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HopQ1 is a client for host 14-3-3 proteins

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Phosphorylation of HopQ1, a Type III Effector from *Pseudomonas syringae*, Creates a Binding Site for Host 14-3-3 Proteins

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Abstract
HopQ1 (Hrp outer protein Q), a type III effector secreted by *Pseudomonas syringae* pv. *phaseolicola*, is widely conserved among diverse genera of plant bacteria. It promotes the development of halo blight in common bean. However, when this same effector is injected into *Nicotiana benthamiana* cells, it is recognized by the immune system and prevents infection. Although the ability to synthesize HopQ1 determines host specificity, the role it plays inside plant cells remains unexplored. Following transient expression *in planta*, HopQ1 was shown to co-purify with host 14-3-3 proteins. The physical interaction between HopQ1 and 14-3-3a was confirmed *in planta* using FRET-FLIM technique. Moreover, mass spectrometric (LC-MS-MS/MS) analyses detected specific phosphorylation of the canonical 14-3-3 binding site (RSXpSXP, pS denotes phosphoserine) located in the N-terminal region of HopQ1. Amino acid substitution within this motif abrogated the association and led to altered subcellular localization of HopQ1. In addition, the mutated HopQ1 protein showed reduced stability *in planta*. These data suggest that the association between host 14-3-3 proteins and HopQ1 is important for modulating the properties of this bacterial effector.
Introduction
A multi-component defense response is initiated when plant pattern recognition receptors perceive microbially-derived structural components (Nürnberger and Brunner, 2002), which are referred to as PAMPs (pathogen-associated molecular patterns). Many bacterial pathogens have developed type III secretion system (TTSS) effectors that can suppress or modulate PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). Effector-triggered immunity (ETI) represents a second layer of defense, whereby plants have evolved mechanisms that rely upon R proteins to sense and respond to cognate TTSS effector. Thus, expression of a specific bacterial effector can either sustain disease in susceptible plants or render the pathogen avirulent in resistant plants that express the corresponding R protein. Several lines of evidence suggest an involvement of scaffold proteins from the 14-3-3 family in mediating these defense responses at various levels (Yang et al., 2009; Oh et al., 2010). Some R proteins have been shown to bind 14-3-3 proteins directly. RPW2.8, which confers resistance to fungal pathogens of Golovinomyces spp., associates specifically with the 14-3-3 isoform lambda (designated GF14λ) from Arabidopsis thaliana (Yang et al., 2009). Moreover, both types of resistance were compromised in Arabidopsis lacking the lambda isoform. Consistently, ectopic overexpression of GF14λ in transgenic Arabidopsis results in enhanced resistance to powdery mildew (Yang et al., 2009). Tobacco N protein, which mediates resistance to Tobacco Mosaic Virus (TMV), also binds 14-3-3 protein (Ueda et al., 2006). The viral p50 replicase helicase domain is the cognate ligand for N protein. Since this domain also interacts with 14-3-3s, it is possible that 14-3-3s might function in the formation of the receptor-ligand recognition complex (Ueda et al., 2006). In addition, the tomato 14-3-3 protein TF7 has been shown to exhibit positive regulation on the MAPK cascade, which is activated rapidly by pathogen recognition (Oh et al., 2010; Oh and Martin, 2011).

There is increasing evidence that many intracellular pathways are regulated by the modulation of scaffold protein properties, rather than the activities of integral components in the signaling cascades (Good et al., 2011). This strategy enables signal transduction to be turned on or off rapidly via the assembly or disassembly of complexes. This same mechanism also allows the intensity and kinetics of a response to be fine-tuned to the stimulus (Good et al., 2011). It was recently suggested that the manipulation of scaffolding may be one strategy employed by
pathogens to interfere with the host defense response. The best-characterized example of scaffolding manipulation is the phytotoxin fusicoccin, which is secreted by the fungus *Fusicoccum amygdali*. Fusicoccin targets a 14-3-3 protein that regulates guard cell H+-ATPases and its activity results in stomatal opening, facilitating pathogen entry (Oecking et al., 1994). Some bacterial virulence factors simply require scaffold proteins to reach their destination within host cells or to become enzymatically active, while others target the host scaffold proteins to suppress defenses. *Yersinia* species secrete the TTSS effector YopK (*Yersinia* outer protein K), which binds to the receptor for activated C kinase (RACK1) in mammals (Thorslund et al., 2011). It is hypothesized that this interaction blocks phagocytosis, allowing efficient extracellular proliferation of the bacteria. *Yersinia* has also acquired the virulence factor (YopM), which mimics eukaryotic scaffolds and forces bridging of host kinases (McDonald et al., 2003). Similarly, enterohemorrhagic *Escherichia coli* strains use EspG to form an artificial complex that effectively reprograms host signaling (Selyunin et al., 2011).

HopQ1 (also known as HopQ1-1, NC_007274.1) is a type III effector that has been acquired recently by *Pseudomonas syringae* strains (Rohmer et al., 2004), whereas its xenologs from *Ralstonia solanacearum* and *Xanthomonas* spp. appear to be ancient. HopQ1 contributes to host specificity but its exact role in pathogenesis remains undefined. This study shows that HopQ1 must undergo a specific phosphorylation event *in planta* as a prerequisite for its binding to host 14-3-3, and that its properties depend upon the formation of the effector-host protein complex.

**Results**

A number of bacterial effectors are activated once inside host cells, upon interaction with host proteins. Analysis *in silico* of *P. syringae* pv. *phaseolicola* 1448A type III effector inventory revealed that most of the effectors possess putative 14-3-3 binding motifs (Supplemental Table S1). This suggests that association with plant 14-3-3 proteins can be important for their function. HopQ1, that contains in the N-terminal domain (Figure 1) a canonical mode 1 14-3-3 binding site [48-53 aa, high stringent Scansite algorithm; http://scansite.mit.edu, (Obenauer et al., 2003)] was selected to test significance of this motif and expressed transiently *in planta*. Leaf samples were collected 2 d after agroinfiltration of *N. benthamiana* and HopQ1 was affinity-purified via its Strep II tag. Mass spectrometric (LC-MS-MS/MS) analyses revealed that
HopQ1 co-purified with nine isoforms of 14-3-3 proteins. Two isoforms of this family, a and c, were identified in all four independent experiments (Table I). The mode 1 14-3-3 binding motif (RSXpSXP, pS denotes phosphoserine contains a phosphoserine and, therefore, it is possible that HopQ1 is phosphorylated in host cells. Mass spectrometric analyses performed on HopQ1 expressed \textit{in planta} showed that a central serine residue in the predicted 14-3-3–binding site (S51) had been phosphorylated in the peptide SKpSAPALLTAAQR (m/z = 687.362+, MASCOT score 85; Figure 2A, B). We detected up to four different putative phosphorylation sites in several independent experiments (data not shown) but only S51 was repeatedly phosphorylated in all of them. As a complementary approach, \textit{in vitro} kinase assays were performed. Recombinant HopQ1 was expressed in \textit{E. coli} and then co-incubated with crude plant extracts, which provided a source of kinase (Supplemental Figure S1). While HopQ1 purified from \textit{E. coli} was not modified (data not shown), mass spectrometry indicated that HopQ1 was phosphorylated when incubated with the crude extracts (Figure 2C, D) and an increase of 80 Da was again detected at the S51 residue within the peptide pSAPALLTAAQR (m/z = 589.792+, MASCOT score 107). To confirm these results, the relevant serine residue was mutated to a phospho-null alanine (HopQ1-S51A) and the mutant construct was expressed in \textit{E. coli}. In the \textit{in vitro} assay, phosphorylation of purified HopQ1-S51A was almost completely abolished, which suggests that S51 represents a major phosphorylation site (Figure 3). In contrast, the modification of HopQ1 at position 49 (HopQ1-S49A) resulted in a partial reduction of the phosphorylation signal and not elimination. The same modification pattern was observed with resistant (\textit{N. benthamiana}) and susceptible (\textit{P. vulgaris}) plants (Supplemental Figure S2). Interestingly, HopQ1 was phosphorylated by extracts from various plants, including non-host species (Supplemental Figure S1). The amino acid sequence surrounding S51 suggests that HopQ1 could be a substrate for a kinase from the CDPK-SnRK superfamily (Hrabak et al., 2003). Consistent with this possibility, representatives of both CPK and SnRK families phosphorylated HopQ1 \textit{in vitro} (Supplemental Figure S3). These findings support the suggestion that an S49A substitution might weaken the consensus site, and collectively these data suggest that HopQ1 S51 might play a central role in interaction with 14-3-3. Consistent with this hypothesis, 14-3-3 proteins were not detected by mass spectrometry when HopQ1-S51A was transiently expressed in \textit{N.}}
*benthamiana* whereas HopQ1-S49A co-purified with the same set of 14-3-3 isoforms as the wild-type HopQ1 (Table II).

