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Úloha vybraných podjednotek komplexu exocyst v odpovědi rostlin na patogena.

The Role of selected exocyst subunits in response of plants to pathogen

Disertační práce

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Contents

List of Abbreviations

ABA -	Abscisic acid
ATG -	*AUTOPHAGY-RELATED GENE*
Avr -	Avirulent protein
Bgh -	*Blumeria graminis* f. sp. *Hordei*
CPK5 -	CALCIUM-DEPENDENT PROTEIN KINASE5
CW -	Cell wall
DAMP -	Damage-Associated Molecular Pattern
ETI -	Effector-triggered immunity
PAMP -	Pathogen-Associated Molecular Pattern
PIP2 -	Phosphatidylinositol-4, 5-bisphosphate
PM -	Plasma membrane
PR -	Pathogenesis-related (protein)
PRR -	Pattern Recognition Receptor
PTI -	PAMP-triggered immunity
Pto DC3000 -	*Pseudomonas syringae* DC3000
RIN4 -	RPM1-interacting protein 4
ROP -	Rac-related GTPases of plants
ROS -	Reactive oxygen species
SA -	Salicylic acid
Y2H -	Yeast two-hybrid (assay)

Abstrakt

V posledních létech se objevuje narůstající počet publikací naznačujících zapojení rostlinné sekretorické dráhy do obrany vůči fytopatogenům. Konkrétně, v rámci současného výzkumu byly detailněji objevovány úlohy rostlinného komplexu exocyst. Přesto, jak přesně exocystem zprostředkovaná exocytóza přispívá k sekreci antimikrobiálních látek a obraně založené na buněčné stěně zůstává neobjasněno. V předkládané práci poskytuji experimentální důkazy a navrhuji další hypotézy o vybraných podjednotkách exocystu v imunitní reakci rostlin. Zvláště ukazuji, že EXO70B1 podjednotka exocystu interaguje s proteinem souvisejícím s imunitou, RIN4. Štěpení RIN4 pomocí AvrRpt2 *Pseudomonas syringae* efektorové proteázy uvolňuje jak RIN4 fragmenty, tak EXO70B1 z plazmatické membrány, když je tranzientně exprimován v listech *Nicotiana benthamiana*. Spekuluji o tom, jak by to mohlo mít vliv na regulaci polarizované depozice kalózy. Ve společně vypracovaném článku jsme také navrhli hypotézu, že EXO70B1-zprostředkovaná autofagická degradace TN2 rezistenčního proteinu zabraňuje jeho hyperaktivaci a vývoji fenotypů mimikujících léze. Kromě toho ve spolupráci s kolegy uvádím údaje o účasti EXO70H4 v sekreci kalózasyntázy PMR4, potřebnou pro depozici křemíku. Představujíc možný konvergentní mechanismus navrhujeme, že EXO70H4 podjednotka exocystu by se mohla podílet také na výstavbě obranné papily. Konečně, ve společně vypracovaném přehledovém článku shrnujeme poznatky a navrhujeme další scénáře pro účast rostlinného exocystu na sekreci v souvislosti s autofagií v obraně rostlin.

Abstract

In the recent years, there has been a growing number of publications indicating at the involvement of plant secretory pathway in defense against phytopathogens. Specifically, roles of plant exocyst complex have been explored in deeper detail in current research. Yet, exactly how exocyst-mediated exocytosis contributes to secretion of antimicrobials and cell wall-based defense remains unclear. In the presented Dissertation, I provide both experimental evidence and devise further hypotheses on selected exocyst's subunits in plant immune reactions. Particularly, I show that EXO70B1 exocyst subunit interacts with immunity-related RIN4 protein. Cleavage of RIN4 by AvrRpt2 *Pseudomonas syringae* effector protease releases both RIN4 fragments and EXO70B1 from the plasma membrane when transiently expressed in *Nicotiana benthamiana* leaves. I speculate on how this might have an implication in regulation of polarized callose deposition. In a co-authored opinion paper, we also hypothesize that EXO70B1-mediated autophagic degradation of TN2 resistance protein prevents its hyperactivation and lesion mimic phenotype development. In addition, in collaboration with my colleagues, I present data on EXO70H4's engagement in PMR4 callose synthase secretion, required for silica deposition. Representing a possible convergent mechanism, we propose that EXO70H4 exocyst subunit might likewise participate in defensive papilla buildup. Finally, in a co-authored review paper, we summarize knowledge and suggest further scenarios for plant exocyst involvement in autophagy-related secretion in plant defense.

Introduction

The exocyst complex and its role in plant cell morphogenesis

Plants are known to have indeterminate growth and development and continue to grow new organs throughout their lives. During the process, new cells are produced through division of stem cells in meristems. Daughter cells arising from these divisions that lose contact with mother stem cells start to adopt their fate, i.e. differentiate from each other. Cell differentiation is driven by both signals related to the cell lineage from which they derive as well as positional information in the gradient of a morphogen or interaction with neighboring cells in the same tissue or tissue layer. As a result, plant cells functionally specialize. This, among other processes, usually involves specific cell shape formation, or cell morphogenesis (reviewed in Schiefelbein, 1994; Fowler and Quatrano, 1997; Scheres, 2001).

Plant cell morphogenesis is a process taking place during the division and differentiation of plant cells, as well as ecological interactions. It is accompanied by changes in the composition and structure of the cell wall and the plasma membrane. For this, coordination of cytoskeleton and secretory pathway activity was shown to be vital (Fowler and Quatrano, 1997). Having perceived directional signals coming from other parts of the plant or from within the tissue layer in which they reside, plant cells develop asymmetry, also known as cell polarity (Scheres, 2001; Costa, 2016). This is manifested by asymmetric distribution of proteins and lipids to distinct parts of the cell. Being in some morphogenic field or contact with neighboring cells, all plants cells show *bona fide* some degree of polarization. The most easily discernible types include those of endodermis, tip growing root hair and pollen tube cells and xylem parenchyma cells with polarly distributed PIN auxin transporters (reviewed e.g. in Nakamura and Grebe, 2017). Recently, separate trafficking pathways have been discovered to establish apical and basal polarities in *Arabidopsis thaliana* (Arabidopsis) root cells (Li *et al.*, 2017*a*). Also, specification of geometric edges of Arabidopsis lateral root cells was shown to rely on RabA5c GTPase-mediated trafficking (Kirchhelle *et al.*, 2016).

One of the key regulators required for plant cell morphogenesis and particularly plant cell polarity establishment and maintenance is the exocyst complex. The exocyst is a protein complex that mediates tethering of vesicles to target membrane before SNARE-mediated fusion (Zárský *et al.*, 2009).

The exocyst was initially described in budding yeast (*Saccharomyces cerevisiae*). First, yeast Sec15 protein was recognized as a part of a larger protein complex and itself as being associated with the plasma membrane (PM) (Bowser and Novick, 1991). Then, the Sec8 protein was identified in the same 19.5S particle containing Sec15 (Bowser *et al.*, 1992). Later, TerBush and Novick discovered through chromatography methods and co-immunoprecipitation a third protein, the Sec6. They also determined that the complex must contain at least eight subunits (TerBush and Novick, 1995). Subsequently, SEC3, SEC5, SEC10, and EXO70 subunits were described to comprise the complex, hereafter termed the

Exocyst (TerBush *et al.*, 1996). Finally, the Exo84 subunit was co-immunoprecipitated with the exocyst components, making it the eighth subunit of the complex. Interestingly, the same report has also shown that the Exo84 specifically localizes to the bud tip connecting mother and daughter yeast cells and that Exo84 depletion results in secretory vesicle accumulation and defect in invertase secretion (Guo *et al.*, 1999*a*). These studies, along with previous experiments showing genetic interaction between the Sec15 and a regulator of vesicle trafficking, the SEC4 GTPase, suggested a role of the exocyst complex in polarized secretion (Salminen and Novick, 1989; Walworth *et al.*, 1992). Since then, there have been numerous studies proving its role in polarized secretion and exocytosis in yeast and mammalian cells (reviewed e.g. in Wu and Guo, 2015). Also, exocyst composition has been decisively corroborated in yeast. The study of Heider *et al.* confirmed that the complex consists of eight subunits with equal stoichiometry (Heider *et al.*, 2016). Importantly, each subunit contains a conserved helical bundle structure, possibly essential for its tethering function during exocytosis (Croteau *et al.*, 2009).

The tethering role of exocyst has been well demonstrated by ectopic targeting of yeast Sec3 subunit. In yeast and mammals, Sec3 and EXO70 exocyst subunits are known to serve as spatial landmarks for the rest of the complex through the interaction with phosphatidylinositol 4,5-bisphosphate (PIP2) on the PM (He *et al.*, 2007; Liu *et al.*, 2007). When translationally fused to Tom20 mitochondrial protein, the Sec3 recruited Sec6 and Sec8 subunits and also secretory vesicles to mitochondria. Similarly, except for Exo70, all other ectopically targeted exocyst subunits tested recruited Sec8 to mitochondria, showed defect in invertase secretion a grew more slowly than control cells (Luo *et al.*, 2014). Based on fluorescent imaging and measurements in yeast, Picco *et al.* were able to reconstruct the 3D architecture of the exocyst complex *in vivo*. According to their data, about 14 exocyst complexes on average should reside at sites of vesicle fusions (Picco *et al.*, 2017).

In plant cells, the exocyst has been implied in processes of cell polarity establishment and maintenance, including pollen tube growth, polarized growth and (upon pollen arrival) hydration of stigmatic papillae, cytokinesis, localized deposition of seed coat pectin, transport of PIN auxin carriers to specific parts of the plasma membrane, development of the periarbuscular membrane, maturation of the trichome cell wall and secretion of callose synthase to the trichome PM, specification of central endodermal PM for subsequent CASP protein delivery, autophagic transport to the vacuole and probably polarized secretion of defense cargo during response to microbial pathogens (Cole *et al.*, 2005; Synek *et al.*, 2006, 2017; Hála *et al.*, 2008; Kulich *et al.*, 2010, 2013, 2015, 2018; Pecenková *et al.*, 2011; Genre *et al.*, 2012; Drdová *et al.*, 2013; Zárský *et al.*, 2013; Rybak *et al.*, 2014; Zhang *et al.*, 2015; Du *et al.*, 2015; Safavian *et al.*, 2015; Tan *et al.*, 2016; Kalmbach *et al.*, 2017). In addition, anisotropic (but not polar) root cell elongation is also affected in Arabidopsis exocyst mutants (Cole *et al.*, 2014).

Contrary to other Eukaryotes, several plant exocyst subunits have expanded into more than one isoform (Cvrčková *et al.*, 2012). EXO70 subunit forms the largest family, with 23 members in Arabidopsis. They are differentially expressed during ontogenesis and in different

tissues, but distinct isoforms are also coexpressed in the same cell (Synek *et al.*, 2006; Li *et al.*, 2010; Cvrčková *et al.*, 2012). These and other observations have led to the proposal of the recycling domain hypothesis, whereby different EXO70 subunits mediate secretion and recycling of vesicles to distinct PM domains. The activated cortical domain and the connected endosomal compartment were thus suggested to constitute a dynamic entity named the recycling domain (Zárský *et al.*, 2009; Zárský and Potocký, 2010; Figure 1).

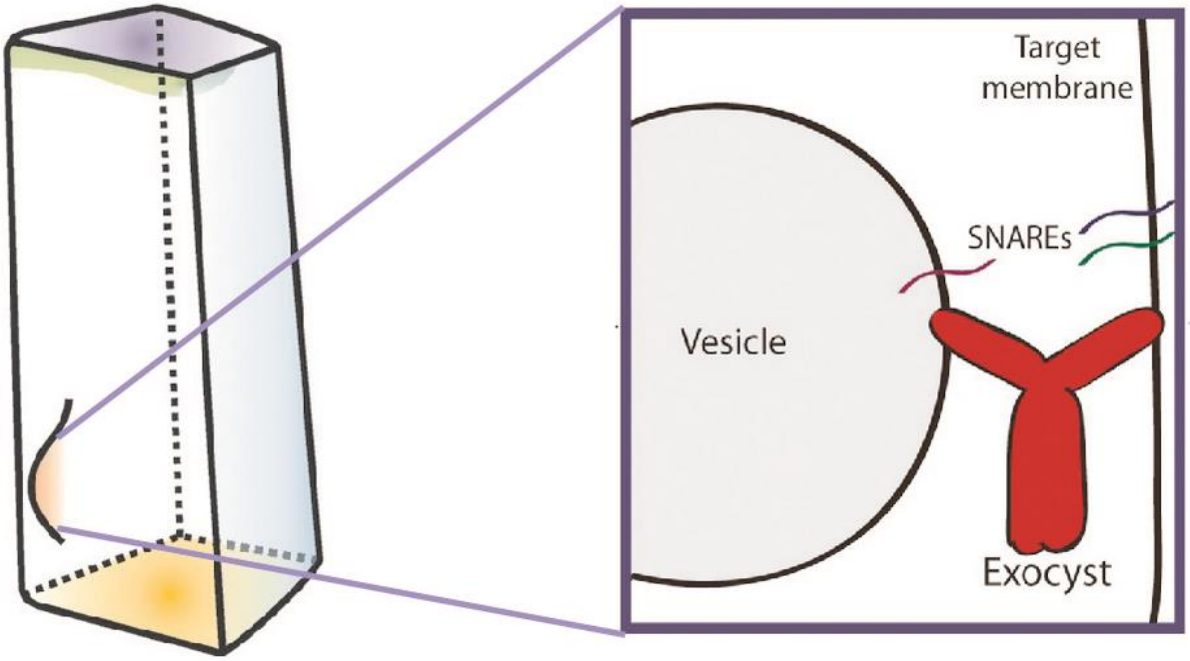

Figure 1. The recycling domain concept and diversification of plant EXO70 paralogs. According to the hypothesis, distinct EXO70 paralogs may be responsible for maintaining multiple recycling domains per cell. Coordination of exocyst and SNARE protein activity for delivery of vesicles to specific parts of the PM might be crucial to the process. Adapted and modified from Zárský et al., 2009 and Heider and Munson, 2012.

An interesting aspect of exocyst function is its regulation by small GTPases. The originally reported Sec15 genetic interaction with Sec4 Rab GTPase was soon confirmed by subcellular fractionation as well as immunoelectron co-localization of both proteins on secretory vesicles (Salminen and Novick, 1989; Finger *et al.*, 1998; Guo *et al.*, 1999*b*). This interaction has been suggested to serve exocyst assembly or activation. Other Rab GTPases were later found to interact with Sec15 in *Drosophila*, too (Wu *et al.*, 2005). Furthermore, the interaction between the activated Cdc42 GTPase of the Rho subfamily and the Sec3 exocyst subunit promotes polarized secretion. Sec3 and Sec5 subunits are mislocalized in *cdc42* mutant yeast and the *cdc42* mutant itself lost polarized growth (Zhang *et al.*, 2001). Ral subfamily GTPases RalA and RalB were also observed to interact with exocyst subunits (Brymora *et al.*, 2001), with implication in diverse cellular processes requiring secretion. Surprisingly, one of the recently-described function of exocyst related to its interaction with activated RalB GTPase is

associated with autophagosome formation and autophagy induction (Bodemann *et al.*, 2011). There are several relevant recent publications illustrating the importance of small GTPases in modulating exocyst function as their effector in polarized secretion. A detailed description of the regulation of exocyst complex by small GTPases in non-plant models is, however, beyond the scope of this dissertation and has been reviewed well elsewhere (Heider and Munson, 2012; Wu and Guo, 2015). Unlike yeast and animal cells, the interaction of plant exocyst SEC3 subunit with GTPases is not direct but rather mediated by ICR1 adaptor in Arabidopsis (Lavy *et al.*, 2007). ICR1 overexpressing line exhibits swollen root hairs and deformed leaf epidermal cells, further supporting its role in polarized exocytosis (Lavy *et al.*, 2007). Also, RIC7 adaptor protein interacts with the active ROP2 GTPase and recruits EXO70B1 exocyst subunit to the PM in *Vicia faba* guard cells (Hong *et al.*, 2016).

Besides small GTPases, exocyst localization is determined by direct interaction with membrane lipids as well as interaction with other proteins. For instance, SEC3 binds several phosphoinositides, consistent with its role as a membrane landmark (Bloch *et al.*, 2016; Li *et al.*, 2017*b*; Ma *et al.*, 2017). Tobacco EXO70B1 subunit co-localizes with phosphatidic acid and PIP2 markers, possibly reflecting its binding capacities. Additionally, tobacco pollen tube PM domains occupied by EXO70A1 and EXO70B1 signals are mutually exclusive, providing further evidence for the recycling domain hypothesis (Zárský *et al.*, 2009; Sekereš *et al.*, 2017). The role of adaptor proteins other than ICR1 and RIC7 in regulation of plant exocyst localization and function will be further discussed in the relevant sections of the Discussion.

Having briefly introduced the plant exocyst complex and its role in polarized secretion, I will now focus on exocyst's contribution to plant immunity, a topic directly related to this thesis.

The role of exocyst in response of plants to pathogen

Soon after the discovery of genes encoding the subunits of the exocyst complex in plant genomes, it was speculated that the plant complex might participate in defense reactions (Elias *et al.*, 2003; Hála *et al.*, 2008). There are two lines of evidence that support this notion. First, plant cell polarization had been known to contribute to plant defense long before the actual publications documented the role of secretory pathway in this process (Schmelzer, 2002). Second, several of the exocyst subunits are not only multiplied in number, but also transcriptionally induced by pathogens or their elicitors (Hruz *et al.*, 2008; Pecenková *et al.*, 2011).

The plant immune system and the role of secretory pathway in plant defense

Plant innate immune system is based on receptors able to recognize conserved molecular structures derived from microbial pathogens. The first layer of defense, termed PAMP-Triggered Immunity (PTI), is activated upon binding of conserved Pathogen-Associated Molecular Patterns (PAMPs) to plant Pattern Recognition Receptors (PRR). Commonly occurring molecules recognizes as PAMPs include those of bacterial flagellin, bacterial elongation factor Tu (EF-Tu) and fungal cell wall component chitin. The resulting signaling cascade leads to buildup of reactive oxygen species (ROS), synthesis of antimicrobial

compounds, production of PATHOGENESIS-RELATED (PR) proteins, deposition of callose into the cell wall and formation of defensive papilla (reviewed in Jones and Dangl, 2006). Papilla contains phenolic compounds, callose and hydroxyproline-rich proteins and represents a physical barrier to invading fungal and oomycete pathogens, as well as extracellularly dwelling bacterial pathogens (Aist, 1976; ThordalChristensen *et al.*, 1997).

A functional secretory pathway is crucial to the process. This is evident from the phenotype of the *pen1* Arabidopsis mutant, defective in SYP121 (PEN1) t-SNARE syntaxin. The *pen1* mutant is compromised in penetration resistance towards non-adapted, non-host fungal pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*), owed to its delayed papilla formation (Collins *et al.*, 2003; Assaad *et al.*, 2004). Likewise, a mutation in the gene coding for SYP132 syntaxin in *Nicotiana benthamiana* tobacco leads to decreased resistance to bacterial pathogen *Pseudomonas syringae* pv. *tabaci* (Kalde *et al.*, 2007).

PEN1 accumulates in papilla during the fungal pathogen attack and this is dependent on GNOM ARF GEF (Guanine nucleotide Exchange Factor) function (Meyer *et al.*, 2009; Nielsen *et al.*, 2012; Nielsen and Thordal-Christensen, 2012). A PEN1 ortholog in barley is ROR2 (REQUIRED FOR MLO-SPECIFIED RESISTANCE2) syntaxin. During the penetration of barley host cell by *Bgh*, YFP-ROR2 disappears from the PM and is redistributed similarly to PEN1 to papilla. ROR2 and callose papillary localization is dependent on ARFA1b/1c small GTPase. At the same time, ARFA1b/1c co-localizes with multivesicular body marker. This is consistent with data in Arabidopsis, where callose and PEN1 deposition to papilla was suggested to rely on GNOM-mediated membrane recycling (Böhlenius *et al.*, 2010; Nielsen *et al.*, 2012).

Many bacterial and fungal pathogens deliver into host plant cells effector proteins able to suppress basal/PTI/preinvasive response. Recognition of these effectors as avirulent proteins in plants expressing corresponding Resistance (R) proteins results in activation of the second layer of defense known as Effector-Triggered Immunity (ETI). This is often accompanied by programed cell death of host cells in the pathogen contact site, thus preventing the pathogen from accessing the nutrients (reviewed in Dangl and Jones, 2001; Jones and Dangl, 2006). There are several gain-of-function mutations of R proteins known that lead to their hyperactivation and spontaneous lesion-mimic phenotype development (reviewed in Pečenková *et al.*, 2016).

Formation of callose plugs in response to *Pseudomonas syringae* infection is dependent on PMR4 callose synthase function (Kim *et al.*, 2005). PMR4 participates in wound and papillary callose deposition (Jacobs *et al.*, 2003). Previous observation as well as our data suggest that both PMR4-GFP and GFP-PMR4 localize to the plasma membrane in barley and Arabidopsis leaf cells, respectively (Blümke *et al.*, 2013; Kulich *et al.*, 2018). Delivery of plasma membrane cargo such as PMR4 relies on intact secretory pathway. It is therefore not surprising that many microbial pathogens suppress PTI by targeting and modification of components of the plant secretory machinery. The fungal toxin brefeldin A (BFA) blocks polarized callose deposition by inhibition of GNOM-dependent membrane recycling (Nielsen

et al., 2012). GNOM is a guanine nucleotide exchange factor for the small GTPase ARF. *P. syringae* effector HopM1 also inhibits callose deposition by induction of degradation of ARF GEF AtMIN7 (Nomura *et al.*, 2006). Interfering with defensive callose deposition thus might to be a common strategy of several plant pathogens.

In addition to secretory pathway, autophagic trafficking has been shown to contribute to plant defense, particularly during the ETI response. A detailed description of multitude of complex, ATG-protein-regulated processes, including engagement in plant defense reactions is, however, provided in author's review paper attached to this dissertation and would be redundant at this place. A possible connection between the autophagy and secretory pathway and the secretion of autophagy pathway-derived compartments to host-plant pathogen interface will be also discussed in the relevant sections of the Discussion.

The exocyst in plant defense

The very first report pointing to plant exocyst involvement in defense reactions was that of Humphry *et al.*, indicating a role for EXO70D3 in *Golovinomyces orontii* fungal resistance (Humphry *et al.*, 2010). This was soon followed by report of Pečenková *et al.*, where EXO70B2 and EXO70H1 subunits were recognized as being important for microbial pathogen resistance. These candidates were selected based on RT-PCR analysis of several EXO70s upon treatment with elicitor peptide elf18. In effect, the *exo70B2* and *exo70H1* Arabidopsis mutants were more sensitive to *Pseudomonas syringae* pv *maculicola* infection than the wild type (WT) plants and *exo70B2* mutants developed abnormal papillae in response to *Bgh* (Pecenková *et al.*, 2011). This was later confirmed by a study demonstrating increased sensitivity of *exo70B2* mutant towards virulent *Pseudomonas syringae* DC3000 (*Pto* DC3000) bacteria and an oomycete pathogen *Hyaloperonospora arabidopsidis*. Importantly, the same article showed a defect in PAMP responses of the *exo70B2* mutant, including impaired ROS production upon Flg22, elf18, chitin and Pep1 elicitor application, and a defect in root growth inhibition by Flg22 peptide. One might therefore speculate that EXO70B2 is engaged in trafficking of PRR receptors such as FLS2, CERK1 and/or PEPR to the PM. Notably, the EXO70B2 is targeted by PUB22 E3 ubiquitin ligase for proteasome-mediated degradation, thus attenuating excessive PAMP-induced signaling (Stegmann *et al.*, 2012).

The closest homolog of EXO70B2 is the EXO70B1 exocyst subunit. In plants, its first function was unexpectedly associated with autophagic transport to the vacuole. The *exo70B1* mutant displays ectopic hypersensitive reaction, manifested by lesion formation, due to SA hyperaccumulation. Also, the *exo70B1* has fewer intravacuolar ATG8-positive bodies and lower levels of anthocyanins in its tissues. Furthermore, ATG8-labeled autophagosomes partially co-localize with EXO70B1 protein and EXO70B1 contains conserved ATG8-Interacting Motifs (AIMs) in its sequence (Tzfadia and Galili, 2013; Cvrčková and Zárský, 2013; Kulich *et al.*, 2013). This, along with the fact that YFP-labeled EXO70B1 co-localizes with anthocyanins in the vacuole, led to a model in which EXO70B1 exocyst complex or subcomplex has been proposed to transport anthocyanins and possibly other secondary metabolites to the vacuole (Kulich *et al.*, 2013; Kulich and Žárský, 2014). Then, defense-

associated roles of EXO70B1 were described. The Arabidopsis *exo70B1* mutant was reported to be less resistant to *Pto* DC3000 than the WT (Stegmann et al., 2013), while it was found to be more resistant to the same pathogen and the *Pto* DC3000 bacterium expressing the AvrRpt2 effector (Zhao et al., 2015). Differences between the two studies were thought to be due to the different growth conditions used; however, the same mutant displays enhanced resistance to the oomycete *H. arabidopsidis* and the adapted powdery mildew *Golovinomyces cichoracearum*, apparently due to the lower threshold for hypersensitive response activation (Stegmann et al., 2013; Zhao et al., 2015).

The lesion formation phenotype of the *exo70B1* mutant was suggested to be a result of TN2 R protein hyperactivation. Unable to bind intact EXO70B1, activated TN2 triggers a signaling cascade involving CALCIUM-DEPENDENT PROTEIN KINASE5 (CPK5) that ultimately leads to H_2O_2 and SA accumulation and hypersensitive response phenotype development (Zhao *et al.*, 2015; Liu *et al.*, 2017). In this scenario, the EXO70B1 is predicted to be manipulated by pathogen effectors and guarded by TN2 protein, capable of eliciting ETI response (Zhao *et al.*, 2015). This would be consistent with its presumed role in defense secretion. Both EXO70B1 and EXO70B2 interact with the SNAP33 protein, a well-known adaptor forming a ternary SNARE complex with PEN1 and VAMP721/VAMP722 syntaxins involved in fungal defense (Kwon *et al.*, 2008; Meyer *et al.*, 2009). Alternatively, TN2 might be under constitutive negative regulation conferred by autophagy-related, EXO70B1-mediated trafficking to the vacuole (Pečenková *et al.*, 2016; see further in the Discussion).

Another EXO70B1's function implied in defense is linked to the regulation of stomatal opening. In this respect, EXO70B1 has been reported to act as a positive regulator of *Pseudomonas*-induced stomatal closure (Stegmann *et al.*, 2013). Stomata are frequently used as entry doors to colonize the leaf mesophyll by microbial pathogens. Outside the phytopathological context, the EXO70B1 is known to be a positive regulator of ABA- and mannitol-induced stomatal closure and a positive regulator of light-induced stomatal opening (Hong *et al.*, 2016; Seo *et al.*, 2016).

Besides EXO70B1 and EXO70B2, the rice exocyst subunits EXO70F2 and EXO70F3 are targeted by Avr-Pii effector derived from *Magnaporthe oryzae* fungus. EXO70F3 is specifically required for Pii R protein-mediated ETI (Fujisaki *et al.*, 2015). It is therefore possible that in the absence of the cognate Pii receptor, *M. oryzae* Avr-Pii effector may have evolved to interfere with EXO70F2/F3 function to promote its own virulence. Similarly, the RXLR AVR1 effector promotes *in planta* growth of *Phytopthora infestans* through stabilization of Sec5 exocyst subunit and suppression of Sec5-mediated callose deposition. The Sec5 subunit itself is also essential for SA-induced PR1 protein secretion and resistance towards *P. infestans* (Du *et al.*, 2015).

A very recent report described a novel interactor of the rice EXO70E1 exocyst subunit, named Bph6. Plants silenced in *Bph6* are more susceptible to brown grasshopper (*Nilaparvata lugens*) feeding. Moreover, rice lines harboring *Bph6* gene are more resistant towards brown planthopper and white-backed planthopper (*Sogatella furcifera*), but not to striped stem borer

(a chewing insect) or the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* strain PXO145. Bph6 stimulates callose deposition into phloem sieve tubes, which should prevent the brown grasshopper from ingesting the phloem sap (Guo *et al.*, 2018). This is a second report describing exocyst's involvement in insect resistance. Previously, Kulich *et al*. showed that *exo70H4* mutant plants support the growth of *Spodoptera littoralis* and *Pieris brassicae* caterpillars less than WT, probably due to their elevated levels of jasmonic acid (Kulich *et al.*, 2015).

Finally, a recent report has suggested a role for several of the *N. benthamiana* exocyst subunits in plant defense. Particularly, Sec5, Sec6 and Sec10 core subunits were found to be required for *P. syringae* resistance. The observed phenotype might be related to defensive callose deposition, as multiple core as well EXO70 subunits mutants are defective in callose secretion in response to *Pst* DC3000 *ΔhrcC* (Du *et al.*, 2018). This might possibly reflect exocyst's function in polarized secretion of PMR4 callose synthase to the PM (see the Discussion). Of note, the publication also describes for the first time a role for the plant exocyst in defense to necrotrophic pathogen. As opposed to biotrophic pathogen infection, silencing of Sec5, Sec6 and Sec10 subunits reduces susceptibility to the necrotrophic fungus *Botrytis cinerea* (Du *et al*., 2018).

