

RESEARCH PAPER

# The role for the exocyst complex subunits Exo70B2 and Exo70H1 in the plant–pathogen interaction

Tamara Pečenková<sup>1,2</sup>, Michal Hála<sup>1</sup>, Ivan Kulich<sup>2</sup>, Daniela Kocourková<sup>1</sup>, Edita Drdová<sup>1</sup>, Matyáš Fendrych<sup>1</sup>, Hana Toupalová<sup>1</sup> and Viktor Žárský<sup>1,2,\*</sup>

<sup>1</sup> Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 263, 16502 Praha 6, Czech Republic

<sup>2</sup> Department of Experimental Plant Biology, Faculty of Sciences, Charles University, Viničná 5, 12844 Praha 2, Czech Republic

\* To whom correspondence should be addressed: E-mail: [zarsky@ueb.cas.cz](mailto:zarsky@ueb.cas.cz)

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## Abstract

Recently, the octameric vesicle-tethering complex exocyst was found in plants and its importance for *Arabidopsis* morphogenesis was demonstrated. Exo70 exocyst subunits in plants, unlike in yeasts and mammals, are represented by a multigene family, comprising 23 members in *Arabidopsis*. For Exo70B2 and Exo70H1 paralogues, transcriptional up-regulation was confirmed on treatment with an elicitor peptide, elf18, derived from the bacterial elongation factor. Their ability to participate in the exocyst complex formation was inferred by the interaction of both the Exo70s with several other exocyst subunits using the yeast two-hybrid system. *Arabidopsis* plants mutated in these two genes were used to analyse their local reaction upon inoculation with *Pseudomonas syringae* pv. *maculicola* and the fungal pathogen *Blumeria graminis* f. sp. *hordei*. The *Pseudomonas* sensitivity test revealed enhanced susceptibility for the two *exo70B2* and one *H1* mutant lines. After *Blumeria* inoculation, an increase in the proportion of abnormal papilla formation, with an unusual wide halo made of vesicle-like structures, was found in *exo70B2* mutants. Intracellular localization of both Exo70 proteins was studied following a GFP fusion assay and *Agrobacterium*-mediated transient expression of the constructs in *Nicotiana benthamiana* leaf epidermis. GFP-Exo70H1 localizes in the vesicle-like structures, while GFP-Exo70B2 is localized mainly in the cytoplasm. It is concluded that both Exo70B2 and Exo70H1 are involved in the response to pathogens, with Exo70B2 having a more important role in cell wall apposition formation related to plant defence.

**Key words:** Elicitor, exocyst, immunity, papilla, pathogen, secretion, vesicles.

## Introduction

Upon pathogen attack the first responses of the plant cell are directed toward the site of the pathogen contact: the cytoskeleton reorganizes and cell wall reinforcements are deposited through the action of the secretory pathway (Schmelzer, 2002; Robatzek, 2007). As shown for the epidermal cells of a resistant potato plant attacked by the fungus *Phytophthora infestans* (the late blight causal agent), changes in cytoplasmic streaming and translocation of the cell nucleus toward the fungal penetration site take place (Schmelzer, 2002). The cell wall defensive appositions called papillae are formed beneath the attempted pathogen penetration sites (Aist, 1976). It was shown that papilla composition varies; however, they commonly contain cal-

lose and phenolic compounds (McLusky *et al.*, 1999). Reactive oxygen species are also involved in papilla formation, where they serve to drive hardening by cross-linking the cell wall components, to intoxicate the pathogen, and to induce defence-related gene expression (Levine *et al.*, 1994; Hüchelhoven *et al.*, 1999). The papilla formation is considered to be an important resistance mechanism that can prevent an unnecessary hypersensitive response, or cell death (Schmelzer, 2002).

For the proper assembly of the papilla, the site-directed synthesis, cytoskeleton-mediated transport, and local secretion to the cell wall have to be highly co-ordinated, meaning that the initiation and maintenance of the new cell polarity

is the key factor for efficient plant defence (Schmelzer, 2002). A crucial role for the secretory pathway in papilla-dependent plant immunity was recently confirmed. Genetic screenings for mutants with increased penetration by *B. graminis*, the powdery mildew causal agent, have identified the *PENETRATION1* (*PEN1* or *SYPI21*) gene which encodes a plasma membrane SNARE protein syntaxin (Sanderfoot *et al.*, 2000; Collins *et al.*, 2003). This increase in mutant sensitivity is the consequence of the role *PEN1* has in the timely assembly of the papillae and focal secretion (Assaad *et al.*, 2004; Kwon *et al.*, 2008).

