQDIECGGGYIK**LLSGYVNQKKFGGDTPYSFMFGPDICGTQT
KK**LHVILSYQGQNYPIKK**ELECETDKLTHFYTFILRPDASYSIWIDG
RERDSGSMYTDWDILPPRKIKAVNAKKPTDWDDREYIENPNDVKPEG
YDSIPREIPDPKAKKPIYWDDEDDGMWKAPK**VPNPAYR**PGAWKRKKI
KNPNYKGKWK**IPWIDNPEFEDDPDLYVLKPIK**YVGIEVWQVKAGSIF
DNILIGDDPDYAKQVIQEVFSHR**EAEKEAFEEAEK**VRKAKEEEEAQR
SREEGERRRRERGRDRHRDRYKRRYHHDYMYDDHDEL

E Sl-CRT3b

MALSKNEKRKSLLLVSSLESFLLVLLLFSIITSSLSEIFFEEQFDDD
WQSRWVKSDWKRSEGKAGSFKHTAGKWAGDPDDKGIQTSTDAKHFAI
SAKIPEFSNKNRTLVVQYSIKLEQDIECGGGYIKLLSGYVNQKKFGG
DTPYSMMFGPDICGTQTKK**LHVILSYQGQNYPIKK**DLQCETDKLTHF
YTFILRPDASYSIWIDGRERDSGSMYTDWDIFPPRKIKDVNAKKPAD
WDDREYIEDPNEVKPEGYESIPKEIPNPKAKKPDHWDDEEDGIWRPP
KIPNPAYKGPWKRKKVKNPNYKGKWKTPWIDNPEFEDDPDLYVLKPI
KYVGIEVWQVKAGSLFDNILICDDPDYAKKVIEEVFANRETEKEAFE
EAEKVRKAKEEEEAQRAREEGERRRRERDRDRGRDRHRDRNKRRYRR
DYDDDYHDEL

F Sl-CRT2

MATRRMKSPSSLHLVAVFSLLVVAAAAEVFFQESFNDGWESRWVKSE
WKKDENMAGEWNHTSGKWNGDANDK**GIQTSEDYRFYAISAEFPEFSN
K**GKNLVFQFSVKHEQKLDCGGGYMKLLSGDVDQKKFGGDTPYSIMFG
PDICGYSTKKVHAILTYNETNHLIKKEVPCETDQLTHVYTFILRPDA
TYSILIDNVEKQSGSLYSDWDILPPKKIKDPSAKKPEDWDDKEFIDD
PEDKKPEGYDDIPEEITDPEAKKPEDWDDEEDGEWTAPTIPNPEYKG
PWKAKKIKNPNYKGKWK**APMIDNPDFKDDPDLYVFPK**LKYVGVELWQ
VK**SGTLFDNVVICDDPEFAK**SIAEETWGKQKDAEKAAFEEAEKKREE
EESKNAPAESDADEDDEADEADSDDADDKSDSKDEDTHDEL

Figure 2. Immunopurification of Cf-4-eGFP reveals ER chaperones as co-purifying proteins. (A) Immunopurification with GFP-affinity beads from *N. benthamiana* leaves transiently expressing Cf-4-eGFP or GUS. After pull-down, beads were boiled in SDS sample buffer and the eluted proteins were separated by SDS-PAGE and Coomassie-stained. Protein bands specific for the Cf-4-eGFP pull-down were present at 140 kDa and 70 kDa (arrows), whereas a 50 kDa protein band also co-purified in the GUS control (asterisk). (B) Coverage map of identified Cf-4-eGFP peptides originating from the protein band migrating at 140 kDa, shown in panel (A). The Cf-4-eGFP protein sequence is shown and underlined is the cloning adaptor sequence that has been inserted between Cf-4 and eGFP. The eGFP sequence is depicted in italics and in bold the identified Cf-4 and eGFP peptides are shown. (C) Coverage map of identified *Nb*BiP peptide sequences originating from the 70 kDa protein as shown in panel (A), projected on the *Sl*BiP1 protein sequence. Sequences of the identified peptides are depicted in bold. Individual amino acids both depicted in bold and underlined indicate the position where the *Nb*BiP sequence differs from the *Sl*BiP1 sequence. (D-F), Coverage maps of *Nb*CRT peptide sequences (depicted in bold) identified upon analysis of total tryptic on-bead digests and projected on the corresponding *Sl*CRT3a (D), *Sl*CRT3b (E) and *Sl*CRT2 (F) protein sequences.

2

BiPs Interact with All Cfs Transiently Expressed in *N. benthamiana*

We studied the tomato *BiP* gene family using the tomato genome sequence (www.solgenomics.net) and identified four genes encoding BiP homologs (one more than in *Arabidopsis*) [25], all containing a typical C-terminal HDEL-type ER-retention signal (Supplemental Fig. S2). We also investigated the *N. benthamiana BiP* family using existing databases to which recently the first draft of the *N. benthamiana* genome sequence was added (www.solgenomics.net), in addition to RNA sequencing data from this plant [26]. This also resulted in the identification of four distinct *NbBiP* genes. However, two *NbBiP* genes, *NbBiP2* and *NbBiP4*, are highly homologous to each other (Supplemental Fig. S2). A phylogenetic tree based on the *Arabidopsis*, tomato and *N. benthamiana* BiP protein sequences is presented in Supplemental Fig. S3A.

To determine whether BiPs only interact with Cf-4 or also with other Cf proteins, we transiently expressed eGFP-tagged Cf-2.2, Cf-4, Cf-4E, Cf-9 and the auto-active Cf-homolog Peru2 all C-terminally fused to eGFP. We were able to express and purify all fusion proteins. However, using αGFP antibody, when expressed in the absence of P19 we detected a much weaker Cf-4-eGFP signal (Figs. 3A and 3B) and only after a longer exposure a Cf-4-eGFP signal was revealed (Fig. 3C). Duplicate blots were incubated with an αBiP antibody, revealing that BiPs co-purified with all Cf proteins, as well as with the auto-active Cf-homolog Peru2 (Fig. 3D). Upon overexposure of the immunoblot, we also detected a BiP signal for Cf-4-eGFP expressed in the absence of P19. So, even at low expression levels we found interaction of the BiPs with Cf-4-eGFP (Fig. 3E). As expected, BiPs did not co-purify with the cytosolic GFP-HemAgglutinin (HA) control fusion protein, a protein which does not pass the ER (Figs. 3F and 3G). Together, these results show that BiPs interact with various Cf proteins and that the BiP ER-QC pathway is used by all of these RLPs.

Silencing of Multiple *BiPs* Causes Lethality in *N. benthamiana*

To investigate the role of the BiP chaperones in Cf-4 biogenesis, we generated Tobacco Rattle Virus (TRV)-based Virus-Induced Gene Silencing (VIGS) constructs to knock-down the encoding genes in *N. benthamiana* expressing *Cf-4*. These plants were then transiently transformed to express Avr4 in order to test whether their responsiveness to this matching effector is compromised. As the tomato genome sequence was already available much earlier than the *N. benthamiana* genome sequence, we used the sequence information of the tomato *BiP* family to generate TRV inserts for VIGS of *BiP1*, *BiP2*, *BiP3* or *BiP4* in *N. benthamiana*. In addition, we generated VIGS fragments simultaneously targeting three *BiPs* (TRV insert *BiP-1, -2* and *-4*) and targeting all four *BiPs* (TRV insert *BiP-1, -2, -3* and *-4*; see Supplemental Fig. S2 and Materials and Methods). Upon examining the *N. benthamiana* genome sequence in combination with the RNA sequencing data [26], we observed that the fragments *BiP1*, *BiP3*, *BiP-1, -2*

and *-4* and *BiP-1*, *-2*, *-3* and *-4* target the corresponding *N. benthamiana* homologs. However, due to the high overall homology between *NbBiP2* and *NbBiP4*, the generated VIGS fragments targeting *BiP2* or *BiP4*, in fact target both genes simultaneously.
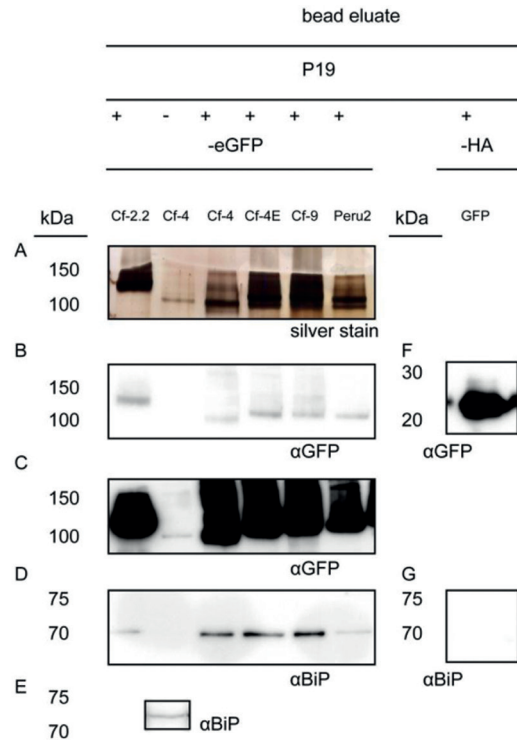

Figure 3. BiP interacts with various Cf proteins. The eGFP-tagged Cf proteins and auto-active Cf-like protein Peru2 and GFP-HA were expressed with P19 (+). Cf-4 was also expressed without P19 (-). Total protein extracts were subjected to immunopurification and proteins present in the eluate of the GFP affinity beads (bead eluate) were separated by 8% SDS-PAGE and silver-stained (A) or analyzed by immunoblotting (B-G). (A) Silver-stained immunopurified Cf proteins and Peru2. (B) Proteins shown in (A), analyzed by immuno blotting using αGFP antibodies, with 1 min of exposure time. (C) Proteins shown in (A), analyzed by immuno blotting using αGFP antibodies, with 5 min of exposure time. Note that Cf-4-eGFP is now also detected when not co-expressed with P19. (D) A duplicate blot as shown in (B) and (C) was incubated with αBiP to reveal BiP as a co-purifying protein. (E) Overexposure of the blot shown in (D). Note that BiP also co-purifies with Cf-4 when expressed in the absence of P19. (F) Immunopurified GFP-HA, as detected by αGFP antibodies. (G) A duplicate blot as in (F), now incubated with αBiP as primary antibody. Note that BiP does not co-purify with GFP-HA. Representative results from three independent experiments are shown.

It has been reported previously that RNA interference-mediated gene silencing of all three *BiP* homologs in *Arabidopsis* is lethal [27]. Therefore we first monitored the *N. benthamiana* plants for aberrant phenotypes over a period of three weeks after inoculation with the different recombinant TRV silencing constructs. VIGS of single *BiPs* did not result in aberrant phenotypes (Supplemental Fig. S4A). Similarly, we did

not observe an aberrant phenotype upon inoculation with TRV:Sl-*BiP2* or TRV:Sl-*BiP4*, which are both targeting *BiP2* and *BiP4* simultaneously (Supplemental Fig. S4A). However, about 10 days after inoculation with the TRV construct targeting all four *BiPs* lethality was observed (Supplemental Fig. S4A). A similar, albeit weaker, phenotype was observed when three *BiPs* (*BiP1*, *BiP2* and *BiP4*) were targeted (Supplemental Fig. S4A). These observations suggest that BiPs act redundantly and that in *N. benthamiana* silencing of multiple *BiPs* severely compromises viability.

To determine whether particular BiPs are specifically required for the biogenesis of Cf-4 proteins, we inoculated *N. benthamiana* expressing *Cf-4* with the different VIGS constructs targeting the individual *BiPs*. TRV:*Cf-4* was included as positive control, whereas TRV:*GUS* served as a negative control. Three weeks after TRV inoculation, fully expanded leaves were transiently transformed to express Avr4 to test the plants for Cf-4 functionality [23]. To check whether the plants were still able to mount an HR, an auto-active variant of the NB-LRR immune receptor Rx (RxD460V) [28] and the pro-apoptotic factor BAX [29] were expressed in the same leaf. Inoculation with the different VIGS constructs targeting the individual *BiPs* did not compromise the Avr4-triggered HR, indicating full Cf-4 functionality. RxD460V- and BAX-triggered cell death also still occurred in these plants (Supplemental Fig. S4B). By contrast, when *Cf-4* itself was targeted a compromised Avr4-triggered HR was observed, whereas the response to RxD460V and BAX was not affected (Supplemental Fig. S4B).

Cf-4 Co-Purifies with the Different Tomato CRT Homologs when Transiently Co-Expressed in *N. benthamiana*

We identified three genes encoding CRT homologs, all carrying an HDEL ER retention motif, in the tomato genome sequence (Supplemental Fig. S5). In *Arabidopsis* also three *CRTs* are present. Interestingly, this species has one *CRT* homolog which is specific for plants (*AtCRT3*) [30-32], whereas tomato has two *CRTs* that are highly homologous to *AtCRT3*. We named these *CRTs SlCRT3a* and *SlCRT3b*. The third tomato *CRT*, *SlCRT2*, is most homologous to *AtCRT1* and *AtCRT2*. We also investigated the *N. benthamiana CRT* family and could clearly distinguish three *NbCRT* homologs. Like tomato, *N. benthamiana* has three *CRT* homologs of which two are most homologous to *AtCRT3* (Supplemental Fig. S5). However, given the fact that we observed single nucleotide polymorphisms in the *N. benthamiana CRTs*, it is very likely that *N. benthamiana* has duplicates of several *CRT* homologs, due to the amphidiploid nature of this plant species. A phylogenetic tree based on the *Arabidopsis*, tomato and *N. benthamiana* CRT protein sequences is presented in Supplemental Fig. S3B.

We generated eGFP-tagged forms of the tomato CRTs (Sl-CRT2-eGFP, Sl-CRT3a-eGFP and Sl-CRT3b-eGFP) and performed co-immunopurifications with Cf-4-Myc to determine

whether the different tomato CRTs interact with this RLP. Confocal laser scanning microscopy revealed that the C-terminal eGFP tag did not affect the subcellular localization of the CRTs, as all CRT-eGFP fusion proteins localized to the ER-network upon transient expression in *N. benthamiana* (Supplemental Fig. S6). Co-expression of the CRT-eGFP fusions with Cf-4-Myc in *N. benthamiana*, followed by immunopurification of the CRTs with GFP-affinity beads, revealed that Cf-4-Myc co-purifies with all three *Sl*CRTs (Figs. 4A and C). The amount of co-purified Cf-4-Myc correlated with the amounts of the different CRTs that were immunopurified, indicating that Cf-4 interacts with all *Sl*CRTs to a similar extent. Cf-4-Myc did not co-purify with the cytosolic GFP-HA protein (Figs. 4B and 4D).
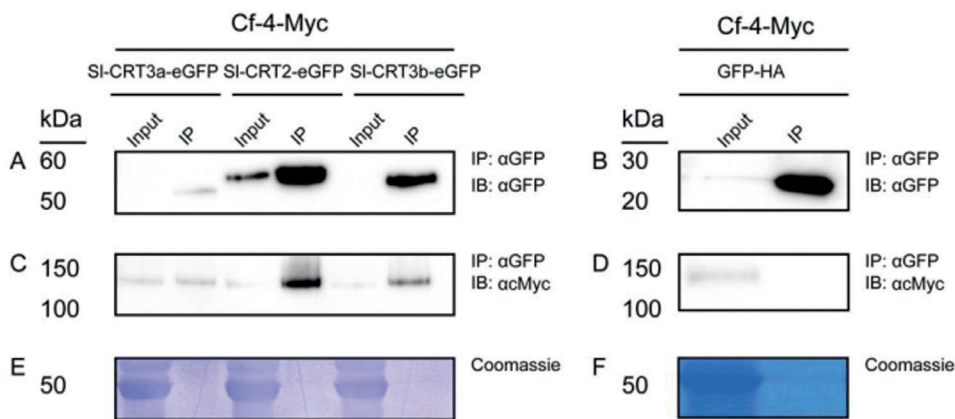

Figure 4. Cf-4 co-purifies with the different tomato CRT homologues. Cf-4-Myc was expressed in combination with Sl-CRT3a-eGFP, Sl-CRT2-eGFP, Sl-CRT3b-eGFP or GFP-HA in *N. benthamiana*. Total protein extracts of the transiently transformed leaf tissue were subjected to immunopurification using GFP-affinity beads. Total proteins (Input) and Immunopurified Proteins (IP) were separated by SDS-PAGE and analyzed by immunoblotting. (A-B) Immunoblots incubated with αGFP antibody, for detection of the immunopurified CRTs (A) or GFP-HA (B). Note that Sl-CRT3a-eGFP is accumulating to much lower amounts of protein than the other Sl-CRTs. (C-D) Immunoblots incubated with αMyc antibody, for detecting co-purified Cf-4-Myc. (E-F) Coomassie-stained blots showing the 50 kDa RuBisCo band present in the total protein extract used as input for the immunopurifications shown in (A-D), confirming equal loading. Representative results from three independent experiments are shown.

*Nb*CRT3a Is Required for Functionality of the Cf-4 Protein in *N. benthamiana*

We next studied the role of the CRTs in Cf-4 function in *N. benthamiana:Cf-4* by VIGS. We generated specific *CRT2*, *CRT3a* and *CRT3b* VIGS constructs based on the sequences of the *CRT* families of tomato and *N. benthamiana*, to establish individual *CRT* gene knock-downs. Upon comparison of the *CRT* sequences of tomato and *N. benthamiana*, we found that for targeting *NbCRT2* and *NbCRT3b*, a VIGS fragment based on the respective orthologous tomato *CRT* sequences can be used (TRV:Sl-*CRT2* and TRV:Sl-*CRT3b*), whereas for targeting *NbCRT3a* a silencing construct based on the

N. benthamiana CRT3a sequence was required (TRV:Nb-*CRT3a*; Supplemental Fig. S5). Q-RT-PCR analysis revealed that the three VIGS constructs knocked-down the expression of the individual *CRTs* (Fig. 5A). We observed that in TRV:Sl-*CRT3b*-inoculated plants, in addition to *NbCRT3b* expression, *NbCRT3a* expression is reduced to some extent. This cross-silencing is likely due to the high overall homology between both *CRT3* sequences (Supplemental Fig. S5). VIGS of the *CRTs* did not result in any aberrant morphological phenotype (Supplemental Fig. S7).
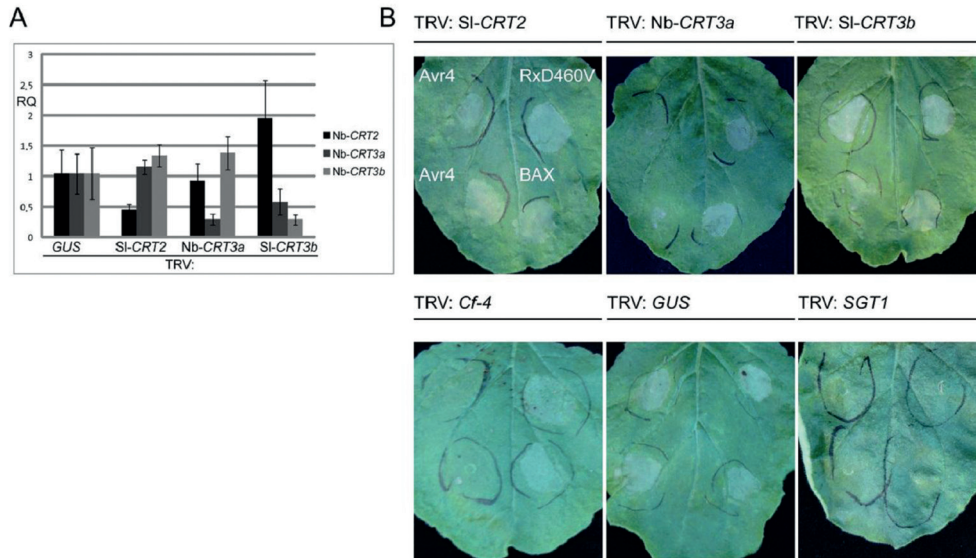
Figure 5. *NbCRT3a* is required for Cf-4 function in *N. benthamiana*. (A) *N. benthamiana* was inoculated with the indicated TRV constructs and relative transcript levels (RQ) of *NbCRT2*, *NbCRT3a* and *NbCRT3b* were determined by Q-RT-PCRs. The expression levels for each *CRT* gene were standardized to their expression levels in the TRV:*GUS*-inoculated plants. All samples were normalized to actin. The standard deviation shows the variation between three technical repeats. (B) Transgenic *N. benthamiana:Cf-4* plants were subjected to VIGS of the *CRTs* by inoculation with the TRV constructs indicated above each panel. TRV:*Cf-4*, TRV:*GUS* and TRV:*SGT1* served as controls. Three weeks after inoculation, Avr4 (in duplicate), auto-active Rx (RxD460V) and BAX were expressed by *Agrobacterium* infiltration in the order indicated in the first panel. Leaves were photographed three days later. Note that similar to *Cf-4*-silenced plants, in the *CRT3a*-silenced plants the response to Avr4 is specifically suppressed. Representative results from three independent experiments are shown. In each independent experiment at least three plants were silenced per TRV construct.

We tested whether silencing of the *CRTs* affects the Cf-4/Avr4-triggered HR. TRV:*Cf-4*, TRV:*GUS* and TRV:*SGT1* inoculations were included as controls. Three weeks after inoculation of *N. benthamiana:Cf-4* plants with the various TRV constructs, leaves were transiently transformed to express Avr4, auto-active Rx (RxD460V) and the pro-apoptotic factor BAX. Interestingly, *CRT3a*-silenced plants showed a strongly reduced Avr4-triggered HR, whereas RxD460V- and BAX-triggered cell death remained unaltered in these plants when compared to the TRV:*GUS*-inoculated plants (Fig. 5B). In fact,

the reduction in responsiveness to Avr4 of the *CRT3a*-silenced plants was similar to that of the TRV:*Cf-4*-inoculated plants. By contrast, *CRT2*- and *CRT3b*-silenced plants still mounted the Avr4-triggered HR (Fig. 5B). Silencing of *SGT1* resulted in loss of the response to all transiently expressed proteins (Fig. 5B). Together, these results indicate that *NbCRT3a* is essential for functionality of the Cf-4 protein in *N. benthamiana*.

SlCRT3a Is Required for Full Cf-4-Mediated Resistance of Tomato to *C. fulvum*

The compromised Cf-4-mediated HR due to knock-down of *CRT3a* expression in *N. benthamiana* prompted us to test whether *CRT3a* is also required for Cf-4-mediated resistance of tomato to an Avr4-producing strain of *C. fulvum*. *Cf-4*-expressing tomato plants were inoculated with the TRV:Sl-*CRT2* and TRV:Sl-*CRT3b* constructs and a TRV construct based on the tomato *CRT3a* sequence (TRV:Sl-*CRT3a*) (Supplemental Fig. S5). Three weeks later the plants were inoculated with a strain of *C. fulvum* secreting Avr4 and transgenic for the *GUS* reporter gene. After another two weeks leaflets were stained for GUS activity to reveal fungal colonization of the leaf tissue. As controls, Cf-4 tomato plants inoculated with TRV:*Cf-4* and TRV:*GUS* were included. We also included fully susceptible MM-Cf-0 tomato plants in each disease assay.

Strikingly, VIGS of *CRT3a* resulted in compromised Cf-4-mediated resistance to *C. fulvum* when compared to TRV:*GUS*-inoculated plants, as observed by the blue spots on the tomato leaflets, indicating fungal colonization of the leaf mesophyll (Fig. 6A). Resistance appeared to be compromised to a level more or less similar to that of Cf-4 tomato silenced for the *Cf-4* gene itself. Leaflets of TRV:Sl-*CRT2*- and TRV:Sl-*CRT3b*-inoculated plants did not show enhanced *C. fulvum* growth when compared to the TRV:*GUS* control (Fig. 6A). Quantitation of the amount of fungal colonization revealed that leaflets of TRV:Sl-*CRT3a*- and TRV:*Cf-4*-inoculated plants are significantly more intensely colonized by *C. fulvum*, than leaflets of the other plants (Fig. 6B).

Cf Proteins Contain Complex-Type *N*-linked Glycans

Having established that knock-down of *CRT3a* gene expression compromises both Cf-4-mediated HR and resistance, we wanted to investigate the molecular basis of this observation. As CRTs are specifically involved in folding and maturation of glycoproteins, we reasoned that silencing of *CRT3a* results in compromised CRT3a-assisted folding, thereby affecting the transport of the Cf-4 protein to the Golgi, which is the cellular compartment where complex-type glycosylation takes place. Hampered entry into the Golgi, then leads to a reduced pool of mature Cf protein containing complex-type *N*-linked glycans. First, we wanted to confirm that functional eGFP-tagged Cf proteins carry complex-type *N*-linked glycans. For this we made use of a polyclonal αHRP antibody directed against HorseRadish Peroxidase (HRP), which is a heavily glycosylated protein containing many complex-type *N*-linked glycans that act as antigens. As a result,

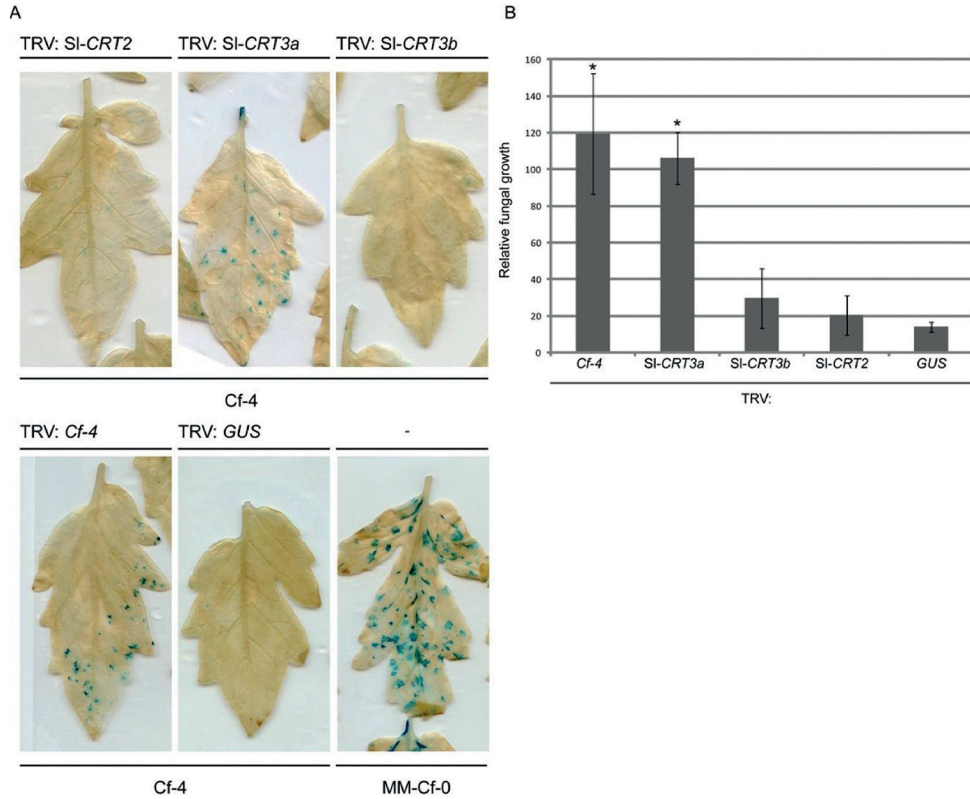the αHRP antibody cross-reacts with proteins that are also subjected to this type of glycosylation [33].

Figure 6. *Sl*CRT3a is required for full Cf-4-mediated resistance of tomato to *C. fulvum*. (A) Cf-4 tomato was inoculated with the indicated TRV constructs and non-inoculated MM-Cf-0 was included as a *C. fulvum*-susceptible control. Three weeks after TRV inoculation of the Cf-4 plants, leaflets of all plants were inoculated with a strain of *C. fulvum* secreting *Avr4* and transgenic for the *GUS* gene. Two weeks later, leaflets were stained for GUS activity to detect fungal colonization, visible as blue-stained spots. (B) Quantification of the colonization by *C. fulvum* of leaflets of Cf-4 tomato inoculated with the different TRV constructs by the ImageJ plug-in Phenotype Quant. The average relative fungal colonization in three independent experiments as shown in (A) was calculated and the error bars represent the standard error of mean (SEM). For each experiment about 30 leaflets were scanned and examined for *C. fulvum* colonization by the quantification software. Asterisks indicate a statistically significant difference as compared to the TRV:*GUS*-inoculated plants (one-way ANOVA, p<0.05). Three independent experiments were performed and representative results are shown.

We immunopurified eGFP-tagged Cf-2.2, Cf-4, Cf-4E, Cf-9 and Peru2 from transiently transformed *N. benthamiana* leaves and after SDS-PAGE and blotting, one blot was incubated with αGFP antibody to detect the total amounts of immunopurified Cf proteins, whereas a duplicate blot was incubated with the αHRP antibody. Incubation with αGFP revealed that similar amounts of the Cf proteins were immunopurified (Fig.

7A). Incubation with αHRP antibody resulted in a clear signal at the expected size of the different Cf proteins, indicating that they all carry complex-type *N*-linked glycans (Fig. 7B). Interestingly, Cf-2.2 and Peru2 show a remarkably stronger signal than the other Cf proteins (Fig. 7B). On this blot, the detected complex glycosylated protein fraction most likely represents the pool of mature and biologically active transiently expressed Cf proteins.
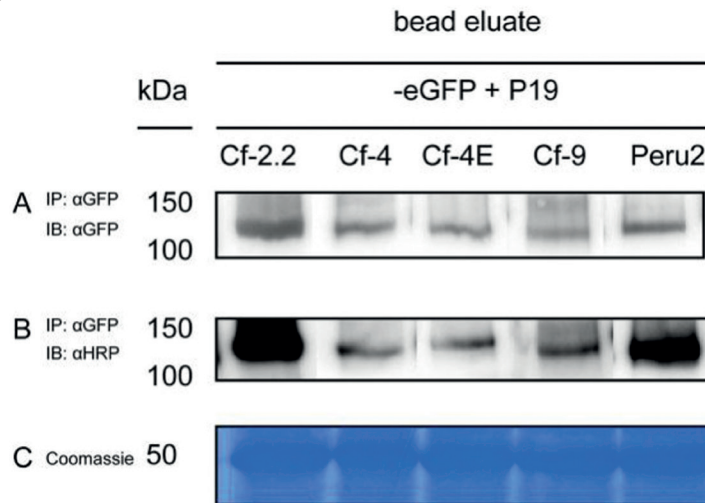

Figure 7. Transiently expressed Cf proteins contain complex-type *N*-linked glycans. Cf proteins and auto-active Peru2, all fused to eGFP, were transiently co-expressed with P19 in *N. benthamiana* and immunopurified using GFP-affinity beads. The bead eluate was divided into two equal volumes and proteins were subjected to SDS-PAGE, followed by immunoblotting. (A) Immunoblot incubated with αGFP antibody to reveal the amounts of purified eGFP-tagged Cf protein. (B) A duplicate of the blot presented in (A), but now incubated with αHRP to show the pool of complex glycosylated Cf protein. (C) Coomassie-stained blot of the total protein extracts used as input for the immunopurifications showing the 50 kDa RuBisCo band to confirm equal loading. Representative results from three independent experiments are shown. For clarity, lanes from the same immunoblot and with the same exposure time were reordered.

NbCRT3a Silencing in *N. benthamiana* Hampers Cf-4 Complex Glycosylation

To investigate whether *NbCRT3a* knock-down indeed reduces the pool of mature Cf protein containing complex-type *N*-linked glycans, we inoculated *N. benthamiana* with the different *CRT* VIGS constructs, including TRV:*GUS* as a control. We then expressed Cf-4-eGFP in silenced leaves, followed by Cf-4 immunopurification, SDS-PAGE and immunoblotting. We determined the total amount of purified Cf-4 protein by incubation with αGFP antibody, whereas the relative amount of complex glycosylated Cf-4-eGFP protein was determined using αHRP antibody. The αGFP blot revealed that we purified similar amounts of Cf-4 protein from different *CRT*-silenced plants (Fig. 8A). Strikingly, VIGS of *CRT3a* almost completely abrogated the accumulation of Cf-4 carrying complex-type glycans (Fig. 8B). This phenomenon was also observed upon VIGS of *CRT3b*, albeit to a much lower extent (Fig. 8B). The pool of Cf-4 carrying complex-type glycans was not

affected when *CRT2* was knocked-down (Fig. 8B). Taken together, these results indicate that *CRT3a* is the most important lectin-like chaperone involved in Cf-4 protein folding, allowing subsequent transport of the protein to the Golgi apparatus for the generation of mature, complex glycosylated Cf-4 proteins.
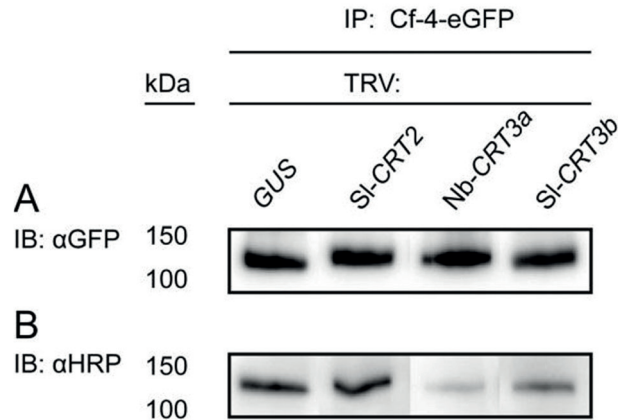
Figure 8. VIGS of *CRT3a* results in a reduction of the pool of Cf-4 protein carrying complex type *N*-linked glycans. Cf-4-eGFP was transiently expressed in *N. benthamiana* leaves, silenced for the different *CRTs* as indicated above the panels. Two days after *Agrobacterium* infiltration, Cf-4-eGFP was immunopurified. The eluate from the beads was divided into two equal volumes and proteins were subjected to SDS-PAGE and immunoblotting. (A) Immunoblot incubated with αGFP antibody to show the total amounts of immunopurified Cf-4-eGFP. (B) A duplicate of the blot presented in (A), but now incubated with αHRP antibody to reveal the pool of mature Cf-4 carrying complex type glycans. Representative results from three independent experiments are shown. For clarity, lanes from the same immunoblot and with the same exposure time were reordered.

DISCUSSION

ER-QC Chaperones Are Involved In Maturation of Transmembrane Immune Receptors
Here we show that the ER chaperones BiP and CRT are *in planta* interactors of Cf proteins (Figs 2,3 and 4) and that CRT3a is the most important calreticulin lectin-type chaperone required for the biogenesis and maturation of complex glycosylated Cf proteins (Fig. 8). Several *Arabidopsis* mutants in the ER-QC pathway were recently identified to be immunocompromised in bacterial infections [13, 34, 35]. Mutants in the folding sensor UGGT, the ERD2b HDEL-receptor and CRT3 were affected in the accumulation of the RLK EFR, which is the receptor for the bacterial Microbe-Associated Molecular Pattern (MAMP) EF-Tu (elf18/26), and subsequent elf18-induced responses [13, 34-37]. Interestingly, *crt1* and *crt2* double mutants were hardly affected in elf18-induced responses, hence it was concluded that from the three *Arabidopsis* CRTs, CRT3 is the

most important homolog for the biogenesis of functional EFR [34, 35]. *Arabidopsis crt3* knock-out mutants were more susceptible to *Pseudomonas syringae* pv tomato DC3000 than *efr* mutants, suggesting that multiple immune receptors require CRT3-mediated ER-QC [35]. In line with this, we observed that silencing of a particular *CRT3* homolog in *N. benthamiana* and tomato, *SlCRT3a*, compromises Cf-4-mediated defence responses. This suggests that Cf-4 is a client of this CRT3 homolog.

Upon silencing of *CRT3a* in *N. benthamiana* we did not observe a reduced accumulation of the Cf-4 protein, despite of compromised Cf-4-mediated responses and a nearly complete loss of the protein pool carrying complex-type *N*-linked glycans (Fig. 8). This suggests that due to lower CRT3a levels, Cf-4 cannot maturate in the Golgi apparatus and therefore does not undergo the full glycosylation required for its functionality. Immature MAMP-receptors are prevented to reach the plasma membrane where they perceive their cognate MAMPs [38]. Hence, Cf-4 transport to the plasma membrane may also be affected when the amount of CRT3a lectin chaperone is limiting. Cf-4 functionality could also be lost because the under-glycosylated Cf-4 protein cannot perceive the Avr4 ligand anymore or has become unstable upon exposure of the extracellular LRRs, which are normally highly glycosylated, to the apoplast.

The reason that we did not observe reduced Cf-4 protein accumulation upon *CRT3a* silencing could be due to the fact that VIGS does not completely knock-out *CRT3a* expression (Fig. 5A). Interestingly, weakly defective *crt3* mutant plants accumulate EFR protein to normal levels, despite the hampered functionality of the MAMP receptor [34]. However, in these mutants elf26 ligand binding to EFR was greatly reduced. Hence, it was speculated that receptor abundance control and quality control can be uncoupled and only the latter was hampered in weak *crt3* mutant plants. In that case our *CRT3a*-silenced *N. benthamiana* plants resemble the weak *crt3 Arabidopsis* mutant. Lowered *CRT3b* expression levels also reduced the Cf-4 pool carrying complex-type *N*-linked glycans to some extent (Fig. 8). As CRT3a and CRT3b are highly homologous (Supplemental Figs. 3B and 5), VIGS of *CRT3b* also somewhat lowered the expression of *CRT3a* due to cross-targeting by the *CRT3b* insert (Fig. 5A). Nevertheless, the mature pool of Cf-4 was sufficiently large and there was no effect on its function (Figs. 5, 6 and 8). We noticed an upregulation of *CRT2* transcription in plants silenced for *CRT3b* (Fig. 5A). This upregulation might be required to compensate for the suppression of several cellular functions of the CRT3s. However, it is unlikely that higher *CRT2* expression levels affect the Cf-4/Avr4-mediated HR. As specific silencing of *CRT3a* strongly suppresses the Cf-4-mediated HR (Fig. 5B) and in this plant *CRT2* expression levels (and also *CRT3b* expression levels) are very similar to the TRV:GUS-inoculated plants, the sufficiently high *CRT3a* expression levels in the *CRT3b*-silenced plant are the reason for an unaffected Cf-4/Avr4-mediated HR (Fig. 5A). In agreement with this, silencing of *CRT2* also does not lead to a reduced Cf-4-mediated HR

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Arabidopsis mutants in the BiP co-chaperone SDF2 were also compromised in EFR signalling due to reduced accumulation of mature EFR protein [13]. In addition to affecting EFR levels, also the accumulation of the RLK FLS2 (Flagellin Sensing-2), responsible for recognition of the bacterial MAMP flagellin and its elicitor-active epitope flg22 [39], was affected to some extent [13]. In rice, Os-BiP3 was reported to interact with the RLK XA21, responsible for resistance to *Xanthomonas oryzae* pv. *oryzae* [40]. Silencing of Os-*BiP3* did not lead to loss of XA21-mediated resistance in rice. However, over-expression of Os-*BiP3* resulted in reduced resistance to the bacterium due to induction of ER-stress and enhanced XA21 proteolysis [40]. It remains to be elucidated whether over-expression of one or more *Sl*BiPs will affect resistance of tomato to *C. fulvum*. Interestingly, two BiP chaperones were found to accumulate during the Cf-4/ Avr4-triggered HR in tomato, suggesting that upregulation of BiPs is required for Cf-4-mediated resistance [41].

Recently, CRT2 and CRT3a of *N. benthamiana* were reported to be involved in effector-triggered immunity. Silencing of *Nb*CRT2 and *Nb*CRT3a reduced the accumulation of the membrane-localized, Induced Receptor Kinase (IRK) that is involved in immunity to Tobacco Mosaic Virus (TMV) mediated by the NB-LRR-type immune receptor *N* [42]. This shows that the IRK requires multiple CRTs for full functionality and accumulation. In addition to a role as a chaperone, *Arabidopsis* CRT2 was shown to regulate salicylic acid-dependent expression of defence genes, with its C-terminal calcium-binding domain and amino acid residue H173 in its N-terminal chaperone domain playing an important role in this regulation [43]. We found that CRT3a is specifically required for the maturation of Cf proteins to functional immune receptors. Together with the role of ER chaperones in EFR/FLS2-mediated MAMP-responses and *N*-mediated resistance, we show that ER-QC is an essential layer of plant immunity, both in MAMP-triggered immunity as well as in effector-triggered immunity.

Regarding the HR assays in *N. benthamiana*:*Cf-4* inoculated with the various TRV constructs, care has to be taken with interpreting the results as Avr4 is expressed by *Agrobacterium* infiltration. The secreted Avr4 protein may also require ER chaperone-assisted folding and this process could also be affected upon VIGS of the different ER-QC chaperones. However, this is unlikely to be the case for the following reasons. Avr4 has only one putative *N*-linked glycosylation site which is not required for Avr4 function as an effector triggering Cf-4-mediated HR [44]. Hence it is unlikely that Avr4 folding heavily depends on the CRTs. Furthermore, a clear decrease in the Cf-4 pool carrying complex *N*-linked glycans was observed upon *CRT3a* silencing in *N. benthamiana*, coinciding with compromised Avr4-triggered HR. Most importantly, in Cf-4-containing tomato, silencing of *CRT3a* leads to compromised resistance to a strain of *C. fulvum* that secretes Avr4 directly into the apoplast upon colonization of the leaflets. We can therefore exclude that hampered folding and/or translocation of Avr4 to the apoplast causes the suppressed HR in *CRT3a*-silenced *N. benthamiana:Cf-4*.

Proper Complex Glycosylation is Required for Cf Protein Function

It was shown earlier that transiently expressed Cf-9 protein is heavily glycosylated on its extracellular LRRs. Furthermore, mutational analysis of the individual *N*-linked glycosylation sites in the LRR domain revealed that all sites, except one, are indeed glycosylated [19, 20]. Glycosylation was found to be required for Cf-9 functionality, as introduction as well as removal of *N*-linked glycosylation sites reduced the Avr9-triggered HR [20]. The complexity of the *N*-linked glycans was not determined in previous experiments. Here we show by using an αHRP antibody that binds to β(1,2)-xylose and α(1,3)-fucose residues [33], that all Cf proteins contain complex-type glycans that are decorated with these residues.

Studies on EFR and FLS2 revealed that nearly all glycosylation sites of these receptors are also being occupied [38, 45]. Mutations in STT3A, a member of the OST complex, led to a loss of EFR-mediated immune responses and elf26 ligand binding, but did not affect FLS2-mediated responses [13, 34, 38]. Similar to the effects of specific mutations of glycosylation sites in Cf-9, *N*-linked glycosylation at specific residues was also found to be important for EFR function. For example, a single mutation at a conserved *N*-linked glycosylation site at position N143 results in an EFR mutant that is unable to bind elf26 and only accumulates to low levels [38]. Recently, two additional glycosylation sites (N342 and N366, respectively) were found to be crucial for EFR functionality [45]. Strikingly, FLS2 functionality is much less dependent on ER-QC and *N*-linked glycosylation [38, 45]. This was explained by proposing that EFR, in an evolutionary perspective, is relatively "younger" than FLS2. Such "young" immune receptors do not easily fold by themselves and are expected to require more stringent ER-QC for their biogenesis [34, 35, 38, 46].

Our data show that Cf proteins also rely heavily on ER-QC, suggesting that also these RLPs have only evolved recently. Indeed, *Cf* genes are arranged in large genomic clusters in which during meiosis cross-overs occur that rapidly generate new Cf-variants [47-49]. When these new Cf proteins detect novel effectors they will be retained in the population. However, these new receptors have not yet been selected for proper intrinsic stability and therefore they heavily rely on ER-QC for their folding and functionality.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tobacco (*Nicotiana tabacum*), wild-type *Nicotiana benthamiana* and transgenic *N. benthamiana:Cf-4* [50] were grown in climate chambers under 16 h of light (150 µmol m^{-2} s^{-1}) at 25˚C and 8 h of darkness at 21˚C, at a relative humidity of ~75%. Tomato (*Solanum lycopersicum*), transgenic for the *Cf-4* resistance gene (Hcr9-4D) [51] were grown in the greenhouse under 16 h of light (100 W m^{-2} supplemental light when the

intensity dropped below 150 W m^{-2}) at 21°C and 8 h of darkness at 19°C, at a relative humidity of ~75%.

Construction of Binary Vectors for *Agrobacterium*-Mediated Expression
The sequences of all oligonucleotide primers used in this study can be found in Supplemental Table S2. Full length *Cf-2.2* was amplified by PCR on cDNA obtained from *Cf-2.2* expressing tomato using primers to13 and to14. *Cf-4* was amplified from plasmid pRH46 [23] using primers to11 and to12 and *Peru2* was amplified from pWL111.5 [24] using primers to22 and to23. PCR fragments of *Cf-2.2*, *Cf-4* and *Peru2* were subsequently cloned into pDONR™201 using Gateway® BP clonase II™ (Invitrogen). *Cf-4E* was amplified with primers to129 and to130 from plasmid pRH471. *Cf-9* was obtained from pRH21 [23] using primers to128 and to129. PCR fragments of *Cf-4E* and *Cf-9* were cloned into pENTR™/D-Topo® (Invitrogen). *SlCRT2*, *SlCRT3a* and *SlCRT3b* were amplified from tomato cDNA using primer combinations to197 and to198, to195 and to196 and to199 and to200, respectively, and cloned into pENTR™/D-Topo®. All pDONR™201 and pENTR™/D-Topo® constructs were sequenced to verify the sequence of the fragments and subsequently fragments were transferred to the binary vector pBIN-KS-35S::-GWY-eGFP (Sol 2095; for C-terminally tagging with eGFP) or pGWB20 [52]), using Gateway® LR Clonase II™, thereby generating pBIN-KS-35S::Cf-2.2-eGFP (Sol 2738), pBIN-KS-35S::Cf-4-eGFP (Sol 2701), pBIN-KS-35S::Cf-4E-eGFP (Sol 2737), pBIN-KS-35S::Cf-9-eGFP (Sol 2736), pBIN-KS-35S::Peru2-eGFP (Sol 2739), pGWB20-Cf-4-Myc (Sol 2783), pBIN-KS-35S::Sl-CRT2-eGFP (Sol 2790), pBIN-KS-35S::Sl-CRT3a-eGFP (Sol 2789) and pBIN-KS-35S::Sl-CRT3b-eGFP (Sol 2791). Binary vectors used to express non-tagged Cf-4 and Cf-9 were pRH48 and pRH21, respectively [23]. All binary vectors were transformed to *Agrobacterium tumefaciens* strain C58C1, carrying helper plasmid pCH32, for *Agrobacterium* infiltration assays. *Agrobacterium* infiltrations were performed as described previously [23].

Immunopurifications of Transiently Expressed Tagged Proteins
Leaves of six-week-old *N. benthamiana* plants were transiently transformed by *Agrobacterium*-mediated expression with pBIN-KS-35S::Cf-eGFP constructs (or the controls pBIN61-GUS or pBIN61-GFP-HA) and the P19 silencing suppressor [53] in a 1:1 ratio at a final O.D.$_{600}$=1 for each construct. For the co-immunopurifications of Cf-4-Myc with the different Sl-CRTs-eGFP fusion proteins, constructs were infiltrated in a 1:1 ratio without P19 at an O.D.$_{600}$=1 of each construct. For the immunopurification of Cf-4-eGFP from TRV-inoculated plants Cf-4-eGFP was infiltrated without P19 at an O.D.$_{600}$=1. Two days after *Agrobacterium* infiltration, leaves were frozen in liquid nitrogen and ground to a fine powder using mortar and pestle. Proteins were extracted using RIPA extraction buffer (150 mM NaCl, 1.0% IGEPAL® CA-630 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), plus one complete protease inhibitor tablet (Roche) per 50 ml extraction buffer (1 gram of leaf tissue (fresh weight) per 2 mL of extraction buffer was

used). Samples were centrifuged at 4°C for 15 min at 18,000g and the supernatant was transferred to a new tube. For mass spectrometry analysis of proteins co-purifying with the Cf proteins, 10 mL of total protein extract was incubated at 4°C for 3 hours with 60 µL (50% slurry) of GFP-trap_A® beads (Chromotek). For other immunopurifications, 2 mL of total protein extract was incubated at 4°C for 1 hour with 15 µL (50% slurry) of the GFP-trap_A® beads. After incubation, beads were collected by 1,000g centrifugation and subsequently washed for 5 times in 1 mL extraction buffer. After each wash step the beads were collected by 1,000g centrifugation. For SDS-PAGE, beads were boiled for 5 min in 60 µL, respectively 15 µL, of 2x SDS loading buffer after the final wash step, and after centrifugation at 18,000g for 5 min the supernatant was loaded on gel.

Tryptic Digestion of Immunopurified Proteins

Immunopurified proteins were separated by 7% SDS-PAGE, after which the gel was stained with SimplyBlue™ Safestain Coomassie solution (Invitrogen) for 30 minutes and shortly destained using 10% ethanol/7.5% acetic acid (v/v) in water. For analysis on the Synapt MS (Waters), protein bands were excised from the gel and subjected to in-gel tryptic digestion. Gel slices were destained in 40% ACN in 0.1 M ammonium bicarbonate for 30 min and after air-drying by vacuum centrifugation (Savant Speedvac, USA), trypsin (10 ng/µL; sequence grade modified, Promega, WI, USA) was added (completely covering the gel slice), followed by overnight incubation at 37°C. Tryptic peptides were subsequently extracted with 50% ACN containing 0.1% TriFluoroAcetic acid (TFA) (Fluka, Buchs, Switzerland) and air-dried by vacuum centrifugation.

In an alternative approach, GFP-trap_A® beads used for the immunopurification were not boiled in SDS loading buffer to release the proteins, but the purified proteins were subjected to on-bead tryptic digestion. Hereto, beads were washed twice with 50 mM ammonium bicarbonate buffer (pH 8), after which the beads were incubated for 1 hour at 60°C with 50 mM DTT in 50 mM ammonium bicarbonate. Subsequently, 50 mM iodoacetamide in 50 mM ammonium bicarbonate was added and beads were incubated at room temperature in the dark for another hour. Next, 50 mM cysteine was added and directly thereafter 1 µL of trypsin (Roche) solution (0.5 µg, in 1 mM HCl) was added to the solution. Beads were incubated overnight at 20°C, after which the digestion was terminated by adding TFA to a final concentration of 0.5% (v/v). For analysis on the Orbitrap XL (Thermo Scientific), proteins were stored at -20°C until further analysis. For analysis on the Synapt MS (Waters), after centrifugation at 15,000g for 10 min, the peptides were cleaned by binding them to a SupelClean™ LC-18 1 ml SPE column (Supelco) equilibrated with 0.1% TFA. Peptides were then eluted with 60% ACN containing 0.1% formic acid (Merck), were dried down by vacuum centrifugation and dissolved in 20 µL of 0.1 M ammonium formate (pH 10) prior to 2-D LC-MS analysis. For technical details on protein identification by mass spectrometry on the Synapt MS and Orbitrap XL and subsequent data processing we refer to the supplementary materials and methods.

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Phylogenetic Analysis of BiP and CRT Families

Phylogenetic analysis of BiP and CRT sequences was performed using the online web service Phylogeny.fr at www.phylogeny.fr [54]. We used MUSCLE for multiple sequence alignments and PhylML for tree building. TreeDyn was used for tree rendering.

Confocal Microscopy

Confocal microscopy was performed on *Agrobacterium* infiltrated *N. benthamiana* leaves, employing a Carl Zeiss LSM 510 META confocal microscope equipped with a Plan-Apochromat 63x/1.4 Oil DIC objective. eGFP and chlorophyll were excited using an argon laser beam at 488 nm and emission from eGFP and chlorophyll was detected using the 505-530 nm band-pass filter and the 650 long-band filter, respectively.