To corroborate these findings, Fluorescence Resonance Energy Transfer (FRET) efficiency was quantified for HopQ1 variants and 14-3-3a, an isoform identified in all co-purification experiments. A fusion protein between 14-3-3a and eCFP (enhanced Cyan Fluorescent Protein) was co-expressed with wild-type HopQ1-eYFP (enhanced Yellow Fluorescent Protein) or HopQ1-S51A-eYFP in the leaves of *N. benthamiana* plants. After 2 days, the average eCFP lifetime was assayed using Fluorescence Lifetime Imaging Microscopy (FLIM) and significant reductions were found when 14-3-3a-eCFP was co-expressed with HopQ1-eYFP (Table III and Supplemental Figure S4). This finding confirms the presence of a physical interaction between 14-3-3a and HopQ1 *in planta*. In contrast, only a minor reduction in eCFP lifetime was observed in the presence of the HopQ1-S51A-eYFP fusion protein, which supports the idea that S51 is involved in binding 14-3-3.

Pull-down assays were used to determine whether HopQ1 phosphorylation is a prerequisite for 14-3-3 binding. Recombinant HopQ1-6xHis protein obtained from *E. coli* was co-incubated with leaf protein extracts from *N. benthamiana* or *P. vulgaris* (Table IV, Supplemental Table SII). Subsequently, HopQ1 was purified and eluates subjected to mass spectrometric analysis. Under such conditions, no 14-3-3s–derived peptides were found. However, when HopQ1-6xHis had been first phosphorylated *in vitro* and then used in the pull-down assay, various 14-3-3s isoforms were detected. In contrast, no 14-3-3s were identified when *in vitro* phosphorylated HopQ1-S51-A was used in the experiments.

In an alternative experiment we analyzed by gel filtration chromatography formation of HopQ1-14-3-3 complex *in vitro* (Figure 4). Recombinant 14-3-3a protein was first incubated with HopQ1-6xHis, either non-phosphorylated or *in vitro* phosphorylated by CPK3. Next, the samples were separated using Superdex 200 10/300GL column. A peak corresponding to HopQ1-14-3-3 complex was detected only in the samples containing phosphorylated HopQ1-6xHIS (Figure 4, orange trace). In contrast, the analyses revealed only presence of 14-3-3 dimer and HopQ1 monomer in the samples containing non-phosphorylated HopQ1. The identity of the peaks was confirmed by SDS-PAGE analysis of the appropriate fractions (data not shown). Collectively these results demonstrate that HopQ1 binds 14-3-3s in a phosphorylation-dependent manner and that this binding occurs both in plants where
it triggers defense responses \((N. benthamiana, \text{ Table IV})\) and in plants where it contributes to increased virulence of the bacteria \((P. vulgaris, \text{ Supplemental Table SII})\).

Since association with 14-3-3 might modify localization of a partner, the subcellular distribution patterns of HopQ1 and HopQ1-S51A were compared. As seen in Figure 5, in epidermal cells of \(N. benthamiana\) HopQ1-eYFP localized almost exclusively to the cytoplasm, whereas HopQ1-S51A-eYFP was directed to the nucleus. Similar pattern was observed in mesophyll cells of \(N. benthamiana\). Thus, interaction with 14-3-3a might either facilitate nuclear export or hinder nuclear import of HopQ1. Interestingly, when 14-3-3a was expressed alone, it accumulated in both the cytosolic and nuclear compartments (Figure 6), but in the presence of ligand, 14-3-3a was detected predominantly in the cytoplasm. In contrast, HopQ1-S51A expression did not affect 14-3-3a distribution. Susceptible and resistant plants showed similar localization patterns. These observations indicate that both interacting partners might be involved in the reciprocal determination of localization.

Another aspect of association to 14-3-3 is revealed by the difference in the steady state levels of C-terminally 3xHA tagged HopQ1 variants when these were transiently expressed in \(N. benthamiana\) under control of the CaMV 35S promoter (Figure 7A). In contrast, HopQ1 and HopQ1-S51A levels were similar when expressed in \(P. syringae\ pv. tabaci\) in a broad host range plasmid under control of the constitutive form of ptac promoter (Figure 7B). This suggests that interaction with 14-3-3 may increase the stability of HopQ1 effector in host tissue. To test this hypothesis, we monitored levels of HopQ1 in protein extracts supplemented with R18, an inhibitor which disrupts 14-3-3-3a ligand associations (Wang et al., 1999). In the first experiment (Figure 7C) crude protein extracts isolated from \(N. benthamiana\) leaves, that transiently expressed HopQ1-Flag protein, were incubated for 1h at 30°C. Subsequently, HopQ1 was detected by immunoblot analysis using an antibody specific to Flag. While HopQ1 steady state level remained unchanged under control conditions, co-incubation with increasing amounts of the R18 proportionally reduced its level. The fact that application of the R18 to the extract decreased HopQ1 level indicates that R18 is able to displace HopQ1 from the complex with endogenous 14-3-3 proteins. We next tested whether application of exogenous 14-3-3a would outcompete the inhibitor. To this end, increasing amounts of the recombinant 14-3-3a were added to the extract, from \(N. benthamiana\) leaves expressing HopQ1-Flag
protein, supplemented with the R18. As shown in Figure 7D, the effect of R18 on the HopQ1 stability could be overcome by exogenous 14-3-3a but not BSA. Similar set of experiments was performed with bean protein extracts (Figure 7E, F). After incubation of the in vitro reconstituted HopQ1-14-3-3a complex for 1h at 30°C with the bean crude extract a band corresponding to intact HopQ1 protein was visible on the immunoblot while in samples containing the R18 this band was not detected (Figure 7E). Additionally, as shown in Figure 7F, application of the phosphorylated HopQ1 to the bean extract resulted in its degradation while the presence of 14-3-3a suppressed this process. Collectively these results suggest that binding to 14-3-3 might protect HopQ1 from degradation by plant proteases (Figure 7 C-F). The fact that we have not found substantial differences in protein levels of HopQ1 and HopQ1-S51A C-terminally fused to eYFP suggests that the presence of longer tags might stabilize the effector in N. benthamiana cells (data not shown).

The efficient plant defense strategy, hypersensitive cell death, is induced when HopQ1 is transiently expressed in Nicotiana tabacum (Figure 8A) in contrast to severe chloroses triggered by the effector in N. benthamiana leaves (Wroblewski et al., 2009). To determine whether modification of the 14-3-3 binding site is critical for HopQ1 recognition by the plant immune system, tobacco leaves were infiltrated with Agrobacterium cultures carrying HopQ1 constructs. As shown in Figure 8A, the hypersensitive response is elicited by both HopQ1 and HopQ1-S51A, which suggests that this protein can function as an avirulence determinant, despite impaired 14-3-3 binding.

HopQ1 is missing from P. syringae pv. syringae B728a (PsyB728a), which is highly virulent in N. benthamiana (Vinatzer et al., 2006). To further test whether the association between HopQ1 and 14-3-3 is involved in the pathogen recognition process, PsyB728a was engineered to express HopQ1 variants. Next, whole N. benthamiana plants were inoculated by dipping in bacterial suspensions and then plants were incubated for 10 days. As observed with the tobacco wildfire pathogen P. syringae pv. tabaci 11528 (Wei et al., 2007), expression of HopQ1 or HopQ1-S51A rendered PsyB728a avirulent (Figure 8B). In contrast, plants infected by the control strain of PsyB728a, which carried the mCherry protein construct, were severely affected and eventually died. In summary, these observations suggest that the mutation that inhibits 14-3-3 binding does not interfere with HopQ1 perception by
tobacco plants. These findings are also consistent with a model in which the association between HopQ1 and 14-3-3 represents a part of the pathogenic strategy. Previous studies demonstrated that HopQ1 expression positively affected growth of _P. syringae_ in common bean cv. Red Mexican (Ferrante et al., 2009). To assess the functional relevance of 14-3-3 binding in HopQ1 virulence we employed a competitive index (CI) assay (Macho et al., 2007). Plasmids expressing HopQ1 variants were introduced to _P. syringae_ pv. _tomato_ DC3000D28E, a strain deficient in 28 effectors. Bean leaves were infiltrated with a mixed inoculum of strains expressing HopQ1 or HopQ1-S51A. At selected time points bacteria were isolated from leaf tissue and plated. To differentiate the strains, resulting colonies were replicated onto new plates containing appropriate antibiotics. CI was calculated as the wild-type - to - mutant ratio within output sample normalized for bacterial load. As shown in Figure 9, the strain expressing wild-type HopQ1 was a superior competitor since the CI was significantly different from 1. The differences between HopQ1 and HopQ1-S51A properties were subtle, yet reproducible. Although modification of the 14-3-3–binding motif did not attenuate virulence dramatically under growth chambers conditions, it may result in a significant reduction of pathogen fitness in the field (Wichmann and Bergelson, 2004).

