The host exocyst complex is targeted by a conserved bacterial type III effector protein that promotes virulence

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ABSTRACT

For most Gram-negative bacteria, pathogenicity largely depends on the type-III secretion system that delivers virulence effectors into eukaryotic cells. The subcellular targets for the majority of these effectors remain unknown. Here, we show that *Xanthomonas campestris*, the causal agent of black rot disease, delivers the highly conserved effector XopP that interacts with host's EXO70 protein. EXO70 is an essential component of the exocyst complex with a role in plant immunity. The XopP/EXO70 interaction is specific and inhibits exocyst-dependent exocytosis without activating a specific plant NLR receptor that guards EXO70. In this way, *Xanthomonas* efficiently inhibits the host's PAMP-triggered immunity (PTI) by blocking exocytosis of PR1, callose deposition and the FLS2 immunity-receptor translocation to the plasma membrane, promoting successful infection.

INTRODUCTION

Pathogens use common strategies to colonize their plant and animal hosts. Pathogenicity, for the majority of the Gram-negative bacterial species, largely depends on the highly conserved Type-III protein Secretion System (T3SS) that delivers virulence proteins, known as “effectors”, into eukaryotic host cells. The type-III effector proteins (T3EPs) interfere with various cellular responses to the pathogen’s benefit including the inhibition of the host’s defence mechanisms (Dangl and Jones, 2019; Duxbury et al., 2016; Jo et al., 2019; Mermigka and Sarris, 2019; Staskawicz et al., 2001; Tampakaki et al., 2010). Several studies have revealed functional similarities among T3EPs from plant and animal pathogenic bacteria, suggesting that they exert similar functions in eukaryotic host cells (Büttner and Bonas, 2003; Jo et al., 2019; Staskawicz et al., 2001).

Plant pathogenic bacteria have evolved multiple T3EPs enabling evasion of recognition by the host’s immunity system, that often appear to be functionally redundant. However, the role(s) and the subcellular targets of these T3EPs remain an important open question in molecular host-microbe interactions and have only partially been elucidated.

Bacterial species of *Xanthomonas* genus are important model species for studies on the bacterial pathogenicity and host adaptation (An et al., 2020; Niño-Liu et al., 2006; Timilsina et al., 2020). This genus consists of a large group of plant-associated Gram-negative bacteria, with a high impact on food production due to severe diseases that 27 *Xanthomonas* species cause on almost 400 monocot and dicot plants (Ryan et al., 2011). *Xanthomonas campestris* pv. *campestris* infects through the vascular system the economically important plant family *Brassicaceae*, which includes the model species *Arabidopsis thaliana* (Fargier et al., 2011). The virulence of most *Xanthomonas* species depends on the presence of the *hrp* gene cluster that encodes the type-III secretion system (T3SS) and a number of T3EPs. Different *Xanthomonas* species or even different strains of the same species reveal divergent numbers of T3EPs. However, most of the sequenced species comprise of a core set of nine effectors (Ryan et al., 2011; Vicente and Holub, 2013). In *Xanthomonas campestris* the core type-III effectorome is limited to only three of these effector proteins, the XopP, XopF1 and XopAL1, indicated as Xops (Xanthomonas outer proteins) (Roux et al., 2015).

XopP was initially identified in *Xanthomonas campestris* pv. *vesicatoria* using the AvrBs2/Bs2 reporter system (Roden et al., 2004). Though of unknown function, it has being implicated in virulence in both monocot and dicot species. A XopP homolog from

Xanthomonas oryzae pv. *oryzae* (Xoo) strongly suppresses peptidoglycan- and chitin-triggered immunity in rice, targeting a positive regulator of plant immunity, a ubiquitin E3 ligase, inhibiting its ligase activity and therefore suppressing Xoo resistance (Ishikawa et al., 2014).

When plants are exposed to pathogenic microbes, different layers of immune responses are activated. Pattern recognition receptors (PRRs) in the plasma membrane recognize conserved domains of pathogens, called pathogen-associated molecular patterns (PAMPs) and activate pattern-triggered immunity (PTI). This includes the activation of signaling cascades that leads to secretion of defense components and callose deposition for cell wall fortification. However, adapted pathogens translocate T3EPs inside the host cell that can inhibit PTI, leading to effector-triggered susceptibility (ETS) (Dangl and Jones, 2019; Duxbury et al., 2016; Jones and Dangl, 2006; Mermigka and Sarris, 2019). In turn, plants have employed intracellular nucleotide-binding domain and leucine-rich repeat (NLR) receptors to detect the effects of specific pathogen T3EPs. Protein domains that function as integrated decoys (IDs), are present in a number of NLRs. These IDs are targeted by the effectors and subsequently lead to initiation of effector-triggered immunity (ETI) (Duxbury et al., 2016; Sarris et al., 2015, 2016).

The exocyst complex is a mediator in plant immunity, tethering the post-Golgi secretory vesicles to the plasma membrane in the early stage of exocytosis, before SNAREs drive fusion of vesicles with membranes (Elias et al., 2003). Consistent with this, components of the complex are targeted by various pathogens. In plants, as in animals and other eukaryotes, the exocyst complex consists of eight subunits; SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70 and EXO84. However, unlike in mammals, in plants the EXO70 subunit has 23 paralogs (Elias et al., 2003; Zhang et al., 2010).

Exocyst has been reported to play an important role in PTI induction and resistance against bacteria, oomycetes and fungi in *Arabidopsis thaliana* (Peenková et al., 2011; Stegmann et al., 2012, 2014). For *Arabidopsis* EXO70B1, it has been shown to be guarded by the NLR TN2, while *exo70B1* mutants activate TN2-mediated ETI-like immunity (Zhao et al., 2015). Furthermore, interaction between AtEXO70B1 and RIN4, a known regulator in defense responses has been reported. The latter is cleaved by AvrRpt2, an effector of *Pseudomonas syringae* and it was hypothesized that this cleavage released both RIN4 and EXO70B1 from plasma membrane to the cytoplasm, inhibiting vesicle tethering (Sabol et al., 2017). Moreover, it has been reported that AvrRpm1, from *Pseudomonas syringae*, phosphorylates RIN4 which subsequently

108 inhibits its interaction to EXO70s, including EXO70B1, leading finally to inhibition of
109 various defense molecules' secretion (Redditt et al., 2019).

110 Here, we report that the conserved T3EP XopP from *Xanthomonas campestris* pv.
111 *campestris* associates with various members of the exocyst complex, including
112 EXO70B1, SEC10 and EXO84B, resulting in inhibition of the exocytosis process and
113 consequently the host's PTI response. We showed that XopP inhibits secretion of the
114 Pathogenesis-related protein 1A (PR1a) in transient expression assays, as well as, the
115 callose deposition in *Arabidopsis* transgenic lines expressing XopP. Likewise, the
116 XopP/EXO70B1 and EXO70B2 interaction affects the EXO70-mediated trafficking of
117 FLS2 to the plasma membrane, however, without activating the TN2 dependent
118 immunity.

119 This is the first report of a *Xanthomonas* core effector that targets members of the
120 exocyst complex, potentially inhibiting its assembly. Our results support that the
121 XopP/AtEXO70 interaction potentially results in steric hindrance, rather than an
122 enzymatic effect, as the molecular mechanism by which the XopP effector anchors to
123 the exocytosis complex and manipulates exocytosis avoids activation of host NLR-
124 dependent immunity.