Immunoblotting and Silver Staining

Immunopurified proteins were separated by 8% SDS-PAGE and transferred to an Immun-Blot® PVDF membrane (Bio-Rad) using overnight electro blotting at 25V. The membrane was incubated with blocking solution containing 5% skimmed milk powder and, to detect GFP fusion proteins, blots were incubated with 1:5,000 diluted αGFP-HRP (Anti-GFP-HRP, 130-091-833, MACS antibodies). For detection of BiP, blots were first incubated with 1:1,000 diluted αBiP (BiP-95, sc-33757, Santa Cruz) and subsequently with 1:2,000 diluted Goat anti Rabbit-HRP (Anti-Rabbit IgG–HRP, A9169, Sigma). For detection of complex glycans, blots were first incubated with 1:2,000 diluted αHRP (Rabbit Anti-Horseradish Peroxidase, 323-005-021, Jackson ImmunoResearch) and then incubated with 1:2,000 diluted Goat anti Rabbit-HRP. Detection of Myc was performed by 1:2,000 diluted cMyc (cMyc 9E10, sc-40, Santa Cruz) and subsequently 1:2,000 diluted anti-Mouse Ig-HRP (Amersham). In-gel silver staining was performed as described previously [55].

Generation of Constructs for Virus-Induced Gene Silencing

Fragments of genes to be used for Virus-Induced Gene Silencing (VIGS) were amplified by PCR on tomato or *N. benthamiana* cDNA. A VIGS fragment for *SlBiP1* was amplified using primers to137 and to138 and a VIGS fragment for *SlBiP2* was amplified using primers to139 and to140. VIGS fragment *SlBiP4* was amplified by primers to141 and to142. All VIGS fragments were cloned into pCR™4-TOPO® (Invitrogen) and the sequences were verified. The VIGS fragments were *Eco*RI-excised from pCR™4-TOPO® and cloned into *Eco*RI-linearized pTRV-RNA2 (pYL156) [56, 57], to generate pTRV:Sl-*BiP1* (Sol 2730), pTRV:Sl-*BiP2* (Sol 2731) and pTRV:Sl-*BiP4* (Sol 2732).

VIGS fragment *SlBiP3* was amplified with primers to201 and to202 and a fragment targeting *SlBiP1, SlBiP2* and *SlBiP4* (*SlBiP-1,-2* and *-4*) was amplified using primer

combination to176 and to177. A fragment for *SlCRT2* was amplified by primers to170 and to171 and a fragment for *SlCRT3b* was amplified with to174 and to175. Fragments *NbCRT3a* and *SlCRT3a* were amplified with primers pairs to213/to214 and to172/to173, respectively. Fragments *SlBiP3*, *SlBiP1, -2* and *-4*, Sl*CRT2*, *NbCRT3a*, *SlCRT3a* and *SlCRT3b* were cloned into pCR™4-TOPO® and sequenced for the correct insert. Fragment *SlBiP1, -2* and *-4* was excised from pCR™4-TOPO® using restriction enzymes *Xba*I and *Bam*HI and cloned into pTRV-RNA2, linearized with the same restriction enzymes, to generate pTRV:Sl-*BiP1, -2* and *-4* (Sol 2771). Fragment *SlBiP3* was excised from pCR™4-TOPO® using restriction enzymes *Bam*HI and *Xho*I and cloned into pTRV-RNA2, linearized with the same restriction enzymes to generate pTRV:Sl-*BiP3* (Sol 2793). The *SlBiP3* fragment was cloned into Sol 2771, linearized with *Bam*HI and *Xho*I, to generate pTRV:Sl-*BiP1, -2, -3* and *-4* (Sol 2792). PCR fragments *SlCRT2*, *NbCRT3a*, *SlCRT3a* and *SlCRT3b* were excised from pCR™4-TOPO® by *Eco*RI and *Bam*HI and cloned in pTRV-RNA2 linearized with the same restriction enzymes to generate pTRV:Sl-*CRT2* (Sol 2768), pTRV:Nb-*CRT3a* (Sol 2803), pTRV:Sl-*CRT3a* (Sol 2769) and pTRV:Sl-*CRT3b* (Sol 2770).

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VIGS in *N. benthamiana*, Q-RT-PCR and HR Assays

Four-week-old wild-type (for Q-RT-PCRs) or transgenic *N. benthamiana:Cf-4* (for HR assay) plants were inoculated by *Agrobacterium*-mediated expression with pTRV-RNA1 and the pTRV-RNA2 constructs described above targeting the different ER-QC genes [56, 57]. TRV:*Cf-4* [58], TRV:PDS [56, 57] and TRV:*GUS* [59] were mixed with pTRV-RNA1 and served as controls. For silencing of *SGT1* we infiltrated the pTV00-derived constructs pBINTRA6 (binary TRV-RNA1 vector) and TRV:*SGT1* [60, 61]. All Agroinoculations were done in a 1:1 ratio with a final O.D.$_{600}$=0.8 for each construct and photographs were taken three weeks after inoculation with the recombinant virus.

For Q-RT-PCR, single leaves of recombinant TRV-inoculated wild-type *N. benthamiana* were harvested per VIGS treatment. Total RNA was extracted from ground leaf material using the NucleoSpin® RNA Plant kit (Macherey-Nagel). First strand cDNA synthesis was performed using M-MLV reverse transcriptase (Promega) and an oligo(dT) primer. Q-RT-PCR was done according to the protocol of the SensiMix™ SYBR kit (Bioline). *NbCRT2* expression was examined using primers to203 and to204, *NbCRT3a* expression with primers to205 and to206 and *NbCRT3b* expression with primers to207 and to208. Endogenous *NbActin* was amplified using primers to58 and to59. Q-RT-PCR was performed on an ABI7300 machine (Applied Biosystems) using the following cycles; 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 45 sec. PCR products were not derived from genomic DNA contamination, as control samples omitting M-MLV did not result in a PCR product after 40 amplification cycles (data not shown).

For the HR assay, three weeks after inoculation of *N. benthamiana:Cf-4* with the various VIGS constructs, mature leaves above the inoculated leaves were infiltrated with *A. tumefaciens* directing expression of Avr4 (pMOG800-Avr4, pRH87) [23] at $O.D._{600=}0.03$ [50], RxD460V (pB1-Rx (AT39-H1) (D460V)), [28] at $O.D._{600}=0.1$ and BAX [29] at $O.D._{600}=0.5$. Three days after *Agrobacterium*-mediated expression of the elicitors, leaves were examined for the development of an HR.

VIGS in Tomato, *Cladosporium fulvum* Disease Assay and Quantification of *C. fulvum* Growth

Cotyledons of ten-day-old Money Maker (MM)-Cf-0 tomato plants transformed with the *Hcr9-4D* (*Cf-4*) gene [51], resistant to Avr4-producing strains of *Cladosporium fulvum* were agroinoculated with the pTRV-RNA1 and the pTRV-RNA2 constructs described above [56, 57]. TRV:*Cf-4* [58], TRV:*PDS* [56, 57], TRV:*GUS* [59] and TRV:*GFP* [62] were mixed with pTRV-RNA1 and served as controls. In *C. fulvum* disease assays, non-agroinoculated Hcr9-4D plants, fully resistant to the fungus, were included and non-agroinoculated MM-Cf-0 plants were included as fully susceptible controls. Agroinoculations were done in a 1:1 pTRV:RNA1 and pTRV:RNA2 ratio, with a final O.D.600=1 for each construct. Four tomato plants per treatment were used in each experiment.

For *C. fulvum* disease assays, three weeks after inoculation with the recombinant TRV viruses, plants were inoculated with conidia of the fungus as described previously [63]. We used *C. fulvum* race 5 (secreting Avr4)-*pGPD:GUS*, expressing the *β-Glucuronidase* (*GUS*) gene under control of the constitutive *GPD* promoter. Two weeks after inoculation with *C. fulvum*, colonization of all inoculated leaflets was studied by an X-glucuronide (Biosynth AG) stain to detect GUS activity. Quantification of blue-stained spots indicating fungal growth was done using the ImageJ plug-in Phenotype Quant in the Clado GUS Quant mode using the default settings [64]. The plug-in determines the total area of blue-stained spots (indicating fungal growth) and the total leaf surface area. Average fungal colonization was calculated for three independent experiments. In each individual experiment the relative fungal growth on non-agroinoculated Hcr9-4D plants was used as a calibrator and all other treatments were normalized to this treatment. Statistical analysis was performed by a one-way ANOVA, followed by a Tukey's post-hoc test.

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Supplemental Data

Supplemental Materials And Methods

Mass Spectrometry for Protein Identification Using the Synapt MS

Two-dimensional Liquid Chromatography-coupled Mass Spectrometry (LC-MS), using both Data-Independent Acquisition (DIA/MS[E]) and Data-Dependent Acquisition (DDA/MS-MS), allowed accurate identification of the immunopurified proteins. For peptide separation a nano Acquity UPLC system (Waters Corporation) was used, employing orthogonal reversed phase separation at high and low pH, respectively. With this 2-D set up, the pool of peptides was eluted from the first dimension XBridge C_{18} trap column (in 20 mM ammonium formate, pH 10) using a discontinuous step gradient of 13% and 45% ACetoNitrile (ACN). For the second dimension, an acidic ACN gradient was applied using a BEH (Ethylene Bridged Hybrid) C_{18} column (75 µm x 25 cm, Waters) and a 65 min linear gradient from 3% to 40% ACN (in 0.1 % formic acid) at 200 nL/min was employed. The eluting peptides were on-line injected into a Synapt Q-TOF MS instrument (Waters Corporation) using a nanospray device coupled to the second dimension column output. The Synapt MS was operated in the positive ion mode with "V-Optics". For the external calibration, [Glu[1]] fibrinopeptide B (1 pmol/uL; Sigma) was delivered as the lock mass compound from a syringe pump to the reference sprayer of the NanoLockSpray source and samples every 30 sec. LC-MS data were collected using the data-independent manner of MS[E], thus alternating the low and elevated collision energy modes for the data acquisition. For data generation, the constant collision energy was 4 eV in the low collision energy MS mode, whereas the collision energy was ramped from 15 to 40 eV during each 1.5 sec data collection cycle in the elevated energy mode. RF (Radio Frequency) was applied to the Q-TOF and was adjusted to ion products with an m/z of 140~1900. Data-dependent acquisition was performed in such a way that the precursor ions were matched with an include-list in terms of m/z and retention time of the peptides. The eluting peptide ions were detected in the MS survey scan (0.6 sec) over a 300-1600 m/z range. The dynamic exclusion window was set at 60 sec with an automatically adjusted collision energy based on the observed precursor m/z and charge state.

Database Search and Data Processing after Mass Spectrometry Analysis on the Synapt MS

LC-MS/MS and MS[E] data were processed using ProteinLynx™ Global Server software (PLGS version 2.5, Waters Corporation) and the resulting list of masses containing all the fragment information was searched against the Tomato_Nico2_Merged protein sequence database, containing sequences from *S. lycopersicum* and *N. benthamiana*. For MS[E], the search was performed using the following parameters: a minimum of five

fragment ions per peptide and a minimum of nine fragment ions per protein, a minimum of one peptide match per protein and a maximum of one missed trypsin cleavage. Furthermore, cysteine carbamydo-methylation and methionine oxidation were chosen as fixed and variable modifications, respectively, and a false discovery threshold of 4% was used. The false discovery rate was determined automatically in PLGS by searching the randomized database. For DDA analysis, the peptide tolerance was set to 30 ppm and a fragment tolerance of 0.05 Da. The AutoMod option was applied as secondary search to the database search results with a maximum of one missed trypsin cleavage and non-specific secondary digest. The AutoMod analysis tool increases protein coverage by taking into account missed trypsin cleavages and non-specific cleavages, post-translational modifications, and amino acid substitutions. Finally, the DDA and MS^E outputs were merged in Excel. Protein identification was considered to be accurate when assigned based on at least two proteotypic peptides typical for proteolysis by trypsin. In the DDA analyses single peptides were only kept if the ladder score was above 50.

Mass Spectrometry for Protein Identification Using the Orbitrap XL
The LC system that was combined with the MS was a nano-Acquity (Waters Corporation) with the solvents 0.1% formic acid in water (A) and 100% ACN (B). Peptides were trapped and washed on a vented trap column (Waters Symmetry, 5 µm beads, 0.180 x 20 mm) with a flow rate of 0.010 mL/min, at 1% B. The reverse phase analytical column used was a Waters BEH 130 C18 column (1.7 µm beads, 0.075 x 250 mm) and a flow rate of 250 nL/min was employed with the following gradient program: 1% B to 5% B in 2 min; 5% B to 40% B in 40 min; 40% B to 80% B in 2 min; 80% B for 5 min and 1% B for 19 min. A Proxeon nano-ion source was used, with 2 kV applied on a stainless steel union holding a 10 µm fused silica spraying emitter. The mass spectrometer (Orbitrap XL, Thermo Scientific) used a data-dependent acquisition program of 6 events: one high resolution scan over *m/z* 300-2,000 at 60,000 resolution, followed by five data-dependent CID events at low resolution in the LTQ. For the high resolution data a lock mass of *m/z* 445.120025 was used [1]. For MS/MS events, a minimum signal of 500 ion counts was required to select the parent ion and an isolation width of 2 Da was used with a normalized collision energy of 35%. The pre-scan option was enabled and charge state recognition was selected for 2+, 3+ and 4+ ions. The chromatography function was used (threshold 1,000 ion counts, peak width 10 sec, correlation 0.9). After an MS/MS event a dynamic exclusion was applied to that *m/z* for 60 sec (over the range – 0.5 to + 1.5 *m/z*).

Database Search and Data Processing after Mass Spectrometry Analysis on the Orbitrap XL

Raw MS data were converted to Mascot Generic Format using Discoverer (Thermo Scientific, Version 1.2.0.207). Charge state deconvolution and de-isotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, version 2.3). Trypsin was specified as the cleavage enzyme and two missed cleavages were permitted. Carbamido-methyl (C) was specified as a fixed modification and variable modifications were oxidation (M), phosphorylation (ST), formyl (N-term) and cation:Na (DE). Mono-isotopic mass values were used and the protein mass was unrestricted. Peptide mass tolerance was ± 10 ppm and fragment mass tolerance was ± 0.8 Da. An in-house database of Solanaceous plant sequences (Sol_effectors 20110601, containing 1,529,535 sequences with a total of 115,957,536 residues) was searched. All Mascot search results were loaded into Scaffold (version Scaffold_3_00_07, Proteome Software Inc.) to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted when greater than 95.0% probability, as specified by the Peptide Prophet algorithm [2]. Protein identifications were accepted when greater than 95.0% probability and containing at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [3]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

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REFERENCES SUPPLEMENTAL MATERIALS AND METHODS

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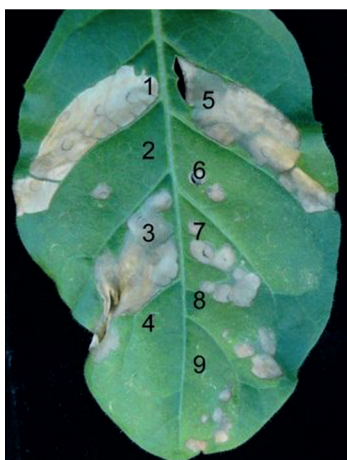**Figure S1.**
Cf-eGFP fusion proteins are functional. Co-expression of Cf-eGFP proteins with matching or non-matching Avr effectors of *C. fulvum* in *N. tabacum* by *Agrobacterium*-mediated expression. (1) Cf-4-eGFP + Avr4; (2) Cf-4-eGFP + Avr9; (3) Cf-4E-eGFP + Avr4E; (4) Cf-4E-eGFP + Avr9; (5) Cf-9-eGFP + Avr9; (6) Cf-9-eGFP + Avr4; (7) Peru2-eGFP; (8) Peru2-eGFP + Avr4; (9) Peru2-eGFP + Avr9.

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                    *        20         *        40         *        60
Sl-BiP1 : ------------------------------------------------------------ :   -
Nb-BiP1 : ------------------------------------------------------------ :   -
Nb-BiP2 : ------------------------------------------------------------ :   -
Nb-BiP4 : ATGCTTCTCAGTTATGATTGGACCAAGTCATCAAACATGACATGCATGAAAACAATCAGC :  60
Sl-BiP2 : ------------------------------------------------------------ :   -
Sl-BiP4 : ------------------------------------------------------------ :   -
Sl-BiP3 : ------------------------------------------------------------ :   -
Nb-BiP3 : ------------------------------------------------------------ :   -


                    *        80         *       100         *       120
Sl-BiP1 : -----------------------ATGGCTGCCTGTTCG-------------------- :  15
Nb-BiP1 : ------------------------------------------------------------ :   -
Nb-BiP2 : ------------------------------------------------------------ :   -
Nb-BiP4 : AACATAGAAGAGAAACGATTCAACGACGAAGATATTGTTCAGAGGCAGTGTTCTGTGGAA : 120
Sl-BiP2 : -----------------------ATGAA-ATCTTGTC-------------------- :  13
Sl-BiP4 : -----------------------ATGG----CTGGTG-------------------- :  10
Sl-BiP3 : -----------------------ATGAGAATGAGAC-------------------- :  13
Nb-BiP3 : -----------------------ATGGTC---GAC-------------------- :  10


                    *       140         *       160         *       180
Sl-BiP1 : ------AGGCGAGGCAATTCTCTCGTCGTG--------------------CTAGCG :  45
Nb-BiP1 : ----------------------ATG----------------------CTACCA :   9
Nb-BiP2 : ------------------------------------------------------------ :   -
Nb-BiP4 : AGAGGTGTCCTTGTGGATCTACGAGTTATGGCTGGTGCGTGGAAGAGACGCGCATCCTTG : 180
Sl-BiP2 : --ACTTATACTT-----TGCACTAATTGGC--------------------TTG :  39
Sl-BiP4 : ------GTGCGTG----GAAGCTAAGTGCA--------------------TCCTGG :  36
Sl-BiP3 : ------GTTCAAAGAATTTCAAGAACTTAG--------------------CATTCTTG :  45
Nb-BiP3 : ------GTTCAAAGGATTTCAAAATTTTAG--------------------CATTCTTG :  42


                    *       200         *       220         *       240
Sl-BiP1 : ATCGTTTTAT------------TAGGCTCTCTTTCT------------------CCACTTT :  76
Nb-BiP1 : A----------------------GTTCTCTTTCT------------------CCACTTT :  28
Nb-BiP2 : ------------------------------------------------------------ :   -
Nb-BiP4 : GTCGTTTTCGCAATCGTCCTCTTCGGGACCTTGTTC------------------CCATTTT : 223
Sl-BiP2 : TTTGGTTTGTCAA---------CAGGGACCTTGTTT------------------CCATTCT :  73
Sl-BiP4 : ATCATCTTGT------------TCGGGTCCTTGTTC------------------CCATATT :  67
Sl-BiP3 : ATTTTGCTTC-----------------TTTTTGCTTCAGAGTTTTGCTTAGGACTAACAG :  88
Nb-BiP3 : ATTTTGCTTC-----------------TCTTGTTTCAGA-----------ACTAACAC :  73


                    *       260         *       280         *       300
Sl-BiP1 : CCAATGCAAAGGAGGA--GCCTACAAAAC-TTGGTAGCCGTTATAGGAATTGATCTTGGGA : 133
Nb-BiP1 : CGATTGCCAAAGAGGA--GACAACCAAAT-TACGGAACAGTTATTGGAATAGACCTTGGGA :  85
Nb-BiP2 : ------------------------------------------------------------ :   -
Nb-BiP4 : CTATAGCTAAAGAGA--AGCTACAAAGT-TAGGAACAGTTATTGGTATAGATCTTGGAA : 280
Sl-BiP2 : CTATAGCTAAAGAGA--AGCTACCAAGT-TAGGAACTGTTATCGGTATAGATCTTGGAA : 130
Sl-BiP4 : CTGTAGCCAACGAGA--AGCTTCCAAGT-TGGGAACAGTTATTGGAATTGATCTTGGAA : 124
Sl-BiP3 : TAGCTGCTGAGAAAAACAGAATCTTGGAACAGTGATTGGAATTGATCTAGGGA : 148
Nb-BiP3 : TAGCAACTGAGAAAAATCAGAACAGAACCTTGGAACAGTGATTGGAATTGATTTAGGGA : 133


                    *       320         *       340         *       360
Sl-BiP1 : CAACCTATTCCTGTGTTGGGGTGTACAA---CAATGGTCATGTGGAGATTATAGCCTAATG : 190
Nb-BiP1 : CTACCTATTCCTGTGTTGGAGTTTATAA---CAATGGTCATGTTGAGATTATAGCCAATG : 142
Nb-BiP2 : ------------------------------------------------------------ :   -
Nb-BiP4 : CAACTTACTCATGTGTTGGTGTCTACAA---CAACGGACATGTTGAAATCATAGCAAATG : 337
Sl-BiP2 : CAACTTATTCTTGTGTTGGAGTTTACAA---CAATGGACATGTTGAGATCATAGCAAATG : 187
Sl-BiP4 : CAACTTACTCATGTGTTGGTGTTTACAA---CAATGGTCATGTTGAAATTATAGCAAATG : 181
Sl-BiP3 : CAACTTATTCTTGTGTGGGTGTATACAAAGGCGAGAATAATGTCGAGATTATAGCGAACG : 208
Nb-BiP3 : CAACATATTCTTGTGTGGGTGTATACAAAGGAGAGAATAACGTCGAGATCATAGCGAACG : 193
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              *         380         *         400         *         420
Sl-BiP1 : ACCAAGGAAACAGGATTACACCATCATGGGTTGCATTCACTGATAACGAGAGATTGATTG : 250
Nb-BiP1 : ACCAGGGAAACCCTATTACGCCTTCATGGGTTGGATTCACCCATGGTGAGAGGTTGATTG : 202
Nb-BiP2 : ------------------------------------------------ATGGT------------- : 5
Nb-BiP4 : ACCAAGGTAATCGTATCACCCCTTCATGGGTAGCCTTCACTGAYGGTGAGAGGCTGATTG : 397
Sl-BiP2 : ACCAAGGTAATCGTATCACCCCTTCATGGGTAGCCTTCACTCATGGTGAGAGACTGATCG : 247
Sl-BiP4 : ACCAAGGTAATCGCATCACCCCTTCATGGGTTTCATTCACACATGGTGAGAGGTTGATTG : 241
Sl-BiP3 : ATCAAGGAAACACAATCACCCCTTCATGGGTTGCATTTACTGACACAGAGAGGTTAATTG : 268
Nb-BiP3 : ATCAAGGGAACCGAATCACCCCTTCATGGGTTGCATTCACTGATACAGAAGGTTAGTTG : 253

              *         440         *         460         *         480
Sl-BiP1 : GTGAGGCTGCCAAGAACCTGGGTGCGGTCAACCCAGAAGAACAATCTTTGATGTTAAGA : 310
Nb-BiP1 : GTGAGGCTGCAAAGAACCAGGCAGCTGTCAATCCCGAAGAACCATCTTTGATGTTAAGA : 262
Nb-BiP2 : ------------------------------------------------TTTGAT----AAT : 14
Nb-BiP4 : GTGAGCCAGCTAAAAATCAGGCTGCTGTTAATCCTGAGAGGACCATATTTGACGTCAAAA : 457
Sl-BiP2 : GTGAGCCAGCTAAAAATCAAGCAGCTGTGTAATCCAGAGAGCGACCGTCTTTGACGTCAAAA : 307
Sl-BiP4 : GTGAGGCAGCAAAAAATCTAGCCGCTGTGAACCCAGAAGCGACCATCTTTGATGTCAAGA : 301
Sl-BiP3 : AGAGCTGCAAAGAATCAAGAGCTCTTAACCCCGAACGTACTATATATGACGTTAAAA : 328
Nb-BiP3 : GAGAGCTGCAAAGAATCAAGCAGCTCTTAACCCGGAACGTACTATATATGATGTTAAAA : 313

              *         500         *         520         *         540
Sl-BiP1 : GGCTGATTGGAAGAAAATTTGAAGATAAGGAAGTACAGAGGGATATGAAGCTGGTACCGT : 370
Nb-BiP1 : GGCTTATTGGAAGAAAAGTTTGAGGACAAGGAAGTTCAGAGGGATATGAAGCTTGTCCCAT : 322
Nb-BiP2 : GACTC--------------TTGA---------------------------CTTATCTC-- : 31
Nb-BiP4 : GACTTATCGGGACAAAGTTTGATGACAAAGAAGTACAAAGGGACAAGAAACTTGTTCCAT : 517
Sl-BiP2 : GACTTCTGGGACAAAGTTTGATGACAAAGAAGTACAAAGGGACATGAAACTTGTTCCCT : 367
Sl-BiP4 : GGCTTATCGGAAACAAAGTTTGAAGACAGAGAAGTTCAAAGGGACATGAAACTTGTTCCGT : 361
Sl-BiP3 : GGCTTATAGGAACGAAGTTTGATGATCCAGAAGTTCAGGAAATTATCAAATGTTGCCTT : 388
Nb-BiP3 : GGCTCATTGGAACGAAGTTTGATGATCAAGAAGTTCAAAAATTATTGATGTTGCCTT : 373

              *         560         *         580         *         600
Sl-BiP1 : ACAAGATTGTCAGCAAGGATGGAAAGCCCTACATACAAGTCAAAATCAAGGATGGAGAAG : 430
Nb-BiP1 : ACAAGATCGTGAACAAGGATGGCGAAGCCTTACATACAAGTCAAAATCAAGGACGGAGAAG : 382
Nb-BiP2 : -------TGTT-------------------TATA-----GTTAAGATCAAAGATGGGGGACA : 61
Nb-BiP4 : ATGAGATTGTTAACAAGGATGGCGAAACCATATATCCAAGTTAAGATCAAAGATGGGGGACA : 577
Sl-BiP2 : ATAAGATTGTTAACAAGGATGGCGAAACCTTATATCCAAGTTAAAATCAAAGATGGGGGACA : 427
Sl-BiP4 : ACAAGATTGTTAACACGGATGGTAAACCATACACCAAGTTAAGATTAAAACGGGGGACA : 421
Sl-BiP3 : TTAAATGTTGTGAACAAAGCTGGCAAGCCTCATGTGGAAGTCAAGATTAAAGAGGGAGATG : 448
Nb-BiP3 : TTGAAGTTGTGAACAAAGATGGCAAGCCTTATGTACAAGTCAAGATTAAAGATGGTGAAG : 433

              *         620         *         640         *         660
Sl-BiP1 : TTAAGGTATTTAGTCCTGAGGAAATCAGCGCCATGATTTTGACAAAGATGAAAGAGACAG : 490
Nb-BiP1 : TTAAGGTATTTAGTCCTGAGGAAATCAGTGCCATGATTTCTGATAAAGATGAAGGAGACAG : 442
Nb-BiP2 : CTAAGGTCTTCAGTCCTGAGGAGATCAGTGCTATGATTCTGACCAAGATGAAAGAAACAG : 121
Nb-BiP4 : CTAAGGTCTTCAGTCCTGAGGAGATCAGTGCTATGATTCTGACCAAGATGAAAGAAACAG : 637
Sl-BiP2 : CCAAGGTCTTCAGTCCTGAGGAGATCAGTGCTATGATTCTGTCAAGATGAAAGAAACAG : 487
Sl-BiP4 : ACAAGGTCTTCACTCCTGAGGAGATCAGTGCCATGATTTTGACCAAGATGAAGGAAACAG : 481
Sl-BiP3 : TTAAAGTTTTTAGCCCAGAGGAAATTAGTGCTATGATTTTGCAGAAAATGAAAGAAACAG : 508
Nb-BiP3 : TGAAAAAGTTTAGCCCAGAAGAAATTAGTGCTATGATCTTGCAAAAAATGAAGGAAACAG : 493

              *         680         *         700         *         720
Sl-BiP1 : CCGAAGCATTCCTTGGGAAGACTATTAAGGATGCTGTGGTCACTGTTCCTGCATATTTCA : 550
Nb-BiP1 : CTGAAGCTTTCCTTGGAAAGAAAATTAAGGATGCTGTAGTAACTGTTCCTGCTTATTTCA : 502
Nb-BiP2 : CAGAAGCTTACCTTGGAAAGAAAATCAAGGATGCAGTTGTCACAGTCCCAGCATACTTCA : 181
Nb-BiP4 : CAGAAGCTTACCTTGGAAAGAAAATCAAGGATGCAGTTGTCACAGTCCCAGCATACTTCA : 697
Sl-BiP2 : CAGAAGCTTTCCTTGGAAAGACAATCAAGGATGCAGTTGTTCCAGTCCCAGCATACTTCA : 547
Sl-BiP4 : CTGAGGCTTTCCTTGGAAAGAAAATCAATGATGCAGTTGTCACTGTTCCCGCTTACTTTA : 541
Sl-BiP3 : CTGAGGCCGTATCTACGGAAATCAATTAAGCACGCTGTCATCACCGTTCCAGCTTATTTA : 568
Nb-BiP3 : CTGACGCTTATTACGGAACTCGATTAAACATGCTGTCATTACCGTTCCAGCTTATTTTA : 553
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                    *         740         *         760         *         780
Sl-BiP1 : ATGATGCACAAAGACAGGCGACGAAAGGATGCTGGTGTGTTATTGCTGGATTAAATGTTGCTA : 610
Nb-BiP1 : ATGACGCGCAAAGGCAGGCTACTAAGGATGCAGGTGTGTTATTGCTGGCTTAAATGTTGCAA : 562
Nb-BiP2 : ATGATGCCCAGAGGCAGGCCACTAAGGATGCAGGTGTGTTATTGCTGGTTTCAACGTGGCAA : 241
Nb-BiP4 : ATGATGCCCAGAGGCAGGCCACTAAGGATGCAGGTGTGTTATTGCTGGTTTCAACGTGGCAA : 757
Sl-BiP2 : ATGATGCCCAGAGGCAGGCCACTAAGGATGCCGGTGTCATTGCTGGTTTCAACGTGGCAA : 607
Sl-BiP4 : ATGATGCCCAGAGGCAGGCCACTAAGGATGCAGGTGTAATCGCTGGACTAAATGTGGCAA : 601
Sl-BiP3 : ATGATGCACAAAGGCAAGCAACCAAGGATGCTGGTACCATACCAGGGCTGAATGTAGTTA : 628
Nb-BiP3 : ATGATGCGCAAAGGCAACGCAACTAAGGATGCTGGCGCCATAGCAGGGTTCAATGTAGTTA : 613

                    *         800         *         820         *         840
Sl-BiP1 : GGATCATTAATGAGCCAACAGTGCAGCCATTGCCTACGGACTGGGATAAGAAAGGTGGTG : 670
Nb-BiP1 : GAATTATTAATGAACCAACAGCAGCAGCCTATTGCTTATGGATTGGACAAGAAAGGTGGCG : 622
Nb-BiP2 : GAATCATTAACGAACCAACTGCAGCTGCCCATTGCCTATGGATTAGATAAGAAAGGTGGTG : 301
Nb-BiP4 : GAATCATTAACGAACCAACTGCAGCTGCCCATTGCCTATGGATTAGATAAGAAAGGTGGTG : 817
Sl-BiP2 : GAATCATTAATGAACCAACTGCAGCTGCCCATTGCCTATGGATTAGATAAGAAAGGTGGTG : 667
Sl-BiP4 : GAATCATTAATGACCGACTGCAGCAACAATTGCCTATGGATTGGATAAGAAAGGTGGTG : 661
Sl-BiP3 : GGATAATTAATGACCGACTGCGGCGCTGCTATAGCCTATGGATTGGATAAGAAAGGGAAAG : 688
Nb-BiP3 : GGATAATAAACGAGCCTACCGCGGCGCTGCAATAGCCTATGGATTGGACAAGAAAGGAAAAG : 673

                    *         860         *         880         *         900
Sl-BiP1 : AGAAGAACATTCTTGTCTTTGACCTTGGTGGTGGGACATTCGATGTCAGTATTCTGACCA : 730
Nb-BiP1 : AGAAGAACATTCTCGTCTTTGATCTTGGTGGTGGGGACATTTGATGTGTCTTGACAA : 682
Nb-BiP2 : AAAAGAACATCCTGGTCTTTGACCTTGGTGGTGGTACATTTGATGTTAGTATTCTCACTA : 361
Nb-BiP4 : AAAAGAACATCCTGGTCTTTGACCTTGGTGGTGGTACATTTGATGTTAGTATTCTCACTA : 877
Sl-BiP2 : AAAAGAACATCCTTGTCTTTGACCTTGGTGGTGGTACATTTGATGTTAGTATCCTTACTA : 727
Sl-BiP4 : AAAAGAACATCCTTGTCTTTGACCTTGGTGGTGGAACATTTGATGTTAGCATCCTCACCA : 721
Sl-BiP3 : AACAGAACATTTTGTGTATGATCTTGGTGGTGGCACATTTGATGTTAGCATACTTAGCA : 748
Nb-BiP3 : AGCAGAATATTTTGTGTATGATCTTGGCGGTGGCACATTTGACGTGAGCGTACTTAGCA : 733

                    *         920         *         940         *         960
Sl-BiP1 : TTGACAATGGTGTATTTGAGGTTCTTGCAACAAATGGTGACACCCATCTTGGAGGTGAGG : 790
Nb-BiP1 : TTGACAATGGTGTGTTTGAGGTTCTGTCCACAAATGGAGATACCCATCTTGGAGGTGAGG : 742
Nb-BiP2 : TTGACAATGGTGTTTTTGAGGTTCTTGCCACAAATGGAGACACTCATCTAGGAGGAGAGG : 421
Nb-BiP4 : TTGACAATGGTGTTTTTGAGGTTCTTGCCACAAATGGAGACACTCATCTAGGAGGAGAGG : 937
Sl-BiP2 : TTGACAATGGTGTTTTTGAGGTTCTTGCCACAAATGGAGACACTCATCTAGGAGGAGAAA : 787
Sl-BiP4 : TCGATAATGGTGTTTTTGAAGTTCTTGCAACAAATGGAGACACTCACCTGGGAG------ : 775
Sl-BiP3 : TTGATAATGGTGTTTTTGAAGTCCTAGCAACAAATGGTAACACTCACTTAGGAGGAGAGG : 808
Nb-BiP3 : TTGACAATGGTGTTTTTGAGGTATTAGCAACCAATGGTAACACTCACTTAGGAGGAGAGG : 793

                    *         980         *         1000        *         1020
Sl-BiP1 : ACTTTGATCAAAGGATTATGGAATACTTCATCAAGTTGATCAAGAAGAAGCATGGAAAGG : 850
Nb-BiP1 : ACTTTGATCAGAGGATTATGGAATACTTTATCAAATTGATTAAGAAGAAGCATGGAAAGG : 802
Nb-BiP2 : ATTTTGACCAGAGCGATTATGGAGTACTTCATTAAATTGATAAAGAAGAAGCATGGAAAGG : 481
Nb-BiP4 : ATTTTGACCAGAGCGATTATGGAGTACTTCATTAAATTGATAAAGAAGAAGCATGGAAAGG : 997
Sl-BiP2 : ATTTTGACCAGAGCGTTATGGAGTATTTCATTAAATTGATAAAGAAGAAGCATGGTAAGG : 847
Sl-BiP4 : ------------AGGATCATGGAGT-------------GATTAAGAAGAAGCATGGGAAGG : 811
Sl-BiP3 : ACTTTGATCATAAACTAATGGACTATTTCATCAAGTTGATCAAGAGGAAATATAGCAAGG : 868
Nb-BiP3 : ATTTTGATCACAGAGATAATGGATTATTTCATCAAGTTGATTAAAAGGAAATATAGCAAGG : 853

                    *         1040        *         1060        *         1080
Sl-BiP1 : ACATCAGCAAGGACAACAGAGCTCTTGGAAAGCTAAGGAGAGAAGCTGAGCGTGCCAAGA : 910
Nb-BiP1 : ACATCAGCAAAGACAACAGAGCTCTTGGTAAGCTAAGGAGAGAAGCTGAGCGTGCCAAGA : 862
Nb-BiP2 : ACATCAGCAAGGATAACAGGGCTTTGGGAAAGCTCAGGAGAGAAGCTGAGCGTGCCAAGA : 541
Nb-BiP4 : ACATCAGCAAGGATAACAGGGCTTTGGGAAAGCTCAGGAGAGAAGCTGAGCGTGCCAAGA : 1057
Sl-BiP2 : ACATTAGCAAGGATAACAAGGCTTTGGGTAAGCTCAGGAGAGAAGCTGAGCGTGCCAAGA : 907
Sl-BiP4 : ACATCAGCAAGGACAACAGAGCTTTGGGTAAGCTTAGGAGAGAAGCTGAGCGTGCCAAGA : 871
Sl-BiP3 : ATATTAGTAGTGACAAGAAAGCTCTTGGAAAGCTTAGAGAGAATGTGAGAGAGCCAAGA : 928
Nb-BiP3 : ATATTAGTAATGACAAGAAAGCTCTAGGAAAACTTAGAAAGGAATGTGAGAGAGCCAAGA : 913
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              *         1100         *         1120         *         1140
Sl-BiP1 : GATCCCTTAGTAGCCAGCACCAAGTCCGCGGTTGAGATTGAATCATTATTTGATGGTACTG : 970
Nb-BiP1 : GGGCACTGAGTAGCCAGCACCAGGTCCGAGTTGAGATTGAATCTCTTTTTGATGGTACTG : 922
Nb-BiP2 : GAGCACTGAGCAGTCAGCATCAAGTCAGGGTTGAGATTGAATCTCTCTTCGATGGTGTAG : 601
Nb-BiP4 : GAGCCGTGAGCAGTCAGCATCAAGTCAGGGTTGAGATTGAATCTCTCTTCGATGGTGTAG : 1117
Sl-BiP2 : GAGCCCTGAGCAGCCAGCATCAGGTCAGGGTAGAGATCGAGTCTCTTTTTGATGGTGTGG : 967
Sl-BiP4 : GATCTCTGAGCAGCCAGCACCAAGTCAGGGTAGAGATCGAATCTCTTTGATGGTGTGG : 931
Sl-BiP3 : GGGCCTTAAGTAACCAGCACCAAGTACGTATCGAGATCGAGTCTCTTTTTCGATGGTGTTG : 988
Nb-BiP3 : GAGCCTTAAGTAACCAGCACCAAGTACGCGTTGAAATCGAGTCTCTTTTTCGATGGTATTG : 973

              *         1160         *         1180         *         1200
Sl-BiP1 : ATTTCTCTGAACCACTGACCCGTGCTCGCTTTGAGGAGTTGAACAATGATCTCTTCAGAA : 1030
Nb-BiP1 : ACTTCTCTGAACCATTAACCCGTGCTCGCTTTGAGGAGTTGAACAATGATCTCTTCAAAA : 982
Nb-BiP2 : ATTTCTCTGAACCACTTACTCGGGCTCGTTTTGAGGAGCTGAACAACGATTTATTCAGGA : 661
Nb-BiP4 : ATTTCTCTGAACCACTTACTCGGGCTCGTTTTGAGGAGCTGAACAACGATTTATTCAGGA : 1177
Sl-BiP2 : ATTTCTCTGAACCACTAACCCGGCTCGTTTTGAGGAGCTGAACAATGATTTGTTCCGTA : 1027
Sl-BiP4 : ATTTCTCCGAAGCACTAACTCGGGCACGTTTTGAGGAGCTAAACAATGATTTGTTTAGAA : 991
Sl-BiP3 : ACTTCTCCGAGCCATTAACAAGGGCGAGATTCGAGGAGTTGAACATGGATTTGTTCAAGA : 1048
Nb-BiP3 : ACTTCTCTGAGCCGTTAACAAGGGCAAGATTCGAGGAATTGAACATGGATTTGTTCAAGA : 1033

              *         1220         *         1240         *         1260
Sl-BiP1 : AGACAATGGGACCTGTCAAGAAGGCAATGGATGATGCTGGCCTACAAAGAACCAGATTG : 1090
Nb-BiP1 : AGACAATGGGACCTGTCAAGAAGGCAATGGAAGATGCTGGCGTGGGAGAGAACCAAATTG : 1042
Nb-BiP2 : AGACAATGGGTCCTGTTAAGAAGGCTATGGAGGATGCTGGACTAGAAAAGAACCAGATTG : 721
Nb-BiP4 : AGACAATGGGTCCTGTTAAGAAGGCTATGGAGGATGCTGGACTAGAAAAGAACCAGATTG : 1237
Sl-BiP2 : AGACGATCACTCCTGTCAAGAAGGCTATGGAGGATGCTGGTCTGGCAAAGAACCAGATTG : 1087
Sl-BiP4 : AGACAATGGGGCCTGTTAAGAAGGCTATGGAGGACGCCGGCGCTAGGGAAGAACCAGATTG : 1051
Sl-BiP3 : AGACAATGGGTCCAGTCAAGAAGGCATTAGAGGATGCAAACTTGAAGAAGACGGATATTG : 1108
Nb-BiP3 : AGACGATCAGTCCAGTCAAGAAGGCATTAGAGGATGCAAATTTGAAGAAGACAGATATTG : 1093

              *         1280         *         1300         *         1320
Sl-BiP1 : ATGACATTGTTCTTGTTGGTGGAAGCACCAGCATTCCTAAGGTGCAACAGCTCCTCAACG : 1150
Nb-BiP1 : ATGAGATAGTTCTTGTTGGTGGAAGCACTAGAATTCCAAAAGTGCAACAACTCCTGAACG : 1102
Nb-BiP2 : ATGAAATTGTTTTTGGTTGGTGGAAGTACCAGGGATTCCTAAAGTTCAACAGCTTTTGAAAG : 781
Nb-BiP4 : ATGAAATTGTTTTTGGTTGGTGGAAGTACCAGGGATTCCTAAAGTTCAACAGCTTTTGAAAG : 1297
Sl-BiP2 : ACGAGATTGTCTTGGTTGGTGGAAGTACCAGGATTCCAAAAGTTCAACAGCTTTTGAAAG : 1147
Sl-BiP4 : ATGAAATTGTTCTTGTTGGTGGAAGTACCAGGATTCCTAAAGTTCAACAGCTCTTTGAAGG : 1111
Sl-BiP3 : ATGAACTCGTGCTTGTTGGTGGAGTACTCGAATTCCAAAGTGCAACAGCTCTTGAAGG : 1168
Nb-BiP3 : ATGAACTCGTGCTTGTTGGTGGCGTACTCGAATTCCAAAGTGCAACAGCTATTGAAGG : 1153

              *         1340         *         1360         *         1380
Sl-BiP1 : ACTATTTTGACGGCAAGGAGCCCACCAAGGGTGTAAATCCTGATGAAGCAGTTGCTTATG : 1210
Nb-BiP1 : ACTATTTTGAAGGGAAGGAGCCCTAACAAGGGTGTGAATCCTGACGAGGCAGTTGCTTATG : 1162
Nb-BiP2 : ATTATTTTGATGGCAAGGAGCCCAACAAGGGTGTCAACCCCGACGAAGCAGTTGCTTATG : 841
Nb-BiP4 : ATTATTTTGATGGCAAGGAGCCCAACAAGGGTGTCAACCCCGACGAAGCAGTTGCTTATG : 1357
Sl-BiP2 : ATTATTTTGATGGCAAGGAGCCTAGCAAAGGTGTCAACCCCGATGAAGCAGTTGCTTATG : 1207
Sl-BiP4 : ATTATTTTGACGGCAACGAACCTAGCAAAGGAGTCAACCCTGACGAAGCAGTTGCTTATG : 1171
Sl-BiP3 : ATTTCTTTGATGGTAAAGAACCTAACAAGGGTGTTAATCCTGACGAAGCTGTAGCTTACG : 1228
Nb-BiP3 : ATTTCTTTGATAGGAAAGAACCAAATAAGGGTGTTAATCCGGACGAAGCTGTAGCTTATG : 1213

              *         1400         *         1420         *         1440
Sl-BiP1 : GTGCTGCTGTTCAAGGTGGTATGCTCAGTGGTGAGGGTGGAGATGAAACTAAAGATATCC : 1270
Nb-BiP1 : GTGCTGCTGTCCAAGGTGGTATCTTAAGTGGAGACGGTGGTGACGAAACTAAAGATATCC : 1222
Nb-BiP2 : GTGCAGCTGTACAAGGAGGAATCTTGAGTGGAGACGGAGAGTGATGAAACCAAAGATATTC : 901
Nb-BiP4 : GTGCAGCTGTACAAGGAGGAATCTTGAGTGGAGACGGAGAGTGATGAAACCAAAGATATTC : 1417
Sl-BiP2 : GTGCAGCTGTACAAGGAGGTATCTTGAGTGGAGAAGGAGTGATGAAACCAAAGATATTC : 1267
Sl-BiP4 : GTGCAGCAGTACAAGGAGGAATTTTGAGTGGCGAGGGTGGTCATGAAACTAAAAATATTC : 1231
Sl-BiP3 : GAGCAGCAATACAAGGAGCAATCCTTGGTGGTGAAGGAGGGGAAGAGACCAAAGATCTTT : 1288
Nb-BiP3 : GAGCAGCAGTACAAGGAGCAATTCTTGGTGGTGAAGGAGGGGAAGAAACCAAAGATCTGT : 1273
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                    *         1460        *         1480        *         1500
Sl-BiP1 : TTCTCCTGGATGTCGCTCCTCTCAGCCTTGGTATTGAAACCGTTGGTGGTGTGATGACCA : 1330
Nb-BiP1 : TTCTCCTGGATGTTGCTCCTCTCAGCCTTGGTATTGAAACTGTTGGTGGCGTGATGACCA : 1282
Nb-BiP2 : TTCTACTGGATGTTGCTCCACTGACTCTTGGTATTGAAACGGTTGGAGGAGTGATGACAA :  961
Nb-BiP4 : TTCTCCTGGATGTTGCTCCATTGACTCTTGGTATTGAAACGGTTGGAGGAGTGATGACAA : 1477
Sl-BiP2 : TTCTCTTGGATGTTGCTCCACTGACTCTTGGTATTGAAACTGTTGGAGGAGTGATGACAA : 1327
Sl-BiP4 : TTCTTCTGGATGTGGCTCCACTTACTCTTGGTATTGAAACTGTTGGCGGAGTGATGACTA : 1291
Sl-BiP3 : TGTTGCTTGATGTGACTCCTCTAAGTCTCGGGAATTGAGACAGTTGGTGGGGTTATGACAA : 1348
Nb-BiP3 : TGTTGCTTGATGTGACTCCGTTAAGTCTTGGAATCGAGACAGTTGGTGGTGTTATGACAA : 1333

                    *         1520        *         1540        *         1560
Sl-BiP1 : AATTGATCCCCAGAAATACCGTTATCCCAACCAAGAAGTCCCAGGTCTTCACCACTTACC : 1390
Nb-BiP1 : AATTGATTCCTAGGAACACTGTCATCCCAACCAAGAAGTCTCAAGTCTTCACTACATACC : 1342
Nb-BiP2 : AGTTGATCCCAAGGAACACCGTTATTCCTACTAAGAAATCTCAAGTCTTCACCACTTACC : 1021
Nb-BiP4 : AGTTGATCCCAAGGAACACTGTTATTCCTACTAAGAAATCTCAAGTTTTCACCACTTACC : 1537
Sl-BiP2 : AGTTGATCCCAAGAAACACTGTCATTCCTACTAAGAAGTCTCAAGTCTTCACCACTTACC : 1387
Sl-BiP4 : AGTTGATCCCAAGAAACACTGTCATTCCTACTAAAAAGTCTCAAGTCTTCACCACTTATA : 1351
Sl-BiP3 : AGTTGATTCCAAGAAACAGTAGGATACCTACCAAGAAAACCCAAGTTTTCACCACCTATC : 1408
Nb-BiP3 : AGTTGATTCCAAGAAACAGTAGGATTCCTGTCAAGAAAACTCAAGTTTTCACCACCTATC : 1393

                    *         1580        *         1600        *         1620
Sl-BiP1 : AAGACCAGCAGACCACTGTCTCCATTCAGGTTTTTGAAGGTGAAAGGAGTCTTACCCAAGG : 1450
Nb-BiP1 : AAGACCAGCAGACCACTGTCACCATTTCGGTTTATGAAGGAGAAACGAGTCTTACTAAGG : 1402
Nb-BiP2 : AGGATCAGCAGACAACCAGTCACAATTTCGGTCTTTGAAGGTGAACGCAGTCTCACAAAGG : 1081
Nb-BiP4 : AGGATCACCAGACAACCAGTGACAATTTCGGTCTTTGAAGGTGAACGCAGTCTCACCAAGG : 1597
Sl-BiP2 : AGGACCAGCAGACAACCGTCACAATTTCGGTCTTTGAAGGTGAACGCAGTATGGTAAAGG : 1447
Sl-BiP4 : AGGATCAACAGACAACCGTCACAATTCAGGTCTTTGAAGGTGAACCTAGCCTCACCAAGG : 1411
Sl-BiP3 : AAGACCAACAAACATCAGTAACAATAAAGGTATATGAAGGTGAACGAGCTTACCCAAGG : 1468
Nb-BiP3 : AAGACCAACAAACATCTGTAACAATTAAGGTATATGAAGGTGAAACGAGCTTGACAAAGG : 1453

                    *         1640        *         1660        *         1680
Sl-BiP1 : ACTGTAGGAATCTCGGGAAGTTTGATCTTACAGGAATTCCTCCAGCTCCAAGGGGGAACCC : 1510
Nb-BiP1 : ACTGCAGGCTCCTTGGAAATTTTGATCTCACCGGAATTCCTCCAGCTCCAAGGGGAACAC : 1462
Nb-BiP2 : ACTGCAGGTTACTTGGAAAATTCGACTTAACTGGAATAGCTCCAGCTCCAAGAGGAACTC : 1141
Nb-BiP4 : ACTGCAGGCTACTTGGGAAATTTGACTTAACTGGAATAGCTCCAGCTCCAAGAGGAACTC : 1657
Sl-BiP2 : ACTGTAGACTACTGGGGAAGTTTGATTTGACAGGAATACCTCCAGCACCAAGAGGAACAC : 1507
Sl-BiP4 : ACTGCAGACAGCTTGGAAAATTTGATTTAACTGCAGTAGCTCCAGCTCCTAGAGGAACTC : 1471
Sl-BiP3 : ATTGTCGCGAACTTGGAAGCTTCGACTTGTCTGGTATACCACCTGCTCCGAGGGGAGTGC : 1528
Nb-BiP3 : ATTGTCGTGAACTTGGAAGCTTTGACTTATCGGGTATACCTCCCGCTCCAAGGGGAGTGC : 1513

                    *         1700        *         1720        *         1740
Sl-BiP1 : CCCAAATTGAGGTCACATTCGAAGTTGATGCCAATGGTATCTTGAATGTCAAAGCAGAAGG : 1570
Nb-BiP1 : CTCAAATTCAGGTTACCTTTGAAGTTGATGCGAAACGGTATTTTCAATGTCAAAGCAGAAG : 1522
Nb-BiP2 : CTCAAATTGAAGTTACATTTGAGGTTGATGCCAACGGTATCCTCAATGTGAAAGCTGAAG : 1201
Nb-BiP4 : CTCAAATTGAAGTTACGTTTGAGGTTGATGCCAACGGTATCCTCAATGTGAAAGCGGAAG : 1717
Sl-BiP2 : CTCAAATTGAAGTAACTTTTGAGGTTGATGCCAATGGTATCCTAAACGTGAAAGCTGAGG : 1567
Sl-BiP4 : CACAGATCGAGGTGACATTTGCAGTTGATGCAAATGGTATTCTCGACGTCAAAGCTGAAG : 1531
Sl-BiP3 : CACAAATTGAGGTGACTTTTCAGGTTGATGAGAATGGAATTCTACATGTAACAGCACAAG : 1588
Nb-BiP3 : CACAGATTGAGGTGAGCTTTGAGATTGATGCGAATGGAATTCTGCAAGTGACAGCACAAG : 1573