**Discussion**

Previous studies have suggested that HopQ1 plays a role in determining the host range of _P. syringae_; however, its mode of action inside the plant cell has remained unclear. This study shows that HopQ1 associates with plant 14-3-3 proteins in a phosphorylation-dependent manner. The HopQ1 sequence contains one putative 14-3-3–binding site of high affinity encompassing the phosphorylated S51. Strikingly, this motif resembles the most typical plant 14-3-3 binding site, with leucine residue at position -5 (with respect to S51 residue), arginine residue at position -3 and serine residue at position -2 (Johnson et al., 2010). A single substitution within this motif (HopQ1-S51A) abrogates 14-3-3 binding (Table II and III and Supplemental Figure S4). The S51 represents possibly the major phosphorylation site of HopQ1 since site-directed mutation that alters this residue almost completely eliminated _in vitro_ phosphorylation of HopQ1 in contrary to other substitutions tested (Supplemental Figure S2). Mass spectrometric analysis of recombinant HopQ1-6xHis produced in _P. syringae_ pv. _tabaci_ DAPP-PG677 did not reveal any phosphopeptides (F. Giska,
unpublished data). This finding indicates that phosphorylation of HopQ1 occurs upon delivery of the effector into plant cells. The context of amino acids surrounding S51 indicates that the 14-3-3 binding site of HopQ1 can be recognized and phosphorylated by plant kinases from CDPK-SnRK family. They are basophilic and have preference for arginine residue at the position -3 (Vlad et al., 2008), and importantly many of them are activated in response to various environmental cues including pathogen attack (Romeis et al., 2001; Kulik et al., 2011). Consistently, HopQ1 is phosphorylated in vitro (Figure S3) by representatives of this family (CPK3 and SnRK2.4) but it deserves to be examine more closely in vivo.

It is assumed that client proteins with two low affinity binding sites bind to dimeric 14-3-3 but not to monomeric 14-3-3 forms, while proteins with a high affinity site bind also to monomeric form (Tzivion et al., 2001). Medium stringency Scansite search and ELM prediction [The Eukaryote Linear Motif resource for Functional Sites in Proteins, http://elm.eu.org/links.html (Gould et al., 2010)] revealed in HopQ1 one (20-34 aa) or two (24-29 aa, 73-78 aa) additional putative 14-3-3 binding sites, respectively (Supplemental Table SI). This suggests that HopQ1 might interact with 14-3-3 monomerically (via the S51-containing motif) or cooperatively with the dimeric form of 14-3-3. Our structural studies (manuscript in preparation) suggest that this latter possibility looks more likely since the stoichiometry of the HopQ1 and 14-3-3 complex seems to be 1:2. However, the motif encompassing S51 is a dominant binding site as a single amino acid substitution that alters this motif completely abolished the interaction with 14-3-3s (Table II, Table III, Table IV). Interestingly, one of the 14-3-3 binding sites overlaps with predicted by ELM phosphodegron sequence (22-29 aa). Phosphorylation within phosphodegrons often initiates the ubiquitination and proteolytic degradation cycle for proteasome substrates. As HopQ1 was more stable than HopQ1-S51A protein (Figure 7) when expressed in N. benthamiana it might suggest that protection of the phosphodegron by 14-3-3 protein could account for the differences in the protein steady state levels. Consistently, disruption of HopQ1-14-3-3 complex by the competitive inhibitor R18 significantly reduced HopQ1 stability in plant extracts (Figure 7). Strikingly, some viruses use the same phosphodegron motif to perturb cellular regulation (Dinkel et al., 2012) suggesting it can also play a similar role in bacterial pathogenesis.

While the HopQ1 mutated to eliminate 14-3-3 binding (HopQ1-S51A) is still recognized by the tobacco surveillance system (Figure 8), its ability to enhance P.
syringae pv. tomato DC3000D28E growth in bean plants is slightly reduced (Figure 9). This finding suggests that while the interaction with 14-3-3s might contribute to HopQ1 virulence, it is not related to avirulence. This suggestion is further supported by the presence of one or more predicted (Gould et al., 2010) 14-3-3–binding sites among HopQ1 xenologs from the genus Xanthomonas (XopQ, Xanthomonas outer protein Q) and from R. solanacearum (RipB, Ralstonia effector injected into plant cells B). In addition, RipB co-purified with isoforms 14-3-3e and 14-3-3i when transiently expressed in N. benthamiana (M. Piechocki, unpublished data). We have not observed drastic reduction in virulence of hopQ1-S51A (Figure 9). It is not unexpected since wild-type HopQ1 caused only a slight increase in P. syringae pv. tabaci DAPP-PG677 growth (5 fold) whereas P. syringae pv. phaseolicola 1448AΔhopQ1 showed no growth attenuation in bean plants (Macho et al., 2012).

HopQ1 associated in vitro and in planta with several 14-3-3 isoforms from various phylogenetic groups and showed no apparent specificity for one isoform. Similarly, the TTSS effector XopN of X. campestris pv. vesicatoria has been shown to bind four tomato 14-3-3 isoforms in the yeast two hybrid system (Kim et al., 2009). However, not necessarily all interactions of HopQ1 with 14-3-3s were direct, since 14-3-3s can form homo- and hetero-dimers. It is often assumed that many 14-3-3 isoforms are functionally redundant while specificity of others can be achieved not only through protein structure but also due to distinct patterns of spatial and temporal expression, dimerization status and phosphorylation (Boer et al., 2012; Paul et al., 2012).

Interaction with 14-3-3 can affect various features of partner proteins: their enzymatic activity, half-life, subcellular localization and ability to bind other proteins. By interacting with 14-3-3, some bacterial pathogens have hijacked these properties. Exoenzyme S (ExoS), an ADP-ribosyl-transferase toxin secreted by the TTSS of Pseudomonas aeruginosa, becomes active upon an interaction with host 14-3-3 proteins (Ottmann et al., 2007). The nuclear accumulation of RolB, a bacterial oncoprotein involved in the pathogenesis of Agrobacterium rhizogenes, depends upon binding to a specific isoform of 14-3-3 (Moriuchi et al., 2004). Based on in silico predictions, HopQ1 is classified as a nucleoside hydrolase; however, this activity could not be detected in the recombinant protein produced in E. coli. The E. coli ribonucleoside hydrolase RihA (Petersen and Møller, 2001) is active under the same conditions (data not shown). Although it might be expected that post-translational modification and/or 14-3-3 binding could result in catalytically-active HopQ1 in planta,
no increase in nucleoside hydrolase activity was found between protein extracts obtained from *N. benthamiana* control plants and plants transiently expressing HopQ1 (data not shown). It remains possible that HopQ1 nucleoside hydrolase activity requires low molecular weight co-factors that were lost during extraction and/or that its activity is exerted on substrates that have not yet been tested. While wild-type HopQ1 localizes primarily to the cytoplasm, HopQ1-S51A accumulates in the nucleus (Figure 5). Thus, the nuclear-cytoplasmic shuttling of HopQ1 might be regulated by its association with 14-3-3. Conversely, since HopQ1 co-purified with 14-3-3 isoforms from various phylogenetic groups and showed no apparent specificity for one isoform, it is possible that 14-3-3s might serve as a virulence target for the bacterial effector. Although members of this protein family are quite abundant, it has been hypothesized that some intracellular processes are regulated by temporal redistribution of 14-3-3s, limiting its availability for other partner proteins (Tzivion et al., 2000). HopQ1 could exert its virulence activity by retaining 14-3-3s in the cytoplasm, thereby interfering with host defense signaling and such a scenario might explain why 14-3-3a is redistributed when co-expressed with HopQ1. On the other hand, binding of 14-3-3s only contributes partially to HopQ1 virulence activity and so this interaction may play an accessory role, possibly in recruiting *bona fide* virulence targets. An association between HopQ1 and 14-3-3 could enable the binding of a putative virulence target via the second subunit of the 14-3-3 dimer. Questions pertaining to the catalytic activity of HopQ1 and whether or not multi-protein complexes are formed in plant cells remain to be explored.