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Results and Discussion

XopP targets EXO70B1 *in vitro* and *in vivo*

NLR-IDs may reveal host proteins targeted by pathogens (Sarris et al., 2016). To further test this hypothesis, we performed a yeast two hybrid (Y2H) screen between conserved IDs from a wide range of NLRs, to detect potential interactors with the *Xanthomonas* core effectorome. We cloned twenty-four conserved plant NLR-IDs into pGBKT7-RFP vector and thirteen effectors, including nine core effectors from *Xanthomonas* species, into pGADT7-RFP vector and conducted a Y2H screen. This revealed several interactions, including an EXO70 interaction with XopP, which we decided to investigate in depth (Fig. 1A, S1D).

XopP is a highly conserved effector (Roux et al., 2015; Ryan et al., 2011; Vicente and Holub, 2013). In general, the activities of T3EPs and their roles in virulence can be masked by redundancy. Nevertheless, a significant reduction in virulence was previously reported for a XopP Tn5gusA5 insertion mutant of the model strain *Xanthomonas campestris* pv. *campestris* 8004. The mutated strain reveals significantly reduced symptoms of black rot on the leaves of Chinese radish (*Raphanus sativus* var. *radiculus*) compared to the wild-type strain Xcc 8004 (Jiang et al., 2009). We repeated the infection assays to Chinese radish plants using the wild-type strain Xcc 8004 and the XopP mutant and we obtained similar results (Fig. S5C).

To determine whether EXO70 is a real target of XopP, we performed a BLASTP analysis using the EXO70-ID as a query. From both protein and nucleotide blast analysis, most hits were EXO70B1. Thus, we cloned EXO70B1 from *Arabidopsis thaliana* in pGBKT7 vector and performed a yeast two hybrid assay with the effector. As shown in Fig. 1B and S1E, AtEXO70B1, but not AtEXO70A1 (splice forms 1 and 2), strongly interacts with XopP, indicating that this could be an authentic target. Our results also indicate a specificity to EXO70B1. To further reinforce this observation, we investigated the co-localization of the two proteins (Sabot et al., 2017). We constructed XopP-mCherry and AtEXO70B1-mCherry fusions at the C-terminus and transiently expressed the constructs under the 35S CamV promoter in *Nicotiana benthamiana* leaves. We observed the same compartmentalization of both XopP and AtEXO70B1 in the cell cytoplasm and inside the nucleus (Fig. S1A).

To validate the interaction in planta, we performed Biomolecular Fluorescence Complementation (BiFC) and co-immunoprecipitation (co-IP) analyses (Fig 1C and 1D, respectively). For BiFC assay, we transiently expressed under the CaMV 35S promoter in *Nicotiana benthamiana* leaves the EXO70B1 or the NLR RRS1-S (used

as a negative control) fused with nVENUS C-terminally, together with the XopP or the AvrRps4 effector from *Pseudomonas syringae* pv. *pisi* (as a negative control), both fused with cCFP C-terminally. EXO70B1 interacted specifically with XopP (Fig. 1Ca) but not with AvrRps4 (Fig. S1B) and the complex is localized to the cytoplasm, but also in the inner periphery of the plasma membrane, as it is shown by spots in 3D projection (Fig. 1Ca''). XopP did not interact with RRS1-S (Fig. 1Cb). For the co-IP assay, we transiently expressed EXO70B1 fused to a tandem 6xHis and 3xFLAG epitope tag (HF) and XopP fused with a -Myc tag and performed co-IP with a-FLAG beads. In Fig. 1D, the interaction of these two proteins is observed, while as a negative control the AvrRps4 effector was used (Fig. S1C, upper and lower lanes, respectively).

To investigate if the two proteins interact also *in vitro*, size exclusion chromatography (SEC) experiment was performed to XopP and EXO70B1 proteins separately and together, using the same type of column (Superdex™ 200 Increase 3.2/300). As shown in Fig. 1E, when the protein solutions were combined at a ratio of 1:1 both proteins were eluted in earlier fraction volumes, in comparison to their own elution profiles. This strongly suggests an *in vitro* complex formation between XopP and EXO70B1.

Furthermore, we investigated the potential co-localization of the XopP itself, as well as the XccXopP/AtEXO70B1 BiFC complex, with the autophagosome marker ATG8-mCherry, a Plasma Membrane (PM) marker and the early/late endosomal marker, DsRed-FYVE, using confocal microscopy. However, we did not manage to show these proteins to be co-localized with any of the above markers, except for PM-marker (Fig. S7A, B).

EXO70B1 specifically interacts with XopP and not with other homologs.

The next step was to determine whether EXO70B1 interacts with other XopP homologs or if this interaction is limited specifically to XopP originating from *Xanthomonas campestris* pv. *campestris* (here after XccXopP). We initially performed a BLASTP search at the NCBI and KEGG in order to identify the XccXopP homologs. Next we performed a phylogenetic analysis of all XopP homologs, using a Maximum-Likelihood method. The analysis was not limited to *Xanthomonas* species, since we identified homologs to *Ralstonia* and *Acidovorax* species. Our results pointed out five subgroups, including the *Ralstonia* group, the *Acidovorax* group and three *Xanthomonas* groups indicated as XopP1, XopP2 and the EXO70 interacting XccXopP groups (Fig. 2A). We amplified from each of the four groups a representative effector to test in a yeast-two hybrid analysis whether they interact with EXO70B1. We used two effectors from

previous work of our lab, the *Xanthomonas oryzae* pv. *oryzicola* XopP1 and XopP2 (Michalopoulou et al., 2018) and *Ralstonia solanacearum* Hlk3 and *Acidovorax citrulli* XopP-homologs. The tested preys are represented in the phylogenetic tree in bold (Fig. 2A). In our analysis, we also included *AtEXO70F1*. Interestingly, EXO70B1 did not interact with any homologs of XopP used, whereas EXO70F1 interacted with XccXopP1 (Fig. 2B), which indicates a specificity regarding XopP/ EXO70 interactions.

EXO70B1 and XopP interact at their N-termini

We sought to further characterize the interaction between EXO70B1 and XccXopP, in order to minimize the area of binding capacity. For this, we constructed three truncations of the plant protein according to domains similarly as for *AtEXO70B2* (Fig. 2C) (Teh O.K. et al., 2019). The truncated proteins were expressed in the yeast vector as baits and tested for interaction with the effector via Y2H. Only AB domain, which corresponds to the first 388 amino acids of the protein, interacts with the effector (Fig.2C), while the expression of the other constructs was verified via western blot analysis (Fig. S3C). We next split the XccXopP in N- and C-terminal fragments (Fig. 2D), using Phyre2 bioinformatics program (Kelley et al., 2016), as there is no information of the effector's structure. Similarly, Y2H showed that EXO70B1 and the effector interact at their N-terminal parts (Fig. 2D). In addition, during the co-IP analysis we observed that two cleaved forms of XopP are present (Fig. S2A). Based on this observation we decided to clone XccXopP minus the first 100 amino acids, leading to construct XccXopP-100 where cleaved forms no longer appear (Fig. 2D and S2A). This is also consistent with previous reports that many T3EPs are cleaved in their first amino acids when entering host cells, in order to be activated (Mudgett et al., 2000; Sohn et al., 2009). Next, we checked that XccXopP-100 continues to interact with EXO70B1 in Y2H assay (Fig. 2D). The XccXopP-100 appears to be uncleaved, and interestingly, in visualization of field-emission SEM (FE-SEM) of a gel-like formation of XccXopP-100 after dialysis in storage buffer in absence of β -mercaptoethanol, we observed the formation of fibrils (Fig. S2B). Similar fibrils were observed in TEM micrographs of XccXopP-100 after overnight dialysis in storage buffer in absence of β -mercaptoethanol (Fig. S2C).