                    *         1760        *         1780        *         1800
Sl-BiP1 : ACAACGGTACTGGAAAAGCAGAGAAGATTACCATTACAAACGACAAGGGGCCGCTTAAGCC : 1630
Nb-BiP1 : ATAAAGGGCACTGGTAAATCAGAGAAGATTACCATTACAAATGACAAGGGCCGCTTAAGCC : 1582
Nb-BiP2 : ATAAAGCCTCTGCAAACTCAGAGAAGATCACCATTACCAATGACAAGGGTCGCTTGAGTC : 1261
Nb-BiP4 : ATAAAGCCTCTGCAAAGTCAGAGAAGATCACCATTACCAATGATAAGGGTCGCTTGAGTC : 1777
Sl-BiP2 : ATAAAGCCTCTGCAAAGTCAGAAAAGATTACCATCACGAATGACAAGGGTCGTTTGAGTC : 1627
Sl-BiP4 : ACAAAGCCTCTGCAAAATCAGAGAAGATTACAATTACCAATGATAAGGTCGCTTGAGTC : 1591
Sl-BiP3 : ACAAAGCAGCTAAGAAGTCGAAATCTATTACCATCACTAGTGACAAGTCGCGTCTAAGCC : 1648
Nb-BiP3 : ACAAAGCAGCTAAGAAGTCCAAATCCATTACCATCACTAGCGACAAGTCGCGTCTAAGCC : 1633
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              *      2180       *      2200       *      2220
Sl-BiP1 : AGCCATGATGAGCTG--------------------------------------------- : 1998
Nb-BiP1 : GGACATGATGAACTATTCCAGAATGATAATTACAACAAAAACAACAAACCCGGTGGAATC : 1992
Nb-BiP2 : TCACATGACGAGCTGTAA------------------------------------------- : 1638
Nb-BiP4 : TCACATGACGAGCTGTAA------------------------------------------- : 2154
Sl-BiP2 : TCACATGATGAGCTGTAA------------------------------------------- : 2004
Sl-BiP4 : ------GATGAGCTGTAA------------------------------------------- : 1860
Sl-BiP3 : GCTTATGATGAATTATAG------------------------------------------- : 2010
Nb-BiP3 : TCTTACGATGAATTGTAG------------------------------------------- : 1995
```

Figure S2. Sequence alignment of the tomato *SlBiP* and *N. benthamiana NbBiP* genes. Residues highlighted in light and dark grey represent identical nucleotides at the indicated position shared between two or three *BiP* homologs, respectively. Residues highlighted in black represent nucleotides that are shared by all *BiP* homologs at the indicated position. Sequences of DNA inserts used for VIGS are indicated by different colours: blue for TRV:Sl-*BiP1*, green for TRV:Sl-*BiP2*, brown for TRV:Sl-*BiP3* and turquoise for TRV:Sl-*BiP4*. In red the Sl-*BiP1* insert that also targets *SlBiP2* and *SlBiP4*, and therefore was used for constructing TRV:Sl-*BiP-1*,*-2* and *-4* is indicated. The DNA insert of TRV:Sl-*BiP-1, -2, -3* and *-4* consists of the red *SlBiP1* fragment plus the brown *SlBiP3* fragment (see materials and methods). All *SlBiP* inserts contain at least a stretch of 21 nucleotides or more that is identical to the corresponding *NbBiP* homolog. Note that TRV:*SlBiP2* and TRV:*SlBiP4* both target *NbBiP2* and *NbBiP4*, as these two *BiP* genes of *N. benthamiana* are highly homologous.
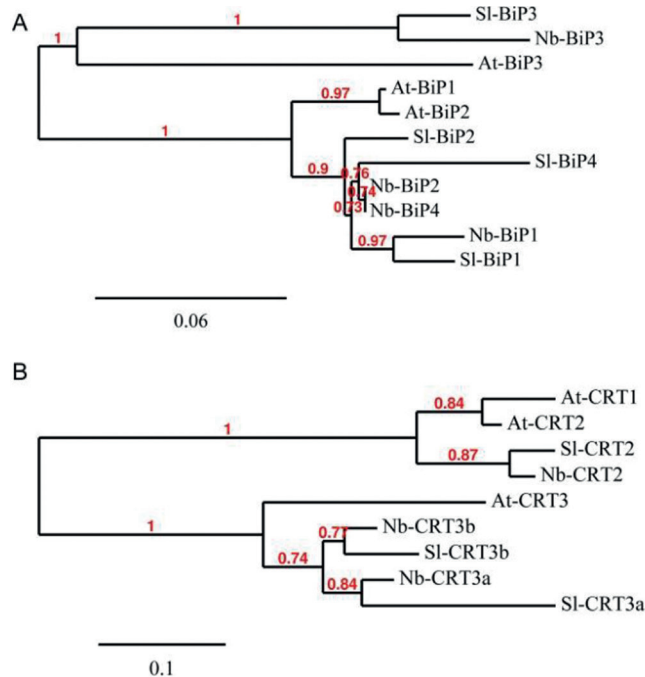
Figure S3. Tomato and *N. benthamiana* contain four BiP homologs and two CRT3 homologs. Phylogenetic trees of *Arabidopsis*, tomato and *N. benthamiana* BiPs (A) and CRTs (B) were constructed using Phylogeny. fr in the "one click" mode. Maximum likelihood values are represented at each node and the relative evolutionary distance is shown below each tree.
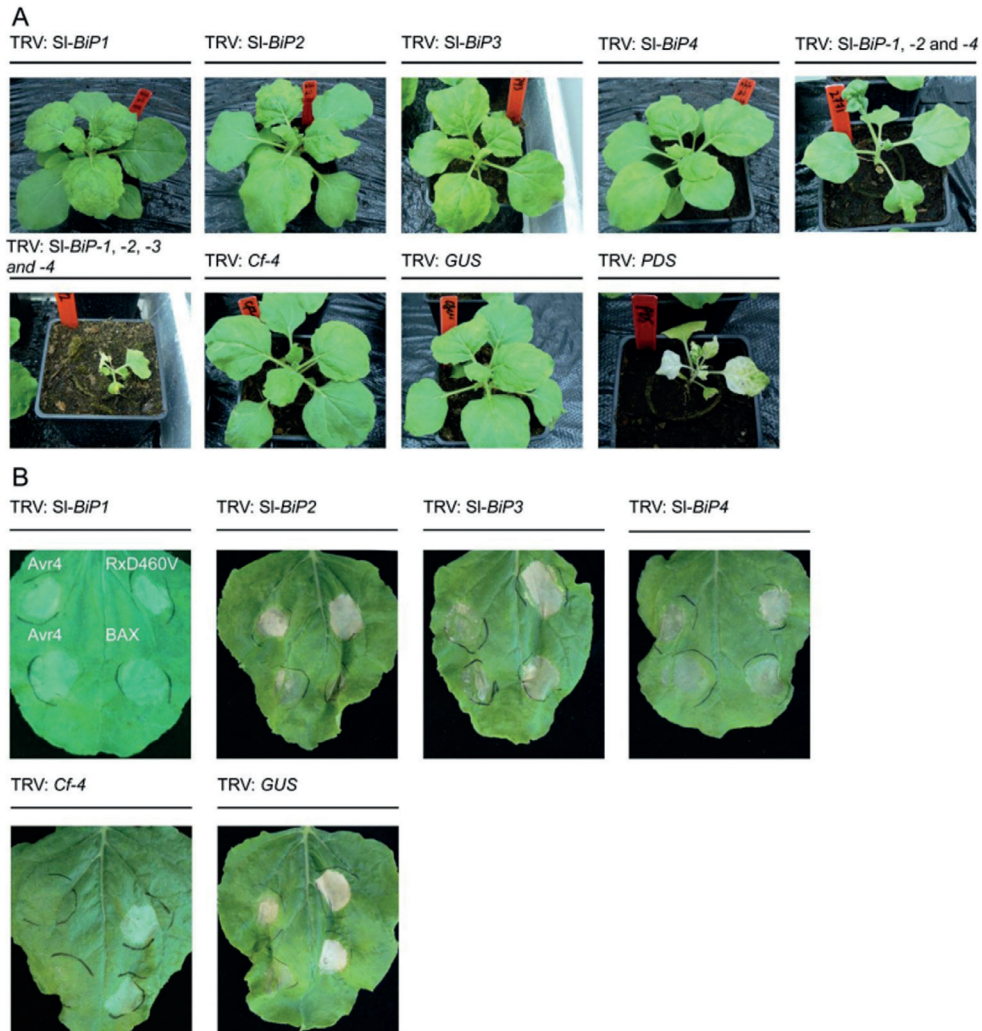
Figure S4. Silencing of individual *BiPs* does not affect Cf-4 function, whereas silencing of multiple *BiPs* causes lethality in *N. benthamiana*. (A) VIGS of multiple *BiPs* causes lethality in *N. benthamiana*. *N. benthamiana* expressing *Cf-4* was subjected to VIGS of *BiP* family members by inoculation with the indicated TRV constructs. TRV:*Cf-4* and TRV:*GUS* served as controls, whereas TRV:*PDS* was included to monitor the progress of silencing, visualized by the typical photobleaching. Plants were photographed three weeks after inoculation with the recombinant virus. (B) Inoculation with TRV carrying inserts targeting the individual *BiPs* does not affect the Avr4-triggered HR. *N. benthamiana* expressing *Cf-4* was inoculated with the indicated TRV constructs and three weeks later Avr4 (in duplicate), auto-active Rx (RxD460V) and BAX were transiently expressed in the order indicated in the first panel. Leaves were photographed three days later. Representative results from three independent experiments are shown. In each experiment at least three plants were inoculated per TRV construct.

```
              *        20         *        40         *        60
Sl-CRT3A : ------------------------------------ATGG-------------------- :   4
Nb-CRT3A : ACGACCCAAAAGTTTTCATAAAAAGAGCAAAATGGCTCACTCTGAGCATAAACCAAGTAG :  60
Sl-CRT3B : ------------------------------ATGGCTCTTTCCAAGAATGAAAAAAGAAA :  29
Nb-CRT3B : --------------ATTTCCACAATGAGGCAAATGGCTCTGTCCAAGCATGAACAAAGAAC :  47
Sl-CRT2  : ------------------------------ATGGCT--------ACTCGACGAATGAAA :  21
Nb-CRT2  : ------GTATTTCGATCTCACAACAGTGCCCATGGCT--------ACTCAACGAAGGGCA :  46

              *        80         *       100         *       120
Sl-CRT3A : ------A---------TTGTC--TAG---------TAGTAGTTTTGCTACTTTTACACT :  38
Nb-CRT3A : ACTGATA---------TTCTCATTGG---------TACTAGTTTTGCTCTTTCTTACACT : 102
Sl-CRT3B : AAGTCTACTACTAGTCTCCTCACTTGAATCATTTCTACTAGTTTTGTTACTGTTTTCAAT :  89
Nb-CRT3B : AAGTCTAAAACTAGTCTTCTCAGCTGAATCATTTCTACTAGTTTTGTTACTTTTCTCACT : 107
Sl-CRT2  : AGCCCTAG--------CTCTCTCCAT-----------CTCGTTGCTGTGTTCTCTCTACT :  62
Nb-CRT2  : AACCCTAG--------CTCTCTCCAT-----------CTAATTACTGTGTTCTCTCTGCT :  87

              *       140         *       160         *       180
Sl-CRT3A : TGTGACTTGTTCAGTATGTGACACCTTCTTTGAAGAACGATTTGATGATGGTTGGCGGAA :  98
Nb-CRT3A : TGTCTCTTCTTCAGTATCTGACATTTTCTTTGAAGAAAGCTTTGATGATGGATGGAGGAG : 162
Sl-CRT3B : TATTACATCTTCATTATCTGAGATCTTCTTTGAAGAACAATTTGATGATGAGTTGGCAGAG : 149
Nb-CRT3B : TCTCAGCTCTTCATTCTCTGAGATCTTTTTTGCAGAACAGTTCGATGATGATTGGCGGAG : 167
Sl-CRT2  : AGTAGTCGCCGCCGCCGCCGAAGTTTTCTTCCAGGAAAGCTTCAATGATGGTTGGGAAAG : 122
Nb-CRT2  : TGTCACTGTTGTCTCCGCTGAGGTCTTCTTCGAGGAGTTTCAACGATGGTTGGGAAAG : 147

              *       200         *       220         *       240
Sl-CRT3A : CAAATGGGTGAAGTCTGAGTGGAAAAGCAGTGAAGGAAAGCAGCGAAATTTAAGCACAC : 158
Nb-CRT3A : CAGATGGGTGAAGTCAGACTGGAAAAATCAGTGAAGGAAAAGCAGGCTCGTTTAACCATAC : 222
Sl-CRT3B : CAGATGGGTGAAGTCTGACTGGAAAAGGAGTGAAGGGAAAGCAGGCTCCTTTAACCATAC : 209
Nb-CRT3B : CAGATGGGTGAAGTCTGACTGGAAAAGGAGTGAAGGGAAAGCAGGTTCATTTAACCATAC : 227
Sl-CRT2  : CAGGTGGGTAAAATCTGAATGGAACGAAAGATGAAAACATGCTGGAGAGTGGAATCACAC : 182
Nb-CRT2  : CAGGTGGGTGAAATCTGAATGGAACGAAAGATGAAAACATGCTGGAGAGTGGAATCACAC : 207

              *       260         *       280         *       300
Sl-CRT3A : AGCGGGCAACTGGCCTGGTGATCCTGATGACAGAGGTCTTCAGAC-AACAAGTGATGCCA : 217
Nb-CRT3A : GGCCGGTACATGGGCTGGTGATCCTGATGACAAAGGTATCCAGAC-AACAAACGATGCCA : 281
Sl-CRT3B : AGCTGGGAAATGGGCTGGTGATCCTGATGACTAAAGGTATTCAGAC-GTCAACCGATGCCA : 268
Nb-CRT3B : GCTGGAAAATGGGCTGGTGATCCTGATGATAAAGGTATTCAGAC-ATCAAGCGATGCCA : 286
Sl-CRT2  : CTCTGGGAAGTGGAATGGTGACGCCAATGACAAAAGGTATTCAGACCAGTGAA-GACTACA : 241
Nb-CRT2  : ATCTGGCAAGTGGAATGGACGCGCTAATGACAAAAGGTATCCAAACCAGTGAG-GACTACA : 266

              *       320         *       340         *       360
Sl-CRT3A : AACATTTGCCCTCTCTGCAAAGATACCGGAATTTAGCAATAAAAACACAACACTGGTTG : 277
Nb-CRT3A : AACATTTGCTGTCTCTGCAAAAATACCGGAATTTAGCAATAAAAACAGAACACTGGTTG : 341
Sl-CRT3B : AACATTTCGCCATCCTGCTACGATACCAGAATTTAGCAACAAGAATAGAACATTGGTTG : 328
Nb-CRT3B : AACATTTCGCCATTTCTGCTACGATACCGGAATTTAGCAACAAGAATAGAACTTTGGTTG : 346
Sl-CRT2  : GGTTCTACGCCATTTCGGCTGAGTTCCCTGAATTTAGTAACAAGGGAAAGAACTTAGTGT : 301
Nb-CRT2  : GGTTCTATGCCATTTCAGCTGAGTTCCCTGAATTTAGCAACAAGGGAAAAACTTAGTGT : 326

              *       380         *       400         *       420
Sl-CRT3A : TACAGTATTCTATTAAGTTTGAGCAAGATATTGAATGTGGAGGAGCGCTACATAAAGCTTC : 337
Nb-CRT3A : TCCAGTATTCTATAAGTTTGAGCAAGATATTGAATGTGGAGGGGGTTACATAAAGCTTC : 401
Sl-CRT3B : TACAGTACTCTATAAAGCTTGAGCAAGACATTGAATGTGGTGGAGGTTACATAAAGCTTC : 388
Nb-CRT3B : TACAATATTCTATAAGTTTGAGCAAGACATTGAGTGTGGTGGAGGTTACATAAAGCTTC : 406
Sl-CRT2  : TCCAGTTCTCTGTTAAGCATGAGCAGAAGCTTGACTGTGGTGGTGGTACATCAAGTTGC : 361
Nb-CRT2  : TCCAGTTCTCGGTGAAGCATGAGCAGAAGCTTGACTGTGGTGGTGGTACATCAAGTTGC : 386

              *       440         *       460         *       480
Sl-CRT3A : TCTCTGGATATGTCAATCAGAAGAAATTCGGAGGAGACACCCCATACAGTTTTATGTTTG : 397
Nb-CRT3A : TCTCTGGATATGTCAATCAGAAGAAATTTGGTGGAGACACTCCATACAGTTTTATGTTTG : 461
Sl-CRT3B : TCTCTGGATATGTCAACCAGAAGAAATTCGGGGGAGACATACTCCTTACAGTTGTGATGTTTG : 448
Nb-CRT3B : TCTCTGGATATGTCAACCAGAAGAAATTTGGGGGAGACACACCCTTACAGTTATGATGTTTG : 466
Sl-CRT2  : TTAGTGGGACGTTGACCAAAAGAAATTCGGTGGTGACACTCCCTACAGTATCATGTTTG : 421
Nb-CRT2  : TTAGTGCGGATGTTGACCAAAAGAAATTCGGTGGTGACACTCCCTACAGCATCATGTTTG : 446
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              *        500         *        520         *        540
Sl-CRT3A : GACCAGATATATGTGGTACACAG-ACAAAGAAACTTCATGTTATACTTTCCTACCAAGGA : 456
Nb-CRT3A : GGCCAGATATCTGTGGTACACAG-ACGAAGAAACTTCATGTTATACTTTCCTACCAAGGC : 520
Sl-CRT3B : GACCGGATATCTGTGGTACACAG-ACGAAGAAACTTCATGTTATACTTTCCTACCAAGGC : 507
Nb-CRT3B : GACCGGATATCTGTGGTACACAG-ACAAGAAACTTCATGTTATGCTTTCCTATCAAGGC : 525
Sl-CRT2  : GACCAGACATCTGTGGC-TACAGTACCAAGAAAGTCCATGCTATTCTCACTTATAACCAG : 480
Nb-CRT2  : GCCCAGACATCTGTGGC-TACAGCACCAAGAAGGTCCATGCTATTATCACTTATAATGAC : 505

              *        560         *        580         *        600
Sl-CRT3A : CAGAATTACCCCATCAAGAAGGAATTAGAATGTGAAACGGACAAATTAAGACATTTCTAC : 516
Nb-CRT3A : CAGAATTACCCCATCAAGAAGGAATTAGAATGTGAAACAGACAAATTAACACATTTCTAC : 580
Sl-CRT3B : CAGAATTATCCAATCAAAAAGGATCTGCAATGTGAGACAGACAAGTTAACCCATTTCTAC : 567
Nb-CRT3B : CAGAATTATCCCATCAAAAAGGATCTACAATGTGAAACAGACAAATTAACCCATTTCTAC : 585
Sl-CRT2  : ACAAACCATTTGATCAAGAAGGAGTCCCATGTGAGACTGATCAGCTGACCCATGTCTAC : 540
Nb-CRT2  : ACAAACCACTTGATCAAGAAGGAAGTACCTTGTGAGACTGATCAACTGACCCATGTCTAC : 565

              *        620         *        640         *        660
Sl-CRT3A : ACCTTCATTCTTAGACCTGATGCATCATACAGCATCTGGATTGATGGTAGAGAAGAGAT : 576
Nb-CRT3A : ACGTTCATTCTTAGACCTGATGCATCCTACAGCATCTGGATTGATGGTCGAGAAGAGAT : 640
Sl-CRT3B : ACATTCATTCTTCTTAGACCTGATGCATCATACAGCATCTGGATAGATGGCCGAGAGAGGGAT : 627
Nb-CRT3B : ACATTCATTCTTAGACCTGATGCATCATACAGCATCTGGATTGATGGTCGA--------- : 636
Sl-CRT2  : ACTTTCATCCTCCGTCCTGATGCCACATACAGTATTCTCATTGACAATGTGGAGAAACAG : 600
Nb-CRT2  : ACTTTCATCCTCCGCCCTGATGCTACATACAGCATTCTCATTGATAATGTGGAGAAACAG : 625

              *        680         *        700         *        720
Sl-CRT3A : TCTGGAAGTATGTACACAGATGGGACATACTTCCTCCCCGCAAAATTAAGGCTGTAAAT : 636
Nb-CRT3A : TCTGGAAGTATGTACACAGACTGGGACACTTCCTCCCCGCAAATCAAGCATGTAAAT : 700
Sl-CRT3B : TCTGGAAGCATGTATACAGCTGGGATATCTTTCCCCCTCGAAAATTAAGATGTAAAT : 687
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : TCTGGTAGCTTGTACTCTGATTGGGACATTCTCCCACCAAAGAAGATCAAGCATCCAAGT : 660
Nb-CRT2  : TCTGGTAGCTTGTACTCTGACTGGGACCTTCTCCCACCAAAGACAATCAAGCATCCAAGT : 685

              *        740         *        760         *        780
Sl-CRT3A : GCGAAAAAGCCTACTGACTGGGATGATAGGCAATATATAGAGAATCCAAATGATGTCAAA : 696
Nb-CRT3A : GCAAAAAAGCCTGCAGACTGGGAAGACAGACAATATATTGAAGATGACCAGATGACATCAAA : 760
Sl-CRT3B : GCGAAAAAGCCTGCAGATTGGGATGATAGACAATATATAGAGGATCCAAACGAGGTCAAA : 747
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : GCCAAGAAACCTGAAGATTGGGATGACAAGGAATTCATTGATGTACCCGAGGATAAGAAG : 720
Nb-CRT2  : GCCAAGAAGCCTGAAGATTGGGATGAGAAGGAATTCATTGATGATCCTGAGGATAAGAAG : 745

              *        800         *        820         *        840
Sl-CRT3A : CCTGAGGGATATGATTCTATTCCAAGAGAGATTCCGGACCCAAAAGCTAAAAAGCCTATT : 756
Nb-CRT3A : CCTGAGGGATATGATTCTATTCCGAGAGAGATTCCAGACCCGGAAAGCTAAAAAGCCAAAT : 820
Sl-CRT3B : CCAGAGGGATATGAATCTATTCCAAAAGAGATCCCTACCCCAAAAGCTAAAAAGCCTGAT : 807
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : CCAGAGGGCTATGATGACATTCCAGAGGAGATAACTGATCCTGAGCCAAGAAGCCAGAG : 780
Nb-CRT2  : CCAGAGGGCTATGATGACATTCCAGAAGAGATAACTGATCCTGATGCCAAGAAGCCAGAG : 805

              *        860         *        880         *        900
Sl-CRT3A : TACTGGGATGACGAAGATGATGGTATGTGGAGGCACCCAAAGTACCTAATCCAGCCATAC : 816
Nb-CRT3A : AACTGGGATGATGAAGAGGATGGTATGTGGAGCCACCCAAAGTACCTAATCCAGCCATAC : 880
Sl-CRT3B : CACTGGGATGATGAACAGGATGGCATCTGGAGACCACCAAAAATTCCTAACCAGCCATAT : 867
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : GACTGGGATGATGAAGAAGATGGTGAATGGACAGCCCCAACCATCCCCAATCCAGAGTAC : 840
Nb-CRT2  : GACTGGGATGATGAAGAAGATGGTGAATGGACAGCCCCAACTATTCCCAACCCCGAGTAC : 865

              *        920         *        940         *        960
Sl-CRT3A : CGGCCGGGTGCATGGAAAAGGAAGAAAATTAAGAACCCCAATTATAAAGGGAAATGGAAG : 876
Nb-CRT3A : CGT---GGTCCATGGAAAAGGAAGAAAATTAAGAATCCCAACTATAAAGGAAAATGGAAG : 937
Sl-CRT3B : AAG---GGTCCATGGAAACGGAAGAAAGTGAAGAACCCTAACTATAAAGCTAAATGGAAG : 924
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : AAG---GGCCCATGGAAGGCAAAGAAAATCAAGAACCCCAACTACAAGGGAAAGTGGAAG : 897
Nb-CRT2  : AAG---GGCCCATGGAAGCCAAAGAAAATTAAGAACCCCAACTACAAGGGGAAGTGGAAG : 922
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                *         980        *        1000        *         1020
Sl-CRT3A : ATCCCCTGGATTGATAATCCAGAATTTGAAGATGATCCTGATCTCTACGTGCTGAAGCCA : 936
Nb-CRT3A : ATTTCCTTGGATTGATAATCCAGAATTTGAAGATGATCCTGATCTCTACGTACTGAAGCCG : 997
Sl-CRT3B : ACCCCTTGGATCGATAATCCAGAATTTGAAGATGATCCTGATCTCTATGTGTTGAAGCCT : 984
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : GCTCCTATGATTGACAACCCAGACTTCAAGGATGACCCAGATCTATATGTTTTCCCAAAA : 957
Nb-CRT2  : GCACCTTTGATTGATAACCCAGACTTCAAAGATGATCCAGATCTCTATGTTTTCCCAAAG : 982

                *         1040        *        1060        *         1080
Sl-CRT3A : ATCAAATATGTCGGTATTGAAGTTTGGCAGGTAAAGGCAGGTTCTATTTTTGATAACATT : 996
Nb-CRT3A : ATCAAATATGTTGCTATCGAAGTTTGGCAGGTAAAGGCAGGTTCTGTTTTTGATAACATT : 1057
Sl-CRT3B : ATCAAATATGTTGCAATCGAAGTTTGGCAGGTGAAGGCAGGTTCTCTATTTGACAACATA : 1044
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : TTGAAGTATGTTGCAGTGGGAGCTGTGGCACAGTGAAATCTGGAACTTTGTTTGACAATGTT : 1017
Nb-CRT2  : CTGAAGTATGTGGCAGTTGACTGTGGCACAGTGAAATCTGGAACCTTGTTTGACAATATT : 1042

                *         1100        *        1120        *         1140
Sl-CRT3A : CTGATTGGCGATGATCCAGATTATGCAAAACAAGTGATTCAGGAAGTGTTTTCCCAT--- : 1053
Nb-CRT3A : TTGGTGTGTGATGAGCCAGATTATGCAAAACAAGTGATTCAGGAAGTGTTGCCAAT--- : 1114
Sl-CRT3B : CTGATATGTGATGACCCTGATTATGCAAAGAAGTGATTGAGGAAGTCTTTGCTAAT--- : 1101
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : GTGATATGCGATGATCCAGAGTTTGCCAAGTCAATTGCAGAGGAAACATGGGGAAAGCAG : 1077
Nb-CRT2  : GTCATATGCGATGATCCAGAGTATGCCAAGGCAATTGCTGAAGAACATGGGGAAAGCAG : 1102

                *         1160        *        1180        *         1200
Sl-CRT3A : AGGGAGGCTGAAAAGGAGGCTTTCGAAGAGGCAGAGAAAGTGAGAAAAGCAAAACAGGAA : 1113
Nb-CRT3A : AGGGAGGCTGAAAAGGAGGCTTTTGAAGAGGCAGAGAAAGTGAGAAAAGCAAGAGAAGAA : 1174
Sl-CRT3B : AGGGAGACTGAAAAGGAGGCTTTTGAGGAGGCAGAGAAAGTGAGAAAAGCAAAACAGGAA : 1161
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : AAAGATGCTGAAAAGGCTGCTTTTGAGGAAGCAGAAAAGAAGAGAGAGGAGGAGGAATCA : 1137
Nb-CRT2  : AAGGATGCGGAAAAGGCTGCTTTTGAGGAAGCAGAAGAAGAGAGAGAGGATGAGGAATCA : 1162

                *         1220        *        1240        *         1260
Sl-CRT3A : GAGGAAGCTCAAAGATCAAGAGAGGAAGCGTGAACCGACGAGACGAGAAAG---------- : 1163
Nb-CRT3A : GAGGAAGCTCAAAGAGCAAGAGAGGAAGCGTGAAACGACGAGACGAGAAAG---------- : 1224
Sl-CRT3B : GAGGAAGCCCAAAGAGCAAGAGAAGAACGTGAAACGACGAGAAGAGAAAGAGATAGAGAT : 1221
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : AAGAATGCTC------CAGCTGAATCTGATGC-CGATGAAGATGACGAGG---------- : 1180
Nb-CRT2  : AAGGATGCTC------CAGCTGATTCTGATGC-CGAGCAAGATGA--------------- : 1200

                *         1280        *        1300        *         1320
Sl-CRT3A : --GGGTAGGGACACACACCGG-GACAGATACAAGAGCCGCTATCACCATGATTATATGTA : 1220
Nb-CRT3A : --GGGTAGGGACCACATCGG-GACAGATACAAGAACCGCTATCACCACGATTATATGGA : 1281
Sl-CRT3B : AGGGGTAGGGACAGGCACACA-GATAGAACAAGAGCCGCTATCGCCGTGATTATGATGA : 1280
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : --CTGATGAGGCTCATTCACATGATGCTGATGACAACTCTGACTCCAAGGAT------GA : 1232
Nb-CRT2  : -----------TCATTCTGATGATGCTGATGACAACTCGGAGTCCAAGGAT------GA : 1242

                *         1340        *        1360        *         1380
Sl-CRT3A : TGACGATCATGATGAACTTAA---------------------------------------- : 1242
Nb-CRT3A : TGACTATCATGATGAACTTAAAATTGGTGTTAA-----GATGATACTACATTTTCCACTC : 1336
Sl-CRT3B : TGACTATCATGATGAACTTGA--------------------------------------- : 1302
Nb-CRT3B : ------------------------------------------------------------ : -
Sl-CRT2  : GGACACACATGATGAACTGTAA------------------------------------- : 1254
Nb-CRT2  : TGAAGCACATGATGAACTGTAAGGTGATTTAAGCTGACAAAGATGTTAGCAAGATTAGCT : 1302
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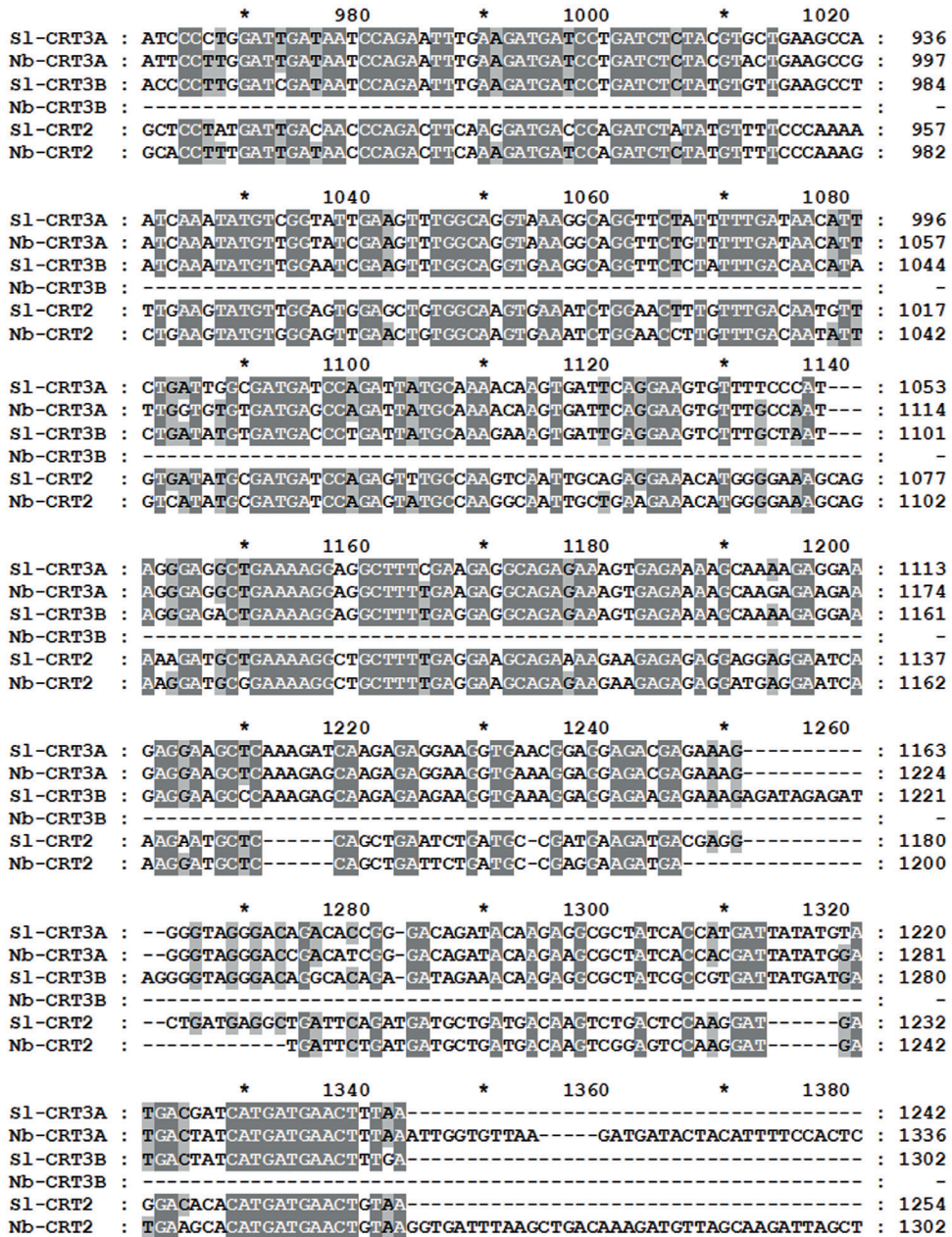
Figure S5. Sequence alignment of the tomato *SlCRT* and *N. benthamiana NbCRT* genes. Residues highlighted in light and dark grey represent identical nucleotides at the indicated position shared between two or three *CRT* homologs, respectively. Residues highlighted in black represent nucleotides that are shared by all *CRT* homologs at the indicated position. Sequences of DNA inserts used for VIGS are indicated in different colours: turquoise for TRV:Sl-*CRT3a* and TRV:Nb-*CRT3a*, green for TRV:Sl-*CRT2* and brown for TRV:Sl-*CRT3b*. Except for the fragment inserted in TRV:Sl-*CRT3a*, all *SlCRT* inserts contain at least a stretch of 21 nucleotides or more that is identical to the corresponding *NbCRT* homolog.

2

SI-CRT2-eGFP

SI-CRT3a-eGFP

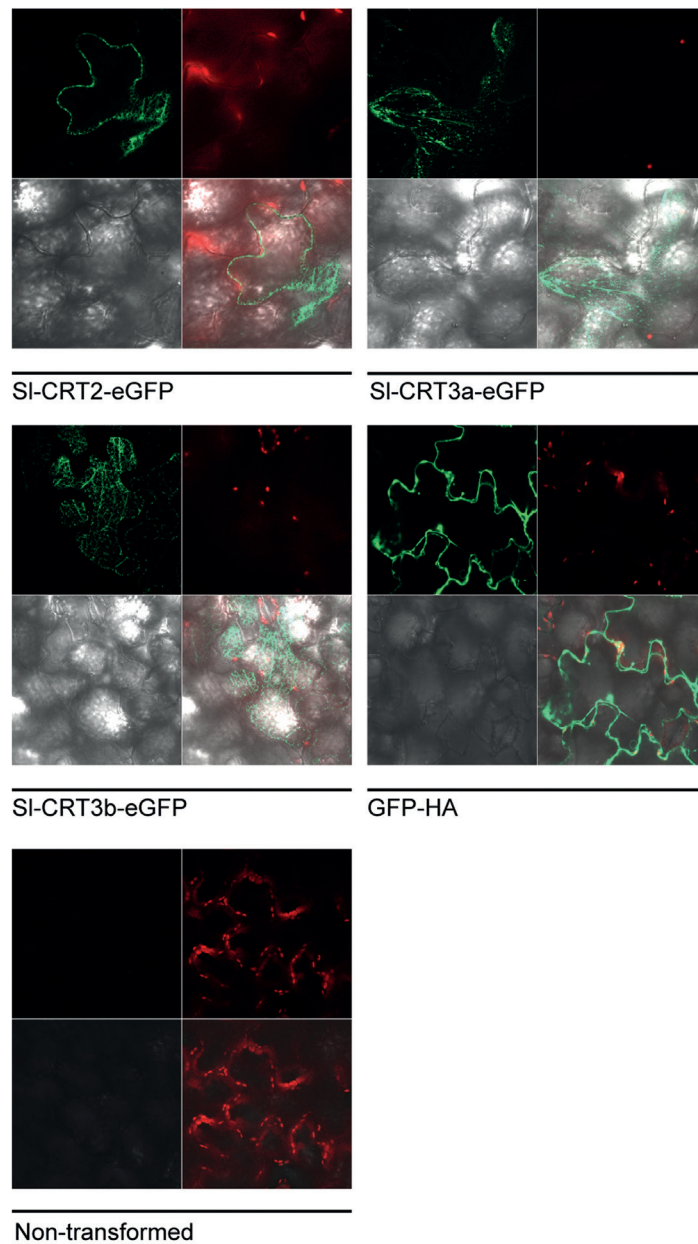SI-CRT3b-eGFP

GFP-HA

Non-transformed

Figure S6. Transiently expressed SI-CRT-eGFP proteins localize to the ER-network. The cellular localization of the indicated SI-CRT-eGFP fusion proteins, transiently expressed using *Agrobacterium* infiltration in *N. benthamiana* epidermal leaf cells, is shown. GFP-HA localizes to the cytoplasm, whereas a non-infiltrated leaf was used as a negative control. Each panel shows the eGFP signal (upper left), the chloroplast signal (upper right), the bright field picture (lower left) and the combined image (lower right).
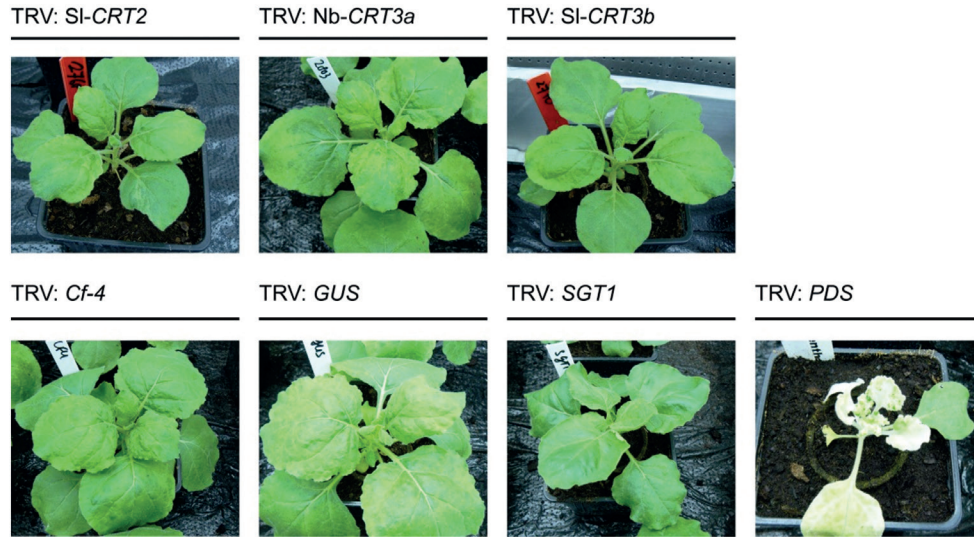
Figure S7. VIGS of the different *NbCRTs* does not result in an aberrant phenotype in *N. benthamiana*. Transgenic *N. benthamiana:Cf-4* was subjected to VIGS of the different *CRTs* by inoculation with the indicated TRV constructs. TRV:*Cf-4*, TRV:*GUS*, TRV:*SGT1* and TRV:*PDS* served as controls. Plants were photographed three weeks after inoculation with the recombinant virus. Similar to inoculation with TRV:*GUS* and TRV:*Cf-4*, VIGS of the *CRTs* did not result in an aberrant phenotype. Silencing of *PDS* lead to the photobleaching phenotype, whereas silencing of *SGT1* typically resulted in stunted plants with dark green, curled leaves. Representative photographs of three independent experiments are shown.

Table S1. Scores of peptides identified by mass spectrometry. Mascot ion scores are presented for the identifications on the Orbitrap XL. ProteinLynx peptide ladder scores are presented for identifications on the Synapt MS.

Protein name	Peptide sequence	Mascot Ion Score	ProteinLynx peptide score
Cf-4-eGFP	STSCCSWDGVHCDETTGQVIELDLR		6.2385
	LDLSYNDFTGSPISPK		7.2696
	FGEFSDLTHLDLSHSSFR		7.1241
	GVIPSEISHLSK		7.1045
	VFHLSDLEFLDLSSNPQLTVR		7.8532
	LKGPIPNSLLNQK		6.6454
	NEYLSHLDLSNNR		6.8697
	ILGNLQTMK		7.0783
	EIDESTGFPEYISDPYDIYYNYLTTISTK		6.3311
	ILDSNMIINLSK		6.9861
	LEHIITTK		5.9407
	GEELFTGVVPILVELDGDVNGHK		7.1039
	SAMPEGYVQER		5.4794
	AEVKFEGDTLVNR		6.0148
	GIDFKEDGNILGHK		7.4862
*Nb*BiP peptides	NGHVEIIANDQGNR		5.3916
	DGKPYIQVK		6.9857
	IKDAVVTVPAYFNDAQR		8.1502
	DAGVIAGLNVAR		8.2218
	IINEPTAAAIAYGLDKK		7.9626
	ARFEELNNDLFR		7.7288
	NQIDEIVLVGGSTR		5.6292
	DYFDGKEPNK		6.9857
	GVNPDEAVAYGAAVQGGILSGEGGDETK		7.5296
	DILLLDVAPLTLGIETVGGVMTK		6.8456
	FDLTGIAPAPR		8.1423
	LSQEEIER		7.0558
	EAEEFAEEDK		6.3662
	EALEWLDDNQSAEKEDYEEK		6.6689
*Nb*CRT2	GIQTSEDYR	46.7	
	FYAISAEFPEFSNK	75.6	
	APMIDNPDFKDDPDLYVFPK	57.2	
	SGTLFDNVVICDDPEFAK	67.8	
*Nb*CRT3a	HFALSAK	40.5	
	FEQDIEcGGGYIK	71.6	
	LHVILSYQGQNYPIKK	64	
	VPNPAYR	56.4	
	IPWIDNPEFEDDPDLYVLKPIK	65.1	
	EAEKEAFEEAEK	74.6	
*Nb*CRT3b	LHVILSYQGQNYPIKK	64	

Table S2. Sequences of all oligonucleotide primers used. Restriction sites used for cloning are underlined.

Primer code	Primer name	Sequence (5'-3') (Restriction sites are underlined)
to11	Cf-4-AttB1-fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGGTTGTGTAAAACTTGTGTTTTT
to12	Cf-4-AttB2-nostop-rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTATATCTTTTCTTGTGCTTTTTCATTTTC
to13	Cf-2.2-AttB1-fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGATGATGGTTTCTAGAAAAGTAGT
to14	Cf-2.2-AttB2-nostop-rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTGAAGCGATTATTTCTTCTTCTGT
to22	Peru2-AttB1-fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGGTTACGTGAAACTTGTGTT
to23	Peru1and2-AttB2-nostop-rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTATATCTTTTCTTGTGCTTTTTCATT
to58	ActinF_am8	TATGGAAACATTGTGCTCAGTGG
to59	ActinR_AM9	CCAGATTCGTCATACTCTGCC
to128	GW-Cf-9-fw	CACCATGGATTGTGTAAAACT
to129	Cf-9/4/4E-nostop-rev	ATATCTTTTCTTGTGCTTTTTCA
to130	Gw-Cf-4/4-E-fw	CACCATGGGTTGTGTAAAACT
to137	qSlBip1 / NtBip1-fw	CAGGTTTTTGAAGGTGAAAGG
to138	qSlBip1/NtBip1-rev	GCAAACTCTTCTGCTTCACGA
to139	qSlBip2(5)-fw	AACTTGCAGACAAGTTA
to140	qSlBip2(5)-rev	CATGTGAATCATCATCC
to141	qSlBiplike-fw	GAAAGAGAAGATTGAAACGGTG
to142	qSlBiplike-rev	TCATCGTCACCAGCACCTGATT
to170	EcoRI-qSLCRT2-fw	AGAATTCGTTGCTTAGTGGAGACGTTG
to171	BamHI-qSLCRT2-rev	AGGATCCTGGCATCAGGACGGAGG
to172	EcoRI-VIGS/qSLCRT1- fw	AGAATTCATGGATTGTCTAGTAGTAGTTT
to173	BamHI-VIGS/qSlCRT1-rev	AGGATCCGCCAGTTGCCCGCTGTG
to174	EcoRI-VIGSSlCRT3-fw	AGAATTCATTTCTACTAGTTTTGTTACTG
to175	BamHI-VIGSSlCRT3-rev	AGGATCCGGTTGACGTCTGAATACCTTTA
to176	NewXbaI-SlBiPall-fw	ATCTAGACTTGTCTTTGACCTTGGTG
to177	NewBamHI-SlBiPall-rev	AGGATCCGCACCATAAGCAACTGC
to195	Sl-CRT1 gwfw	CACCATGGATTGTCTAGTAGTAGTTTTGC
to196	Sl-CRT1 nostoprev	AAGTTCATCATGATCGTCATACA
to197	Sl-CRT2 gwfw	CACCATGGCTACTCGACGAATGAAAAG
to198	Sl-CRT2 nostoprev	CAGTTCATCATGTGTGTCC
to199	Sl-CRT3 gwfw	CACCATGGCTCTTTCCAAGAATG
to200	Sl-CRT3 nostoprev	AAGTTCATCATGATAGTCATCATC
to201	BamHi-SlBiP3(4)fw	AGGATCCGGGTGTATACAAAGGGGAGA
to202	XhoI-SlBiP3(4)rev	ACTCGAGGATAATTTTCTGAACTTCTGG
to203	Nb CRTa1864fw:	GAAAACATGGCTGGAGAGTGG
to204	Nb CRTa1864rev:	TTTGGTCAACATCCCCACTA
to205	Nb CRTa7179fw:	TAAGCACACGGCCGGCAC
to206	Nb CRTa7179rev:	ATTTGTCTGTTTCACATTCTAAT
to207	Nb CRTa95466+a94604fw	TTTCGCCATTTCTGCTAAGG
to208	Nb CRTa95466+94604rev	TCCAAACATCATACTGTAAGGG
to213	EcoRI-NbCRT1vigsfw	AGAATTCATGGCTCACTCTGAGC
to214	BamHI-NbCRt1vigsrev	AGGATCCCCATCCATCATCAAAGC

83
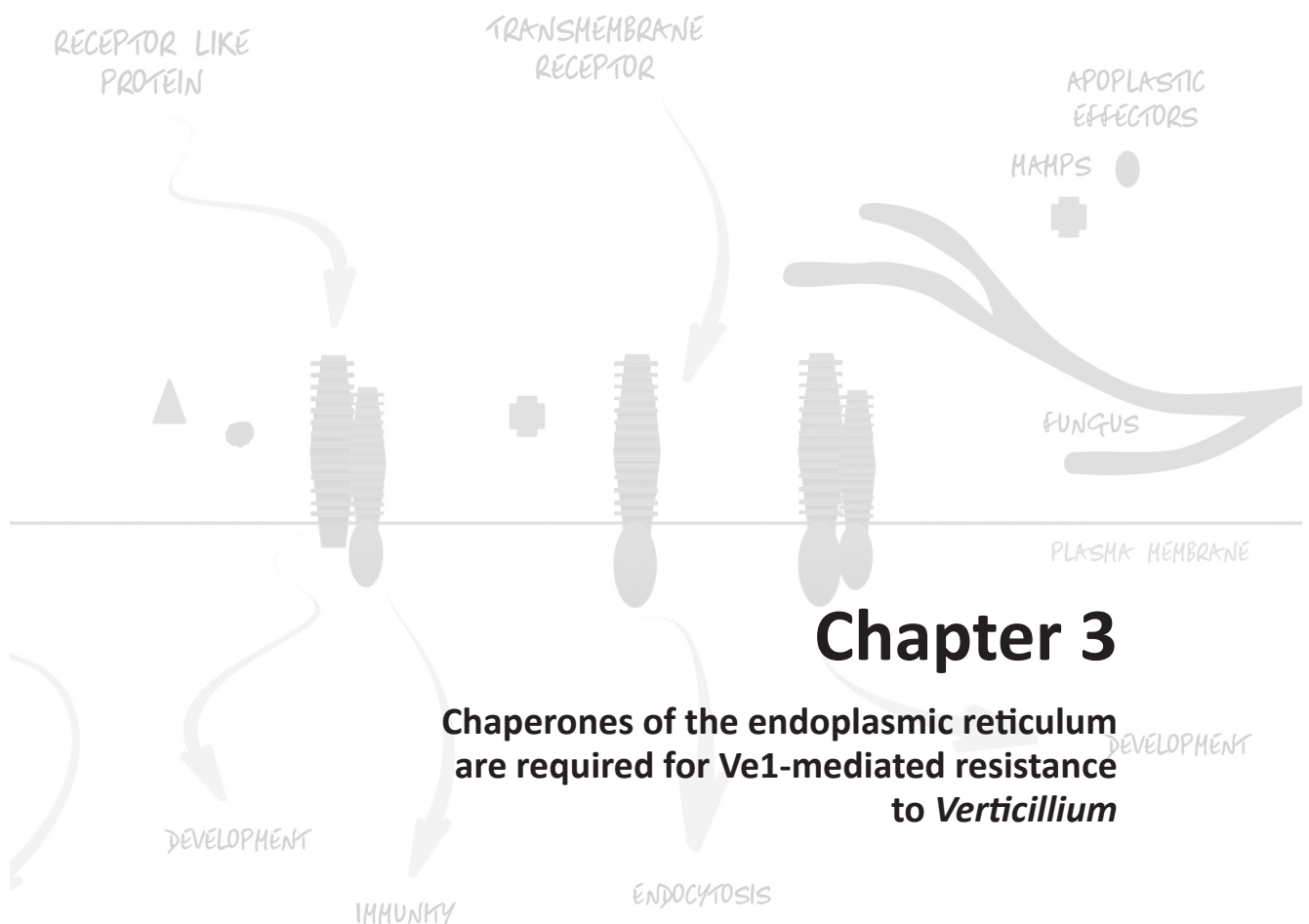
Chapter 3

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

Liebrand, T.W.H.[*], Kombrink, A.[*], Zhang, Z., Sklenar, J., Jones, A.M.E., Robatzek, S., Thomma, B.P.H.J.[#], and Joosten, M.H.A.J[#]. **Chaperones of the endoplasmic reticulum are required for Ve1-mediated resistance to *Verticillium*.** *Mol Plant Pathol* (2013) 15: *109-117*
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[#]These authors contributed equally

Abstract

The tomato Receptor-Like Protein (RLP) Ve1 mediates resistance to the vascular fungal pathogen *Verticillium dahliae*. To identify proteins required for Ve1 function, we transiently expressed and immunopurified functional Ve1-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 CalReTiulin (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 was 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.

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INTRODUCTION

Recognition of microbial patterns or damage-associated host molecules by immune receptors results in the activation of immune responses in plants [1-3]. These immune receptors are either localized in the cytoplasm or at the cell surface [1, 4, 5]. Examples of well-studied cell surface receptors that are involved in pathogen recognition are the *Arabidopsis* (*Arabidopsis thaliana*) Receptor-Like Kinases (RLKs) Flagellin Sensing-2 (FLS2) and the EF-Tu Receptor (EFR), which are responsible for recognition of bacterial flagellin and elongation factor-Tu, respectively [6, 7]. Another important class of plant cell-surface receptors are the Receptor-Like Proteins (RLPs). Similar to most RLKs, most RLPs carry extracellular Leucine-Rich Repeats (LRRs), but they lack a cytoplasmic kinase domain [8]. Examples of RLPs that play a role in immunity are the tomato (*Solanum lycopersicum*) Cf proteins [9, 10], Ve1 [11], LeEIX2 [12], *Arabidopsis* AtRLP30 [13], apple (*Malus domestica*) HcrVf2 [14] and rapeseed (*Brassica napus*) LepR3 [15].