**Materials and Methods**

**Plant material.** *Nicotiana benthamiana*, *Nicotiana tabacum* cv. Xanthi-nc and *Phaseolus vulgaris* cv. Red Mexican plants were grown in soil under controlled environmental conditions (21°C, 16 h light, 8 h dark), as described previously (Talarczyk et al., 2002).

**Recombinant protein preparation.** A sequence encoding HopQ1 (NC_007274.1) from *Pseudomonas syringae* pv. *phaseolicola* 1448A was cloned into the pET30a vector. For site-directed mutagenesis, target plasmids carrying hopQ1 were PCR-amplified with two phosphorylated primers. The primers were designed so that they annealed back to back with the plasmid, and one primer carried the desired
mutations. Following amplification with Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA; www.thermoscientific.com), templates were removed by digestion with DpnI (Thermo Fisher Scientific) and mutated PCR products were circularized with T4 DNA ligase (Thermo Fisher Scientific). The resulting plasmids were transformed into *Escherichia coli* DH5α and clones were screened by sequencing. Full-length cDNA encoding AtCPK3 (NC_003075) was cloned into the pGEX-6P-2 vector. GST-tagged AtSnRK2.4 (NC_003070.9) was a kind gift from Dr. G. Dobrowolska (Bucholc et al., 2011). Full-length cDNA encoding Nt14-3-3a-1 (AB119466) was first cloned into pGEM-T-easy Vector System and then re-cloned into pASK-IBA3 to express Nt14-3-3a-1 C-terminally fused with Strep-tag II. Recombinant protein expression was performed in *E. coli* (BL21 Rosetta) cells, induced either with 0.25 mM IPTG or 0.43 μM anhydrotetracycline hydrochloride (tet promoter), at 18°C for 4 h. His- and glutathione S-transferase (GST)-tagged recombinant proteins were purified using HisTrap and GSTrap HP columns, respectively (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA; www.gehealthcare.com). Strep-tagged proteins were purified using Strep-Tactin Macroprep columns (IBA GmbH, Göttingen, Germany; http://www.iba-go.com).

**Protein analysis.** Four leaf discs (diameter 1.2 cm) were collected and extracted in buffer containing 100 mM Tris HCl pH 8.0, 150 μM NaCl, 0.2% (v/v) Triton X-100 and protease inhibitor cocktail (BioShop Canada Inc, Burlington, Ontario, Canada, http://www.bioshopcanada.com). Protein content was measured by the Bradford method using a commercially available reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA; www.bio-rad.com). Protein samples were fractionated by 12.5% SDS-PAGE and subjected to immunoblot analysis using specific primary antibodies: monoclonal mouse anti-Flag (1:3000, SIGMA-ALDRICH), anti-HA (1:3000, Santa Cruz Biotechnology, www.scbt.com), anti-His (1:3000 for colorimetric or 1:10000 for chemiluminescent assay, SIGMA-ALDRICH), anti-GFP (1:3000, Covance, www.covance.com) and secondary anti-mouse antibodies conjugated either to alkaline phosphatase or horseradish peroxidase (both 1:10000, SIGMA-ALDRICH). Strep-tagged proteins were detected using Strep-Tactin AP conjugate (1:2000, IBA GmbH). Immunoblots were developed using the NBT/BCIP colorimetric detection kit from Roche Applied Science (Mannheim, Germany, www.roche-applied-science.com) or ECL Plus Western Blotting Reagents (GE Healthcare).
**In vitro phosphorylation assay.** Purified recombinant HopQ1-6xHis variants (~3 µg) were incubated with the appropriate recombinant protein kinase (~0.3 µg) or crude plant protein extract (~2 µg) in reaction buffer (40 mM Tris HCl [pH 8.0], 10 mM MgCl₂, 1 mM DTT, 0.1% [v/v] Triton X-100) containing 50 µM ATP supplemented with 1.5 µCi (γ³²P)ATP. For CPK3 kinase assays, 0.25 mM CaCl₂ was added to the buffer. Reactions were carried out at 30°C for 30 min and terminated by adding 4x Laemmli sample buffer. Samples were resolved by 12.5% SDS-PAGE and analyzed with a Phosphorimager or by autoradiography. For mass spectrometry, samples were prepared as described above but using non-radioactive ATP. For pull-down assays, phosphorylated HopQ1 was obtained by incubating 2 mg HopQ1 and 200 µg CPK3 kinase under the conditions described above.

**Strep-tag II affinity purification.** DNA fragments encoding HopQ1 gene variants were cloned into the pROK2 vector containing a (6xHis)-(Ala-Ala)-Strep-tag II-encoding sequence at the 3’-end. Next, the constructs were electroporated into A. tumefaciens (GV3101) cells, which were then infiltrated into 4-week-old N. benthamiana leaves, as described previously (Romeis et al., 2001). Infiltrated leaves were collected after 48 h and ground in liquid nitrogen. Samples were then thawed in Ex-Strep buffer (100 mM Tris HCl [pH 8.0], 150 mM NaCl, 10 mM DTT, 2 mM AEBSF, 5 µg·ml⁻¹ leupeptin, 5 µg·ml⁻¹ bestatin, 50 mM NaF, 1% [v/v] Phosphatase Inhibitor Cocktail 1 [SIGMA-ALDRICH, St. Louis, MO, USA; www.sigmaaldrich.com], 0.5% [v/v] Triton X-100 and 200 µg·ml⁻¹ avidin) and purified using Strep-Tactin MacroPrep (IBA GmbH, Göttingen, Germany; http://www.iba-go.com).

**Flag affinity purification.** RipB (YP_002258475) was amplified using purified Ralstonia solanacearum DNA as a template (a gift from Dr. Stephane Genin) and cloned into pENTR/D-TOPO vector and then moved using LR clonase into the appropriate pGWB vector. Following transient expression in N. benthamiana RipB-Flag was purified using EZ view Red Anti-FLAG M2 Affinity Gel (SIGMA-ALDRICH) and the samples were subjected to mass spectrometry analysis.

**Mass spectrometry analysis.** Samples were reduced with 100 mM DTT for 30 min at 56°C and then alkylated with iodoacetamide in darkness for 45 min at RT.
Following overnight digestion with sequencing grade-modified trypsin (Promega Corporation, Madison, WI, USA; www.promega.com) and gel separation, the resulting peptides were eluted from the gel with 0.1% TFA and 2% ACN. Liquid chromatography (LC)-mass spectrometry (MS) analyses were carried out using a nano-Acquity (Waters Corp., Milford, MA, USA; www.waters.com) LC system coupled to an LTQ FTICR (Thermo Fisher Scientific) mass spectrometer. Spectrometer parameters were as follows: capillary voltage, 2.5 kV; cone, 40 V; N₂ gas flow, 0; and range, 300–2000 (m/z). The spectrometer was calibrated on a weekly basis using Calmix (caffeine, MRFA, Ultramark 1621). Samples were loaded from the autosampler tray (cooled to 10°C) to the pre-column (Symmetry C18, 180 µm × 20 mm, 5 µm; Waters) using a mobile phase of 100% MilliQ H₂O acidified by 0.1% formic acid. The peptides were then transferred to a nano-UPLC column (BEH130 C18, 75 µm × 250 mm, 1.7 µm; Waters) with a gradient of 5–30% acetonitrile, 0.1% FA over 45 min. The column outlet was coupled directly to the ESI ion source of the LTQ-FTICR (Thermo Fisher Scientific) mass spectrometer, working in the data-dependent mode to perform the switch automatically between MS and MS/MS. A blank run preceded each analysis to ensure the absence of cross contamination between samples.

**Database search procedures.** After preprocessing of the raw data with Mascot Distiller software (version 2.1.1, Matrix Science), output lists of precursor and product ions were compared to the NCBInr database using the Mascot database search engine (v 2.1, Matrix Science). Search parameters included semi-trypsin enzyme specificity, one missed cleavage site, Cys carbamidomethyl fixed modification and variable modifications including Met oxidation and phosphorylation of S, T or Y residues. The protein mass and taxonomy were unrestricted, peptide mass tolerance was 20 ppm and MS/MS tolerance was 0.8 Da. Proteins containing peptides with Mascot cut-off scores >50, which indicated identity or extensive homology (p < 0.05) of peptides, were considered positive identifications.