In yeast and mammals, it has been shown that EXO70B1 interacts with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) of the plasma membrane (PM) through its basic residues, localized at C-terminus (Wu and Guo, 2015). Recently, this association with the PM has been extended also for plants. Endosidin2 (ES2), a small molecule, inhibited the EXO70A1 binding to the PM (Zhang et al., 2016). From these

data and according to our results, we conclude that XopP does not target the C-terminal part of EXO70B1 to directly inhibit this association although we cannot exclude that the effector's binding at the N-terminus of EXO70B1 might change its whole conformation.

XccXopP associates with *Arabidopsis* EXO70F1 and EXO70B2 but not with the Exo70B1 from monocots

EXO70B1 is a conserved part of the exocyst complex, being implicated in immune responses to different pathogens and autophagy (Kulich et al., 2013; Stegmann et al., 2014; Zhao et al., 2015). In an attempt to investigate if the XccXopP also targets other EXO70s that are also involved in immune responses (Peenková et al., 2011; Stegmann et al., 2012), we chose two paralogs of *exo70B1*, the phylogenetically close *AtExo70B2* and the phylogenetically distant *AtEXO70F1* for Y2H analyses (Stegmann et al., 2012). We have already seen that *AtEXO70F1* interacted with *XccXopP1* (Fig. 2B). Moreover, we checked the intraspecies specificity of the effector; and we cloned *Exo70B1* from the monocot *Oryza brachyantha*, a wild-crop relative of common rice. Interestingly, during the cloning process, we discovered a smaller, not annotated version of the protein, lacking the first 234 amino acids. We cloned this short version of *Obexo70B1* from cDNA (termed *ObEXO70B1_small*). Notably, the large version of the protein was not expressed in our conditions and needed to be cloned using gDNA (termed *ObEXO70B1_large*) (Fig. S3A). All EXO70B1 proteins were expressed as baits. In a Y2H screen we observed that both *AtEXO70B2* and *AtEXO70F1* interact with *XccXopP*. However, both *ObEXO70B1_small* and *ObEXO70B1_large* do not interact with the effector (Fig. S4A). *ObEXO70B1_small* and large protein expression was verified via western blot analysis (Fig. S3B). This finding led us to perform an amino acid alignment between all *XccXopP* interacting and non-interacting EXO70s, including the NLR-ID, in the hope of identifying possible amino acid changes in the non-interacting protein. We focused on the N-terminal domain of *AtEXO70B1* which interacts with *XccXopP*. For the alignment we used only the *ObEXO70B1_small* protein sequence. Even if the N-terminal part of the proteins seems to be quite divergent, we identified one crucial amino acid change, from lysine to glutamate, that seems to be conserved among the *XccXopP*-interacting homologs but appears to be mutated in *ObEXO70B1_small* (Fig. S4B).

To test if glutamate is an essential amino acid for the *XccXopP*/EXO70 interaction, we constructed mutated forms of *ObEXO70B1_small* and *AtEXO70B1*, corresponding to

K134E and E347K, respectively and tested again for interaction with XccXopP in a two-hybrid analysis in *Saccharomyces cerevisiae* (Fig. S4C). Interestingly, we observed a partial gain of interaction between the mutated ObEXO70B1_small^{K134E} and the effector. However, the mutated AtEXO70B1^{E347K} still interacted with XccXopP. Thus, glutamate seems to be a necessary but not sufficient amino acid for XccXopP/EXO70 interaction, since its mutation does not abolish the interaction. Future structural studies will reveal the exact nature of this interaction.

In plants, the EXO70 subunit of the exocyst complex comprises a large number of paralogs. For instance, in *Arabidopsis thaliana* there are 23 different EXO70 isoforms. This led to the hypothesis that different EXO70 subunits have specialized functions inside the cells and may take part of specific cargoed-vesicle exocytosis (Saeed et al., 2019; Sekereš et al., 2017). The fact that XopP associates with EXO70s from different phylogenetic clades could indicate that this core effector of *Xanthomonas* species might target the exocytosis of different cargos, such as antimicrobial compounds, callose or other membrane and lipid features.

XccXopP inhibits the exocytosis of Pathogenesis-Related protein 1A (PR1a)

The exocyst complex mediates the tethering of secretory vesicles to the cell plasma membrane (Guo et al., 1999). Under pathogen attack, this complex also contributes to efficient immunity by sending the TGN-originating secretory vesicles or MVBs originating from the process of autophagy, to the site of the attack (Pečenkova et al., 2017). Furthermore, it has been reported that the exocyst is required for secretion of the Pathogenesis-Related protein 1A (PR1a) (Du et al., 2015; Gu and Innes, 2012; Hammond-Kosack et al., 1994). Our finding that XccXopP targets AtEXO70B1, AtEXO70B2 and AtEXO70F1 (Fig. 1B; S4A), led us to speculate that the effector may act as an inhibitor of exocytosis during immune activation. To check our hypothesis we initially investigated if the PR1a secretion is compromised by XccXopP. For this we used the Chp7 serine protease effector from *Clavibacter michiganensis subsp. sepedonicus* that has been shown to induce an apoplastic Hypersensitive Response (HR) in *Nicotiana tabacum* only when it is targeted to the apoplast via the PR1a secretion peptide, but not intracellularly (Lu et al., 2015). We transiently co-expressed the PR1sp-Chp7 construct together with XccXopP effector in *Nicotiana tabacum* leaves. Both constructs were expressed using the 35S CaMV promoter. As a negative control we used the GUS protein. Three days post infiltration (dpi), an HR was observed in the leaves where the PR1sp-Chp7 was co-expressed with GUS, but not

along with *XccXopP* (Fig. 3A). To rule out any possibility of *XccXopP* being mediated as a general cytoplasmic HR inhibitor we performed a co-expression of *XccXopP* with the *XcvXopQ* effector that induces cytoplasmic HR in *Nicotiana tabacum* leaves (Adlung et al., 2016). Similarly, to rule out any potential interaction of *XccXopP* with Chp7, we performed a Y2H analysis between *XccXopP* as a bait and the Chp7 as a prey. As it is shown in Fig S5A and B, the *XccXopP* did not inhibit the *XcvXopQ*-dependent cytoplasmic HR, nor associated with Chp7 in a Y2H assay, respectively. Overall, these results indicate that *XccXopP* inhibits the PR1a exocytosis process, probably via interacting with EXO70B1 or any other(s) member(s) of the exocytosis complex.