The biogenesis of functional transmembrane receptors requires proper folding and glycosylation. In addition, 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 correctly take place, Endoplasmic Reticulum (ER)-resident chaperones regulate ER-Quality Control (ER-QC) [16-18]. 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) [19-21]. The major function of these chaperones in ER-QC is to prevent the accumulation of unfolded proteins through assisting in protein folding and initiating ER-stress signalling when unfolded proteins start to accumulate [17, 22]. 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 Asn (N) residues of nascent glycoproteins [23]. The subsequent action of glucosidases renders the glycoproteins accessible for CRT/CNX-assisted folding [16]. A third ER-QC pathway is supported by protein disulphide isomerases and involves the formation of disulphide bridges between free thiol groups present in the client protein [16, 24]. 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 [16, 25]. Consequently, a number of *Arabidopsis* mutants in ER-chaperone components are immuno-compromised for bacterial infections. *sdf2* mutants for example showed reduced immunity mediated by the EF-Tu Receptor (EFR) [20]. Similarly, *Arabidopsis* mutants in the folding sensor UDP Glc Glycoprotein glucosylTransferase (UGGT), the ERD2b HDEL receptor, and CRT3 were compromised in EFR-mediated defence responses [20, 26-28].

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The tomato Ve1 receptor is an LRR-RLP that mediates resistance to the fungal vascular wilt pathogens *Verticillium dahliae* and *V. albo-atrum* [11, 29, 30]. Ve1 provides resistance to race 1 strains of these pathogens upon perception of the secreted Ave1 effector protein [31, 32]. Another group of defence-associated LRR-RLPs are the tomato Cf proteins, involved in resistance to the fungal biotrophic leaf pathogen *Cladosporium fulvum* [9]. Recently, it was discovered that Cf proteins interact with ER-QC chaperones that are required for proper Cf function [33]. Furthermore, it was found that both tomato and *Nicotiana benthamiana* contain four *BiP* homologs and three CRT-like chaperones and that Cf-4 especially depends on CRT3a for its biogenesis [33]. CRT3a is an isoform of the plant-specific CRT3-class [28] and silencing of *CRT3a* in *N. benthamiana* resulted in a reduced pool of mature complex glycosylated Cf-4 protein, whereas the total Cf-4 protein pool was not affected. Silencing of individual *BiPs* did not have an effect on Cf-4 function and silencing of multiple *BiPs* led to lethality in *N. benthamiana* [33].

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 Ve1 glycosylation or a suppression of the Ve1-mediated Hypersensitive Response (HR) upon 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 requirement 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 was generated [31, 32] 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 [33]. Two peptides were found to match *N. benthamiana* (*Nb*) CRT2 (one of the three *Nb*CRTs) and 32 peptides matched one or more of the four *Nb*BiP homologs (Table S1). A list of additional co-purifying proteins besides the BiPs and CRTs is presented in Table S2. In an alternative approach, Ve1-eGFP was immunopurified from *N. benthamiana* after its transient expression and the immunoprecipitate was run on

SDS gel and blotted. The blot was incubated with αBiP, detecting endogenous *Nb*BiPs, revealing a clear band at the expected molecular weight of the four *Nb*BiP proteins (Fig. 1A). Collectively, these results show that the *Nb*CRTs and *Nb*BiPs interact with Ve1 upon transient expression in *N. benthamiana*.

Previously, C-terminally-tagged eGFP fusion proteins of the *S. lycopersicum* (*Sl*) CRTs were generated [33] and we here investigated whether Ve1 interacts with the three individual *Sl*CRTs. Therefore, *Sl*CRT2, *Sl*CRT3a and *Sl*CRT3b, all fused to eGFP, were transiently co-expressed with the fusion protein Ve1-HemAgglutinin (Ve1-HA) by *Agrobacterium* infiltration in *N. benthamiana*. CRT immunoprecipitation using GFP-affinity beads, followed by detection of Ve1-HA using αHA antibody revealed that Ve1 interacts with all three *Sl*CRTs, as Ve1-HA is co-immunopurified in all cases (Fig. 1B).

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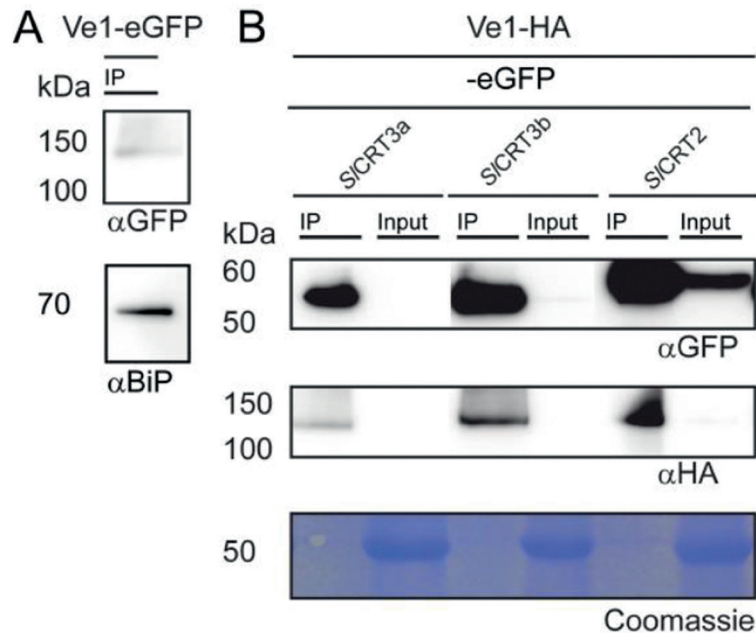
Figure 1. BiPs of *N. benthamiana* co-purify with Ve1 and Ve1 co-purifies with tomato CRTs. Ve1-eGFP was expressed in *N. benthamiana* (A) and Ve1-HA was co-expressed with *Sl*CRT2-eGFP, *Sl*CRT3a-eGFP or *Sl*CRT3b-eGFP in *N. benthamiana* (B). Total protein extracts of the agroinfiltrated leaf tissue were subjected to immunopurification using GFP-affinity beads. Total proteins (Input) and Immunopurified Proteins (IP) were separated by SDS-PAGE and blotted. Blots were incubated with αGFP antibody to detect immunopurified Ve1-eGFP (A) or *Sl*CRT-eGFP (B) fusion proteins. Duplicate blots were incubated with αBiP to detect co-immunopurifying BiPs (A) or with αHA antibody to detect co-immunopurifying 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 picture is shown.

Targeting of Tomato *BiPs* by Virus-Induced Gene Silencing (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 [11]. 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*) [33]. As a control, plants were inoculated with TRV:*GFP*. Two weeks after inoculation with the recombinant viruses, plants were either inoculated with a *V. dahliae* strain expressing *Ave1* or mock-inoculated. Subsequently, plants were monitored for a period of two weeks for stunted growth, which is a typical disease symptom caused by *V. dahliae* infection. Targeting of *SlBiP1* to *SlBiP3* resulted in stunted growth of Motelle plants upon infection with *V. dahliae*, whereas targeting of *SlBiP4* did not compromise Ve1 resistance (Fig. 2). As expected, TRV:*GFP*-inoculation did not result in compromised growth of the plants upon inoculation with *V. dahliae*, showing that these plants remained fully resistant to the fungus (Fig. 2). We determined the efficiency of *BiP* silencing by qRT-PCR with specific primers for the individual *BiPs* (Fig. S1A). We detected knock-down of *BiP1* and *BiP2* in lines inoculated with TRV:Sl-*BiP1* and TRV:Sl-*BiP2*, respectively (Fig. S1A). However, no reduced expression of *SlBiP3* and *SlBiP4* was detected upon inoculation with TRV constructs targeting these genes. This is most likely because expression levels of *SlBiP3* and *SlBiP4* are much lower compared to *SlBiP1* and *SlBiP2* (not shown).
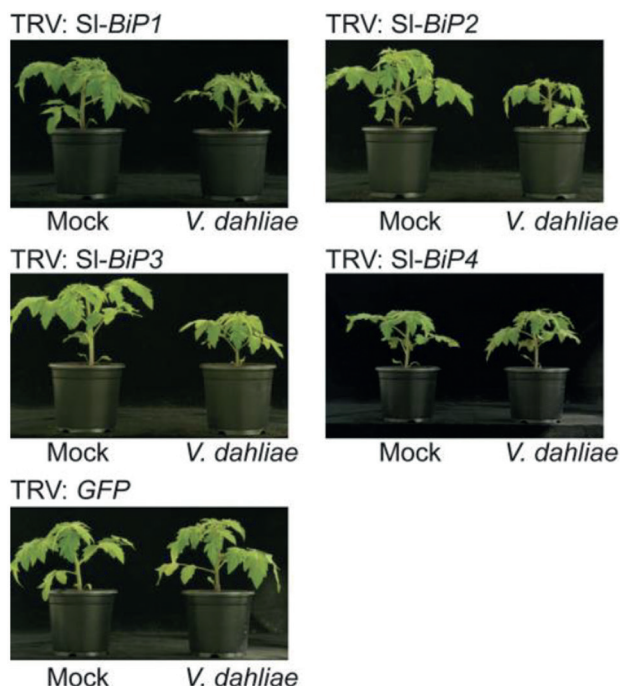
Figure 2.
Targeting of individual *BiPs* by Virus-Induced Gene Silencing (VIGS) results in compromised Ve1-mediated resistance to *V. dahliae*. Tomato cultivar Motelle, carrying the *Ve1* resistance gene, was subjected to VIGS by inoculation with the indicated TRV:Sl-*BiP* constructs. TRV:*GFP* served 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 as compared to the mock-inoculated plants. Three independent experiments were performed and a representative picture 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:Sl-*CRT2*, TRV:Sl-*CRT3a* and TRV:Sl-*CRT3b* [33], targeting the expression of the different *SlCRTs*, 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 upon 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 *SlCRTs* in the TRV:Sl-*CRT*-inoculated plants was shown by qRT-PCR (Fig. S1B).
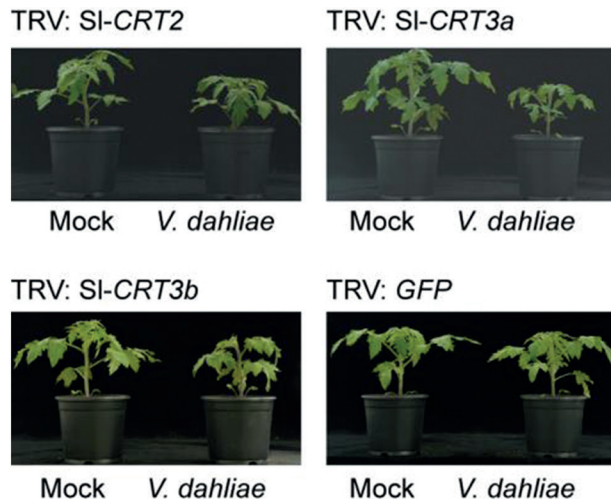
Figure 3.
Targeting of individual *CRTs* by VIGS results in compromised Ve1-mediated resistance to *V. dahliae*. Tomato cultivar Motelle, carrying the *Ve1* resistance gene, was subjected to VIGS by inoculation with the indicated TRV constructs. TRV:*GFP* served as a control. Two weeks after inoculation 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 as compared to the mock-inoculated plants. Three independent experiments were performed and a representative picture is shown.

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Targeting 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 [33]. To reveal whether the *N*-linked complex glycosylation status of Ve1 changes upon targeting the expression of the different *CRTs*, we inoculated *N. benthamiana* and *N. tabacum* cultivar Samsun with the TRV constructs TRV:Sl-*CRT2*, TRV:Nb-*CRT3a* and TRV:Sl-*CRT3b* that target the different *N. benthamiana* *CRTs* [33]. In addition, TRV:*GUS* was used as a control. *N. 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* [31, 32]. Next, Ve1-eGFP was transiently expressed by *Agrobacterium* infiltration in 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 αGFP 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 [34]. Consequently, this antibody cross-reacts with other proteins that are also complex *N*-linked glycosylated [33, 34]. 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 αGFP 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 αHRP revealed that the pool of complex *N*-linked glycosylated Ve1-eGFP is equal, regardless of which TRV construct was used for the inoculation of the plants, both for *N. benthamiana* and *N. tabacum* (Figs. 4A and B). As expected, *N. benthamiana* plants inoculated with TRV:Nb-*CRT3a* showed a strongly reduced complex glycosylation on Cf-4-eGFP (Fig. 4B) [33]. To a lesser extent, this was also observed for plants inoculated with TRV: Sl-*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 (Figs. 4 A and B). This is in contrast to Cf-4, of which the amount of complex glycosylated protein is strongly reduced when *SlCRT3a* is targeted. Hence, the compromised resistance to *V. dahliae* upon targeting of the different *CRTs* is likely not due to 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 knock-down of the *CRTs* and *BiPs* compromises the Ve1-triggered HR, *N. tabacum* (cv Samsun) (*Nt*) was inoculated with TRV: Sl-*BiP1*, TRV: Sl-*BiP2*, TRV: Sl-*BiP3* and TRV: Sl-*BiP4* targeting the different *N. benthamiana BiPs* [33], as well as with TRV:Sl-*CRT2*, TRV:Nb-*CRT3a* and TRV:Sl-*CRT3b* targeting the *CRTs*. Although its genome has not been sequenced yet, 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 homologs of *N. tabacum*. Three weeks post viral inoculations, 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 homologs of the RLK SOBIR1, required for the Ve1-mediated HR in *N. tabacum* [35].
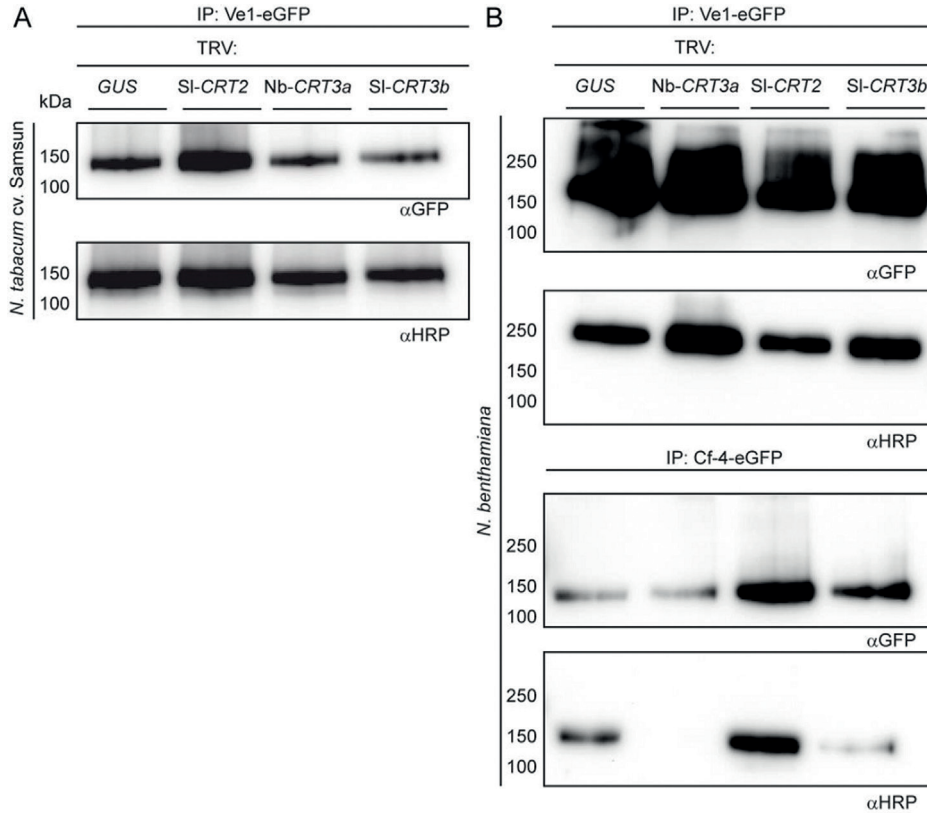

Figure 4. Ve1-eGFP contains complex *N*-linked glycans and VIGS of the different *CRTs* does not affect Ve1 glycosylation. Ve1-eGFP was transiently expressed in (A) *N. tabacum* (cultivar Samsun) or (B) in *N. benthamiana*, silenced for the different *CRTs*. As a control in (B), Cf-4-eGFP was also transiently expressed in *N. benthamiana* silenced for the different *CRTs*. The fusion proteins were subsequently immunopurified using GFP-affinity beads, run on SDS gel and blotted. Blots were incubated with αGFP antibody to reveal the total amount of immunopurified Ve1 and Cf-4 proteins and duplicate blots were incubated with αHRP antibody to reveal the pool of mature, complex glycosylated purified protein. The experiment was performed twice with similar results and a representative picture is shown.

Remarkably, targeting of the different *BiP* and *CRT* homologs 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 (Fig. 2 and Fig. 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).
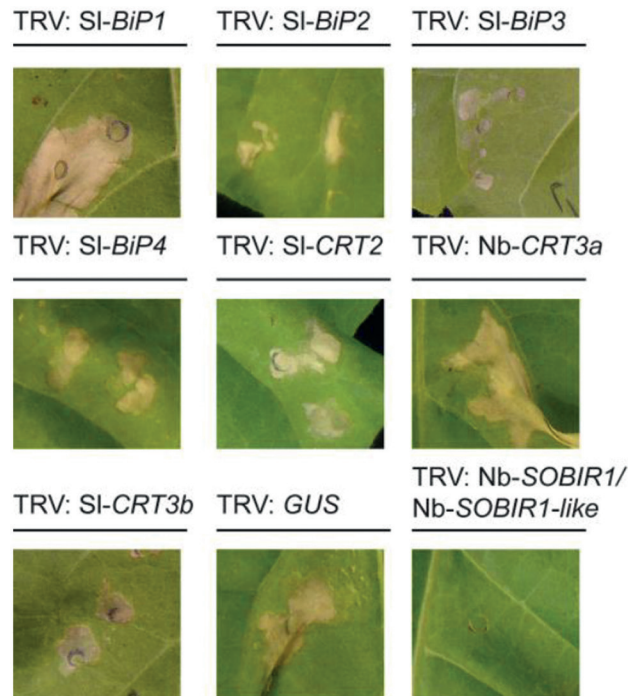
Figure 5. Targeting the individual *BiPs* and *CRTs* in *N. tabacum* does not lead to a compromised Ve1-mediated HR. *N. tabacum* cv. Samsun was subjected to VIGS by inoculation with the indicated constructs. Three weeks later, Ve1 was transiently coexpressed with the matching ligand Ave1. Photographs of infiltrated spots were taken another four days later. TRV: *GUS* and TRV: Nb-*SOBIR1*/Nb-*SOBIR1-like* were included as controls. Three independent experiments were performed and representative pictures are shown.

DISCUSSION

Ve1 is a transmembrane RLP carrying extracellular LRRs that mediates resistance to race 1 strains of the vascular wilt fungus *V. dahliae* and *V. albo-atrum* [11, 29, 30]. Hence, Ve1 requires post-translational modifications and ER-QC-assisted folding before being guided to the plasma membrane [16]. 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 show 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 was recently shown that Ve1-mediated resistance in *Arabidopsis* does not require the HR [36].

Recently, it was shown that tomato Cf proteins carry complex *N*-linked glycans and interact with various ER-QC chaperones [33]. For example, Cf-4 was found to interact with BiP and CRTs in co-immunopurification assays. In addition, silencing of the *N. benthamiana* and tomato *CRT3a* homologs strongly compromised Cf-4-mediated immune responses [33]. 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 [37].

Ve1 function does not seem to depend on one particular CRT. In fact, targeting the three *SlCRTs* 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 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) [33]. Interestingly, Ve1 is still complex glycosylated upon targeting *CRT3a* and the Ve1 protein accumulates to similar levels as in control plants (Fig. 4). Additionally, the Ve1-triggered HR in TRV: Nb-*CRT3a*-inoculated *N. tabacum* plants was still intact. Thus, a reduced accumulation of complex glycosylated Ve1 likely not explains the compromised Ve1-mediated resistance.

Therefore, the question remains 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 was shown to be involved in salicylic acid-dependent expression of defence genes [38]. 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 [11, 35, 39], 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. Whereas Cf-4 relies strongly on *Sl*CRT3a but not on *Sl*CRT2 or *Sl*CRT3b [33], 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 were shown to be required for accumulation of the plasma membrane-localized Induced Receptor Kinase (IRK), which is involved in N-mediated resistance to tobacco mosaic virus [40]. In rice (*Oryza sativa*) *Os*BiP3 interacts with the plasma membrane-localized RLK Xa21 involved in resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*. In

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this case, overexpression of *Os*BiP3 resulted in enhanced resistance to the bacterium [41]. In *Arabidopsis*, mutant forms of the brassinosteroid receptor BRI1 are retained in the ER by CRT3 [42-44]. Furthermore, mutations in a number of ER-QC chaperones strongly compromise functionality of the immune receptor EFR in *Arabidopsis* [20, 26, 27, 45]. In particular, *crt3* mutants accumulate EFR to reduced amounts. In contrast, FLS2 accumulation and downstream responses were hardly affected in these ER-QC chaperone mutants. Perturbation of the *N*-linked glycosylation status of EFR and FLS2 revealed that under-glycosylation strongly affects EFR function, whereas this is not the case for FLS2 [18, 46, 47]. Interestingly, weakly defective *crt3 Arabidopsis* mutants accumulate EFR to wild-type levels but display compromised EFR-triggered responses, showing that abundance control and quality control of the EFR receptor can be uncoupled [27]. This may also explain why Ve1-mediated defence is reduced upon 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.

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Materials And Methods

Plant Material and Growth Conditions
N. benthamiana, N. tabacum cultivar Samsun and tomato (*Solanum lycopersicum*) cultivar Motelle plants, carrying the *Ve1* gene, were grown in the greenhouse under 16h of light at 21°C and 8h of darkness at 19°C. Supplemental light of 100 W m^{-2} was applied when the light intensity dropped below 150 W m^{-2}. 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 [32, 33]. pB7K40-Ve1-HA, p35S:Ve1 and pSOLl292-Ave1 have been described elsewhere [11, 32, 48]. Plasmids directing the expression of the *Sl*CRT-eGFP fusions and all TRV constructs for VIGS experiments have been described previously [32, 33]. All binary plasmids were transformed to *Agrobacterium tumefaciens* strain C58C1, carrying helper plasmid pCH32, for *Agrobacterium* infiltration assays. *Agrobacterium* infiltrations were performed as described previously [32, 49].

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 [11, 32, 33]. We used *Agrobacterium* inoculation to express both pTRV:RNA1 and pTRV:RNA2 [50]. *V. dahliae* race 1 strain

JR2 was used for inoculation of tomato. Fungal inoculations and disease scoring were performed as described previously [11, 39]. *Agrobacterium*-mediated co-expression of p35S:Ve1 and pSOLl292-Ave1 in TRV-inoculated N. tabacum cv. Samsun was performed at an final O.D.600 = 1 for each construct [32].

RNA Extraction and qRT-PCR

For qRT-PCR, leaves of four plants inoculated with a TRV-silencing construct were grinded and subjected to RNA extraction plus first strand cDNA synthesis as described before [33]. Primers matching the individual *SlBiPs*, *SlCRTs* and *SlActin* can be found in Table S3. qRT-PCR was performed as described previously [33].

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 previously described for Cf-4-eGFP [33]. In brief, proteins were extracted using extraction buffer containing 150 mM NaCl, 1% IGEPAL CA-630 (NP40), 50 mM Tris, pH8, and one tablet of protease inhibitor cocktail (Roche) per 50 ml. One g 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). The beads were incubated with the protein extract for one hour, 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), as described previously [33].

Co-Immunopurifications and Immunoblotting

Co-immunopurifications were performed as described before [33]. We used 15 µl (50% slurry) of GFP_TrapA affinity beads for the immunopurifications. Beads were washed five times with the extraction buffer before eluting with SDS loading buffer. After blotting to PVDF-membrane (Bio-Rad), the membranes were incubated with either αGFP (anti-GFP-HRP, 130-091-833, MACS antibodies), αHA (anti-HA-peroxidase clone 3F10, 12013819001, Roche) or with αHRP (rabbit anti-HRP, 323-005-021, Jackson ImmunoResearch) as a primary antibody, and goat anti-rabbit-HRP (anti-rabbit IgG-HRP, A9169, Sigma) as a secondary antibody. All antibodies were used in the dilution recommended by the manufacturer.

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Acknowledgements

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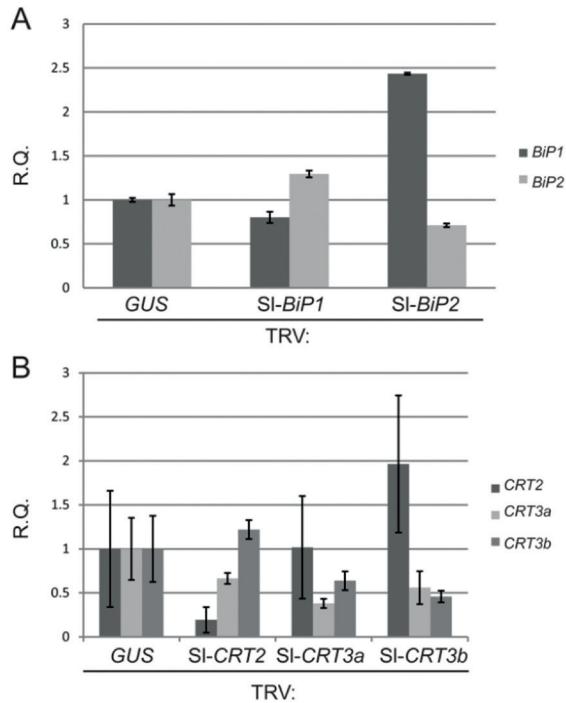
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Supplemental Data

Figure S1.
Determination of the expression levels of *SlBiPs* (A) and *SlCRTs* (B) in TRV-inoculated tomato plants. Tomato plants were inoculated with the indicated TRV-silencing contructs and relative transcript levels of *SlBiP1* and *SlBiP2* (A) as wel as of *SlCRT2*, *SlCRT3a* and *SlCRT3b* (B) were determined by 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 ER-resident chaperones, identified by mass-spectrometry of a tryptic digest of immunopurified Ve1-eGFP, transiently expressed in *N. benthamiana*.

Protein name*	Peptide sequence	Mascot Ion score
*Nb*CRT2	FYAISAEFPEFSNK	91.1
*Nb*CRT2	YVGVELWQVK	42.8
*Nb*BiP2/ *Nb*BiP4	ALSSQHQVRVEIESLFDGVDFSEPLTR	71.5
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4	AMEDAGLEKNQIDEIVLVGGSTR	68.1
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4	DAGVIAGLNVAR	78.7
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4	DILLLDVAPLTLGIETVGGVMTK	31.9
*Nb*BiP2 / *Nb*BiP4	DYFDGKEPNK	44.4
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4	EAEEFAEEDKKVK	57.1
*Nb*BiP2 / *Nb*BiP4	ETAEAYLGKK	36
*Nb*BiP2 / *Nb*BiP4	FDLTGIAPAPR	79.9
*Nb*BiP2 / *Nb*BiP4	FEELNNDLFR	65.9
*Nb*BiP1/ *Nb*BiP4	GHVEIIANDQGNR	67.3
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4/ *Nb*BiP3	IINEPTAAAIAYGLDK	70.6
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4	IKDAVVTVPAYFNDAQR	95.8
*Nb*BiP2/ *Nb*BiP4	IKDGETKVFSPEEISAMILTK	52
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4	IMEYFIK	39.3
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4	ITITNDKGR	51.9
*Nb*BiP1	ITPSWVAFTDGER	56.7
*Nb*BiP4	KFDDKEVQR	61.3
*Nb*BiP1/ *Nb*BiP4	LIGEAAKNQAAVNPER	102.4
*Nb*BiP2/ *Nb*BiP4	LLGKFDLTGIAPAPR	43.9
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4	LSQEEIER	56.8
*Nb*BiP1/ *Nb*BiP4	LVPYEIVNKDGKPYIQVK	55.6
*Nb*BiP2/ *Nb*BiP4	MKETAEAYLGKK	48.8
NbBiP1/ *Nb*BiP4	NGHVEIIANDQGNR	77.7
*Nb*BiP1/ *Nb*BiP4	NQAAVNPER	62.5
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4	NQIDEIVLVGGSTR	50.2
*Nb*BiP2/ *Nb*BiP4	NSLETYVYNMR	77.6
*Nb*BiP1/ *Nb*BiP2/ *Nb*BiP4	QATKDAGVIAGLNVAR	102.8
*Nb*BiP2/ *Nb*BiP4	SGGAPGGASEESNEDDDSHDEL	82.9
*Nb*BiP2/ *Nb*BiP4	SQVFTTYQDQQTTVTISVFEGER	51.4
*Nb*BiP1/ *Nb*BiP4	TIFDVKR	48.9
*Nb*BiP2/ *Nb*BiP4	VEIESLFDGVDFSEPLTR	117.8
*Nb*BiP2/ *Nb*BiP4	VFSPEEISAMILTK	103.8
*Nb*BiP2/ *Nb*BiP4	VQQLLKDYFDGKEPNK	52.8

* Multiple protein names are shown when no distinction between the different homologs, based on the peptide sequence could be made.

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Table S2. Additional Ve1-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 the GFP-HA control immunopurification.

Protein name	Ve1-eGFP	GFP-HA
Ve1-AttB2-eGFP	156	24*
UniRef100_P09043 Cluster: Glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor; *Nicotiana tabacum*	32	6
Homologue to UP|RCA2_TOBAC (Q40565) Ribulose bisphosphate carboxylase/ oxygenase activase 2	26	4
SubName: Full=F7G19.5 protein;	23	0
Receptor-like protein kinase [*Nicotiana tabacum*]; SOBIR1	18	0
Calnexin-like protein; *Solanum lycopersicum*	17	0
Homologue to Beta-carbonic anhydrase; *Nicotiana tabacum*	16	5
Similar to UP|Q4W5U7_LYCES (Q4W5U7) Calnexin-like protein, partial (96%)	16	0
Full=Ubiquitin monomer protein	15	0
ATP synthase subunit beta (ATPase subunit beta) (ATP synthase F1 sector subunit beta)	15	0
Similar to Predicted protein; *Physcomitrella patens* subsp. *patens*	15	0
Calnexin-like protein [*Lycopersicon esculentum* (Tomato)]; St_TC167880 homologue to Calnexin-like protein	14	0
RecName: Full=ATP synthase subunit alpha, chloroplastic; EC=3.6.3.14	14	0
SubName: Full=Putative uncharacterized protein;	13	0
(REVERSE SENSE) similar to Calnexin-like protein; *Solanum lycopersicum*	12	0
UP|G3PB_TOBAC (P09044) Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor (NADP-dependent glyceraldehyde phosphate dehydrogenase subunit B)	12	0
DnaK protein, putative; *Oryza sativa* (japonica cultivar-group)	11	0
Calnexin-like protein [*Lycopersicon esculentum* (Tomato)]	10	0
Homologue to UniRef100_Q40565 Cluster: Ribulose bisphosphate carboxylase/ oxygenase activase 2, chloroplast precursor; n=1; *Nicotiana tabacum*	10	4
Similar to UP|SDF2_ARATH (Q93ZE8) Stromal cell-derived factor 2-like protein precursor (SDF2-like protein), BP745599 MIR *Medicago truncatula* (Barrel medic)	9	0
Similar to UP|ENPL_CATRO (P35016) Endoplasmin homolog precursor (GRP94 homolog)	9	0
UP|PGKH_TOBAC (Q42961) Phosphoglycerate kinase, chloroplast precursor	8	0
Similar to GB|AAM78044.1|21928031|AY125534 At3g62600-F26K9_30 (*Arabidopsis thaliana*), ERDJ3B	8	0
Homologue to ADP,ATP carrier protein, mitochondrial precursor; TA9847_4100 *Solanum tuberosum*, Cluster: ADP-ATP carrier protein	6	0
Chloroplast oxygen-evolving protein 16 kDa subunit; *Nicotiana benthamiana*	5	2
Homologue to Fructose-bisphosphate aldolase; *Nicotiana paniculata* TA8126_4100 Cluster: Plastidic aldolase NPALDP1	5	0
Homologue to UP|Q9XG67_TOBAC (Q9XG67) Glyceraldehyde-3-phosphate dehydrogenase, complete TA13063_4097 Cluster: Glyceraldehyde-3-phosphate dehydrogenase	5	0
Similar to Protein disulfide isomerase; *Ipomoea batatas* TA11473_4100 Cluster: Protein disulfide isomerase; Ipomoea	4	0

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Homologue to Chloroplast pigment-binding protein CP26; *Nicotiana tabacum* TA8052_4100 Cluster: Chloroplast light-harvesting complex II	4	0
TA9218_4100 Homologue to Glycolate oxidase; *Mesembryanthemum crystallinum*, Cluster: Glycolate oxidase; *Brassica*	4	0
Elongation factor TuB, chloroplast precursor [*Nicotiana sylvestris* (Wood tobacco)]	4	0
TA6683_4072 Similar to Predicted protein; *Physcomitrella patens* subsp. *Patens,* Heat shock protein DnaJ, N-terminal *Medicago*	3	0
ATP synthase epsilon chain (ATP synthase F1 sector epsilon subunit)	3	0
26S protease regulatory subunit 6A homolog (TAT-binding protein homolog 1) (TBP-1) (Mg(2+)-dependent ATPase 1); *Solanum lycopersicum*	3	0
Homologue to ATP synthase gamma chain, chloroplast precursor; *Nicotiana tabacum*	3	0
TA48920_4081 Cluster: Putative UDP-glucose:glycoprotein	3	0
TA1494_62890 Similar to RuBisCO large subunit-binding protein subunit alpha, chloroplast precursor; *Pisum sativum*	3	0
Homologue to 26S proteasome subunit 4-like; *Solanum tuberosum*	3	0
Homologue to UP|Q8LSZ2_TOBAC (Q8LSZ2) NADPH: protochlorophyllide oxidoreductase	3	0
OSJNBa0060N03.12 protein [*Oryza sativa* (japonica cultivar-group)]	2	0
Stress responsive cyclophilin [*Solanum commersonii* (Commerson's wild potato)]	2	0
Receptor-like protein kinase; *Nicotiana tabacum*; SOBIR1	2	0
Unnamed protein product [*Nicotiana tabacum*]	2	0
Unknown protein	2	0
Homologue to AW618772 Hypothetical protein *Solanum tuberosum* (Potato)	2	0
(REVERSE SENSE) similar to Protein disulfide-isomerase precursor; *Datisca glomerata*	2	0
Homologue to 26S proteasome AAA-ATPase subunit RPT4a; *Solanum tuberosum* TA9909_4100	2	0
Homologue to Oxygen-evolving enhancer protein 2-2, chloroplast precursor; *Nicotiana tabacum*	2	0
Chloroplast photosystem II 22 kDa component; *Nicotiana benthamiana*	2	0
Phosphoribulokinase, chloroplast precursor; *Mesembryanthemum crystallinum*	2	0
Homologue to UP|Q8H9D4_SOLTU (Q8H9D4) 26S proteasome AAA-ATPase subunit RPT4a	2	0
Homologue to UP|ENO_LYCES (P26300) Enolase (2-phosphoglycerate dehydratase) (2-phospho-D-glycerate hydrolyase), *Lycopersicon esculentum* (Tomato)	2	0
Homologue to UP|PRS7_ARATH (Q9SSB5) 26S protease regulatory subunit 7 (26S proteasome subunit 7) (Regulatory particle triple-A ATPase subunit 1a)	2	0
Homologue to UP|P93775_9LAMI (P93775) Actin, complete	2	0
Homologue to UP|P93570_SOLTU (P93570) Chaperonin-60 beta subunit precursor	2	0
UP|IF410_TOBAC (P41382) Eukaryotic initiation factor (ATP-dependent RNA helicase eIF4A-10) (eIF-4A-10)	2	0
Homologue to UP|O80414_ORYSA (O80414) Mitochondrial phosphate transporter, partial (87%); *Oryza sativa*	2	0
SubName: Full=Catalase	2	0

* Peptides from the GFP-HA control are matched to Ve1-eGFP as the GFP sequence is shared between the two

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Table S3. List of oligonucleotide primers used.

Primer code	Sequence (5'-3')	Target sequence
to286	AAGAATCAAATCAACGAC	*SlBiP1*
to287	TGCACCGCCACTTGGG	*SlBIP1*
to288	GGTTGATGCCAATGGTATCCTAA	*SlBIP2*
to289	GGCTGTTTCGGGCATCAACTC	*SlBIP2*
to290	AGCTAAGAAGTCGAAATCT	*SlBIP3*
to291	AAGAGCGCTCTCTATCTTCTCTCTG	*SlBIP3*
to292	TAAGATTAAAGACGGGGAGAAC	*SlBIP4*
to293	GATTCTTGCCACATTTAGT	*SlBIP4*
to187	CATTGACAATGTGGAGAAACAG	*SlCRT2*
to188	CCTCTGGCTTCTTATCCTCG	*SlCRT2*
to189	TAATCCAGCATACCGGCCG	*SlCRT3a*
to190	TACCTGCCAAACTTCAATACCG	*SlCRT3a*
to185	CATGTATACAGACTGGGATATCT	*SlCRT3b*
to186	AATTTTTGGTGGTCTCCAGATG	*SlCRT3b*
to58	TATGGAAACATTGTGCTCAGTGG	*SlActin*
to59	CCAGATTCGTCATACTCTGCC	*SlActin*
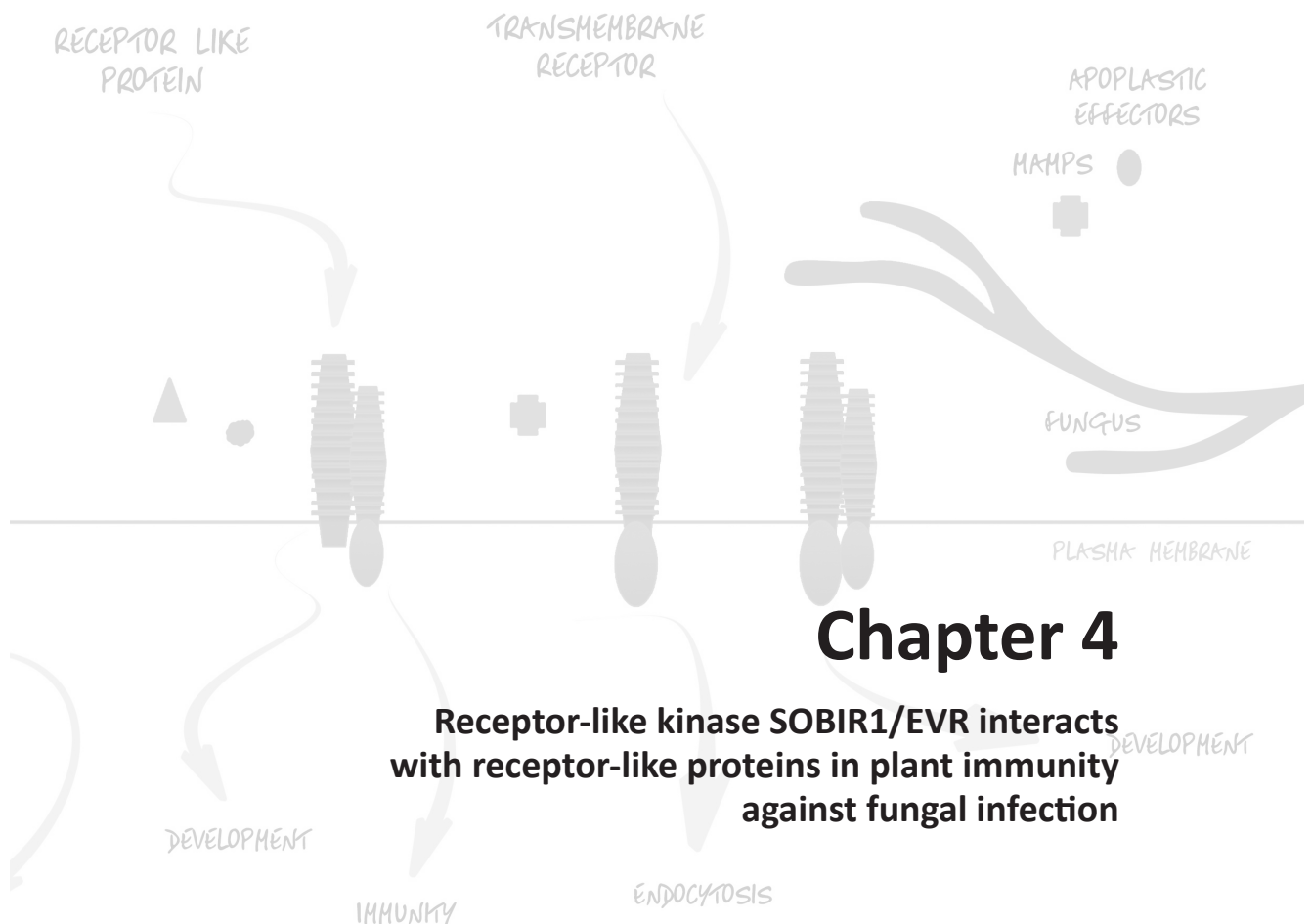
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Chapter 4

Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection

Liebrand, T.W.H., van den Berg, G.C.M., Zhang, Z., Smit, P., Cordewener, J.H.G., America, A.H.P., Sklenar, J., Jones, A.M.E., Tameling, W.I.L., Robatzek, S., Thomma, B.P.H.J., and Joosten, M.H.A.J. **The receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection.** *Proc Natl Acad Sci USA* (2013) 110: 10010-10015

Abstract

The plant immune system is activated by microbial patterns that are detected as non-self molecules or by modified host patterns that are perceived as danger signals. Such patterns are recognized by immune receptors that are cytoplasmic or localized at the plasma membrane. Cell surface receptors are represented by Receptor-Like Kinases (RLKs) that frequently contain extracellular leucine-rich repeats and an intracellular kinase domain for activation of downstream signalling, as well as Receptor-Like Proteins (RLPs) that lack this signalling domain. It is therefore hypothesized that RLKs are required for RLPs to activate downstream signalling. The RLPs Cf-4 and Ve1 of tomato (*Solanum lycopersicum*) mediate resistance to the fungal pathogens *Cladosporium fulvum* and *Verticillium dahliae*, respectively. Despite their importance, the mechanism by which these immune receptors mediate downstream signalling upon recognition of their matching ligand, Avr4 and Ave1, remained enigmatic. Here we show that the tomato ortholog of the *Arabidopsis thaliana* RLK "Suppressor Of BIR1-1/EVeRshed (SOBIR1/EVR)" and its close homolog *Sl*SOBIR1-like, interact *in planta* with both Cf-4 and Ve1 and are required for the Cf-4- and Ve1-mediated hypersensitive response and immunity. Tomato SOBIR1/EVR interacts with most of the tested RLPs, but not with the RLKs FLS2, SERK1, SERK3a/BAK1 and CLV1. SOBIR1/EVR is required for stability of the Cf-4 and Ve1 receptors, supporting our observation that these RLPs are present in a complex with SOBIR1/EVR *in planta*. We show that SOBIR1/EVR is essential for RLP-mediated immunity and propose that the protein functions as a regulatory RLK of this type of cell surface receptors.

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INTRODUCTION

Plants rely on an innate immune system, which is activated upon recognition of pathogen-derived non-self molecules, or host-derived damage products [1, 2]. Conserved "Microbe-Associated Molecular Patterns" (MAMPs) are perceived by "Pattern Recognition Receptors" (PRRs) that activate MAMP-Triggered Immunity (MTI). Pathogenic microbes promote virulence by secretion of effector proteins, many of which suppress MTI [3, 4]. In resistant plants, these effector proteins are detected by resistance proteins that activate Effector-Triggered Immunity (ETI), frequently resulting in the Hypersensitive Response (HR), a localized programmed host cell death response [1]. Conceptually, MTI and ETI function in a similar fashion by employing immune receptors that mount a suitable defence response to halt pathogen ingress upon recognition of appropriate ligands that betray pathogen presence [5].

Most PRRs have been identified as transmembrane Receptor-Like Kinases (RLKs) that frequently contain an extracellular Leucine-Rich Repeat (LRR) domain or Lysin-Motif (LysM) for ligand recognition and an intracellular kinase domain for activation of downstream signalling [6]. The LysM-RLK Chitin Elicitor Receptor Kinase-1 (CERK1) mediates immunity against fungi by recognizing fungal chitin [7, 8], whereas the LRR-RLKs Flagellin Sensing-2 (FLS2) and EF-Tu Receptor (EFR) are involved in recognition of bacterial flagellin and the elongation-factor Tu protein, respectively [9, 10]. Upon ligand recognition, both FLS2 and EFR form a receptor complex with the LRR-RLK Somatic Embryogenesis Receptor Kinase-3/BRI1-Associated receptor Kinase-1 (SERK3/BAK1) and its close paralog BAK1-like-1 (BKK1) [11-13]. The trans-phosphorylation events that follow, together with the dissociation of the cytoplasmic kinase Botrytis-Induced Kinase-1 (BIK1) from the receptor complex, subsequently activate downstream defence signalling [14, 15]. SERK3/BAK1 is not involved in ligand binding to FLS2 and EFR but rather plays a role in downstream signalling upon its recruitment by FLS2 and EFR after ligand binding [2]. Hence, SERK3/BAK1 and BKK1 likely function as signal enhancers and can be regarded as co-regulatory RLKs in FLS2- and EFR-mediated immunity [6, 16].

Receptor-Like Proteins (RLPs) form a second major class of cell-surface receptors in plants. RLPs are structurally similar to RLKs but lack a cytoplasmic kinase domain [17, 18]. RLPs function in defence, such as the Cf proteins and Ve1, as well as in development [18]. Examples of the latter are Clavata2 (CLV2) that plays a role in meristem maintenance, whereas stomatal patterning is regulated by Too Many Mouths (TMM) [18]. As RLPs lack a cytoplasmic kinase domain, it is anticipated that proteins containing such a domain are recruited to activate downstream signalling [19, 20]. Indeed, *Arabidopsis thaliana* CLV2 forms a complex with the transmembrane kinase Coryne and the LRR-RLK CLV1 [21-23], whereas TMM requires the LRR-RLK Erecta to activate downstream signalling [24].

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In tomato (*Solanum lycopersicum*), resistance to specific races of the fungal pathogens *Cladosporium fulvum* (causing leaf mold disease) and *Verticillium dahliae* (causing vascular wilt disease) is mediated by LRR-containing RLPs [25, 26]. Cf proteins confer immunity upon recognition of *C. fulvum* race-specific secreted effectors (also referred to as Avirulence (Avr) proteins) [27], whereas Ve1 recognizes the Ave1 effector protein secreted by race 1 *V. dahliae* strains [28]. Cf-9 was the first identified RLP [19] and since its discovery several attempts have been made to understand Cf-mediated defence signalling by identifying Cf-interacting proteins. Yeast-two hybrid analyses resulted in the isolation of several potential interactors of the cytoplasmic C-terminus of Cf-9 [29-31]. However, an RLK or Coryne-like protein, recruited by Cf proteins and providing a cytoplasmic kinase domain through which Cf-mediated signalling would occur, remained to be identified [20]. Recently, by immunopurification of a functional Cf-4-enhanced Green Fluorescent Protein (eGFP) fusion protein from plants, we identified Endoplasmic Reticulum (ER)-resident chaperones as *in planta* interactors of Cf proteins that are required for Cf protein biogenesis [32]. Here, following a similar approach, we describe the identification of the tomato ortholog of the *Arabidopsis* RLK "Suppressor Of BIR1-1/EVeRshed (SOBIR1/EVR)" (hereafter referred to as SOBIR1) [33, 34] and its close homolog SOBIR1-like, as Cf interactors. Interestingly, both tomato homologs and *Arabidopsis* SOBIR1 interact with Ve1, in addition to Cf-4 and we show that SOBIR1 is required for Cf-2-, Cf-4- and Ve1-mediated immunity. Our work reveals an essential role for SOBIR1 in the plant immune response activated by two distinct RLPs involved in resistance to fungal pathogens and suggests that SOBIR1 functions as an essential regulatory RLK of this type of cell surface receptors.

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RESULTS

Identification of Tomato SOBIR1 and SOBIR1-like as Interactors of Cf-4 and Ve1

To identify novel Cf-interacting proteins, we stably transformed Money Maker (MM)-Cf-0 tomato, lacking *Cf* resistance genes to *C. fulvum*, with a construct driving constitutive expression of a Cf-4-eGFP fusion protein [32]. Transgenic Line (TL) TL3 showed recognition resulting in a specific HR upon infiltration with the *C. fulvum* Avr4 effector, whereas TL21 did not show a response to Avr4 (Fig. S1*A*). Cf-4-eGFP was successfully immunopurified from TL3, whereas the fusion protein could not be detected in TL21 (Fig. S1*B*). To identify proteins copurifying with Cf-4, tryptic on-bead digestion of the purified proteins was performed and the generated peptides were analyzed by mass spectrometry. Interestingly, in the sample originating from TL3 but not in the one from TL21, in addition to peptides originating from Cf-4-eGFP itself, peptides matching to two tomato RLKs encoded by Solyc06g071810.1.1 and Solyc03g111800.2.1 were identified (Table S1). The alignments presented in Fig. S2*A* show that the amino acid sequences

of these tomato RLKs are highly homologous to each other (~74% identical) and are closely related to the *Arabidopsis* RLK SOBIR (~60% identity). Both tomato RLKs are more distantly related to *Sl*SERK3a/BAK1 (~25% identical) [33, 34]. Fig. S2*B* also shows that the nucleotide sequences of both tomato RLKs and *At*SOBIR1 are very similar throughout their coding regions. Hence, we named the genes encoding the two tomato RLKs *SlSOBIR1* and *SlSOBIR1-like*. Similar to *At*SOBIR1, *Sl*SOBIR1 and *Sl*SOBIR1-like have five predicted LRRs, in contrast to *Sl*SERK3a/BAK1, which has only four LRRs. The SOBIR1 sequences of tomato and *Arabidopsis* are highly similar both in their extracellular LRR and cytoplasmic kinase domains, whereas the homology of SOBIR1 to *Sl*SERK3a/BAK1 is mostly restricted to their kinase domains (Fig. S2*A*). No peptides originating from any other RLKs were identified in the peptide sample originating from TL3.

Cf-4-eGFP is also functional in *Nicotiana benthamiana* [32] and immunopurification of transiently expressed Cf-4-eGFP from this plant also yielded peptides from copurifying RLKs potentially matching SOBIR1 and SOBIR1-like (Table S2). The presence of *SlSOBIR1* orthologs in *N. benthamiana* and *N. tabacum* was assessed by searching public databases, indeed revealing two candidate *N. benthamiana* homologs, referred to as *NbSOBIR1* and *NbSOBIR1-like*, and one *N. tabacum* homolog (*NtSOBIR1*) (Fig. S2*C*). To also identify proteins interacting with Ve1, eGFP-tagged Ve1 [35] was immunopurified upon its transient expression in *N. benthamiana*. Also for this RLP, peptides matching *Nb*SOBIR1 and *Nb*SOBIR1-like were identified, while again no peptides from other RLKs were detected (Table S3).