RIN4 as a regulator of plant defense responses

One of the debated regulators of PTI immunity is the Arabidopsis RPM1-interacting protein 4 (RIN4). There are many publications describing various aspects of RIN4 biology. In this section, I will mainly focus on those relevant to the topic of polarized secretion.

RIN4 belongs to a larger family of proteins named NOI (for their nitrate-induced domains) that share either one or both conserved NOI domains with a conserved cleavage site (termed RCS) for the bacterial protease AvrRpt2 (Afzal *et al.*, 2011). In the presence of the corresponding RPS2 receptor protein, cleavage of RIN4 at RCS sites by AvrRpt2 *Pseudomonas syringae* protease induces ETI and thereby limits the growth of bacteria (Axtell and Staskawicz, 2003). Besides AvrRpt2, RIN4 is a target of at least three other effector proteins in Arabidopsis that induce either phosphorylation or ADP-ribosylation, and thus, presumably, inhibit the PTI response.

Mutation of RIN4 enhances Flg22- and *Pseudomonas syringae* DC3000 *hrcC*-induced callose deposition (Kim *et al.*, 2005, 2009), whereas RIN4 overexpression suppresses it (Kim *et al.*, 2005; Afzal *et al.*, 2011; Chung *et al.*, 2014). According to these data, RIN4 would seem to act as a negative regulator of pathogen-induced callose deposition. However, upon Flg22 treatment, cleavage of RIN4 by AvrRpt2 was shown to release RIN4 fragments that suppress callose deposition and support enhanced growth of the *P. syringae hrcC* mutant (Afzal *et al.*, 2011). Induced expression of the same effector in Arabidopsis (in *rps2* background) supported the growth of virulent Pto DC3000 strain, probably independently of MAP kinase inhibition (Eschen-Lippold *et al.*, 2016). Moreover, AvrB, another effector derived from *P. syringae* that induces phosphorylation of RIN4 at specific threonine residue, also blocks Pto DC3000-induced callose deposition (Chung *et al.*, 2014). Similarly, injection of Pto effector

AvrRpm1 into plant cells which also induces RIN4 phosphorylation, blocks callose deposition and supports growth of the *P. syringae hrcC* mutant (Kim *et al.*, 2005, 2009; see Table 1). Therefore, RIN4 is rather a positive regulator of defensive callose deposition. Targeting by bacterial effectors of RIN4 suppresses callose deposition and induces hypersensitive cell death in plants expressing respective R proteins.
Given the role of secretory pathway in callose deposition, RIN4's putative regulation of the process and its targeting by effectors makes it a promising target for inspecting its contribution to exocyst-mediated secretion. Indeed, while looking for Arabidopsis exocyst subunit interactors using a yeast two-hybrid (Y2H) screen, we found a binding partner for some of the EXO70 subunits which belongs to the NOI protein family.

Hypotheses

Based on the fact that cleavage and phosphorylation of RIN4 suppresses callose deposition, both exocyst and RIN4 operate at the plasma membrane (Takemoto and Jones, 2005), exocyst has been shown to participate in defensive callose deposition and on our previous data showing NOI6-exocyst interactions, I hypothesized that RIN4 might regulate localization and/or function of exocyst or its individual subunits. This would be especially relevant in the defense context.

Furthermore, after my colleagues described a role of EXO70H4 exocyst subunit in secondary cell wall thickening and loss of rings and callose in the Arabidopsis *exo70H4* mutant trichome, we speculated that EXO70H4-mediated secretion might be necessary for callose synthase delivery to the PM (Kulich *et al.*, 2015). This would also be relevant for polarized secretion during the defense, as cell wall papilla contains callose that is synthesized by PMR4 callose synthase.

Finally, considering the confronting explanations concerning EXO70B1's function in autophagy and immunity, we devised a hypothesis where EXO70B1 mediates autophagic delivery of TN2 R protein to the vacuole for subsequent degradation. This would provide an alternative explanation for the observed rescue of *exo70B1* mutant by *tn2* mutation (Zhao *et al.*, 2015). This, however, awaits further experimental verification.

Table 1: Summary of RIN4-regulated defense phenotypes with references to relevant publications.

Condition	Genetic background	Callose phenotype	Reference
RIN4 overexpression	Col 0 (WT RIN4 present)	Deposition suppressed	Afzal *et al*., 2011; Kim *et al*., 2005
RIN4 fragments (AvrRpt2)		Deposition suppressed	
Phosphorylation of RIN4 (AvrB)	RIN4 complemented *rpm1 rps2 rin4* mutant	Deposition suppressed	Chung *et al*., 2014
Phosphorylation of RIN4 (AvrRpm1)	*rpm1*	Deposition suppressed	Kim *et al*., 2005
rin4 mutant	*rpm1 rps2*	Deposition enhanced	Kim *et al*., 2005

Aims of the Thesis

The rationale behind the research leading to the articles and hypotheses presented in this dissertation follows the data from my previously published diploma thesis as well as general focus of our laboratory.

The thesis aims to answer the following points:

- The relationship between the exocyst and RIN4 protein.

  In my diploma thesis, I performed Y2H analyses following the initial Y2H screen data obtained by my supervisor and discovered the NOI6 protein as an interactor of several of the exocyst subunits. NOI6 is a homolog of the thoroughly-studied RIN4 protein (see the Introduction). Moreover, public proteomic data suggested an interaction between the RIN4 and EXO70B1 and EXO70E2 exocyst subunits (Afzal *et al.*, 2013). Based on this reasoning, my aim in this thesis is to investigate the relationship between the exocyst and RIN4 protein. In order to achieve this aim, I will try to confirm the indicated interactions, examine the localization pattern of the candidate subunit(s) and determine their biological role in polarized secretion.

- The role of EXO70H4 subunit in trichome callose deposition.

  As already mentioned in the Introduction, the EXO70H4 exocyst subunit might play a role in PMR4 callose synthase delivery to the PM. To investigate this possibility, I will join a collaborative research effort aiming to test the localization and interaction between the two proteins in Arabidopsis trichome model system.

- The development of a hypothesis on the role of exocyst proteins in autophagic trafficking and its connection to plant immunity.

  In this aim, I try will in the first place to lay a conceptual framework for the future research that would address the question. This will specifically include a cooperative, extensive literature review and preparation of a review article with a goal of clarifying and reconciling often conflicting results concerning the role of autophagy in plant defense. Also, a baseline position for the EXO70B1 subunit in the process needs to be considered with respect to the knowledge obtained in this search.

Published Papers

During my PhD studies, I published and co-authored the following papers:

1. **Sabol P,** Kulich I, Žárský V. 2017**.** RIN4 recruits the exocyst subunit EXO70B1 to the plasma membrane. Journal of Experimental Botany 68, 3253–3265.

   Contribution to the paper: With a help from my supervisor and my PI, I designed the experimental setup and performed all the experiments.

2. Kulich I, Vojtíková Z, **Sabol P**, Ortmannová J, Neděla V, Tihlaříková E, Žárský V. 2018. Exocyst subunit EXO70H4 has a specific role in callose synthase secretion and silica accumulation. Plant Physiology 176, 2040 - 2051.

   Contribution to the paper: In this research, I cloned the coding of the PMR4 callose synthase, isolated the proteins and did the Western blot from Flg22- and chitin-treated Arabidopsis plants to assess the EXO70H4 upregulation, and participated in manuscript editing.

3. Pečenková T, **Sabol P**, Kulich I, Ortmannová J, Žárský V. 2016. Constitutive Negative Regulation of R Proteins in Arabidopsis also via Autophagy Related Pathway? Frontiers in Plant Science 7, 260.

   Contribution to the paper: I contributed to writing and editing of this Hypothesis and Theory paper.

4. Pecenková T, Markovic V, **Sabol P**, Kulich I, Žárský V. 2017. Exocyst and autophagy-related membrane trafficking in plants. Journal of Experimental Botany 69, 47–57.

   Contribution to the paper: I participated in writing and editing of this review paper.

The aforementioned publications are listed on the following pages.

Journal of Experimental Botany
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This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

RESEARCH PAPER

RIN4 recruits the exocyst subunit EXO70B1 to the plasma membrane

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Abstract

The exocyst is a conserved vesicle-tethering complex with principal roles in cell polarity and morphogenesis. Several studies point to its involvement in polarized secretion during microbial pathogen defense. In this context, we have found an interaction between the Arabidopsis EXO70B1 exocyst subunit, a protein which was previously associated with both the defense response and autophagy, and RPM1 INTERACTING PROTEIN 4 (RIN4), the best studied member of the NOI protein family and a known regulator of plant defense pathways. Interestingly, fragments of RIN4 mimicking the cleavage caused by the *Pseudomonas syringae* effector protease, AvrRpt2, fail to interact strongly with EXO70B1. We observed that transiently expressed RIN4, but not the plasma membrane (PM) protein aquaporin PIP2, recruits EXO70B1 to the PM. Unlike EXO70B1, RIN4 does not recruit the core exocyst subunit SEC6 to the PM under these conditions. Furthermore, the AvrRpt2 effector protease delivered by *P. syringae* is able to release both RIN4 and EXO70B1 to the cytoplasm. We present a model for how RIN4 might regulate the localization and putative function of EXO70B1 and speculate on the role the AvrRpt2 protease might have in the regulation of this defense response.

Key words: Autophagy, EXO70B1, EXO70B2, exocyst, plant immunity, RIN4, secretion.

Introduction

The exocyst is a conserved protein complex involved in tethering secretory vesicles to the destination/target membrane. In plants, it is implicated in diverse cellular processes that lead to the asymmetric distribution of proteins and membranes within the cell (Elias *et al.*, 2003; Zárský *et al.*, 2009, 2013). Therefore, it is involved in processes of cell polarity establishment including root hair and pollen tube growth, polarized growth of stigmatic papillae, cytokinesis, localized deposition of seed coat pectin, transport of PIN auxin carriers to specific parts of the plasma membrane (PM), development of the periarbuscular membrane, maturation of the trichome cell wall, and probably polarized secretion of defense cargo during response to microbial pathogens (Synek *et al.*, 2006; Hála *et al.*, 2008; Kulich *et al.*, 2010, 2015; Pecenková *et al.*, 2011; Genre *et al.*, 2012; Drdová *et al.*, 2013; Zárský *et al.*, 2013; Zhang *et al.*, 2015). The molecular mechanism of exocyst action is believed to involve the tethering of secretory vesicles to the target membrane prior to SNARE-mediated fusion (Heider and Munson, 2012). Recently, a novel function for the exocyst subcomplex containing EXO70B1, SEC5, and EXO84 subunits has been assigned to autophagic membrane transport to the vacuole (Kulich *et al.*, 2013). Despite some progress, the previously described role for the EXO70E2 subunit in unconventional protein secretion and its relationship to the autophagic pathway remain controversial (Lin *et al.*, 2015). In yeast, EXO70 and SEC3 subunits are hypothesized

to serve as landmarks for membrane targeting of the complex (Wu and Guo, 2015). Unlike yeast and mammals, plant genomes encode many isoforms of the EXO70 subunit (Elias *et al.*, 2003; Cvrčková *et al.*, 2012). They are differentially expressed during ontogenesis and in different tissues (Synek *et al.*, 2006; Li *et al.*, 2010), but several EXO70s are expressed in the same cell type, leading to the hypothesis that they might contribute to the definition of specific cortical PM domains within the same cell (Zárský *et al.*, 2009). It is also possible that some of them may have adopted functions different from vesicle tethering during exocytosis. According to the publicly available data, several of the isoforms are transcriptionally up-regulated by pathogens or pathogen elicitors (Hruz *et al.*, 2008), and there is further evidence that points to a substantial contribution of the exocyst to plant defense against microbial pathogens (Pecenková *et al.*, 2011; Stegmann *et al.*, 2012). This engagement in the competition with parasites is also the best explanation for the extraordinary dynamics of EXO70 family evolution (Pecenková *et al.*, 2011; Cvrčková *et al.*, 2012).

It is therefore not surprising that a growing number of studies indicate a direct involvement of the exocyst complex in polarized secretion and signaling during the defense against microbial pathogens. While the *exo70H1* Arabidopsis mutant is more susceptible to infection by the bacterial pathogen *Pseudomonas syringae* pv *maculicola*, the *exo70B2* mutant is more susceptible to the virulent *Pseudomonas syringae* DC3000 (Pto DC3000), *P. syringae* pv *maculicola*, and the oomycete infection caused by *Hyaloperonospora arabidopsidis* (Pecenková *et al.*, 2011; Stegmann *et al.*, 2012). In addition, *exo70B2* forms abnormal papillae during the infection caused by the fungal pathogen *Blumeria graminis* f. sp. *hordei*. The closest homolog of EXO70B2 is the EXO70B1 exocyst subunit, which, besides its role in autophagy, is also engaged in defense against phytopathogens. The Arabidopsis *exo70B1* mutant was reported to be less resistant to Pto DC3000 than the wild type (Stegmann *et al.*, 2013), while it was found to be more resistant to the same pathogen and the Pto DC3000 bacterium expressing the AvrRpt2 effector (Zhao *et al.*, 2015). Differences between the two studies were thought to be due to the different growth conditions used; however, the same mutant displays enhanced resistance to the oomycete *H. arabidopsidis* and the adapted powdery mildew *Golovinomyces cichoracearum*, apparently due to the lower threshold for hypersensitive response activation (Stegmann *et al.*, 2013; Zhao *et al.*, 2015). One member of the cereal-specific EXO70 subfamily is involved in defense against *B. graminis* (Ostertag *et al.*, 2013), and also a core subunit was reported to be targeted by pathogen effectors—the SEC5 exocyst subunit of *Nicotiana benthamiana* interacts with the *Phytophtora infestans* AVR1 effector, resulting in increased sensitivity of the host plant towards the pathogen (Du *et al.*, 2015).

While looking for Arabidopsis exocyst subunit interactors using a yeast two-hybrid (Y2H) screen, we found a binding partner for some of the EXO70 subunits which belongs to the NOI protein family. Based on this observation, we also tested the RPM1 INTERACTING PROTEIN 4 (RIN4) protein for possible exocyst subunit interactions and found that it interacted with the EXO70B1 exocyst subunit. RIN4 is a member of a larger NOI protein family that shares either one or both conserved NOI (nitrate-induced) domains with a conserved cleavage site [termed the RIN4 cleavage site (RCS)] for the bacterial protease AvrRpt2 (Afzal *et al.*, 2013). Besides AvrRpt2, RIN4 is a target of at least three other effector proteins in Arabidopsis that induce either phosphorylation or ADP-ribosylation, and thus, presumably, inhibit the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) response. In the presence of the corresponding resistance (R) proteins, however, effector-triggered immunity (ETI) is initiated, which often culminates in a hypersensitive cell death response. In the case of AvrRpt2, cleavage of RIN4 at RCSs activates the RPS2 R protein (Mackey *et al.*, 2002; Wilton *et al.*, 2010; Afzal *et al.*, 2011). RIN4 homologs in soybean form a heteromeric complex in which only some components interact with the soybean RPS2 ortholog (Selote and Kachroo, 2010). Recently, a new study showed that the AvrRpt2 protease is also able to inhibit PTI independently of RIN4 (Eschen-Lippold *et al.*, 2016).

Although it is speculated that the role of the putative NOI–exocyst interaction is important for pathogen responses in plants (Da Cunha, 2009; Afzal *et al.*, 2013), the actual mechanism through which RIN4 and possibly other NOI proteins influence exocyst function during a defense response is unknown. Because both RIN4 and the exocyst operate at the PM, we investigated the role RIN4 has in the regulation of exocyst localization or function. Moreover, due to the autophagic origin of some of the cellular compartments involved in polarized secretion during the defense response, we speculate that the autophagic and putative defense secretion roles of EXO70B1 might be connected.

Materials and methods

Co-immunoprecipitation

For co-immunoprecipitation (co-IP) experiments, we used 2–3 leaves of *N. benthamiana* transiently expressing red fluorescent protein (RFP)–EXO70B1, green fluorescent protein (GFP)–RIN4, and free yellow fluorescent protein (YFP) under the control of the ubiquitin promoter (in pUB vectors; Grefen *et al.*, 2010), RFP–EXO70B2 under the control of the 35S promoter (pH7WGR2 vector; Pecenková *et al.*, 2011), and from leaves from plants infiltrated with P19 vector only as a negative control. For protein extraction, we ground 3–4 agroinfiltrated leaves in liquid nitrogen and transferred the resulting powder into the ice-cold extraction buffer with added protease inhibitors (Roche). We vortexed the lysates briefly and centrifuged them at 3700 *g*, 4 °C for 15 min, and subsequently centrifuged the supernatant for another 10 min at 10 000 *g*, 4 °C. We then used a μMACS GFP Isolation Kit (Miltenyi) for co-IP. We used a modified extraction buffer based on the Miltenyi kit Lysis Buffer that contained only 0.1% Triton X-100 (compared with 1% in kit buffer), as we experienced non-specific binding of proteins to the magnetic beads at high Triton X-100 concentration as previously described (Avila *et al.*, 2015). Eluates were resolved on a 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. Proteins were then detected on the membrane using anti-RFP (ChromoTek), anti-hemagglutinin (HA; Cell Signaling), and horseradish peroxidase (HRP)-conjugated anti-GFP (Miltenyi) antibodies.

Transient expression of proteins in Nicotiana benthamiana

Transient expression was performed as previously described (Pecenková *et al.*, 2011) with slight modification to the composition of the infiltration buffer (50 mM MES pH 5.6, 2 mM Na_3PO_4, 0.5% glucose, and 100 µM acetosyringone). For co-IP, we extracted proteins from leaves 3 d after infiltration. For microscopic observations, we used GFP–RIN4 under the control of the ubiquitin promoter, RFP–EXO70B1 (coding sequence) under the control of the ubiquitin promoter, and RFP–EXO70B2 under the control of the 35S promoter (pH7WGR2 vector; used in Pecenková *et al.*, 2011). We cloned the GFP–RIN4 construct from a cDNA clone in the pUNI51 vector obtained from the Arabidopsis Biological Resource Center, into the pENTR1A vector and then into the pUBN-GFP destination vector (Grefen *et al.*, 2010). We also cloned the EXO70B1 coding sequence into the pENTR1A vector using primers in Table 1, and then into the pUBN-RFP destination vector (Grefen *et al.*, 2010). The fluorescent constructs were observed in plants 2–3 d after infiltration. For co-localization analysis, we employed a 35S:PIP2;1–GFP construct (in the pGWB5 vector) as a PM-localized protein (Boursiac *et al.*, 2005). For further recruitment studies, we cloned EXO70B1 with a C-terminal cyan fluorescent protein (CFP) under the 35S promoter into the pB7m34GW MultiSite Gateway destination vector. The 35S:SEC6–RFP construct was kindly provided by Tamara Pečenková, and the GFP–SYP121 construct by Mike Blatt's laboratory.

Cloning of EXO70B1–mRuby2 with the EXO70B1 promoter

The EXO70B1 coding sequence together with the EXO70B1 promoter was cloned into the Gateway® TagRFP-AS-N vector (Evrogen) using the primers in Table 1. The TagRFP sequence was then replaced by mRuby2 from the cDNA3.1-Clover-mRuby2 vector using the primers in Table 1. Vector pcDNA3.1-Clover-mRuby2 was a gift from Kurt Beam (Addgene plasmid # 49089). The LR reaction was performed subsequently between this vector and a modified pBGW vector (Karimi *et al.*, 2002) containing the transcription terminator. To clone the transcription terminator into the pBGW vector, a *Pst*I fragment (with attr2 and a terminator) of the pUB destination vector (Grefen *et al.*, 2010) was cloned into the original pBGW vector.

Yeast two-hybrid assay

NOI6 and different RIN4 constructs were cloned into the pGADT7 vector using the primers in Table 1. All the other exocyst constructs and the Y2H assay have been described previously (Hála *et al.*, 2008). Briefly, yeast transformed with the respective constructs were plated first on plates without leucine and tryptophan and then in a 10× dilution series starting with OD_{600nm}=0.1 on plates without leucine, tryptophan, histidine, and adenine to test for protein–protein interactions.

Confocal microscopy analysis

For image acquisition, we used a Leica TCS SP2 confocal microscope with a ×63/1.2 water immersion objective, a Zeiss LSM 880 confocal microscope with a ×63/1.2 water immersion objective, and a Nikon TE200e with a Yokogawa Andor spinning disc unit. The GFP constructs were excited at 488 nm and detected at 505–530 nm, and RFP constructs were excited at 560 nm and detected at 600–620 nm.

Quantification of fluorescence signals

To calculate membrane to cytoplasm fluorescent signal ratios, we measured the fluorescence intensity in *N. benthamiana* cells transiently expressing the respective fluorescent constructs. For this, we constructed *Z*-stacks of cells from which we further chose projections only from the medial plane of the cell to avoid mistaking the PM surface for a cytoplasmic signal. Using the Fiji software (Schindelin *et al.*, 2012), we first measured the raw integrated density divided by the region of interest (ROI) area, and then calculated the average value of this ratio from five different regions within a cell. We determined the membrane to cytoplasm ratio as a mean value from 11 cells for each combination of constructs.

Pseudomonas syringae *assay*

We first infiltrated tobacco leaves with *Agrobacterium* strains harboring the different constructs described above. One day after agroinfiltration, we grew an overnight liquid culture of Pto DC3000 strains HrpH– (a mutant for the type III secretion system) and AvrRpt2 in LB medium with appropriate antibiotics. Cultures were pelleted and washed with 10 mM $MgCl_2$ to remove the antibiotics, and then diluted to the final $OD_{600\,nm}$=0.1 in 10 mM $MgCl_2$. We infiltrated this solution into already agroinfiltrated leaves and observed the fluorescence 18–20 h later. No obvious cell death symptoms were macroscopically visible in such treated leaves.

DTT treatment

For the evaluation of the effect of DTT on protein localization, we infiltrated a 50 mM aqueous DTT solution into *Agrobacterium*-infiltrated *N. benthamiana* leaves 3 d after agroinfiltration and observed the fluorescence signal 3 h after DTT infiltration.

Results

Exocyst subunit EXO70B1 interacts with RIN4, but not with RIN4 fragments

In our Y2H screen, we identified the NOI6 protein as an interactor of the EXO70A1 exocyst subunit (see Supplementary

Table 1. *List of primers*

RFP-EXO70B1	B1 CDS ECOrevstop	AAAGTCGACATGGCGGAGAATGGTGAAG
	B1cdsSAL1	TTTGAATTCCTTCATTTTCTTCCCGTGGTAGTC
RCS2_C in pGADT7 (without the C-terminus)	RIN4_RCS2_C_T7_for	TGCGGATCCTCATGGACTGGGACGAGAACA
with the C-terminus	RIN4_RCS2_C_T7_rev	CAAGTCGACTTCATGAGGAAGTGTTGTTCG
	RIN4 FR pGAD for	AGGGATCCCCATGGACTGGGAC
RIN4 CDS in pGADT7	RIN4 full pGAD for	AGGGATCCCCATGGCACGTTC
	RIN4 full pGAD rev	CAGTCGACCTCATTTTCCTCCAAAG
RIN4 N-RCS2 in pENTR1A	RIN4_N_RCS2_SalI	TTCGTCGACCATCAAACCGAATTTAGGCACCACT
	RIN4_N_RCS2_BamHI	GAGGGATCCCCATGGCACGTTCGAATGTAC
EXO70B1 coding sequence with promoter	B1 for	CGGGAATTCAGAGGATAGGAATATATAAAT
	B1 rev	ACCGTCGACTTTCTTCCCGTGG
mRuby2 cloning into TagRFP-AS-N	mRUBY2 AGEI for	ACCGGTAATGGTGTCTAAGGGCGAAGAG
	mRUBY2 NOT1 rev	TTTGCGGCCGCTTACTTGTACAGCTCGTCCATCC

Fig. S1 at *JXB* online). NOI6 (At5g64850) is a member of a larger family of NOI proteins in Arabidopsis (for a recent review, see Afzal *et al.*, 2013). This led us to test whether any of the subunits of the Arabidopsis exocyst complex would interact with the RIN4 protein, which is the most studied member of the NOI family. We therefore cloned the RIN4 coding sequence into a Y2H prey vector, several of the exocyst subunits into bait vectors, and subsequently tested for

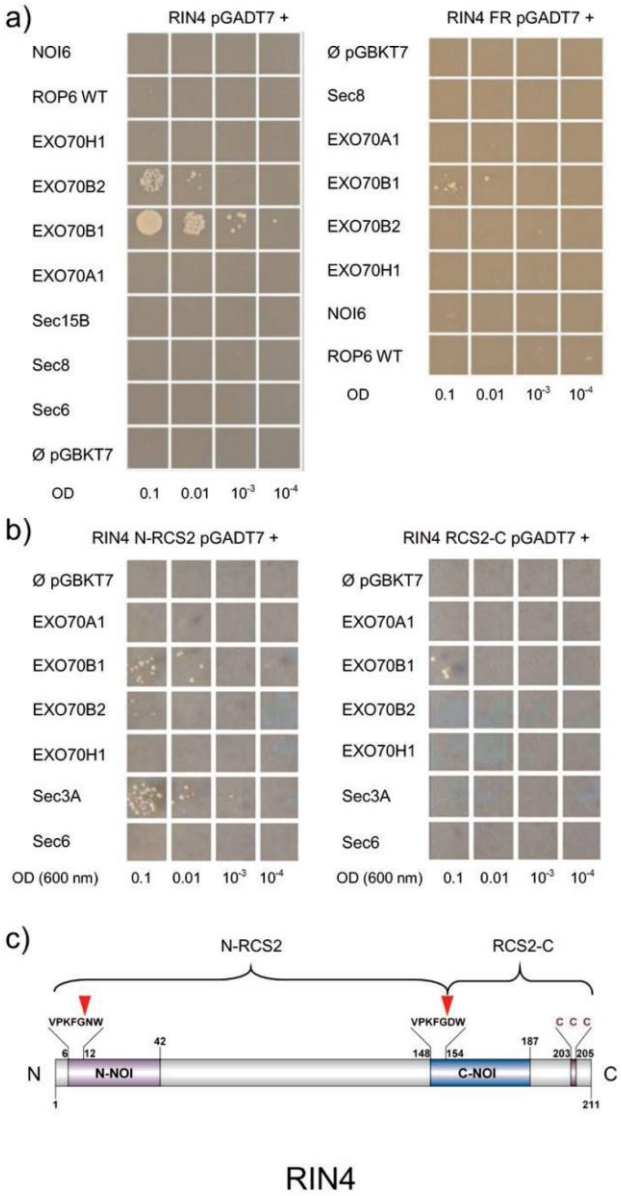

Fig. 1. Yeast-two hybrid assays showing interactions of exocyst subunits with RIN4. (a) Full-length RIN4 protein (on the left) and the C-terminal membrane-anchored RIN4 fragment (on the right). (b) An N-terminal fragment and a C-terminal fragment without palmitoylation sites. SEC3A yeasts autoactivate expression of selection markers (as shown by Hála *et al.*, 2008). (c) The overall structure of RIN4. The AvrRpt2 cleavage consensus sequences (RCS) within NOI domains are shown above the bar scheme, with actual cleavage sites indicated by triangles. C-terminal cysteine residues responsible for membrane anchoring are shown as CCC above the bar scheme. The range of N- (N-RCS2) and C- (RCS2-C) terminal RIN4 fragments used in Y2H experiments is also depicted above the bar scheme of the protein. The model of RIN4 was drawn with CSS-Palm software (Ren *et al.*, 2008). (This figure is available in colour at *JXB* online.)

pairwise interactions. Interestingly, we observed an interaction between the full-length RIN4 protein and EXO70B1. A weak interaction was also observed with the EXO70B2 subunit (Fig. 1a). These are two closely related paralogs within the B clade of the Arabidopsis EXO70 family that share some sequence similarity at the protein level (for a recent review, see Cvrčková *et al.*, 2012). None of the other EXO70 paralogs tested showed this interaction with RIN4.

When the bacterial pathogen *P. syringae* injects its AvrRpt2 effector protein into the plant cell, it cleaves RIN4 at two well-conserved cleavage sites, which produces three fragments; two fragments are subsequently released into the cytoplasm and suppress PTI (Afzal *et al.*, 2011). We therefore tested the interaction with a C-terminal RIN4 fragment that mimics cleavage by the AvrRpt2 protease at the C–NOI cleavage site (Kim *et al.*, 2005; Afzal *et al.*, 2011). Interactions with this cleavage product were lost and only EXO70B1 showed a very weak interaction. Similarly, the N-terminal RIN4 fragment that mimics cleavage at the N–NOI cleavage site also interacts weakly with only EXO70B1, although somewhat more strongly than the C-terminal fragment (Fig. 1b).