**HopQ1-14-3-3a complex formation and size exclusion chromatography.** Recombinant HopQ1 tagged with 6xHis and CPK3 kinase fused to GST were expressed in *E. coli* and purified by affinity chromatography. Then HopQ1-6xHis was in vitro phosphorylated by CPK3. To check phosphorylation status of HopQ1 the
reaction mixture was separated by SDS-PAGE and stained with Pro-Q Diamond. To remove the CPK3 kinase, the reaction was loaded onto GST-binding column. HopQ1 was further purified by ion-exchange chromatography (Q-Sepharose column, GE Healthcare) and size-exclusion chromatography (Superdex 200 10/300GL, GE Healthcare). As control non-phosphorylated form of HopQ1-6xHis was purified using affinity chromatography, ion exchange chromatography and size exclusion chromatography. The recombinant 14-3-3a protein was produced in E. coli in fusion with Strep-tag II, and purified by affinity chromatography and ion-exchange chromatography. To reconstitute the complex, phosphorylated HopQ1 was mixed with 14-3-3a at 2:1 mass ratio, and incubated in buffer containing 20mM Tris-HCl, 150 mM NaCl, 5mM DTT, pH 8.0 for 2 h at 4°C on a rotator. Non-phosphorylated HopQ1 was incubated with 14-3-3a under the same conditions. To check formation of the complex both reaction mixtures were analyzed by size exclusion chromatography in buffer containing 20mM Tris-HCl, 150 mM NaCl, pH 8.0. To determine the retention volume for the unbound 14-3-3a fraction, a sample containing only 14-3-3a was run.

**Constructs for subcellular localization experiments and FRET-FLIM measurements.** HopQ1 sequence variants or full-length cDNA encoding Nt14-3-3a were cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA; www.invitrogen.com). PCR amplification was performed using reverse primers without the native stop codons to fuse ORFs in-frame with sequences encoding C-terminal tags. To generate expression clones, LR recombination was performed using the entry clones obtained and appropriate destination vectors from the pGWB Series (Nakagawa et al., 2007; Nakagawa et al., 2007), i.e., pGWB441, pGWB444 and pGWB454. To generate a construct expressing eCFP_eYFP chimeric fusion protein as a positive control, the eYFP cDNA was cloned into pSAT6-eCFP and subcloned into the destination vector PZP-RCS2. Subsequently, *Agrobacterium* cultures containing the constructs were infiltrated into *N. benthamiana* leaves and, after 72 h, tissues were analyzed using confocal laser scanning microscopy.

**Microbombardment.** Leaves from 2- to 4-week-old plants were used to determine the subcellular locations of HopQ1-eYFP and 14-3-3a-mRFP fusion proteins in *P. vulgaris*. For transient gene expression in epidermal cells, plasmid DNA (2 µg) was
adsorbed onto tungsten M17 particles (diameter 1.1 µm, 350 µg) and then microbombardment was performed at a pressure of 1100 psi using a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories Inc., Hercules, CA, USA; www.bio-rad.com). Tissues were analyzed 24 h after bombardment.

Confocal laser scanning fluorescence microscopy. Transient intracellular fluorescence was observed by confocal laser scanning microscopy using a Nikon TE2000E EZ-C1 inverted confocal microscope equipped with 60x oil immersion objective lens (numerical aperture = 1.4). eYFP was excited with the 488 nm line from an argon ion laser and images of mRFP were obtained using a 543 nm HeNe laser excitation. eYFP and mRFP fluorescence signals were detected using the 515/30 and 605/75 emission filters, respectively. Scanning was performed in sequential mode to prevent bleed-through. Images were collected in z-stack series at 0.5 µm focus interval and carefully processed using freeware ImageJ. For publication, single optical sections with distinctly visible nucleoli were selected to ensure that similar focal planes were compared for all tested variants. Fluorescence Lifetime Imaging Microscopy (FLIM) was performed, as described previously (Kwaaitaal et al., 2010).

Analysis of HopQ1 steady-state levels. To generate clones expressing HopQ1 variants C-terminally tagged with 3xHA or Flag epitopes, LR recombination was performed using the appropriate pENTR/D-TOPO constructs and destination vectors from the pGWB series.

To check protein levels of HopQ1 variants transiently expressed in N. benthamiana leaves, Agrobacterium cultures containing the hopQ1-3xHA constructs were co-infiltrated with the strains expressing GFP and after 48 h tissues were analyzed by immunodetection using anti-HA and anti-GFP antibodies.

To analyze steady-state level of HopQ1 variants in P. syringae, P. syringae pv. tabaci DAPP-PG677 (kind gift of Dr. B. Vinatzer) strain was transformed with pBBR1MCS-2 plasmids encoding hopQ1 or hopQ1-S51A in fusion with 6xHis epitope. The strains were cultured overnight in KB medium. Subsequently, 1.5 ml bacterial cultures were collected and centrifuged. The pellets were suspended in 300 µl PBS buffer. The samples were subjected to immunoblot analysis using anti-His antibody. The strain carrying pBBR1MCS-2 encoding mcherry gene was used as a control.
To demonstrate role of 14-3-3 binding in HopQ1 stability, a competitive antagonist peptide R18 (Enzo Life Sciences, Exeter, UK, www.enzolifesciences.com) was used in the next two experiments. *Agrobacterium* cultures containing the hopQ1-Flag constructs were infiltrated into *N. benthamiana* leaves and, after 48 h, tissues were collected, ground in liquid nitrogen and thawed in buffer: 50 mM Tris-HCl, 75 mM NaCl, 50 mM NaF, 1x Phosphatase inhibitor cocktail (Sigma), 5 mM DTT, 0.2% Triton, pH 7.0. Samples containing 50 µg crude plant protein extract were supplemented with 5 µg recombinant 14-3-3a-Strep-tag II protein produced in *E. coli* and/or R18 peptide at concentration 5 mM, 150 mM and 300 mM or alternatively with 300 mM R18 and 10, 25 or 50 µg 14-3-3a-Strep-tag II, as indicated. Following incubation for 60 minutes at 30°C, the samples were fractionated by 12.5% SDS-PAGE and subjected to immunoblot analyses with anti-Flag antibody or Strep-Tactin AP conjugate.

For studies of HopQ1 stability in extracts from *P. vulgaris*, 5 µg the *in vitro* reconstituted HopQ1-14-3-3a complex was added to 50 µg bean crude protein extract (obtained, as above described). Then, R18 peptide at concentration 300 mM was added to the appropriate samples. In another experiment 10 µg *in vitro* phosphorylated HopQ1 was added to 50 µg bean crude protein extract and supplemented with 12 µg 14-3-3a or BSA. In both experiments, the samples were incubated for 60 minutes at 30°C, fractionated by 12.5% SDS-PAGE and subjected to immunoblot analyses with anti-His antibody or Strep-Tactin AP conjugate.

**Assessment of the hypersensitive response in *N. tabacum***. *Agrobacterium* cultures (OD_{600}=1) containing pGWB411-derived plasmids were infiltrated into fully-expanded leaves of 6-week-old *N. tabacum* cv. Xanthi-nc plants. After 48 h, symptoms of HR were observed and photographed.

**Pseudomonas syringae strains and inoculation.** The broad host-range plasmids pBBR1MCS-2 and pBBR1MCS-5 (Kovach et al., 1994) were used to express *hopq1* variants in *P. syringae*. The tac promoter was amplified from pGBT30 using a forward primer that added a SacI restriction site to the 5’ end and a reverse primer that added a ribosome-binding site in front of the BamHI restriction site at the 3’ end of the product. The promoter-containing fragment was cloned into pBBR1MCS-2. *hopq1, hopq1-S51A* or *mcherry* sequences were amplified with primers that introduced
BamHI and XhoI sites to the opposite ends of the products and then these fragments were cloned downstream of the tac promoter. The constructs were electroporated into \textit{P. syringae pv. syringae} B728a and \textit{P. syringae pv. tomato} DC3000D28E (kind gifts of Drs. J. Greenberg and A. Collmer, respectively). The strains were maintained on nutrient agar and stored at 18°C. Bacteria were prepared for inoculation, as described previously (Krzymowska et al., 2007), with the exception that following centrifugation at 3,500 \textit{g} for 10 min, the pellet was washed once and resuspended in sterile 10 mM MgCl$_2$. The bacterial suspension was adjusted to OD$_{600}$=0.2 and further diluted (assuming OD$_{600}$=0.2 corresponds to ca. 10$^8$ CFU ml$^{-1}$), as indicated. Bacterial titers were checked by plating.