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Tomato SOBIR1 and SOBIR1-like, and *Arabidopsis* SOBIR1 Interact with Cf-4 and Ve1
C-terminally Myc epitope-tagged versions of the tomato and *Arabidopsis* SOBIR1 RLKs (*Sl*SOBIR1-Myc, *Sl*SOBIR1-like-Myc and *At*SOBIR1-Myc) were generated to perform coimmunopurification experiments with Cf and Ve1. Transient coexpression in *N. benthamiana* revealed that all three SOBIR1 proteins interact with Cf-4 and Ve1 (Fig. 1 and Fig. S1*C*). Coexpression of constructs encoding *Sl*SOBIR1-eGFP and Cf-4-Myc similarly revealed interaction of Cf-4-Myc with *Sl*SOBIR1-eGFP (Fig. S3*A*). We then examined whether the SOBIR1 proteins also interact with RLKs known to be involved in defence and/or development. Interestingly, immunopurification of C-terminally (e) GFP-tagged *Sl*SERK1, *Sl*SERK3a/BAK1 [36], *Sl*FLS2 [37] or *At*CLV1 [38], coexpressed with Myc-tagged SOBIR1 or SOBIR1-like proteins, did not copurify with SOBIR1 (Fig. 1 and Fig. S1*C*).
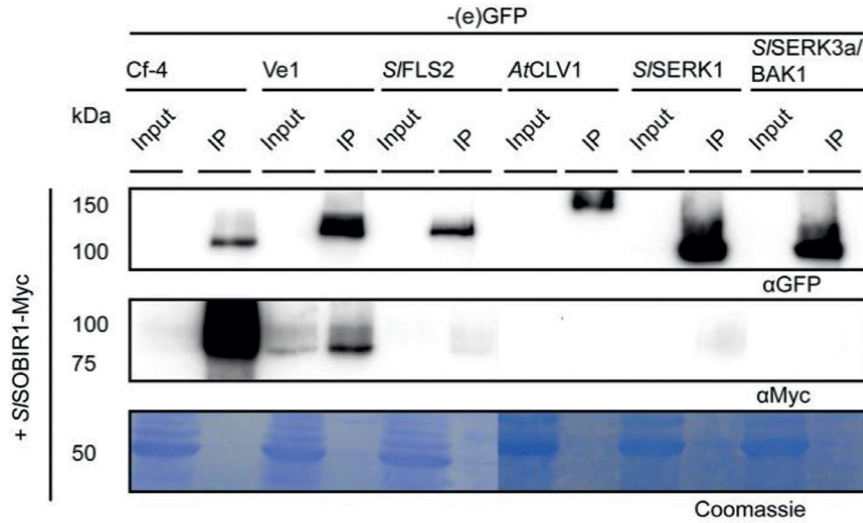
Figure 1. Tomato *Sl*SOBIR1 interacts with Cf-4 and Ve1, but not with various RLKs. Tagged versions of Cf-4, Ve1, *At*CLV1, *Sl*SERK1, *Sl*SERK3a/BAK1 and *Sl*FLS2 (all fused to eGFP, except for *Sl*FLS2, which was fused to GFP) were coexpressed with *Sl*SOBIR1-Myc in *N. benthamiana*. Total protein extracts of transiently transformed leaf tissue were subjected to immunopurification using GFP-affinity beads. Total proteins (Input) and Immunopurified Proteins (IP) were subjected to SDS-PAGE and blotted. Blots were incubated with αGFP antibody to detect the immunopurified (e)GFP fusion proteins and incubated with αMyc antibody to detect coimmunopurifying SOBIR1-Myc proteins. Coomassie-stained blots showing the 50-kDa Rubisco band present in the input samples confirm equal loading. Representative results for three independent experiments are shown.

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To determine whether SOBIR1 requires a functional kinase domain for interaction with Cf-4, the core catalytic Aspartate (D) of its conserved RD kinase motif was substituted to an Asparagine (N) residue. For all tested RLKs containing the catalytic D, among which is SERK3a/BAK1, this mutation causes a loss of kinase activity [39]. Interestingly, C-terminally Myc-tagged *Sl*SOBIR1^{D473N}, *Sl*SOBIR1-like^{D486N} and *At*SOBIR1^{D489N} all still interact with Cf-4-eGFP, showing that kinase activity of SOBIR1 is not required for interaction with the RLP (Fig. S3*B*). It was subsequently tested whether the presence of the Cf-4 ligand, Avr4, would lead to loss of the interaction between SOBIR1 and Cf-4. Cf-4-eGFP and *Sl*SOBIR1-Myc were transiently coexpressed with Avr4 or the non-recognized effector Avr9 infiltrated at two different optical densities. Interaction between Cf-4 and *Sl*SOBIR1 was still observed in the presence of Avr4 and Avr9, indicating that the Cf-4/*Sl*SOBIR1 complex does not dissociate upon recognition of Avr4 by Cf-4 (Fig. S3*C*). We further studied whether *Sl*SOBIR1 forms homodimers and/or heterodimerizes with *Sl*SOBIR1-like or *At*SOBIR1. For this, *Sl*SOBIR1-eGFP was coexpressed with *Sl*SOBIR1-Myc, *Sl*SOBIR1-like-Myc or *At*SOBIR1-Myc, whereas coexpression with Cf-4-Myc was used as a control. Upon pull down of *Sl*SOBIR1-eGFP, Cf-4-Myc strongly copurified with

the RLK. However, we did not observe copurification of *Sl*SOBIR1-Myc, *Sl*SOBIR1-like-Myc, or *At*SOBIR1-Myc, indicating that SOBIR1 does not form homo- or heterodimers with *Sl*SOBIR1-like or *At*SOBIR1 (Fig. S3*D*).

*Sl*SOBIR1 Localizes to the Plasma Membrane and Cytoplasmic Vesicles

It has been reported that *At*SOBIR1-YFP, when expressed under control of its own promoter in *Arabidopsis*, localizes to the plasma membrane and internal membrane compartments of epidermal leaf petiole cells and epidermal root cells [33]. Confocal-laser scanning microscopy performed on *N. benthamiana* epidermal leaf cells transiently expressing *Sl*SOBIR1-eGFP under control of the 35S promoter revealed that *Sl*SOBIR1 mainly localizes to the plasma membrane (Fig. S4*A*). In addition, fluorescence signals were observed in mobile cytoplasmic vesicles (Fig. S4*A*). As previously shown, the GFP-HA control protein localizes to the cytoplasm and nucleus, whereas *Sl*FLS2-GFP localizes to the plasma membrane [37] (Figs. S4*B-D*).

Targeting *SOBIR1* Compromises the Cf-4/Avr4- and Ve1/Ave1-Induced HR

The observation that the two SOBIR1 homologs from tomato and *N. benthamiana* interact with Cf-4 and Ve1 (Fig. 1, Fig. S1*C* and Tables S1, S2 and S3) suggests that both proteins play a role in Cf-4- and Ve1-mediated defence signalling in Solanaceous plants. Therefore, recombinant Tobacco Rattle Virus (TRV)-based Virus-Induced Gene Silencing (VIGS) constructs were generated to target expression of the *NbSOBIR1* homologs, either individually or simultaneously (Fig. S2*C*), and transgenic *N. benthamiana* expressing *Cf-4* was inoculated with the different TRV constructs. Three weeks after viral inoculations, plants were transiently transformed to express *Avr4* [40]. Inoculation with TRV: *NbSOBIR1/NbSOBIR1-like* resulted in a severely compromised Avr4-triggered HR, similar to inoculation with a TRV construct targeting *Cf-4* itself (TRV: *Cf-4*) (Fig. 2). The Avr4-triggered HR was also strongly compromised when *NbSOBIR1* was targeted. When *NbSOBIR1-like* was targeted, the HR was affected to a much lesser extent (Fig. 2). qRT-PCRs revealed that expression of *NbSOBIR1* was strongly reduced upon inoculation with TRV: *NbSOBIR1/NbSOBIR1-like* or TRV: *NbSOBIR1*, as compared to inoculation with TRV: *GUS* (Figs. S5*A* and S5*B*). Interestingly, we did not detect transcripts of *NbSOBIR1-like* in TRV: *GUS*- or TRV: *NbSOBIR1/NbSOBIR1-like*-inoculated plants, suggesting that *NbSOBIR1-like* is not expressed or at a very low level. We therefore reasoned that the slight reduction of the Avr4-triggered HR upon inoculation of *N. benthamiana:Cf-4* with TRV: *NbSOBIR1-like* (Fig. 2) could be attributed to cross-silencing of *NbSOBIR1* by the TRV: *NbSOBIR1-like* construct. Indeed, qRT-PCR confirmed that *NbSOBIR1* expression levels were ~30% reduced upon inoculation with TRV: *NbSOBIR1-like* (Fig. S5*B*). Altogether these results indicate that *NbSOBIR1* is the RLK that is required for the Cf-4-mediated HR in *N. benthamiana*. The Cf homolog *Peru2* from *S. peruvianum* is auto-active in *N. benthamiana*, causing an effector-independent HR when transiently expressed [41].

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Interestingly, the Peru2-eGFP-triggered HR was also strongly compromised upon expression in TRV: *NbSOBIR1/NbSOBIR1-like*-inoculated *N. benthamiana* plants (Fig. S6*A*). To check whether the silenced plants were still able to mount programmed cell death, fully expanded leaves were also transiently transformed to express an auto-active variant of the NB-LRR immune receptor Rx (RxD460V) [42] and the pro-apoptotic factor BAX [43]. Since RxD460V and BAX still triggered a strong cell death, we concluded that the ability of the plants to mount programmed cell death was not compromised (Fig. 2).
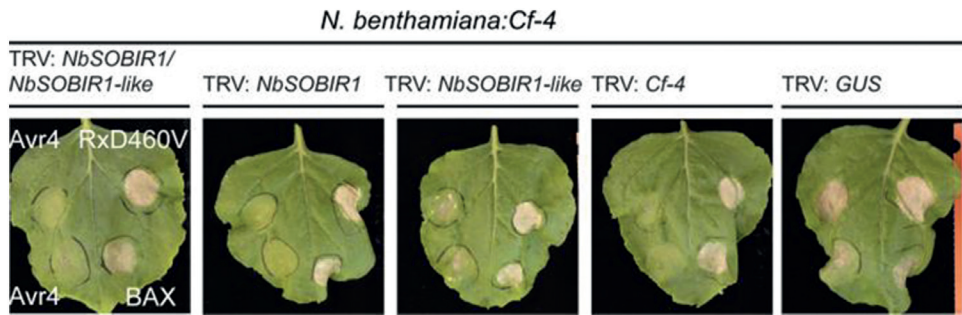
Figure 2. SOBIR1 is required for Cf-4-mediated HR. Transgenic *N. benthamiana*:*Cf-4* plants were subjected to VIGS by inoculation with the TRV constructs indicated above each panel. TRV: *NbSOBIR1/NbSOBIR1-like* targets *NbSOBIR1* and *NbSOBIR1-like* simultaneously. TRV: *Cf-4* and TRV: *GUS* served as controls. Three weeks after TRV inoculation, Avr4 (in duplicate), auto-active Rx (RxD460V) and BAX were transiently expressed in the order indicated in the upper left panel, and leaves were photographed three days later. The experiment was performed three times with three plants for each TRV construct and representative pictures for the experiment are shown.

Unlike in *N. benthamiana*, coexpression of *Ve1* with *Ave1* triggers an HR in *N. tabacum*, a plant for which TRV-based VIGS was recently established [28, 35]. *N. tabacum* plants (cultivar Samsun) were inoculated with TRV: *NbSOBIR1/NbSOBIR1-like*, which also targets the *NtSOBIR1* homolog (Fig. S2*C*) and TRV: *EDS1* as a positive control, as EDS1 is required for Ve1-mediated immunity [26]. Inoculation with TRV: *GFP* was included as a negative control. We used the TRV: *NbSOBIR1/NbSOBIR1-like* construct as we anticipated that *N. tabacum*, of which the currently available genome sequence is very similar to that of *N. benthamiana*, may contain an *NtSOBIR1-like* homologue in addition to *Nt*SOBIR1, although we did not identify an *NtSOBIR1-like* candidate in public databases. Three weeks after inoculation with the different recombinant TRV constructs, *Ve1* and *Ave1* were coexpressed, revealing that plants inoculated with the VIGS constructs targeting *NtSOBIR1/NtSOBIR1-like* and *EDS1* did not mount an HR, in contrast to the TRV: *GFP*-inoculated plants (Fig. S6*B*). Taken together, these results show that *SOBIR1* is required for Cf-4- and Peru2-mediated HR in *N. benthamiana*, and Ve1-mediated HR in *N. tabacum*.

Kinase activity of SOBIR1 is required for Cf-4-mediated HR

To determine whether SOBIR1 requires a functional kinase domain for the Cf-4-mediated HR, we inoculated *N. benthamiana:Cf-4* with TRV: *NbSOBIR1/NbSOBIR1-like*. These plants were then spot-infiltrated to transiently express the combinations *Avr4* and *AtSOBIR1-Myc* or *Avr4* and *AtSOBIR1^{D489N}-Myc*. As a control, *GUS* was expressed in combination with *Avr4*. We reasoned that *AtSOBIR1* would not be targeted by this RNA silencing as there is not sufficient sequence homology between the *NbSOBIR1* genes and *AtSOBIR1*, and therefore *AtSOBIR1*, being a functional homologue of *Nb*SOBIR1, would complement the loss of *NbSOBIR1* and reconstitute the Avr4-triggered HR. However, if SOBIR1 kinase activity is required for Cf-4-mediated HR, *AtSOBIR1^{D489N}-Myc* would not be able to complement.

Coexpression of *GUS* with *Avr4* in the *NbSOBIR1*-silenced plants did not restore the Cf-4-mediated HR (Fig. S5*C*). When *AtSOBIR1-Myc* was coexpressed with *Avr4*, an HR was observed. However, when the kinase-dead mutant *AtSOBIR1^{D489N}-Myc* was coexpressed with *Avr4*, the Avr4-triggered HR was strongly compromised, indicating that SOBIR1 kinase activity is required for Cf-4-mediated HR (Fig. S5*C*). RT-PCR analysis showed that full-length *AtSOBIR1-Myc* and *AtSOBIR1^{D489N}-Myc* transcripts were present in the plants inoculated with TRV: *NbSOBIR1/NbSOBIR1-like* (Fig. S5*D*), confirming that *Arabidopsis* SOBIR1 is indeed not targeted by the VIGS construct. These results show that *At*SOBIR1 complements *Nb*SOBIR1 and the C-terminal Myc epitope tag does not appear to affect *At*SOBIR1 function with respect to its role in Cf-4-mediated HR. Importantly, these results show that SOBIR1 kinase activity is required for the Cf-4-mediated HR.

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SOBIR1 Is Required for Cf- and Ve1-Mediated Resistance to *C. fulvum* and *V. dahliae*, Respectively

To determine whether SOBIR1 is required for Cf-4-mediated resistance of tomato to *C. fulvum*, TRV constructs targeting tomato *SlSOBIR1* and *SlSOBIR1-like* individually or both genes simultaneously, were generated (Fig. S2*B*). As a positive control plants were inoculated with TRV: *Cf-4*, whereas TRV: *GUS*-inoculation served as a negative control. *Cf-4*-expressing tomato was inoculated with the different TRV constructs and three weeks later, plants were inoculated with a race 5 strain of *C. fulvum*, expressing *Avr4* and the *GUS* reporter gene. To detect fungal colonization, leaflets were GUS-stained after two weeks. Inoculation with constructs targeting the two *SlSOBIR1* homologs either individually or simultaneously, resulted in increased fungal colonization as indicated by the much higher number of successful colonization attempts as compared to the TRV: *GUS*-inoculated plants. This shows that both tomato SOBIR1 homologs contribute to Cf-4-mediated resistance (Fig. 3). We also targeted both *SlSOBIR1* homologs in tomato expressing *Cf-2.2* and inoculated these plants with the same *C. fulvum* strain as used above, as this race 5 strain also expresses *Avr2*. Also here, increased fungal colonization was observed as compared to the TRV: *GUS* control (Fig. S6*C*).
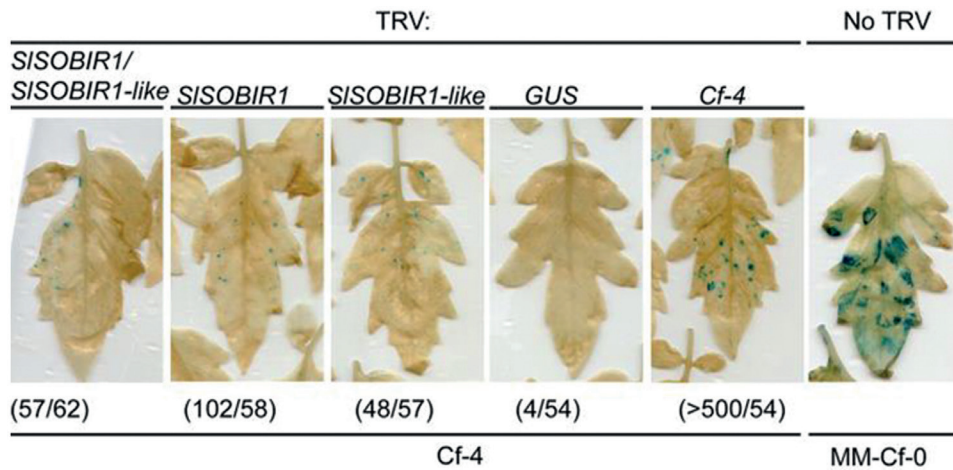
Figure 3. Targeting *SOBIR1* and *SOBIR1-like* suppresses Cf-4-mediated resistance of tomato. Cf-4 tomato was inoculated with the indicated TRV constructs and three weeks later plants were inoculated with an Avr4-secreting, *GUS*-transgenic strain of *C. fulvum*. A non TRV-inoculated susceptible MM-Cf-0 plant was included as control. Two weeks later, leaflets were stained for GUS activity to detect *C. fulvum* colonization. For the Cf-4 tomato plants, the amount of successful colonization attempts (blue spots) versus the total amount of leaflets analyzed for that particular experiment, is indicated between parentheses. The experiment was performed three times and representative pictures are shown.

To test the role of the *Sl*SOBIR1 homologs in resistance to *V. dahliae,* tomato cultivar Motelle that carries the *Ve1* gene was also inoculated with TRV: *SlSOBIR1*, TRV: *SlSOBIR1-like* and TRV: *SlSOBIR1/SlSOBIR1-like*. As controls, plants were inoculated with TRV: *Ve1* and TRV: *GFP*. Three weeks after TRV inoculation, plants were either inoculated with a race 1 strain of *V. dahliae* expressing Ave1 or mock-treated and subsequently monitored for development of disease symptoms (e.g. stunted growth and reduced canopy area). Targeting of the two *Sl*SOBIR1 homologs either individually or simultaneously, as well as *Ve1* itself, resulted in clear stunting and a strongly reduced canopy area compared to the mock-treated plants. These disease symptoms were not observed in plants inoculated with TRV: *GFP* (Fig. S6*D*).

SOBIR1 Is Required for Ve1-Mediated Resistance to *V. dahliae* in *Arabidopsis*

Ve1 provides resistance to *V. dahliae* when introduced in *Arabidopsis* [44]. To study the requirement of *At*SOBIR1 for Ve1-mediated resistance in this plant, we tested whether Ve1 still mediates resistance to *V. dahliae* in an *Arabidopsis sobir1-1* mutant [34]. Similar to the Col-0 wild-type, the *sobir1-1* mutant is susceptible to *V. dahliae* race 1, as shown by the stunted appearance and chlorosis upon fungal infection (Fig. 4). When transformed with the *Ve1* gene, the Col-0 wild-type gains resistance to *V. dahliae* race 1 (Fig. 4). Strikingly, when the *Ve1* gene was introduced into the *sobir1-1* mutant background,

the plants did not gain resistance to the pathogen, as stunting and chlorosis was still observed after inoculation with the fungus (Fig. 4). Quantitative measurement of fungal biomass confirmed these results, as only in the Col-0 wild-type plants transformed with Ve1 fungal colonization was very limited (Fig. S7*A*). This indicates that in addition to its requirement in tomato, SOBIR1 is also required for Ve1-mediated resistance to *V. dahliae* in *Arabidopsis*.

Targeting *SOBIR1* in *N. benthamiana* Leads to Reduced Cf-4 and Ve1 Protein Levels
To investigate whether targeting *SOBIR1* affects Cf-4 and Ve1 protein levels, we inoculated *N. benthamiana* with TRV: *NbSOBIR1/NbSOBIR1-like*, or the control TRV: *GUS*, and after three weeks fully expanded leaves were transiently transformed to individually express eGFP-tagged Cf-4 or Ve1. Subsequently, the steady-state levels of the RLPs were determined by their immunopurification and detection by immunoblotting. Both Cf-4 and Ve1 protein levels were strongly reduced upon targeting *SOBIR1*, when compared to the TRV: *GUS*-inoculated plants indicating that SOBIR1 is required for the accumulation of Cf-4 and Ve1, and thus stabilizes these RLPs (Fig. 5). RT-PCRs revealed that *Cf-4* and *Ve1* are normally expressed in plants inoculated with TRV: *NbSOBIR1/NbSOBIR1-like* and TRV: *GUS*, indicating that reduced accumulation of the Cf-4 and Ve1 proteins is not due to reduced expression levels (Fig. S7*B*).
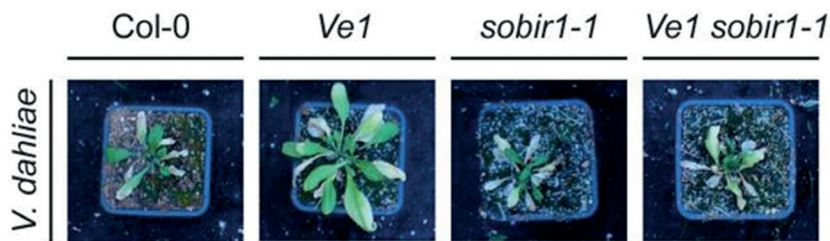

Figure 4. Ve1 is not functional in an *Arabidopsis sobir1-1* mutant background. *Arabidopsis* ecotype Col-0 is susceptible to *V. dahliae* race 1 expressing Ave1 (Col-0). When transformed with the *Ve1* gene, expressed under control of the 35S promoter, Col-0 gains resistance to the fungus (*Ve1*). Similar to the Col-0 wild-type, *sobir1* mutants are susceptible (*sobir1-1*), whereas *sobir1-1* mutant plants transformed with *Ve1* remain susceptible to the fungus (*Ve1 sobir1-1*). The inoculation experiments and qRT-PCR quantifications (Fig. S7*A*) were performed three times, with similar results. A representative picture is shown.
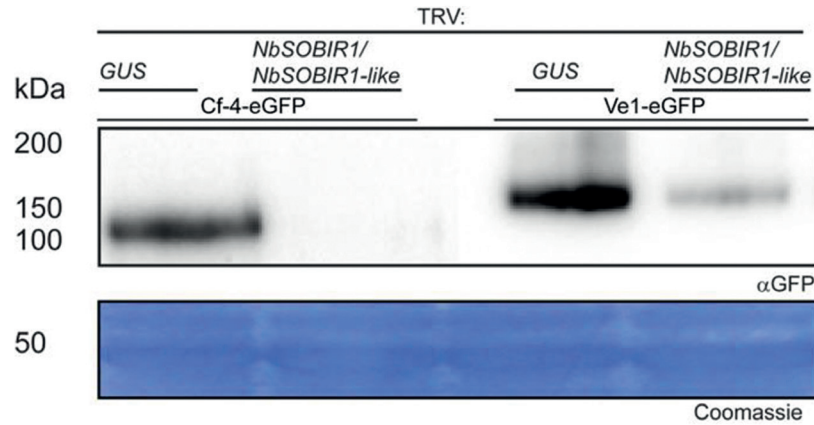
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Figure 5. SOBIR1 is required for the accumulation of Cf-4 and Ve1 proteins. Cf-4 and Ve1, fused to eGFP, were expressed in leaves of *N. benthamiana* subjected to VIGS by inoculation with the indicated TRV constructs. Transiently expressed fusion proteins were immunopurified, subjected to SDS-PAGE and blots were incubated with αGFP antibody for detection of the expressed proteins. The Coomassie-stained blot shows the 50-kDa Rubisco band present in the input samples to confirm equal loading. The experiment was repeated three times with similar results, a representative picture is shown.

The Tomato SOBIR1 Homologs Interact with a Broad Range of RLPs

To test whether the tomato SOBIR1 homologs interact with additional RLPs, Cf-2.2, Cf-4E, Cf-9 and the Cf-like protein Peru2 from *S. peruvianum* were coexpressed as eGFP fusions with *Sl*SOBIR1-Myc or *Sl*SOBIR1-like-Myc in *N. benthamiana*. This revealed that both SOBIR1 homologs copurify with the various Cf proteins (Fig. S8*A*). We expanded our study and examined whether more distantly related tomato RLPs also interact with the tomato SOBIR1 homologs. We fused *Sl*EIX2 [45], mediating perception of the ethylene-inducing xylanase from *Trichoderma viride*, and the closest tomato orthologs of *Arabidopsis* CLV2 (Solyc04g056640.1), TMM (Solyc12g042760.1) and the Suppressor of Npr1-1, Constitutive 2 (SNC2) (Solyc02g072250.1) [46] to eGFP and coexpressed them with the Myc-tagged SOBIR1 homologs in *N. benthamiana*. Immunopurification of the RLPs revealed that *Sl*EIX2, *Sl*CLV2 and *Sl*TMM, but not *Sl*SNC2, interact with *Sl*SOBIR1 and *Sl*SOBIR1-like (Fig. S8*B*).

Discussion

For signal initiation by Cf proteins a mechanistic model was proposed based on the early model of the Clavata1 (CLV1) signalling pathway, in which the RLP CLV2 interacts with the RLK CLV1. This RLK acts as a co-receptor that allows binding of the extracellular endogenous ligand CLV3 and subsequently mediates downstream signalling through its kinase domain [20, 47]. Here, we report that the RLK SOBIR1 interacts with various RLPs

of tomato, including the Cf proteins, Ve1 and *Sl*EIX2 which are all involved in immunity, as well as the tomato homologs of *Arabidopsis Sl*TMM and *Sl*CLV2 which are involved in development (Figs. 1, S1*C*, S8). However, not all RLPs interact with SOBIR1 as is exemplified by *Sl*SNC2 (Fig. S8*B*). In addition, no interaction of SOBIR1 with any of the tested RLKs was found (Fig. 1 and S1*C*). We show that SOBIR1 is required for Cf-2.2, Cf-4- and Ve1-mediated immune responses (Figs. 2, 3, 4, S5 and S6).

SOBIR1 was initially identified in a suppressor screen of the *Arabidopsis bak1-interacting receptor-like kinase 1* (*bir1-1*) mutant, and was referred to as Suppressor Of BIR1-1, 1 [34]. BIR1 encodes another RLK, which interacts with SERK3/BAK1, and the *bir1-1* mutant shows a constitutive defence phenotype, indicating that BIR1 is a negative regulator of defence responses. The *bir1-1* phenotype is suppressed by the *sobir1-1* mutation, suggesting that SOBIR1 is a positive regulator of defence signalling [34]. In line with this, overexpression of *SOBIR1* in *Arabidopsis* leads to constitutive defence activation [34]. Although no direct interaction between SOBIR1 and BIR1 was observed, it was hypothesized that BIR1 functions in a signal transduction pathway that is dependent on SOBIR1 and which promotes pathogen resistance and cell death [34]. As mentioned above, a mutation in *AtSOBIR1* suppresses the *bir1-1* phenotype, whereas an additional mutation in *AtPAD4* fully reverts the *bir1-1 sobir1-1* mutant phenotype back to that of wild-type plants. It was suggested that BIR1 regulates two parallel pathways; one involving resistance proteins that are dependent on PAD4, such as the TIR-NB-LRRs, and one involving another class of resistance proteins requiring SOBIR1 [34]. We propose that the RLPs are members of this latter class of resistance proteins.

We also observed *in planta* interaction of SOBIR1 with RLPs involved in development. Indeed, a role of SOBIR1 in development has been described. *Arabidopsis* mutants in the gene encoding the ADP-ribosylation factor GTPase-activating protein NEVershed (NEV) show impaired floral organ shedding after flowering [48]. A screen for mutations in *nev* plants that restore organ shedding identified a mutation in *SOBIR1* resulting in premature floral organ shedding. Hence, the name EVeRshed (EVR) was coined as a synonym for this RLK, which in this case functions as an inhibitor of abscission [33]. Since SOBIR1/EVR was found to localize to the plasma membrane and cytoplasmic vesicles, it was proposed that the RLK regulates the signalling and internalization of other ligand-binding RLKs involved in floral organ shedding [33]. Interestingly, when transiently expressed in *N. benthamiana*, we likewise found *Sl*SOBIR1-eGFP to localize to the plasma membrane and mobile, cytoplasmic vesicles (Fig. S4). Similar to SOBIR1, also SERK3/BAK1 plays a role both in development and defence and this RLK was initially identified as an interactor of the RLK BRI1, which is involved in Brassinosteroid (BR) perception and signalling [49, 50]. SERK3/BAK1 was also identified to act as a regulator of the RLK-type PRRs FLS2 [11, 13], EFR [12] and PEP1 Receptor protein-1 (PEPR1), an RLK involved in perceiving endogenous peptides [51]. Since Cf and Ve1 interact with SOBIR1 *in planta* and require SOBIR1 for mediating HR and resistance, it is tempting to speculate that SOBIR1 is

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involved in signalling and possible internalization of RLP-containing immune receptor complexes, similar to the function of SERK3/BAK1 in relation to RLKs involved in defence [52].

The current paradigm for several LRR-RLK-type PRRs is their rapid heterodimerization with SERK3/BAK1 upon ligand perception [11-13]. By contrast, interaction between SOBIR1 and the various RLPs studied here is ligand-independent, as we did not coexpress the corresponding ligands in most of our coimmunopurification experiments and still detected copurification of SOBIR1 with the RLPs (Fig. 1, S1*C*, S8). In addition, the presence of Avr4 did not affect the interaction of Cf-4 with SOBIR1 (Fig. S3*C*). Through mutation of its highly conserved RD motif, we showed that a functional SOBIR1 kinase domain is required for Cf-4-dependent HR (Fig. S5*C*), but not for interaction with Cf-4 (Fig. S3*B*). Possibly, the phosphorylation status of SOBIR1 changes upon ligand perception by Cf proteins, thereby allowing additional proteins to associate with the complex. Such proteins could be the previously identified Cf interactors Cf-9–Interacting ThioredoXin (CITRX) [31], the protein kinase Avr9/Cf-9–Induced Kinase 1 (ACIK1) [29], the Soluble N-ethylmaleimide-sensitive factor Adaptor protein REceptor (SNARE) protein Vesicle-Associated Protein 27 (VAP27) [30], and RLKs that reside in the active Cf-containing receptor complex. For example, recently it was shown that SERK1 is also required for Cf-4-mediated resistance of tomato. Furthermore, SERK1 and SERK3/BAK1 are both required for full Ve1-mediated resistance [26, 44]. Because SOBIR1 constitutively interacts with a broad range of RLPs, either involved in defence or in development, it may be that SOBIR1 functions as a scaffold protein stabilizing receptor complexes in which RLPs take part. Alternatively, SOBIR1 could play a role as an integral part of the signalling pathway triggered by RLPs involved in different processes. In that case downstream signalling specificity might be determined by the particular phosphorylation status of the cytoplasmic kinase domain of this regulatory RLK. For example, recent characterization of the *bak1-5* mutation in *Arabidopsis* revealed that the function of SERK3/BAK1 in MTI, the BR response and cell death control can be mechanistically uncoupled [39]. The *bak1-5* mutation is in the kinase domain of SERK3/BAK1 and results in strongly impaired FLS2- and EFR-mediated immune signalling but does not affect BR signalling and the control of the cell death response [39]. Such a situation might also hold for SOBIR1 in relation to signalling triggered by the different RLPs.

Altogether, our studies support the existence of a SOBIR1/RLP complex *in planta*, in which SOBIR1 is required for RLP-mediated immunity against two fungal pathogens that exhibit a different life-style. SOBIR1 appears to function as a regulatory RLK for RLP-containing immune receptor complexes in plants. Future experiments focusing on the cell biology of SOBIR1 and determination of its phosphorylation status and downstream interactors, in the presence and absence of the ligand that is perceived by the interacting RLP, should specify the precise role of SOBIR1 in RLP-containing signalling complexes.

Materials And Methods

Plant Materials and Growth
Growth conditions for *Nicotiana benthamiana*, *Arabidopsis thaliana* and *Solanum lycopersicum* (tomato) are described in SUPPLEMENTAL MATERIALS AND METHODS.

Primers and Vector Construction
Sequences of primers and corresponding targets can be found in Table S4. Construction of plasmids containing Cf-2.2, Cf-4, Cf-4E, Cf-9, Peru2 and Ve1, C-terminally fused to either eGFP or the Myc epitope-tag, has been described previously [32, 35]. The construction of additional vectors for *A. tumefaciens*-mediated transient transformation and VIGS is described in SUPPLEMENTAL MATERIALS AND METHODS.

Plant Transformations
Plasmid pBIN-KS-35S::Cf-4-eGFP (Sol 2701) [32] was used for transformation of tomato MM-Cf-0, which does not carry a functional *Cf-4* gene. Transformations and plant selections were performed as described in SUPPLEMENTAL MATERIALS AND METHODS.

Protein Immunopurification and Identification
Immunopurifications were essentially performed following the protocol described previously with minor modifications [32]. Immunopurifications from stable transgenic tomato expressing Cf-4-eGFP were performed as described in SUPPLEMENTAL MATERIALS AND METHODS.

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VIGS and Disease Assays
VIGS experiments in *N. benthamiana,* tobacco and tomato were performed as described previously [32, 35]. *C. fulvum* disease assays were performed as described before [32] and *V. dahliae* disease assays were performed as described in SUPPLEMENTAL MATERIALS AND METHODS.

Acknowledgements

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Supplemental Data

Supplemental Materials And Methods

Plant Growth Conditions

N. benthamiana, *N. benthamiana:Cf-4* [1] and *N. tabacum* were grown under 16 hours of light at 25°C and 8 hours of darkness at 21°C in climate chambers with a relative humidity of ~75%. *Arabidopsis*, tomato (*S. lycopersicum*) cv. Money Maker (MM) and cv. Motelle, as well as *Cf.2.2-* [2] and *Cf-4* (*Hcr9-4D*)-transgenic MM plants [3] were grown in the greenhouse under 16 hours of light at 21°C and 8 hours of darkness at 19°C. The relative humidity in the greenhouse was ~75%.

Plant Transformations

Plasmid pBIN-KS-35S::Cf-4-eGFP (Sol 2701) [4] was used for transformation of tomato MM-Cf-0, which does not carry a functional *Cf-4* gene. Transformations were performed as described before [5]. Using a qRT-PCR-based method [6], plants carrying only single- or two-copy transgenes were selected. To test for the presence of functional Cf-4, leaflets of transgenic tomato plants were infiltrated with apoplastic fluid from leaflets of a MM-Cf-0 plant colonized by an Avr4-secreting strain of *C. fulvum* using a syringe without needle. Arabidopsis mutant Col-0 *sobir1-1* [7] was transformed with p35S::*Ve1* as described previously [8]. Four homozygous single insert lines expressing *Ve1* were selected based on segregation and qRT-PCR analysis and used in *V. dahliae* disease assays.

Binary Vectors for *A. tumefaciens*-Mediated Transient Transformation

Sequences of primers and corresponding targets can be found in Table S4. Construction of plasmids containing Cf-2.2, Cf-4, Cf-4E, Cf-9, Peru2 and Ve1, C-terminally fused to either eGFP or the Myc epitope-tag, has been described previously [4, 9]. *Sl*FLS2-GFP was expressed from pCAMBIA2300-FLS2p::*Le*FLS2-GFP [10]. For novel constructs, coding regions were amplified from cDNA. PCR fragment *SlSERK3a/BAK1* was cloned in pDONR201 using Gateway BP Clonase II (Invitrogen). Fragments *SlSERK1*, *SlEIX2*, *SlSOBIR1*, *SlSOBIR1-like* and *AtSOBIR1* were cloned in pENTR/D-Topo (Invitrogen). Plasmid pENTR/D-Topo containing *AtCLV1* has been described previously [11]. To generate mutations in the SOBIR1 kinase domain, pENTR/D-Topo vectors containing *SlSOBIR1*, *SlSOBIR1-like* and *AtSOBIR1* coding regions were PCR-amplified with primers introducing a mismatch nucleotide to generate a D to N codon change. After

amplification, the methylated parental plasmid was digested using *Dpn*I. All pDONR201 and pENTR/D-Topo clones were sequenced and subsequently fragments were transferred to the binary transformation vector pBIN-KS-35S::GWY-eGFP (Sol 2095; for C-terminally tagging with eGFP) or pGWB20 [12] (for C-terminally tagging with the Myc epitope), using Gateway LR Clonase II (Invitrogen). This resulted in plasmids pBIN-KS-35S::At-CLV1-eGFP (Sol 2824), pBIN-KS-35S::Sl-CLV2-eGFP (Sol 2782), pBIN-KS-35S::Sl-EIX2-eGFP (Sol 2863), pBIN-KS-35S::Sl-SNC2-eGFP (Sol 3109), pBIN-KS-35S::Sl-TMM-eGFP (Sol 3110), pBIN-KS-35S::Sl-SOBIR1-eGFP (Sol 2774), pGWB20-Sl-SOBIR1-Myc (Sol 2754), pGWB20-Sl-SOBIR1-like-Myc (Sol 2752), pGWB20-At-SOBIR1-Myc (Sol 2849), pGWB20-Sl-SOBIR1D473N-Myc (Sol 2878), pGWB20-Sl-SOBIR1-likeD486N-Myc (Sol 2879) and pGWB20-At-SOBIR1D489N-Myc (Sol 2880). Avr4 was expressed from pMOG800-Avr4 and Avr9 from pMOG800-Avr9 [13]. GFP-HA was expressed from pBIN61-GFP-HA [14]. All binary plasmids were transformed to *Agrobacterium tumefaciens* strain C58C1, carrying helper plasmid pCH32. Infiltration of *Agrobacterium* into plant leaves (agroinfiltration) was performed as described previously at an O.D.$_{600}$ = 1, unless indicated otherwise [13].

Protein Identification by Immunopurification, Followed by Tryptic Digest and Mass Spectrometry

Immunopurifications from tomato and *N. benthamiana* were essentially performed following the protocol described previously with minor modifications [4]. For immunopurifications from the transgenic tomato lines expressing Cf-4-eGFP young, not fully expanded, leaves of 6-week-old plants were taken. Proteins were extracted using Extraction Buffer (EB) (150 mM NaCl, 1% IGEPAL CA-630 (NP40), 50 mM Tris, pH8, plus one tablet of protease inhibitor cocktail (Roche) per 50 ml of extraction buffer). To 1 g of leaf material (fresh weight) 2 ml of EB was added. Subsequently, a total protein extract of 10 ml was subjected to immunopurification by adding 60 µl (50% slurry) of GFP_TrapA beads (Chromotek) and incubation while shaking for one hour [4]. Beads were then washed five times with EB. Tryptic on-bead digestion was followed by mass-spectrometry using either the Synapt MS (Waters) or the Orbitrap XL (Thermo Scientific) [4].

Coimmunopurifications and Immunoblotting

Coimmunopurifications were performed as described previously [4]. Two ml of protein extract was incubated for 1 hour with 15 µl GFP_TrapA beads (50% slurry) and beads were washed five times with EB. Protein blots were developed using either αGFP-HRP (130-091-833, MACS antibodies) or αcMyc (cMyc 9E10, sc-40, Santa Cruz) with αMouse-HRP (Amersham) as a secondary antibody.

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Confocal Microscopy

Confocal microscopy was performed on agroinfiltrated *N. benthamiana* leaves as described previously [4].

Generation of VIGS Constructs

Fragments to be used for VIGS were PCR amplified from *N. benthamiana* or tomato cDNA (Table S4). All fragments were cloned into pCR4-TOPO (Invitrogen) and sequenced. The tomato fragments were then excised from pCR4-TOPO using restriction enzymes *Xba*I and *Bam*HI and cloned into pTRV2: RNA2 (pYL156) [15] that was linearized with the same enzymes, to generate pTRV2: *SlSOBIR1* (Sol 2756), pTRV2: *SlSOBIR1-like* (Sol 2755) and pTRV2: *SlSOBIR1/SlSOBIR1-like* (Sol 2779). Fragment *NbSOBIR1* was excised from pCR4-TOPO using enzymes *Eco*RI and *Bam*HI and cloned into pTRV2: RNA2, linearized with the same enzymes, to generate pTRV2: *NbSOBIR1* (Sol 2850). VIGS fragment *NbSOBIR1-like* was excised from pCR4-TOPO using *Bam*HI and *Xho*I and cloned into pTRV2: RNA2, linearized with the same enzymes, to generate pTRV2: *NbSOBIR1-like* (Sol 2851). Fragment *NbSOBIR1-like* was also cloned into Sol 2850, linearized with *Bam*HI and *Xho*I, to generate pTRV2: *NbSOBIR1/NbSOBIR1-like* (Sol 2854).

VIGS in *N. benthamiana* and Tobacco and HR Assays

VIGS experiments in *N. benthamiana:Cf-4*, wild-type *N. benthamiana* and *N. tabacum* cv. Samsun were performed as described previously [4, 9]. In brief, four-week-old plants were inoculated by agroinfiltration with pTRV: RNA1 and pTRV: RNA2 [15]. TRV: *Cf-4* [16], TRV: *EDS1* [9], TRV: *GUS* [17], TRV: GFP [18] and TRV: *PDS* [15] were included as controls.

For HR assays, three weeks after virus inoculations mature leaves were agroinfiltrated to individually express Avr4 at an O.D.$_{600}$ = 0.03 [1], RxD460V (pB1-Rx (AT39-H1; D460V)) [19] at an O.D.$_{600}$ = 0.1, BAX [20] at an O.D.$_{600}$ = 0.5 and Peru2-eGFP at an O.D.$_{600}$ = 1. For complementation analysis with *At*SOBIR1-Myc and the respective D489N kinase mutant, constructs driving expression of these proteins, in addition to GUS, were coexpressed with Avr4 (O.D.$_{600}$ = 0.03) at an O.D.$_{600}$ of 0.5, in *N. benthamiana:Cf-4*. In tobacco, Ve1 (pMOG800-Ve1) [6] and Ave1 (pFAST-Ave1) [9] were transiently coexpressed in leaf sections at an O.D.600 of 2 for each construct. Three days after agroinfiltration leaves were examined for development of an HR.

RNA extraction, cDNA synthesis and (q)RT-PCR analysis

For qRT-PCRs, RNA was isolated from *N. benthamiana* inoculated with the various TRV constructs. For RT-PCRs, mature leaves of TRV-inoculated plants were either agroinfiltrated with Cf-4-eGFP or Ve1-eGFP, or they were not transiently transformed. RNA extraction, cDNA synthesis and qRT-PCR were performed as described before [4]. For RT-PCR analysis, the amount of cycles is indicated. The primer combinations used can be found in Table S4.

VIGS in Tomato, and *C. fulvum* and *V. dahliae* Disease Assays

Tomato was subjected to VIGS as described previously [4]. For *C. fulvum* disease assays, tomato plants transformed with the *Hcr9-4D* (*Cf-4*) or *Cf-2.2* gene and fully resistant to a race 5 strain of *C. fulvum* (secreting both Avr4 and Avr2), were subjected to agroinoculation with various recombinant TRV VIGS constructs targeting *SlSOBIR1(-like)*, *Cf-4* or *GUS*. Non-agroinoculated MM-Cf-0 plants served as fully susceptible controls. For *V. dahliae* disease assays, tomato cultivar Motelle carrying the *Ve1* gene and resistant to *V. dahliae* race 1 strains expressing Ave1, was used. TRV: *Ve1* and TRV: *GFP* were used as controls. Four to six tomato plants were used per treatment in each experiment. Leaf canopy area measurements were performed as described before [21] and for each plant the canopy area was calculated. The average canopy area of *V. dahliae*-inoculated plants, compared to control plants, was calculated for three independent biological repeats.

C. fulvum inoculations were performed as described previously [4]. *C. fulvum* race 5-pGPD:*GUS* and constitutively expressing the *GUS* reporter gene, was used for inoculations [22]. *V. dahliae* disease assays on tomato and *Arabidopsis*, as well as quantification of *V. dahliae* biomass by qRT-PCRs, were performed as described previously [6, 8]. *V. dahliae* race 1 strain JR2, expressing Ave1, was used in the various disease assays.

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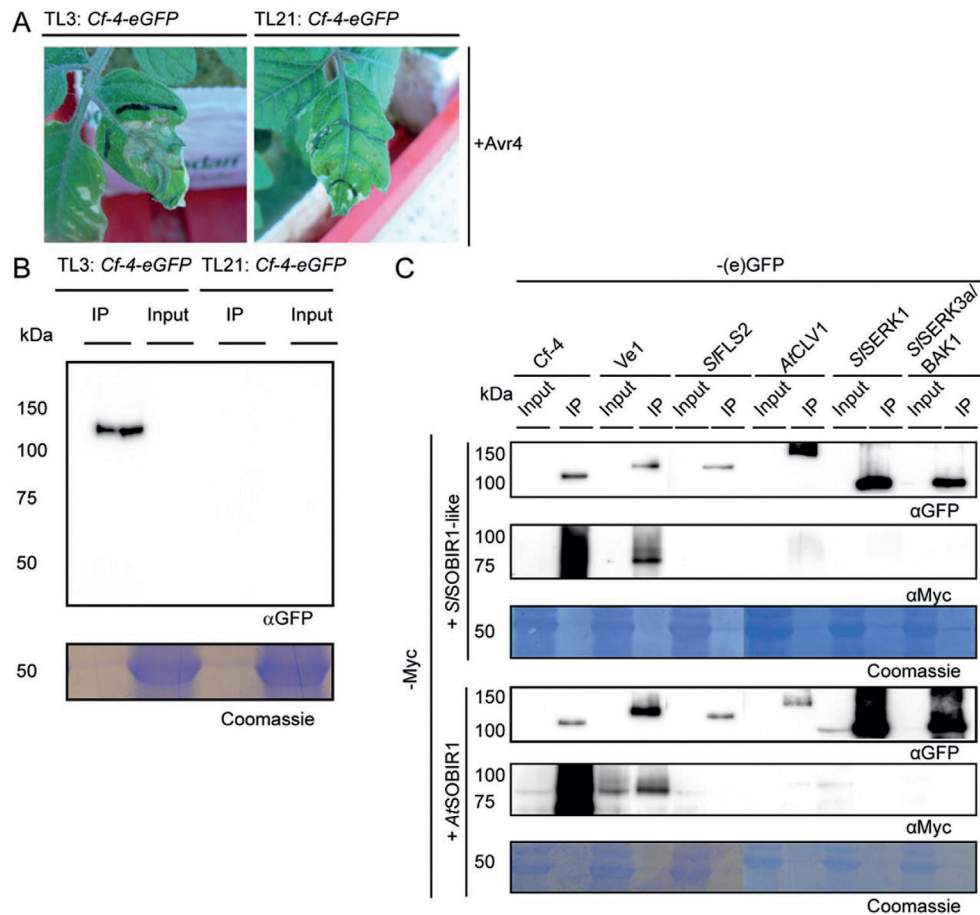
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Figure S1. Cf-4-eGFP is functional and efficiently immunopurified from stably transformed tomato and tomato *Sl*SOBIR1-like and *Arabidopsis At*SOBIR1 interact with Cf-4 and Ve1, but not with various RLKs. (A) Leaflets of the transgenic lines TL3 and TL21, stably transformed with the Cf-4-eGFP gene under control of the 35S promoter, were infiltrated with apoplastic fluid obtained from leaflets of susceptible Money Maker-Cf-0 plants colonized by *C. fulvum* secreting Avr4. TL3 mounted an HR by two days after infiltration, in contrast to line TL21, which did not respond to the Avr4 infiltration. (B) Cf-4-eGFP is detectably immunopurified from TL3, but not from TL21. Total protein extracts were subjected to immunopurification by using GFP-affinity beads and total proteins (Input) and Immunopurified Proteins (IP) were subjected to SDS-PAGE and analyzed by immunoblotting using αGFP antibody. The Coomassie-stained blot shows the 50-kDa Rubisco band present in the input samples to confirm equal loading. (C) Tagged versions of Cf-4, Ve1, *At*CLV1, *Sl*SERK1, *Sl*SERK3a/BAK1 and *Sl*FLS2 (all fused to eGFP, except for *Sl*FLS2, which was fused to GFP) were coexpressed with *Sl*SOBIR1-like-Myc and *At*SOBIR1-Myc in *N. benthamiana*. Total protein extracts of transiently transformed leaf tissue were subjected to immunopurification using GFP-affinity beads. Total proteins (Input) and Immunopurified Proteins (IP) were subjected to SDS-PAGE and blotted. Blots were incubated with αGFP antibody to detect the immunopurified (e)GFP fusion proteins and incubated with αMyc antibody to detect coimmunopurifying SOBIR1-Myc proteins. Coomassie-stained blots showing the 50-kDa Rubisco band present in the input samples confirm equal loading. Representative results for three independent experiments are shown.