The overall polarity of eukaryotic cells strongly depends on the ability of the cell to deliver newly synthesized proteins, membrane lipids, and/or cell wall components to discrete sites on the cell surface by localized exocytosis, thus enabling the cell to keep an asymmetry of the plasma membrane composition and to grow or differentiate in certain directions. Polarized exocytosis involves the delivery, docking, and fusion of secretory vesicles with the target plasma membrane and all these phases are regulated by small GTPases, especially from the Rab and Rho families (Novick and Zerial, 1997; review by Ridley, 2006). The tethering of exocytotic vesicles is mediated by a multi-protein complex, the exocyst (Guo *et al.*, 1999). The exocyst was discovered in yeast and has also been well studied in animals as an effector of small Rab and Rho GTPases in the regulation of cellular polarity (reviewed in Hsu *et al.*, 2004). It consists of eight subunits: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p, all of them being mostly hydrophilic proteins which associate via GTPases and membrane lipids, partly with the vesicle and some with the target plasma membrane domain forming the full complex, possibly during the tethering step (TerBush *et al.*, 1996; Guo *et al.*, 1999). After the vesicles are properly tethered to the plasma membrane, the final step of exocytosis mediated by interaction of integral membrane proteins v-SNARE and t-SNARE (SNARE, soluble N-ethylmaleimide-sensitive fusion attachment protein receptors) can be executed, leading to the fusion of the bilayers of the secretory vesicle and the plasma membrane (Rothman, 1994).

Recently, the exocyst was found as a true complex in plants and its importance for *Arabidopsis* morphogenesis was analysed using a combination of biochemical, genetic, and cytological approaches (Hála *et al.*, 2008). The first indirect interaction of a small GTPase with a plant exocyst subunit was identified: *Arabidopsis* Sec3 interacts with plant-specific Rho GTPases (ROPs) via binding to the adaptor protein ICR1 *in vivo* (Lavy *et al.*, 2007). Work with the maize *sec3* mutant (*roothairless1*) and our work with *sec5*, *sec6*, *sec8*, *sec15*, and *exo70A1* *Arabidopsis* mutants, show the failure of mutant root hairs and pollen tubes to elongate properly in the polarized fashion (Cole *et al.* 2005; Wen *et al.*, 2005; Synek *et al.* 2006; Hála *et al.* 2008, Žárský *et al.*, 2009). *Arabidopsis* *exo70A1* and *exo84b* mutants also display other defects in cell division and growth (e.g. in the leaves and hypocotyls) implying that the exocyst is an important participant in plant cytokinesis regulation (Synek

*et al.*, 2006; Fendrych *et al.*, 2010). Interestingly, in contrast to yeast and mammals, which have only one Exo70 gene, plants have a large Exo70 gene family consisting of 23 members in *Arabidopsis*. They can be phylogenetically clustered into three groups and further into nine sub-groups (Eliáš *et al.*, 2003; Synek *et al.*, 2006).

All these studies point to the plant exocyst complex as an important component in the regulation of polarized cell growth; a role that might also strongly influence the capability of plant cells to react to pathogen attack. Therefore, there was a need to determine if the expression of exocyst subunits is responsive to pathogen stimuli and if they might be involved in the non-host cell wall-based defence. The transcriptional responses of several *Arabidopsis* Exo70s after an elicitor treatment, and the defence behaviour of the two selected Exo70 mutants after challenge with two model pathogens, the bacterium *Pseudomonas syringae* pv. *maculicola* and barley powdery mildew *Blumeria graminis* f. sp. *Hordei*, were analysed. Interaction of Exo70B2 and H1 with several other exocyst subunits was also found using the two-hybrid interaction in yeast. Our data show that exocyst subunits Exo70B2 and Exo70H1 (possibly in concert with the exocyst itself) are non-redundantly involved in plant defence against pathogen attacks.