We used confocal microscopy to further test if *XccXopP* inhibits PR1a exocytosis. After N-terminally fusing the PR1 secretion peptide with the chimeric RFP-GFP protein (35S::PR1sp-RFP-GFP), we transiently co-expressed the 35S::PR1sp-RFP-GFP in *Nicotiana benthamiana* leaves with and without *XccXopP* effector using the GUS protein as a negative control. When the RFP-GFP chimeric protein lacking a cell secretion peptide is expressed, it reveals a nuclear-cytoplasmic localization, so, both RFP and GFP signals are detected (Fig. 4B). Contrariwise, when the chimeric RFP-GFP protein was targeted to the apoplast via the PR1sp, we predominantly observed RFP signal, since the GFP is almost quenched in the acidic environment of the apoplast (Fig. 4B). When *XccXopP* was co-expressed with PR1sp-RFP-GFP both fluorescent signals were co-localized near the plasma membrane in vesicle-like formations, indicating the inhibition of the chimeric PR1sp-RFP-GFP translocation to the apoplast (Fig. 4B; Fig. S5F).

***XccXopP* inhibits the membrane translocation of the transmembrane immunity receptor FLS2.**

It has been recently reported that both the *Arabidopsis thaliana* EXO70B1 and EXO70B2 regulate the trafficking of the transmembrane immunity receptor FLS2 to the plasma membrane (PM) (Wang et al., 2020). FLS2 receptor is responsible for the recognition of the bacterial flagellin (flg22) epitope and the activation of the flagellin-dependent PTI in *Arabidopsis* (Wang et al., 2020). We wanted to determine whether *XccXopP* acts as a negative regulator of the EXO70B-dependent FLS2 translocation to the PM, leading to reduced FLS2 accumulation at the PM. To investigate this, we analyzed the fluorescence intensity of the chimeric FLS2-GFP protein itself or when co-expressed with *XccXopP*-mCherry, in *N. benthamiana* plants. When *XccXopP* is

present, we observed a significant reduction of the FLS2 signaling in PM (Fig. 4A, B). We categorized our results in two subgroups (Fig. 4A). In subgroup I (the most abundant subgroup) and subgroup II, we observed a reduction in FLS2 signaling about 50% and 100%, respectively.

XccXopP targets the EXO70B avoiding activation of its guarding NLR immune receptor

XccXopP is the first *Xanthomonas* effector shown to target an exocyst component to inhibit the exocytosis process, although it has been previously shown for AVR1 effector, from *Phytophthora infestans* to interact with the exocyst component Sec5 disturbing vesicle trafficking (Du et al., 2015). Similarly, the AvrPii, a fungal effector from *Magnaporthe oryzae* has been reported to interact with EXO70F2 and F3 of rice (Fujisaki et al., 2015). Recently, it was reported that the EXO70B1 is guarded in *Arabidopsis* by the NLR immune receptor TN2. The ubiquitination of EXO70B1 by the AvrPtoB effector from *Pseudomonas syringae*, leads to a TN2 activation and HR induction in *Arabidopsis* and transiently in *N. tabacum* (Wang et al., 2019).

In order to investigate if the XccXopP is able to activate TN2 immunity, we cloned the Arabidopsis TN2 resistance gene (AtTN2-YFP) and its homolog from *Brassica napus* (BnTN2-YFP). We transiently expressed in *Nicotiana tabacum* cv. Petit Gerard leaves the TN2s alone or with AtEXO70B1. Our results revealed an HR induction by the BnTN2 alone (Fig. 4E) but not by the AtTN2 (data not shown). We verified the expression levels for both TN2 proteins using confocal microscopy (Fig. S5E). The BnTN2-triggered HR was abolished when co-expressed with AtEXO70B1 in a 1:1 ratio (Fig. 4E). Interestingly, and unlike AvrPtoB, the XccXopP did not activate the TN2-dependent HR when transiently co-expressed in *N. tabacum* (Fig. 4E). This result led us to assume that XccXopP does not damage EXO70B1 protein. This was expected since no cleaved forms of the protein appeared in the co-IP assay (Fig. 1D). We believe that XccXopP associates with the exocyst component, potentially deploying a different mechanism to inhibit exocytosis.

To further verify our observation, we generated *Arabidopsis thaliana* transgenic lines expressing the XccXopP-mCherry under the regulation of the Arabidopsis Rubisco promoter and the NOS terminator, in cultivar Col-0. Our transgenic lines did not reveal any TN2-dependent phenotype (Fig. S5D), unlike the AvrPtoB transgenics which were lethal due to TN2 activation (Wang et al., 2019).

XccXopP inhibits cell wall callose deposition through exocyst targeting.

When PAMPs are recognized by PRR receptors, cellular events such as activation of MAP kinases, production of ROS and callose deposition are activated as a regular PTI response. Callose deposition is a common target for many pathogens, including several *Xanthomonas* species, which inhibit flg22-callose deposition at the cell wall, however with an unknown mechanism for the majority of them (Popov et al., 2016). Furthermore, it has been reported that the callose secretion/deposition in plants involves the exocyst complex (Du et al., 2015; Nielsen et al., 2012; Robatzek, 2007; Xu and Mendgen, 1994).

We evaluated callose deposition in *Arabidopsis thaliana* transgenic lines that stably express XccXopP, using T1 plants of three of our transgenic lines to further study callose deposition levels (Fig. 4C,D), upon eliciting PTI with 20 μ m flg-22. We compared our results with WT *Arabidopsis* Col-0 plants and noticed that in XopP-lines 15-2 and 22-1, almost no callose is secreted, while in XopP-line 20.6 the callose deposition levels are half of the respective WT Col-0 treated plants (Fig. 4C,D). Based on these results we evaluated the XccXopP-mCherry expression levels in all three lines using confocal microscopy. We observed that callose deposition inversely correlates with XccXopP-mCherry expression levels (Fig. 4C). Taken together, these data indicate that XccXopP inhibits the exocytosis process responsible for the callose deposition in our transgenic *Arabidopsis*.

XccXopP inhibits the exocytosis through association with multiple components of the exocyst complex.

The exocyst complex has been shown to form two sub-complexes (I and II), consisting of the components Sec3, Sec5, Sec6, Sec8 and Sec10, Sec15, EXO70, EXO84, respectively (Heider et al., 2016; Mei et al., 2018). Moreover, the assembly between the exocyst components is conserved from *Saccharomyces cerevisiae* to mouse and the two sub-complexes arrive at the PM independently in mammalian cells, being able to carry vesicles without assembling in an octameric complex (Ahmed et al., 2018). In yeast, Sec10 and Sec15 form a stable pair, which then requires EXO84 to associate with EXO70 and form the full sub-complex II. Whether this model is precisely reproducible in plants remains to be discovered. To test if XccXopP has a “larger” target such as a whole sub-complex of the exocyst, we cloned the other seven exocyst components of *Arabidopsis thaliana* (including the *Sec15A*, *Sec15B*, *Exo84B* and *Exo84C* paralogs) as preys in pGADT7 vector. We investigated, in Y2H, the