A

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                    *          80          *         100          *         120
SlSOBIR1   : ---MTSNIHFFI----LYVVSIFLFVQAR-----LNLYPPDHAALLLVQKDLGIISVN--      :  46
SlSOBIR1-l : MTFTASYIHLSF----LSFFTVILLVQAK-----LNLYSPDHSALLLVQKGLGIPAQRNV      :  51
AtSOBIR1   : MAVPTGSANLFIRPLIAVLSFLLLSSFVSSVEWLDIDSSLKALQVIETELGVNSQRSS      :  60
SlSERK3a/B : MDQSVLAIWVFI-----CLIGLLFNISPVAG----NAEGDALNALKTNLADPNSVLQSWD      :  51

                    *          80          *         100          *         120
SlSOBIR1   : ----NPCTLAGISCERRPGNTT--QVVRVTRIVFRSNGIKGTLSSAIGKLTELKELSISD      : 100
SlSOBIR1-l : L--ENPCNSVGISCEKRLINNS--NLRVTRVVFKSYGIKGTLSPAIGRLSELKELSIQN      : 107
AtSOBIR1   : ASDVNPCGRRGVFCERRHSATTGEYVLRVTRLVYRSRSITGTISPVIGMLSELKELTISN      : 120
SlSERK3a/B : ATLVNPCTWFHVTCNNE---------NSVTRVDLGNANISGQIVLQCLQLQKIQYLELYS      : 102

                    *         140          *         160          *         180
SlSOBIR1   : NQLSEQIPVQIIDCRKLEILQIQRNRFSGKIPSELSALNRLRIVDFSSNEFSGNLDFLKY      : 160
SlSOBIR1-l : NKLFDRIPTEIVDSRKLEILNLNQNQFSGKVPELSSLVRIRILDIGSNKLSGNLNFLKY      : 167
AtSOBIR1   : NQLVNAVPVDIISCKQLEVLDLRKNRFSGQIPGNFSSLSRLRILDISSNKLSGNLNFLKN      : 180
SlSERK3a/B : NNISGRIPNELGNLTEIVSLDIYLNNLNCPIPPSTGRLQKLRFLRLNNNSLN--------      : 154

                    *         200          *         220          *         240
SlSOBIR1   : FPNLEKLSLADNMFTGKIDFSLKSFRNLRFINISGNSFLEGPVPVMSQIEHLSADLNRKN      : 220
SlSOBIR1-l : FPNLEKLSLADNMFTGRIPQSIKSFRNLRLLNISDNSFLEGCTVPVVSQVEHLSTDLNRKD      : 227
AtSOBIR1   : LRNLENLSVANNLFSGKIPEQIVSFHNLRFFDFSGNRYLEGCPAPVMSSIKLQISPHQT--      : 238
SlSERK3a/B : --------------EGIPMSITTIVALQVLDLSNN-HITGLVPVNGSFSLFT-------      : 191

                    *         260          *         280          *         300
SlSOBIR1   : GVPKRYILAENSTR---ISAMGPASVP--APADAPVNRVVPAMHKRKNKKR-KLRSWFLC      : 274
SlSOBIR1-l : YVLKRYILAENATRPRHSPAMAPESKSRYAQAPGPSTVVVPVKKDKHNKIRRKVGAWILC      : 287
AtSOBIR1   : ----RHILAETPTS---SPTNKPNNSTT-SKAPKGAPKPGKLKKKKKKSKKKVAAWILC      : 290
SlSERK3a/B : -----------------PISFANNQLEVPPVSPPPLPPTESSSSVGNSATGAIAGGVA      : 234

                    *         320          *         340          *         360
SlSOBIR1   : FLAGTFACGISAVICSLLFKMVMFFVRRGNNDSSLTIYSPLIKKAEDLAFLEKEDGVASL      : 334
SlSOBIR1-l : FFAGVFACGLSALIFSVLCKVLMFLIRGIRNDPGLTIFSPLIKKAEHLAFLENEDGLESL      : 347
AtSOBIR1   : FVVGAIGCTISGFVFSVLFKLIIQAIRGSEKPPGPSIFSPLIKKAEDLAFLENEEALASL      : 350
SlSERK3a/B : AGAALLFAAPAIFIAWWRRKPQDHFFDVPAEEDPEVHLGCLKRFSLRELQVASDNFSNR      : 294

                    *         380          *         400          *         420
SlSOBIR1   : EMIGKGGCGEVYRAELPGSNGKIIAIKKIIQSPMDAAEITEEDTKALNKKMRQVKSEIQI      : 394
SlSOBIR1-l : ELIGQGGCGKVYKAALPGSDGKIIAVKKIIQPPKDAAELTEEDSKAMNKKMRQIKSEIKI      : 407
AtSOBIR1   : EIIGRGGCGEVFKAELPGSNGKIIAVKKVIQPPKDADELTDEDSKFLNKKMRQIRSEINT      : 410
SlSERK3a/B : NILGRGGFGKVYKGRL--ADGSLVAVKRLKEERTQGGEI-----------QFQTEVEM      : 339

                    *         440          *         460          *         480
SlSOBIR1   : VGQIRHRNLLPLLAHMPRPDCHYLVYEYMKNGSLQDILQQVTEGTRELDWLGRHRIAAGV      : 454
SlSOBIR1-l : VGQIRHRNLLPLLAHMPRPDCHYLVYEYMKNGSLQDTLQVREGKRELDWSARHRIAMGI      : 467
AtSOBIR1   : VGHIRHRNLLPLLAHVSRPECHYLVYEYMEKGSLQDILTDVQAGNQIMWPARHKIALGI      : 470
SlSERK3a/B : ISMAVHRNLIRLRGFCMTPTERVLVYPYMENGSVASRLRERPESEPPLDWPKRKRIALGS      : 399

                    *         500          *         520          *         540
SlSOBIR1   : AAGLEYLHINHTQRIIHRDLKPANILLDDDMEARVADFGLAKAVPDAHTHITTSNVAGTV      : 514
SlSOBIR1-l : AAGLEYLHINHTQRIIHRDLKPGNVLLDDDMEARIADFGLAKAVPDAHTHITTSNVAGTI      : 527
AtSOBIR1   : AAGLEYLHMDHNPRIIHRDLKPANVLLDDDMEARISDFGLAKAMPDAVTHITTSHVAGTV      : 530
SlSERK3a/B : ARGLAYLHDHCDPKIIHRDVKAANILLDEEFEAVVGDFGLAKLMDYKDTHVTT-AVRGTI      : 458
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                         *         560         *         580         *         600
SlSOBIR1     : GFIAPEYYQTLKFTDKCDIYSFGVVIAVLVIGKGPSD-DFFQHTSEMSLVKWLRNVMTSD : 573
SlSOBIR1-l   : GYIAPEYHQTLKFTDKCDIYSFGVLIGVLVMGKIPSD-EFFQNTSEMSLVKWMRNVMTSE : 586
AtSOBIR1     : GYIAPEFYQTHKFTDKCDIYSFGVILGILVIGKIPSD-EFFQHTDEMSLIKWMRNIITSE : 589
SlSERK3a/B   : GHIAPEYLSTGKSSEKTDVFGYGVMILELVTGQRAFDLARLANDDDVMLLDWVKGLLKDK : 518


                         *         620         *         640         *         660
SlSOBIR1     : DPKIAIDPKLIGNGYDEQMLLVLKIACFCTLDNPKERPNSKDVRCMLTQIKH-------- : 625
SlSOBIR1-l   : DPNRAIDPKLMGNGNEDQMLLVLKIACFCTMENPKERPNSKDVRCMLQIKH-------- : 638
AtSOBIR1     : NPSLAIDPKLMDQGFDEQMLLVLKIACYCTLDDPKQRPNSKDVRIMLSQIKH-------- : 641
SlSERK3a/B   : KYETLVDADLQGNYNEEEVEQLIQVALLCTQSTPTERPKMSEVVRMLEGDGLAERWEEWQ : 578


                         *         680         *
SlSOBIR1     : ------------------------------------ :  -
SlSOBIR1-l   : ------------------------------------ :  -
AtSOBIR1     : ------------------------------------ :  -
SlSERK3a/B   : KEEMFRQDYNHVHHPHTDWIIADSTSNIRPDELSGPR : 615
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B

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                         *         20          *         40          *         60
SlSOBIR1     : ATGAC---------------------------------TTCGAATATCCACTTTTTTC :  25
SlSOBIR1-li  : ATGACC--------------------------TTCACAGCCTCATATATACACCTTTCTT :  34
AtSOBIR1     : ATGGCTGTTCCCACGGGAAGCGCCAATCTCTTTCTCCGGCCTCTCATCCTCGCCGTTCTC :  60


                         *         80          *         100         *         120
SlSOBIR1     : TTTTATACGTTGTGAGCCTTTTCCTTTTTGT---TCAAGCAAGATTGAATCTTTATCCAC :  82
SlSOBIR1-li  : TTTTGTCTTTTTTCACTGTCATCCTTCTTGT---TCAAGCAAAACTAAACCTTTACTCAC :  91
AtSOBIR1     : TCCTTTCTTCTTCTCTCTTCATTCGTCTCCTCTGTTGAGT--GGCTCGATATCGATTCCT : 118


                         *         140         *         160         *         180
SlSOBIR1     : CAGATCATGCTGCAACTCTTGCTTGTACAAAAGGACTTAGGCATC------------ATTT : 130
SlSOBIR1-li  : CTGATCACAGTGCTCTTTTGCTTGTCCAAAAAGGCTTAGGCATCCCTGCTCAGCCCAATG : 151
AtSOBIR1     : CAGATCTCAAAGCTCTTCAGGTCATCGAAACACAGCTCGGCAGTCAACAGTCAACGTTCTT : 178


                         *         200         *         220         *         240
SlSOBIR1     : C------TGTTAACAACCCGTGTACGCTCGCAGGAATATCGTGCGACCGTAGACCGGGTA : 184
SlSOBIR1-li  : T------TCTTGAGAACCCATGCAACTCTGTTGGAATATCATGCGAAAACGACTCA-TA : 204
AtSOBIR1     : CGGCCAGTGATGTCAACCCTTGCGGTCGCCGAGGAGTTTTCTGCGAGAGACGGCACTCTG : 238


                         *         260         *         280         *         300
SlSOBIR1     : ACACAACACAAGT-TG-----TGAGAGTTACCCGTATTGTATTCAGATCCAACGGATTAA : 238
SlSOBIR1-li  : A-ACAATTCAAATGTGC----TAAGAGTCACAAGGGTTGTCTTCAAATCCTACGGGTTGA : 259
AtSOBIR1     : CCACCACCGGACAATACGTTCTCCGTGTCACGAGCCTCGTCTACCGATCCAGGACCTTGA : 298


                         *         320         *         340         *         360
SlSOBIR1     : AGGGCAACTTTGTCTTCTGCTATTGCAAACTCACTGAGCTCAAAGAGCTTTCTCGTTCCG : 298
SlSOBIR1-li  : AGGGTACTCTGTCTCCTGCCATTGGGAGGCTTTCTGAGCTCAAAGAGCTGTCCCTCCAA : 319
AtSOBIR1     : CGGGGACTATCTCTCCGGTCATCGGAATGTTGTCGGAGCTCAAGGAACTTACTTGTCTA : 358


                         *         380         *         400         *         420
SlSOBIR1     : ACAATCAACTATCTGAACAAATCCCGCGTTCAGATTATTGATTGTCGGAAGTTAGAGATTC : 358
SlSOBIR1-li  : ACAACAAACTCTTTGACAGAATACCAACTGAAATAGTTGACTCCACAAAATTGGAAATCT : 379
AtSOBIR1     : ATAACCAGTTGGTCAACGCTGTTCCGGTGGATATCTTAAGCTGTAACCAACTTGAAGTTC : 418
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                       *         440         *         460         *         480
SlSOBIR1    : TTCAACTTCAAAGGAACAGATTTTCTGGGAAGATTCCATCCGAATTGTCAGCTTTAAACC :  418
SlSOBIR1-li : TGAACCTTCAAAACAACCAATTTTCTGGCAAAGTCCCACCTGAATTATCATCTCTAGTCC :  439
AtSOBIR1    : TTGATCTTCGCAAAAATAGATTTTCCGGTCAGATTCCGGGAAACTTTTCTAGTTTGAGCC :  478

                       *         500         *         520         *         540
SlSOBIR1    : GTTTAAGGATAGTTGACTTTTCATCGAATGAGTTTTCGGGGAATCTTGATTTCTTGAAGT :  478
SlSOBIR1-li : GACTTCGTATCCTTGACCTTGGCTCTAATGAGTTATCTGGGAACCTGAACTTCTTGAAAT :  499
AtSOBIR1    : GTCTTCGAATCCTTGATCTCTCTTCCGAACAAGTTATCCGGGAACTTGAATTTCTTGAAGA :  538

                       *         560         *         580         *         600
SlSOBIR1    : ACTTTCCTAATCTGGAAAAACTGTCTCTGGCTGATAATATGTTCACTGGAAAAATACCAT :  538
SlSOBIR1-li : ACTTTCCTAACCTTGAAAAACTCTCCCTTGCTGATAACATGTTTACTGGCAGAATACCTC :  559
AtSOBIR1    : ATCTACGCAATCTGGAGAATCTCTCTGTTGCAAACAATCTCTTCTCAGGCAAAATCCCGG :  598

                       *         620         *         640         *         660
SlSOBIR1    : TTTCATTGAAATCTTTCCGAAATCTTCGTTTTCTCAACATTTCAGGCAATAGTTTCCTTG :  598
SlSOBIR1-li : AATCCTTGAAATCTTTCAGAAATCTCCGGCTCCTCAACATTTCAGACAATAGTTTCCTTG :  619
AtSOBIR1    : AACAAATTGTGTCGTTCCACAATCTCCGGTTCTTTGATTTCTCCGGAAACCGGTATTTGC :  658

                       *         680         *         700         *         720
SlSOBIR1    : AAGGTCCAGTACCTGTCATGAGCCAAATTGAGCATTTATCAGCAGATTTGAATCGAAAAA :  658
SlSOBIR1-li : AAGGTACGGTGCCTGTCGTGAGTCAAGTTGAGCACTTATCAACAGACTTAAATCGAAAAG :  679
AtSOBIR1    : AAGGTCCGCCTCCGGTTATGAGCAGTATCAAGCTTCADACATCTCCACATCAAACAA :  715

                       *         740         *         760         *         780
SlSOBIR1    : ATGGCGTTCCTAAACGTTACATTCTAGCTGAGAATTCAACAAGGATAT---------CTG :  709
SlSOBIR1-li : ATTATGTTCTTAAGCGTTACATTCTTGCTGGAACGCAACAAGGCCACGGCACAGCCCTG :  739
AtSOBIR1    : GACACATTCTT--GC-TGAAACTCCAACTTCAAGTCCCAGGAACAAAC--------CCAA :  764

                       *         800         *         820         *         840
SlSOBIR1    : CAATGGGACCTGC-TTCGGT--TCCAG-----CTCCAGCTCCAGCACCT-GT-CAATCGT :  759
SlSOBIR1-li : CAATGGCACCCGAGTCCAAA--TCAGATATGCCCAAGCTCCAG-ACCTAGTACAGTTGT :  797
AtSOBIR1    : CAACAGCACT-ACATCAAAAGCTCCAAAGG-----GAGCTCCAAAACCAGGG-AAGTTGA :  817

                       *         860         *         880         *         900
SlSOBIR1    : GTTGTACCGGCCATGCA---TAAACGTAAGAACAAGAAAAGGAAGTTACGATCTTGCTTT :  816
SlSOBIR1-li : A--GTACCAGTAAAAAAGACAAACATAACAACATCACAAGGAAGGTAGGCGCCTGGATT :  855
AtSOBIR1    : A----------GAAGAAG---AAGACAAGACAAGAAGAAGAAATAGCGCCATGGATC :  864

                       *         920         *         940         *         960
SlSOBIR1    : CTTGGTGTTTCCTAGCTGGAACTTTTGCTGGGGCTATATCTCTGTCCTCTGTTCCTTGCTA :  876
SlSOBIR1-li : CTTGGATTCTTTTGCTGGAGCTTTTCGCTGGGGGCTTATCAGCGTTGATCTTCTCCGTCCTC :  915
AtSOBIR1    : TTAGGGTTTGTCGTTGGAGCCATAGGAGGAACAATCTCTGGGTTTGTCTTTCTCGCTCTTG :  924

                       *         980         *        1000         *        1020
SlSOBIR1    : TTCAACATGGTAATGTTCTTCGTAAGAAGAGG-GAATAACGATTCAAGTTTAACGATATA :  935
SlSOBIR1-li : TGCAACGTGCTTATGTTTTTGATCAGAGGGATCAGAAATGATCCAGGCTTAACAATTTT :  974
AtSOBIR1    : TTCAAATTGATTATTCAAGCAATAGAGGATCAGAAAAACCA-CCAGGTCCTTCCATATT :  983

                       *        1040         *        1060         *        1080
SlSOBIR1    : CAGTCCACTGATCAAGAAGAGCCGAGGACTTGGCTTTCTTAGAGAACGAAGATGGAGTGGC :  995
SlSOBIR1-li : CAGTCCATTGATCAAGAAAGCCGACCACTTGGCCTTTCTGGAGAATGAAGATGGATTGGA : 1034
AtSOBIR1    : CAGCCCATTGATCAAGAAAGCTGAACGATTTAGCTTTCTTCGGAGAATGAACGAAGCGTTACC : 1043
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                   *         980          *        1000         *        1020
SlSOBIR1    : TTCAAGATGGTAATGTTCTTCGTAAGAAGAGG-GAATAACGATTCAAGTTTAACCATATA :  935
SlSOBIR1-li : TGCAAGCGTGCTTATGTTTTTGATCAGAGGGAT-CAGAAATGATCCAGGCTTAACAATTTT :  974
AtSOBIR1    : TTCAAATTGATTATTCAAGCAATAAGAGGATCAGAAAAACCA-CCAGGTCCTTCCATATT :  983

                   *        1040          *        1060         *        1080
SlSOBIR1    : CAGTCCACTGATCAAGAAAGCCGAGGACTTGGCTTTCTTAGAGAAGGAAGATGGAGTGGC :  995
SlSOBIR1-li : CAGTCCATTGATTAAGAAAGCCGAGCCACTTGGCCTTTCTGGAGAATGAAGATGGATTGGA : 1034
AtSOBIR1    : CAGCCCATTGATCAAGAAAGCTGAAGATTTAGCTTTCTTGGAGAATGAAGAAGCGTTAGC : 1043

                   *        1100          *        1120         *        1140
SlSOBIR1    : ATCGCTTGAAATGATTGGAAAAGGTGGCTGTGGAGAAGTTTTATAGCAGAGCTACCGGG : 1055
SlSOBIR1-li : ATCACTACAACTCATTGGGCAAGGTGGATGTGGAAAAGTTTATAAAGCTGCATTACCTGG : 1094
AtSOBIR1    : TTCTCTCGGACGATCATAGGAAGAGGAGGTTGTGGAGAAGTTTTCAAAGCTGAACTACCAGG : 1103

                   *        1160          *        1180         *        1200
SlSOBIR1    : GAGTAATGGGAAGATTATAGCTATAAAGAAGATTATCCAATCCCCAATGGATGCTGCAGA : 1115
SlSOBIR1-li : AAGTGACGGAAAGATTATAGCTGTAAAGAAGATCATACAACCACCAAAGGATGCTGCAGA : 1154
AtSOBIR1    : AAGCAATGGGAAGATCATAGCTGTGAAGAAAGTGATCCAACCGCCTAAAGACGCCGATGA : 1163

                   *        1220          *        1240         *        1260
SlSOBIR1    : GATCACCGAGGAAGATACTAAGGCGTTGAACAAGAAAATGCGTCAAGTTAAATCAGAAAT : 1175
SlSOBIR1-li : ACTCACTGAGGAAGATAGCAAGGCTATGAATAAGAAAATGCGCCAGATTAAATCAGAAAT : 1214
AtSOBIR1    : ACTAACAGATGAAGATTCCAAGTTTCTGAACAAGAAAATGAGGCAAATTAGATCCGAGAT : 1223

                   *        1280          *        1300         *        1320
SlSOBIR1    : TCAAAATTGTAGGTCAAATCAGACACCGGAATCTGCTTCCATTACTCGCGCATATGCCAAG : 1235
SlSOBIR1-li : CAAAATTGTAGGTCAAATCAGACACCGGAATTTGCTTCCCTACTGGCGCATATGCCAAG : 1274
AtSOBIR1    : CAACACAGTCGCGCCATATCCGGCACCGGAATCTTCTCCCATTGTTAGCACACGTGTCAAG : 1283

                   *        1340          *        1360         *        1380
SlSOBIR1    : GCCAGACTGTCATTACTTGGTCTACGAATACATGAAAAATGGGAGTTTACAGGATATCCT : 1295
SlSOBIR1-li : ACCAGACTGCCACTACTTGGTCTATGAGTACATGAAAAATGGGAGCTTACAGGATACCCT : 1334
AtSOBIR1    : ACCCGACTGCCACTACCTCGTTTACGAGTACATGGAAAGCGGGAGTTTGCAAGATATATT : 1343

                   *        1400          *        1420         *        1440
SlSOBIR1    : GCAGCAAGTCACAGAAGGCACAAGGGAATTAGATTGGTTGGGACGACACAGAATTGCAGC : 1355
SlSOBIR1-li : CCAGCAAGTCACAGAGAGGGGAAACGGGAACTAGATTGGTCGGCACGTCACCGAATTGCAAT : 1394
AtSOBIR1    : CACTGACGTACAAGCTGCAAATCAAGAACTAATGTGGCCAGCGACGCACCAGATTGCTTT : 1403

                   *        1460          *        1480         *        1500
SlSOBIR1    : GGGGGTAGCTGCTGGTCTCGAGTATCTCCATATAAACCATACTCAACGCATAATTCACAG : 1415
SlSOBIR1-li : GGGAATAGCTGCTGGACTCGAGTATCTCCATATAAATCATACTCAGCGTATAATTCACAG : 1454
AtSOBIR1    : AGGTATAGCTGCAGGGCTTGAGTACCTTCACATGGATCATAACCCACGAATCATTCACAG : 1463

                   *        1520          *        1540         *        1560
SlSOBIR1    : AGATTTAAAGCCAGCAAATATCCTACTTGATGATGACATGGAAGCTCGAGTTGCTGATTT : 1475
SlSOBIR1-li : AGATCTAAAGCCAGGCAATGTCCTCCTGGATGATGACATGGAAGCTCGAATTGCGGATTT : 1514
AtSOBIR1    : AGACTTAAAGCCAGCCAATGTTCTTCTTGATGATGACATGGAAGCCAGAATTTCAGATTT : 1523

                   *        1580          *        1600         *        1620
SlSOBIR1    : TGGCCTTGCAAAGGCAGTTCCAGATGCTCATACACACATATCACCACTTCAAATGTTGCAGG : 1535
SlSOBIR1-li : TGGCCTTGCAAAGGCTGTCCCAGATGCTCATACACATATTACAACTTCAAATGTGGCAGG : 1574
AtSOBIR1    : CGGACTCGCAAAGGCAATGCCAGATGCAGTCACACATATTACAACCTCGCATGTTGCAGG : 1583
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                     *        1640       *        1660       *        1680
SlSOBIR1      : AACTGTTGGATTTATTGCACCAGAATATTATCAGACACTGAAGTTTACAGATAAGTGTGA : 1595
SlSOBIR1-li   : AACTATAGGATACATCGCTCCAGAATATCTCAGACACTGAAGTTCACTGATAAGTGTGA : 1634
AtSOBIR1      : TACTGTGGATACATAGCACCAGAGTTTTATCAAACCCACAAGTTCACAGATAAATGTGA : 1643

                     *        1700       *        1720       *        1740
SlSOBIR1      : TATATACAGCTTTGGTGTGGTGCTAGCTGTGTTGGTTATCGGTAAGGGTCCATCGGATGA : 1655
SlSOBIR1-li   : TATATACAGCTTTGGGGTGCTGCTAGGCGTGCTAGTTATCGGAAAGCTTCCATCTGATGA : 1694
AtSOBIR1      : TATCTATAGTTTTGGAGTGATTCTTGGGATTCTGGTGATTGGGAAGCTTCCTTCAGATGA : 1703

                     *        1760       *        1780       *        1800
SlSOBIR1      : TTTTTTCCAACATACTTCTGAGATGAGTTTAGTTAAGTGGCTGAGGAATGTAATGACTTC : 1715
SlSOBIR1-li   : GTTCTTCCAAAACACGTCTGAGATGAGTTTAGTGAAATGGATGAGAAATGTCATGACTTC : 1754
AtSOBIR1      : GTTTTTTCAGCATACTGACGAGATGAGTCTGATAAAGTGGATGAGGAACATAATAACATC : 1763

                     *        1820       *        1840       *        1860
SlSOBIR1      : TGACGATCCTAAAATAGCAATTGATCCTAAGCTTATAGGAAATGGATACGACGAACAAAT : 1775
SlSOBIR1-li   : TGAGGATCCAAAACAGAGCAATTGATCCAAAGCTGATGGGTAATGGAAATGAGGACCAAAT : 1814
AtSOBIR1      : GGAAAATCCTAGCCTTGCGATTGATCCGAAGTTGATGGATCAAGGATTCGATGAACAGAT : 1823

                     *        1880       *        1900       *        1920
SlSOBIR1      : GCTTTTTGGTTCTCAAGATAGCTTGCTTTTTGTACTTCTCGACAATCCAAAGGAGAGGCCTAA : 1835
SlSOBIR1-li   : GCTTTTTGGTTCTCAAGATTGCCTTTTTGTACCATGGACAATCCCAAGGAGAGGCCTAA : 1874
AtSOBIR1      : GCTTCTGGTTCTCAAGATCGCCTGTTACTGTACTTTGGATGATCCAAAACGAAGGGCCGAA : 1883

                     *        1940       *        1960
SlSOBIR1      : CAGTAAGGATGTTAGGTGCATGTTAACTCAGATCAAGCATTAG : 1878
SlSOBIR1-li   : TAGTAAGGATGTTAGGTGCATGTTGATGCAGATCAAGCATTAA : 1917
AtSOBIR1      : CAGTAAAGATGTCAGGACCATGTTCTCCCAGATCAAGCACTAG : 1926
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                    *        20        *        40        *
NbSOBIR1    : ATGGCCTTCACTGCTTCACAAATTCACTTCTTTTTCTTCTCACTTTTCGCCTTTT : 55
NtSOBIR1    : ATGGCCTTCACTGCTTCACAAATCCACTTCTTTTTCATCTCCCTTTTCGCCCTTT : 55
NbSOBIR1-l  : ATGGCCTCCACTACCTCCCATATTCACCTATCTCTTCTGTCTCTCTTCACTCTTA : 54

                   60        *        80        *        100       *
NbSOBIR1    : TACTTATTGCTGTTCAAGCAAGACTGAATCTTTATCCACCAGATCATGCTGCACT : 110
NtSOBIR1    : TACTTATT---GTTCAAGCAAAACTAAATCTCTATCCACCAGATCATGCTGCACT : 107
NbSOBIR1-l  : TCCTTCAT---GTTCAAGCAAGAATCAACCTTTACTCGCCAGATTATAGTGCTCT : 106

                   120       *        140       *        160
NbSOBIR1    : TTTGCTTGTCCAAAAAGACTTGGGCATCCAAGGTCAACGCATTGC--------AC : 157
NtSOBIR1    : TTTGCTTGTCCAAAAAGACTTGGGCATCCAAGGTCAACGCATTGC--------AC : 154
NbSOBIR1-l  : TTTGGTTGTCCGAAAAGGCTTAGACGTCCCTAGACAACTTAGTGCTATAGAGAAC : 161

                 *        180       *        200       *        220
NbSOBIR1    : TT-TGCAACTCTGCAACAATATCCTGTGAAAGGCGAAAGGCAAACAGAACACAAT : 211
NtSOBIR1    : TT-TGCAACTCTGTAGCAGTATCCTGCGAAAGGCGAAAGGCAAACAGAACACAGT : 208
NbSOBIR1-l  : CCATGCAATTCTGTTGGAATATCATGTGAACGACGACTCACAAACAATTCATATG : 216
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                          *         240         *         260         *
NbSOBIR1   : TGTTGAGAGTCACCCGTATTGACTTCAGATCCAGTGGATTGAGTGGAACTTTATC : 266
NtSOBIR1   : TGTTGAGAGTCACCCGTATTGACTTCAGATCCAATGGATTGAGTGGAACTTTGTC : 263
NbSOBIR1-1 : TGCTTAGAGTCACAGGGGTTGTTTTCAAGTCCTATGAACTGAGGGCAACTCTATC : 271


              280         *         300         *         320         *
NbSOBIR1   : TCCTGCCATTGGAAAAACTTTCTGTGCTCAAAGAACTCTCTCTTCCAAACAACCAA : 321
NtSOBIR1   : TCCTGCCATTGGCAAACTTTCTGAGCTCAAAGAGCTCTCTCTTCCAAACAACCGA : 318
NbSOBIR1-1 : TCCTGCCATTTGCAAGCTTTCTGAGTTCAAAAAATTGTCCCTACAAAACAACAAG : 326

                340         *         360         *         380
NbSOBIR1   : CTCTTTGACCAAATCCCAGTTCAGATTCTTGAT-TGCCGTAAACTGGAGATTCTT : 375
NtSOBIR1   : CTCTTTGACCAAATCCCAGTTCAGATTCTCGAC-TGCCGTAAACTGGAGATCCTT : 372
NbSOBIR1-1 : CTCTTTGACAGAATCCCAACTCAAATTGTTGAGGTGCCGAAAATTGGAAATCTTG : 381


                *         400         *         420         *         440
NbSOBIR1   : GACCTTGGAAACAATCTGTTTTCTGGGAAAGTTCCATCTGAATTATCATCTCTAC : 430
NtSOBIR1   : GACCTTGGAAACAATCTGTTTTCTGGGAAAGTTACATCTGAATTATCATCTCTAC : 427
NbSOBIR1-1 : AACCTTCAAAACAACCAGTTTTCTGGCAAAGTCCCATCTGAATTATCATCTCTTG : 436


                  *         460         *         480         *
NbSOBIR1   : TCCGCCTTCGAATTCTTGATCTTTCTTCAAATGAGTTTTCTGGGAATCTTAACTT : 485
NtSOBIR1   : TCCGCCTTCGAATTCTTGATCTTTCTTCAAATGAGTTGTCTGGGAATCTTAACTT : 482
NbSOBIR1-1 : TCCGCCTTCGAATCCTTGACCTCTCTTCTAATGAATTATCAGGGAACCTCAATTT : 491


            500         *         520         *         540         *
NbSOBIR1   : CTTGAAGTATTTTCCCAACTTAGAAAAACTCTCTTTAGCTGATAACATGTTCACT : 540
NtSOBIR1   : CTTGAAGTATTTTCCCAACTTAGAAAAACTCTCTTTAGCTGATAACATGTTCACT : 537
NbSOBIR1-1 : CTTGAAATACTTTCCCAACCTTGAAAATTTGTCCCTTGCTGATAACATGTTCATT : 546


              560         *         580         *         600
NbSOBIR1   : GGAAAAATACCCCCTTCTTTGAAATCTTTTAGGAATCTCCGTATCCTTAATATTT : 595
NtSOBIR1   : GGAAAAATACCCCCTTCTTTGAAATCTTTCAGAAATCTCCGTTTCCTTAATATTT : 592
NbSOBIR1-1 : GACAAAATACCTCAATCCTTGAAATCTTTCAGAAATCTCCGGTTCCTCAACATTT : 601


                *         620         *         640         *         660
NbSOBIR1   : CAGGAAACAGTTTTCTTGAAGGTCATGTGCCTGTTATGAGTCAAGTTGAGCACTT : 650
NtSOBIR1   : CAGGAAACAGTTTTCTTGAAGGTCCTGTGCCTGTTATGAGTCAAGTTGAGCACTT : 647
NbSOBIR1-1 : CAGGGAATAGTTTTCTTTAAGGTCCGGTGCCTTTCATGAGTCAAGTTAAGCACTT : 656


                *         680         *         700         *
NbSOBIR1   : GTCAGCAGAATTGGATCAGCAC---TTCGTTCCAAAACGTTACATTCTTGCTGAA : 702
NtSOBIR1   : GTCAGCAGATTTGGATCGGAACACTTCGTTCCAAAACGTTACATTCTTGCTGAA : 702
NbSOBIR1-1 : ATCAGCAGACTTGAATCGAAAGATTATGTTCTTAAACGTTACATTCTTGCTGAA : 711
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                720          *         740          *         760          *
NbSOBIR1    : AATTCAACAAGGTCAAATCAGATATCAGCACTGGCACCTAATTCCAATTCAGGAA : 757
NtSOBIR1    : AATTCGACAAGCTCAAATCAGATATCAGCACTGGCACCTAATTCCAATTCAGGAA : 757
NbSOBIR1-l  : AACTCAACAAGGTCAAGTCACAAATCTGCAATGGCACCACAGTCCTATTCAGGAA : 766

                780          *         800          *         820
NbSOBIR1    : ATGCCCCAGCTCCAGCACCGAGTCATAATGTCACTCCAATCCATAAACATAGTAA : 812
NtSOBIR1    : ATGCCCCAGCTCCAGCACCGAGTCATAATGTCGCTCCAATCCATAAAAGTAATAA : 812
NbSOBIR1-l  : ATGCCCCGGCTCCAGGACCCAGTGCAATTGTAGTACCAGTACACAAACATAACAA : 821

                  *         840          *         860          *         880
NbSOBIR1    : CAGGAAGAAAAGGAAAGTCAGAGCGTGGTTACTTGGTTTCTTTGCTGGTTCTTTC : 867
NtSOBIR1    : CAAGAAGAAAAGGAAAGTCAGAGCGTGGTTTCTTGGTTTCTTTGCTGGTTCTTTC : 867
NbSOBIR1-l  : CACA---AAAAAGAAGGTAAGCATATGGATTCTTGGATTCCTGGCTGGATCTTTT : 873

                    *         900          *         920          *
NbSOBIR1    : GCAGGAGCTATATCTGCAGTACTCTTATCAGTTCTTTTTAAGCTGGTCATGTTTT : 922
NtSOBIR1    : GCAGGAGCTATATCTGCAGTACTCTTATCAGTTCTCTTTAAGCTGGTAATGTTTT : 922
NbSOBIR1-l  : GCAGGGATCTTATCTGC-GTGGTCTTATCCTTGCTCTTCAAGGTGGTTATGATTA : 927

                940          *         960          *         980          *
NbSOBIR1    : TTGTCCGAAAGGGAAAGACTGATGGAACTTTAACAATATACAGTCCACTGATTAA : 977
NtSOBIR1    : TCGTCCGAAAGGGAAAGACTGATGGAACTTTAACAATATACAGTCCACTGATTAA : 977
NbSOBIR1-l  : TCG---GAGGGAGCAAGGATGATTCAGGTATAACAATTTTCAGTCCATTGATTAA : 979

                1000         *        1020          *       1040
NbSOBIR1    : GAAAGCCGAGGATTTGGCCTTCTTAGAGACAGAAGATGGAGTAGCATCACTTGAA : 1032
NtSOBIR1    : GAAAGCCGAGGATTTGGCCTTCTTAGAGAAAGAAGATGGAGTAGCATCACTTGAA : 1032
NbSOBIR1-l  : GAAAGC------------------------------------------------- : 985

                  *        1060          *       1080          *       1100
NbSOBIR1    : ATGATTGGAAAAGGTGGATGCGGAGAAGTTTATAGAGCTGAGTTACCGGGAAGTA : 1087
NtSOBIR1    : ATGATTGGAAAAGGTGGATGTGGAGAAGTTTATAGAGCTGAGTTACCAGGAAGTA : 1087
NbSOBIR1-l  : ------------------------------------AGAGTTACCAGGAAGTA : 1002

                    *        1120          *       1140          *
NbSOBIR1    : ATGGGAAGATTATAGCTATAAAGAAGATTATACAACCCCCAATGGATGCTGCAGA : 1142
NtSOBIR1    : ATGGGAAGATTATAGCTATAAAGAAGATTTTACAACCCCCAATGGATGCTGCAGA : 1142
NbSOBIR1-l  : ATGGAAAGATTATTGCTATAAAGAAGATCGTAGAACCCCCAAAGGATGCTGCAGA : 1057

                1160          *       1180          *       1200          *
NbSOBIR1    : ACTCACCGAGGAAGATACCAAGGCTTTGAACAAAAAAATGCGACAAGTAAAATCA : 1197
NtSOBIR1    : ACTCGCCGAGGAAGATACCAAGGCTTTGAACAAGAAAATGCGACAGGTAAAATCA : 1197
NbSOBIR1-l  : ACTCACTGAGGAAGATAGCAAGGCTTTGAATATTAAAATGTGCCAGACCAAATCA : 1112
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                 1220         *      1240         *      1260
NbSOBIR1    : GAAATTCAAATTCTTGGTCAAATCAGACACACAGGAATCTGCTTCCCCTACTGGCAC : 1252
NtSOBIR1    : GAAATTCAAATTCTTGGTCAAATCAGACACACAGGAATTTGCTTCCCCTACTGGCAC : 1252
NbSOBIR1-1  : GAAATTAAAATTGTAGGTCAAATAAGACACACAGGAATTTGCTTCCTCTAATAGCAC : 1167

                        *      1280         *      1300         *      1320
NbSOBIR1    : ATATGCCTAGGCCAGACTGCCATTACTTGGTATATGAATATATGAAAAATGGGAG : 1307
NtSOBIR1    : ATATGCCTAGGCCGGACTGCCATTACTTGGTATATGAATATATGAAAAATGGGAG : 1307
NbSOBIR1-1  : ATATGCCAAGACCTGACTGCCATTACTTGGTCCGTGAGTACATGAAAAATGGGAG : 1222

                       *      1340         *      1360         *
NbSOBIR1    : CTTACAAGATATCCTCCAGCAAGTCACAGAAGGGACAAGGGAATTAGATTGGTTG : 1362
NtSOBIR1    : CTTACAAGATATCCTCCAGCAAGTCACAGAAGGGACAAGGGAATTAGATTGGTTG : 1362
NbSOBIR1-1  : CTTACAGGATGCCGTCCAGCAAGTCACGGAGGGGACACGGGAAATAGATTGGTCG : 1277

                1380         *      1400         *      1420         *
NbSOBIR1    : GGACGACACCGAATTGCAGTAGGGATAGCTTCTGGACTTGAGTATCTCCATATAA : 1417
NtSOBIR1    : GGACGACACCGAATTGCAGTAGGGATAGCTACTGGACTTGAGTATCTCCATATAA : 1417
NbSOBIR1-1  : GCACGTCATCGAATTGCAGTCGGGATCGCTGCTGGGCTTGAGTATCTTCATGTAA : 1332

                  1440         *      1460         *      1480
NbSOBIR1    : ACCACAGTCAATGCATAATTCACAGAGATCTAAAGCCAGCAAATGTCCTTCTTGA : 1472
NtSOBIR1    : ACCACAGTCAATGCATAATTCACAGAGATCTAAAGCCAGCAAATGTCCTTCTTGA : 1472
NbSOBIR1-1  : ATCATAGTCAGCGTACAATTCACAGGGATCTAAAGCCAGCAAATGTCCTTCTTG- : 1386

                     *      1500         *      1520         *      1540
NbSOBIR1    : CGATGATATGGAAGCTCGAATTGCTGATTTTGGACTTGCAAAAGCACTCCCAGAT : 1527
NtSOBIR1    : CGATGATATGGAAGCTCGAATTGCTGATTTTGGACTTGCAAAGGCAGTTCCAGAT : 1527
NbSOBIR1-1  : ------------------------------------CGAAAGCAATCCCAGAA : 1403

                        *      1560         *      1580         *
NbSOBIR1    : GCCCATACACATGTTACGACTTCAAATGTTGCAGGAACTGTGGGATATATTGCAC : 1582
NtSOBIR1    : GCCCATACACATGTTACGACTTCAAATGTTGCAGGAACTGTGGGATATATTGCAC : 1582
NbSOBIR1-1  : TCCCTTACGCTTTTCA--ACTTCACACGTGGTAGGAACTTTACGGATACATTGCAC : 1456

                1600         *      1620         *      1640         *
NbSOBIR1    : CAGAATACCATCAGACACTGAAGTTTACGGGTAAGTGTGATATATACAGCTTTGG : 1637
NtSOBIR1    : CAGAATACCATCAGACACTGAAGTTTACGGATAAGTGTGATATATACAGCTTTGG : 1637
NbSOBIR1-1  : CAGAATATTACCAGACCGTTAAGTTCACAGATAAGTGTGATATATACAGCTTCGG : 1511

                     1660         *      1680         *      1700
NbSOBIR1    : TGTGGTGTTGGCAGTGCTGGTTATAGGAAAACTTCCATCAGATGAATTTTTCCAG : 1692
NtSOBIR1    : TGTGGTGTTAGCAGTGCTGGTTATAGGAAAGCTTCCATCAGATGAATTTTTCCAG : 1692
NbSOBIR1-1  : GATGCTGCTAGGCGTGCTAGTTATGGGAAAGTTTCCCTCTGATGAGCTCTTCCAG : 1566
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                    *         1720         *         1740         *         1760
NbSOBIR1    : CACACGCCTGAGATGAGTTTAGTGAAGTGGCTGAGAAATGTAATGACTTCTGAGG : 1747
NtSOBIR1    : CACACGCCTGAGATGAGTTTAGTGAAGTGGCTGAGAAATGTAATGACTTCCGAGG : 1747
NbSOBIR1-l  : CCGGTTTCTGGGATGGGTTTAGTGAAATGGATGAGAAATGTCACGACTTCTGAGA : 1621

                          *         1780         *         1800         *
NbSOBIR1    : ATCCGAAAAGGGCAATTGATTCAAAGCTGATAGGAAATGGATTTGAGGAGCAAA- : 1801
NtSOBIR1    : ATCCGAAAAGGGCAATTGATCCAAAGCTGATAGGAAGTGGATTTGAGGAGCAAA- : 1801
NbSOBIR1-l  : ATCCAAAAAGAGCAATTGATCCAAAGCTGATGGGTAATGGGTATGAGGAGCAAAA : 1676

                    1820         *         1840         *         1860         *
NbSOBIR1    : TGCTTTTGGTTCTCAAGATAGCTTGCTTTTGTACTCTGGAAAATCCAAAGGAGAG : 1856
NtSOBIR1    : TGCTTTTGGTTCTCAAGATCGCTTGCTTTTGTACACTGGAAAATCCAAAGGAGAG : 1856
NbSOBIR1-l  : TGCTTTTGGTTCTCAAGATTGCCTGCTTTTGCTCACTGGATAATGCAAAGGAGAG : 1731

                       1880         *         1900         *         1920
NbSOBIR1    : GCCTAACAGTAAGGATGTTAGGTGTATGTTAACTCAGATCAAGCATTAG------ : 1905
NtSOBIR1    : GCCTAACAGTAAGGATGTTAGGTGTATGTTAACTCAGATCAAGCATTA------- : 1904
NbSOBIR1-l  : GCCTAACAGTAAGGATGTTAGATGCATGTTAACTCAGATCAAGCCTCAAGTAGGT : 1786

                    *
NbSOBIR1    : --------- :    -
NtSOBIR1    : --------- :    -
NbSOBIR1-l  : AGAGGATAA : 1795
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Figure S2. Alignments of SOBIR1 sequences from tomato, *Arabidopsis*, *N. benthamiana* and tobacco. (A) Amino acid sequence alignment of *Sl*SOBIR1 (Solyc06g071810.1.1), *Sl*SOBIR1-like (*Sl*SOBIR1-l; (Solyc03g111800.2.1), *At*SOBIR1 and *Sl*SERK3a/BAK1 (*Sl*SERK3a/B). Amino acid residues highlighted in black and dark grey represent identical residues in all four or three of the aligned protein sequences, respectively. Residues in light grey or white represent residues present in two sequences or are unique residues, respectively. The conserved RD motif as well as the conserved residues K377 and E407 of *At*SOBIR1, present in the kinase domain of all four proteins are underlined. (B) Nucleotide sequence alignment of tomato *SlSOBIR1 SlSOBIR1-like* (*SlSOBIR1-li*) and *Arabidopsis AtSOBIR1* (AT2G31880.1) coding regions. Residues highlighted in black and grey represent identical nucleotides in all three, or two of the aligned coding regions, respectively. Nucleotide sequences used for the TRV-based VIGS constructs are indicated by different colours: TRV: *SlSOBIR1*, green; TRV: *SlSOBIR1-like*, blue; TRV: *SlSOBIR1/SlSOBIR1-like*, red. (C) Nucleotide sequence alignment of *N. benthamiana NbSOBIR1*, tobacco *NtSOBIR1* (SGN-U441568) and *NbSOBIR1-like* (*NbSOBIR1-l*) coding regions. The available genomic sequence was used to obtain *Nb*SOBIR1 and *Nb*SOBIR1-like sequences and EST data provided the *Nt*SOBIR1 coding sequence. Intron sequences were manually removed from the *NbSOBIR1-like* sequence. Residues highlighted in black and grey represent identical nucleotides in all three, or two of the aligned coding regions, respectively. Nucleotide sequences used for the TRV-based VIGS constructs are indicated by different colours: TRV: *NbSOBIR1*, green; TRV: *NbSOBIR1-like*, blue. TRV: *NbSOBIR1/NbSOBIR1-like* contains both of these fragments (*NbSOBIR1* and *NbSOBIR1-like*).

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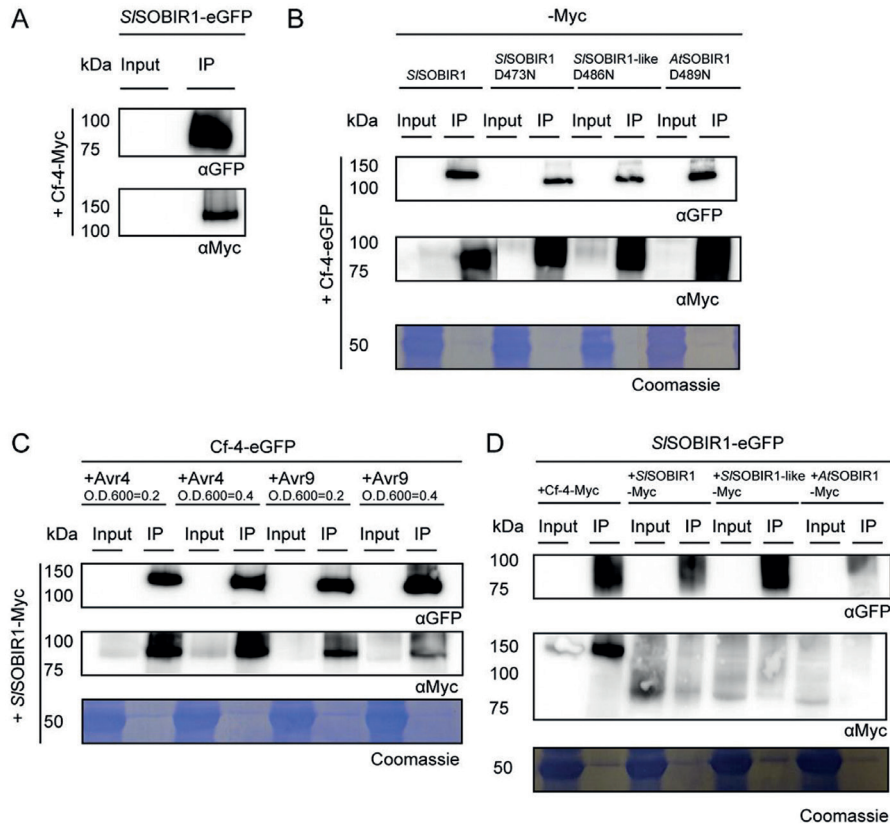
Figure S3. Cf-4 interacts with *Sl*SOBIR1 in a reverse immunoprecipitation assay, with SOBIR1 mutants in the RD motif of the kinase domain, with *Sl*SOBIR1 in the presence of Avr4, and SOBIR1 does not form homo- or heterodimers. (A) *Sl*SOBIR1-eGFP was coexpressed with Cf-4-Myc in *N. benthamiana* and total protein extract was subjected to immunopurification using GFP-affinity beads. The total protein extract (Input) and Immunopurified Proteins (IP) were subjected to SDS-PAGE and blotted. Blots were incubated with αGFP antibody to detect immunopurified *Sl*SOBIR1-eGFP, whereas coimmunopurified Cf-4-Myc was detected using αMyc. (B) Cf-4-eGFP was coexpressed with *Sl*SOBIR1-Myc, *Sl*SOBIR1[D473N]-Myc, *Sl*SOBIR1-like[D486N]-Myc and *At*SOBIR1[D489N]-Myc in *N. benthamiana*. Immunopurifications and detection of proteins were performed as described under (*A*). (C) Cf-4-eGFP was coexpressed with *Sl*SOBIR1-Myc in the presence of Avr4 or Avr9 (the O.D.$_{600}$ of the *A. tumefaciens* cultures was either 0.2 or 0.4) in *N. benthamiana*. Leaves were harvested one day after agroinfiltration, which was just before the onset of an HR in the Avr4 agroinfiltrated leaves. Immunopurifications and detection of proteins were performed as described under (A). (D) *Sl*SOBIR1-eGFP was coexpressed with Cf-4-Myc, *Sl*SOBIR1-Myc, *Sl*SOBIR1-like-Myc and *At*SOBIR1-Myc. After two days, *Sl*SOBIR1-eGFP was immunopurified and the samples were analyzed for co-purification of the Myc-tagged versions. Immunopurifications and detection of proteins were performed as described under (A). All assays were performed twice and a representative picture for each experiment is shown.
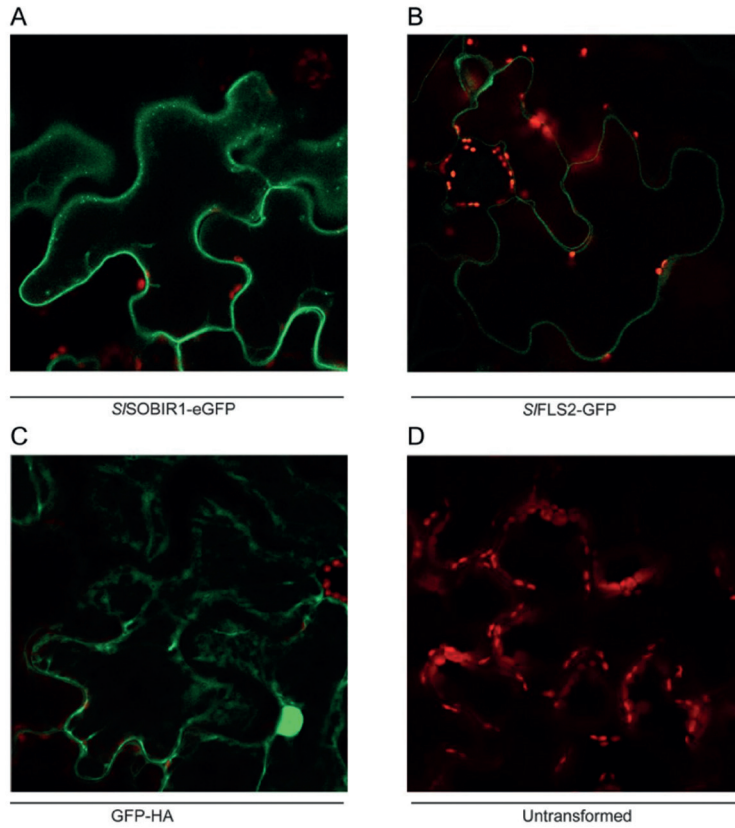
Figure S4. *Sl*SOBIR1-eGFP localizes to the plasma membrane and cytoplasmic vesicles. Proteins were transiently expressed in *N. benthamiana* epidermal leaf cells and their subcellular localization is shown. Each panel shows a combination of the eGFP signal (green) and the chloroplast signal (red). (A) *Sl*SOBIR1-eGFP localizes to the plasma membrane. When focusing on top of the plasma membrane, *Sl*SOBIR1-eGFP is also detected in distinct spots that resemble cytoplasmic vesicles. (B) *Sl*FLS2-GFP typically localizes to the plasma membrane. (C) GFP-HA localizes to the cytosol and the nucleus. (D) An untransformed plant was used as a negative control. Representative pictures for three independent experiments are shown.