## Materials and methods

### Plant material

The *Arabidopsis thaliana* T-DNA insertional mutant lines that were used in this study were obtained from NASC, namely: SALK\_091877 and SAIL\_339-D07 (At1g07000, Exo70B2) and SALK\_042456 (At3g55150, Exo70H1). The seeds were surface-sterilized and plated onto half-strength Murashige and Skoog medium. For the purpose of semi-quantitative RT-PCR after PAMP (elf18) treatment, *A. thaliana* Col-0 was used. The plants were propagated *in vitro* for 7–10 d, and used for RT-PCR, or were transferred into Jiffy tablets and cultivated in a growth chamber under the same cultivation conditions (21 °C, 16 h light d<sup>-1</sup>, with a light intensity of 70 μmol m<sup>-2</sup> s<sup>-1</sup>). For the purpose of *Pseudomonas* infection, plants were cultivated in Sanyo MLR 351H growth chamber (23 °C, short-day conditions of 10/14 h, 75% humidity, and a light intensity of 50 μmol m<sup>-2</sup> s<sup>-1</sup> emitted by 40 W Sanyo electric lamps).

### Elf18 treatment and RT-PCR

The N terminus of bacterial elongation factor Tu, comprising the first N-acetylated 18 amino acids, Ac-SKEKFERTKPHVNVG-TIG, termed elf18, was synthesized (Vidia Ltd., Czech Republic). The elf18 was used to treat the 12-d-old *A. thaliana* plants, wild-type ecotype Col-0, grown on vertical 0.5 MS plates (21 °C, 16 h light d<sup>-1</sup>). Prior to treatment, plants were transferred into liquid 0.5 MS medium (two flasks with five plants in 10 ml, two replicas of each) and left for 2 d on an orbital shaker, under the same temperature and light conditions. The elf18 peptide in the final 5 μM concentration (diluted in deionized water) was added to two sets of plants, while two other sets were mock treated. Six hours later, the material was harvested.

The RNA was isolated using the RNeasyPlant kit (Qiagen). The RNAs obtained (1 μg) were subjected to DNase treatment (Gibco; according to the manufacturer's instructions) and subsequent reverse transcription using the oligodT primer (Transcriptor High

Fidelity cDNA Synthesis Kit, Roche Applied Science; according to the manufacturer's instructions). PCR was performed with *Taq* DNA polymerase (Fermentas), in 25 cycles. The primers used for RT-PCR analysis were adjusted so that the amplified area was of approximately 500 bp (see Supplementary Table S1 at *JXB* online).

#### Yeast-two hybrid assay

The Exo70B2 and H1 cDNA (obtained from Riken) were cloned into both pGADT7 and pGBKT7 vectors (Clontech Laboratories, Inc.) in the case of Exo70B2, and into pGBKT7 in the case of H1. In addition, SNAP33 cDNA obtained by RT-PCR was cloned into pGADT7. All the other exocyst constructs used have already been described by Hála *et al.* (2008). Yeasts that were transformed with pairs of plasmids were grown on  $-Leu/-Trp$  selective media. Murine p53 (binding domain) and SV40 large T-antigen (activation domain), provided by the manufacturer, were used as a positive control. Different pGBTs with pGAD with an inserted non-coding piece of vector pENTR3C were used as negative controls. Approximately 1 mm<sup>2</sup> of positive colonies from  $-Leu/-Trp$  plates was resuspended in 200  $\mu$ l of sterile water, diluted 20 $\times$  and 400 $\times$ , and subsequently plated onto  $-Leu/-Trp/-His/-Ade$  plate.

#### Inoculation of the plant material with *P. syringae* pv. *maculicola*

For *P. syringae* pv. *maculicola* strain 795 (Davis *et al.*, 1991) inoculation, plants were cultivated in climate chamber for 5 weeks under the short-day regime (10/14 h light/dark, 23 °C, 75% humidity). The mutant *npr1-1*, a gift from Saskia van Wees (Utrecht University), was used as a positive control. Bacteria were prepared as described by Katagiri *et al.* (2002). Briefly, freshly inoculated plates (2–3-d-old; LB with 25 mg l<sup>-1</sup> streptomycin) were used to prepare the liquid overnight culture, without streptomycin, with shaking of 130 rpm on 28 °C. Bacteria were centrifuged at 2500 g for 10 min and pellet resuspended in 10 mM MgCl<sub>2</sub>. According to the OD<sub>600</sub>, the culture was diluted to approximately 3 $\times$ 10<sup>5</sup> cfu ml<sup>-1</sup> (approximate OD<sub>600</sub>=0.0006).

Rosette leaves were inoculated with diluted bacteria using a syringe—four leaves for six plants of each of the lines tested. Three days later, leaf discs of the same size (6 mm in diameter) were cut from all the inoculated leaves, homogenized in 1 ml of 10 mM MgCl<sub>2</sub>, and diluted 1:10 several times. For three subsequent dilutions, 10  $\mu$ l, in duplicates, were plated onto LB with streptomycin. Two days later, the number of colonies was counted.