interactions of *AtEXO70B1* with the other exocyst components and with itself and we found that *EXO70B1* interacts with *SEC5A*, *SEC15B*, *EXO84B* as it was previously reported (Kulich et al., 2013; Zhao et al., 2015), as well as, with itself (Fig. S6A, C). Our results reveal that the interactions of *EXO70B1* with the other three exocyst components are mediated by the first 388 amino acids of *EXO70B1* (AB truncation) (Fig. S6E). We also noticed a weak interaction between *EXO70B1* and *SEC3A*, only when we transformed the AH109 yeast strain with both plasmids but not in PJ69 yeast strain. These results are in accordance with (Zhao et al., 2015). Also, using Y2H assays we investigated the interactions between the components of sub-complex II and we found that similarly to yeast and mammals, *SEC15B* interacts with both *SEC10* and *EXO84B* (Fig. S6F). Our results are consistent with a previously reported Y2H screening (Hála et al., 2008; Picco et al., 2017). Furthermore, we validated *in planta* all the above interactions, using BiFC assay (Fig. S6B, F). Notably, *XccXopP* seems to interact with two more components of the exocyst complex, the *SEC10* and *EXO84B*, and in fact they belong to the same sub-complex that also includes *EXO70B1* (Fig. S6A). We validated our results obtained from Y2H analysis, using *in planta* BiFC assays, where *XccXopP* and *SEC10* appear to associate in specific foci (Fig. S6B), resembling the vesicle-like formations observed when PR1-RFP-GFP's exocytosis is inhibited (Fig. 3B; S5F). We also investigated which protein domains of *XccXopP* are essential for the association with both *SEC10* and *EXO84B*, using Y2H assays. We noticed that, similarly to *EXO70B1*, the N-terminal region of *XccXopP* is responsible for these associations (Fig. S6D). Additionally, we investigated our hypothesis that *XccXopP* could act as an inhibitor of the assembling of the two exocyst sub-complexes or the disassembly of sub-complex II, inhibiting *EXO70B1* (SC II) and *SEC5A* (SC I) interaction (Mei et al., 2018) or inhibiting the association of SC II components (Mei et al., 2018), respectively. To investigate our first hypothesis, we cloned the *AtExo70B1* in MCS I and *XccXopP* in MCS II of pBridge vector and transformed the Yeast-3-Hybrid (Y3H) compatible AH109 yeast strain, along with pGADT7 expressing *SEC5A* and we investigated the potential inhibition in Y3H assay. When L-Methionine was absent from the yeast growing medium, *XccXopP* was induced by the MET25 promoter and partially inhibited the *EXO70B1*-*SEC5A* association (Fig. 5). To test our second hypothesis, we cloned the *AtSec15B* in MCS I and *XccXopP* in MCS II of pBridge vector and we performed a Y3H assay between *SEC15B* and its interactors *EXO70B1*, *SEC10* and *EXO84B*. We observed a partial inhibition of the association between *SEC15B* and *SEC10* (Fig. 5), while, *SEC15B* did not interact with *EXO70B1* when transformed as bait and prey, respectively. Thus, we tested the potential inhibition generating the opposite constructs, where *EXO70B1* is

cloned as a bait in pBridge and SEC15B is cloned as a prey in pGADT7, vectors respectively. As we present in Fig. 5, we observed a partial inhibition by *XccXopP*. Furthermore, we tested the inhibition of SEC15B and EXO70B1 interaction by *XccXopP*, via triple co-IP analysis. We cloned *Sec15B* fused to HF C-terminal epitope tag and *Exo70B1* and *XccXopP* fused to -myc C-terminal epitope tag. We performed a triple IP with FLAG-beads and we checked the EXO70B1 levels via western blot analysis, in presence and absence of *XccXopP*. As it is shown in Fig. S7C, we did not succeed to visualize the inhibition effect in presence of *XccXopP*. However, it is noteworthy that *XccXopP* co-IPed with EXO70B1, although it does not directly interact with SEC15B. From this assay we cannot observe the inhibition we observed in Y3H assay. This can be explained, since co-IP does not represent a quantitative method and it is difficult to see the inhibition we observed in Y3H assays.

Our overall data, can reasonably lead us to speculate that the effector acts as an intermediate molecule, inhibiting sub-complex II from forming a functional complex with sub-complex I. Future structural studies will examine if the effector can mimic EXO70B1 and if they share a similar structure and dimerize (Fig.S6A, C), enabling *XccXopP* to replace it with a non-functional component at the sub-complex II (Fig. 6).

Conclusion

This work reveals a bacterial pathogenic strategy to hijack plant exocytosis through exocyst targeting to inhibit PTI, however, avoiding NLR-dependent immunity activation. Considering the contribution of *XopP* to *X. campestris* infection, it seems to play an important role in the development of bacterial black rot disease. As a consequence, the exocyst complex appears as a plant susceptibility component during *X. campestris* infection that could be a potential target for the development of strategies to generate resistance against bacterial diseases.

Materials and Methods

Plants

Nicotiana species

Nicotiana benthamiana and *Nicotiana tabacum* cv Petit Gerard and N34-4 plants, used in our study, were grown in greenhouse conditions.

Arabidopsis thaliana

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used in our study as wild type. *Arabidopsis* seeds were stratified at 4°C in water for 3 days before sowing in soil and then were grown in greenhouse conditions. To generate constructs for conditional expression of XopP effector from *Xanthomonas campestris* pv. *campestris* in Col-0 WT background, we inserted with Golden Gate cloning in series the native Rubisco promoter, the gene, a C-terminal mCherry epitope tag and NOS terminator into pICSL86955OD vector (Engler et al., 2008). The plants were transformed with the floral dip method (Clough and Bent, 1999). The transformants were selected with BASTA. In brief, plants were sprayed from a short distance with BASTA (Bayer, 120 ppm Glufosinate – ammonium) twice in a week and the survivals were screened through PCR.

Escherichia coli

E. coli (Stellar or DH10b strains) were grown on LB plates with the appropriate antibiotic at 37°C and kept at 4°C for up to two weeks. For liquid cultures, a bacterial scrape was inoculated in liquid LB with appropriate antibiotic and grown at 37°C with shaking at 200 rpm approximately.

Agrobacterium tumefaciens

A. tumefaciens (AGL1, C58C1 and GV3101 strains) were grown on LB plates with appropriate antibiotics at 28°C and kept at 4°C for up to two weeks. For liquid cultures, a bacterial scrape was inoculated in 5 ml LB with appropriate antibiotic and grown at 28°C with shaking at 200 rpm approximately.

Bacterial strains

Xcc8004 WT, as well as 060E08 (mutant strain for XopP effector) were grown at 28°C in LB plates with appropriate antibiotics (Jiang et al., 2009).

Cloning and Constructs

For all constructs, used in the study, we used Golden Gate (GG) cloning and Phusion-HF polymerase (NEB). All constructs generated were verified by Sanger sequencing. EXO70-ID was amplified from pDONR221_Exo70_C_C119, given from Prof. M. Moscou (TSL), with STOP codon using BsaI sites and cloned to pGBKT7-RFP GG compatible vector for Y2H assay. The EXO70-ID is a fusion of RGH2 NLR of *Hordeum vulgare* ssp. *vulgare* cv. Baronnesse. *AtExo70B1* and *AtExo70F1*, as there are intronless genes, were amplified from genomic DNA (from Col-0 plants) in three parts, domesticating the internal BsaI sites, either with STOP codon for Y2H assay or without STOP codon for tagging in plants and cloned to pBluescript GG compatible vector. For both analyses, GG reaction was performed with pGBKT7-RFP as a final vector for Y2H assay or pICH86988 with a C-terminal tag for plant assays. *AtExo70B2* was given to us in pUG41308 vector from Prof. Marco Trujillo (University of Freiburg) and subcloned to pGBKT7-RFP vector for Y2H assay. *ObExo70B1_small* was amplified from cDNA, in two parts, domesticating the internal BsaI site either with STOP codon for Y2H assay or without STOP codon for tagging in plants. The *ObExo70B1_large* was amplified from gDNA, first amplifying the whole ORF with the 5' UTR and then performed nested PCR, with an internal FW primer. The PCR fragments were cloned firstly to pBluescript vector and then transferred in pGBKT7-RFP vector for Y2H assay or pICH86988 fused to a C-terminal epitope tag for plant assays. XopP was amplified from genomic DNA of *Xanthomonas campestris* pv. *campestris* ATCC33913 in two parts, domesticating the internal BsaI site with STOP codon for Y2H assay and without STOP codon for tagging in plants. *XopP1* and *XopP2* were amplified from genomic DNA of *Xanthomonas oryzae* pv. *oryzicola* WHRI 5234 (NCPB 1585), its draft genome being recently sequenced (Michalopoulou et al., 2018). *Hlk3* was amplified from genomic DNA of *Ralstonia solanacearum* GMI1000, a kind donation of Prof. Nemo Peeters (INRA-CNRS). *AcXopP* was amplified from genomic DNA of *Acidovorax citrulli*, a kind donation of Prof. Goumas (HMU). All the above PCR fragments were cloned in pBluescript vector and then subcloned to pGADT7-RFP GG compatible vector for Y2H assay or to pICH86988 fused to a C-terminal tag for in planta assays.