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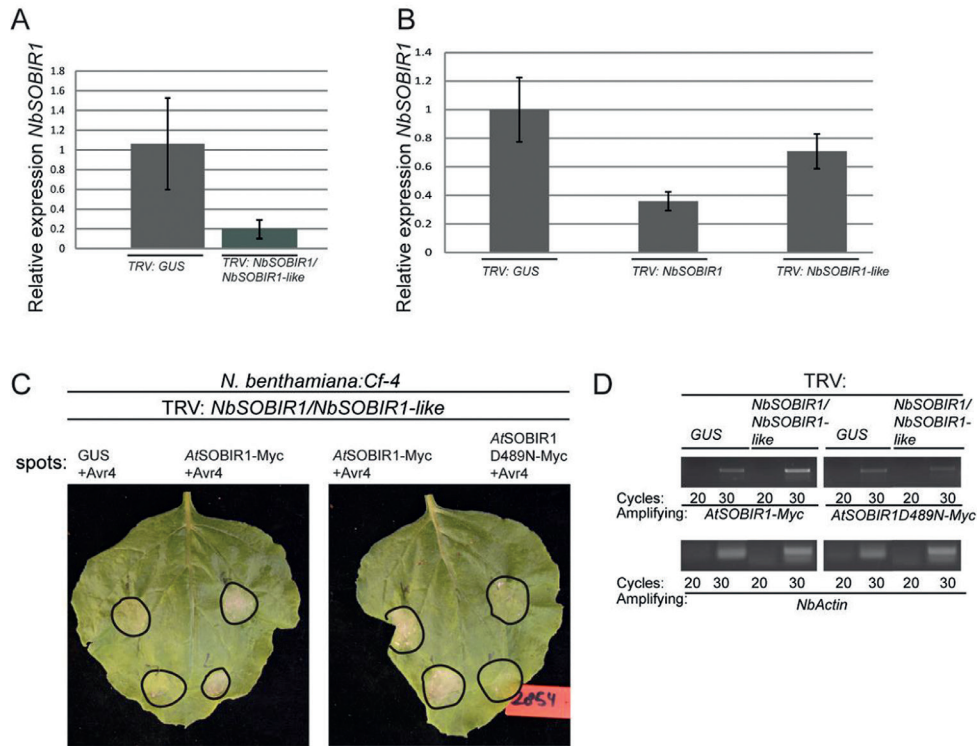
Figure S5. SOBIR1 kinase activity is essential for its role in Cf-4-mediated HR. (A) Expression of *NbSOBIR1* is efficiently knocked-down by inoculation of *N. benthamiana* with TRV: *NbSOBIR1/NbSOBIR1-like*. (B) Expression of *NbSOBIR1* is efficiently knocked-down by inoculation of *N. benthamiana* with TRV: *NbSOBIR1*. Inoculation with TRV: *NbSOBIR1-like* results in slight cross-silencing of *NbSOBIR1* expression. For (A) and (B), plants were inoculated with TRV: *GUS* or the indicated constructs targeting *Nb*SOBIR1s and relative expression of *NbSOBIR1* was determined by qRT-PCR for each sample. Samples were normalized to endogenous *NbActin*. Standard deviations show the variation between three technical repeats. In total two biological experiments were performed with similar results and a representative result is shown. (C) Kinase activity of SOBIR1 is required for Cf-4-mediated HR. Transgenic *N. benthamiana*:*Cf-4* plants were subjected to VIGS by inoculation with TRV: *NbSOBIR1/NbSOBIR1-like*. Three weeks later, mature leaves were agroinfiltrated to transiently express the combinations *GUS + Avr4*, *AtSOBIR1-Myc + Avr4* and *AtSOBIR1^{D489N}-Myc + Avr4*, as indicated. *GUS*, *AtSOBIR1-Myc* and *AtSOBIR1^{D489N}-Myc* were infiltrated at a final O.D.$_{600}$ = 0.7 and Avr4 at O.D.$_{600}$ = 0.03. (D) RT-PCR analysis confirms that *AtSOBIR1-Myc* and *AtSOBIR1^{D489N}-Myc* are not targeted by TRV: *NbSOBIR1/NbSOBIR1-like*-inoculation of *N. benthamiana*. Three weeks after inoculation with the indicated TRV constructs, leaves were agroinfiltrated with *AtSOBIR1-Myc* or *AtSOBIR1^{D489N}-Myc*. RNA was isolated, transcribed into cDNA and used as a template for RT-PCRs using primers amplifying *AtSOBIR1* or endogenous *NbActin*. The amount of PCR cycles is indicated.
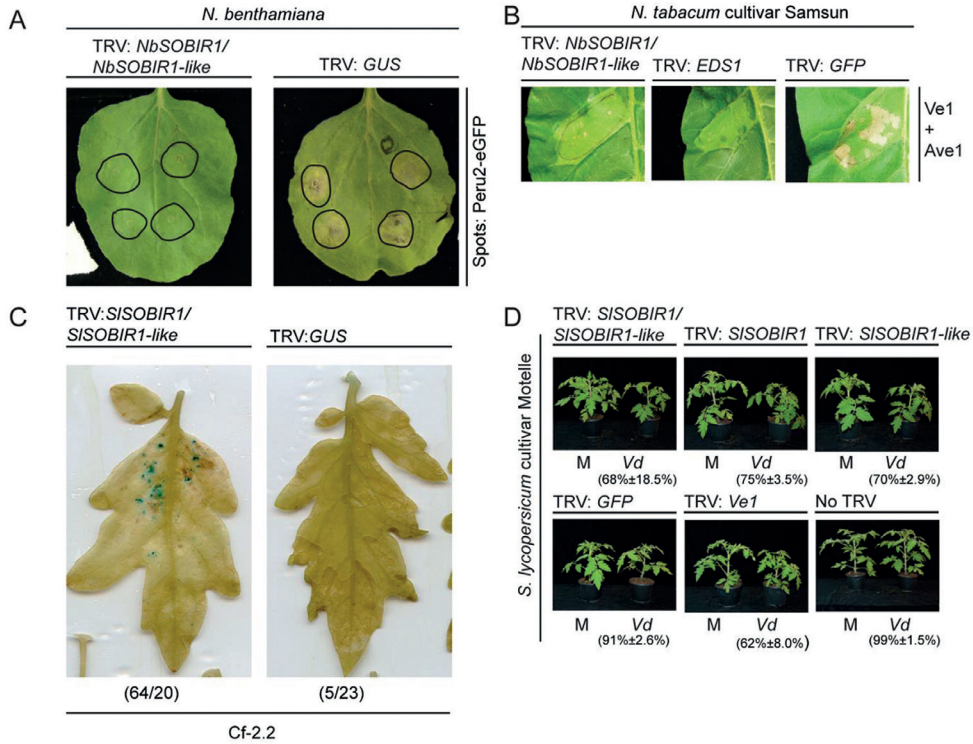
**Figure S6.** SOBIR1 is required for auto-activity of the Cf homolog Peru2, Ve1-mediated HR in tobacco and Cf-2.2- and Ve1- mediated resistance in tomato. (A) Peru2 auto-activity is compromised upon silencing of *NbSOBIR1. N. benthamiana* plants were subjected to VIGS by inoculation with TRV: *NbSOBIR1/NbSOBIR1-like*. Three weeks after TRV inoculation, Peru2-eGFP was transiently expressed by agroinfiltration at four sites in the leaves and leaves were photographed three days later. (B) The Ve1-mediated HR is compromised upon targeting *Nt*SOBIR1 homologs in tobacco. *N. tabacum* cultivar Samsun was subjected to VIGS by inoculation with the TRV constructs indicated above each panel. TRV: *EDS1* and TRV: *GFP* served as controls. Three weeks after TRV inoculation, Ve1 and Ave1 were coexpressed and leaves were photographed five days later. The experiments were performed three times with three plants for each TRV construct and representative pictures for the experiments are shown. (C) Targeting *SlSOBIR1* and *SlSOBIR1-like* suppresses Cf-2.2-mediated resistance to *C. fulvum* expressing Avr2. *Cf-2.2*-expressing tomato was inoculated with TRV:*SlSOBIR1*/*SlSOBIR1-like* or TRV:*GUS* and three weeks later plants were inoculated with an Avr2-secreting, *GUS*-transgenic strain of *C. fulvum*. Two weeks later, leaflets were stained for GUS activity to detect *C. fulvum* colonization. The amount of successful colonization attempts (blue spots) versus the total amount of leaflets analyzed for that particular treatment, is indicated between parentheses. (D) Tomato cultivar Motelle (carrying *Ve1*) was subjected to VIGS by inoculation with the constructs indicated above each panel. TRV: *GFP*, TRV: *Ve1*, and non TRV-inoculated Motelle plants were included as controls. Each panel shows a mock (M)-treated plant on the left and a *V. dahliae* (*Vd*)-inoculated plant on the right. Stunting of the *V. dahliae*-inoculated plants, as compared to the mock-treated plants indicates compromised resistance. Percentages between parentheses indicate the average canopy area (and standard deviations) of *V. dahliae*-inoculated plants in three independent experiments compared to the control mock treatment, for which the canopy area was set to 100%. Representative photographs for three independent experiments are shown.

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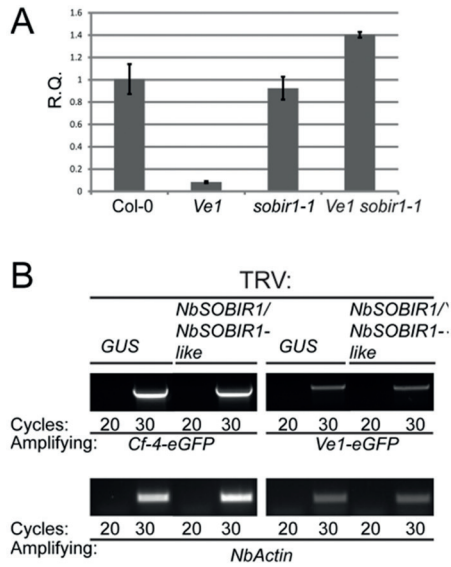
Figure S7.
Quantification of *V. dahliae* biomass in *Arabidopsis sobir1-1* and *sobir1-1 Ve1*-complemented lines and expression of Cf-4- and Ve1- in *N. benthamiana* silenced for the *NbSOBIR1* homologs (A) Relative Quantity (R.Q.) of fungal biomass present in the plants shown in Fig 4, as determined by qRT-PCR. Standard deviations show the difference between technical repeats. The inoculation experiments and qRT-PCR quantifications were performed three times, with similar results. (B) *Cf-4-eGFP* and *Ve1-eGFP* are expressed in *N. benthamiana* silenced for the *NbSOBIR1* homologs. *N. benthamiana* was subjected to VIGS by inoculation with the indicated TRV constructs and subsequently transiently transformed to express *Cf-4-eGFP* or *Ve1-eGFP*. RNA was isolated, transcribed into cDNA and used as a template for RT-PCRs using primers amplifying *Cf-4*, *Ve1* and endogenous *NbActin*, respectively. The amount of PCR cycles is indicated.

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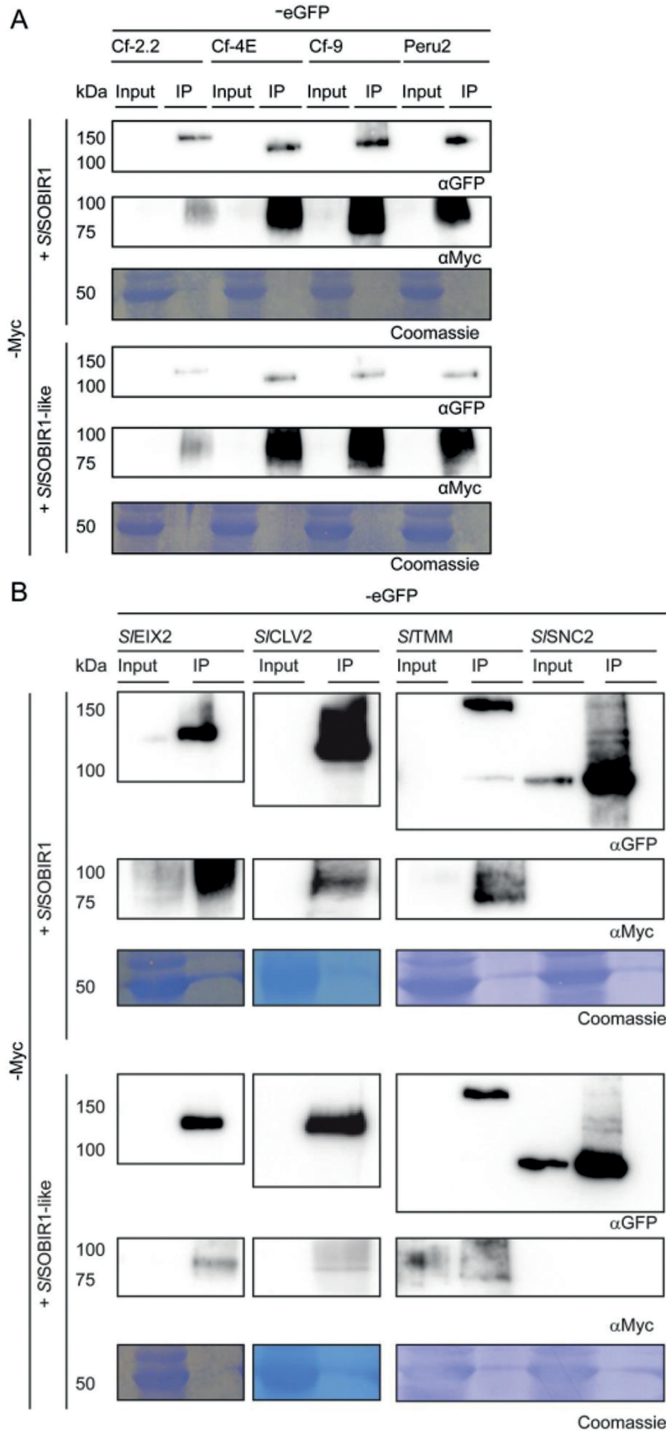
Figure S8.
*Sl*SOBIR1 and *Sl*SOBIR1-like interact with all Cf proteins tested and with the RLPs *Sl*EIX2, *Sl*CLV2 and *Sl*TMM, but not with *Sl*SNC2. (A) Cf-2.2, Cf-4E, Cf-9 and the auto-active Cf homolog Peru2, all C-terminally fused to eGFP, were coexpressed with *Sl*SOBIR1-Myc and *Sl*SOBIR1-like-Myc in *N. benthamiana* and the proteins fused to eGFP were immunopurified using GFP affinity beads. Total proteins (Input) and Immunopurified Proteins (IP) were subjected to SDS-PAGE and blotted. Blots were incubated with αGFP antibody to detect the immunopurified proteins fused to eGFP and αMyc antibody was used for detection of coimmunopurified *Sl*SOBIR1-Myc and *Sl*SOBIR1-like-Myc. Coomassie-stained blots showing the 50-kDa Rubisco band present in the input confirm equal loading. The assay was performed twice and a representative picture is shown. (B) The indicated RLPs, fused to eGFP, were coexpressed with *Sl*SOBIR1-Myc or *Sl*SOBIR1-like-Myc in *N. benthamiana* and immunopurified using GFP-affinity beads. Total proteins (Input) and Immunopurified Proteins (IP) were subjected to SDS-PAGE and blotted. Blots were incubated with αGFP antibody to detect the immunopurified RLP-eGFP fusion proteins and coimmunopurified *Sl*SOBIR1-Myc and *Sl*SOBIR1-like-Myc were detected using αMyc antibody. Coomassie-stained blots showing the 50-kDa Rubisco band present in the input confirm equal loading. The assay was performed three times and a representative picture is shown.

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Table S1. Sequences and ProteinLynx scores of peptides specifically matching Solyc06g071810.1.1, referred to as *Sl*SOBIR1, Solyc03g111800.2.1, referred to as *Sl*SOBIR1-like and Cf-4-eGFP, identified by mass-spectrometry using the Synapt MS, of a tryptic digest of Cf-4-eGFP immunopurified from transgenic tomato line TL3.

Protein name	Peptide sequence	ProteinLynx score
*Sl*SOBIR1	DLKPANILLDDDMEAR	40.9
*Sl*SOBIR1	EDGVASLEMIGK	62.3
*Sl*SOBIR1	IIQSPMDAAEITEEDTK	53.5
*Sl*SOBIR1	KAEDLAFLEK	78.9
*Sl*SOBIR1	VADFGLAK	62.2
*Sl*SOBIR1-like	DAAELTEEDSK	65.1
*Sl*SOBIR1-like	DLKPGNVLLDDDMEAR	57.0
*Sl*SOBIR1-like	IADFGLAK	66.7
*Sl*SOBIR1-like	NDPGLTIFSPLIK	42.7
*Sl*SOBIR1-like	NVMTSEDPNR	50.9
Cf-4-eGFP	EIDESTGFPEYISDPYDIYYNYLTTISTK	28.7
Cf-4-eGFP	FEGHIPSIIGDLVGLR	39.8
Cf-4-eGFP	FGEFSDLTHLDLSHSSFR	74.3
Cf-4-eGFP	GPIPNSLLNQK	60.3
Cf-4-eGFP	ILDSNMIINLSK	78.3
Cf-4-eGFP	ILGNLQTMK	64.7
Cf-4-eGFP	ISLNELTFGPHNFELLLK	59.0
Cf-4-eGFP	LDLSYNDFTGSPISPK	52.7
Cf-4-eGFP	LYLYNVNIDDR	55.6
Cf-4-eGFP	NEYLSHLDLSNNR	65.3
Cf-4-eGFP	SSGNTNLFMGLQILDLSSNGFSGNLPER	30.9

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Table S2. Sequences, ProteinLynx and Mascot ion scores of peptides specifically matching *Nb*SOBIR1 or matching both *Nb*SOBIR1 and *Nb*SOBIR1-like, identified by mass-spectrometry of a tryptic digest of immunopurified Cf-4-eGFP, transiently expressed in *N. benthamiana*.

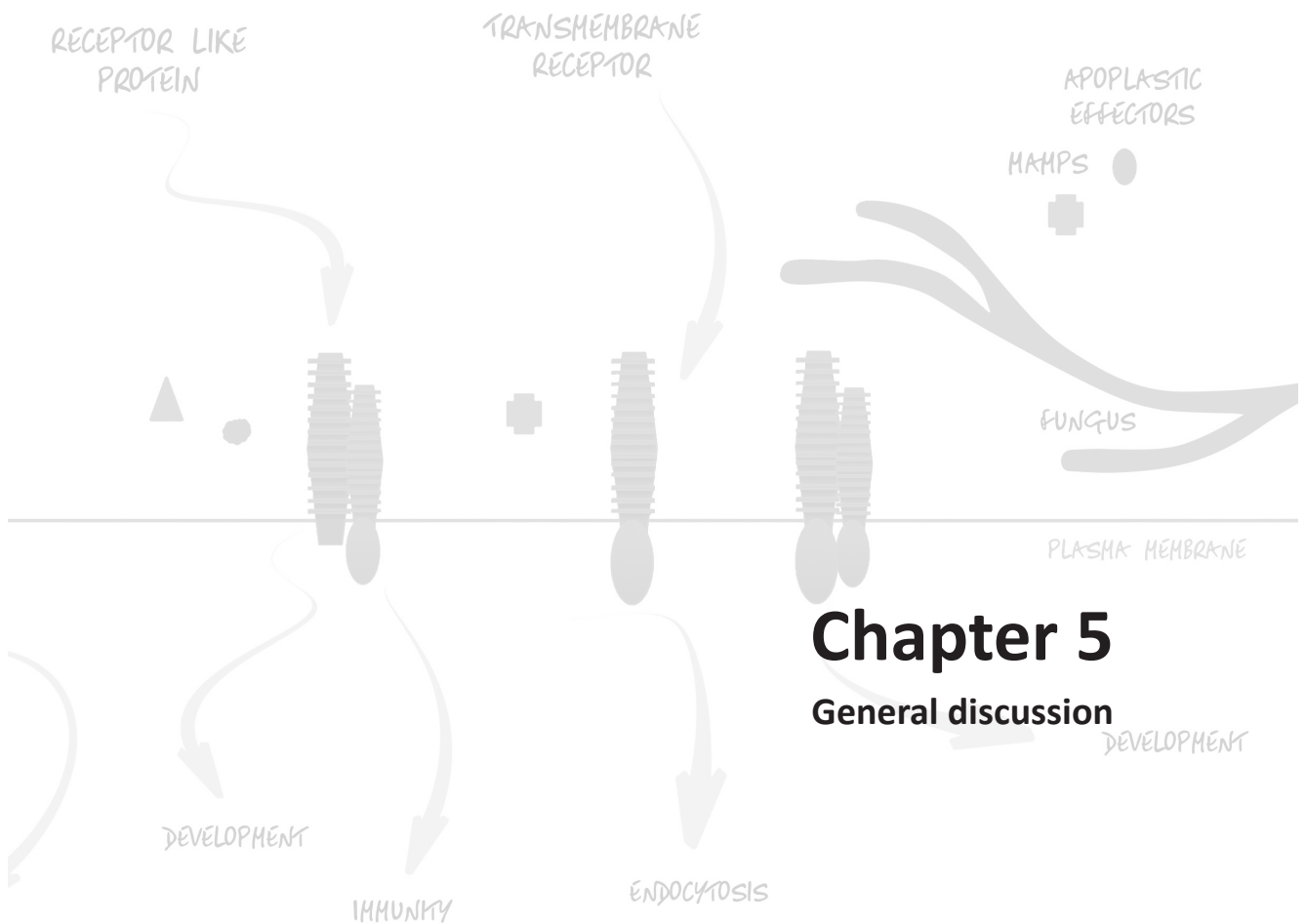| Protein name*	Peptide sequence	Mascot ion score[†]	ProteinLynx score[‡]
*Nb*SOBIR1/SOBIR1-like	ASMPAPAPAPVNR		29.3
*Nb*SOBIR1/SOBIR1-like	DGSLQDILQQVTEGTR		32.3
*Nb*SOBIR1/SOBIR1-like	ELDWLGR		59.0
*Nb*SOBIR1/SOBIR1-like	LNLYPPDHAALLLVQK		33.3
*Nb*SOBIR1/SOBIR1-like	LSLADNMFTGK		69.8
*Nb*SOBIR1/SOBIR1-like	NGSLQDILQQVTEGTQ		48.4
*Nb*SOBIR1/SOBIR1-like	NGSLQDILQQVTEGTR		78.5
*Nb*SOBIR1/SOBIR1-like	NHTQRI		45.5
*Nb*SOBIR1/SOBIR1-like	NLEKLSLADN		31.6
*Nb*SOBIR1/SOBIR1-like	YFPNLEK		56.4
*Nb*SOBIR1	LNLYPPDHAALLLVQK	50.8	
*Nb*SOBIR1	KLEILDLGNNLFSGK	59.4	
*Nb*SOBIR1	SEIQILGQIR	68	
*Nb*SOBIR1	NGSLQDILQQVTEGTR	100.5	
*Nb*SOBIR1	DLKPANVLLDDDMEAR	96.4	
*Nb*SOBIR1	LPSDEFFQHTPEMSLVK	98.3	
*Nb*SOBIR1	NVMTSEDPK	74.7	
*Nb*SOBIR1/SOBIR1-like	LIGSGFEEQMLLVLK	62.4	
*Nb*SOBIR1	IACFCTLENPK	74.7	
*Nb*SOBIR1	DLGIQGQR	38	
*Nb*SOBIR1/SOBIR1-like	SNGLSGTLSPAIGK	82.7	
*Nb*SOBIR1	ILDLSSNELSGNLNFLK	96	
*Nb*SOBIR1	GKTDGSLTIYSPLIK	108.6	
*Nb*SOBIR1	GGCGEVYRAELPGSNGK	49.9	
*Nb*SOBIR1/SOBIR1-like	KILQPPMDAAELAEEDTKALNK	61.2	
*Nb*SOBIR1/SOBIR1-like	AVDPDAHTHVTTSNVAGTVGYIAPEYHQTLK	50.2	

*For identifications with ProteinLynx, peptides were matched on the translated tomato genome sequence. Due to the difference in sequence between *Sl*SOBIR1 and *Nb*SOBIR1 homologs, no distinction between *Nb*SOBIR1 and *Nb*SOBIR1-like can be made. Mascot identifications were based on translated tobacco EST sequences and peptides can match either *Nb*SOBIR1 or *Nb*SOBIR1-like. When specifically matching to the corresponding *N. benthamiana* homolog, the protein name is indicated. When no distinction can be made, *Nb*SOBIR1/SOBIR1-like is indicated.

[†]Mascot ion scores are presented for the peptides that were identified on the Orbitrap XL.

[‡]ProteinLynx peptide ladder scores are presented for the peptides that were identified on the Synapt MS.

Table S3. Sequences and Mascot ion scores of peptides specifically matching *Nb*SOBIR1, *Nb*SOBIR1-like or both, and Ve1-eGFP, identified by mass-spectrometry using the Orbitrap XL, of a tryptic digest of immunopurified Ve1-eGFP transiently expressed in *N. benthamiana*.

Protein name*	Peptide sequence	Mascot ion score
*Nb*SOBIR1-like	ILDLSSNELSGLNFLK	83.6
*Nb*SOBIR1	KLEILDLGNNLFSGK	76.2
*Nb*SOBIR1	GKTDGSLTIYSPLIK	109.2
*Nb*SOBIR1/SOBIR1-like	ILQPPMDAAELAEEDTK	62.8
*Nb*SOBIR1	DLKPANVLLDDDMEAR	82.7
*Nb*SOBIR1	AVPDAHTHVTTSNVAGTVGYIAPEYHQTLK	45.9
*Nb*SOBIR1	NVMTSEDPKR	45.9
*Nb*SOBIR1	LPSDEFFQHTPEMSLVK	46.9
*Nb*SOBIR1	LSLADNMFTGK	83.8
*Nb*SOBIR1	SNGLSGTLSPAIGK	49.8
*Nb*SOBIR1	TDGSLTIYSPLIK	74
Ve1-eGFP	SLLLQFKGSLQYDSTLSKK	35.7
Ve1-eGFP	YLNLSNAGFVGQIPITLSR	65
Ve1-eGFP	LVTLDLSTILPFFDQPLK	58.7
Ve1-eGFP	DCQISGPLDESLSK	55.5
Ve1-eGFP	GSIPIFFR	38.2
Ve1-eGFP	LELSNCNFYGSIPSTMANLR	113.3
Ve1-eGFP	KLTYLDLSRN	47.9
Ve1-eGFP	RNQFVGQVD	62
Ve1-eGFP	VLSLSSNFFR	63
Ve1-eGFP	LGVLNLGNNK	63.4
Ve1-eGFP	LLEVLNVGNNR	75.1
Ve1-eGFP	SNLVVLDLHSNR	88.6
Ve1-eGFP	SIVNCKLLEVLNVGNNR	77.4
Ve1-eGFP	GMMVADDYVETGR	81.1
Ve1-eGFP	LYYQDTVTLTIK	75.6
Ve1-eGFP	VFTSIDFSSNR	73.2
Ve1-eGFP	GEELFTGVVPILVELDGDVNGHK	78.7
Ve1-eGFP	FSVGEGEGDATYGKLTLK	42.8
Ve1-eGFP	MPEGYVQER	601.1
Ve1-eGFP	YPDHMKQHDFFK	54.1

*Peptides were identified based on translated tobacco EST sequences and can match either *Nb*SOBIR1 or *Nb*SOBIR1-like. When specifically matching to the corresponding *N. benthamiana* homolog, the protein name is indicated. When no distinction can be made, *Nb*SOBIR1/SOBIR1-like is indicated.

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Table S4. Sequences of oligonucleotide primers used in this study.

Primer code	Sequence (5'-3')	Target sequence
Ro1	CACCATGGCTCCATTGTTCCTCTC	*SlSNC2*
Ro2	TTTACAACATTTGGACAATAAAAC	*SlSNC2*
Ro6	CACCATGGCCCTTTTTCTCTCAATA	*SlTMM*
Ro7	CAACAGACAAACTAGAACAAAAA	*SlTMM*
to11	GGGGACCACTTTGTACAAGAAAGCTGGGTTATATCTTTTCTTGTGCTTTTTCATTTTC	*AttB1-Cf-4* RT-PCR
to12	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGATGATGGTTTCTAGAAAAGTAGT	*AttB2-Cf-4* RT-PCR
to118	CACCATGAAAATGATGGCAAC	*Ve1* RT-PCR
to119	CTTTCTTGAAAACCAAAGCAAG	*Ve1* RT-PCR
to156	ATGCTTGATCTGAGTTAACA	*SlSOBIR1*
to157	CACCATGACTTCGAATATC	*SlSOBIR1*
to164	CACCATGACCTTCACAGCCTC	*SlSOBIR1-like*
to165	ATGCTTGATCTGCATCAACATGC	*SlSOBIR1-like*
to166	ATCTAGATTCGTAAGAAGAGG	VIGS fragment *SlSOBIR1*
to167	AGGATCCGAATTTCTGATTTAAC	VIGS fragment *SlSOBIR1*
to168	ATCTAGAGAACGCAACAAGGC	VIGS fragment *SlSOBIR1-like*
to169	AGGATCCAGAGGACGGAGAAG	VIGS fragment *SlSOBIR1-like*
to178	ATCTAGAATCAGACACTGAAGTTTAC	VIGS fragment *SlSOBIR1/SlSOBIR1-like*
to179	AGGATCCATCTTGAGAACCAAAAG	VIGS fragment *SlSOBIR1/SlSOBIR1-like*
to180	CACCATGGCAGAATCACTTGTTGAAC	*SlCLV2*
to181	ACCTGCTAAATTTTTTGTTTGC	*SlCLV2*
to239	CACCATGGCTGTTCCCACGGGAA	*AtSOBIR1* and RT-PCR
to240	GTGCTTGATCTGGGACAACATGG	*AtSOBIR1* and RT-PCR
to241	AGAATTCAATCTTTATCCACCAGATCATGC	VIGS fragment *NbSOBIR1*
to242	AGGATCCCAGAAAGTTTTCCAATGGCAG	VIGS fragment *NbSOBIR1*
to243	AGGATCCTTGGAAATCTTGAACCTTC	VIGS fragment *NbSOBIR1-like*
to244	ACTCGAGGATTTCTGAAAGATTTCAAG	VIGS fragment *NbSOBIR1-like*
to250	CACCATGGGCAAAAGAACTAATCCA	*SlEIX2*
to251	GTTCCTTAGCTTTCCCTTCAG	*SlEIX2*
to257	CTCAACGCATAATTCACAGAAATTTAAAGCCAGC	*SlSOBIR1D473N* mutation
to258	GCTGGCTTTAAATTTCTGTGAATTATGCGTTGAG	*SlSOBIR1D473N* mutation
to259	CTCAGCGTATAATTCACAGAAATCTAAAGCCAGGC	*SlSOBIR1-likeD486N* mutation
to260	GCCTGGCTTTAGATTTCTGTGAATTATACGCTGAG	*SlSOBIR1-likeD486N* mutation
to261	CCCACGAATCATTCACAGAAACTTAAAGCCAGCCAATG	*AtSOBIR1D489N* mutation
to262	CATTGGCTGGCTTTAAGTTTCTGTGAATGATTCGTGGG	*AtSOBIR1D489N* mutation
to266	CTTAGAAAAACTCTCTTTAGC	*NbSOBIR1* qRT-PCR

to267	TATGGATTGGAGTGACATTATG	*NbSOBIR1* qRT-PCR
to272	GCAATTGTAGTACCAGTACAC	*NbSOBIR1-like* qRT-PCR
to273	AATCAATGGACTGAAAAC	*NbSOBIR1-like* qRT-PCR
to45	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGG ATCAGTCGGTGTTGGCGA	*SlSERK3a/BAK1*
to46	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTTG GCCCTGACAACTCATCCG	*SlSERK3a/BAK1*
to58	TATGGAAACATTGTGCTCAGTGG	*NbActin* RT-PCR and qRT-PCR
to59	CCAGATTCGTCATACTCTGCC	*NbActin* RT-PCR and qRT-PCR
to88	CACCATGGTGAAGGTGATGGAGAA	*SlSERK1*
to89	CCTTGGACCCGATAATTCAAC	*SlSERK1*
ITS1-F	AAAGTTTTAATGGTTCGCTAAGA	*V. dahliae* qRT-PCR
ST-VE1-R	CTTGGTCATTTAGAGGAAGTAA	*V. dahliae* qRT-PCR
AtRubF	GCAAGTGTTGGGTTCAAAGCTGGTG	*Arabidopsis Rubisco* qRT-PCR
AtRubR	CCAGGTTGAGGAGTTACTGGAATGCTG	*Arabidopsis Rubisco* qRT-PCR

4

RECEPTOR LIKE
PROTEIN

TRANSMEMBRANE
RECEPTOR

APOPLASTIC
EFFECTORS

MAMPS

FUNGUS

PLASMA MEMBRANE

Chapter 5

General discussion

DEVELOPMENT

DEVELOPMENT

IMMUNITY

ENDOCYTOSIS

Abstract

Leucine-Rich Repeat-Receptor-Like Proteins (LRR-RLPs) are ubiquitous cell-surface receptors lacking a cytoplasmic signalling domain. For most of these LRR-RLPs it remained enigmatic how they activate cellular responses upon ligand perception. Recently, the LRR-Receptor-Like Kinase (LRR-RLK) Supressor Of BIR1-1 (SOBIR1) was shown to be essential for triggering defence responses by certain LRR-RLPs that act as immune receptors. Next to SOBIR1, the regulatory LRR-RLK BRI1-Associated Kinase-1 (BAK1) is also required for LRR-RLP function. Here we compare the roles of SOBIR1 and BAK1 as regulatory LRR-RLKs in immunity and development. BAK1 plays a general regulatory role in plasma membrane-associated receptor complexes, consisting of LRR-RLPs and/or LRR-RLKs. On the other hand, SOBIR1 appears to be specifically required for functioning of receptor complexes containing LRR-RLPs.

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Receptor-Like Proteins Require a Signalling Partner

Plants need to sense outside cues to respond adequately to changing environmental conditions, extracellular signals and invading micro-organisms. Thereto, plant cells are decorated with cell-surface receptors that recognize different ligands and subsequently activate signal-transduction cascades resulting in a tailored response [1-7] (Intermezzo 1). A specific class of Plasma Membrane (PM)-localized receptors are Receptor-Like Proteins (RLPs) that carry an extracellular ligand-binding domain, but lack any obvious cytoplasmic signalling-competent moiety. Besides their very short cytoplasmic tail and their transmembrane domain, these RLPs contain in most cases extracellular Leucine-Rich Repeats (LRRs) [4, 8] (Intermezzo 2). In 1994 the first plant LRR-RLP was identified, which is the resistance protein Cf-9 from tomato (*Solanum lycopersicum*, *Sl*) conferring resistance to strains of the biotrophic pathogenic fungus *Cladosporium fulvum* secreting the matching effector protein Avr9 [9-11]. Because LRR-RLPs lack a kinase domain, it was anticipated that they need to interact with proteins that contain such a domain, like transmembrane Receptor-Like Kinases (RLKs), in order to form a signalling-competent receptor complex [9, 11, 12]. This model was confirmed for some LRR-RLPs involved in plant development [13-16]. However, for most LRR-RLPs, e.g. those involved in disease resistance, an interacting transmembrane kinase remained to be found. Recently, the LRR-RLK Suppressor Of BIR1-1 (SOBIR1) [17], which is also known as EVeRshed (EVR) [18], was found to specifically interact with LRR-RLPs involved in plant immunity and development. Furthermore, SOBIR1 was shown to be required for LRR-RLP function [19, 20]. Here we discuss the putative role of SOBIR1 in PM-associated receptor complexes containing LRR-RLPs. In addition, we compare the specific involvement of SOBIR1 in LRR-RLP-containing complexes to the general role of the LRR-RLK BRI1-Associated kinase-1 (BAK1) (also referred to as SERK3) and related members of the family of Somatic Embryogenesis Receptor Kinases (SERKs) in the functioning of PM-associated receptor complexes [21].

Intermezzo 1. Plant Innate Immunity

Recognition of structurally conserved Microbe-Associated Molecular Patterns (MAMPs) or host-derived Damage-Associated Molecular Patterns (DAMPs) triggers an innate immune response in plants [22]. These MAMPs or DAMPs are perceived by transmembrane cell-surface proteins, such as Receptor-Like Kinases (RLKs) and Receptor-Like Proteins (RLPs) that function as Pattern Recognition Receptors (PRRs), that subsequently activate downstream defence responses. In the case of MAMP perception the response of the plant is referred to as MAMP-Triggered Immunity (MTI) [23]. Secreted effector proteins of pathogens can suppress MTI, either by interfering with MAMP recognition or by modulating cellular immune responses triggered upon MAMP detection [24-27]. In response, plants can detect pathogen effectors that localize to the cytoplasm via Nucleotide-Binding (NB)-LRR proteins, whereas effectors that are

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secreted into the apoplast can be perceived by transmembrane proteins that structurally resemble PRRs [9, 25]. These events initiate a second-layer of plant immunity, referred to as Effector-Triggered Immunity (ETI) [25, 28, 29]. Basically, DAMP-triggered immunity, MTI and ETI activate similar defence responses, although they utilize different immune receptors that perceive the pathogen and subsequently halt its proliferation [30].

Intermezzo 2. Examples of LRR-RLPs Involved in Plant Immunity
Similar to RLKs, most RLPs are predicted to localize to the PM [4]. RLPs carry only a very short cytoplasmic domain [4, 8] and can have extracellular LRRs, but alternatively may also possess extracellular Lysine Motifs (LysM) [4, 31]. A subset of RLPs lacks both a transmembrane domain and a cytoplasmic part and is attached to the PM through a Glycosyl Phosphatidyl Inositol-(GPI)-anchor [31, 32]. In *Arabidopsis*, 57 genes encoding transmembrane RLPs with extracellular LRRs have been identified [8, 33], whereas tomato contains a total of 176 genes that encode LRR-RLPs [34].

For a limited, but rapidly growing, set of LRR-RLPs a role in plant immunity has been reported. In addition to the Cf proteins, Ve1, EIX1 and EIX2, in apple the LRR-RLP HcrVf2 (Homologue of *Cladosporium fulvum* resistance of the Vf region-2) is involved in resistance to the pathogenic fungus *Venturia inaequalis* [35]. Furthermore, a reverse genetics screen on the 57 Arabidopsis LRR-RLP-encoding genes revealed that *RLP18* and *RLP30* play a role in immunity against a bacterial pathogen [33]. Interestingly, RLP30 was recently shown to recognize the proteinaceous MAMP SsE1 of the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* [20]. An *Arabidopsis* mutant in the LRR-RLP-encoding gene *SNC2 (Suppressor of NPR1, Constitutive-2)* shows a constitutive defence phenotype, indicating this LRR-RLP is a suppressor of immune responses [36, 37]. Recently, the *Arabidopsis* LRR-RLP RFO2 (Resistance to *Fusarium Oxysporum*-2) has been shown to be involved in resistance to the fungus *Fusarium oxysporum* [38], whereas the *Arabidopsis* ReMAX (Receptor of eMAX)/RLP1 receptor recognizes the *Xanthomonas* MAMP eMAX (Enigmatic MAMP of *Xanthomonas*) [39]. In wheat (*Triticum aestivum*), the *TaRLP1.1* gene codes for an LRR-RLP that contributes resistance to stripe rust caused by *Puccinia striiformis* f. sp. *tritici* [40]. Finally, the LepR3 protein of rapeseed (*Brassica napus*) is an LRR-RLP required for resistance to the pathogenic fungus *Leptosphaeria maculans* [41].

SOBIR1 is Required for LRR-RLP Function
The finding that Cf proteins interact with the tomato orthologs of the *Arabidopsis thaliana* (*At*) SOBIR1 (At2g31880) *in vivo* [19], established that these LRR-RLPs indeed interact with a possible signalling-competent LRR-RLK partner. Tomato expresses two redundant homologs of *At*SOBIR1, named *Sl*SOBIR1 (Solyc06g071810) and *Sl*SOBIR1-like (Solyc03g111800). This redundancy likely explains why these two LRR-RLKs were not

identified in genetic screens aimed at identifying components required for Cf function [19, 42, 43]. Probably, these two SOBIR1 homologs are ohnologs that emerged from the most recent Whole-Genome Triplication (WGT) event in the *Solanum* lineage [44]. In line with this, two SOBIR1 ohnologs are also present in the potato (*Solanum tuberosum*, *St*) genome (*StSOBIR1*, Sotub06g029250 and *StSOBIR1-like,* Sotub03g023250).

The tomato SOBIR1 homologs do not only interact with Cf proteins, but also with the tomato LRR-RLPs Ethylene-Inducing Xylanase receptor-2 (EIX2) and Ve1 [19]. Ve1 recognizes the secreted effector Ave1 of the vascular pathogen *Verticillium dahliae* [45, 46], whereas EIX2 acts as a receptor of Ethylene-Inducing Xylanase (EIX) [47]. Furthermore, the tomato SOBIR1 homologs interact with the closest tomato homologs of the *Arabidopsis* LRR-RLPs Clavata2 (CLV2) and Too Many Mouths (TMM), which are both involved in development, but not with the closest tomato homolog of the *Arabidopsis* LRR-RLP Suppressor of NPR1-1, Constitutive-2 (SNC2) [19]. NPR1 (Non-expressor of Pathogenesis-Related genes-1) is crucial for perception and downstream signalling of the defence hormone salicylic acid [48]. SNC2 is an LRR-RLP of which the encoding gene was identified in a mutational screen for suppressors of the *Arabidopsis npr1* mutant phenotype [36]. Interestingly, it was recently shown that *At*SNC2 requires the transmembrane ankyrin repeat protein Bian DA-1 (BDA1) for its function [37]. Future studies should indicate whether BDA1 has a broader role in LRR-RLP function.

Follow-up studies on SOBIR1 confirmed that it is required for LRR-RLP-triggered immunity. For example, co-silencing of the two tomato *SOBIR1* homologs in Cf-4- or Ve1-containing tomato resulted in compromised resistance to *C. fulvum* secreting Avr4 and to *V. dahliae* secreting Ave1, respectively [19]. Correspondingly, the Cf-4-mediated Hypersensitive Response (HR), which is a type of defence-associated programmed cell death, was compromised in *N. benthamiana* upon simultaneously silencing of the two *NbSOBIR1* homologs [19]. Furthermore, introduction of the *Ve1* gene into an *Arabidopsis sobir1-1* knock-out mutant did not result in gain of resistance to *V. dahliae* [19]*,* as seen for introducing *Ve1* in wild-type *Arabidopsis* [49]. *Arabidopsis* and tomato represent the two major eudicot clades, the Eurosids and the Asterids, respectively. Hence, the requirement of SOBIR1 homologs in LRR-RLP function appears to be widely conserved amongst the eudicots.

Recently, two independent studies confirmed the importance of SOBIR1 in LRR-RLP function. Following a forward genetics approach, *Arabidopsis* RLP30 was found to perceive the *Sclerotinia sclerotiorum* Elicitor-1 (SsE1) elicitor from *Sclerotinia sclerotiorum* and to be required for resistance against this necrotrophic fungal pathogen [20]. RLP30 function was found to depend on SOBIR1, as *Arabidopsis sobir1* mutants were strongly compromised in RLP30-mediated immunity [20]. Another *Arabidopsis* LRR-RLP that was found to interact with SOBIR1 is Responsiveness to *Botrytis* PolyGalacturonase-1 (RBPG1). RBPG1 specifically recognizes fungal endopolygalacturonases and requires

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*At*SOBIR1 for ligand-induced cell death (Lisha Zhang and Jan A.L. van Kan, personal communication). Together, these data point to an important and common role for SOBIR1 as a regulatory LRR-RLK in LRR-RLP-containing complexes. As SOBIR1 interacts with various LRR-RLPs, either involved in defence or in development, and both in tomato and in Arabidopsis, SOBIR1 emerges as being an ancient regulatory LRR-RLK that has co-evolved with the various LRR-RLPs in different eudicots.

BAK1 is a Central Regulator of Innate Immunity

Upon perception of so-called Microbe-Associated Molecular Patterns (MAMPs), the LRR-RLKs Flagellin-Sensing-2 (FLS2) and the Elongation Factor-Tu Receptor (EFR), which function as pattern recognition receptors (PRRs), rapidly form a complex with BAK1 [21, 50-53]. It was recently shown that BAK1 acts as a co-receptor with FLS2, as in addition to a direct interaction with FLS2, the LRRs of BAK1 bind to the C-terminus of flg22 that is bound to the FLS2 ectodomain [54]. Upon complex formation of FLS2 and EFR with BAK1, trans-phosphorylation events occur between these PRRs and BAK1, after which the receptor-like cytoplasmic kinase (RLCK) Botrytis-Induced Kinase-1 (BIK1) that is released from FLS2 and EFR mediates downstream defence signalling [55-58]. This shows that the kinase activity of BAK1 is required for immunity triggered by two different MAMP receptors [50, 59]. Therefore, in addition to being a co-receptor, BAK1 also acts as a regulatory LRR-RLK as BAK1 participates in downstream signalling enhancing the initial signal [22, 23, 59]. BAK1 is not the only SERK family member that interacts with FLS2 and EFR, as SERK4 (also known as BAK1-Like-1; BKK1) and possibly additional SERKs, also interact with these PRRs [51].

BAK1, SERK4 and BIK1 also interact with the *Arabidopsis* LRR-RLKs PEP-Receptor-1 (PEPR1) and PEPR2, which both perceive the Damage-Associated Molecular Pattern (DAMP) signal peptide PEP1 and are required for PEPR1/2-triggered signalling [51, 60-63]. Remarkably, the *Arabidopsis* Chitin Elicitor Receptor Kinase-1 (CERK1) also interacts with BIK1 but apparently not with BAK1, suggesting that the requirements of this Lysin Motif (LysM)-RLK for immune signalling only partially overlap with those of LRR-RLKs [56].

Interestingly, in addition to the requirement of SOBIR1, Cf- and Ve1-mediated immunity is also dependent on members of the SERK family [49, 64]. For example, Ve1 requires BAK1 for full functionality in tomato [64], whereas Cf-4-mediated resistance to *C. fulvum* is compromised upon silencing of tomato *SERK1* [49]. Moreover, Ve1- mediated resistance to *V. dahliae* is compromised in *Arabidopsis bak1* and *serk1* mutants [49]. RLP30 function was also compromised in *Arabidopsis bak1* mutants [20], whereas RBPG1-induced cell death was found to be independent of BAK1 (Lisha Zhang and Jan A.L. van Kan, personal communication). BAK1 is also required for the immune response triggered

by the tomato LRR-RLPs EIX1 and EIX2 [65]. Both EIX1 and EIX2 mediate recognition of the fungal elicitor EIX, but in this particular case EIX1 attenuates EIX2-triggered defence responses by interacting with BAK1 [65]. Various studies showed that BAK1 is required to mount a defence response against different strains of the bacterium *Pseudomonas*, the oomycetes *Phytophthora infestans* and *Hyaloperonospora arabidopsidis* and the fungi *V. dahliae* and *S. sclerotiorum* [20, 50-52, 64, 66]. In conclusion, BAK1 and other SERKs play a role in defence signalling mediated by LRR-RLKs and LRR-RLPs against a wide variety of pathogens, highlighting the central role of this family of LRR-RLKs in plant immunity.

SOBIR1 also Plays a Broad Role in Immunity

SOBIR1 was originally found to play a role in plant innate immunity by performing a screen for suppressors of the LRR-RLK BIR1 (BAK1-Interacting RLK-1) [17]. BIR1 interacts with and is phosphorylated by BAK1 [17, 67]. It was proposed that BIR1 functions as a negative regulator of defence-associated LRR-RLKs. Consequently, an *Arabidopsis bir1-1* T-DNA insertion mutant shows a constitutive defence phenotype, including dwarfism. A suppressor screen of this *bir1-1* phenotype yielded mutations in the LRR-RLK *SOBIR1* that restore the growth and defence phenotype, hence its name SUPPRESSOR OF BIR1-1 [17]. This suggests that *SOBIR1* acts as a positive regulator of defence, which is normally inhibited by BIR1 in wild-type *Arabidopsis*. In agreement with this observation, over-expression of *AtSOBIR1* in *Arabidopsis* results in a constitutive defence phenotype [17]. Interestingly, this phenotype depends on the heterotrimeric G-protein subunit *Arabidopsis* G protein β-subunit-1 (AGB1), which is needed for a full MAMP response [68]. AGB1 is localized at the PM, suggesting that it may function together with RLKs. However, initial experiments failed to demonstrate interaction between this subunit and the kinase domains of SOBIR1, BAK1, FLS2, CERK1 or EFR [68].

Interestingly, *AtSOBIR1* homologues are transcriptionally regulated upon activation of defence responses by a broad range of pathogens and MAMPs. For example, *AtSOBIR1* expression is induced upon perception of the bacterial flagellin-derived peptide flg22 ligand by FLS2 in *Arabidopsis* cell cultures and seedlings [69]. Likewise, *AtSOBIR1* is induced upon activation of the EFR receptor [70]. However, *Arabidopsis sobir1* mutants are not compromised in flg22-induced MAMP responses [20], suggesting that SOBIR1 is not directly involved in FLS2-mediated immunity. Additional transcriptome studies also revealed that the *SOBIR1* gene is transcriptionally regulated upon exposure of plants to biotic stress. For example, the apple (*Malus x domestica*) homolog of *SOBIR1* is differentially expressed in apple trees resistant to bacterial fire blight disease upon *Erwinia amylovora* infection [71]. Similarly, a *SOBIR1* homolog of white mustard (*Sinapis alba*) is upregulated upon infection with the fungus *Alternaria brassicicola* [72]. In *Arabidopsis* various viruses also cause induction of *SOBIR1* expression [73, 74]. Finally, *SOBIR1* is also proposed to play a role in Systemic Acquired Resistance (SAR) [75].

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The observation that *SOBIR1* is transcriptionally regulated upon challenge by various pathogens, next to its specific role in LRR-RLP function, renders SOBIR1 a likely target for pathogen effectors perturbing plant immunity. Indeed, recent results indicate that effectors secreted by the bacterial pathogen *Pseudomonas syringae* interact with tomato SOBIR1, suggesting that SOBIR1 function is vulnerable to inactivation by effectors (Sorina C. Popescu, personal communication). This result is confirmed by the observation that SOBIR1 binds to a *Pseudomonas*-derived molecule using phage-display technology [76]. Overall, the results of the aforementioned studies suggest that SOBIR1 plays an important and broad role in immunity of plants to pathogens.

BAK1 and SOBIR1 Have Roles Outside Immunity
Together with other SERK members, BAK1 regulates various developmental processes in plants [21, 53, 77]. For example, BAK1 interacts in a ligand-dependent manner with the LRR-RLK BRassinosteroid Insensitive-1 (BRI1) that functions as a receptor for endogenous brassinosteroid hormones [78, 79]. SERK1 and SERK4 also interact with BRI1 and function redundantly with BAK1 in this respect [80-82]. A comprehensive genetic study showed that multiple SERKs are required for BRI1 function [83]. Just recently, it was found that also in this case BAK1, in addition to SERK1, acts as a co-receptor with BRI1, as the LRRs of both BAK1 and SERK1 specifically interact with the BRI1-bound brassinosteroid hormone [84, 85]. The downstream RLCK BIK1 also interacts with BRI1 and negatively regulates BRI1 responses [86]. Sequential trans-phosphorylation events between BRI1, BIK1 and BAK1, followed by the release of BIK1, are required to specifically activate downstream brassinosteroid signalling [86, 87]. Besides participating in brassinosteroid signalling, SERK1 and SERK2 play a role in pollen formation, and consequently a *serk1;serk2* double mutant is male-sterile [88, 89]. Independent of brassinosteroid signalling, SERKs act in *Arabidopsis* root development [90, 91] and BAK1 is involved in photo-morphogenesis [92]. Additionally, BAK1 and SERK4 play a role in containment of cell death, which may in part be related to a role of these SERKs in plant immunity [53, 80, 93].

SERK1 has also been reported to be involved in floral organ abscission in *Arabidopsis*, as a mutation in *SERK1* reverts the *nevershed* (*nev*) mutant phenotype, which shows defects in floral organ shedding, cellular trafficking and Golgi architecture [94, 95]. *NEV* encodes an ADP-Ribosylation Factor GTPase-Activating Protein (ARF GAP). Strikingly, not only a mutation in *SERK1* suppresses the *nev* phenotype, but also mutations in *SOBIR1 (EVR)* revert the *nev* phenotype (*nev evr-1* and *nev evr-2*) [18]. In transgenic *Arabidopsis*, SOBIR1 fused to Yellow Fluorescent Protein (YFP) is present in the abscission zone of floral organs, the floral style, and the petioles of cauline leaves [18]. Both SERK1 and SOBIR1 appear to play their role downstream of NEV in floral abscission and function either in a parallel pathway or upstream of the LRR-RLKs HAESA (HAE) and HAESA-Like-2

(HSL2) [18, 94]. Additionally, it was found that both HAE and SOBIR1 interact with the RLCK CaST away (CST). This RLCK belongs to class VII of the RLCKs that also comprises BIK1. It has been proposed that CST aids in the assembly and internalization of receptor complexes involved in floral abscission [96]. It remains to be elucidated whether SOBIR1 also recruits CST for performing its regulatory role in immunity, or whether in this case another downstream interactor is involved.

Since SOBIR1 was found to specifically interact with LRR-RLPs [19], the results described above suggest that also in floral organ shedding one or more LRR-RLPs are involved. Besides the demonstrated roles of SOBIR1 in innate immunity and development, *SOBIR1* expression is induced by exposure of *Arabidopsis* suspension cells to high light stress [97] and an LRR-RLK survey amongst *Arabidopsis* T-DNA insertion mutants revealed a role for SOBIR1 in tolerance to high auxin and high salt concentrations [98].