#### Inoculation of the plant material with *B. graminis* f. sp. *hordei*

The inoculation of mutant lines was performed in collaboration with the laboratory of Ulrich Hildebrandt, University of Wuerzburg. Approximately 4-week-old plants (before the appearance of the first inflorescence) were used for inoculation by *Blumeria graminis* f. sp. *hordei* in a settling tower. Conidia from infected barley leaves were then blown into the tower using pressurized air to ensure their even distribution at 50 conidia cm<sup>-2</sup>. After several minutes the conidia had settled and the plants were moved to a growth chamber that was kept closed under the hothouse to achieve a relative humidity of at least 90% all the time. Two days later, leaves were detached and treated as described in Ringelmann *et al.* (2009). Briefly, to de-stain leaves, they were placed with their inoculated surface up onto Whatman 3MM paper moistened with ethanol:acetic acid (3:1, v/v) in a Petri dish until bleached and then transferred to filter paper moistened with lactoglycerol (lactic acid:glycerol:water, 1:1:1, by vol.) for 3 h. Then, fungal structures were stained for 30 min by carefully pipetting a few droplets of trypan-blue (Merck, Darmstadt, Germany) as 0.05% (w/v) in acetic acid:glycerol:water (1:1:1, by vol.) onto the inoculated leaf surface.

#### Histochemical examination of infected material

The responses to pathogens was monitored and inspected by microscope and documented by photography. The visualization of germinated spores was achieved by trypan-blue staining of fungal structures (see above). Microscopic analysis of papillae was done using Olympus BX51 microscope with an attached DP50 camera (Olympus). The confocal microscopy (Zeiss LSM 5Duo) was used for the thorough and detailed analysis of the papilla structure. The quantification of papillae stages was performed by counting 50–100 germinated spores from one leaf; for each WT and mutant line 4–5 leaves (originating from three different plants) were evaluated. The total number of evaluated spores on one leaf was considered as 100% and the mean was determined for these 4–5 values.

#### Transient expression in *N. benthamiana* of fluorescently tagged chimeric constructs

For both Exo70B2 and Exo70H1, cDNAs from pENTR3C vector were transferred by a recombinant LR reaction of Gateway cloning (Invitrogen) that is catalysed by the enzyme mixture LR Clonase into pGWB6 (Nakagawa *et al.*, 2007) vector, placing the cDNA in-frame with GFP on the N-terminus, under the control of the 35S promoter. This way, the constructs obtained were used for the transformation of *A. tumefaciens*, strain GV3103. Overnight cultures were diluted in infiltration buffer (10 mM MES, 10 mM MgCl<sub>2</sub>) until OD 0.1 and used for infiltration by syringe of the abaxial *N. benthamiana* leaf surface. The similar approach was used for FRET analysis, where, in addition, Exo70B2 and Exo70H1 were cloned into vector pH7WGR2, allowing N-terminal fusion with RFP. More details on performing FRET analysis can be found in Supplementary Fig. S3 at *JXB* online.

#### Confocal microscopy

After approximately 36 h from the time of infiltration, the first observation under the Zeiss LSM Duo confocal microscope, using the 40 $\times$  water-corrected objective, was performed. Excitation of red-shifted GFP was committed with the laser beam of wavelength of 488 nm.

#### Statistical evaluation

All the images were quantitatively and statistically evaluated with the aid of image-processing software. For the measurements and quantification of papillae sizes, software AnalySIS (Soft Imaging System GmbH, Germany) and ImageJ (Wayne Rasband, NIH, USA) were used. The numerical data obtained were processed using Microsoft Excel. To assess the significance of the data obtained, ANOVA analysis available on <http://www.physics.csbsju.edu/stats/> was performed.