We confirmed EXO70B1 Y2H interactions with the full-length RIN4 using a co-IP assay with transiently expressed

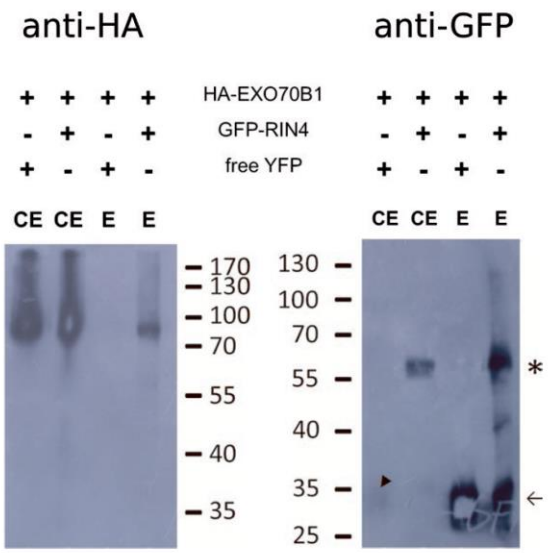

Fig. 2. Co-immunoprecipitation assay (co-IP) showing an interaction between HA-EXO70B1 and GFP–RIN4 proteins transiently expressed in *N. benthamiana*. Proteins were immunoprecipitated with anti-GFP magnetic beads, and EXO70B1 was detected with anti-HA antibody. Experiments were performed three times with similar results. Marker sizes are shown in kDa next to the blots. Left: detection of HA-EXO70B1 in total cell extracts (CE) from plants co-expressing free YFP; in total cell extracts from plants co-expressing GFP–RIN4, in eluate (E) after co-IP with free YFP, and in eluate after co-IP with GFP–RIN4. Right: detection of GFP/YFP in plants expressing HA-EXO70B1 and co-expressing YFP in total cell extract (CE), GFP–RIN4 in total cell extract, YFP in eluate (E) after co-IP, and GFP–RIN4 in eluate after co-IP. The arrow marks bands corresponding to free YFP, and the asterisk marks the position of GFP–RIN4. (This figure is available in colour at *JXB* online.)

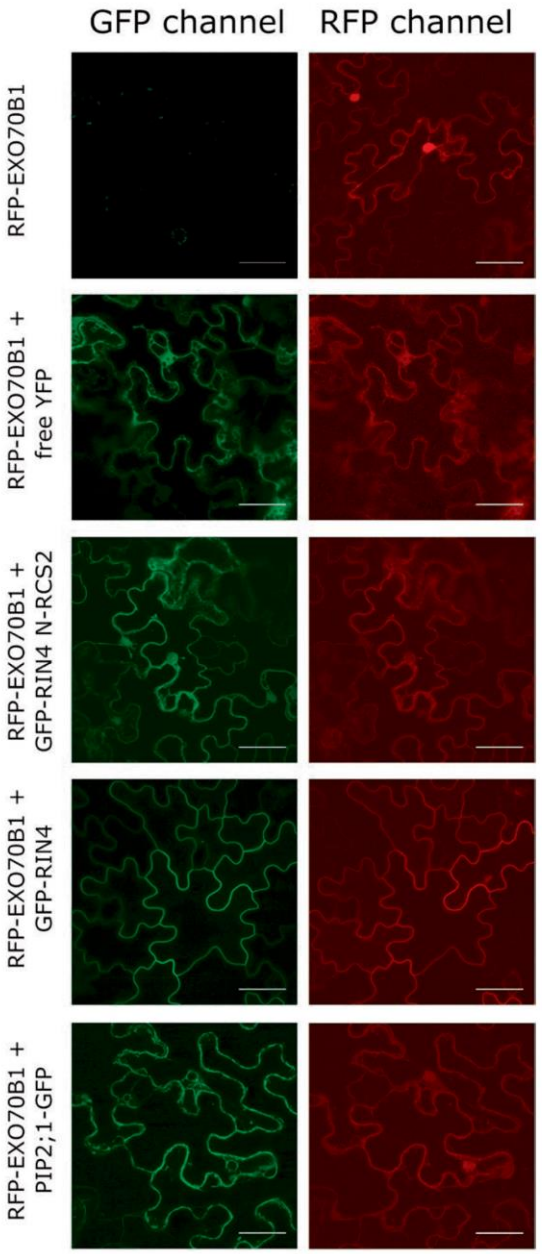

Fig. 3. RIN4 recruits EXO70B1 to the plasma membrane. Shown are the confocal microscopy images of fluorescently labeled constructs transiently expressed in *N. benthamiana* leaves. While RFP–EXO70B1 alone (upper two panels) localized mainly to the cytoplasm and nucleus, when co-expressed with GFP–RIN4, RFP–EXO70B1 was almost exclusively localized to the plasma membrane. In contrast to RIN4, aquaporin PIP2;1–GFP did not change the subcellular localization of RFP–EXO70B1. RFP–EXO70B1 without RIN4 co-localized in the cytoplasm with both free YFP and the N-terminal RIN4 fragment (N-RCS2). Scale bars=50 μm.

proteins in *N. benthamiana* leaves. With anti-GFP magnetic beads, we were able to precipitate HA-tagged EXO70B1 protein together with GFP–RIN4, but not with the free YFP control (Fig. 2). Together, our data indicate an interaction between EXO70B1 and RIN4 proteins.

RIN4 recruits EXO70B1 to the PM

As the RIN4 protein is localized to the PM and one of the putative functions of EXO70B1 would be the targeting of vesicles to the pathogen attack site based on its immunity-related phenotypes (Stegmann *et al.*, 2012; Zhao *et al.*, 2015), we hypothesized that RIN4 might be required for the EXO70B1 protein and possibly also the whole exocyst complex function at the PM. Due to the presence of several NOI proteins in the Arabidopsis proteome with potential functional redundancy and the difficulty of working with multiple gene knockouts, we used the *N. benthamiana* heterologous model system for transient expression and cellular dynamic studies. When we expressed RFP–EXO70B1 under the ubiquitin promoter in *N. benthamiana* leaves, we observed a strong fluorescence signal in the cytoplasm, nucleus, and at the PM, essentially in accordance with the previously published localization patterns (Hong *et al.*, 2016; Fig. 3). We confirmed the cytoplasmic localization of RFP–EXO70B1 by co-expression with both free YFP and the previously published cytoplasmic N-terminal GFP–RIN4 fragment, which is normally released to the cytoplasm after AvrRpt2 cleavage at the RCS2 cleavage site (Takemoto and Jones, 2005; Afzal *et al.*, 2011).

Interestingly, when we co-expressed RFP–EXO70B1 with GFP–RIN4 under the ubiquitin promoter, EXO70B1 localized almost exclusively to the PM (Fig. 3). This localization was dependent on the strength of GFP–RIN4 expression, with stronger expression resulting in more RIN4 protein on the PM, and subsequently more EXO70B1 on the PM. Quantification of membrane to cytoplasm fluorescence ratios revealed a significant difference between EXO70B1 PM localization when EXO70B1 was expressed alone as compared with when EXO70B1 was co-expressed with RIN4 (Fig. 6a). We further wanted to know whether the effect of EXO70B1 recruitment to the PM was specific for the RIN4 protein. We therefore expressed another PM-localized protein, PIP2;1 aquaporin,

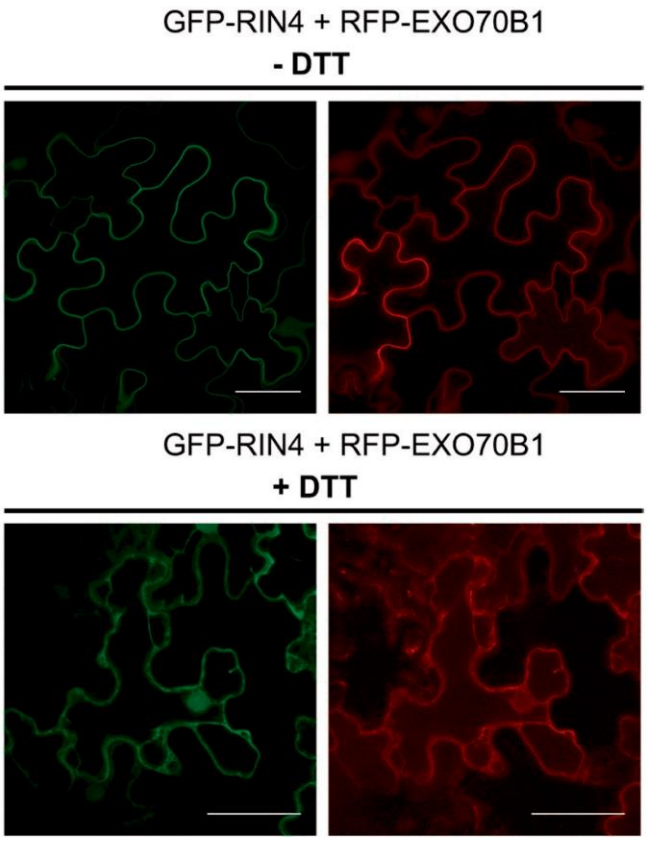

Fig. 4. DTT causes relocalization of both GFP–RIN4 and RFP–EXO70B1 to the cytoplasm. Shown are the confocal microscopy images of fluorescently labeled constructs transiently expressed in *N. benthamiana* leaves. Upper panels depict control cells; lower panels show cells treated with 50 mM DTT. Scale bars=50 µm.

and checked whether it influenced EXO70B1 localization. When PIP2;1–GFP was expressed transiently in *N. benthamiana* under the 35S promoter, it localized not only to the PM, but also to the cytoplasm and a structure possibly representing the endoplasmic reticulum. Nevertheless, when we co-expressed RFP–EXO70B1 together with PIP2;1–GFP, EXO70B1 localization was still cytoplasmic (Fig. 3), confirming that RIN4 specifically recruits EXO70B1 to the PM. Similarly, EXO70B1 partially co-localized with GFP–SYP121 expressed under the ubiquitin promoter in the PM. However, GFP–SYP121 did not recruit EXO70B1 to the PM (Supplementary Fig. S2).

Application of DTT for 3 h by infiltration into *N. benthamiana* leaves caused relocalization of GFP–RIN4 to the cytoplasm, possibly due to interference with palmitoylation of C-terminal cysteinyl residues (Fig. 4). Although we cannot rule out a general effect of DTT on disulfide bond formation and, thus, the tertiary structure of the protein, disulfide bond prediction software did not predict any disulfide bonds in RIN4 or EXO70B1 proteins, while the other model predicted some disulfide bonds in EXO70B1 only (Ceroni *et al.*, 2006; Ferrè and Clote, 2006). Nevertheless, RFP–EXO70B1 also translocated from the membrane to the cytoplasm under these conditions, as compared with the untreated control cells. These results suggest that RIN4 is mainly responsible for membrane localization of EXO70B1.

Pseudomonas *AvrRpt2 releases both RIN4 and EXO70B1 from the PM to the cytoplasm*

Based on our interaction and localization data, we reasoned that the delivery of the AvrRpt2 protease, which cleaves RIN4 into fragments (Afzal *et al.*, 2011), could also release EXO70B1 from the PM. Indeed, we observed RFP–EXO70B1 signal mostly in the cytoplasm in most of the fluorescent agroinfiltrated *N. benthamiana* leaf cells after we co-infiltrated a *P. syrinage* strain expressing the AvrRpt2 protease. Both GFP–RIN4 and RFP–EXO70B1 were released to the cytoplasm in cells where AvrRpt2 had been delivered. In contrast, both GFP–RIN4 and RFP–EXO70B1 stayed at the PM when we co-infiltrated the *P. syringae* mutant strain HrpH– that is deficient in the type III secretion system (Fig. 5). These results support the notion that PM

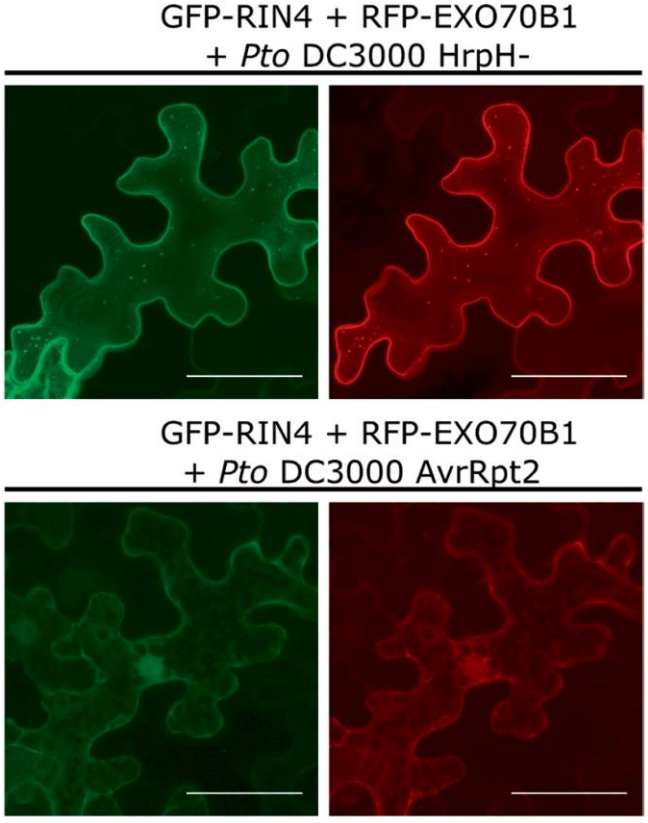

Fig. 5. *P. syringae* AvrRpt2 causes relocalization of both GFP–RIN4 and RFP–EXO70B1 to the cytoplasm. Shown are *Z* projections of confocal images of cells transiently expressing GFP–RIN4 (left panels) together with RFP–EXO70B1 (right panels) that were co-infiltrated either with the mutant strain of *Pseudomonas* (*Pto* DC3000 HrpH–; upper two panels) or with a strain harboring the AvrRpt2 protease (*Pto* DC3000 AvrRpt2; lower two panels). See the Materials and Methods for further details. Scale bars=50 μm.

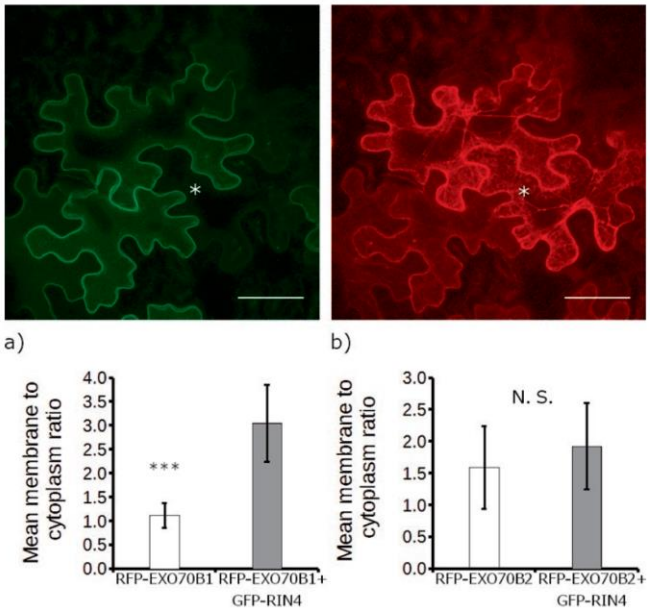

Fig. 6. RIN4 does not recruit EXO70B2 to the plasma membrane. GFP–RIN4 (upper left panel) was overexpressed together with RFP–EXO70B2 (upper right panel) in *N. benthamiana* leaves. Asterisks mark a cell expressing RFP–EXO70B2 only. Panels are *Z* projections of 17 confocal sections. Scale bar=50 μm. Quantification of the membrane to cytoplasm fluorescence ratio from constructs transiently expressed in *N. benthamiana* leaves is shown in the bottom part of the figure. Fluorescence was measured as described in the Materials and Methods in the cytoplasmic strands and the membrane portion of the cells. Shown are the average and the SD (error bars) from 11 cells for each combination. Cytoplasmic strands were seen only occasionally in (a) with most RFP–EXO70B1 signal being on the plasma membrane when co-expressed with GFP–RIN4. The average ratio for this combination is therefore underestimated. Three asterisks denote a significant difference as determined by a *t*-test (*P*-value <0.001) and N.S. indicates a difference that is not statistically significant (*t*-test *P*-value >0.05).

localization of EXO70B1 is to a large extent dependent on intact RIN4 protein.

RIN4 does not recruit EXO70B2 to the PM

The closest homolog of EXO70B1 in Arabidopsis is the EXO70B2 exocyst subunit, which also showed some interaction with RIN4 in our Y2H assay (Fig. 1). We therefore asked if EXO70B2 is also recruited by RIN4 to the PM. When transiently expressed under the 35S promoter in *N. benthamiana* leaves, EXO70B2 localized mostly to the cytoplasm, as previously reported (Pecenková *et al.*, 2011), with some weak PM signal. Unlike EXO70B1, RFP–EXO70B2 stayed in the cytoplasm even when it was co-expressed with GFP–RIN4 (Fig. 6). Additionally, fluorescence signal quantification did not show any significant difference between EXO70B2 expressed alone and EXO70B2 co-expressed with RIN4 (Fig. 6b). Therefore, EXO70B2 is not recruited to the PM by RIN4.

SEC6 core exocyst subunit is also not recruited to the PM by RIN4

To determine whether RIN4 recruits EXO70B1 to the PM as a part of the exocyst holocomplex, we investigated SEC6 core

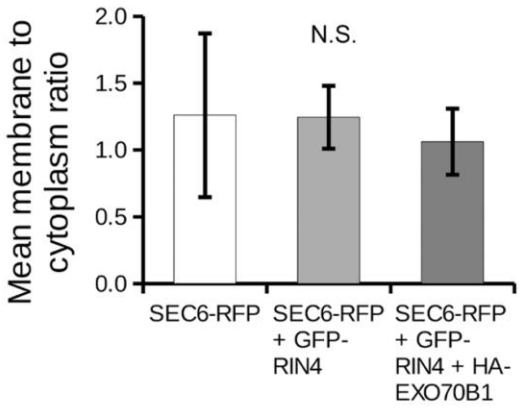

Fig. 7. Neither GFP–RIN4 nor GFP–RIN4 co-expressed with HA-EXO70B1 recruit SEC6–RFP to the PM. Shown is the quantification of membrane to cytoplasm fluorescence ratio of constructs transiently expressed in *N. benthamiana* leaves. Fluorescence was measured as described in the Materials and Methods in the cytoplasmic strands and the membrane portion of the cell. Depicted are the means and the SD (error bars) from eight (SEC6–RFP alone and with GFP–RIN4) and nine (SEC6–RFP with GFP–RIN4 and HA-EXO70B1) cells. Differences between the means are not statistically significant (N.S.; ANOVA *P*-value=0.599).

exocyst subunit subcellular localization. SEC6–RFP (Fendrych *et al.*, 2013) was expressed transiently under the 35S promoter in *N. benthamiana* leaves and localized almost exclusively to the cytoplasm with minimal PM signal. When co-expressed with GFP–RIN4, SEC6–RFP remained in the cytoplasm. We previously observed that SEC6 interacts with EXO70B1 (Kulich *et al.*, 2013) and therefore wondered if SEC6 PM localization would require co-expression of the *Arabidopsis thaliana* EXO70B1. However, when we co-expressed SEC6–RFP together with GFP–RIN4 and HA-tagged EXO70B1, SEC6–RFP still showed a mostly cytoplasmic signal (Fig. 7). Along with HA-EXO70B1, we also cloned and transiently expressed EXO70B1 with a C-terminal CFP together with GFP–RIN4 and SEC6–RFP; however, in this combination, GFP–RIN4 was unable to recruit SEC6–RFP to the PM (Fig. 8). Thus, neither GFP–RIN4 alone nor combined with HA-EXO70B1 or EXO70B1–CFP is sufficient to recruit SEC6–RFP to the PM.

In Arabidopsis, RIN4 recruits EXO70B1 to the PM in guard cells

Finally, we were interested in determining how the cellular localization of the RFP-tagged EXO70B1 exocyst subunit is influenced by GFP–RIN4 expression in Arabidopsis. In the wild-type (WT) background, RIN4, and possibly other NOI proteins that might interact with EXO70B1 and influence its localization, is endogenously expressed. We therefore did not expect a large change in EXO70B1 localization in WT Arabidopsis after GFP–RIN4 overexpression. This turned out to be the case for epidermal pavement cells of the first true leaves in 7-day-old Arabidopsis seedlings, where RFP–EXO70B1 expressed under the ubiquitin promoter localized mostly to the PM (Fig. 9). This localization remained unchanged even in plants co-expressing GFP–RIN4 under the ubiquitin promoter. Surprisingly, this pattern was quite distinct from RFP–EXO70B1 localization in stomatal guard cells, where it localized mostly to the cytoplasm and the nucleus, similar to the localization in the *N. benthamiana* transient system, where EXO70B1 was recruited to the PM in plants overexpressing GFP–RIN4 (Fig. 9).

To see the effect of *rin4* mutation on EXO70B1 PM localization, we cloned the EXO70B1–mRuby2 construct under its own promoter and compared its localization in the WT and *rin4 rps2* mutant. Consistent with the redundancy in the NOI protein family, we did not observe any difference in localization between the WT and *rin4 rps2* mutant cotyledon cells of 7-day-old seedlings. EXO70B1–mRuby2 localized to the PM and the cytoplasm in both genotypes (Supplementary Fig. S3). In comparison with the ubiquitin promoter-driven construct, however, we were able to see rarely and only cytoplasmic EXO70B1 signal in guard cells. This could be due to a lower level of expression of this construct, but could also point to a distinct form of regulation of EXO70B1 protein levels in guard cells.

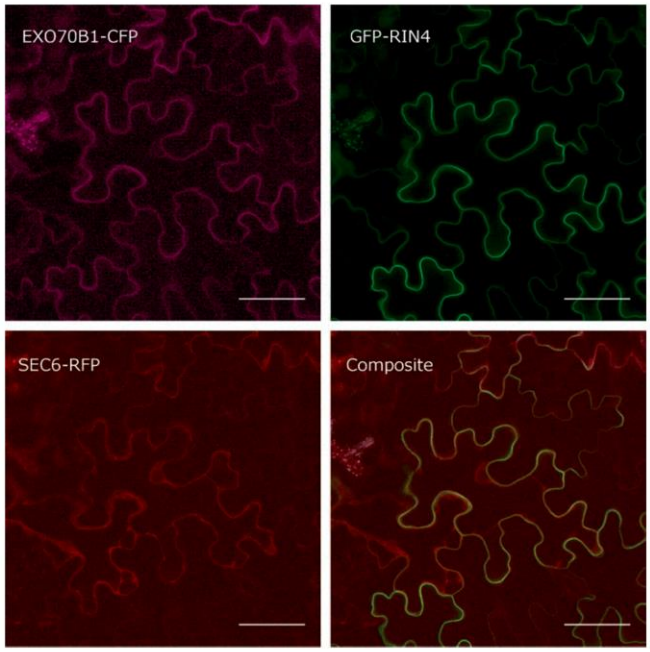

Fig. 8. RIN4 does not recruit SEC6 even in the presence of EXO70B1. EXO70B1–CFP was co-expressed with GFP–RIN4 and SEC6–RFP transiently in *N. benthamiana* leaves. Shown are the confocal images and composite images. Cytoplasmic strands are clearly visible in the SEC6–RFP channel. Scale bar=50 μm.

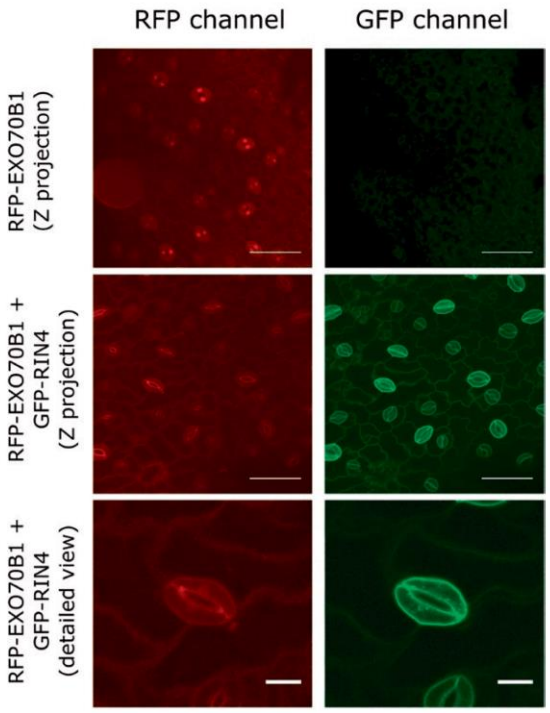

Fig. 9. RIN4 recruits EXO70B1 to the PM in Arabidopsis stomatal guard cells. Confocal images of epidermal pavement and guard cells of the first true leaves of Arabidopsis seedlings show red fluorescence of RFP–EXO70B1 and green fluorescence of GFP–RIN4. Note the cytoplasmic and nuclear signal when RFP–EXO70B1 is expressed alone. Images in the upper two rows are maximal projections of 24 (RFP–EXO70B1) and nine (RFP–EXO70B1+GFP–RIN4) optical sections, respectively. The scale bar is 50 μm for the images in the upper two rows and 10 μm for the third row.

Discussion

Mechanisms related to RIN4 function in plant immunity interactions are not fully understood despite quite large efforts from the research community. In this article, we present new data on EXO70B1 localization with respect to its immunity-related function. We show by both Y2H and co-IP assays that the EXO70B1 exocyst subunit interacts with RIN4 and that this interaction is almost lost when RIN4 is cleaved into fragments. This suggests that the interaction site for EXO70B1 lies within the AvrRpt2 protease cleavage motifs of RIN4, or that they at least contribute to the 3D structure recognized by EXO70B1. Furthermore, as the N-terminal RIN4 fragment released to the cytoplasm upon cleavage interacts somewhat more strongly with EXO70B1 than the C-terminal fragment, we might speculate that the relocalization of the N-terminal part of RIN4 may prevent proper PM targeting of EXO70B1 in a dominant negative manner. Indeed, we observed RFP–EXO70B1 signal mainly in the cytoplasm when we co-infiltrated the *P. syringae* strain containing AvrRpt2 protease as compared with the mutant strain control. Although less specific, DTT results further corroborate the hypothesis that EXO70B1 PM localization is dependent, at least in the *N. benthamiana* heterologous system, on the RIN4 protein. This is also in agreement with public proteomic data, according to which EXO70B1 as well as EXO70E2 exocyst subunits are predicted to interact with RIN4 protein (Da Cunha, 2009; Afzal *et al.*, 2013).

Interestingly and in contrast to EXO70B1, its closest homolog EXO70B2 is not recruited to the PM by RIN4. This corresponds well with Y2H data, which showed that EXO70B2 interacts only weakly with the full-length RIN4 protein. Despite the fact the interaction data and quantification of the PM to cytoplasm ratio for EXO70B2 do not support the RIN4-dependent localization hypothesis, we cannot completely rule out a contribution of RIN4 to EXO70B2 localization and function in an immunity context, especially in a homologous Arabidopsis system. EXO70B2 is known to play a role in defense against microbial pathogens (Pecenková *et al.*, 2011; Stegmann *et al.*, 2012). Alternatively, EXO70B2 might use different proteins from the NOI family as adaptors for PM targeting. This would be consistent with the apparent lack of phospatidylinositol-4,5-bisphosphate-binding sites on the C-terminus of EXO70B2 (Zárský *et al.*, 2009), which are responsible for binding to the PM.

Localization of fluorescent constructs showed that only RIN4, but not the aquaporin PIP2;1, targets EXO70B1 to the PM, indicating that RIN4 specifically pulls EXO70B1 to the PM. As the Arabidopsis genome encodes several NOI proteins, these might also contribute to EXO70B1 localization and function; however, there are no NOI proteins predicted that share substantial amino acid similarity with RIN4 in the *N. benthamiana* genome. We were therefore able to show that RIN4 is specifically sufficient to localize EXO70B1 to the PM. Our preliminary data also indicate that NOI6 interacts with EXO70B1 (Supplementary Fig. S1); the precise roles of this and possibly other NOI proteins in exocyst localization have yet to be determined. A similar influence on exocyst localization has been hypothesized for several proteins in different developmental and/or environmental contexts (see below).

Unlike EXO70B1 and EXO70B2 exocyst subunits, SEC6 does not interact with RIN4 in our Y2H assay. Consistently, we did not observe any recruitment of SEC6–RFP to the PM by GFP–RIN4. SEC6 was shown to interact with EXO70B1 (Kulich *et al.*, 2013); however, this Y2H interaction was rather weak and this is probably why we did not observe any SEC6 recruitment even when HA-EXO70B1 was co-infiltrated along with GFP–RIN4. Alternatively, the RFP fluorescent tag on the C-terminus of SEC6 may prevent *in vivo* interaction between HA-EXO70B1 and SEC6–RFP, or SEC6 and RIN4 may compete for a common binding site of EXO70B1. Furthermore, SEC6 may require other Arabidopsis exocyst subunits not present in *N. benthamiana* for its interaction and subsequent recruitment. Unlike *N. benthamiana*, in Arabidopsis SEC6–RFP localizes to the PM and its localization is EXO70A1 dependent (Fendrych *et al.*, 2013). Therefore, we cannot currently assess the contribution of RIN4 to exocyst holocomplex PM recruitment. However, as in the case of ROP2–RIC7 EXO70B1 PM recruitment, the

rest of the complex need not follow EXO70B1. The exocyst has been recently described to exist as a stable octameric complex in yeast cells (Heider *et al.*, 2016). Yet, the EXO70 subunit alone is capable of inducing membrane curvature independently of the rest of the complex (Zhao *et al.*, 2013). The notion that particular EXO70 isoforms present in plant cells could act separately to support specific membrane trafficking events (Zárský *et al.*, 2013) would further explain why RIN4 recruits only EXO70B1 but not SEC6.