Transmembrane proteins, such as LRR-RLKs and LRR-RLPs are synthesised in the Endoplasmic Reticulum (ER) where different ER chaperones contribute to ER-Quality Control (ER-QC)-mediated folding (Intermezzo 3). Next the transmembrane receptors are transported to the PM by the cellular trafficking machinery. Subsequently, receptor complexes can be removed from the PM by endocytosis [99]. Interestingly, SOBIR1 might be involved in endosomal trafficking, as *Sl*SOBIR1-eGFP localizes to mobile cytoplasmic vesicles in transiently transformed *N. benthamiana* epidermal leaf cells [19]. It has been proposed [18] that SOBIR1 might promote the internalization of receptor complexes, which would limit the amount of receptors available at the PM, thereby inhibiting abscission. Furthermore, it was suggested that SOBIR1 might regulate Golgi dynamics during cellular stress, which for example occurs during abscission and challenge by pathogens [18]. It remains to be elucidated whether the Cf and Ve1 proteins are subject to endocytosis and if so, whether SOBIR1 and/or SERK proteins are required for their internalisation.

An important observation in this context is that co-silencing of the *SOBIR1* homologs in *N. benthamiana* reduces Cf-4 and Ve1 protein levels in a transient expression assay [19]. This is in contrast to the observation that in *Arabidopsis bak1* mutants, BAK1-interacting LRR-RLKs still accumulate to levels seen in wild-type plants [50, 52, 83]. Possibly, reduction of SOBIR1 levels prevents accumulation of LRR-RLP-containing complexes by triggering their degradation via the ER-degradation pathway or by allowing their trafficking to lytic vacuoles [100]. In an alternative model, SOBIR1 does not direct LRR-RLP cellular trafficking, but functions as a scaffold protein that is generally required for accumulation of LRR-RLPs.

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Intermezzo 3. Endoplasmic Reticulum-Quality Control of RLKs and RLPs Involved in Plant Immunity

Before being transported to their functional cellular destination, synthesis of transmembrane receptors takes place in the Endoplasmic Reticulum (ER) [100]. Additionally, many of these receptors undergo post-translational modifications such as *N*-linked glycosylation [101]. To prevent secretion of immature and incorrectly folded proteins from the ER, strict Quality Control (QC) mechanisms must exist [100, 102]. A number of ER-QC pathways have been identified in plants and these pathways rely on different ER-localized chaperones. One pathway relies on the HSP40 Binding Proteins (BiPs) that act in concert with HSP40-like co-chaperones containing J domains (ERdj3) and Stromal-Derived Factor-2 (SDF2) [103-105]. A second pathway monitors the *N*-linked status of client proteins and is mediated by the lectin chaperones CalNeXin (CNXs) and CalReTiculins (CRTs) [100-102, 106]. Protein disulphide isomerases mediate the formation of disulphide bridges and form the third pathway of ER-QC [107, 108]. Eventually, terminally misfolded proteins are degraded by a mechanism known as ER-associated protein degradation [100, 109]. In addition, a number of these chaperones are involved in activation of ER-stress signalling and aid in initiating the unfolded protein response [110-112], a response that prevents accumulation of large amounts of unfolded proteins by transcriptionally upregulation of ER-QC chaperones.

Recently, a number of *Arabidopsis* mutants involved in the *N*-linked glycosylation pathway and ER-QC were found to be immunocompromised to bacterial infections. For example, mutants in subunits of the OligoSaccharylTransferase (OST) complex, such as OST3/6 and STT3A (STaurosporin and Temperature-sensitive-3A), as well as in the folding sensor protein UDP-glucose Glycoprotein Glucosyl Transferase (UGGT), Glucosidase II, the HDEL receptor ERD2b, SDF2 and CRT3 all have reduced responses mediated by EFR to the elf18 epitope of elongation factor-Tu [104, 113-117]. The mechanism underlying the reduced EFR functionality in these mutants is in most cases a reduction in accumulation of the receptor [104, 113-117]. Remarkably, accumulation of- and immune responses mediated by FLS2, another MAMP receptor, are hardly compromised in these mutants [104, 113-117]. Both, EFR and FLS2 are *N*-linked glycosylated on their LRR domains [117, 118]. However, it was found that EFR is much more vulnerable to hypoglycosylation than FLS2, as revealed by functional studies in the *Arabidopsis stt3a* mutant and by directional mutagenesis of critical Asn (N) residues of Asn-X-Ser/Thr signatures [117, 118]. Also in rice ER-QC chaperones play an important role in immunity as over-expression of *BiPs* and knock-down of *SDF2* homologs compromise resistance mediated by the RLK Xa21 [119, 120].

The Cf proteins of tomato contain numerous *N*-linked glycosylation sites in their extracellular LRR domain and, as determined for Cf-9, nearly all are being glycosylated and many are required for full functionality although the complexity of glycosylation remained to be determined [121, 122]. Using an antibody specifically recognizing

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complex-type glycans we were able show that different Cf proteins contain complex-type *N*-linked glycans decorated with β(1,2)-Xylose and α(1,3)-Fucose residues [123]. Similarly, we found that another tomato LRR-RLP, Ve1, also contains this complex-type *N*-linked glycosylation [124].

Furthermore it was found that Cf proteins and Ve1 interact with the different tomato CRTs as well as BiP chaperones involved in ER-QC. However, whereas Cf-4 strongly requires specifically tomato CRT3a for its complex *N*-linked glycosylation, the Avr4-triggered HR and mediating resistance against *C. fulvum* [123], Ve1 requires the presence of nearly all tomato BiP and CRT homologs for mediating resistance against *V. dahliae* [124]. Besides, Ve1 complex glycosylation was unaltered upon knock-down of the different CRTs despite reduced Ve1-mediated defence. Knock-down of multiple *BiPs* in *N. benthamiana* proved to be lethal and the effect of *BiP* knock-down on Cf-4-mediated resistance remains to be elucidated [123]. However, two BiPs were found to be upregulated during the Cf-4/Avr4-triggered Hypersensitive Response (HR) and may thus play an important role in Cf-signalling [125]. Also the HDEL receptors ERD2a and ERD2b appear to play a role in the Cf-mediated responses as silencing of these genes increases the Cf-9-triggered HR, possibly due to enhanced secretion receptor from the ER [126]. Knock-down of the individual *BiPs* in Ve1-expressing tomato resulted in a reduced Ve1-mediated resistance to *V. dahliae* for three out of the four tomato *BiPs* [124].

In contrast to Cf-4, which is heavily underglycosylated upon knock-down of CRT3a in *N. benthamiana*, Ve1 does not seem to be underglycosylated upon knock-down of the different CRTs. This somehow resembles weak *crt3* mutants in *Arabidopsis* that still accumulate EFR but show reduced EFR-triggered responses [114]. Combined, these results underline significant differences between Cf-4 and Ve1 in requirement of ER-QC chaperones for the biogenesis to functional receptors, more or less similar as observed for the RLKs FLS2 and EFR. Interestingly, a recent study showed that *N. benthamiana* CRT3a is also required for the response to the *Phytophthora infestans* INF1 elicitor and it was speculated that INF1 would be perceived by a transmembrane receptor requiring stringent CRT3a-mediated glycosylation [127].

It is worth noting that ER-QC chaperones of plants appear to have acquired specific roles. The plant specific CRT3 homolog seems to have a particular function in immunity [128], and indeed EFR, Cf-4, Ve1 and the putative INF1-receptor all require the presence of a functional CRT3 homolog. Apparently, important regulatory RLKs such as BAK1, required for FLS2, EFR and Ve1 function [49-51, 64] do not heavily depend on ER-QC as FLS2 responses requiring BAK1 still occur in many of the *Arabidopsis* ER-QC mutants [104, 113-117]. Additionally, it is worth to note that different immune receptors have different dependencies on ER-QC. The above mentioned examples clearly show a strong requirement in ER-QC for a number of cell-surface localized receptors, yet there

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is a much less strong requirement for FLS2. As an explanation for this phenomenon, it is hypothesized that in an evolutionary perspective relative young receptors would require a more stringent ER-QC compared to relative older receptors [22, 113, 114, 117]. Since EFR only occurs in members of the Brassicaceae in contrast to FLS2 which occurs in more plant families [22, 113, 114, 117], this may suggests that EFR requires a more stringent QC. Similarly the tomato Cf and Ve1 RLPs are not broadly present in the plant kingdom and furthermore reside in genomic clusters where cross-overs may rapidly occur to generate new variants that may require stringent ER-QC [64, 129-131].

SOBIR1 and BAK1 Trigger Responses Related to Defence or Development

SOBIR1 is a dual-specificity kinase, as its kinase domain auto-phosphorylates on both Tyr and Ser/Thr residues [18]. An important question that remains to be answered is whether SOBIR1 kinase activity is required for its regulatory role in Cf-4 function, similar to the requirement of BAK1 kinase activity for its function in LRR-RLK signalling [59]. It is important to note that a kinase-dead version of *At*SOBIR1 does not complement a knock-down of *NbSOBIR1* in transgenic *N. benthamiana* expressing Cf-4, while wild-type *At*SOBIR1 does [19]. Moreover, the *Arabidopsis bak1-5* mutant allele was recently characterised [59]. Intriguingly, this allele carries a mutation in the kinase domain of BAK1, which severely compromises the MAMP-triggered responses, but does not affect BRI1-mediated responses and the capacity of BAK1 to control cell death. Hence, the development- and defence-associated functions of BAK1 are uncoupled in this *bak1-5* mutant. Thus, differential phosphorylation of the kinase domain of this regulatory LRR-RLK might determine which downstream signalling components are recruited and what the outcome will be of the activated signal-transduction cascade. In line with this, it was suggested that the downstream RLCK BIK1 is differentially phosphorylated upon activation by either BRI1 or FLS2 [86], which might explain the different cellular responses upon activation of these receptors that both recruit BAK1. With respect to LRR-RLP function, it remains to be elucidated whether differential phosphorylation of the SOBIR1 kinase domain occurs and whether SOBIR1 is directly involved in signalling by LRR-RLPs.

Roles of BAK1 and SOBIR1 in Receptor Complexes

With the identification of SOBIR1 as a regulatory LRR-RLK for LRR-RLP function, yet another generic component of PM-associated receptor complexes has been unveiled. Over the last years our insight into the functioning of many receptor complexes has increased dramatically. When dissecting different receptor complexes, current data indicate that it all boils down to many different ligand-detecting receptors requiring the regulatory LRR-RLKs BAK1 and/or SOBIR1.

Fig. 1 provides an overview of various processes related to plant development and defence regulated by receptor complexes that (i) reside at the PM and (ii) require BAK1 and/or SOBIR1 as regulatory LRR-RLKs. First, BRI1 and the PRRs FLS2 and EFR are shown, which mediate developmental responses to brassinosteroids and defence-related responses to the MAMPs flagellin and Elongation Factor-Tu (EF-Tu), respectively (Fig. 1A and 1B). Both BRI1 and the PRRs interact with BIK1 prior to ligand perception and form a ligand-induced complex with BAK1 (as well as with related SERKs). Activated BRI1 phosphorylates the negative regulators BIK1 and BRI1-Kinase Inhibitor-1 (BKI), resulting in the dissociation of these proteins from the kinase domain of BRI1 [86, 132]. BRI1 also associates with BRI1 Signalling Kinases (BSKs) and Constitutive Differential Growth-1 (CDG1), which are RLCKs of the classes XII and VII, respectively [133, 134]. Upon activation of BRI1, the BSKs are released from the receptor complex and initiate downstream signalling (Fig. 1A).

FLS2 constitutively interacts with BSK1 [135], as well as with the DENN (Differentially Expressed in Normal and Neoplasmic cells) domain-containing protein SCD1 (Stomatal Cytokinesis-Defective-1) (Fig. 1B) [136]. MAMP-Triggered Immunity (MTI) is subsequently initiated by rapid trans-phosphorylation and release of the kinase BIK1 [23, 55-57]. Perception of flg22 by FLS2 triggers BAK1-dependent endocytosis of the PRR [137, 138]. The Plant U-Box (PUB) E3-ubiquitin ligases PUB12 and PUB13 are subsequently phosphorylated by BAK1 and ubiquitinate FLS2, leading to its degradation and dampening of flagellin-induced responses [139] (Fig. 1B). So far, no LRR-RLPs appear to be involved in these processes and current data indicate no direct role for SOBIR1 in these signalling pathways [19, 20].

Fig. 1C presents the situation in which plants are not challenged by a pathogen. In this case BAK1 (and the related SERKs) do not interact with ligand-perceiving transmembrane receptors like FLS2, but instead associate with the negative regulator BIR1, preventing the initiation of a defence response [17]. BIR1 (maybe in a complex with BAK1/SERKs) also suppresses SOBIR1 function [17] and thereby possibly negatively regulates LRR-RLP-mediated responses.

Fig. 1D illustrates the possible roles of SOBIR1 in LRR-RLP-mediated immunity, taking Cf-4 as an example. The interaction between Cf-4 and SOBIR1 is Avr4-independent [19] and upon Avr4 recognition the phosphorylation status of the SOBIR1 kinase domain might change, resulting in the recruitment of SERKs and trans-phosphorylation events between SOBIR1 and the SERKs, after which downstream signalling is triggered by the receptor complex. In this model, SOBIR1 actively takes part in downstream defence signalling. Alternatively, SOBIR1 acts as a scaffold for the LRR-RLP facilitating its accumulation. In this scenario, the recruited SERKs solely mediate downstream defence signalling.

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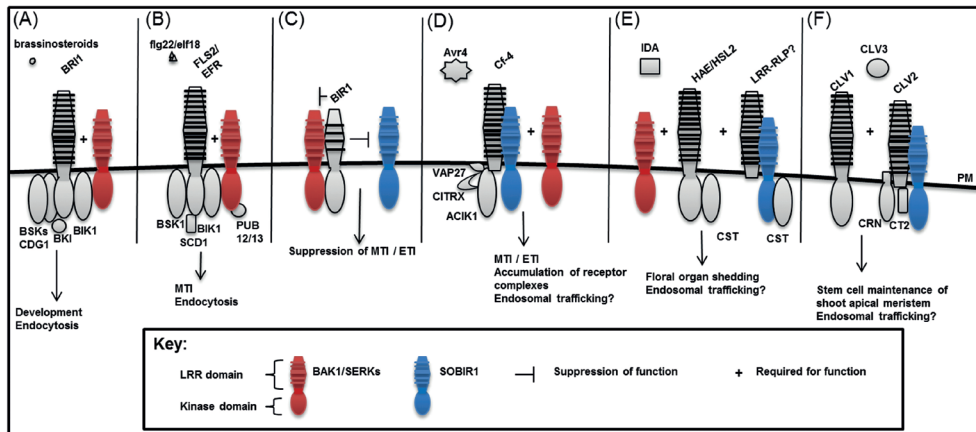
Figure 1. The involvement of SOBIR1 and BAK1/SERKs in PM-localised LRR-RLP- and LRR-RLK-containing complexes. (A) The LRR-RLK BRI1 perceives brassinosteroid hormones and forms a ligand-induced complex with the co-receptor BAK1 and other SERKs also acting as co-receptors [21, 84, 85, 145-147]. (B) The PRRs FLS2 and EFR mediate immunity against bacteria. Upon perception of the MAMPs flagellin and EF-Tu, respectively, or elicitor-active peptides derived from these MAMPs (flg22 and elf18, respectively) these PRRs form a complex with BAK1 and additional SERKs probably through BAK1/SERK interaction with the PRR-bound ligand [21, 23, 51, 54]. (C) In the situation where the plant is not challenged by a pathogen, the LRR-RLK BIR1 is present in a complex with BAK1/SERKs. BIR1 has been shown to genetically interact with SOBIR1 to suppress plant immune responses [17]. (D) Cf-4 (and other LRR-RLPs) constitutively interact with SOBIR1 [19]. Members of the SERK family are also required for LRR-RLP function [27, 29, 30, 47]. (E) The LRR-RLKs HAE and HSL2 are positive regulators of abscission and are predicted to mediate perception of the small secreted endogenous peptide IDA [144]. Here SOBIR1 is referred to as EVeRshed (EVR) and acts as an inhibitor of abscission [18], possibly via interaction with an LRR-RLP. (F) The LRR-RLK CLV1 and the LRR-RLP CLV2 independently perceive the small secreted peptide ligand CLV3 in the shoot apical meristem [13-15]. CLV2 was also found to interact with SOBIR1 [19], suggesting that SOBIR1 also plays a role in the functioning of CLV2-containing complexes. See text for further details.

In addition to the SERKs, various cytoplasmic proteins have been found to play a role in Cf function. Examples are the class VII RLCK ACIK1 (Avr9-Cf9-Induced Kinase-1) [140], VAP27 (VAMP-Associated Protein-27) [141] and CITRIX (Cf-9-InTeRacting thIoredoXin) [142, 143]. Similar models can be drawn for other defence-associated LRR-RLPs with which SOBIR1 interacts, such as Ve1 or EIX2 that recognise the *V. dahliae* effector Ave1 or the MAMP EIX, respectively.

Fig. 1E shows the involvement of SERK1 and SOBIR1 (EVR) in the abscission of floral organs that is initiated upon perception of the predicted endogenous ligand Inflorescence Deficient in Abscission (IDA) by the LRR-RLKs HAE and HSL2 [144]. In a *nevershed* background, both *sobir1* and *serk1* mutants restore floral organ shedding, indicating the requirement of these LRR-RLKs in this process [18, 94]. The class VII RLCK CST interacts with HAE/HSL2 as well as with SOBIR1 and the latter may aid in receptor complex assembly and endocytosis [96]. So far, it is not known whether LRR-RLPs play a role in the abscission of floral organs.

Fig. 1F illustrates the presence of SOBIR1 in the CLV complex. SOBIR1 interacts with CLV2 [19] and its role in this complex remains to be elucidated. Both the LRR-RLK CLV1 and the LRR-RLP CLV2 independently affect shoot apical meristem size upon detection of the endogenous secreted CLV3 peptide by negatively regulating *WUSCHEL* gene expression [148]. CLV2 function was shown to also require the transmembrane kinase domain-containing protein CoRyNe (CRN) [13-15, 149] and CT2 (Compact plant-2), which is a predicted α-subunit of a hetero-trimeric GTP-binding protein [150].

Overall, a picture emerges supporting the involvement of at least two conserved regulatory LRR-RLKs in a plethora of PM-associated receptor complexes. Future studies should address to what extent these two types of regulatory LRR-RLKs provide specificity in downstream signalling. It will be essential to identify partners of BAK1 and SOBIR1 before and after activation of PM-associated receptor complexes that can explain this specificity. In addition, it should be investigated whether specific changes in the phosphorylation status occur upon ligand perception by the different ligand-binding receptors associating with these regulatory LRR-RLKs. Such studies will advance our understanding of how these regulatory LRR-RLKs contribute to the specificity of the downstream responses initiated by different ligand-binding receptors.

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Summary

Receptors present at the plasma membrane play a crucial role in resistance of plants to extracellular pathogens. In tomato (*Solanum lycopersicum*) plasma membrane-localized Cf proteins mediate resistance to the biotrophic fungal leaf pathogen *Cladosporium fulvum* through recognition of secreted fungal effector proteins, also known as Avirulence (Avr) proteins. Ve1, another plasma membrane receptor of tomato, mediates resistance to strains of the vascular fungal pathogen *Verticillium dahliae* secreting the Ave1 effector. Both Cf proteins and Ve1 carry an extracellular Leucine-Rich Repeat (LRR) domain, have a transmembrane domain and carry a short cytoplasmic tail lacking any obvious signalling domains. These proteins are referred to as LRR-Receptor-Like Proteins (LRR-RLPs). In contrast to LRR-RLPs, another well-studied class of plasma membrane-localized receptors involved in resistance to pathogens, the LRR-Receptor-Like Kinases (LRR-RLKs), carry a cytoplasmic signalling domain consisting of a kinase. This domain is thought to facilitate downstream signalling upon pathogen perception by the extracellular LRR domain. Ever since the discovery of the first LRR-RLP in 1994, which was the Cf-9 protein, it remained enigmatic how this class of proteins transmits a downstream defence signal upon effector recognition. The work described in this thesis was initiated with the aim to obtain more insight in the molecular mechanisms by which LRR-RLPs involved in resistance to extracellular fungi trigger plant immunity upon perception of the invading pathogen. In addition to obtaining detailed information on the biogenesis and maturation of these LRR-RLPs, a putative regulatory LRR-RLK (referred to as SOBIR1) of this class of extracellular receptors was identified. This LRR-RLK is proposed to facilitate accumulation and subcellular trafficking of LRR-RLPs, whereas the receptor can also be directly involved in cellular signalling upon effector perception by the interacting LRR-RLP.

Chapter 1 is a general introduction outlining the biological background of the plant innate immune system and its perturbation by successful pathogens. The chapter focuses on plasma membrane receptor complexes involved in resistance to pathogens and concludes with an overview of the information available on Cf- and Ve1-mediated immune signalling, at the start of this thesis work.

The extracellular LRRs of the Cf proteins and Ve1 are complex *N*-linked glycosylated and this domain is therefore expected to be subjected to the activity of Endoplasmic Reticulum (ER) chaperones for folding and Quality Control (QC). Recently, it was shown that a number of LRR-RLKs, involved in defence against bacterial pathogens, depend on specific ER-QC chaperones for their proper glycosylation and accumulation. Plants mutated in these ER-QC chaperones are consequently compromised in their resistance to bacterial pathogens. **Chapter 2** describes the identification of the ER-localized Binding Proteins (BiPs) and CalReTiculins (CRTs) as interactors of the Cf-4 receptor protein. A functional Cf-4-enhanced Green Fluorescent Protein (Cf-4-eGFP) fusion

protein was generated and transiently expressed by *Agrobacterium tumefaciens*-mediated infiltration in leaves of the plant species *Nicotiana benthamiana*, followed by immunopurification of the fusion protein and mass spectrometry-based identification of copurifying proteins. Amongst the copurifying proteins were ER-resident chaperones of the BiP and CRT families. Silencing experiments proved that BiPs are redundantly required for Cf-4-mediated immunity, whereas silencing of multiple *BiPs* simultaneously is lethal to the plant. Regarding the CRTs it was discovered that knock-down of one particular *CRT* homolog, *CRT3a*, results in strongly compromised Cf-4-mediated resistance in tomato. Likewise, the Cf-4-triggered Hypersensitive Response (HR) was also compromised upon knock-down of *CRT3a* in *N. benthamiana*. By determining the status of complex *N*-linked glycosylation of Cf-4 upon silencing of the different individual *CRTs*, it was found that knock-down of specifically *CRT3a* strongly reduces complex glycosylation of Cf-4, providing a molecular explanation for the reduced Cf-4 functionality upon silencing of the gene encoding this chaperone. The outcome of the work described in this chapter demonstrates the stringent ER-QC requirements of Cf-4 for its maturation to a functional receptor. In this chapter the importance of proper ER-QC for a number of plant innate immune receptors of the LRR-RLK and LRR-RLP class is discussed.

In **Chapter 3** the requirement of the different BiP and CRT ER-QC chaperones for Ve1-mediated resistance to *V. dahliae* was studied. Remarkably, and in contrast to what is the case for Cf-4, silencing of nearly all individual *BiPs* and *CRTs* resulted in reduced Ve1-mediated resistance of tomato to *V. dahliae* strains secreting Ave1. This suggests that Ve1-mediated resistance requires a stringent ER-QC mediated by the various BiP and CRT chaperones. Interestingly, in tobacco, the Ve1-mediated HR was not compromised and Ve1 still showed complex *N*-linked glycosylation upon silencing of the individual genes encoding the ER-QC chaperones. These results demonstrate that Cf-4 and Ve1 have different requirements for BiP- and CRT-mediated ER-QC. It remains to be elucidated how silencing of the *BiPs* and *CRTs* actually compromises Ve1-mediated resistance. A possibility is that downstream signalling components, not required for Ve1-mediated HR in tobacco, are strongly affected in tomato by silencing the various genes encoding the ER chaperones.

In an early model describing Cf-mediated signalling, it was proposed that the LRR-RLP might interact with one or multiple LRR-RLKs to form a signalling-competent receptor complex. Such a model has nowadays been confirmed for a number of LRR-RLPs involved in plant development. However, for LRR-RLPs involved in plant defence, such as the Cf proteins and Ve1, the downstream signalling mechanism remained a mystery. **Chapter 4** describes the identification of the LRR-RLK SOBIR1 as an interactor of Cf-4 and Ve1, as well as of other Cf proteins and of additional LRR-RLPs involved in defence. Furthermore, SOBIR1 was found to interact with LRR-RLPs involved in development. For these studies, Cf-4 was immunopurified from transgenic tomato stably expressing Cf-4-

eGFP, as well as from *N. benthamiana* upon its transient expression. Similarly, the Ve1-eGFP fusion protein was transiently expressed in *N. benthamiana* and immunopurified. Mass-spectrometry of the immunoprecipitates revealed SOBIR1 as an interactor of both Cf-4 and Ve1. Interestingly, tomato was found to contain two *SOBIR1* homologs, *SOBIR1* and *SOBIR1-like*, whereas *Arabidopsis thaliana* (*Arabidopsis*) contains only one *SOBIR1* gene. Co-immunoprecipitation assays revealed that SOBIR1 interacts specifically with a number of LRR-RLPs involved in defence or in development, but not with a number of LRR-RLKs that were tested. Silencing of *SOBIR1* homologs compromises Cf-2-, Cf-4- and Ve1-mediated immunity in tomato. Additionally, Cf-4- and Ve1-mediated HR in *Nicotiana* species was compromised upon silencing of *SOBIR1*. Further genetic evidence for the requirement of SOBIR1 in LRR-RLP-mediated immune responses came from studies in *Arabidopsis*. Similar to the wild-type, a *Ve1*-transgenic *Arabidopsis sobir1* knock-out mutant was susceptible to an *Ave1*-expressing *V. dahliae* strain, whereas *Ve1* in an *Arabidopsis* wild-type background does provide resistance to the fungus. SOBIR1 kinase activity is likely required for LRR-RLP-mediated defence responses, as a kinase-dead variant of the LRR-RLK from *Arabidopsis* does not restore the loss of Cf-4/Avr4-triggered HR in a complementation assay in *N. benthamiana*. It was also observed that SOBIR1 is crucial for stable accumulation of LRR-RLPs, as silencing of *SOBIR1* leads to a strongly reduced accumulation of the Cf-4 and Ve1 proteins.

The identification of SOBIR1 as an LRR-RLK required for Cf-4 and Ve1 function provides the first evidence that the LRR-RLPs Cf-4 and Ve1 form a receptor complex in which LRR-RLKs take part, thereby confirming the early model. Before the start of the studies of which the results are described in this thesis, SOBIR1 was already known to be a positive regulator of plant defence responses. Furthermore, the LRR-RLK (in this case referred to as Evershed) had been described to be involved in floral organ shedding, which is a developmental process. The fact that we found SOBIR1 to interact with LRR-RLPs involved in either defence or development, suggests that SOBIR1 functions as a regulatory LRR-RLK in many different LRR-RLP-mediated processes. This observation is somehow reminiscent of what has been described for the LRR-RLK SERK3/BAK1 (Somatic Embryogenesis Receptor Kinase-3/BRI1-Associated Kinase-1), a member of the SERK family of LRR-RLKs, as SERK3/BAK1 functions as regulatory LRR-RLK in plant defence and developmental processes triggered by ligand-binding LRR-RLKs. **Chapter 5** summarizes and discusses the major findings described in this thesis and places them in a broader perspective. A comprehensive overview of our current understanding of plasma membrane-localized receptor complexes in which SOBIR1 and/or SERK3/BAK1 take part is provided and the chapter particularly focuses on a comparison of the putative regulatory roles of the LRR-RLKs SOBIR1 and SERK3/BAK1 in plasma membrane-localized receptor complexes. The data obtained in this thesis support a model in which these two RLKs both have specific and conserved functions in plant innate immune responses and development. It is proposed that SOBIR1 specifically

functions as regulatory LRR-RLK for ligand-binding LRR-RLPs, whereas SERK3/BAK1 (and other SERK family members) function as regulatory LRR-RLKs for ligand-binding LRR-RLKs and LRR-RLPs. Alternatively, SOBIR1 might act as an LRR-RLP-specific scaffold protein that facilitates their stabilisation and, in this scenario, is indirectly involved in signalling by these LRR-RLPs.

Samenvatting

Plasmamembraan receptoren spelen een belangrijke rol bij de afweerreactie van planten tegen extracellulaire ziekteverwekkers. De op het plasmamembraan aanwezige Cf receptoren van tomaat (*Solanum lycopersicum*) herkennen extracellulaire effectoren, de zogenaamde avirulentie (Avr) eiwitten die worden uitgescheiden door de schimmel *Cladosporium fulvum*. *C. fulvum* is de veroorzaker van de bladvlekkenziekte van tomaat en wanneer een *C. fulvum* effectoreiwit door een Cf receptor wordt herkend treedt er een afweerreactie in werking die verdere infectie van de plant door de schimmel verhindert. Een andere plasmamembraan receptor van tomaat is Ve1. Ve1 herkent het Ave1 effectoreiwit dat wordt uitgescheiden door bepaalde isolaten van de schimmel *Verticillium dahliae*. *V. dahliae* is een ziekteverwekker die groeit in het vaatweefsel van tomaat en die de verwelkingsziekte veroorzaakt.

De Cf receptoren en Ve1 hebben een zeer overeenkomstige structuur. Ze bezitten een extracellulair Leucine-Rich Repeat (LRR) domein, een transmembraan domein en een klein cytoplasmatisch domein dat geen homologie vertoont met signaleringsdomeinen bekend van andere eiwitten. Dit type plantenreceptoren staat bekend als LRR-RLPs (Receptor-Like Proteins). In tegenstelling tot LRR-RLPs hebben LRR-RLKs (Receptor-Like Kinases) een cytoplasmatisch signaleringsdomein, bestaande uit een kinase. LRR-RLKs zijn betrokken bij zeer veel processen, waaronder afweerreacties tegen ziekteverwekkers. Het kinase domein speelt daarbij een cruciale rol, omdat het een signaaltransductie cascade in gang kan zetten die uiteindelijk leidt tot resistentie. Al sinds de ontdekking van de eerste LRR-RLP in 1994, de receptor Cf-9, is het een mysterie hoe deze groep van plasmamembraan receptoren een cytoplasmatisch signaal in gang kan zetten op het moment dat een schimmeleiwit wordt herkend. Het werk zoals beschreven in dit proefschrift, is uitgevoerd met als doel meer inzicht te krijgen in het moleculaire mechanisme waarmee LRR-RLPs die betrokken zijn bij resistentie tegen schimmels, de immuniteit van planten kunnen activeren na herkenning van de binnendringende ziekteverwekker. Naast het verkrijgen van gedetailleerde informatie over de biogenese en maturatie van deze LRR-RLPs, werd er een mogelijk regulerende LRR-RLK (genaamd SOBIR1) van dit type extracellulaire receptors geïdentificeerd. Deze LRR-RLK is mogelijk specifiek betrokken bij de accumulatie en subcellulaire lokalisatie van LRR-RLPs, maar zou ook een directe rol kunnen spelen in het activeren van een cytoplasmatisch signaal na perceptie van een effector door de LRR-RLP.

Hoofdstuk 1 bestaat uit een algemene inleiding over de biologische achtergrond van het immuunsysteem van planten. Hierbij ligt de focus op een beschrijving van de samenstelling en het functioneren van receptorcomplexen die aanwezig zijn op de plasmamembraan. Het hoofdstuk eindigt met een beschrijving van wat bekend was over de moleculaire mechanismen waarmee Cf en Ve1 eiwitten de afweerreactie tegen schimmels activeren, op het moment dat het onderzoek zoals beschreven in dit proefschrift begon.

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Het extracellulaire LRR domein van zowel de Cf receptoren als Ve1 wordt geglycosyleerd in het Endoplasmatisch Reticulum (ER). Een groep van chaperonne-eiwitten die aanwezig zijn in het ER, spelen een belangrijke rol bij de vouwing en kwaliteitscontrole van geglycosyleerde eiwitten. Recentelijk is aangetoond dat een aantal LRR-RLKs betrokken bij de afweerreactie tegen bacteriële ziekteverwekkers afhankelijk is van specifieke ER chaperonnes voor hun correcte glycosylering en accumulatie. Planten waarin de genen coderend voor deze chaperonnes waren gemuteerd, bleken gehinderd te zijn in hun afweer tegen bacteriële ziekteverwekkers. **Hoofdstuk 2** beschrijft de identificatie van chaperonnes van het ER behorend tot de klasse van de BiPs (Binding Proteins) en de CRTs (CalReTiculins) als Cf-4-interacterende eiwitten. Hiertoe werd een Cf-4-enhanced Green Fluorescent Protein (Cf-4-eGFP) fusie-eiwit gegenereerd, dat door middel van *Agrobacterium tumefaciens* infiltratie tot expressie werd gebracht in de modelplant *Nicotiana benthamiana*. Hierna werden immuno-zuiveringen van het fusie-eiwit uitgevoerd, gevolgd door het identificeren van meezuiverende Cf-4-eGFP-interacterende eiwitten met behulp van massa-spectrometrie. Het bleek dat de ER chaperonnes van de BiP- en CRT-families tussen de geïdentificeerde eiwitten aanwezig waren. Uit experimenten waarbij in *N. benthamiana* de expressie van de verantwoordelijke genen werd onderdrukt, bleek dat BiPs elkaars rol in het functioneren van Cf-4 over kunnen nemen. Het onderdrukken van de expressie van meerdere *BiP* genen tegelijk bleek lethaal te zijn voor de plant. Wat betreft de CRT familie bleek het onderdrukken van de expressie van één specifieke *CRT*, genaamd *CRT3a*, te resulteren in een sterk verminderde resistentie van tomaat tegen *C. fulvum* en een verminderde door Cf-4 geactiveerde overgevoeligheidsreactie (HR). Door de mate van complexe glycosylering van Cf-4 te bestuderen na onderdrukking van *CRT3a* expressie bleek dat Cf-4 minder complex geglycosyleerd was. Dit is een moleculaire verklaring voor de verminderde functionaliteit van het Cf-4 eiwit. De resultaten van dit hoofdstuk demonstreren dat Cf-4 sterk afhankelijk is van ER chaperonnes en dat glycosylering zeer belangrijk is voor de functionaliteit van het Cf-4 eiwit. Verder tonen de resultaten van het onderzoek, zoals beschreven in dit hoofdstuk, het belang van een correcte ER kwaliteitscontrole voor transmembraan receptoren die een rol spelen bij de afweerreactie van planten.

In **hoofdstuk 3** wordt het belang van de verschillende BiP en CRT eiwitten beschreven voor de door Ve1 geactiveerde resistentie tegen *V. dahliae*. In tegenstelling tot de situatie bij Cf-4, bleek dat het onderdrukken van de expressie van bijna alle individuele *BiPs* en *CRTs* resulteerde in een verminderde resistentie van tomaat tegen *V. dahliae*. Deze resultaten suggereren dat ook Ve1 sterk afhankelijk is van ER kwaliteitscontrole door de BiP en CRT chaperonnes. Opmerkelijk was dat de HR die door Ve1 wordt geactiveerd in tabak, niet verminderd was wanneer de expressie van de individuele chaperonnes werd onderdrukt. Verder bleek dat ook de glycosylering van Ve1 niet verminderd was. Deze resultaten demonstreren dat Cf-4 en Ve1 een verschillende mate van afhankelijkheid hebben van de diverse ER chaperonnes voor hun functionaliteit. Het is nog onduidelijk

hoe het onderdrukken van de expressie van de verschillende *BiPs* en *CRTs* exact zorgt voor een verminderde door Ve1 geactiveerde resistentie van tomaat. Een mogelijkheid is dat in tomaat belangrijke signaaltransductie componenten, die niet nodig zijn voor de door Ve1 geactiveerde HR in tabak, sterk in hun functioneren worden beïnvloed wanneer de expressie van de *BiPs* en *CRTs* is onderdrukt.

In een eerder model dat het mogelijke signaleringsmechanisme van Cf receptoren beschrijft werd voorgesteld dat de LRR-RLP wellicht interacteert met één of meerdere LRR-RLKs om zo een functioneel complex te vormen dat een signaal kan afgeven aan het cytoplasma. Dit model bleek overeen te komen met de samenstelling van een aantal LRR-RLP complexen betrokken bij diverse ontwikkelingsprocessen in planten. Voor LRR-RLPs betrokken bij afweerreacties tegen ziekteverwekkers, zoals de Cf receptoren en Ve1, bleef de samenstelling van het signaleringcomplex echter onbekend. **Hoofdstuk 4** beschrijft de identificatie van de LRR-RLK SOBIR1 als een interacterend eiwit van Cf-4 en Ve1. Deze LRR-RLK interacteert bovendien met andere Cf receptoren en LRR-RLPs betrokken bij afweerreacties alsmede met LRR-RLPs betrokken bij plantontwikkeling. Voor het identificeren van SOBIR1 werd Cf-4 geïsoleerd door middel van immuno-zuiveringen uit bladeren van transgene Cf-4-eGFP tomatenplanten. Bovendien werd het fusie-eiwit gezuiverd na expressie in *N. benthamiana* met behulp van *Agrobacterium* infiltraties. Op eenzelfde manier werd het Ve1-eGFP fusie-eiwit gezuiverd uit *N. benthamiana*. Door middel van massa-spectrometrie op de verkregen monsters werd SOBIR1 geïdentificeerd als een meezuiverend, interacterend eiwit van beide LRR-RLPs. Opmerkelijk is dat tomaat twee homologen bezit van *SOBIR1*, genaamd *SOBIR1* en *SOBIR1-like*, terwijl de modelplant *Arabidopsis thaliana* (*Arabidopsis*) slechts één *SOBIR1* gen heeft. Door middel van co-immuno-zuiveringsexperimenten werd ontdekt dat de SOBIR1 homologen specifiek interacteren met diverse LRR-RLPs, maar niet met een aantal LRR-RLKs die werden getest. Het onderdrukken van de expressie van *SOBIR1* verminderde de mate van Cf-2-, Cf-4- en Ve1-geactiveerde resistentie van tomaat. Daarnaast bleek dat de Cf-4- en Ve1-geactiveerde HR in *Nicotiana* verminderd is na onderdrukking van *SOBIR1* genexpressie. Additioneel genetisch bewijs dat SOBIR1 betrokken is bij de immuunrespons die geactiveerd wordt door LRR-RLPs, werd verkregen door studies gedaan in *Arabidopsis*. Een *Ve1*-transgene *Arabidopsis sobir1* mutant, waarin het gen coderend voor de LRR-RLK niet meer functioneel was, bleek niet resistent te zijn tegen een *V. dahliae* isolaat dat Ave1 produceert, terwijl Ve1 getransformeerd naar wild-type *Arabidopsis* wel resistentie geeft tegen de schimmel. De enzymatische activiteit van het cytoplasmatische kinase domein van SOBIR1 is zeer waarschijnlijk nodig voor zijn functie, want een SOBIR1 eiwit met een enzymatisch inactief kinase domein is niet in staat het verlies van de door Cf-4/Avr4-geactiveerde HR, als gevolg van endogeen *SOBIR1* silencing, te herstellen in een complementatie-test in *N. benthamiana*. Ten slotte werd ontdekt dat SOBIR1 van cruciaal belang is voor de accumulatie van de interacterende LRR-RLPs. Het onderdrukken van de expressie van

SOBIR1 zorgde namelijk voor een sterk verminderde accumulatie van de Cf-4 en Ve1 eiwitten.

De identificatie van SOBIR1 als LRR-RLK nodig voor Cf-4 en Ve1 functie is het eerste bewijs dat de LRR-RLPs Cf-4 en Ve1 een receptorcomplex vormen waarvan LRR-RLKs deel uitmaken. Daarmee is een eerder model dat de mogelijke samenstelling van Cf receptorcomplexen beschrijft bevestigd. Bij de aanvang van dit promotieonderzoek was SOBIR1 al beschreven als een positieve regulator van de afweerreactie van planten. Daarnaast was al bekend dat SOBIR1 (in dit geval Evershed genoemd) betrokken is bij de ontwikkeling van planten, namelijk bij het afstoten (abscissie; "shedding") van bloembladen. Het feit dat in deze studie SOBIR1 is geïdentificeerd als een interacterende LRR-RLK van LRR-RLPs suggereert dat SOBIR1 functioneert als een regulerende LRR-RLK bij verschillende processen die door LRR-RLPs worden geactiveerd. Een dergelijke functie vertoont overeenkomsten met rol van SERK3/BAK1 (Somatic Embryogenesis Receptor Kinase-3/BRI1-Associated Kinase-1), een LRR-RLK dat lid is van de SERK familie van LRR-RLKs. SERK3/BAK1 functioneert namelijk als regulerende LRR-RLK voor ligand-bindende LRR-RLKs die een rol spelen bij de afweerreactie of bij ontwikkelingsprocessen van planten. **Hoofdstuk 5** bediscussieert de belangrijkste bevindingen van dit proefschrift en plaatst ze in een breder perspectief. Er wordt een uitgebreid overzicht gegeven van het huidige inzicht in de samenstelling en het functioneren van receptorcomplexen die aanwezig zijn op het plasmamembraan en er wordt in het bijzonder aandacht besteed aan een vergelijking tussen de mogelijk regulerende rol van de LRR-RLKs SOBIR1 en SERK3/BAK1 in plasmamembraan-gelokaliseerde receptorcomplexen. Onze resultaten ondersteunen een model waarin deze twee LRR-RLKs specifieke en geconserveerde functies hebben in de immuniteit en ontwikkeling van planten. Mogelijk functioneert SOBIR1 als een regulerende LRR-RLK specifiek voor LRR-RLPs, terwijl SERK3/BAK1 (en andere SERKs) als regulerende LRR-RLK functioneert voor zowel ligand-bindende LRR-RLKs als LRR-RLPs. In een alternatief model is SOBIR1 niet direct betrokken bij het signaleringsmechanisme van LRR-RLPs, maar zorgt het voor de stabiliteit van receptorcomplexen waarvan LRR-RLPs deel uit maken.

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Thomas

Curriculum vitae

Thomas Willem Hendrik Liebrand werd op 13 januari 1986 geboren te Zevenaar. In 2003 behaalde hij het havodiploma aan het Liemers College te Zevenaar. In hetzelfde jaar begon hij met de hlo-opleiding Laboratoriumtechniek met de specialisatie Plantenbiotechnologie aan de Hogeschool Larenstein te Velp. Gedurende deze vierjarige opleiding fuseerde deze opleiding met de hlo-opleidingen van de Hogeschool van Arnhem en Nijmegen (HAN) te Nijmegen. Tijdens zijn hbo-studie werd in 2006 een stageproject uitgevoerd aan de vakgroep *Plant-Microbe Interactions* van de Universiteit Utrecht. Onder begeleiding van dr. Sjoerd van der Ent deed hij onderzoek naar transcriptiefactoren die een rol spelen bij bacterieel geïnduceerde systemische resistentie in de modelplant *Arabidopsis thaliana*. In 2007 voerde hij een afstudeerproject uit bij *Plant Research International* te Wageningen. Onder begeleiding van dr. Jan Schaart werkte hij aan een systeem om genetisch gemodificeerde planten selectiemerker vrij te maken met behulp van genetische recombinatie. Na het succesvol afronden van zijn hlo-opleiding begon hij in datzelfde jaar met de MSc-opleiding *Plant Biotechnology* aan de Wageningen Universiteit. Zijn afstudeervak werd in 2008 uitgevoerd bij de vakgroep Fytopathologie. Onder begeleiding van dr. Wladimir Tameling en dr. ir. Matthieu Joosten werd onderzoek gedaan naar de cytoplasmatische immuunreceptor NRC1 van tomaat. Begin 2009 werd een klein afstudeervak uitgevoerd binnen de onderzoeksgroep van dr. Silke Robatzek bij het Max-Planck Instituut voor plantenveredeling te Keulen, alwaar gewerkt werd aan het opzetten van een *split-ubiquitin-yeast-two-hybrid*-systeem voor het vinden van eiwitinteractoren van de Cf-4-immuunreceptor van tomaat. Zijn MSc-studie rondde hij *cum laude* af. Eind 2009 begon Thomas met een promotieonderzoek bij de vakgroep Fytopathologie van de Wageningen Universiteit. Onder begeleiding van prof. dr. ir. Pierre de Wit en dr. ir. Matthieu Joosten werd onderzocht hoe zogenaamde *Receptor-Like Proteins* (zoals de Cf-4-immuunreceptor) van tomaat functioneren in het bieden van resistentie tegen ziekteverwekkende schimmels. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

LIST OF PUBLICATIONS

Liebrand, T.W.H., Van den Burg, H.A. and Joosten, M.H.A.J. Two for all: receptor-associated kinases SOBIR1 and BAK1. *Trends in Plant Sci*, in press

Zhang, L., Kars, I., Essenstam, B., Liebrand, T.W.H., Wagemakers, L., Elberse, J., Tagkalaki, P., Tjoitang, D., Van den Ackerveken, G., and Van Kan, J.A.L. Fungal endopolygalacturonases are recognized as MAMPs by the Arabidopsis Receptor-Like Protein RBPG1. *Plant Physiol,* in press

Liebrand, T.W.H.[*], Kombrink, A.[*], Zhang, Z., Sklenar, J., Jones, A.M.E., Robatzek, S., Thomma, B.P.H.J., and Joosten, M.H.A.J. Chaperones of the endoplasmic reticulum are required for Ve1-mediated resistance to *Verticillium*. *Mol Plant Pathol* (2013) 15: 109-117

Liebrand, T.W.H., Van den Berg, G.C.M., Zhang, Z., Smit, P., Cordewener, J.H.G., America, A.H.P., Sklenar, J., Jones, A.M.E., Tameling, W.I.L., Robatzek, S., Thomma, B.P.H.J., and Joosten, M.H.A.J. The receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection. *Proc Natl Acad Sci USA* (2013) 110: 10010-10015

Liebrand, T.W.H., Smit, P., Abd-El-Haliem, A., De Jonge, R., Cordewener, J.H.G., America, A.H.P., Sklenar, J., Jones, A.M.E., Robatzek, S., Thomma, B.P.H.J., Tameling, W.I.L., and Joosten, M.H.A.J. Endoplasmic reticulum-quality control chaperones facilitate the biogenesis of Cf receptor-like proteins involved in pathogen resistance of tomato. *Plant Physiol* (2012) 159: 1819-1833

[*] Shared first authorship

Education Statement Of The Graduate School
Experimental Plant Sciences

The Graduate School **EXPERIMENTAL PLANT SCIENCES**
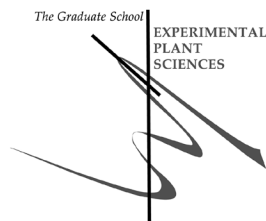
Issued to: **Thomas Liebrand**

Date: **21 February 2014**

Group: **Phytopathology, Wageningen University & Research Centre**

1) Start-up phase	*date*
▶ **First presentation of your project**	
Identification and charaterization of Cf-4 interacting proteins	Apr 16, 2010
▶ **Writing or rewriting a project proposal**	
▶ **Writing a review or book chapter**	
Two for all: the receptor-associated kinases SOBIR1 and BAK1, Trends in Plant Science, Nov 2013, doi:10.1016/j.tplants.2013.10.003	Oct 2013
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	

Subtotal Start-up Phase **7.5 credits***

2) Scientific Exposure	*date*
▶ **EPS PhD student days**	
European PhD Student Retreat in Exprimental Plant Sciences, MPI, Cologne, Germany	Apr 15 - 17, 2010
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student day, Wageningen University	May 20, 2011
European PhD Student Retreat in Exprimental Plant Sciences, Orsay-Paris, France	Jul 05 - 08, 2011
European PhD Student Retreat in Exprimental Plant Sciences, JIC, Norwich, United Kingdom	Aug 15 - 17, 2012
EPS PhD student day, University of Amsterdam	Nov 30, 2012
ExPectationS (EPS career day), Wageningen University	Feb 01, 2013
▶ **EPS theme symposia**	
EPS theme symposia 2 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 15, 2010
EPS theme symposia 2 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 03, 2011
EPS theme symposia 2 'Interactions between Plants and Biotic Agents', Wageningen University	Feb 10, 2012
EPS theme symposia 2 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 24, 2013
▶ **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19 - 20, 2010

▶	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04 - 05, 2011
	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02 - 03, 2012
	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 22 - 23, 2013

▶ **Seminars (series), workshops and symposia**

Invited seminars (Christiane Gebhardt, Peter Moffett, Paul Birch, Felix Mauch, Brande Wulff, Heribert Hirt, Naoto Sibuya, Regine Kahmann, Klaus Harter)	Feb - Dec 2010
EPS workshop Endomembrane signalling, Amsterdam	Jul 02, 2010
CBSG workshop genome mining in tomato	Oct 26, 2010
Joint MPI Marburg Phytopathology meeting	Oct 28 - 29, 2010
Invited seminar Rosie Bradshaw	Aug 04, 2011
Invited seminars (David Baulcombe, Larry Griffing, Ralph Panstruga)	Oct 10, 2012
Invited seminars (Brian Staskawicz, David Weller, Silke Robatzek)	May - Oct 2013

▶ **Seminar plus**

▶ **International symposia and congresses**

Keystone Meeting: Receptors and Signaling in Plant Development and Biotic Interactions, Tahoe, CA, USA	Mar 14 - 19, 2010
Keystone meeting: Plant Immunity: Pathways and Translation, Big Sky, Montana, USA	Apr 07 - 12, 2013

▶ **Presentations**

Poster Keystone Meeting "Receptors and Signalling"	Mar 14 - 19, 2010
Poster CBSG summit 2011	Feb 01, 2011
Presentation Joint MPI Marburg Phytopathology meeting	Oct 28, 2010
Presentation CBSG clustermeeting pathogenomics 2010	Nov 21, 2010
Presentation CBSG proteomics Hotel summit 2010	Dec 13, 2010
Presentation ALW meeting EPS Lunteren 4-5 april 2011	Apr 05, 2011
Presentation CBSG clustermeeting pathogenomics 2011	Oct 20, 2011
Lecture (45min) at HAN university for 3th year Bachelor students: Basic concepts in plant-pathogen interactions	Nov 29, 2011
Lecture (45min) at HAN university for 3th year Bachelor students: Identification of proteins required for Cf function	Nov 29, 2011
Presentation CBSG proteomics Hotel summit 2011	Dec 14, 2011
Presentation CBSG summit 2012	Mar 01, 2012
Presentation CBSG clustermeeting pathogenomics 2012	Oct 25, 2012
Presentation CBSG proteomics Hotel summit 2012	Jan 19, 2013
Presentation CBSG summit 2013	Feb 11, 2013
Presentation Keystone meeting Plant Immunity: Pathways and Translation	Apr 10, 2013

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►	**IAB interview**	Nov 15, 2012
►	**Excursions**	
	CBSG matchmaking event (excursion to Rijk Zwaan and Monsanto)	Oct 18, 2012

Subtotal Scientific Exposure   29.9 credits*

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	VLAG PhD course proteomics	Apr 18 - 21, 2011
	EPS autumn school 'Host Microbe Interactomics'	Nov 01 - 03, 2011
►	**Journal club**	
	Member of literature discussion group at Phytopathology	2009 - 2013
►	**Individual research training**	
	Individual training (PRI Bioscience): Progenesis LC-MS and ProteinLynx Global Server; LC-MS data analysis.	2010 - 2011

Subtotal In-Depth Studies   6.2 credits*

4) Personal development		*date*
►	**Skill training courses**	
	Moral dilemmas in daily scientific practices	Feb 15 - 18, 2011
	Techniques for Writing and Presenting a Scientific Paper (TWP)	Jul 03 - 06, 2012
	Career Orientation	Mar 2013
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	

Subtotal Personal Development   3.9 credits*

TOTAL NUMBER OF CREDIT POINTS*	**47.5**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

* A credit represents a normative study load of 28 hours of study.