For avirulence assays, bacterial suspensions were adjusted to 10$^8$ CFU ml$^{-1}$ in milliQ water and supplemented with Silwet L-77 (0.02%). Five-week-old \textit{N. benthamiana} plants were dip-inoculated by inverting whole plants into bacterial suspensions and gently agitating for 30 s. Following inoculation, plants were placed immediately under a plastic dome to maintain high humidity levels for 24 h. Development of symptoms was assessed within 10 days.

For virulence assays, \textit{P. syringae pv. tomato} DC3000D28E derivatives expressing HopQ1 variants were mixed at equal CFUs prior to inoculation (10$^5$ CFU ml$^{-1}$). Bacterial suspensions were infiltrated into leaves of 2-week-old \textit{P. vulgaris} cv. Red Mexican plants using a needleless hypodermic syringe. At selected time points, two 1 cm-diameter leaf discs were cut from infiltrated zones in each plant. Discs were superficially sterilized with 70% ethanol for 1 min, rinsed with sterile water for 1 min and then ground in 300 µl 10 mM MgCl$_2$. Serial dilutions were plated onto KB agar plates. The bacteria were grown at 28°C and after 2 days replicated onto plates containing either kanamycin or gentamicin, which enabled strain differentiation and CFU counting. The competitive index (CI) was calculated as described previously (Macho et al., 2007). CI was defined as the ratio of the strain carrying wild-type hopQ1 to the strain expressing hopQ1S51A within the outputs samples, divided by the corresponding ratios in the input inocula. Data are reported as means ± standard deviation (SD). Mean results were calculated from five plants for each variant.

**Statistical analysis.** Data are reported as the mean ± standard deviation (SD). The results were compared statistically by using a two-tailed Student t test, and
differences were considered significant if P values were <0.05 or as indicated in the legends.

**Pull-down assays.** Recombinant HopQ1 (0.5 mg) was phosphorylated in vitro and incubated with 30 ml plant extracts (1 µg µl⁻¹) for 2 h at 4°C in buffer containing 50 mM NaH₂PO₄, 150 mM NaCl, 2 mM AEBSF, 5 µg·ml⁻¹ leupeptin, 5 µg·ml⁻¹ bestatin, 50 mM NaF, 1% (v/v) Phosphatase Inhibitor Cocktail 1, 0.5% Triton X-100 and 20 mM imidazole (pH 8.0). Samples were loaded onto a HisTrap HP column, washed with 20 column volumes of His-tag wash buffer (50 mM NaH₂PO₄, 150 mM NaCl and 20 mM imidazole [pH 8.0]), and then bound proteins were eluted by the addition of 500 mM imidazole. Mass spectrometry was used to identify proteins that co-purified with HopQ1. Non-phosphorylated 6xHis-HopQ1 was used as a negative control.

**Supplemental Data**

Following materials are available in the online version of this article.

**Supplemental Table SI.** 14-3-3 binding motifs are ubiquitously present in TTSS effectors from *P. syringae* pv. *phaseolicola* 1448A.

**Supplemental Table SII.** Various 14-3-3s isoforms from *P. vulgaris* interact with recombinant HopQ1-6xHis in a phosphorylation-dependent manner.

**Supplemental Table SIII.** List of primers used for constructions.

**Supplemental Figure S1.** Kinase activity capable of phosphorylating HopQ1 is ubiquitously conserved in plants.

**Supplemental Figure S2.** Serine 51 is the major phosphorylation site of HopQ1.

**Supplemental Figure S3.** CPK3 and SnRK2 phosphorylate HopQ1 in vitro.

**Supplemental Figure S4.** Interaction between HopQ1 variants and 14-3-3a in planta.

**References**


Tzivion G, Shen YH, Zhu J (2001) 14-3-3 proteins; bringing new definitions to scaffolding. Oncogene 20: 6331-6338


Figure legends

**Figure 1.** The 14-3-3 binding site is conserved in HopQ1, the TTSS effector from *Pseudomonas syringae*, and XopQ, its xenolog from *Xanthomonas* spp. The canonical mode-1 14-3-3–interacting motif is shaded in gray and the putative phosphoserine is highlighted in red.

**Figure 2.** The predicted 14-3-3–binding motif of HopQ1 is phosphorylated by plant kinases. (A, B) HopQ1-Strep-tag II fusion protein expressed in *N. benthamiana* leaves was affinity-purified and subjected to LC-MS-MS/MS analysis. (C, D) HopQ1-6xHis expressed in bacteria was incubated with total protein extracts from *N. benthamiana* and then affinity-purified and analyzed by LC-MS-MS/MS. Panels A and C show the fragmentation spectra with peak assignment to b, y, b-H$_3$PO$_4$ and y-H$_3$PO$_4$, with “–P” denoting loss of a H$_3$PO$_4$ group. Major signals of the MS/MS spectra are identified by their corresponding fragment tags of the b, y series, but also of the y-H$_3$PO$_4$ and b-H$_3$PO$_4$ series, which were expected in the case of a phosphorylated peptide. Panels B and D show the peptide sequences with daughter ions of the b, y, b-H$_3$PO$_4$ and y-H$_3$PO$_4$ series found in the spectra. In the peptide derived from HopQ1 phosphorylated *in vitro* (C, D), the presence of the full series of b2-b10 fragments and the expected b-H$_3$PO$_4$ series allows for unequivocal localization of the phosphate at Ser 51. In the peptide derived from HopQ1 expressed *in planta* (A, B), the majority of b and b-H$_3$PO$_4$ pairs are also present. In addition, the presence of strong y, y-PO$_3$ and y-H$_3$PO$_4$ signals indicate that the site of phosphorylation is S51 and not S49.

**Figure 3.** Serine 51 plays a critical role in the phosphorylation of HopQ1. Recombinant HopQ1 variants with a C-terminal 6xHis epitope were incubated with leaf protein extracts from *N. benthamiana* in buffer containing [γ³²P]ATP. Samples were resolved by SDS-PAGE and analyzed by autoradiography. Only residual phosphorylation was detected for HopQ1 mutated at position 51. As a loading control the Coomassie Brilliant Blue (CBB) stained gel is shown in lower panel. The experiment was performed three times with similar results.

**Figure 4.** HopQ1 binds to 14-3-3a in phosphorylation dependent manner. Representative gel filtration runs on a Superdex 200 column. Recombinant HopQ1 with a C-terminal 6xHis epitope was incubated with recombinant CPK3 prior binding
to 14-3-3a (orange trace). As a control non-phosphorylated HopQ1 was used (green trace). Under conditions used (5mM DTT) HopQ1 exists as a monomer (elution volumes 14.68, 14.69). 14-3-3a was eluted as dimers (peaks 13.63, 13.72). Recombinant CPK3 was removed from the reaction by affinity capture on GST-column. The experiment was performed twice with similar results.

Figure 5. Subcellular localization of HopQ1 variants. Confocal images of representative *N. benthamiana* leaf epidermal cells (upper panel) or mesophyll cells (lower panel) transiently expressing either wild-type HopQ1-eYFP (left panel) or HopQ1-S51A-eYFP (right panel). White arrowheads indicate the nuclei. Scale bars represent 10 µm. The pictures were taken 72 h after agroinfiltration. For each variant ca. 50 transformed cells were examined.

Figure 6. Co-expression of 14-3-3a and HopQ1 affects nuclear-cytoplasmic partitioning of the binding partners. (A) *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* strains carrying constructs encoding 14-3-3a-mRFP and variants of HopQ1 fused to eYFP. For each variant ca. 50 transformed cells were examined. (B) The same constructs were transiently co-expressed in *P. vulgaris* epidermal cells via particle bombardment. In both plant species, 14-3-3a-mRFP localized to the cytoplasm and nucleus. Co-expression with wild-type HopQ1-eYFP resulted in the relocation of 14-3-3a-mRFP from the nucleus to the cytoplasm. A non-interacting form of HopQ1 (HopQ1-S51A-eYFP) shows highly increased nuclear accumulation and does not alter the localization of 14-3-3a-mRFP. For each variant ca. 20 transformed cells were examined. White arrowheads indicate the nuclei. Scale bars represent 10 µm.