Truncation forms of *AtEXO70B1* were generated with the help of Prof. Marco Trujillo, using *AtEXO70B2* as a template, due to high sequence similarity. Using pGBKT7-*AtEXO70B1* as a template, PCR fragments from 1 to 388 amino acids (AB), from 319 to 511 amino acids (BC) and from 389 to 625 amino acids (CD) were generated, including START and STOP codons and were cloned to pBluescript vector and then subcloned to pGBKT7-RFP for Y2H assays. Truncation forms of XopP were generated using Phyre2 bioinformatics program. Using pGADT7: XopP as a template, PCR

fragments from 1 to 420 amino acids and from 421 to 733 amino acids, including START and STOP codons were amplified and cloned to pBluescript vector and then subcloned to pGADT7-RFP vector, for Y2H assay. For amplification of XopP minus the 100 first amino acids (XopP-100), an internal FW primer was used and was cloned from template pGADT7: XopP. AvrRps4 effector from *Pseudomonas syringae* pv. *pis* was used from a previous study (Sarris et al., 2015).

Chp7 construct, used in the study, was given by Professor Jane Glazebrook (Lu et al., 2015). pMDC:spC7HPB was verified by restriction endonucleases digestion and transferred to AGL-1 *Agrobacterium tumefaciens* strain for plant assays (Lu et al., 2015). XopQ was amplified from genomic DNA of *Xanthomonas campestris* pv. *vesicatoria*, a kind donation of Prof. Goumas (HMU) and cloned to pICH86988 fused to a YFP C-terminal epitope tag. For the generation of PR1-RFP-GFP, PR1 secretion peptide was amplified from pMDC:spC7HPB construct using as a FW primer 5' AAGGTCTCGccATGGGATTTGTTCTCTTTTCACAATTGC 3' and as a RV primer 5' AAGGTCTCACATTGAATTTTGGGCACGGCAAGAGTG. The PCR fragment was firstly cloned into pBluescript vector and sequence was verified by sequence analysis. The final construct was cloned into pICSL86900_OD under the expression of CamV 35S promoter and Nos terminator. FLS2-GFP construct, used in this study, was a kind donation of Dr. Katarzyna Rybak (LMU, Institute of Genetics), cloned in pCambia vector and transformed in GV3101 strain of *Agrobacterium tumefaciens*. Tn2 NLR gene was amplified from *Arabidopsis thaliana* Brassica napus zs11 gDNAs and cloned to pBluescript vector and later subcloned to pICH86988, fused C-terminally to YFP epitope tag for in planta assays. The remaining exocyst components (Sec3A, Sec5A, Sec6, Sec8, Sec10, Sec15A, Sec15B, Exo84B and Exo84C) were amplified from *A. thaliana* cDNA and cloned into pBluescript vector and later to pGADT7 vector for Y2H assays or pICH86988 fused C-terminally to an epitope tag for in planta assays (stated in the text). DsRed-FYVE confocal marker was kindly given to us in a C58C1 *Agrobacterium* strain from Prof. František Baluška (University of Bonn). Plasma-Membrane-RFP, Tonoplast-RFP and Ubi10::mCherry::Atg8A::Alli mCherry confocal markers were kindly given to us in AGL-1 (but for Atg8a which was in GV3101) *Agrobacterium* strains from Prof. Yasin Dagdas (Gregor Mendel Institute of molecular plant biology).

Phylogenetic analysis of XopP

In KEGG, using *Xanthomonas campestris* pv. *campestris* XopP (XCC_1247) in search term , we identified the orthologs from various pathogens, including *Xanthomonas*

species, *Ralstonia* and *Acidovorax*. The annotated protein sequences of 73 orthologs were manually downloaded from KEGG and two of them were recovered from sequence analysis of draft genome annotation of *Xanthomonas oryzae* pv. *oryzicola* WHRI 5234 (NCPB 1585) (Michalopoulou et al., 2018). Identity percentage of orthologs used did not drop down from 24,4%. Moreover, in order to confirm that the resultant orthologs are indeed homologous to Xcc_1247, we did reverse Blastp in NCBI for each of the 73 protein sequences. From this search, we excluded nine protein sequences. All 67 proteins were aligned via MEGA5. MEGA5 was also used to construct the maximum likelihood phylogenetic tree using the Poisson correction model (Tamura et al., 2011; ZUCKERKANDL and PAULING, 1965).

Y2H and Y3H assays

The vectors used for Y2H assay were pGBKT7-RFP and pGADT7-RFP, which were GG compatible, kindly given to us from Dr. Marc Youles (TSL). RFP is a dual selection marker, along with the antibiotic resistance gene, being utilized for inserting the gene of interest. All plant proteins were cloned as baits (in pGBKT7 vector) and bacterial effectors were cloned as preys (in pGADT7 vector), otherwise being stated. A standard two-hybrid screen was carried out with the yeast reporter strain PJ694a (or otherwise stated where we used AH109), using all the necessary negative controls (bait with empty pGADT7-RFP, prey with empty pGBKT7-RFP and empty pGBKT7-RFP and pGADT7-RFP) for each transformation pair. As a positive control, a known interaction was used from a previous study (Sarris et al., 2015). The selection of the plasmids transformation was and yeast-two hybrid analysis was performed with selection of the auxotrophic markers leucine, tryptophan and adenine, respectively. Accordingly, the vectors used for Y3H assay were pBridge, kindly given to us by Prof. Subba Rao Gangi Setty (Indian Institute of Science Bangalore) and pGADT7-RFP vectors. EXO70B1 and XopP were cloned in MCS I and II sites of pBridge, respectively. AH109 yeast strain was transformed with both vectors and the two-hybrid analysis was performed with selection of the auxotrophic marker adenine with supplementation of 1mM Methionine in the medium, for suppressing XopP expression. The three hybrid analysis was performed in medium lacking leucine, tryptophan, adenine and methionine. All yeast strains left to grow in 30°C.