ROP GTPases are one of the main regulators of cell polarity establishment. The role of GEF and GAP proteins as ROP GTPases regulators in defense against plant pathogens is well established (Huesmann *et al.*, 2011; Yamaguchi and Kawasaki, 2012). The SEC3 subunit interacts with activated ROP GTPase through the ICR1 scaffold protein (Lavy *et al.*, 2007). A recent study also revealed another ROP GTPase-associated protein as an exocyst interactor. RIC7 interacts with active ROP2 GTPase and pulls EXO70B1 to the PM in *Vicia faba* guard cells. Here, RIC7 seems to regulate stomatal opening negatively, probably through negative regulation of EXO70B1 localization and thus function, which is suggested by the retarded light-induced stomatal opening in the *exo70B1-1* mutant (Hong *et al.*, 2016). During arbuscular mycorrhizal symbiosis, plant Vapyrins might also act as scaffold proteins and thus recruit EXO70I to or maintain it at the tip of periarbuscular membranes (Zhang *et al.*, 2015). There is yet another example of exocyst recruitment that has recently been observed in differentiating xylem vessel cells, where the VETH2–COG complex recruits EXO70A1 to cortical microtubules (Oda *et al.*, 2015). Currently it is not known whether the exocyst interacts with any other adaptor proteins besides ICR1, RIC7, and possibly Vapyrins. NOI proteins may be good candidates for exocyst adaptors.

Interestingly, in the WT background, RFP–EXO70B1 localization changes only in stomatal guard cells when GFP–RIN4 is co-expressed in stably transformed Arabidopsis lines. Liu *et al.* (2009) showed that while the *RIN4* gene is expressed more in stomatal cells than in the whole leaf, the RIN4 protein is less abundant in stomata than in the whole leaf. Therefore, overexpressing *RIN4* under the ubiquitin promoter may enhance the level of RIN4 protein in guard cells and induce the recruitment of EXO70B1 to the PM. The fact that GFP–RIN4 recruits RFP–EXO70B1 to the PM only in guard cells might also suggest a distinct form of regulation. Here, the ROP2–RIC7 pathway was also shown to influence EXO70B1 PM localization (Hong *et al.*, 2016; see above). Our results seem to contradict this report, showing that RFP–EXO70B1 localized to the cytoplasm and nucleus in open stomatal guard cells in Arabidopsis seedlings. In contrast, Hong *et al.* reported a recruitment of DsRed-labeled EXO70B1 to the PM in open stomata in the light in *V. faba*. However, Hong *et al.* did observe EXO70B1 in the cytoplasm of open stomatal guard cells when they used a C-terminal sGFP fusion in Arabidopsis. Also, unlike our study, they used the constitutive 35S promoter for their overexpression studies. Nevertheless, our data suggest an additional layer of regulation of EXO70B1 localization in guard cells. Besides ROP2–RIC7, RIN4 seems to influence EXO70B1 localization in guard cells with possible implications in plant–pathogen interaction. A very recent report has also suggested abscisic acid-dependent EXO70B1 ubiquitination and degradation in guard cells (Seo *et al.*, 2016), which further highlights the complexity of the regulation of EXO70B1 function in stomata.

What might be the biological role for PM recruitment of EXO70B1? Considering the roles of EXO70B1 in plant immunity and autophagic transport (Kulich *et al.*, 2013; Stegmann *et al.*, 2013), one might speculate that these two functions are actually connected. There are two lines of evidence that hint at this conclusion. First, the exocyst is known to operate at the PM where it is believed to mediate tethering of defense-related secretory vesicles to the pathogen attack site, where it could co-operate with RIN4. Cleavage of RIN4 by the AvrRpt2 protease would consequently release both RIN4 fragments and EXO70B1 to the cytoplasm, blocking the tethering of secretory vesicles or compartments. Despite some ambiguity of the published data (compare Stegmann *et al.*, 2013; Zhao *et al.*, 2015), the *exo70B1* mutant has been shown by one study to be more resistant to AvrRpt2-expressing *Pseudomonas* (Zhao *et al.*, 2015). We believe this could be due to elevated levels of salicylic acid in this mutant, which, along with the activation of RPS2 resistance protein, triggers a strong hypersensitive response (Kulich *et al.*, 2013). Secondly, multivesicular bodies, which have been described to fuse with the PM at the pathogen attack site as a means of defense (An *et al.*, 2006), are speculated to originate with a contribution from the autophagy pathway (Katsiarimpa *et al.*, 2013; Kulich *et al.*, 2013). Thus, we speculate that EXO70B1 is recruited to the PM by RIN4, where it can participate in the tethering of secretory vesicles or autophagic compartments to the pathogen attack site. We are currently investigating which compartments are involved in this defense pathway, and to what extent the autophagic function of EXO70B1 contributes to this pathway. Upon flg22 treatment, the cleavage of RIN4 by AvrRpt2 was shown to release RIN4 fragments that suppress callose deposition and support enhanced growth of the *P. syringae hrcC* mutant (Afzal *et al.*, 2011). Currently, we cannot say whether EXO70B1 contributes to this pathogen-induced callose deposition. Further research is obviously needed to gain more insight into how secretory and autophagy pathways in plant cells are co-ordinated to ward off microbial pathogens.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Yeast-two hybrid assays showing the interaction between exocyst subunits and NOI6.

Fig. S2. SYP121 does not recruit EXO70B1 to the plasma membrane in *N. benthamiana* leaf cells.

Fig. S3. EXO70B1–mRuby2 localizes to the PM and cytoplasm in both WT and the *rin4 rps2* mutant.

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Exocyst Subunit EXO70H4 Has a Specific Role in Callose Synthase Secretion and Silica Accumulation[1][OPEN]

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Biogenesis of the plant secondary cell wall involves many important aspects, such as phenolic compound deposition and often silica encrustation. Previously, we demonstrated the importance of the exocyst subunit EXO70H4 for biogenesis of the trichome secondary cell wall, namely for deposition of the autofluorescent and callose-rich cell wall layer. Here, we reveal that EXO70H4-driven cell wall biogenesis is constitutively active in the mature trichome, but also can be activated elsewhere upon pathogen attack, giving this study a broader significance with an overlap into phytopathology. To address the specificity of EXO70H4 among the EXO70 family, we complemented the *exo70H4-1* mutant by 18 different Arabidopsis (*Arabidopsis thaliana*) EXO70 paralogs subcloned under the EXO70H4 promoter. Only EXO70H4 had the capacity to rescue the *exo70H4-1* trichome phenotype. Callose deposition phenotype of *exo70H4-1* mutant is caused by impaired secretion of PMR4, a callose synthase responsible for the synthesis of callose in the trichome. PMR4 colocalizes with EXO70H4 on plasma membrane microdomains that do not develop in the *exo70H4-1* mutant. Using energy-dispersive x-ray microanalysis, we show that both EXO70H4- and PMR4-dependent callose deposition in the trichome are essential for cell wall silicification.

The exocyst is a protein complex conserved across all eukaryotes, composed of eight subunits with a rod-like shape. Its main function described in yeast is tethering secretory vesicles to the plasma membrane (PM; Munson and Novick, 2006). Two exocyst subunits, SEC3 and EXO70, were described as spatial landmarks for polarized secretion in yeast. It was clearly shown that SEC3 is capable of working as a landmark by itself, whereas EXO70 likely requires additional signaling factors to work (Luo et al., 2014), such as small GTPases (Wu et al., 2010). The rest of the complex, also referred to as the exocyst core, is associated with secretory vesicles and is regulated via RAB GTPase interactions (Robinson et al., 1999). Most of the time, all exocyst subunits form a relatively stable holocomplex in yeast (Heider et al., 2016; Picco et al., 2017). The interaction of the exocyst core with SEC3 and EXO70 subunits at the PM mediates the tethering of the vesicle to the membrane, followed by the SNARE protein-mediated fusion of the vesicle with the membrane (Heider and Munson, 2012; Yue et al., 2017). Besides conventional roles, the animal EXO70 protein was shown to induce membrane curvature (Zhao et al., 2013).

In comparison with yeast and mammalian genomes, which encode only one or two EXO70s, land plant genomes encode a high number of EXO70 paralogs, which likely emerged during land colonization. The Arabidopsis (*Arabidopsis thaliana*) genome encodes 23 EXO70 paralogs (Elias et al., 2003; Cvrčková et al., 2012). EXO70A1, the most basal EXO70, has been shown to be involved in the secretion of membrane proteins such as PIN1 or BRI1 (Drdová et al., 2013). It

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also is crucial for the proper development of the Casparian strip (Kalmbach et al., 2017). EXO70A1 also is recruited to the microtubules decorating xylem secondary cell wall thickenings in a COG/VETH-dependent manner, where EXO70A1 recruits the exocyst complex and is responsible for the development of the cell wall thickenings (Oda et al., 2015; Vukašinović et al., 2017). Another example of the exocyst recruitment was shown on EXO70B1, which is recruited to the PM by RIN4 (Sabol et al., 2017). Thus, besides direct lipid interaction, plant EXO70s also seem to be recruited by other proteins. Besides secretion, the EXO70B1 subunit functions in autophagy (Kulich et al., 2013), immune responses (Stegmann et al., 2013; Zhao et al., 2015), and stomatal opening (Hong et al., 2016; Seo et al., 2016). EXO70H1 and EXO70B2 were shown to facilitate the defense papilla buildup (Pecenková et al., 2011). Recently, we showed a role of the EXO70H4 paralog in Arabidopsis trichome cell wall maturation (Kulich et al., 2015).

Arabidopsis leaf trichomes are unicellular outgrowths with very specific polarized shape and two to four (but most frequently three) sharp branches. Their shape is easily and visibly distorted by numerous mutations, which, together with their large size and good accessibility, led to their popularity as a model for studies of plant cell morphogenesis (Hülskamp et al., 1994). They have been especially vital for studies of microtubule- and actin-dependent morphogenetic events (Saedler et al., 2004; Tian et al., 2015), but also serve as a model for deposition of cell wall components (Sinlapadech et al., 2007; Bischoff et al., 2010).

We previously demonstrated that the Arabidopsis trichome cell wall consists of two distinct domains, the basal thin cell wall of the bulb and apical thick cell wall. These domains are separated by a callose-rich structure named the Ortmannian ring (OR). Only the apical cell wall domain consists of an outer and inner layer. We showed that the development of the inner cell wall layer depends on the exocyst subunit EXO70H4 (Kulich et al., 2015). However, we did not provide a detailed description, subcellular localization, and the functional context of EXO70H4. Here, we focused on the EXO70H4-dependent callose deposition, as the lack of callose is one of the most prominent defects of the *exo70H4-1* mutant and because callose deposition is of great interest due to its involvement in plant immunity.

Callose is a cell wall polymer, β-1-3-glucan that is involved in many plant developmental processes, such as cell plate formation (Hong et al., 2001), the development of phloem (Bonke et al., 2003), stomata (Guseman et al., 2010), pollen, and pollen tube, as well as regulations of plasmodesmatal permeability (Iglesias and Meins, 2000). It also is deposited in response to abiotic and biotic stress stimuli like wounding, pathogen invasion, or heavy metals exposure (Frye and Innes, 1998; Blümke et al., 2013; Ellinger et al., 2013, 2014).

In contrast to cellulose, a β-1-4-glucan that is crystallized into permanent microfibrils, callose is deposited as an amorphous plug, and often only transiently, because it is specifically and rapidly degraded by the hydrolytic enzymes β-1-3-glucanases (Levy et al., 2007). Callose is synthesized at the PM by large glucan synthase complexes called callose synthases (CalS) or glucan synthase-like (GSL). The Arabidopsis genome contains 12 CalS genes, which fall into two groups (Verma and Hong, 2001). *CalS* from the first group contain up to 50 exons and are some of the longest genes found in the Arabidopsis genome. The second group consists of *CalS11* (*GSL1*) and *CalS12* (*GSL5*), containing two and three exons, respectively (Hong et al., 2001). Different CalS are expressed in a tissue-specific way in response to diverse physiological conditions (Dong et al., 2008).

It is supposed that callose synthases are transported to the sites of callose synthesis by vesicle trafficking and that the cytoskeleton and endomembrane system are necessary for callose synthase distribution, transport, and positioning (Cai et al., 2011; Drakakaki et al., 2012). Despite pilot observations of callose synthase transport by vesicle-like bodies (Cai et al., 2011; Drakakaki et al., 2012; Nielsen et al., 2012), strong evidence is missing.

In this study, we worked with two callose synthase proteins: CalS9 (GSL10) and CalS12. CalS9 is known to act in male gametophyte development with mutants defective in pollen mitotic division (Töller et al., 2008; Huang et al., 2009), and it is one of the most transcribed callose synthases in the trichome (Jakoby et al., 2008). CalS12, also known as powdery mildew resistant 4 (PMR4), is a stress-induced callose synthase (Vogel and Somerville, 2000). Knockout mutants lack pathogen-induced callose deposits (Jacobs et al., 2003; Ellinger et al., 2013). One of the possible roles for the callose deposits may be inducing mechanical stiffness in the cell wall by supporting its silicification.

Silica is a nonessential micronutrient absorbed by plants in the form of silicic acid, $Si(OH)_4$, and deposited in different amounts into cell walls of various tissues and structures. Typically, trichome cell walls of many plants are encrusted with silica. Some observations suggest that silica deposition may be related to callose synthesis. The co-occurrence of callose and silica deposition was previously shown in several plant species, including silica hyperaccumulators, for example, in the common horsetail (*Equisetum arvense*; Law and Exley, 2011), in epidermal trichomes of numerous species (Waterkeyn and Dupont, 1982), *Selaginella* (Webster, 1992), and Arabidopsis (Brugiére and Exley, 2017). Callose was suggested as an inducing rather than catalyzing element of silicification, operating as a supportive matrix for the specific condensation of silicic acid into silica nanoparticles (Brugiére and Exley, 2017). There also is evidence that carbohydrates other than callose can act as organic matrices for silicification (Perry et al., 1987; Leroux et al., 2013; Guerriero et al., 2016), but also that in some cases no carbohydrates are needed (Hodson, 2016).

Despite this considerable evidence for the dependency of silica deposition on callose synthesis, a comprehensive analysis was still missing. In this report, we

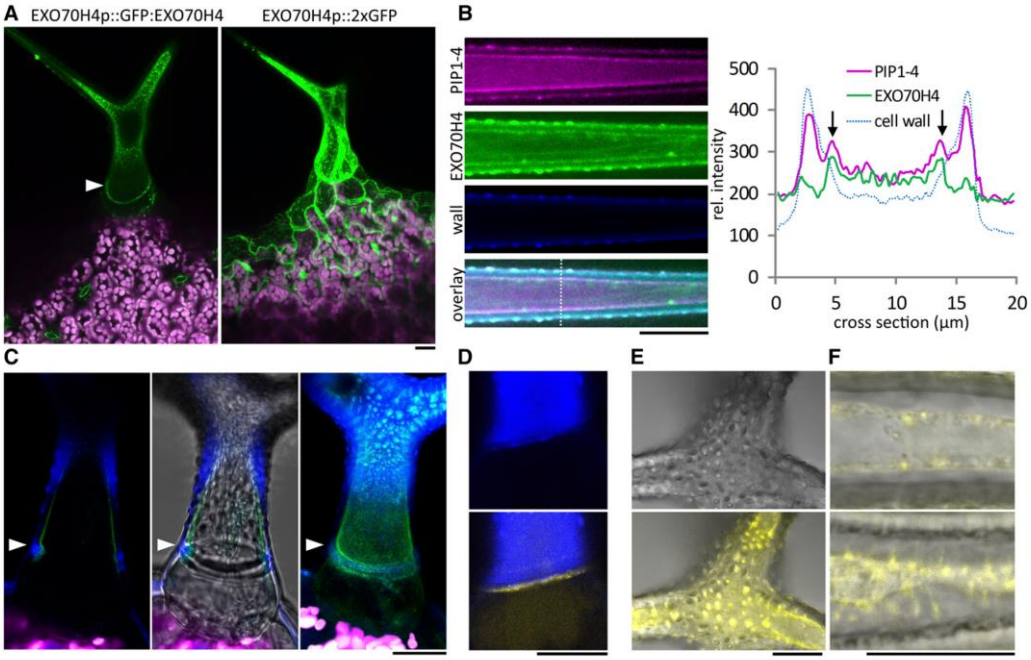

Figure 1. EXO70H4 localization in the trichome. A, EXO70H4 (green) under its native promoter is only visible in the trichome (left), but the EXO70H4 promoter is active also in other epidermal cells (right). Magenta represents chlorophyll; arrowhead points at the OR. B, EXO70H4 colocalizes with PM marker PIP1-4. Graph on the right represents plot of the profile depicted as white dotted line on the left. C, EXO70H4p::mGFP:EXO70H4 (green) localizes to the OR (white arrowheads) and the apical domain above the OR, which produces a highly autofluorescent cell wall (blue). Chlorophyll is in magenta. Left, Single section; middle, single section with transmission; right, z projection. D, A detail of the OR labeled by mCH-EXO70H4 (yellow) and the border of the apical autofluorescent cell wall domain (blue) and the basal domain. Top, Autofluorescence; bottom, autofluorescence and mCH-EXO70H4. E, Detailed view of the mCH-EXO70H4-positive cell wall ingrowths. Top, Transmission; bottom, transmission and mCH-EXO70H4. F, Plasmolysis of a trichome with labeled mCH-EXO70H4, without (top) and with (bottom) developed cell wall ingrowths. The differential attachment of the PM to the cell wall is visible. Bars = 20 μm.

demonstrate that callose is indispensable for silica deposition in Arabidopsis trichomes.

RESULTS

EXO70H4 Is Localized to the OR and a PM Domain above It

To observe the localization of the EXO70H4 protein, we generated native promoter-driven constructs with an N-terminal GFP and with mCHERRY (GFP-EXO70H4, mCH-EXO70H4). These constructs were proven to be functional, as they fully complemented the *exo70H4-1* mutation described previously (Kulich et al., 2015). In the absence of stress, EXO70H4 constructs were exclusively expressed in the trichome; however, the EXO70H4 promoter also was active in other epidermal cells, showing the capacity of EXO70H4 to be activated elsewhere (Fig. 1A). This also was true for other EXO70 paralogs expressed under the EXO70H4 promoter (see further), suggesting posttranscriptional regulation of EXO70H4. To show whether GFP-EXO70H4 localizes to specific PM domains, we looked for colocalization with the well-established PM marker mCHERRY-PIP1-4 (Geldner et al., 2009; Fig. 1B). Unfortunately, PIP1-4 is almost completely degraded in the mature trichome.

The localization of EXO70H4 very well matches the callose-rich and autofluorescent cell wall shown in Kulich et al. (2015). The XFP-EXO70H4 signal is always present at the OR and above it (apical domain) throughout trichome cell wall development (Fig. 1, A, C, and D). The presence of the EXO70H4 signal was always accompanied by cell wall autofluorescence. The basal PM domain beneath the OR is devoid of GFP-EXO70H4 (Fig. 1, A and D). Identical results were obtained using the mCHERRY construct mCH-EXO70H4 (Fig. 1, D–F). In young trichomes, the

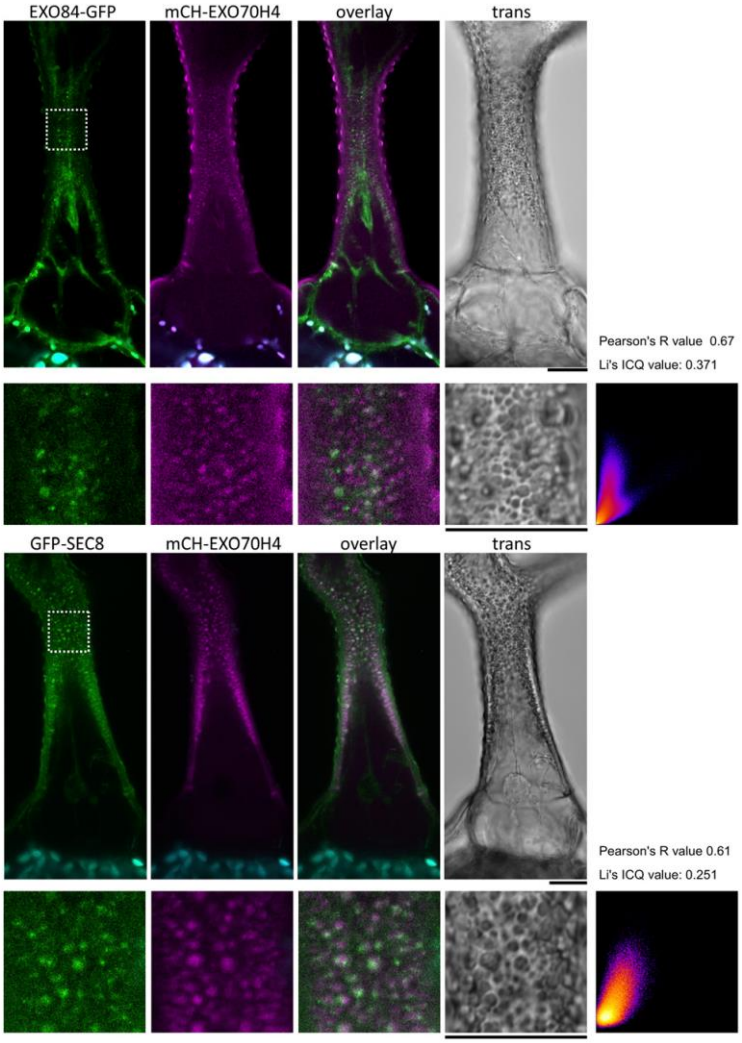

Figure 2. Colocalization of the core exocyst subunits with EXO70H4. SEC8 and EXO84 under their own natural promoters largely localize to the cytoplasm and partially localize to the EXO70H4-positive compartments. The ratio of both signals changes over time. Bars = 20 μm.

signal above the OR is homogeneous. In older trichomes, the signal becomes more speckled and accumulates at the differential interference contrast-visible cell wall ingrowths, which are a common feature of older trichomes (Fig. 1E). Upon plasmolysis, the mCH-EXO70H4 signal is easily separated from the cell wall of the young trichomes but is attached to the cell wall that has developed ingrowths along cytoplasmic strands (Fig. 1F). To confirm that these are actual cell wall ingrowths, we investigated the trichome cell wall from the inside using environmental scanning electron microscopy (ESEM; Supplemental Fig. S1).

On these ingrowths, mCH-EXO70H4 transiently colocalizes with the core exocyst subunits GFP-SEC8 and EXO84-GFP expressed under their respective natural promoters (Fig. 2). The core exocyst subunit signal is, however, visible also in the cytoplasm and in other membrane domains, suggesting their general function in secretion. These data fit with our previous yeast two-hybrid study, where EXO70H4 also physically

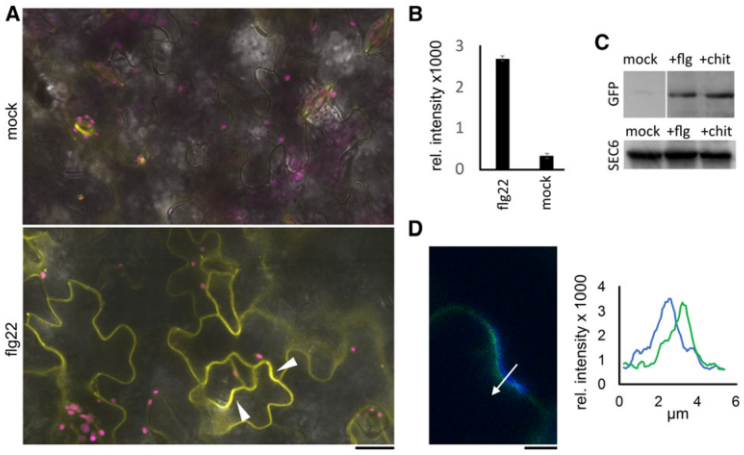

Figure 3. EXO70H4 up-regulation by flg22 in the leaf pavement cells. A, Representative images of leaves 5 h after flg22 treatment (spraying by 1 μM flg22 in 0.05% Silwet and 0.05% Silwet as control). Yellow, mCH-EXO70H4; magenta, chlorophyll; grays, transmission. Arrowheads depict domains with enchiched EXO70H4 signal. Scale bar = 20 μm. B, Quantification of the EXO70H4 signal intensity out of 10 plants and 100 cells. Similar results were obtained in three independent replicates, with both GFP- and mCHERRY-labeled constructs. C, Up-regulation of EXO70H4 in total cell extract from whole rosettes of GFP-EXO70H4 plants, 5 h after flg22 and chitin treatment. The white line is where one empty lane was left on the gel due to overflow. SEC6 was used as a loading control. D, GFP-EXO70H4 (green) forms a microdomain with enriched signal. This is accompanied by development of cell wall autofluorescence (blue). The arrow shows the position of the plot profile in D. Scale bar = 5 μm. Right, Plot profile demonstrating spatial separation of EXO70H4 and cell wall autofluorescence.

interacted with the Arabidopsis exocyst subunits SEC5A, SEC6, and EXO84b (Kulich et al., 2015).

Flg22 Induces EXO70H4 Expression in Epidermal Pavement Cells

Because the composition of the trichome inner cell wall layer highly resembles the pathogen-induced cell wall appositions, we investigated whether there is a possible function of EXO70H4 beyond the trichome. As shown in Figure 1A, the EXO70H4 promoter has a capacity to drive EXO70H4 expression in other epidermal cells, but the EXO70H4 protein is absent. Public microarray data suggest an elevation of the *EXO70H4* mRNA signal upon flg22 treatment. Therefore, we applied 1 μM flg22 by spraying on the mature Arabidopsis rosettes (24 d old). Four hours after the treatment, the first visible signal appeared in the epidermal pavement cells and peaked approximately 5 h after induction (Fig. 3A). The signal disappeared again 12 h after induction. We got similar results with both GFP and mCHERRY lines and quantified the fluorescence intensity (Fig. 3B). These results are supported by the western blot analysis of the total cell extract using an anti-GFP antibody and document similar up-regulation using chitin as elicitor (Fig. 3C). In many cells, the EXO70H4 signal was not evenly distributed across the cell surface and formed small domains of higher signal intensity. These domains developed cell wall autofluorescence similar to the autofluorescence of the trichome apical cell wall (Fig. 3D).

EXO70H4 Differs from Other Arabidopsis EXO70 Paralogs in Its Function and Subcellular Localization

To learn more about the specificity of EXO70H4 function, we performed a cross-complementation analysis, in which we subcloned multiple EXO70 paralogs under the EXO70H4 promoter. We subcloned 18 different EXO70 paralogs in the same fashion under EXO70H4 promoter (EXO70H4p::GFP:EXO70XY). We selected at least one gene from each subfamily (A–G) and all members of the subfamily H. Then, we transformed these constructs into the *exo70H4-1* mutant background and observed the development of trichome autofluorescence (Fig. 4, A and B) and trichome callose deposition (Supplemental Fig. S2). Both of these parameters provided us with identical results. Apart from EXO70H4, no other paralog could restore either the callose or autofluorescence, suggesting that the function of EXO70H4 is highly specific.

Most EXO70 paralogs showed no PM localization in the trichome (Supplemental Fig. S3). While EXO70A1 was uniformly distributed over the whole PM of the trichome (even beneath the OR; Fig. 4C), EXO70B1 and most of the other EXO70 paralogs showed cytoplasmic and nuclear localization.