Figure 7. Interaction with 14-3-3 proteins increases HopQ1 stability in plants. (A) The presence of Serine 51 affects HopQ1 steady-state level in planta. Wild-type HopQ1 or HopQ1-S51A proteins carrying C-terminal 3xHA epitopes were transiently co-expressed with GFP in *N. benthamiana* leaves. Crude protein extracts (20 µg proteins per lane) were isolated from leaves 48h after agroinfiltration. HopQ1 variants were detected by immunoblot analysis using an antibody specific to HA. As an expression control the level of GFP was checked using anti-GFP antibody. The experiment was repeated twice. (B) Modification of 14-3-3 binding motif does not
change HopQ1 stability in *P. syringae*. C-terminally His-tagged HopQ1 variants were expressed in *P. syringae* pv. *tabaci* DAPP-PG677. Crude protein extracts prepared from overnight bacterial cultures were fractionated on a 12.5% SDS-PAGE and subjected to immunoblot analysis using a specific anti-His antibody. Equal protein loading was shown by Ponceau Red staining. The experiment was repeated twice. (C) R18, the inhibitor of 14-3-3 binding affects HopQ1 stability in plant extracts. Wild-type HopQ1 protein carrying a C-terminal Flag epitope was transiently expressed in *N. benthamiana* leaves. Crude protein extract was supplemented with recombinant 14-3-3a-Strep II protein isolated from *E. coli* or R18 peptide at various concentrations, as indicated. HopQ1 was detected by immunoblot analysis using specific primary antibodies. The level of 14-3-3a was monitored using Strep-Tactin AP conjugate. Equal protein loading was shown by Ponceau Red staining. (D) Application of 14-3-3a reverts the effect of R18 on HopQ1 stability. Wild-type HopQ1 protein carrying a C-terminal Flag epitope was transiently expressed in *N. benthamiana* leaves. Crude protein extract was supplemented with R18 and increasing amounts of recombinant 14-3-3a-Strep II protein isolated from *E. coli*, as indicated. HopQ1 was detected by immunoblot analysis using specific primary antibodies. The level of 14-3-3a was monitored using Strep-Tactin AP conjugate. Equal protein loading was shown by Ponceau Red staining. (E) Assembled in vitro complex of HopQ1-6xHis and 14-3-3a-Strep II was incubated with bean crude protein extract in the absence or presence of the R18. HopQ1 and 14-3-3a were detected by immunoblot analyses using anti-His antibodies or Strep-Tactin AP conjugate, respectively. Equal protein loading was shown by Ponceau Red staining. (F) In vitro phosphorylated HopQ1-6xHis was added to bean crude extract and incubated in the presence of 14-3-3a or BSA. HopQ1 and 14-3-3a were detected by immunoblot analyses using anti-His antibodies or Strep-Tactin AP conjugate, respectively. Equal protein loading was shown by Ponceau Red staining.

**Figure 8.** HopQ1 interaction with 14-3-3s is not critical for its perception by host plants. (A) Binary vectors encoding either wild-type HopQ1 or HopQ1-S51A were introduced via agroinfiltration into leaves of *Nicotiana tabacum*. Hypersensitive response developed in the infiltrated area within 48h, in response to both *Agrobacterium* strains. In contrast, no macroscopic signs of tissue necrotization developed in control leaves expressing GFP. (B) *Nicotiana benthamiana* plants were
inoculated with *Pseudomonas syringae* pv. *syringae* B728a strains carrying pBBR1-MCS2 derivatives, which express either HopQ1, HopQ1-S51A or Cherry protein, as a control. Disease symptoms developed only in control plants treated with *P. syringae* pv. *syringae* B728a encoding Cherry protein. The pictures were taken 10 days post-inoculation. The experiment was performed twice, with similar results.

**Figure 9.** Assessment of virulence properties of HopQ1 effector mutated to eliminate 14-3-3 binding. Bean leaves were inoculated with *P. syringae* pv. *tomato* DC3000D28E (~10^5 CFU ml^-1) strains expressing HopQ1 or HopQ1-S51A. Immediately prior to infiltration, bacteria were mixed in a 1:1 ratio. Two and six days post inoculation, two leaf discs per plant were cut out from the infiltrated zones, ground in sterile 10 mM MgCl₂, diluted and plated on KB medium. Bacterial strains were distinguished by a selectable marker. The competitive index was calculated as the ratio of bacteria expressing the wild-type HopQ1 to bacteria expressing the mutated HopQ1 isolated from plant leaf and normalized to the input titers of the bacteria. Asterisks indicate that the index is significantly different from one, as established using Student's *t*-test (*P*<0.01). The experiment was performed three times with similar results.
Tables

**Table I.** LC-MS-MS/MS analysis data showing 14-3-3 family isoforms that co-purify with HopQ1-Strep II transiently expressed in *N. benthamiana* leaves.

<table>
<thead>
<tr>
<th>14-3-3 isoform</th>
<th>Sequence coverage in separate experiments(^{(a)})</th>
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<tbody>
<tr>
<td>a</td>
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<td>14</td>
</tr>
<tr>
<td>b</td>
<td>40</td>
<td>29</td>
</tr>
<tr>
<td>c</td>
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<tr>
<td>i</td>
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</table>

\(^{(a)}\) Protein coverage parameters calculated by Mascot software are shown for each experiment. Protein coverage parameters for HopQ1 in these experiments were 61, 36, 23 and 65, respectively.

\(^{(b)}\) Protein coverage ‘0’ refers to the 14-3-3 isoform that has not been identified by LC-MS-MS/MS analysis in this experiment.
Table II. LC-MS-MS/MS analysis data showing 14-3-3 family isoforms that co-purify with variants of HopQ1-Strep II transiently expressed in *N. benthamiana* leaves.

<table>
<thead>
<tr>
<th>14-3-3 isoform</th>
<th>HopQ1-S51A (53, 43)</th>
<th>HopQ1-S49A (45,63)</th>
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<tr>
<td>a</td>
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<td>b</td>
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<tr>
<td>c</td>
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\(^{(a)}\) Protein coverage parameters calculated by Mascot software are shown for each experiment. Protein coverage parameters for HopQ1-S51A and HopQ1-S49A in these experiments are shown in parentheses.

\(^{(b)}\) Protein coverage ‘0’ refers to the 14-3-3 isoform that has not been identified by LC-MS-MS/MS analysis in this experiment.
Table III. FRET-FLIM analysis showing that HopQ1 S51 is critical for 14-3-3a binding.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>$\tau$ (a) [ns]</th>
<th>$E$ (b) [%]</th>
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<td>eCFP</td>
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<td>eCFP-eYFP$^{(c)}$</td>
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<td>HopQ1-S51A-eYFP</td>
<td>2.23 ± 0.05</td>
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</table>

(a) $\tau$, mean donor lifetimes ± SD in the presence ($\tau_{DA}$) or absence of the acceptor ($\tau_D$).

(b) $E$, FRET efficiency, $E=1-(\tau_{DA}/\tau_D)100\%$.

(c) The positive control, eCFP-eYFP fusion protein, establishes a maximal detectable level of FRET under imaging conditions used.
Table IV. Various 14-3-3s isoforms from *N. benthamiana* interact with recombinant HopQ1-6xHis in a phosphorylation-dependent manner.

<table>
<thead>
<tr>
<th>14-3-3 isoform</th>
<th>Non-phosphorylated HopQ1-6xHis (a)</th>
<th>In vitro phosphorylated HopQ1-6xHis (b)</th>
<th>In vitro phosphorylated HopQ1-S51A-6xHis (b)</th>
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<tbody>
<tr>
<td>a</td>
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<td>47&lt;sup&gt;(c)&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>b</td>
<td>0</td>
<td>0&lt;sup&gt;(c)&lt;/sup&gt;</td>
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<tr>
<td>c</td>
<td>0</td>
<td>44</td>
<td>0</td>
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</tr>
<tr>
<td>i</td>
<td>0</td>
<td>23</td>
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</table>

<sup>(a)</sup> Recombinant HopQ1-6xHis purified from *E. coli* was incubated with *N. benthamiana* crude protein extract. After consecutive purification step the sample was subjected to LC-MS-MS/MS analysis. Protein coverage for HopQ1 was 87.

<sup>(b)</sup> Recombinant HopQ1-6xHis and HopQ1-S51A-6xHis were phosphorylated *in vitro* prior to incubation with *N. benthamiana* crude protein extract. After consecutive purification steps the samples were subjected to LC-MS-MS/MS analysis. Protein coverage parameters for HopQ1 and HopQ1-S51A in these experiments were 83 and 86, respectively.

<sup>(c)</sup> Protein coverage parameters of 14-3-3 isoforms that co-purified with HopQ1-6xHis calculated by Mascot software following LC-MS-MS/MS analysis.