Subcellular co-/localizations and BiFC

N. benthamiana was infiltrated with *Agrobacterium* strains at OD₆₀₀ = 0.5 expressing indicated constructs. Confocal markers for co-localization assays were expressed at OD₆₀₀ = 0.4. For subcellular co-/localizations and BiFC, leaf discs were analyzed 3

609 dpi. All images were captured in a Leica SP8 confocal microscope (facility of IMBB).
 610 For subcellular localizations, all indicated constructs were fused to a C-terminal eCFP,
 611 YFP or mCherry epitope tags, unless for the confocal markers which were given to us
 612 already cloned with DsRed, mCherry or RFP epitope tags. For BiFC, all indicated
 613 constructs were fused to a C-terminal nVENUS or nCERULEAN or cCFP epitope tags.
 614 All samples were imaged with a 40x water objective, otherwise marked. The confocal
 615 images were processed with ImageJ. Excitation of GFP was made using the 488 nm
 616 of Argon laser and emission of spectra was collected at 496-520 nm. Excitation of YFP
 617 was made using the 514 nm of Argon laser and emission of spectra was collected at
 618 520-550 nm. Excitation of mCherry, DsRed and RFP was made using the 561 nm laser
 619 and emission of spectra was collected at 593-628, 573-616 or 573-620 nm,
 620 respectively. Excitation of eCFP was made at 405 nm and emission of spectra was
 621 collected at 463-487 nm. Chlorophyll was detected between 653 and 676 nm and
 622 excited from 561 nm. Gain was at 100%, otherwise marked. Quantification of GFP
 623 signal from infiltrations with FLS2-GFP was processed with Image J software, as
 624 described in [https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-](https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html)
 625 [fluorescence-using-imagej.html](https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html).

626 **HR assay**

627 For HR assay with PR1sp-Chp7 in presence or absence of XopP *N. tabacum* cv. Petit
 628 Gerard were infiltrated with *Agrobacterium* C58C1 or AGL-1 strains at OD₆₀₀ = 0.1 for
 629 PR1sp-Chp7 and OD₆₀₀ = 0.4 for GUS and XopP. For HR assay with *At-* or *Bn*TN2
 630 constructs, *N. tabacum* cv. N34-4 and Petit Gerard were infiltrated with *Agrobacterium*
 631 C58C1 strain of TN2, EXO70B1 or XopP at OD₆₀₀ = 0.5 (equimolar ratio).

632 **Callose deposition**

633 Leaves from 4-week-old *Arabidopsis* plants were infiltrated with 20 µM of flg22 in 10
 634 mM of MgCl₂ or mock solution and removed 12 h after infiltration. Callose staining,
 635 image acquisition, and processing were carried out as described in (Lin Jin, 2017) with
 636 a change in destaining. In brief, clearing leaves from chlorophyll was carried out with
 637 acetic acid and ethanol in a 1:3 ratio overnight (12 hours). Callose visualization was
 638 made in confocal SP8 microscope in a 10x dry objective with excitation from 405 nm
 639 and emission between 490 and 530 nm. Images were processed as described in (Lin
 640 Jin, 2017) with Image J software and a graph was created depicting Callose
 641 deposits/mm², from 6 pictures per genotype and treatment. Some samples with
 642 extreme values were removed from the quantification.

643 **Protein extraction and Co-immunoprecipitation**

N. benthamiana was infiltrated with *Agrobacterium* strains at OD₆₀₀ = 0.5 expressing indicated constructs. Agro-infiltrated leaves were grinded 72 hpi with liquid nitrogen and proteins were extracted in GTEN buffer (10% glycerol, 25 mM Tris-Cl pH7.5, 1 mM EDTA, 150 mM NaCl) supplemented freshly with 10mM DTT, protease inhibitor cocktail (Calbiochem) and 0,15% v/v NP-40. After 10 min centrifugation at 4°C, supernatant was collected in clean microcentrifuge tube and incubated 2 h at 4°C with a-flag mouse antibody (Sigma), following 1 h incubation at 4°C with PureProteomeProtein A/G Magnetic Beads Mix (Millipore). Beads were washed with GTEN buffer, before adding 1x SDS loading dye and boiling the samples for 5 minutes. Total protein from yeast transformations was extracted according to (Horvath et al., 1994).

SDS PAGE and Immunoblotting

Protein samples from plant leaves were separated at 8% SDS PAGE, electro-blotted at PVDF membrane (Millipore), then blocked for 1 h with 5% w/v milk in TBS and incubated for 1 h with primary antibodies a-flag 1:4000 (Sigma) or a-myc 1:4000 (Cell Signalling). Membranes were then incubated for 1 h with secondary antibody a-mouse HRP conjugate 1:10000 (Cell Signalling) and chemiluminescent substrate ultra (Millipore) was applied for 5 min before camera detection in Sapphire Biomolecular Imager (Azure Biosystems). Total protein lysate from yeast transformations were separated in 10% or 12% SDS PAGE, electro-blotted at PVDF membrane (Millipore), then blocked for 90 minutes with 5% w/v milk in TBS supplemented with 0,05% Tween-20 and incubated for 1 h with the primary antibody a-Gal4-DBD 1:500 (sc-510, Santa Cruz Biotechnology). The rest procedure is similar as in proteins extracted from plant leaves.

Protein expression conditions in *E.coli*

Different fragments of XopP protein were produced (Fig. S2A), several of which were insoluble. We determined, via multiple alignment of XopP homologues in *Xanthomonas* genus, that the only soluble construct, until now, is the fragment lacking the first 100 N-terminal residues (molecular weight 71.092 Da). In case of EXO70B1, its full length of 624 amino acid residues (molecular weight 70.639 Da) was a soluble construct.

XopP-100 gene fragment was cloned and inserted into the pET26b (ColE1 plasmids) vector carrying a C-terminal 6-His epitope tag and transformed into the *E.coli* strain BL21. A sufficient amount of soluble protein was obtained after induction using the

following conditions: Cells were grown in LB medium containing 30 µg/ml kanamycin until an OD₆₀₀ value of 0.6 was reached. The culture was induced with 0.4 mM IPTG and left to grow at 20°C overnight. The cell paste was re-suspended in 100 ml lysis buffer containing 25 mM Tris pH=8.0, 300 mM NaCl, 5 mM imidazole and 15 mM β-mercaptoethanol, and homogenized. After adding protease inhibitors (20 µg/ml leupeptin, 1 mM PMSF and 150 µg/ml benzamidine), the solution was sonicated for 5 min. The precipitate was subsequently removed by centrifugation at 12,000 rpm at 4°C for 45 min. Purification was carried out using His-tag affinity chromatography at 4°C with a 8-ml Ni-NTA Qiagen column pre-equilibrated in lysis buffer and initially washed stepwise with 10, 20 and 30 mM imidazole. With a subsequent increase in imidazole concentration, the protein started eluting at 100 mM imidazole. Fractions containing the protein were dialyzed against the storage buffer containing 25 mM Tris pH=8, 100 mM NaCl and 10 mM β-mercaptoethanol and concentrated to approximately 2.8 mg/ml, for subsequent crystallization experiments. In case of *Exo70B1*, the full length gene was cloned and inserted into the LIC 1.10 vector carrying a N-terminal 6-His-SUMO3 tag, and transformed into the *E.coli* strain Rosetta™ (DE3). The same protocol was followed and the fractions containing the protein were dialyzed against the storage buffer (25 mM Tris pH=8, 100 mM NaCl, 10 mM β-mercaptoethanol and 0.5 mM SENP-2 protease). Subsequently, a reverse purification protocol was carried out using His-tag affinity chromatography, and the fractions containing the protein were concentrated to approximately 8.5 mg/ml.