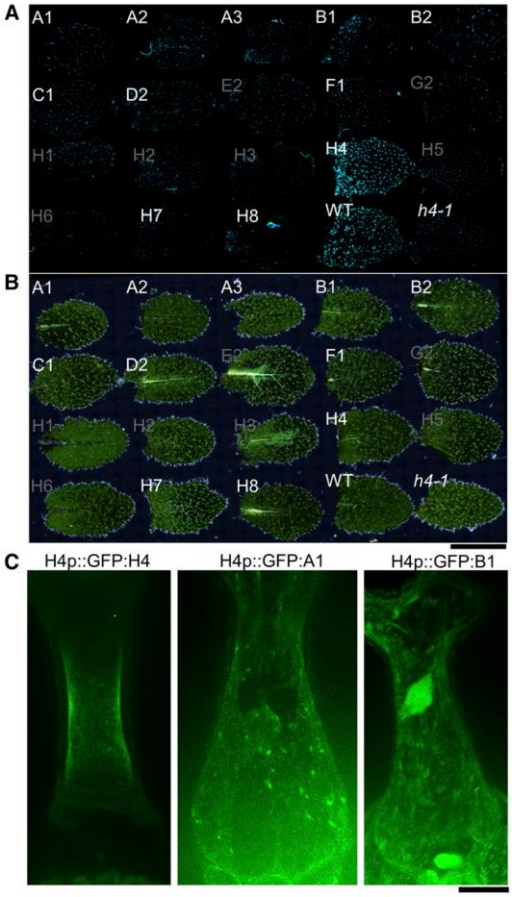

Figure 4. Complementation of the *exo70H4-1* mutant by multiple EXO70 paralogs. A, Autofluorescence of trichomes on leaves of individual transgenic lines (A1 stands for *exo70H4-1* complemented by EXO70H4p::GFP:EXO70A1, etc.), plus wild-type control (WT) and *exo70H4-1* mutant (*h4-1*). Gray letters label lines where no GFP signal was observed in the trichome. B, Dark-field view of A. Large images (A and B) consist of 475 tiles. Scale bar = 500 μm. C, Localization of selected EXO70 paralogs under the EXO70H4 promoter in *exo70H4-1* background (H4p::GFP:H4 stands for EXO70H4p::GFP:EXO70H4, etc.). Localizations of all 18 tested EXO70 paralogs are shown in Supplemental Figure S3.

Despite using an identical experimental setup, some of the EXO70 proteins (H1, E2, G2, H2, H3, H5, and H6) were not detected in the trichome, and therefore, we cannot exclude that some of these have the capacity to complement EXO70H4 on the protein level (Supplemental Fig. S3). This may be due to microRNA (miRNA) regulation of the EXO70 mRNA. We identified several miRNAs that may interfere with EXO70. The number of predicted interfering RNAs (based on Yi et al. [2015] database) is enhanced in the EXO70H subfamily (Supplemental Table S1).

EXO70H4 Is Essential for PM Localization of Callose Synthases in the Trichome

Since we showed that callose is absent from the *exo70H4-1* mutant trichomes (Kulich et al., 2015), we further investigated the callose synthase delivery to the PM. We worked with two callose synthases and generated ubiquitin promoter-driven callose synthase constructs UBQ::GFP:PMR4 (GFP-PMR4) and UBQ::GFP:CALS9 (GFP-CALS9). Here, we focus on PMR4, which is essential for the callose production in the trichome. We obtained similar results using CALS9. These can be found in the supplements (Supplemental Fig. S4).

As reported previously, *pmr4* mutants lack pathogen-induced callose deposits (Jacobs et al., 2003; Ellinger et al., 2013). In our hands, *pmr4* trichomes also lacked the visible callose, stained with aniline blue (Fig. 5A). This phenotype was complemented by transforming the *pmr4* mutant plants with GFP-PMR4 (Fig. 5B), demonstrating the functionality of this construct. In contrast to *exo70H4-1*, *pmr4* trichomes are mechanically stiff and show wild type-like autofluorescence (Fig. 5C).

Next, we introduced UBQ::GFP:PMR4 into the wild type and the *exo70H4-1* mutant line. In the wild-type trichome, the signal of both callose synthases was visible in immobile membrane speckles at the PM and also on mobile membrane bodies, possibly representing multiple steps of the secretory pathway (Fig. 6A). To separate mobile PMR4 fraction from the immobile PM dots, we performed time-series imaging and minimal-intensity projections (Fig. 6B). In the *exo70H4-1* mutant plants, the signal from the PM speckles was lost, suggesting a secretory defect of both of these callose synthases (Fig. 6, B–D).

By colocalization of EXO70H4p::mCHERRY:EXO70H4 with UBQ::GFP:PMR4, we show that the callose synthase speckles also are EXO70H4 positive (Fig. 6A). As described above, the signal of callose synthases was first visible in smaller transient speckles on the PM of young trichomes and developed later into large speckles. Taken together, our data show the dependency of callose synthase secretion on the EXO70H4 protein.

Silica Accumulation Is Dependent on Callose Deposition and Thus on the EXO70H4-Dependent PMR4 Secretion

While doing our ESEM studies of untreated biological samples (Tihlaříková et al., 2013; Neděla et al., 2015), we applied energy-dispersive x-ray spectroscopy for a semiquantitative analysis of elements in the trichome. Surprisingly, apart from heavy metals, we noticed a significant amount of silica in the domain above the OR.

This silica encrustation was absent in *exo70H4-1* mutant trichomes. To determine whether this was a direct effect of the *exo70H4-1* mutation, we next included a *pmr4* mutant (which lacks callose in the trichome), and we increased the amount of silica in the soil by watering with sodium silicate solution (final concentration 2 mM). As shown in Figure 7, both the *pmr4* and the *exo70H4-1* mutants had dramatically reduced silica levels in the trichomes. Therefore, we conclude that callose deposition is essential for cell wall silicification and that the *exo70H4-1* silica phenotype is a secondary phenotype, contingent on the impaired callose synthase delivery and the subsequent absence of callose synthesis.

DISCUSSION

In our previous study, we demonstrated the EXO70H4-dependent development of the callose-rich cell wall in the Arabidopsis trichome. Here, we show that EXO70H4 acts by promoting the secretion of callose synthase. Surprisingly, no other EXO70 subcloned under the EXO70H4 promoter was able to complement the *exo70H4-1* phenotype, despite high sequence similarity of some paralogs (Cvrčková et al., 2012). Since EXO70 proteins in general act as spatial landmarks for secretion, it is likely that the specificity of EXO70 paralogs reflects their differential target binding capacities. EXO70H4 decorates a specific PM subdomain in the trichome, while the most ancestral EXO70A1 localizes all over the trichome PM. This is consistent with the previous model of multiple recycling domains within one cell (Zárský et al., 2009). We also observed that some of the EXO70 paralogs with cytoplasmic localization in the trichome localize to the PM in the pavement cells (e.g. EXO70B1), suggesting differential regulatory mechanisms.

The cross-complementation analysis suggests that the EXO70H4-positive trichome PM domains have a highly distinctive character and cannot be recognized by other EXO70 paralogs. Previously, it was proposed that the EXO70 paralogs differ just in their expression pattern (Li et al., 2010). In this study, we demonstrate that there is functional divergence between the paralogs. This also is supported by other studies showing specific EXO70 roles (Kulich et al., 2013; Zhao et al., 2015) and recently by the specific PM domain localizations of NtEXO70A1 and NtEXO70B1 in tobacco (*Nicotiana tabacum*) pollen tubes (Sekereš et al., 2017).

Since EXO70 proteins are putative landmarks for secretion, the specificity of EXO70 paralogs could mainly be determined by different localization signals. For example, during xylogenesis, EXO70A1 localization to cortical microtubules is maintained by COG-VETH proteins (Oda et al., 2015; Vukašinović et al., 2017). Also, as we showed recently, EXO70B1 PM localization can be achieved by protein-protein interaction with NOI family proteins (Sabol et al., 2017). We speculate that a similar mechanism, but with different proteins, may be responsible for the specific localization of many EXO70 paralogs, causing their functional diversity. Whether the process of EXO70H4 localization is mediated by specific lipid-binding properties or by interacting proteins is the subject of our follow-up study.

The initially homogenous PM signal of GFP-EXO70H4 develops later into stable speckles, which then form ingrowths of the cell wall. Similar behavior of the exocyst subunits was observed during xylogenesis, where EXO70A1-tagRFP first localized dispersedly to the PM and later on gradually organized into a bundled pattern (Vukašinović et al., 2017). Such stabilization of the polarity was observed previously in budding yeast (*Saccharomyces cerevisiae*; Brennwald and Rossi, 2007). The mechanism of this stabilization in plants is not yet known, but in our opinion it may be achieved by a positive feedback loop, whereby the original EXO70 attracts vesicles with more exocyst subunits.

Unfortunately, not all the EXO70 constructs in our cross-complementation study were expressed in the trichome. We explain this by a predicted RNA interference regulation, which is common among stress-induced transcripts (Sunkar and Zhu, 2004), or by ubiquitination, which was previously manifested as a possible step in EXO70 protein regulation (Samuel et al., 2009; Stegmann et al., 2012; Seo et al., 2016). Very likely, EXO70s are subjected to a high degree of regulation at multiple levels.

Here, we show a secretory defect of the *exo70H4-1* mutation on one type of cargo: two callose synthases, which both localized and behaved similarly despite their functional classification. Of these, only CALS12 was biologically relevant for callose synthesis in the trichome. More cargo affected by the *exo70H4-1* mutation must exist, since the trichomes lacking only callose and silica in the case of the *pmr4* mutation are still mechanically relatively stiff and accumulate autofluorescent compounds and metals, unlike *exo70H4-1* mutants.

The inner cell wall of the trichome shares many similarities with the pathogen-induced cell wall (being rich in callose, silica, and phenolic compounds; Russo and Bushnell, 1989; Ghanmi et al., 2004), and as we show here, EXO70H4 has the capacity to contribute to such a cell wall biogenesis, since the EXO70H4 protein appears in nontrichome cells upon bacterial elicitor treatment, allowing PMR4 and other cargo secretion and callose synthesis. Deposited callose thereafter acts as a matrix for silica accumulation, which is known to modulate physical properties of the cell wall and acts as an important line of defense against fungal pathogens (Ghanmi et al., 2004; Fauteux et al., 2006; Vivancos et al., 2015). The exact relationship between callose and the silica accumulation was enigmatic for a long time, although it was clear that these processes are related (Law and Exley, 2011; Exley, 2015). First evidence that callose may be essential for silica deposition was provided recently (Brugiére and Exley, 2017), using chemical staining of silica. In our study, we extend these

observations with quantitative and statistically processed data. We also show that callose is not essential for the accumulation of phenolic compounds in the cell wall in contrast to some observations that lignification precedes silicification (Zhang et al., 2013).

While Arabidopsis trichomes contain relatively little silica, trichomes of species such as nettle (*Urtica dioica*) are well known for their silicified cell wall (Sangster and Hodson, 2007). Cucumber (*Cucumis sativus*) trichomes also contain silica, and supplementing plants with silica leads to physically stiffer trichomes (Samuels, 1993). In cucumber, basal cells of the trichomes (sometimes referred to as cells surrounding the trichome or the trichome spine) were the site of maximal silica deposition (Samuels et al., 1991a, 1991b; Chérif et al., 1992). Transcriptomic analyses have revealed that wild-type cucumber contains 406-fold more *CsEXO70H4* transcript than the trichome-less *tbh* mutant (Chen et al., 2014). This suggests that the mechanisms we describe in the Arabidopsis trichome may have more general implications for eurosids.

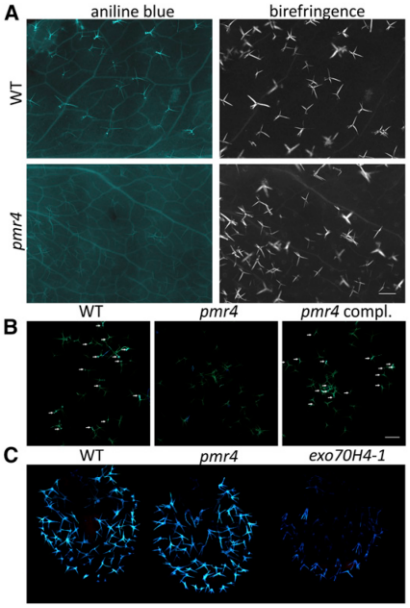

Figure 5. Trichome phenotype of *pmr4* mutant. A, Aniline blue staining of callose (left) and cellulose birefringence in polarized light (right) for *pmr4* (top) and wild type (WT; bottom) leaves. B, Aniline blue staining of callose on isolated trichomes of wild type, *pmr4*, and *pmr4* mutant complemented with UBQ::GFP:PMR4 construct (*pmr4* compl.); arrows point to trichomes with developed callose structures. Scale bar = 500 μm. C, Autofluorescence of phenolic compounds in broken (dead) trichomes of wild type, *pmr4*, and *exo70H4-1*, respectively.

MATERIALS AND METHODS

Plant Material and Growth

If not indicated otherwise, plants were grown in standard growth chamber conditions (long day 16 h:8 h, 100 μM photosynthetically active radiation $m^{-2} s^{-1}$). LT 36W/958 T8 BIOVITAL NARVA fluorescent tubes were used. These contain a UV-B peak. All plants were grown in Jiffy soil pellets. The *exo70H4-1* mutant was described previously (Kulich et al., 2015), as well as the *pmr4-1* mutant (Vogel and Somerville, 2000). As a control, a wild-type sibling of the *exo70H4-1* mutant was used. As a control for the *exo70H4-1* × *rdr6-12* double mutant, *rdr6-12* (Peragine et al., 2004) was used.

Callose Staining and Autofluorescence Visualization

To stain for callose, whole leaves were washed for 3 h in acetic acid:ethanol (1:3) solution, washed three times in deionized water, and incubated overnight in aniline blue solution (150 mM KH_2PO_4 and 0.01% [w/v] aniline blue, pH 9.5). Trichomes were then imaged on leaves or brushed off and imaged.

For autofluorescence development, leaves were placed in between two microscopy slides and dried up overnight. Fifth and sixth leaves of the 24- to 28-d-old rosettes were used for the aniline blue staining and the third youngest visible leaf for autofluorescence observations.

PAMP Treatments

Flg22 (1 μM) or chitosan solution (150 mg/L) with 0.05% Silwet Star wetting agent was sprayed onto the 24-d-old Arabidopsis (*Arabidopsis thaliana*) rosettes stably transformed with XFP-EXO70H4. Silwet Star (0.05%) was used as a control. Signal was observed 4 to 5 h after the treatment. This experiment was done in triplicate. Spraying was critical factor for the EXO70H4 up-regulation. Protein extract from whole rosettes was used for the western blot analysis, using primary anti-GFP and anti-SEC6 antibodies (Agrisera; AS15 2987 and AS13 2686, respectively).

Construct and Transgenic Line Preparation

For the cloning of all EXO70 paralogs, a multisite gateway approach was used. The EXO70H4 promoter (1 kb upstream) was subcloned into pDONORP4-P1r. GFP in pEN-L1-F-L2 (GFP) was obtained from Karimi et al. (2007). pEN-L1-mCherry-L2 was obtained from Mylle et al. (2013); however, since this construct had a stop codon, we used it as a template to generate a new pDONOR 221-mCherry construct. EXO70 coding sequences were amplified in one or two steps (with att extension primers) and subcloned into the pDONOR P2R-P3 using Gateway BP clonase (Invitrogen). Then, multisite reactions were performed using the EXO70H4 promoter, GFP, EXO70 coding sequence, and destination vector pB7m34GW (BASTA plant selection; Karimi et al., 2007). pDONOR vectors were sequenced using M13 primers. The destination binary constructs were sequenced using M13 primers and two GFP primers (see primer list in Supplemental Table S2). To clone callose synthase PMR4, the genomic fragment was subcloned into pDONOR 221 using Gateway BP clonase and then transferred into the pUBN-GFP vector (Grefen et al., 2010) using Gateway LR clonase. Since the CalS9 genomic fragment is too long to amplify, we isolated the cDNA. This was stitched together from two parts, as none of transcripts had all 42 introns properly spliced. GFP-SEC8 and EXO84b-GFP lines were described previously (Fendrych et al., 2013). All primers in this study are listed in Supplemental Table S2.

All prepared constructs were electroporated into *Agrobacterium tumefaciens* GV3101 competent cells. The floral dip method (Clough and Bent, 1998) of plant transformation was used, and the transformants were selected on soil by spraying with BASTA (150 mg/L of glufosinate-NH_4). At least five individual transformants were observed in each experiment, and at least two biological replicates were made.

Light Microscopy

Zeiss LSM880 with C-Apochromat 40×/1.2 W Korr FCS M27 objective was used for Figures 1, 4, and 5 [GFP (488): 508–540 nm, chlorophyll (488) 650–721 nm, cell wall autofluorescence (405) 426–502, mCHERRY (561) 597–641]. For Figure 4C and Supplemental Figures S3 and S4, we used

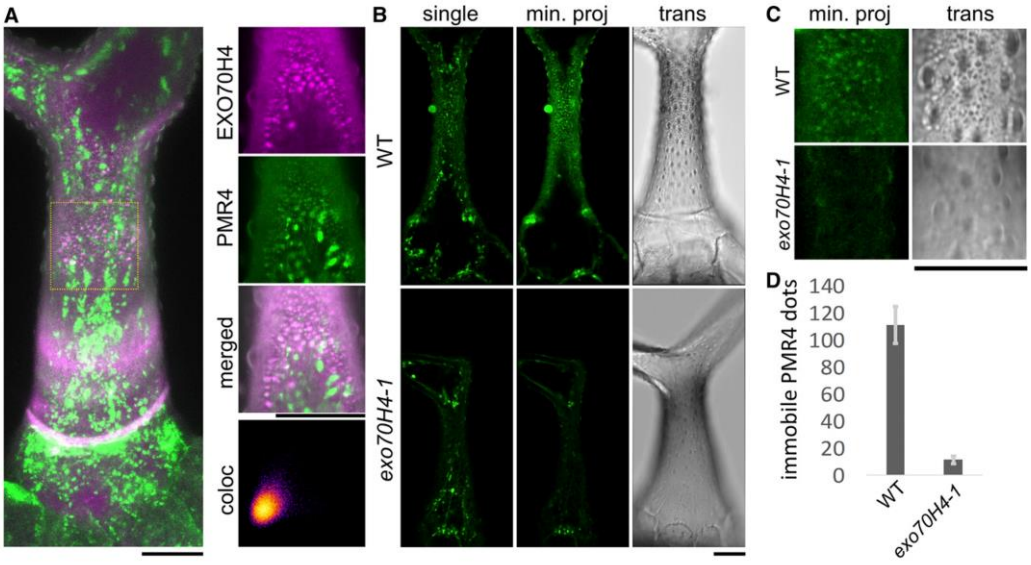

Figure 6. EXO70H4 recruits PMR4 to the ingrowths of the trichome cell wall. A, Left, Overall view of the trichome expressing mCH-EXO70H4 (magenta) and GFP-PMR4 (green). Projection of 14 sections. Yellow dotted square depicts detailed view on the right. Right, Detailed view of a single section with cell wall ingrowths decorated by EXO70H4 and PMR4 with a 2D histogram for the colocalization (Pearson's R value, 0.35; Li's ICQ value, 0,127). B, PMR4-positive cell wall ingrowths are absent in the *exo70H4-1* mutant. In the single frame, both the mobile endomembrane fraction and the immobile ingrowths are visible. The mobile fraction is reduced by minimal intensity projection (min. proj.). C, A detail of the ingrowths from B. D, Quantification of the immobile dots in a blind study using the minimal intensity projections. WT, Wild type; bars = 20 μm.

Yokogawa CSU-X1 on Nikon Ti-698 E platform, Agilent MLC400 laser box, and iXON camera (Andor) using filter stringent cubes for GFP and RFP. For Figure 4, A and B, and Supplemental Figure S2, Nikon Eclipse 90i with PlanApo 4×/0.2 objective and Nikon DsFi 2 camera were used. Images were processed using the Fiji platform (Schindelin et al., 2012).

ESEM and Energy-Dispersive X-Ray Microanalysis

For a semiquantitative energy-dispersive x-ray microanalysis (EDS), four trichomes from eight leaves were selected for each of the three samples (wild type, *pmr4*, and *exo70H4-1*), air dried, and placed on a carbon pad. To maximize

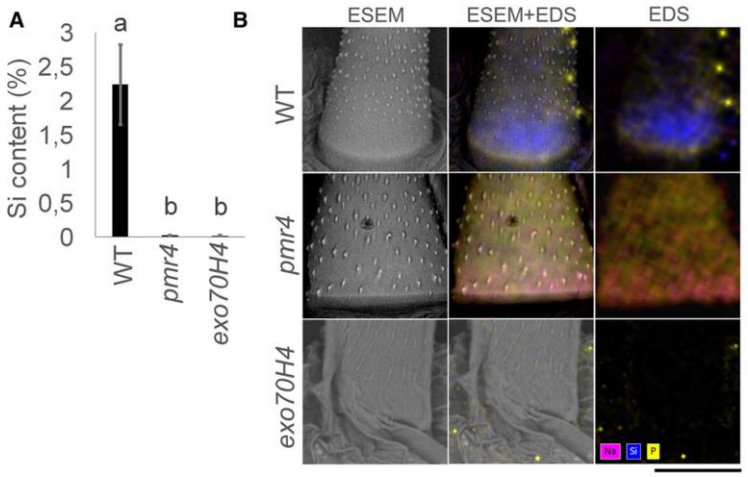

Figure 7. The presence of silica in Arabidopsis trichomes is dependent on callose. A, Quantification of the silica content in multiple genotypes grown on a soil containing 2 mM sodium silicate. Error bars represent SES. Letters above the bars: mean significant differences (HSD Tukey post hoc test, $P < 0.01$). B, Examples of trichomes accumulating silicon (blue signal). The accumulation is prominent in the apical domain above the OR. Note that the *exo70H4-1* cell wall collapsed due to its mechanical properties. Elements such as sodium (magenta) and phosphate (yellow) also are indicated. Scale bar = 30 μm. WT, Wild type.

detection efficiency and accuracy of the analysis, trichomes were selected according to their shape and shadowing in x-ray maps using a solid-state detector of backscattered electrons and fast EDS mapping. Dried, chemical treatment-free, and conductive coating-free samples were imaged and analyzed using ESEM Quanta 650 FEG equipped with EDS silicon drift detector Bruker Quantax 400 XFlash 6/60 under beam energy 10 keV, beam current 100 pA, working distance 10 mm, and water vapor pressure 100 Pa. The ratio of silicon in the sample was calculated as the median values obtained from all trichomes for each sample.

The cell wall of the mature Arabidopsis trichome was in situ opened using two Kleindiek micromanipulators MM3A-EM, thus without any manipulation, cutting, or contamination outside the specimen chamber of ESEM. The inner surface of the trichome (Supplemental Fig. S1) was imaged using gaseous secondary electron detector under beam energy 10 keV, beam current 50 pA, working distance 11.5 mm, and water vapor pressure 170 Pa.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. ESEM view of the inner structure of the fresh mature Arabidopsis trichome cell wall.

Supplemental Figure S2. Complementation of the *exo70H4-1* mutant by multiple EXO70 paralogs.

Supplemental Figure S3. Overview of EXO70 paralog localization in the trichome.

Supplemental Figure S4. EXO70H4-dependent Cals9 delivery.

Supplemental Table S1. RNA interference of EXO70 paralogs.

Supplemental Table S2. List of primers used in this study.

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Constitutive Negative Regulation of R Proteins in *Arabidopsis* also via Autophagy Related Pathway?

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Even though resistance (R) genes are among the most studied components of the plant immunity, there remain still a lot of aspects to be explained about the regulation of their function. Many gain-of-function mutants of R genes and loss-of-function of their regulators often demonstrate up-regulated defense responses in combination with dwarf stature and/or spontaneous leaf lesions formation. For most of these mutants, phenotypes are a consequence of an ectopic activation of R genes. Based on the compilation and comparison of published results in this field, we have concluded that the constitutively activated defense phenotypes recurrently arise by disruption of tight, constitutive and multilevel negative control of some of R proteins that might involve also their targeting to the autophagy pathway. This mode of R protein regulation is supported also by protein–protein interactions listed in available databases, as well as *in silico* search for autophagy machinery interacting motifs. The suggested model could resolve some explanatory discrepancies found in the studies of the immunity responses of autophagy mutants.

Keywords: resistance, autophagy, R, Avr, ETI, dwarf, lesions, exocyst

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INTRODUCTION

There are several approaches how to study and classify the plant immunity related events, and the most widespread is division of the plant immunity into two modes – a pathogen-associated molecular patterns (PAMPs) triggered immunity (PTI), which is triggered usually by recognition of structural components of pathogen on the surface of the host cell, and effector triggered immunity (ETI; Jones and Dangl, 2006). These two defense modes employ basically the same means, but PTI is more general and mild, while ETI is much stronger and more efficient. ETI is triggered by the direct or indirect interaction between a specific disease resistance (R) protein and a corresponding avirulence (Avr) protein of pathogen and is accompanied by a number of changes within the plant – production of reactive oxygen species (ROS) by an oxidative burst, accumulation of the salicylic acid (SA), and the transcriptional activation of genes involved in defense response, that lead to a possible final stage – localized programmed cell death called the hypersensitive response (HR; Pontier et al., 1998; review in McDowell and Woffenden, 2003; Vlot et al., 2008).

Disease resistance (R) genes are central components of the plant immune response. All R proteins contain at least some of basic motifs – either Toll/interleukin-1 receptor (TIR) or coiled-coil (CC) structure on the N terminal part, nucleotide-binding site (NBS), leucine-rich repeat

(LRR), protein kinase and transmembrane domains (review by Martin, 1999; Liu et al., 2007). There are 145 putative genes encoding a product with a TIR domain and 51 with CC domain predicted in the *Arabidopsis thaliana* Col-0 genome (Meyers et al., 2003; Jacob et al., 2013). Majority encode proteins with TIR, NBS, and LRR domains, making the TNL group; some genes encode proteins with TIR and NBS domains but no LRR domain (TN genes) and some encode proteins with a TIR domain only (TX genes; Meyers et al., 2003; Nandety et al., 2013). Besides CC-NBS-LRR containing proteins which make CNL group, there are also four proteins that have NBS motifs similar to CNLs, but lack a CC motif (Meyers et al., 2003).

There are several important molecules involved in signaling downstream the successful R-Avr recognition – ENHANCED DISEASE SENSITIVITY 1 (EDS1), PHYTOALEXIN DEFICIENT 4 (PAD4), NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1) and SENESCENCE ASSOCIATED GENE 101 (SAG101), which are essential for the accomplishment of HR and for the accumulation of the SA. EDS1, PAD4 and SAG101 are involved in transferring signals mainly from TNL proteins, while CNL pathway mostly relies on signaling through NDR1 (Century et al., 1997; Feys et al., 2001; He and Gan, 2002; Wagner et al., 2013).

In *Arabidopsis* mutants in genes coding for R and R-associated proteins, along with defense related deviations, two other most frequent phenotypes are a dwarf stature and a spontaneous HR lesion formation; many times present even simultaneously (**Table 1**). Rarely, a lethal phenotype occurs as well, even though no developmental function for these genes has been found so far. We could notice that for most of the R genes mutants, described phenotypes are a consequence of their activation, in some cases even a gain of function mutations (GOF). Based on the comparison of different studies of plant immunity, our hypothesis aims to suggest a model in which the hyper immune phenotypes arise as a result of disruption of tight, multistep and constitutive negative control of R proteins that possibly involves also their inactivation by the autophagy pathway.

OF DWARFS AND LESIONS

It was shown that mutants with over activated R protein dependent defense response develop mostly two phenotypes – dwarfism and/or necrotic leaf lesions (reviewed e.g., in Lorrain et al., 2003 and Janda and Ruelland, 2014). For instance, in plants overexpressing a CNL gene *ACTIVATED DISEASE RESISTANCE 1* (*ADR1*), a constitutive defense response and a dwarf phenotype were found (Grant et al., 2003). A TNL protein SUPPRESSOR OF NPR1 CONSTITUTIVE 1 (SNC1) was found to be overactive in the *bonzai1-1* (*bon1-1*) mutant which also shows a constitutive defense response and reduced plant size (Yang and Hua, 2004). Along with *bon1*, several other autoimmune dwarf mutations were found to be suppressed by mutation of SNC1 locus; namely in *BON1-ASSOCIATED PROTEIN* (*bap1*), *BAK1-INTERACTING RECEPTOR-LIKE KINASE 1* (*bir1*), *SUPPRESSOR OF RPS4-RLD 1* (*srfr1*), *CONSTITUTIVE EXPRESSER OF PATHOGENESIS-RELATED GENE* (*cpr1*) and *MITOGEN-ACTIVATED PROTEIN KINASE 1* (*mpk1*; review in Gou and Hua, 2012). Plants overexpressing a TIR-X gene At2g32140 show also dwarf phenotype and activated expression of defense-related genes (Kato et al., 2014). This phenotype was dependent on EDS1, PAD4, and partially dependent on SALICYLIC ACID INDUCTION DEFICIENT 2 (SID2).