<sup>(d)</sup> Protein coverage ‘0’ refers to the 14-3-3 isoform that has not been identified by LC-MS-MS/MS analysis in this experiment. The experiment was performed twice.
**Figure 1.** The 14-3-3 binding site is conserved in HopQ1, the TTSS effector from *Pseudomonas syringae*, and XopQ, its xenolog from *Xanthomonas* spp. The canonical mode-1 14-3-3 interacting motif is shaded in gray and the putative phosphoserine is highlighted in red.
Figure 2. The predicted 14-3-3 binding motif of HopQ1 is phosphorylated by plant kinases. (A, B) HopQ1-Strep-tag II fusion protein expressed in *N. benthamiana* leaves was affinity-purified and subjected to LC-MS-MS/MS analysis. (C, D) HopQ1-6xHis expressed in bacteria was incubated with total protein extracts from *N. benthamiana* and then affinity-purified and analyzed by LC-MS-MS/MS. Panels A and C show the fragmentation spectra with peak assignment to b, y, b-H$_3$PO$_4$ and y-H$_3$PO$_4$, with “P” denoting loss of a H$_3$PO$_4$ group. Major signals of the MS/MS spectra are identified by their corresponding fragment tags of the b, y series, but also of the y-H$_3$PO$_4$ and b-H$_3$PO$_4$ series, which were expected in the case of a phosphorylated peptide. Panels B and D show the peptide sequences with daughter ions of the b, y, b-H$_3$PO$_4$ and y-H$_3$PO$_4$ series found in the spectra. In the peptide derived from HopQ1 phosphorylated *in vitro* (C, D), the presence of the full series of b2-b10 fragments and the expected b-H$_3$PO$_4$ series allows for unequivocal localization of the phosphate at Ser 51. In the peptide derived from HopQ1 expressed *in planta* (A, B), the majority of b and b-H$_3$PO$_4$ pairs are also present. In addition, the presence of strong y, y-PO$_3$ and y-H$_3$PO$_4$ signals indicate that the site of phosphorylation is S51 and not S49.
Figure 3. Serine 51 plays a critical role in the phosphorylation of HopQ1. Recombinant HopQ1 variants with a C-terminal 6xHis epitope were incubated with leaf protein extracts from *N. benthamiana* in buffer containing [*γ^32P*]ATP. Samples were resolved by SDS-PAGE and analyzed by autoradiography. Only residual phosphorylation was detected for HopQ1 mutated at position 51. As a loading control the Coomassie Brilliant Blue (CBB) stained gel is shown in lower panel. The experiment was performed three times with similar results.
**Figure 4.** HopQ1 binds to 14-3-3a in phosphorylation dependent manner. Representative gel filtration runs on a Superdex 200 column. Recombinant HopQ1 with a C-terminal 6xHis epitope was incubated with recombinant CPK3 prior binding to 14-3-3a (orange trace). As a control non-phosphorylated HopQ1 was used (green trace). Under conditions used (5mM DTT) HopQ1 exists as a monomer (elution volumes 14.68, 14.69). 14-3-3a was eluted as dimers (peaks 13.63, 13.72). Recombinant CPK3 was removed from the reaction by affinity capture on GST-column. The experiment was performed twice with similar results.
Figure 5. Subcellular localization of HopQ1 variants. Confocal images of representative *N. benthamiana* leaf epidermal cells (upper panel) or mesophyll cells (lower panel) transiently expressing either wild-type HopQ1-eYFP (left panel) or HopQ1-S51A-eYFP (right panel). White arrowheads indicate the nuclei. Scale bars represent 10 µm. The pictures were taken 72 h after agroinfiltration. For each variant ca. 50 transformed cells were examined.
Figure 6. Co-expression of 14-3-3a and HopQ1 affects nuclear-cytoplasmic partitioning of the binding partners. (A) N. benthamiana leaves were co-infiltrated with A. tumefaciens strains carrying constructs encoding 14-3-3a-mRFP and variants of HopQ1 fused to eYFP. For each variant ca. 50 transformed cells were examined. (B) The same constructs were transiently co-expressed in P. vulgaris epidermal cells via particle bombardment. In both plant species, 14-3-3a-mRFP localized to the cytoplasm and nucleus. Co-expression with wild-type HopQ1-eYFP resulted in the relocation of 14-3-3a-mRFP from the nucleus to the cytoplasm. A non-interacting form of HopQ1 (HopQ1-S51A-eYFP) shows highly increased nuclear accumulation and does not alter the localization of 14-3-3a-mRFP. For each variant ca. 20 transformed cells were examined. White arrowheads indicate the nuclei. Scale bars represent 10 μm.
Figure 7. The presence of Serine 51 affects HopQ1 stability in planta. (A) Wild-type HopQ1 or HopQ1-S51A proteins carrying C-terminal 3xHA epitopes were transiently co-expressed with GFP in N. benthamiana leaves. Crude protein extracts (20 µg proteins per lane) were isolated from leaves 48h after agroinfiltration. HopQ1 variants were detected by immunoblot analysis using an antibody specific to HA. As an expression control the level of GFP was checked using anti-GFP antibody. The experiment was repeated twice. (B) C-terminally His-tagged HopQ1 variants were expressed in P. syringae pv. tabaci DAPP-PG677. Crude protein extracts prepared from overnight bacterial cultures were fractionated on a 12.5% SDS-PAGE and subjected to immunoblot analysis using a specific anti-His antibody. Equal protein loading was shown by Ponceau Red staining. The experiment was repeated twice. (C) R18, the inhibitor of 14-3-3 binding affects HopQ1 stability in plant extracts. Wild-type HopQ1 protein carrying a C-terminal Flag epitope was transiently expressed in N. benthamiana leaves. Crude protein extract was supplemented with recombinant 14-3-3-Strep II protein isolated from E. coli or R18 peptide at various concentrations, as indicated. HopQ1 and 14-3-3a were detected by immunoblot analysis using specific primary antibody. Equal protein loading was shown by Ponceau Red staining. (D) Application of 14-3-3a reverts the effect of R18 on HopQ1 stability. Wild-type HopQ1 protein carrying a C-terminal Flag epitope was transiently expressed in N. benthamiana leaves. Crude protein extract was supplemented with R18 and increasing amounts of recombinant 14-3-3a-Strep II protein isolated from E. coli, as indicated. HopQ1 was detected by immunoblot analysis using specific primary antibodies. The level of 14-3-3a was monitored using Strep-Tactin AP conjugate. Equal protein loading was shown by Ponceau Red staining. (E) Assembled in vitro complex of HopQ1-6xHis and 14-3-3a-Strep II was incubated with bean crude protein extract in the absence or presence of the R18. HopQ1 and 14-3-3a were detected by immunoblot analyses using anti-His antibodies or Strep-Tactin AP conjugate, respectively. Equal protein loading was shown by Ponceau Red staining. (F) In vitro phosphorylated HopQ1-6xHis was added to bean crude extract and incubated in the presence of 14-3-3a or BSA. HopQ1 and 14-3-3a were detected by immunoblot analyses using anti-His antibodies or Strep-Tactin AP conjugate, respectively. Equal protein loading was shown by Ponceau Red staining.
Figure 8. HopQ1 interaction with 14-3-3s is not critical for its perception by host plants. (A) Binary vectors encoding either wild-type HopQ1 or HopQ1-S51A were introduced via agroinfiltration into leaves of *Nicotiana tabacum*. Hypersensitive response developed in the infiltrated area within 48h, in response to both *Agrobacterium* strains. In contrast, no macroscopic signs of tissue necrotization developed in control leaves expressing GFP. (B) *Nicotiana benthamiana* plants were inoculated with *Pseudomonas syringae* pv. *syringae* B728a strains carrying pBBR1-MCS2 derivatives, which express either HopQ1, HopQ1-S51A or Cherry protein, as a control. Disease symptoms developed only in control plants treated with *P. syringae* pv. *syringae* B728a encoding Cherry protein. The pictures were taken 10 days post-inoculation. The experiments were performed three times with similar results.
Figure 9. Assessment of virulence properties of HopQ1 effector mutated to eliminate 14-3-3 binding. Bean leaves were inoculated with *P. syringae* pv. *tomato* DC3000D28E (~10⁵ CFU ml⁻¹) strains expressing HopQ1 or HopQ1-S51A. Immediately prior to infiltration, bacteria were mixed in a 1:1 ratio. Two and six days post inoculation, two leaf discs per plant were cut out from the infiltrated zones, ground in sterile 10 mM MgCl₂, diluted and plated on KB medium. Bacterial strains were distinguished by a selectable marker. The competitive index was calculated as the ratio of bacteria expressing the wild-type HopQ1 to bacteria expressing the mutated HopQ1 isolated from plant leaf and normalized to the input titers of the bacteria. Asterisks indicate that the index is significantly different from one, as established using Student's *t*-test (*P*=0.01)The experiment was performed three times with similar results.