Field-Emission Scanning Electron Microscopy (FE-SEM)

The XopP-100 fragment was purified by His-tag affinity chromatography. Fractions containing the protein were dialyzed against the storage buffer in absence of β-mercaptoethanol overnight. The gel-like form that came up, was deposited on a covered glass and air-dried overnight. The sample was then covered with 10 nm of Au/Pd sputtering and observed using a JEOL JEM-2100 transmission electron microscope at 20 kV.

Transmission Electron Microscopy (TEM)

5 µl of XopP-100 sample was deposited onto a formvar/carbon-coated electron microscopy grid for 2 min. After removing the excess with filter paper, 2% w/v uranyl acetate was used in order the sample to be stained, for 2 min. The observations were conducted with a JEOL JEM-2100 transmission electron microscope at 80 kV

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Authors Contributions

V.A.M. carried out most of the experiments; K.K., D.K., M.K. and P.H.N.C. purified recombinant proteins and performed *in vitro* interaction assays; G.M. performed additional experiments and contributed to organizing some of the experiments in the Lab. V.A.M. performed *Arabidopsis* transformation; P.F.S. designed the research and supervised the project; P.F.S. and V.A.M. initiated the project; V.A.M. P.F.S. and J.D.G.J. analysed the data/results. P.F.S., V.A.M. and J.D.G.J. wrote the manuscript. All authors have read and approved the manuscript.

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Figures and Figure Legends

Figure 1. XopP targets EXO70B1 *in vitro* and *in vivo*.

A. XccXopP targets an EXO70 integrated domain (ID) in a Y2H screen.

B. Interaction of AtEXO70B1 with XccXopP effector in Y2H screen. All yeast combinations grow in SC-LW medium and only AtEXO70B1 with the effector survives in SC-LWA, in contrast to AtEXO70A1 (both spliceforms).

C. In planta validation of AtEXO70B1/XccXopP interaction using a BiFC assay, in *N. benthamiana* plants. EXO70B1 and XccXopP were fused C-terminally with nVENUS and cCFP epitope tags respectively and YFP signal was observed in confocal microscopy 3dpi (a, a''). A 3D projection of Z-stack of the interaction is shown in a''. As negative controls, another plant protein (AtRRS1-S) was used with XccXopP (b, b') and no YFP signal was observed. Bars= 15μm.

D. In planta validation of AtEXO70B1/XccXopP interaction in *N. benthamiana* plants using a co-immunoprecipitation (co-IP) assay. EXO70B1 and XccXopP were fused C-terminally with HF (6xHis and 3xFLAG) and -myc respectively and an IP-flag was performed. XccXopP interacts with EXO70B1 as shown in the western-blot with anti-myc.

E. SEC elution profiles for XccXopP (blue), Exo70B1 (orange) and XopP/EXO70B1 complex (grey).

Figure 3. XccXopP inhibits the exocytosis of Pathogenesis-Related protein 1A (PR1A).

A. HR inhibition assay. XccXopP inhibits the exocytosis of the chimeric protein PR1a-Chp7, to the apoplast. Inhibition of HR (left lower lane) appears 3dpi in *N. tabacum* leaves. GUS protein was used as an HR negative control. Expression of GUS or XopP itself does not produce any HR (right lanes). The experiment has been repeated three times with identical results (total plants used in which HR was inhibited = 11/11).

B. The chimeric protein RFP-GFP is expressed in the cytoplasm (upper panel). The PR1a secretion peptide fusion to RFP-GFP chimera (PR1sp-RFP-GFP) results in exocytosis to the apoplast of the RFP-GFP (white arrows, middle panel). Only RFP is visualized, as GFP is quenched in the acidic environment of the apoplast. When XccXopP is expressed (eCFP channel) along with PR1sp-RFP-GFP, the RFP-GFP exocytosis is inhibited and it is relocalized to the cytoplasm, as both fluorescence proteins are visible and localized in vesicle-like formations (white arrowheads). The pictures are taken in a confocal microscope, 3dpi of *N. benthamiana* leaves. Bars = 10 μ m. The experiment was repeated three times with similar results.

Figure 4. XopP inhibits the membrane translocation of the transmembrane immunity receptor FLS2 and Callose deposition, without activating TN2-mediated immunity.

A, B. Visualization (A) and quantification (B) of representative pictures of expression of FLS2 receptor fused C-terminally to GFP tag in the plasma membrane. The FLS2-GFP is co-expressed with XccXopP fused to mCherry tag (left) or expressed alone (right). In samples of subgroup-I, half of the FLS2 membrane localization is observed, while in samples of subgroup-II almost no FLS2 is visible in the plasma membrane, while specific foci are present, resemble vesicle-like formations that cannot be exocytosed (white arrowheads). The pictures are taken in a confocal microscope 3dpi in *N. benthamiana* leaves and are representatives from total 40 cells. Bars = 10 μm. The intensity measurement has been made with Image J software and the number of cells appear above its column.

C, D. Visualization (C) and quantification (D) of callose deposition after elicitation with 20μm flg22 (middle lane) or mock solution (upper lane), in T1 lines of transgenic *Arabidopsis thaliana* (15-2, 22-1 and 20-6) overexpressing XccXOPP. In the lower lane, XccXopP expression is shown in confocal microscope and it is in accordance with the levels of Callose secretion. In line 20.6, the XccXopP expression is almost absent. Bars = 15μm. The quantification has been made with Image J software and data represent the mean +/- SD.

E. Transient expression of the NLR immune receptors *BnTN2* and *AtEXO70B1*, and *XccXopP* in *N. tabacum* leaves. *BnTN2* triggers an HR, which is suppressed by *AtEXO70B1*. *BnTN2* co-expressed with *XccXopP* also induces an HR reaction, while the combination of all three constructs when co-expressed, no HR reaction is observed after 72hpi. The experiment has been repeated three times with identical results.

	pMET:: XccXopP	BD- AtEXO 70B1	BD- AtSEC 15B	AD- AtSec 15B	AD- AtExo o70B 1	AD- AtSec 5A	AD- AtSec1 0	BD (E V)	AD (E V)	
1063										
1064										
1065										
1066	+	+	-			-	-	-	+
1067	+	+	-			+	-	-	-
1068	+	-	-			-	-	-	+
1069	+	-	+			-	-	-	+
1070	+	-	+			-	+	-	-
1071	+	+	-	+		-	-	-	-
1072	+	-	+	-	+	-	-	-	-
1073	+	-	+	-	+	-	-	-	-
1074										-LW
1075										-LWM
										-LWA
										-LWAM

Figure 5. XccXopP inhibits the EXO70B1/SEC5A, SEC15B and SEC15B/SEC10 associations in yeast-three-hybrid (Y3H) assays.

XccXopP interferes with EXO70B1/SEC5A, EXO70B1/SEC15B and SEC15B/SEC10 associations in a yeast-three-hybrid analysis. The constructs were transformed in the Y3H compatible yeast strain AH109. EXO70B1 associates with SEC5A and SEC15B and the latter associates with SEC10, as it is shown in medium lacking leucine, tryptophan and adenine. When methionine is absent from the medium, XccXopP is expressed by a MET25 inducible promoter and the above associations are inhibited.

Figure 6. Schematical Abstract

XccXopP manipulates the exocytosis via associating with members of sub-complex II of exocyst, affecting its proper assembly with the sub-complex I.