HR-like spontaneous leaf necrotic lesions were found to be even more frequently associated with the mutations in R genes and constitutively activated immunity. For instance, a GOF mutant in TNL RPP4 locus called *chilling sensitive 2* (*chs2*) shows lesions in the low temperature conditions (Huang et al., 2010). GOF *Arabidopsis* mutant in the other *CHS* gene, *chs3-1,* which encodes an unconventional disease resistance (R) protein belonging to the TIR-NB-LRR class with a zinc-binding LIM domain (Lin-11, Isl-1 and Mec-3 domains) at the carboxyl terminus, shows arrested growth, chlorosis and constitutively activated defence responses at 16°C (Yang et al., 2010). A mutant in TNL gene *ssi4* develops chlorotic lesions which can be suppressed by high humidity (Shirano et al., 2002; Zhou et al., 2004). In addition, there are several examples of mutants with spontaneous lesions induction which are suppressed by mutations in loci encoding R proteins of CNL type – *ACTIVATED DISEASE RESISTANCE 1 – adr1*, *adr1-l1* and *adr1-l2* suppress *LESION SIMULATING DISEASE 1* (*lsd1*) by down regulating SA signaling (Bonardi et al., 2011; Roberts et al., 2013). Likewise, when a putative TNL encoded by *LAZARUS 5* (*LAZ5*) gene is mutated, *accelerated cell death 11* (*acd11*) lesion phenotype can be suppressed (Palma et al., 2010). It was also shown, that in the absence of the copine-like proteins BON1 and BON3 function, several R-like genes of the TNL/TN type were found to trigger lesion cell death (LCD; Li et al., 2009). Mutation in *SUPPRESSOR OF MKK1 MKK2 2* (*summ2*) which encodes putative NB-LRR, suppresses lesions formation and dwarfism of mutants of MAP kinase pathway *mkk1/mkk2 and mpk4* (Kong et al., 2012).

There are genes coding for other defense related components that when mutated trigger the same constitutive immunity activation and dwarf or/and lesion mimic phenotypes – e.g., *CONSTITUTIVE EXPRESSOR OF PATHOGENESIS-RELATED GENE 1* (*CPR1*), *SUPPRESSOR OF SALICYLIC ACID INSENSITIVITY OF NPR1-5* 2 (*SSI2*), *DEFENSE NO DEATH* 1 (*DND1*), TYPE III *PHOSPHATIDYLINOSITOL-4-KINASES* β*1*β*2* (*PI4KIII*β1β2) (Bowling et al., 1994; Yu et al., 1998; Zhang et al., 2003; Sekine et al., 2004; Gou et al., 2012; Sasek et al., 2014). As a regular aspect of these mutants' phenotype deviations, hyper accumulation of SA was observed.

LETHALITY OF THE HUB

Overactive immunity can disturb plant growth and fitness, and in an extreme case, this can be deleterious. Unexpectedly, an embryo lethal phenotype was found for LOF mutation of a defense related gene *RPM1-INTERACTING PROTEIN 4* (*RIN4*). Being evolutionarily conserved protein in plants, RIN4 is targeted to the plasma membrane by C-terminal acylation, and is required for the activation of a CNL *RESISTANCE TO*

TABLE 1 | List of *Arabidopsis* mutants related to R proteins hyper activity causing dwarf and lesion mimic phenotypes.

Gene	Name	Function category	Related mutant phenotypes	Reference
acd11	*accelerated cell death 11*	Sphingosine transfer protein	Lesions	Brodersen et al., 2002
adr1	*activated disease resistance 1*	CNL	Lesions suppression, dwarf oe	Grant et al., 2003
adr1-l1	*activated disease resistance 1-like 1*	CNL	Lesions suppression, dwarf oe	Collier et al., 2011
adr1-l2	*activated disease resistance 1-like 2*	CNL	Lesions suppression	Bonardi et al., 2011
atg5	*autophagy related gene 5*	Autophagy, ubiquitin ligase	Early senescence	Thompson et al., 2005
atg6	*autophagy related gene 6/Beclin1*	Autophagy activation	Pollen-lethality	Fujiki et al., 2007
atg7	*autophagy related gene 7*	Autophagy, ubiquitin activating enzyme	Defense-related	Doelling et al., 2002
atg8	*autophagy related gene 8*	Ubiquitin-like protein, cargo recruitment	/	Ketelaar et al., 2004
bak1	*brassinosteroid-insensitive associated 1*	Receptor-like protein kinase	Semidwarf	Li et al., 2002
bap1	*bon1-associated protein*	Calcium-dependent phospholipid-binding	/	Hua et al., 2001
bir1	*bak1-interacting receptor-like kinase 1*	Receptor-like protein kinase	Dwarf	Gao et al., 2009
bon1	*bonzai1*	Copine-like, membrane trafficking	Dwarf	Hua et al., 2001
bon2	*bonzai2*	Copine-like, membrane trafficking	Dwarf	Yang et al., 2006
bon3	*bonzai3*	Copine-like, membrane trafficking	Dwarf	Yang et al., 2006
chs2	*chilling-sensitive 2*	TNL	Lesions	Huang et al., 2010
chs3	*chilling-sensitive 3*	TNL	Lesions	Yang et al., 2010
cpr1	*constitutive expresser of pathogenesis-related gene*	F-box protein	Dwarf	Gou et al., 2012
dnd1	*defense no death 1*	Cyclic nucleotide-gated ion channel	Dwarf	Yu et al., 1998
eds1	*enhanced disease sensitivity 1*	R related signaling	Lesions suppression	Rogers and Ausubel, 1997
exo70A1	*exo70A1*	Membrane trafficking	Dwarf	Synek et al., 2006
exo70B1	*exo70B1*	Membrane trafficking	Lesions	Kulich et al., 2013
fls2	*flagellin-sensitive 2*	Receptor-like protein kinase	Defense related	Gomez-Gomez and Boller, 2000
laz4	*lazarus 4*	Membrane trafficking	Lesion suppression	Munch et al., 2015
laz5	*lazarus 5*	R protein	Lesions suppression	Palma et al., 2010
lsd1	*lesion simulating disease 1*	Cell death related	Lesions	Kliebenstein et al., 1999
mkk1/mkk2	*mitogen-activated protein kinase kinase kinase 1/2*	Signaling	Dwarf, lesions	Qiu et al., 2008
mpk1	*mitogen-activated protein kinase 1*	Signaling	Dwarf	Bartels et al., 2009
mpk4	*mitogen-activated protein kinase 4*	Signaling	Dwarf, lesions	Petersen et al., 2000
ndr1	*non-race specific disease resistance 1*	R related signaling	Lesions suppression	Century et al., 1995
pad4	*phytoalexin deficient 4*	R related signaling	Lesions suppression	Jirage et al., 1999
rar1	*required for mlo12 resistance 1*	R related signaling	Lesions suppression	Azevedo et al., 2002
rin4	*rpm1-interacting protein 4*	Immunity related	Embryo lethal	Mackey et al., 2002
rpm1	*resistance to p. syringae pv maculicola 1*	R protein	Defense related	Debener et al., 1991
rps2	*pesistant to p. syringae 2*	R protein	Defene related	Yu et al., 1993
sag101	*senescence associated gene 101*	R related signaling	/	Feys et al., 2005
sgt1b	*suppressor of g-two allele of skp1*	R related signaling	Lesions suppression	Azevedo et al., 2002
sid2	*salicylic acid insensitive 2*	SA synthesis	Defense related	Nawrath and Metraux, 1999
slh1	*sensitive to low humidity 1*	R protein	Lesions	Noutoshi et al., 2005
snc1	*suppressor of npr1 constitutive 1*	R protein	Dwarfism suppression	Li et al., 2010
srfr1	*suppressor of rps4-rld 1*	Tetratricopeptide repeat domain containing	Dwarf	Kim et al., 2010
ssi2	*suppressor of SA insensitivity of npr1-5 2*	Stearoyl-ACP desaturase	Dwarf, lesions	Sekine et al., 2004

(Continued)

TABLE 1 | Continued

Gene	Name	Function category	Related mutant phenotypes	Reference
ssi4	*Suppressor of SA insensitivity of npr1-5 4*	R protein	Dwarf	Shirano et al., 2002
summ2	*Suppressor of mkk1 mkk2 2*	R protein	Dwarfism and lesions suppression	Zhang et al., 2012
syp121/syp122	*Syntaxin 121/syntaxin 122*	Membrane trafficking	Dwarf, lesions	Zhang et al., 2008
syp23	*Syntaxin 23*	Membrane trafficking	Semi-dwarf	Ohtomo et al., 2005
syp31	*Syntaxin 31*	Membrane trafficking	/	Chatre et al., 2009
TN2	*TIR-NBS 2*	R protein	Lesions suppression	Zhao et al., 2015
TX At2g32140	/	R protein	Dwarf	Kato et al., 2014

PSEUDOMONAS SYRINGAE PV. MACULICOLA 1 (RPM1; Kim et al., 2005; Takemoto and Jones, 2005). RIN4 is phosphorylated upon infection with *P. syringae* expressing either AvrB or AvrRpm1 (Mackey et al., 2002). RIN4 is also involved in the activation of another CNL type R protein *RESISTANCE TO P. SYRINGAE 2* (RPS2) by putative Cys protease AvrRpt2 of *P. syringae*, which causes posttranscriptional cleavage and disappearance of RIN4 and this is required for full RPS2 activation (Axtell and Staskawicz, 2003; Mackey et al., 2003). Interestingly, in coimmunoprecipitation experiments, RIN4 was found to associate with RPM1, RPS2 as well as with pathogen recognition receptor (PRR) FLAGELLIN-SENSITIVE 2 (FLS2), creating thus a physical link between PTI and ETI (Qi et al., 2011). The *rin4* null mutation lethality is rescued in a *rin4rps2* double mutant, indicating that RIN4 negatively regulates inappropriate activation of RPS2 (Mackey et al., 2003). In addition, fragments of RIN4, including those produced by AvrRpt2, each containing a nitrate-induced (NOI) domain specific for plants, suppress PTI, also in the *rpm1/rps2/rin4* mutant background, and activate a cell death response in the wild type (Afzal et al., 2011).

MEMBRANE TRAFFICKING AND THE R PROTEINS-DEPENDENT IMMUNITY

Surprisingly, several basic regulators expected to function in the endomembrane trafficking and membrane fusion events, such as SNARE and exocyst proteins, might be also connected to the regulation of activity of R proteins. For instance, the dwarf and lesion-mimic double mutant of plasma membrane syntaxins SYP121 and SYP122 constitutively expresses the SA signaling pathway- as well as other known pathogen-responsive genes (Zhang et al., 2008). The same study shows that based on the suppressor mutant analysis of *syp121 syp122*, PAD4 is of key importance for the lesion development. Mutant alleles of signaling mediators of both TNL and CNL-type resistances *EDS1*, *NDR1*, *REQUIRED FOR MLO12 RESISTANCE 1* (*RAR1*) and *SUPPRESSOR OF G-TWO ALLELE OF SKP1* (*SGT1b*) partially rescued the lesion-mimic phenotype. Interestingly, the double mutant was crossed to the autophagy *atg7* mutant, however, as there was no effect of this mutation on the appearance of lesions, authors concluded that the autophagy does not play a role in this process (Azevedo et al., 2002; Zhang et al., 2008).

Recently, *exo70B1* loss-of-function mutant was found to develop spontaneous leaf lesions, over-express defense responses genes and show enhanced resistance to fungal, oomycete and bacterial pathogens (Kulich et al., 2013; Stegmann et al., 2013). Unexpectedly, its function is not related to the secretion of secretory vesicles to the plasma membrane; instead, EXO70B1 positive compartments were found to end in the central vacuole and to co-localize with autophagosomal marker ATG8f. In a screen for mutants that suppress *exo70B1* phenotype, nine alleles of TIR-NBS2 (TN2) were identified, suggesting that loss-of-function of EXO70B1 leads to activation of this TN protein (Zhao et al., 2015). It was also shown that TN2 interacts with EXO70B1 in yeast and *in planta*. However, it is not known whether TN2 directly monitors EXO70B1 integrity (as proposed by Zhao et al., 2015) or whether EXO70B1 is only required for autophagic transport to the vacuole and subsequent degradation of TN2. EXO70B1-mediated autophagy-related transport to the vacuole might be participating in TN2 degradation. Both scenarios would explain the observed phenotype.

Additionally, recent work confirmed the importance of membrane trafficking in the plant cell death lesion suppression – *lazarus 4* (*laz4*) was found to be mutated in one of three *VACUOLAR PROTEIN SORTING 35* (*VPS35*) genes which code for a subunit of the retromer complex functioning in endosomal protein sorting and vacuolar trafficking – esp. of retrograde retrieval of vacuolar sorting receptors. These results also showed that the retromer deficiency impairs endosomal sorting of immune components and targeting of vacuolar cargo (Munch et al., 2015).

Interestingly, the endosomal compartment may be as well the site of R-Avr proteins interaction – potato R3A and *Phytophtora infestans* effector AVR3a interact and relocalize from the cytoplasm to endocytotic compartment from where they turn on HR signaling (Engelhardt et al., 2012).

Even though it was not described for plants so far, we can expect that the both endosomes and autophagy related membrane trafficking will provide pathogens an opportunity to manipulate both for the purposes of more successful infection. Such an example was recently described for human epithelium-*Salmonella* interaction – at early stages of *S. typhimurium* infection, autophagy is used to seal endosomal membranes damaged by *Salmonella* secretion system during host cell invasion, but later it is also necessary for the further progression of *Salmonella* infection (Kreibich et al., 2015).

FROM AUTOPHAGY TO IMMUNITY

Autophagy is a bulk degradation by which cell/organism recycles nutrients, deals with stress, clears off dysfunctional organelles, aggregates etc. (Levine and Klionsky, 2004). Several types of autophagy have been reported, including macroautophagy, which is present in many organisms including fungi, animals and plants. This process relies on the concerted action of autophagy-related (*ATG*) genes encoded proteins to form first phagophore, to promote phagophore enclosure into autophagosome, and to deliver autophagosomes to the vacuole or lysosomes to release the autophagic bodies for eventual breakdown (Li and Vierstra, 2012; Reggiori and Klionsky, 2013).

When *Arabidopsis* mutants are disrupted in *ATG* genes represented by single loci, they grow normally under non-stress conditions, but are hypersensitive to nitrogen and carbon starvation (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005). However, unlike other non-plant organisms, *Arabidopsis* has nine *ATG8* and two *ATG12* gene isoforms, which makes the study of their role more difficult and suggests that the autophagic process in plants is more complicated than in other organisms. Some of its complexity is reflected in the role of autophagy in plant immunity.

The importance of autophagy in the plant immunity was first demonstrated in Liu et al. (2005) – it was found that the autophagy was required to restrict the spread of plant HR cell death. The activation of hypersensitive cell death via the R gene RPM1 upon infection with bacteria also led to cell death beyond the borders of the infection site in plants silenced for *atg6/Beclin1* (Patel and Dinesh-Kumar, 2008). It was concluded that autophagy prevents unrestricted HR cell death and that functions as a pro-survival pathway in plant–pathogen interactions. All of these observations and conclusions were based on experimenting with older *Arabidopsis* plants and on tissues surrounding the actual infection sites, a few days after local infection.

However, a pro-death function of autophagy during HR cell death was reported as well (Hofius et al., 2009). Autophagy was found to be triggered by some, but not all types of R proteins in the infected tissue and its surroundings. HR cell death triggered by R proteins RPS4, RPP1 and RPM1 was significantly suppressed in *atg* (autophagy) mutants; especially the first two of them which signal through EDS1 signaling component. In this case, cell death was monitored in the actual infection site, in the range of hours after inoculation (Hofius et al., 2009).

Yoshimoto et al. (2009), found no deviations in RPM1-triggered cell death beyond the initial infection site in younger atg mutants. However, in older *atg* mutants such as *atg5*, they observed lesions in non-infected tissues 6–9 days after infection. Interestingly, these effects were suppressed by removal of the SA and by mutations in SA signaling hub "non-expressor of *PR* genes" – NPR1. The authors proposed that autophagy negatively regulates the cell death by controlling NPR1-dependent SA signaling. In contrast to younger leaves, older *atg* mutant leaves contain higher levels of toxic metabolites, disrupted organelles and oxidized proteins which contribute to the cell death spread (Yoshimoto et al., 2009). This could be as well explained as a combination of effects of different sets of genes involved in the adult plant resistance and ineffective autophagy (Carviel et al., 2009). Scientists tried to explain and integrate these conflicting results obtained from studies on HR lesions of *atg* mutants. Zhou et al. (2014) propose that autophagy suppresses SA and ROS signaling amplification loop that leads to cell death, while in the resistance to necrotrophic pathogens it promotes JA signaling. Consistently, a recent hypothesis suggests that SA is not only an autophagy inducer, but also a cargo for autophagy-related ER to vacuole membrane transport and catabolism (Kulich and Zarsky, 2014). Recently, a model was worked out in which the autophagy is both initiator and executioner of cell death and is placed downstream of the R protein activation, and supposed to help the cell to deal with the ER stress provoked by a heavy load with pathogenesis related proteins (PRs, review in Minina et al., 2014).

CONCLUSION AND PERSPECTIVES

Here we show that most of the observed defects in *Arabidopsis* R protein regulator mutants are a direct or indirect consequence of non-pathogen related ectopic R protein activation. It thus seems conceivable that the plant constantly down regulates R protein function, and when this constitutive negative regulation is disturbed, the R proteins are activated and spontaneously signal the non-existent pathogen attack. Based on the example of *rin4* mutant lethality we could speculate, that, similarly to other organisms, the proper function of the negative control might be set already in the earliest stages of development. The plant innate immunity has to be kept as low as possible when it is not necessary in order to prevent high energy costs of defense, and yet in the state of alertness which will allow its fast, in fact instantaneous, activation. We believe that the best way to achieve this is to keep these components (i.e., in our case R genes) transcribed and translated on a sufficient basic level, but to keep their function tightly under negative control which will prevent undesired overactive autoimmunity. How could be this achieved? There are many examples of negative controls involved at various stages of defense that include ubiquitination and proteolysis, phosphorylation of proteins, as well as redox dependent changes in protein multimerization and localization (e.g., Trujillo et al., 2008; Anderson et al., 2011; Vogelmann et al., 2012). We suggest that one of the mechanisms to achieve this is also targeting of defense machinery components – here especially R proteins – to the autophagy pathway for degradation (**Figure 1**). Once the R protein is recruited by autophagy machinery into the autophagosome, it might share the destiny of other autophagic cargos – transport to the vacuole and degradation. We speculate that along with proteins the autophagy related degradation process might destroy also other molecules including signaling relevant molecules as ROS or SA. After the interaction of R protein with its counterpart Avr, R protein is protected against this autophagy-dependent degradation and can interact with downstream components and trigger ETI. This model may be valid also for indirect R-Avr interactions; e.g., the proposed R protein guard function (reviewed e. g. in Spoel and Dong, 2012)

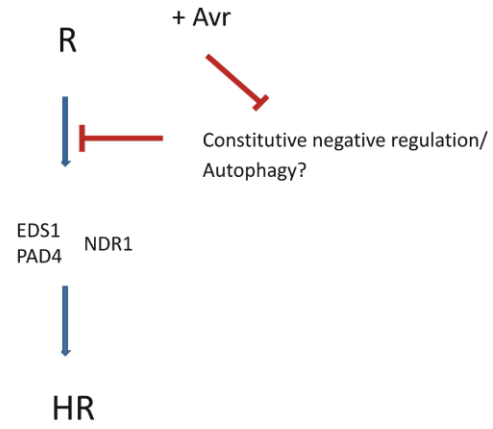

FIGURE 1 | The plant constitutively negatively regulates R protein function. The interaction R-Avr serves mainly to release this negative regulation. The important component of negative regulation is autophagy; when it is non-efficient, R proteins spontaneously over activate defense responses.

could be based on the avoidance of this negative regulation after the recognition of the changed status of the guardee. It should be stressed that we certainly expect other ways of regulations of R proteins to exist, such as a switch from inactive to active state of R protein upon Avr recognition, as well as other ways of negative regulation.

Based on our hypothesis one would expect that autophagy mutants should copy the phenotype of *exo70B1* mutant, having at least some R proteins constitutively activated. While some of the mutants in the autophagy pathway indeed show similar phenotypic deviations (e.g., early senescence and yellowing, sensitivity to starvation, as well as SA hyperaccumulation in *atg2* and *atg5* mutants; Yoshimoto et al., 2009; Wang et al., 2011), others seem to display only early senescence phenotype and cell death phenotypes only after starvation induction (like *atg7* mutant). It also seems that some subunits of autophagy machinery might be more important for the negative regulation of the immunity, while others, e.g., ATG7 and ATG9, in the execution of HR (Hofius et al., 2009; Minina et al., 2014). It should be, however, noted that autophagy proteins (and EXO70B1) have been also implicated in diverse cellular processes independently of their roles in autophagy.

We also expect that, pathogen effectors might have evolved to manipulate and hijack this negative regulation and worsen the plant defense – recently, a *Phytophtora infestans* effector PexRD54 has been shown to outcompete the autophagy cargo receptor Joka and enhance virulence of this pathogen. Interestingly, PexRD54 does this probably through the activation of selective autophagy. Joka could participate in the removal of plant or pathogen molecules that negatively affect host defenses. As authors of the study speculate, PexRD54 would thus counteract the positive role of Joka2-mediated selective autophagy in pathogen defense. An alternative, but not exclusive explanation based on our hypothesis would be that PexRD54 at the same time stimulates the selective autophagy of R proteins capable of detecting it and thus promotes pathogen virulence (Dagdas et al., 2016). Already the report of Engelhardt et al. (2012) demonstrated the capability of cytoplasmic R protein to be recruited to endomembranes, but not for degradation, rather for the purpose of activation. However, this is not exclusive with our model – the interaction of R3A and Avr3A might release the negative regulation of R3A and switch on the HR. This interaction is obviously indirect and requires an intermediate connected to ARA6/ARA7 marked endosomes. It is possible that this activation evolved from the mechanisms of negative regulation. More information on R3A and Avr3A interactors could help to solve this ambivalent situation.

We found an indirect support for our hypothesis in the autopagy-related events described for mammalian cells – it is known from experiments performed on HeLa cells that endocytosed plasma membrane contributes to ATG12–ATG5-ATG16L1-positive/ATG8-negative phagophore precursor vesicles by both clathrin-dependent and -independent routes (Moreau and Rubinsztein, 2012). The subsequent maturation of these small phagophore precursors into phagophores (ATG12–ATG5-ATG16L1-positive/ATG8-positive) is assisted by SNARE-mediated homotypic fusion that increases their size. Additionally, *Arabidopsis* BON1/2/3 belong to copine proteins, a family of ubiquitous Ca(2+)-dependent, phospholipid-binding proteins that are known to be involved in animal membrane trafficking events (Tomsig and Creutz, 2002), and in *Dictyostelium* localize to plasma membrane, contractile vacuoles, organelles of the endolysosomal pathway, and phagosomes (Damer et al., 2005). Therefore, besides confirmed role of EXO70B1 in autophagy and regulation of TN2 activity, very probably SNARE and BON proteins could implement similar role in autophagy-related membrane targeting and membrane fusion events leading to the negative control of R proteins.

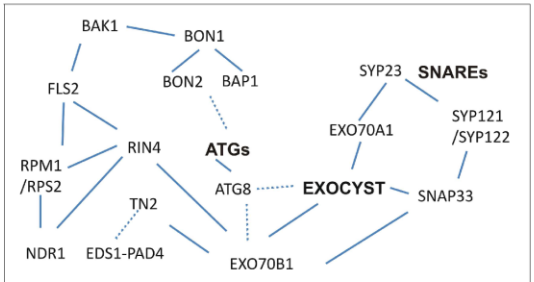

FIGURE 2 | Interactions of PTI, ETI and ATG related proteins with SNAREs and exocyst. The RIN4 interacts with PTI receptor FLS2 and associated proteins, which further interact with BON1, BON2 and BAP. Additionally, RIN4 and related R-proteins may be in the same complex. R proteins and autophagy functions are connected by exocyst and SNARE interactions. (Experimentally confirmed interactions are visualized with lines, non-confirmed with dashed lines).

We found further support for this hypothesis in the connection between assumed autophagy regulating proteins and R proteins, as well as other key molecules of the both PTI and ETI immune response, in the web of protein-protein interactions that are available in Biogrid and PPIN databases (Stark et al., 2006; Mukhtar et al., 2011; **Figure 2**; **Table 1**). The components of PAMP-sensing complexes interact with RIN4, which further interacts with R proteins. Mainly through mediating kinase BAK1, they are connected and interact as well with BON1, BON2 and BAP. RIN4 interacts with R proteins as well as with EXO70B1 (Afzal et al., 2013). Besides its capability to interact with other exocyst and SNARE proteins, EXO70B1, together with 20 other paralogs of *Arabidopsis* EXO70 exocyst subunits, possess ATG8 interacting motives, which indicates that the autophagy machinery and exocyst complex functions are multiply connected (Cvrčková and Zárský, 2013; Tzfadia and Galili, 2013; Sabol et al., in preparation). Thus, in the vicinity of plasma membrane, and depending on membrane trafficking which involves SNARE, exocyst and autophagy complex proteins, a tight control of R protein activation allows the immunity to be kept low but in a constant alert.

Recently, a role for EXO70F3 of *Oryza sativa* in immunity against *Magnaporthe oryzae* was found – OsEXO70F3 appears to play a crucial role in immunity triggered by Pii, suggesting a role for this EXO70 paralog as a decoy or helper in Pii/Avr-Pii interaction (Fujisaki et al., 2015). It may be true that pathogen effectors target these and other exocyst subunits in order to suppress defense, however, we don't consider it to be mutually exclusive with our hypothesis.

Our model could help to better understand and reconcile conflicting aspects of autophagy in the plant immunity (Teh and Hofius, 2014): in the infection sites, R-Avr recognition prevents R protein targeting to inactivation/destruction pathway and triggers the ETI, and with the increased distance from the infection site, declining concentration of Avr protein allows the autophagy to overtake again a control over R protein. In *atg* mutants, the existing constitutive immunity activation results in spontaneous HR lesions formation; but after the pathogen attack, in the case of younger leaves, in addition to R protein deregulation, R is further activated by Avr recognition, which makes cells more resistant and lesions smaller. Or, under conditions with additional stresses, as in the case also of older leaves, because of coincidence between consequences of ineffective autophagy of *atg* mutants and Avr-enhanced over activation of R proteins, less Avr is needed for HR threshold to be crossed and lesions spread farther.

Our model's aim is to focus on one aspect only – a possibility of a negative regulation of some NLRs/innate immunity related proteins by autophagy in plants. However, there are many difficulties that will have to be overcome in order to confirm its validity. Part of difficulties is coming from the complexity of autophagy machinery and a large number of ATG proteins that have also been implicated in diverse cellular processes independently of their roles in autophagy. Autophagy machinery is also difficult to study separately from other endomembrane compartments, especially by using pharmacological treatments. For instance, wortmannin, which is often used for these purposes, is rather pleiotropic drug – dependent on cell type and concentration it affects different types of phosphoinositide kinases, having thus multiple interference with endomembrane dynamics.

To conclude, plants have mechanisms to downregulate R proteins function, and when they are attacked by an appropriate Avr carrying pathogen, the R proteins are stabilized, activating defense responses. This would also mean that R proteins are capable of immunity activation without Avr and that the interaction R-Avr serves mainly to release R proteins negative regulation. The disturbance of the basic autophagy machinery has pleiotropic effects on many plant functions including development and is influenced by growth conditions, abiotic stresses and senescence, hence it is very difficult to study effects of *atg* mutants that would concern specifically defense responses. We believe that in the near future, R proteins studies will bring more information on the regulation of their activity including proteins that target them for the suggested autophagy destruction.

AUTHOR CONTRIBUTIONS

TP did a compilation of data on R-related dwarf and lesion mimic mutants and most of the writing; PS did the RIN4-related data mining and text editing; IK explained the connection to autophagy; JO delt with membrane traficking chapter; VZ did the most of text editing and integrating as well as the finalization of the manuscript.

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REVIEW PAPER

Exocyst and autophagy-related membrane trafficking in plants

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Abstract

Endomembrane traffic in eukaryotic cells functions partially as a means of communication; delivery of membrane in one direction has to be balanced with a reduction at the other end. This effect is typically the case during the defence against pathogens. To combat pathogens, cellular growth and differentiation are suppressed, while endomembrane traffic is poised towards limiting the pathogen attack. The octameric exocyst vesicle-tethering complex was originally discovered as a factor facilitating vesicle-targeting and vesicle-plasma membrane (PM) fusion during exocytosis prior to and possibly during SNARE complex formation. Interestingly, it was recently implicated both in animals and plants in autophagy membrane traffic. In animal cells, the exocyst is integrated into the mTOR-regulated energy metabolism stress/starvation pathway, participating in the formation and especially initiation of an autophagosome. In plants, the first functional link was to autophagy-related anthocyanin import to the vacuole and to starvation. In this concise review, we summarize the current knowledge of exocyst functions in autophagy and defence in plants that might involve unconventional secretion and compare it with animal conditions. Formation of different exocyst complexes during undisturbed cell growth, as opposed to periods of cellular stress reactions involving autophagy, might contribute to the coordination of endomembrane trafficking pathways.

Keywords: Autophagy, endomembranes, exocyst, plant defence, secretory transport, UPS.

Introduction: stress and autophagy in eukaryotes

Plants in nature are constantly exposed to various stresses, including starvation and attacks by bacterial and fungal intruders. In these situations and during developmental senescence-related nutrient remobilization, autophagy is an important contributor to the overall fitness of plant populations. In recent years, new insights into the molecular machinery of autophagy in plant cells have been gained (Doelling *et al.*, 2002; Hanaoka *et al.*, 2002; Rose *et al.*, 2006; review in Michaeli *et al.*, 2016), including a surprising contribution of otherwise canonical exocytotic vesicles tethering exocyst complex (Kulich *et al.*, 2013; Tzfadia and Galili, 2013), similar to what has been previously found for the animal exocyst subcomplex (Bodemann *et al.*, 2011). In

Abbreviations: ABA, abscisic acid; AIC, anthocyanin inducible conditions; ATG, *AUTOPHAGY-RELATED GENE*; AVI, anthocyanin vacuolar inclusions; Avr, avirulent protein; CATCHR, complexes associated with tethering containing helical rrods; ER, endoplasmic reticulum; ETI, effector-triggered immunity; GST, glutathione transferase enzyme; LECA, last eukaryotic common ancestor; LOF, loss-of-function (allele); MHC, major histocompatibility complex; mTOR, mammalian target of rapamycin; MVB/LE, multivesicular body/late endosome; NBR1, neighbor of BRCA1; OA, oxalic acid; PCD, programmed cell death; PI3K, phosphatidylinositol-3-kinase; PI(4,5)P2, phosphatidylinositol-4, 5-bisphosphate; PM, plasma membrane; PRR, pattern recognition receptor; PTI, PAMP-triggered immunity; ROP, Rac-related GTPases of plants; SA, salicylic acid; TFEB, transcription factor EB; TLR, toll-like receptor; UPS, unconventional protein secretion

this review, we will summarize and compare what is known about the exocyst components in autophagy in plants versus animals, discuss possible general involvement of autophagy-related membrane trafficking in secondary metabolite transport in plants, and focus especially on the biotic stress context of exocyst functions in plants.

In mammals, the core of the autophagic machinery functions to produce an isolation membrane, a phagophore, and involves the ULK complex, the Beclin 1/class III phosphatidylinositol-3-kinase (PI3K) complex, two transmembrane proteins, ATG9 and vacuole membrane protein 1 (VMP1), two ubiquitin-conjugation systems, ATG12 and ATG8/LC3, and proteins that mediate fusion between autophagosomes and lysosomes (Yang and Klionsky, 2010). Similarly, the same assembly of autophagy machinery has been found for the evolutionary distant *Saccharomyces cerevisiae* (Noda *et al.*, 2009; Hurley and Schulman, 2014; Stjepanovic *et al.*, 2014).

The existence of modular and complex autophagy machinery structures implies a complex method of regulation, since many of these autophagy components are directly controlled by cellular stress signals. On the other hand, even though autophagy is induced by a variety of stimuli, with multiple sensory inputs, the activation pathways converge to several key points of autophagy induction. The best studied is the case of nutrient deprivation-induced autophagy that is, in mammals, regulated by mTORC1, which is a mammalian target of rapamycin (mTOR) complex 1; mTORC1 is a polyprotein complex that contains mTOR, Raptor, mLST8/GßL, Deptor and PRAS40. mTORC1 interacts with the ULK1-containing autophagy complex and, upon starvation, mTORC1 dissociates from the ULK complex, leading to dephosphorylation of specific residues within ULK1 or ULK2 and ATG13, which are normally phosphorylated by mTORC1, further resulting in ULK activation (Efeyan and Sabatini, 2013; Mizushima, 2010). ULK subsequently activates downstream components of the autophagic machinery, probably by phosphorylation of the Beclin 1/Class III PI3K complex subunit (He and Levine, 2010).

mTORC1-mediated autophagy regulation may be one of the most finely tuned intracellular mechanisms. There are several feedback mechanisms, both positive and negative, that ensure the proper level of autophagy activation (Neufeld, 2010; Behrends *et al.*, 2010; Efeyan and Sabatini, 2013). In addition, it has been previously found that the inactivation of mTORC1 results in dephosphorylation of transcription factor EB (TFEB; Martina *et al.*, 2012, Roczniak-Ferguson *et al.*, 2012); TFEB then translocates to the nucleus, where it induces the transcription of target genes involved in lipid catabolism, fatty acid oxidation, and ketogenesis (Settembre *et al.*, 2012).

In addition to nutritional stress, ER stress, hypoxia, redox stress, and mitochondrial damage are well-known inducers of autophagy (reviewed in Kroemer *et al.*, 2010). In addition, importantly, autophagy was found to play a role in infection and inflammation (Sumpter and Levine, 2010). Mammalian cells utilize autophagy to combat several pathogenic bacteria, such as *Mycobacteriaceae*, *Streptococcus pyogenes*, and *Salmonella typhimurium*. Bacteria are engulfed by the autophagy machinery and subsequently degraded in lysosomes (xenophagy; Cemma and Brumell, 2012). Autophagy is activated by recognition of the pathogen or pathogen-associated component by pattern recognition receptors that, among many other families, include toll-like receptors (TLRs) as well as NOD-like receptors (NLRs) that activate signalling via the inflammasome (Sumpter and Levine, 2010). Conversely, autophagy contributes to antigen processing and major histocompatibility complex (MHC) class II, and probably MHC class I, presentation, linking the innate and adaptive immune mechanisms (Münz *et al.*, 2009; Heikamp and Powell, 2012; Paul and Schaefer, 2012). However, several bacterial species evolved to counteract this host defence mechanism by elaborating replication niches from it (Münz *et al.*, 2009). For instance, at the *Shigella flexneri* entry site, ATG16L1 is recruited by the nucleotide-binding oligomerization domain (NOD) pattern recognition receptors (PRRs). However, completion of the autophagy process is prevented by the effector-induced blockage of LC3 recruitment and effector-mediated escape from LC3-positive vacuoles (Baxt and Goldberg, 2014; Campbell-Valois *et al.*, 2015).

Autophagy also plays a role in the homeostatic balance of the inflammatory response following pathogen clearance. There is increasing evidence that autophagy and inflammasome signalling are counter-regulatory and that inflammasomes can be degraded by autophagy (Galluzzi *et al.*, 2015). Intriguingly, pathogens are also able to exploit this regulatory point. For instance, the recognition of *Pseudomonas aeruginosa* induces autophagy via TLR4 and its adaptor TRIF; however, this is suppressed by *P. aeruginosa*-mediated activation of the NLRC4 inflammasome. NLRC4-associated caspase-1 activity cleaves TRIF, resulting in inhibition of autophagy and production of type I interferon (Jabir *et al.*, 2014; Jabir *et al.*, 2015).

The core autophagic machinery is also completely conserved in plants (Contento *et al.*, 2005; Thompson *et al.*, 2005; Sláviková *et al.*, 2005; Rose *et al.*, 2006). Studies of plant autophagy might be complicated due to the expansion in the number of paralogs of plant canonical autophagy regulating proteins, relating to autophagy-related genes (ATGs) originally described in yeast. Special attention has been devoted to autophagy-related peroxisome and chloroplast turnover in plants, as well as the impact of the latter on the efficiency of plant immunity (Dong and Chen, 2013; Shibata *et al.*, 2013; Lee *et al.*, 2014; Izumi and Nakamura, 2017). As for complex autophagy regulation, especially in the case of the mTOR homologue, plant studies are lagging behind that of yeast and animal models (Díaz-Troya *et al.*, 2008; Montané and Menand, 2013). As there are numerous recent reviews on autophagy in plants and detailed comparative lists of ATG gene conservation (fo example Kim *et al.*, 2012; Michaeli *et al.*, 2016), we will focus here specifically on what is known about the molecular connections of exocyst-to-autophagy machinery in animals compared with plants.

The exocyst in autophagy: connections to ATG machinery and comparison of plants versus animals

The exocyst is an evolutionarily conserved octameric vesicle tethering complex of the CATCHR type (complexes

associated with tethering containing helical rods), originally discovered in the context of exocytosis in yeast (Novick *et al.*, 1980; TerBush, 1996) but later also found to function in exocytosis in animals and plants (for recent review see Heider and Munson, 2012; Zárský *et al.*, 2013; Wu and Guo, 2015). All eight subunits, SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70, and EXO84, are present in all known eukaryotic branches of life (Koumandou *et al.*, 2007). The exocyst functions before SNARE-mediated fusion and facilitates SNARE complex formation, resulting in fusion between the vesicle and the target membrane. The exocyst thus seems to work as a membrane fusion accelerator (Heider and Munson, 2012; Wu and Guo, 2015). The land plant exocyst specifically features a multiplicity of EXO70 subunits encoded within the same organism, from three in liverwort to up to 47 in rice (Cvrčková *et al.*, 2012; Rawat *et al.*, 2017). There is already sufficient data to conclude that different EXO70 isoforms and therefore EXO70-specific complexes might have specific functions even within the same cell (Zárský *et al.*, 2013; Kulich *et al.*, 2013; Sekereš *et al.*, 2017, Synek *et al.*, 2017). Engagement of the exocyst in autophagy has not been observed in yeast, but, as we will discuss in this review, it has been found in multicellular animals and plants; in both, it also seems to be connected to innate immunity defence. As exocytosis and autophagy are endomembrane-trafficking processes, in this review's conclusion we will discuss the potential function of the exocyst as a stoichiometric coordinator of endomembrane traffic.

The engagement of the mammalian exocyst subcomplex in autophagy initiation was discovered in relation to the analysis of the function of small RAL GTPases in phagophore isolation membrane biogenesis (Bodemann *et al.*, 2011). Distinct exocyst subcomplexes interacting with ULK1, BECLIN-1, and ATG5/ATG12 complexes are implicated in autophagy regulation. In the initial phase of mammalian phagophore formation, RALB GTPase interacts with the exocyst subunit EXO84, resulting in the release of exocyst subunit SEC5 from an ULK1-inhibiting exocyst subcomplex at the Golgi apparatus (GA) of approximately 500 kDa in size and activation of ULK1 and Beclin1-VPS34 PI3P kinase on the EXO84-containing exocyst subcomplex, both of which are required for phagophore isolation membrane formation and maturation (Bodemann *et al.*, 2011; review in Antonioli *et al.*, 2017). An EXO84-containing exocyst subcomplex, without SEC5, of approximately 700 kDa is characterized not only by active ULK1 and BECLIN-1 but also by LC3 (Bodemann *et al.*, 2011; review in Antonioli *et al.*, 2017). Using a yeast two-hybrid screen to test for direct interactions between the mammalian exocyst complex proteins and autophagy factors, FIP200, ATG14, and RUBICON were identified (Bodemann *et al.*, 2011). Interestingly, in mammals, the interaction of activated RALB with a SEC5-containing exocyst subcomplex stimulates an innate immune response dependent on TBK1-IRF3 signalling (Simicek *et al.*, 2013). The switch between RALB engagement in either SEC5- or EXO84-containing exocyst subcomplexes is regulated by ubiquitination: ubiquitinated RALB interacts with the SEC5 subcomplex in innate immunity, while deubiquitinated RALB GTPase interacts with the EXO84 exocyst subcomplex participating in autophagosome initiation. The pro-apoptotic STK38 kinase, in interaction with EXO84, BECLIN-1, and RALB kinase, functions as an autophagy-promoting factor (Joffre *et al.*, 2015). In cells depleted in RALB, STK38 is hyperactivated and leads to apoptosis activation (Joffre *et al.*, 2015). The EGFR/LAPTM4B kinase complex associates with SEC5 and thus stimulates autophagy via activation of BECLIN-1 and by removing RUBICON (Tan *et al.*, 2015).

Also endocytosis contributes to phagophore formation in animal cells; endocytosed HeLa cell PM contributes to ATG12-ATG5-ATG16L1-positive/ATG8-negative phagophore precursor vesicles via both clathrin-dependent and independent routes (Moreau and Rubinsztein, 2012). The subsequent maturation of these small phagophore precursors into phagophores, which are ATG12-ATG5-ATG16L1-positive/ATG8-positive, is assisted by SNARE-mediated homotypic fusion that increases their size. However, these aspects are hitherto not resolved in plant endomembrane biology. It will be interesting to investigate if and how SNAREs and exocysts cooperate in the role of commitment to autophagy and whether they are connected to ability of SNAREs to exert an inhibitory function and engage in non-canonical compartmental targeting (for example Grefen *et al.*, 2010; De Benedictis *et al.*, 2013; Uematsu *et al.*, 2017).

Much less is known about the mechanisms linking the exocyst and autophagy in plants. An unexpected engagement of the *Arabidopsis thaliana* EXO70B1 exocyst subunit, one of 23 paralogs in Arabidopsis, in autophagy-related anthocyanin transport to the vacuole was observed in a seminal report by Kulich *et al.* (2013). The EXO70B1/anthocyanin-positive compartment colocalized with an Arabidopsis ATG8 marker and was also spotted in the vacuole after alkalinisation by Concanamycin A (Kulich *et al.*, 2013); note there are nine ATG8 genes in the Arabidopsis genome.

EXO70B1 loss-of-function (LOF) mutant cells accumulated significantly more paramural bodies compared to the wild-type and accumulated significantly less anthocyanins. As with some other autophagy Arabidopsis mutants, *Atexo70B1* also displays nutrition/energy-dependent, that is light and nitrate, spontaneous leaf necrotic lesion formation. EXO70B1 does interact with the core exocyst subunits SEC5 and EXO84, as evidenced from the yeast two-hybrid assay and co-immunoprecipitation studies, where a SEC6 subunit was also recovered. When crossed, the *exo70B1xsec5a* double mutant exhibited an enhanced autophagy phenotype (Kulich *et al.*, 2013). Another member of the EXO70 family of Arabidopsis was also implicated in autophagy; upon ectopic expression, the EXO70E2 paralog localizes to autophagy-like double membrane structures, even in animal cells (Ding *et al.*, 2014b), and is extruded to the apoplast in an exosome-like way (Wang *et al.*, 2010). Though interpretation of this process is controversial, its relation to the autophagy pathway upon its induction is clearly indicated (Lin *et al.*, 2015). While there are no reports centred on exocyst subunit phosphorylation in plants, a review of publications and the public Arabidopsis phosphoproteome database, PhosPhAt 4.0, reveals that it is obvious that, as in animals, plant exocyst subunits are phosphorylated, including EXO70B1, EXO84b,

SEC5A, and SEC10 (Jones *et al.*, 2009). Specific kinases and their target motifs are therefore also expected to be involved in exocyst regulation, including in relation to autophagy.

It is obvious that, in contrast to animal cells, autophagy-specific versions of exocysts in plants will also be defined by a specific EXO70 paralog. Different exocyst complexes or subcomplexes will be thus characterized by the presence of a specific EXO70 isoform. This may be true not only for targeting to different PM-cortical domains within the same cell (Kulich *et al.*, 2013; Sekereš *et al.*, 2017) but also for autophagy-associated versions of the plant exocyst (Zárský *et al.*, 2013). The possible concurrent presence of both SEC5 and EXO84 exocyst subunits in a complex related to autophagy-mediated anthocyanin transport to the vacuole also implies differences between plant and animal exocyst subcomplexes and complexes involved in autophagy (Kulich *et al.*, 2013). The differences between RHO and Ral GTPases in animals and ROP (Rac-related) GTPases of plants are also relevant for speculation regarding exocyst regulation of autophagy in plants. The only small GTPase implied in autophagy regulation in Arabidopsis is currently RAB-G3b (Kwon *et al.*, 2013). It is expected that the aforementioned phosphorylation-dephosphorylation pathways will also be implied in plant exocyst-autophagy relationship dynamics. The question of whether exocyst engagement in autophagy is a last eukaryotic common ancestor (LECA) feature and was subsequently lost in yeast or a result of convergent evolution is currently not possible to reasonably address and will be resolved in the future.

The exocyst in secondary metabolite transport

Although autophagy has been described as a degradation pathway for unwanted or damaged cell material, recent studies imply that autophagy-related transport also mediates trafficking of phytochemicals in plant cells (Brillouet *et al.*, 2013; Kulich *et al.*, 2013; Michaeli *et al.*, 2014)

A good example of cargo transported by autophagy-related direct endoplasmic reticulum (ER) transport to the vacuole are anthocyanins - pigment molecules rendering plant cells purple, which attract pollinating insects to flowers and protect leaves against damage from ultraviolet light (Poustka *et al.*, 2007; Pourcel *et al.*, 2010). Several models of anthocyanin transport in Arabidopsis have been proposed. The ligandin transport model involves direct transmembrane transport of anthocyanins via specific importers with the help of glutathione transferase enzymes (GST) (Poustka *et al.*, 2007; Sun *et al.*, 2012). The vesicular transport model postulates that anthocyanins imported into the ER lumen are transported via vesicles to the vacuole (Hsieh *et al.*, 2007; Pourcel *et al.*, 2010; Gomez *et al.*, 2011). However, vesicular transport of anthocyanins may be mediated by autophagy-like pathways in which two types of autophagy are involved: macroautophagy and microautophagy (Pourcel *et al.*, 2010; Kulich and Žárský, 2014; Chanoca *et al.*, 2015). Macroautophagy, the most well-known autophagic pathway, involves sequestration of cytoplasmic cargo by double membrane structures called autophagosomes. Autophagosomes are then delivered to the vacuole. The outer membrane of the autophagosome fuses with the tonoplast, whereas the inner membrane, together with the cargo, named the autophagic body, is released into the vacuole lumen. In microautophagy, the autophagic body is formed when cytoplasmic cargo is engulfed directly via tonoplast invagination, followed by secession of the membrane (Bassham *et al.*, 2007). The macroautophagy-like model of anthocyanin transport proposes that ER portions filled with anthocyanins are engulfed by autophagosomes and delivered to the vacuole following the formation of autophagic bodies, which fuse together and form anthocyanin vacuolar inclusions (AVIs). This model is based mostly on the observation of AVIs, which are reduced in *atg5* and *atg10* autophagy mutant plants 4 and 5 days after germination and on colocalization studies between anthocyanins and ER markers (Poustka *et al.*, 2007; Pourcel *et al.*, 2010). The microautophagy-like model proposes that anthocyanin aggregates that are in close proximity to the cytoplasmic surface of the vacuole are tightly pressed against the tonoplast and engulfed following the formation of AVIs after membrane secession (Chanoca *et al.*, 2015). We proposed a similar model, where anthocyanin transport is partially mediated by autophagic tubes previously described in yeast (Müller *et al.*, 2000; Kulich and Žárský, 2014). Autophagic tubes are formed when anthocyanin-rich ER subdomains develop within tonoplast invaginations deep inside the vacuole. These tubes are branched and from their tips autophagic bodies bud and then pinch off to form AVIs inside the vacuole. Despite results showing that autophagy is involved in anthocyanin transport to the vacuole, the molecular mechanisms of this process are still unknown. It is known that in yeast and animal cells, the microautophagy pathway relies on core autophagic machinery (Müller *et al.*, 2000; Krick *et al.*, 2008). In yeast, it seems that Atg-dependent macroautophagy is a prerequisite for microautophagy. Some authors have speculated that fusion of macroautophagosomes with the tonoplast is required to supply the vacuole with the excess membrane needed for microautophagy (Müller *et al.*, 2000). The relationship between autophagy and AVI density inside the vacuole upon anthocyanin inducible conditions (AIC) was examined by Chanoca *et al.* (2015), and, interestingly, the authors did not find any statistically significant differences in AVI density between *atg5* mutant and control plants. Moreover, seedlings treated with an autophagy inhibitor, wortmannin - an inhibitor of phosphatidylinositol 3-kinase (Zheng *et al.*, 2014) - showed no significant changes in the number of AVIs in comparison with the control group. These results indicate that microautophagy-related AVI formation during anthocyanin uptake does not rely on an autophagic pathway. To address this question, examination of *atg* mutant plants other than *atg5* is required. At this point, it is relevant to discuss the involvement of an exocyst complex in autophagy-related anthocyanin transport to the vacuole. We have already discussed that the EXO70B1 exocyst subunit, along with SEC5, EXO84B, and possibly SEC6, forms a subcomplex that plays a role in autophagy-related transport to the vacuole. EXO70B1 colocalizes with both anthocyanins and ATG8f inside the vacuole and in *exo70B1* mutant plants, vesicular transport of anthocyanins is likely compromised (Kulich

et al., 2013). Moreover, mutant plants showed a reduced number of neutral red-stained bodies (NRSB), which are closely related to AVIs in the central vacuole (Kulich and Žárský, 2014). Interaction between Arabidopsis exocyst subunits and core autophagy proteins is highly expected, since bioinformatic screens of exocyst subunits revealed numerous ATG8 interacting motifs (AIMs) within them, with five possible AIMs in the EXO70B1 amino acid sequence (Tzfadia and Galili, 2013). We therefore can speculate that EXO70B1 may contribute to macroautophagy-like anthocyanin transport to the vacuole through direct interaction with ATG8, together with SEC5, EXO84b, and SEC6. Based on work by Chanoca *et al.* (2015), it is possible that mechanisms other than autophagy may be involved in tonoplast protrusions during microautophagy-like anthocyanin uptake. If true, one can speculate regarding the contribution of the EXO70B1 subunit independently of other exocyst subunits and core autophagy proteins in the induction of tonoplast curvature (Fig. 1). It was shown by computer modelling that EXO70 causes clustering of PI(4,5)P2 in yeast (Pleskot *et al.*, 2015). A connection between EXO70 proteins and membranes organization was also found in mammalian cells, where EXO70 either via ARP2/3 dependent F-actin stimulation (Zuo *et al.*, 2006; Wang *et al.*, 2004) or oligomerization of EXO70 subunits alone induces negative membrane curvature resulting in PM protrusions (Zhao *et al.*, 2013).

Although the EXO70B1 subunit represents a candidate for the lipid binding related process, we cannot exclude the involvement of other EXO70 paralogs in *Arabidopsis*, since many (but not all) of them harbour C-terminal conserved predicted phosphatidylinositol-4, 5-bisphosphate (PI(4,5)P2) binding sites (Zárský *et al.*, 2009). The Arabidopsis EXO70E2 isoform was implicated in such a process using animal cells as a model (Ding *et al.*, 2014*b*). Assessment of anthocyanin synthesis and content dynamics, as well as the analysis of tonoplast dynamics and morphology under AIC in different *exo70* and autophagy mutant plants, should provide an insight into the possible molecular mechanisms of this trafficking pathway in the future. As we discussed earlier, it is very likely that other secondary metabolites and even phytohormones, such as abscisic acid (ABA) and salicylic acid (SA), might also be cargo in a similar pathway (Kulich and Žárský, 2014). As plant cell walls are a known sink for secondary metabolites, including anthocyanins (Bautista-Ortín *et al.*, 2016), it is quite possible that this specific form of autophagy-related containers might be targeted to the cell wall/apoplast in an exosome-like secretion pathway as well (Kulich and Žárský, 2014).

Exocyst and autophagy in defence

Plants respond to challenges imposed by biotic factors/stressors in a complex way that may be generally classified into two types of defence: pathogen-associated molecular patterns (PAMPs) triggered immunity (PTI), which is usually triggered by recognition of structural components of a pathogen on the surface of the host cell, and effector-triggered immunity (ETI), which is triggered by the direct or indirect interaction between a plant specific disease resistance R protein and a corresponding avirulence (Avr) protein of the pathogen (Jones and Dangl, 2006).

The contribution of autophagy to the defence capacity of plants has been studied over the last decade. However, it has been found that the autophagy role in plant immunity against avirulent *Pseudomonas syringae* is ambiguous. Depending on the age of leaves and duration of infection, autophagy either helps to restrict the hypersensitive (HR) cell death, as a part of ETI, in infected tissue and thus has a pro-life function (Liu *et al.*, 2005; Patel and Dinesh-Kumar, 2008), or it has the opposite function, a pro-death function, as it has been reported that HR cell death triggered by R proteins RPS4, RPP1, and RPM1 was significantly suppressed in *atg* mutants (Hofius *et al.*, 2009; Table 1). In addition, Yoshimoto *et al.* (2009) found no deviations in RPM1-triggered cell death beyond the initial infection site in younger *atg* mutants. However, in older *atg* mutants such as *atg5*, they observed lesions in non-infected tissues 6–9 d after the infection; this effect could be suppressed by downregulation of SA or by LOF mutation in the SA signalling hub non-expressor of PR genes (NPR1). In agreement with the reported SA-dependence of the actual phenotypes of plants with non-functional autophagy, Yoshimoto *et al.* (2009) proposed that autophagy suppresses the SA- and ROS-signalling positive feedback amplification loop that leads to cell death, while in the resistance to necrotrophic pathogens, it promotes jasmonic acid signalling.

It has been recently shown that three isoforms of wheat ATG6 play a role in powdery mildew immunity. All three isoforms were upregulated by infection with *Blumeria graminis* f. sp. *tritici* and knockdowns of atg6 isoforms compromised the resistance of resistant lines and significantly enhanced basal resistance of susceptible plants (Yue *et al.*, 2015).

Autophagy has been found to be a target for pathogen effectors, which hijack the autophagic machinery to improve the success of infection. PexRD54, an effector from the Irish potato famine pathogen *Phytophthora infestans*, binds host autophagy protein ATG8CL to stimulate autophagosome formation; PexRD54 out-competes the host autophagy cargo receptor Joka2 out of ATG8CL complexes to counteract host defences (Dagdas *et al.*, 2016). The case of the Arabidopsis necrotrophic fungal pathogen *Sclerotinia sclerotiorum* is also very interesting; the wild-type form of this pathogen hijacks cell death pathways of the host through oxalic acid (OA), thus causing extended apoptosis. However, an OA-deficient mutant triggers a restricted cell death phenotype in the host, which exhibits markers associated with plant hypersensitive responses, including callose deposition and a pronounced oxidative burst (Kabbage *et al.*, 2013). However, when an OA-deficient mutant was used to infect *atg7* and *atg8* mutants, the pathogenicity of this interaction was restored, indicating that *S. sclerotiorum* employs OA to suppress autophagy and to put host cell death machinery under its own control (Kabbage *et al.*, 2013).

Recently, autophagy was found to be employed by plants in antiviral immunity. The autophagy cargo receptor neighbour of BRCA1 (NBR1) specifically targets nonassembled and virus particle-forming capsid proteins of the cauliflower

mosaic virus to mediate their autophagy-dependent degradation (Hafrén *et al.*, 2017). Accordingly, it has been reported that autophagy also targets geminivirus-virulent factors for degradation (Haxim *et al.*, 2017).

It has been shown that the exocyst complex plays a role in response to biotic stresses, mainly on the level of the cell wall papillae formation, a defensive cell wall thickening, in a non-host interaction with *Blumeria graminis* f. sp. *hordei*, and in a general defence response against pathogenic *P. syringae* and *Hyaloperonospora arabidopsidis* (Pecenková *et al.*, 2011; Stegmann *et al.*, 2012; Stegmann *et al.*, 2013; Zhao *et al.*, 2015). In Arabidopsis, the defence role so far has been demonstrated only for isoforms of the EXO70 exocyst subunit, namely EXO70B1, B2, and H1 (Table 1). The knockout mutations in the *EXO70B2* and *EXO70H1* genes have been found to have an attenuating effect on plant defence capability, which is in agreement with the fact that these genes are highly upregulated upon the elicitor treatment or pathogen inoculation (Hruz *et al.*, 2008; Pecenková *et al.*, 2011; Stegmann *et al.*, 2012). However, the situation with the mutant in the autophagy-related isoform EXO70B1, the isoform most closely related to EXO70B2, has been found to be more complicated (Kulich *et al.*, 2013; Stegmann *et al.*, 2013; Zhao *et al.*, 2015). This mutant develops spontaneous hypersensitive-like leaf lesions, a phenomenon often associated with *atg* mutants in plants, and displays partial loss of anthocyanin vacuole accumulation (Kulich *et al.*, 2013). Analysis of EXO70B1 localization, colocalization with an ATG8 marker, and dynamics together clearly implicated its participation in the autophagy process (Kulich *et al.*, 2013). In the screen for suppressor mutations of spontaneous hypersensitive-like leaf lesion formation, nine alleles of R-related protein TIR-NBS2 (TN2) were identified, suggesting that LOF of EXO70B1 leads to the activation of this R protein (Zhao *et al.*, 2015). Accordingly, the *exo70B1* mutant has been found to be more resistant to AvrRpt2 expressing *P. syringae* and to *H. arabidopsidis* (Stegmann *et al.*, 2013; Zhao *et al.*, 2015). It has also been shown that TN2 directly interacts with EXO70B1; however, it remains unresolved as to whether TN2 directly monitors EXO70B1 integrity, as proposed by Zhao *et al.* (2015), or whether EXO70B1 is required for autophagic transport to the vacuole and subsequent degradation of TN2, thus operating in the possible autophagy-related negative regulation of immunity, as proposed in Pecenková *et al.* (2016). This is consistent with the fact that many gain-of-function mutants of R genes and LOF of their regulators often demonstrate upregulated defence responses. It means that their phenotypes are a consequence of an ectopic activation of R genes or rather a failure of constitutive and multilevel negative control of R proteins that might also involve their targeting to the autophagy pathway (Pecenková *et al.*, 2016). In addition, it has recently been shown that the interaction between EXO70B1 and ETI component effector receptor TN2 also encompasses calcium-dependent protein kinase 5 (CPK5; Liu *et al.*, 2017).

Still, one has to be cautious when interpreting EXO70B1 defence-associated phenotypes. Stegmann *et al.* used spray inoculation to assess the growth of bacteria in the *exo70B1* mutant (Stegmann *et al.*, 2013), discovering that they are more susceptible to this pathogen, which may be the consequence of stomatal behaviour in conditions of cultivation and inoculation methods used in the study, for example prolonged high humidity. They thus also assayed stomata function in this mutant. A role for EXO70B1 in stomatal dynamics has been

Table 1. *Autophagy- and exocyst-related genes involved in plant defence*

Gene	Host plant	Microorganism	Defence role	References
ATG5	*A. thaliana*	*P. s. t. DC3000 AvrRpm1*	Pro-death	Hofius *et al.*, 2009
ATG6	*A. thaliana*	*P. s. t. DC3000 AvrRpm1*	Pro-life	Patel and Dinesh-Kumar, 2008
	T. aestivum	*B. graminis*	Resistance	Yue *et al.*, 2015
ATG7	*A. thaliana*	*P. s. t. DC3000 AvrRpm1*	Pro-death	Hofius *et al.*, 2009
		S. sclerotiorum	Cell death restriction	Kabbage *et al.*, 2013
ATG8	*N. benthamiana*	*P. infestans*	Resistance	Dagdas *et al.*, 2016
	A. thaliana	*S. sclerotiorum*	Cell death restriction	Kabbage *et al.*, 2013
ATG9	*A. thaliana*	*P. s. t. DC3000 AvrRpm1*	Pro-death	Hofius *et al.*, 2009
NBR1	*A. thaliana*	*Pararetrovirus*	Anti-viral	Hafrén *et al.*, 2017
EXO70B1	*A. thaliana*	*P. s. DC3000*	Resistance	Stegmann *et al.*, 2013
		H. arabidopsidis	Susceptibility/defence supression	Stegmann *et al.*, 2013
		P. s. t. DC3000 AvrRpt2	Susceptibility/defence supression	Zhao *et al.*, 2015
EXO70B2	*A. thaliana*	*P. s. pv. maculicola*	Resistance	Pecenková *et al.*, 2011
		B. graminis	Papilla formation	Pecenková *et al.*, 2011
		P. s. t. DC3000	Resistance	Stegmann *et al.*, 2012
		H. arabidopsidis	Resistance	Stegmann *et al.*, 2012
EXO70H1	*A. thaliana*	*P. s. pv. maculicola*	Resistance	Pecenková *et al.*, 2011
EXO70F1	*H. vulgare*	*B. graminis*	Penetration resistance	Ostertag *et al.*, 2013
EXO70F2	*O. sativa*	*M. oryzae*	Supposed guardee	Fujisaki *et al.*, 2015
EXO70F3	*O. sativa*	*M. oryzae*	Supposed guardee	Fujisaki *et al.*, 2015
SEC5	*N. benthamiana*	*P. infestans*	Resistance	Du *et al.*, 2015
EXO70I	*M. truncatula*	*G. versiforme*	Arbuscule development	Zhang *et al.*, 2015
EXO70J	*G. max*	*Sinorhizobium sp.*	Nodule development	Wang *et al.*, 2016

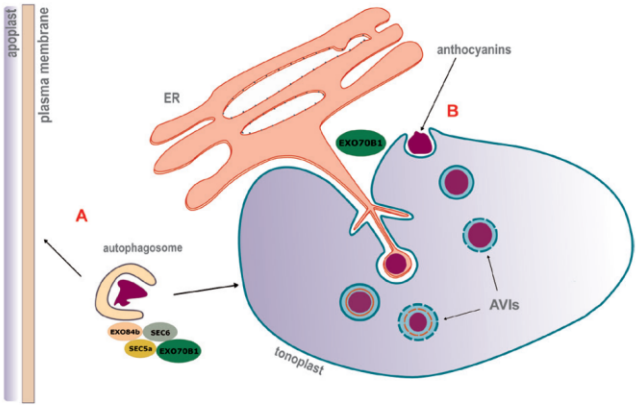

Fig. 1. Two models of exocyst involvement in anthocyanin transport to the vacuole. (A) Exocyst in macroautophagy-like anthocyanin transport to the vacuole. EXO70B1 contributes to early stages of macroautophagy through a direct interaction with ATG8 and later hypothetically provides tethering, together with at least with SEC5, EXO84b, and SEC6, of autophagosome-like vesicles to the tonoplast. (B) Exocyst in microautophagy-like anthocyanin transport to the vacuole. Anthocyanin aggregates, which are in close proximity with the cytoplasmic surface of the vacuole, are tightly pressed against the tonoplast and engulfed. This is followed by membrane secession and formation of AVIs. Anthocyanins can be hypothetically transported to the vacuole by means of ER-derived tonoplast invaginating tubes that could result in AVI formation after pinching off. In both cases, the involvement of exocyst complex-independent EXO70B1 in modulation of membrane curvatures is possible.

recently reported; EXO70B1 plays a role in the negative regulation of light-induced stomata opening and ABA-induced stomata closures, as a subject of inhibition and negative regulation, respectively (Hong *et al.*, 2016; Seo *et al.*, 2016).

Thus, by employing different EXO70 isoforms, the exocyst might be an important component of early stages of plant defence response, which include mainly pathogen perception, signalling and defensive cell wall fortification (EXO70B2), as well as later stages that may involve both PTI and ETI (EXO70B1; reviewed in Pecenková *et al.*, 2016). The suggested 'division of labour' between EXO70B1 – note some plant species have only one EXO70B isoform - and B2 Arabidopsis paralogs has been recently supported by the fact that EXO70B1 in contrast to EXO70B2 interacts with RIN4, a known component and possible cross-point of PTI and ETI in/activation and a target of pathogen effectors (Afzal *et al.*, 2011). EXO70B1 is also recruited by RIN4 to the plasma membrane, unlike EXO70B2 (Sabol *et al.*, 2017). At this moment, it is not clear whether the two isoforms can operate in the same compartment, namely the autophagy-vacuole interface, as found for EXO70B1, or the multivesicular body (MVB)/exosome, as suggested for EXO70B2. It is known that in animal cells and tissues, autophagosomes and MVBs contribute to each others function via membrane fusion between them (González *et al.*, 2017; Shin *et al.*, 2017) and to the formation of exosomes (Hessvik *et al.*, 2016). MVBs, which have been proposed to fuse with PM at the pathogen attack site to participate in the defence (An *et al.*, 2006), are speculated to form with contribution from the autophagy pathway (Katsiarimpa *et al.*, 2013; Kulich *et al.*, 2013; Fig. 2).

Plant exocyst immunity function is not restricted to EXO70B1, EXO70B2, and EXO70H1. It has been shown before that when the EXO70F1-like exocyst subunit of barley was transiently silenced, the efficiency of *B. graminis* penetration was enhanced (Ostertag *et al.*, 2013). In addition, the rice EXO70 isoforms EXO70F2 and EXO70F3 have been detected in a protein complex isolated in a co-immunoprecipitation experiment where rice pathogen *Magnaporthe oryzae* AVR-Pii was used as bait (Fujisaki *et al.*, 2015). It has been suggested that the cytosolic rice counterpart of AVR-Pii guards EXO70F3 and responds when pathogen effectors disturb this interaction (Fujisaki *et al.*, 2015).

The only core exocyst subunit currently directly implied in the plant-pathogen recognition is the SEC5 subunit in *Solanaceae*. *Phytophthora infestans* effector AVR1 directly binds to *Solanaceae* SEC5, interferes with vesicle trafficking, and suppresses PR1 secretion, defensive callose depositions biogenesis, and SEC5-dependent cell death (Du *et al.*, 2015).

The exocyst also functions in the establishment of a beneficial relationship with microbes. In *Medicago truncatula*, multiple exocytotic markers, including the exocyst, have been found to accumulate at the sites of perifungal membrane biogenesis in arbuscular mycorrhizas (Genre *et al.*, 2012). In addition, *exo70I* mutants of *M. truncatula* are unable to support normal arbuscule development and, accordingly, during arbuscule branching, EXO70I is located in spatially restricted zones adjacent to the periarbuscular membrane formation (Zhang *et al.*, 2015). In addition, EXO70s were found to be important for symbiosis establishment with nitrogen fixing soil bacteria (Wang *et al.*, 2016). Leguminous plants are the only ones to contain an EXO70J clade with the characteristic transmembrane domain (Chi *et al.*, 2015). Three isoforms of soybean EXO70Js have been found to be involved in nodule formation (Wang *et al.*, 2016). Interestingly, the identity of such symbiotic membranes is different from regular PM, even if they are continuous, and it is speculated that, as in the case of the cell

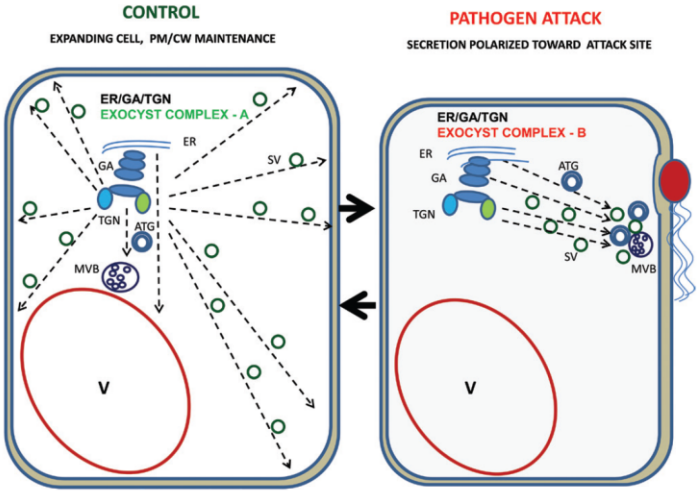

Fig. 2. Model for the function of different exocyst complexes in secretion. By employing different EXO70 isoforms, the exocyst ensures secretory requirements under normal conditions, such as cell growth and/or plasma membrane or cell wall maintenance, and transport into the vacuole (complex 'A'), and in host-microbe interactions (complex 'B'). During biotic interactions of the host plant and biotrophic or hemibiotrophic pathogens, namely fungi and bacteria or endosymbionts, the exocyst contributes to efficient immunity by redirecting secretion to the site of pathogen attack and/or by specialized pathogen infection-induced unconventional secretion of cargo originating from autophagy and MVB compartments. CW, cell wall; ER, endoplasmatic reticulum; GA, Golgi apparatus; PM, plasma membrane; TGN, trans-Golgi network; SV, secretory vesicles; MBV, multivesicular bodies; ATG, autophagosome).

plate, the whole secretory pathway is redirected to the site of perifungal membrane biogenesis, including endocytosis/late endosome (LE)/prevacuolar compartments (Harrison and Ivanov 2017; Fig. 2).

To conclude, by employing different EXO70 isoforms, the plant exocyst ensures the specialized secretory requirements in host-microbe interactions. In cases of interactions of the host plant and biotrophic or hemibiotrophic pathogens studied so far, the exocyst contributes to the efficiency of immunity, either via constitutively expressed exocyst versions related mostly to cell expansion, fo example the exocyst complex with EXO70A1 subunit, or exocyst versions that are specifically equipped by upregulated EXO70s after pathogen infection (Pecenková *et al.*, 2011; Zárský *et al.*, 2013). The LOF mutant of the highly expressed (as noted on Genevestigator) and also autophagy-related isoform EXO70B1, without additional environmental stresses, is more resistant to biotrophic and hemibiotrophic pathogens in line with other autophagy-related mutants that have impaired regulation of SA related immunity. We speculate that the closely related and pathogen-induced isoform EXO70B2 might be involved in the specific autophagy-related production of exosomes for the apoplast-defence-associated reinforcements in papillae (Fig. 2). The capacity of exocyst mutants to fight necrotrophic pathogens remains so far unexplored but might be especially important for better understanding of the EXO70B1-related exocyst role in autophagy and immunity.

Conclusions: possible cross-regulation of exocytosis versus autophagy and unconventional protein secretion via the exocyst as coordinator

Unconventional protein secretion related to autophagy-derived membrane containers, including autophagosomes and MVB/LE, has also come to light in plant cell biology (Ding *et al.*, 2014a; Robinson *et al.*, 2016). Here, we hypothesize that the Arabidopsis exocyst complex versions with either EXO70B1 or EXO70B2 or EXO70E2 subunits might be involved in unconventional secretion via their putative function in different forms of plant cell autophagy, which might eventually result in autophagosomes and MVB/LE, namely exosomes and secretion that probably significantly contribute to both plant defence against pathogens and the establishment of biotic interactions. However, importantly for plants, such autophagy-related pathways may also function in secondary metabolite transport.

Recently, Farhan *et al.* (2017) proposed an analogy between the secretory pathway as a biosynthetic branch and autophagy as a degradative branch of the same cellular endomembrane system, contributing in a dynamic way to overall endomembrane homeostasis. Moreover, they stress that intimate links between these two branches involve certain regulators shared by both pathways. Recent discoveries of the functional input of versions of the exocyst complex not only in conventional exocytosis regulation, including the possibility of regulating an early secretory pathway at the ER via SEC10-translocon interaction (Choi *et al.*, 2012), but also in autophagy allow

one to consider an interesting option: to see the exocyst as a coordinator of endomembrane dynamics under different cell growth/developmental conditions. In animals, the balance between exocytosis- versus autophagy-related forms of the exocyst complex seems to be regulated mostly by specific phosphorylation and RAL GTPase interactions regulated by the overall metabolic status or defence activation, while in land plants, exchange of the EXO70 isoforms might be a dominant regulatory switch under similar conditions but also in secondary metabolite/phytohormones transport - some plant EXO70s are known targets of specific E3 ligase ubiquitination and degradation (Zárský *et al.*, 2013). As all forms of exocyst complexes/subcomplexes compete for at least some common core subunits, the exocyst may be implicated as a stoichiometric coordinator of endomembrane dynamics; subunits involved in autophagy will not be available for exocytosis and vice versa, potentially contributing to the fine-tuning of some endomembrane transport processes depending on the cell environment conditions (Zárský *et al.*, 2013; Luini *et al.*, 2014; Farhan *et al.*, 2017).

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Discussion

The role of the exocyst complex in plant-pathogen interaction has been well documented by several of the recent studies. Despite the emerging progress, however, the mechanisms of exocyst-mediated secretion in plant defense remain largely unclear. Particularly, the nature of transported cargo(es), interaction between the exocyst and SNARE machinery during tethering and contribution of different EXO70 paralogs to defense-related secretion have not been sufficiently explored. Furthermore, it stays unresolved whether the EXO70B1 subunit's function in autophagic trafficking contributes to its immunity-associated phenotypes.

To address these questions, I set out to investigate the relationship between the exocyst and RIN4 protein (Aim #1), in collaboration the role of the EXO70H4 subunit in trichome papillary callose deposition (Aim #2) and, with significant contribution from my colleagues, to devise a hypothesis that would explain the role of EXO70B1 in autophagy and TN2-regulated immunity (Aim #3).

Even though data indicating at an interaction between the EXO70B1 and RIN4 were previously available, a direct evidence that would scrutinize their relationship thoroughly had been lacking (Da Cunha, 2009). In the included research paper, I provided for the first time a direct evidence not only for the interaction between the two proteins, but also for cleavage of RIN4 by bacterial protease and subsequent release of both partners from the PM (Da Cunha, 2009; Sabol *et al.*, 2017).

Given the fact that RIN4 interacts with EXO70B1 and recruits it to the PM and that cleavage of RIN4 reduces callose deposition, one might speculate that EXO70B1 is involved in RIN4-mediated PMR4 callose synthase delivery to the PM and subsequent callose secretion in response to *P. syringae* infection (Da Cunha, 2009; Sabol et al., 2017). This notion is further supported by the role of EXO70B1 in plant defense. Despite some ambiguity of the published data (compare Stegmann *et al.*, 2013; Zhao *et al.*, 2015), the *exo70B1* mutant has been shown by one study to be more resistant to the AvrRpt2-expressing *Pseudomonas* (Zhao et al., 2016). However, this might be explained by elevated levels of salicylic acid (SA) in this mutant (Kulich et al., 2013), which, along with the activation of RPS2 resistance protein, might trigger a strong hypersensitive response. The other report (Stegmann et al., 2013) has indicated that *exo70B1* mutant is more sensitive to the virulent *P. syringae* DC3000 infection. Therefore, EXO70B1 probably acts as a positive regulator of *P. syringae*-related defense. Still, one has to be cautious when interpreting the EXO70B1 defense-associated phenotypes. Stegmann *et al*. used spray inoculation to assess the growth of bacteria in *exo70B1* mutant (Stegmann et al., 2013). They thus assayed stomata function in this mutant. Since *exo70B1* has elevated levels of SA, the effect on bacterial growth may have been an indirect consequence of an influence of SA on stomata. It is also possible that the stomatal function in *exo70B1* mutant might be affected on the level of endomembrane dynamics of guard or subsidiary cells.

Interestingly, RIN4's transcription is upregulated by salicylic acid (SA) and downregulated by methyl-jasmonate and inoculation with the necrotrophic pathogen *Alternaria brassicicola*. Furthermore, its expression is enhanced by application of Flg22 and EF-Tu elicitors. Consistently, RIN4 is coexpressed with SA signaling component NPR1 and, most importantly, with vesicle secretion pathway syntaxins SNAP33 and SYP121 as well as exocyst subunit EXO70B2 (Hruz *et al.*, 2008). EXO70B2 was not found to be recruited to the PM by RIN4 (Sabol *et al*., 2017), however, our unpublished data indicate that it is involved in papilla development in response to a fungal pathogen. During this defense, callose is deposited to the papilla. RIN4 is also upregulated in response to fungal pathogens and *P. syringae* bacteria (Hruz *et al.*, 2008). It is therefore possible that RIN4 might co-operate with both EXO70B1 and EXO70B2 exocyst subunits, probably in different defense contexts.

RIN4 might function as an adaptor for EXO70B1-mediated polarized secretion. Yet, my data show that EXO70B1 is not mislocalized in *rin4 rps2* mutant. This is probably due to the fact that EXO70B1 interacts with NOI6 protein as well. Besides, according to my Y2H results, the NOI6 also interacts with EXO70A1 subunit (Sabol *et al.*, 2017). EXO70A1 is not known to have any defense-related functions and is rather implicated in housekeeping (polarized) secretion (Synek *et al.*, 2006; Chong *et al.*, 2010; Pecenková *et al.*, 2017; Du *et al.*, 2018). Different EXO70s have been suggested to bind to the core exocyst complex, giving rise to distinct holocomplexes. This, through the interaction with different adaptors, might lead to differential targeting of exocyst complexes (Zárský *et al.*, 2013). For instance, VETH-COG complex recruits EXO70A1 subunit to cortical microtubules (Oda et al., 2015; Vukašinović *et al.*, 2017). Also, some EXO70 isoforms may have adopted functions outside the canonical complex. Unfortunately, the expansion of EXO70 and NOI Arabidopsis protein families further complicates their research. Besides adaptors, other factors also determine EXO70B1's localization pattern. Among them, binding lipid partners seem to have a prominent role (see the Introduction).

The suggested importance of EXO70-RIN4 interaction has been further supported by a recent preprint paper revealing the presence of an integrated NOI domain in the rice Pii-2 R protein. *Magnaporthe oryzae* AVR-Pii effector binds to the rice EXO70F3 and thus possibly promotes fungal virulence (Fujisaki *et al.*, 2015). At the same time, EXO70F3 interacts with NOI motif of the Pii-2 receptor. Mutation of either the EXO70F3 or the core NOI motif in the Pii-2 domain blocks AVR-Pii effector-initiated ETI. In this way, Pii-2 receptor protein might monitor EXO70F3 integrity and modification by fungal AVR-Pii effector and trigger ETI (Fujisaki *et al.*, 2017).

EXO70B1 is also involved in autophagosome formation or delivery to the vacuole (Kulich et al., 2013). Multivesicular bodies, which have been described to fuse with PM at the pathogen attack site as a means of defense (An *et al.*, 2006), are speculated to originate with contribution from the autophagy pathway (Katsiarimpa *et al.*, 2013; Kulich et al., 2013). Although autophagy-deficient *atg5* mutant does not have altered PTI responses including Flg22-induced callose deposition, it accumulates SA in higher levels than the WT (Lenz *et al.*, 2011), which might account for the sustained callose deposition of this mutant. Indeed, a

pretreatment of SA increased Flg22-dependent callose deposition in WT but not *npr1* SA signaling mutant, indicating that SA primes callose deposition through NPR1 (Yi *et al.*, 2014). Moreover, the requirement of the autophagy pathway for the formation of callose plugs may not be absolute and there might be other pathway activated in *atg5* SA-accumulating mutant. To resolve this, the detection of callose secretion in *atg5 sid2* (SA synthesis mutant) or *atg5 NahG* and *atg9* mutant in response to *Pto ΔhrcC* infection would be necessary.

In addition, the function of the EXO70B1 subunit in PTI has been proposed to be manipulated by as yet-unexplored pathogen effector(s). This has been suggested to lead to the activation of the TN2 R protein, with which EXO70B1 physically interacts. Authors of the study also explain the lesion formation phenotype of *exo70B1* mutant by an ectopic activation of TN2 protein (Zhao *et al.*, 2015).

A hypothesis developed by my colleague Tamara Pečenková and published in an opinion co-authored paper provides an alternative explanation to the process. In this scenario, the R proteins are under constitutive negative regulation exerted also by autophagy pathway. This would provide a novel, additional layer of control. Also, according to this model, R proteins would be released from this negative regulation upon binding the cognate Avr effector. Therefore, the rescue of the *exo70B1* mutant by a mutation in TN2 R protein or its downstream signaling component CPK5 might be explained by impaired autophagic trafficking of TN2 to the vacuole for degradation in the *exo70B1* mutant (Pečenková *et al.*, 2016; Liu *et al.*, 2017).

Though conceived by my colleagues and with little contribution from my experiments, the EXO70H4 paper addressing the role of this subunit in the trichome cell wall maturation highlights an important point in this discussion. Taking part in the secondary cell wall maturation, the EXO70H4 shares some features with components of the secretory pathway involved in defense response. Particularly, we were able to show that EXO70H4 transports PMR4 callose synthase to the PM for subsequent callose deposition. Also, my experiment confirmed the upregulation of EXO70H4 expression by chitin and Flg22 elicitors. Furthermore, our data demonstrate the EXO70H4 signal appearance in epidermal pavement cells upon Flg22 treatment, with specific enrichment in membrane microdomains (Kulich *et al.*, 2018). One can speculate that these microdomains with enhanced cell wall (CW) autofluorescence represent sites for future callose deposition, a characteristic response to Flg22 and bacteria. In the same way, the chitin upregulation of this isoform might reflect its potential engagement in defensive papilla formation.

Similarly, a non-branched 1,3-β-D-$(Glc)_6$ glucan extracted from the fungal cell wall and chemically similar to (and upon digestion of plant cell wall components also possibly derived from) callose, was recently shown to significantly upregulate EXO70H4 as well as some other Arabidopsis EXO70 isoforms. Interestingly, this upregulation was dependent on CERK1 immunity receptor and was lost in *cerk1-2* mutant (Mélida *et al.*, 2018). CERK1 was also reported to directly bind the chitin oligosaccharide, the major component of fungal cell wall

(Liu *et al.*, 2012). Consistently, according to the publicly available microarray data, EXO70H4 is known to be significantly upregulated by chitin (Hruz *et al.*, 2008). These results further corroborate the idea of EXO70H4 being upregulated by components released from damaged plant cell wall recognized as DAMPs and by fungal cell wall PAMPs and subsequently engaged in PTI response presumably involving localized callose deposition. Interestingly, the PMR4 callose synthase interacts with and is directly regulated by RabA4c GTPase, highlighting the importance of secretory pathway regulators in polarized secretion during defense (Ellinger *et al.*, 2014). Our unpublished data also indicate that Arabidopsis SEC15 interacts with some Rab GTPases, reinforcing the notion that exocyst participates in polarized callose deposition (Martina Růžičková, personal communication). Further experiments are, however, needed to test these hypotheses.

Importantly, silencing of all the core *N. benthamiana* exocyst subunits and some of the EXO70 isoforms, including EXO70H and EXO70B families, reduces callose deposition in response to *Pto ΔhrcC*. Only some of these lines, however, show impaired resistance towards *P. syringae* pv *syringae* isolate B728a (Du *et al.*, 2018; see the Introduction). While growth of the virulent *Pto* DC3000 is independent on the PMR4 function, growth of the type III secretion system-deficient strain *Pto ΔhrcC* unable to deliver effectors into host plant cell is enhanced in *pmr4* mutant (Kim *et al.*, 2005; Clay *et al.*, 2009). *Pseudomonas syringae* bacteria therefore manipulate callose deposition to support their own growth (see also the Introduction). This is also consistent with a study showing diminished callose deposition in response to *Pto* DC3000 as compared with *Pto ΔhrcC* mutant. Interestingly, AvrPto effector of the *Pto* DC3000 effector repertoire is at the same time able to suppress the expression of secreted defense cargo (Hauck et al., 2003). Why only some of the silenced exocyst subunits with diminished callose deposition manifest also decreased resistance towards *P. syringae* pv *syringae* B728a and whether an alternative immune response pathway is activated in the others remains to be investigated. Also, different EXO70s may contribute to callose secretion through distinct mechanisms. While EXO70B2 might participate in PM delivery of PAMP receptors for signaling leading to subsequent callose deposition (Stegmann *et al.*, 2012), one can speculate that EXO70H4 possibly directly transports PMR4 to pathogen infection site and thus contributes to sustained callose deposition.

Besides callose, the PR1 protein is another documented defense cargo whose secretion is dependent on exocyst subunit (Du *et al.*, 2015; see the Introduction). Whether specific EXO70 isoforms take part in this process as well, however, has yet to be resolved. Preliminary results from our laboratory indicate a role for EXO70B2 in PR1 secretion (Tamara Pečenková). A contribution from EXO70B1 protein might be difficult to interpret owed to SA hyperaccumulation and PR1 upregulation in the *exo70B1* mutant (see earlier). Similarly, the rice *Ossec3a* mutant accumulates SA and PR1 protein to higher levels than WT, contributing to its enhanced *M. oryzae* resistance (Ma *et al.*, 2017). Crossing of *exo70B1* and *Ossec3a* mutants with SA-synthesis or signaling mutants should help address the question. Also, whether EXO70B2 and/or EXO70B1 exocyst subunits regulate FYVE-marker-labeled multivesicular bodies- and/or autophagy-pathway derived compartments-mediated secretion of PR1 has yet to be determined (Pečenková *et al.*, 2017). Moreover, not all plant families

have two EXO70B isoforms (Cvrčková *et al.*, 2012). It is therefore possible that EXO70B1 and EXO70B2 paralogs may have partially overlapping functions. Yet, our preliminary data clearly indicate that EXO70B2 cannot functionally complement loss of EXO70B1 (Vedrana Marković and Ivan Kulich, personal communication).

Exocyst subunits other than those cited in this Dissertation might also play a role in microbial pathogen defense-associated secretion. For instance, EXO70E2, a homolog of the rice EXO70E1, is known to interact with RIN4. EXO70E2 is also upregulated by pathogens and elicitors. However, how the contentious EXO70E2-labeled EXPO compartments relate to autophagosomes and whether they mediate secretion of defense-related cargoes remains to be determined (Hruz *et al.*, 2008; Wang *et al.*, 2010). Also, whether distinct EXO70s cooperate during the tethering or whether their role differentiate in space and time awaits further exploration.

Conclusion and Perspectives

Despite the great multiplication and a potential redundancy within the EXO70 Arabidopsis exocyst subfamily, I could show a specific role for EXO70B1 and EXO70H4 subunits. Both appear to have defense-related functions, albeit in seemingly different contexts. Besides, they also perform some *bona fide* isoform-specific tasks. While EXO70B1 participates in presumed RIN4-dependent secretion of defense cargo to the plasma membrane and autophagy-mediated transport to the vacuole, the EXO70H4 subunit is responsible for callose synthase secretion to the PM.

One of the newly discovered aspects of *Pseudomonas*-Arabidopsis interaction presented in this dissertation is the interference with RIN4's adaptor function by AvrRpt2 effector protease, possibly influencing EXO70B1-mediated secretion of antimicrobial cargo. PMR4 callose synthase and PR1 proteins might be good cargo candidates for this pathway.

In conclusion, I was able to accomplish the objectives defined in the Aims. The reported experiments do not defy the presented hypotheses. Yet, many questions remain open as to the role of different EXO70s in plant immunity. Particularly, further experiments are required to elucidate the EXO70H4's function in trichome papillae formation, the conclusions of which might later lead to expansion of knowledge of also defensive papilla formation. Also, putative EXO70B1-mediated vacuolar trafficking of TN2 needs to be tested. Similarly, how the discovered interaction between the EXO70B1 and RIN4 protein regulates polarized secretion in response to microbial plant pathogens and what is the transported defense cargo represent intriguing open questions that require further investigation.

The presented work provides a basis and a framework for future oriented research. Having described the actual or presumed roles of selected plant exocyst subunits in defense against phytopathogens, I hope to elaborate on the topic to expand the knowledge of plant cell polarization.

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