## Results

### Up-regulation of selected putative exocyst subunits encoding mRNAs upon pathogen stimuli

Based on our analysis of publicly available expression data, it was concluded that some *Arabidopsis* exocyst subunits expression levels are highly responsive to treatments with different pathogens or elicitor molecules (data from the current version of the Genevestigator database; Zimmermann *et al.*, 2004). Most apparent in this respect is the situation with the *Arabidopsis* Exo70 family of exocyst subunits. Exo70H1 and Exo70B2 mRNAs are strongly

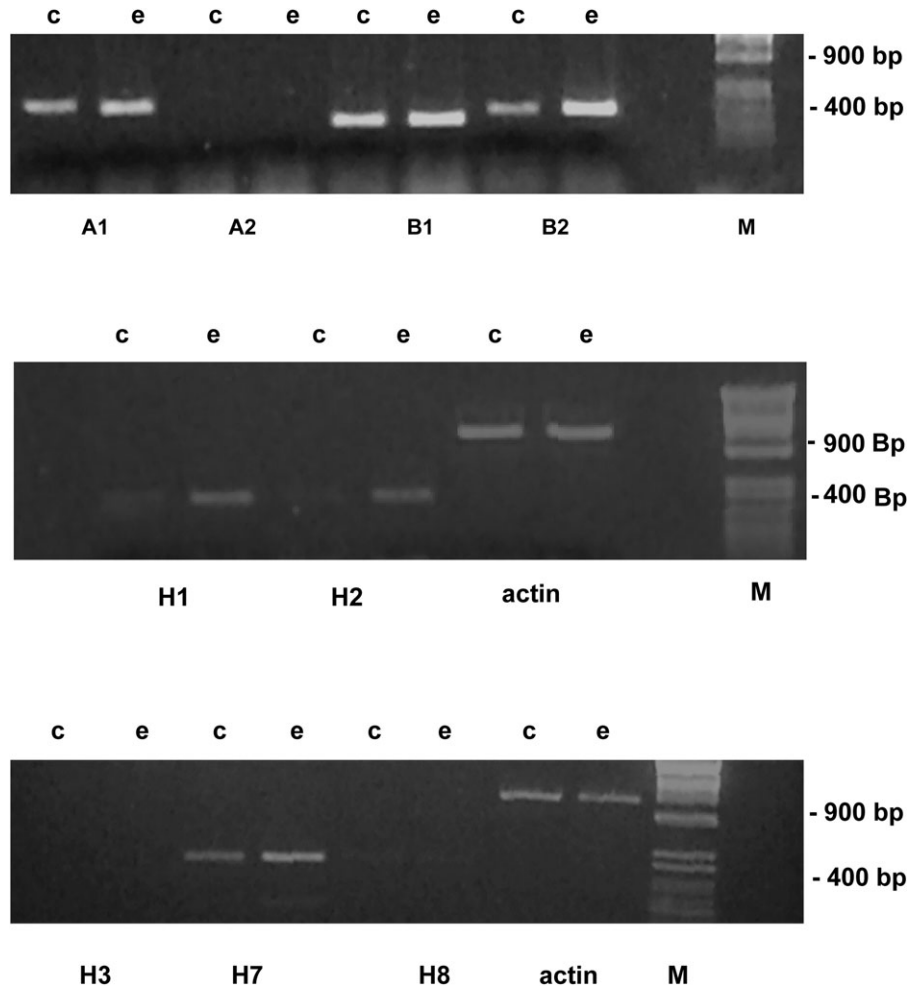
up-regulated upon stimulation with the fungus *B. graminis* f. sp. *hordei*, *Pseudomonas syringae*, and elicitors, e.g. flg22 or elf18 (annotated in the works of Zipfel *et al.*, 2004; Thilmony *et al.*, 2006). The Exo70A1 exocyst subunit encoding mRNA, the one most closely related to yeast and mammalian Exo70s (Synek *et al.*, 2006) is less responsive to pathogens (see Supplementary Fig. S1 at *JXB* online).

Analysis of the transcriptional response of selected Exo70 *Arabidopsis* genes to one of the well-studied elicitors, or pathogen-associated molecular patterns (PAMPs), the N terminal 18 amino acids of bacterial elongation factor Tu, modified by an acetylation on the first residue, termed elf18 (Ac-SKEKFERTKPHVNVGTIG; Kunze *et al.*, 2004) was made. Synthetic elf18 peptide was used to treat 12-d-old *A. thaliana* plants, ecotype Col-0, for 6 h, in the final 5 µM concentration of elicitor. After RNA isolation and reverse transcription, semi-quantitative PCR was performed (see the Materials and methods for details). It could be proved that Exo70B2, H1, H2, and H7 RNAs were strongly up-regulated by the elf18 treatment, while A1 and B1 were less responsive (Fig. 1). For Exo70A2, H3, and H8 no visible

bands were obtained, which is in accordance with their tissue-specific expression.

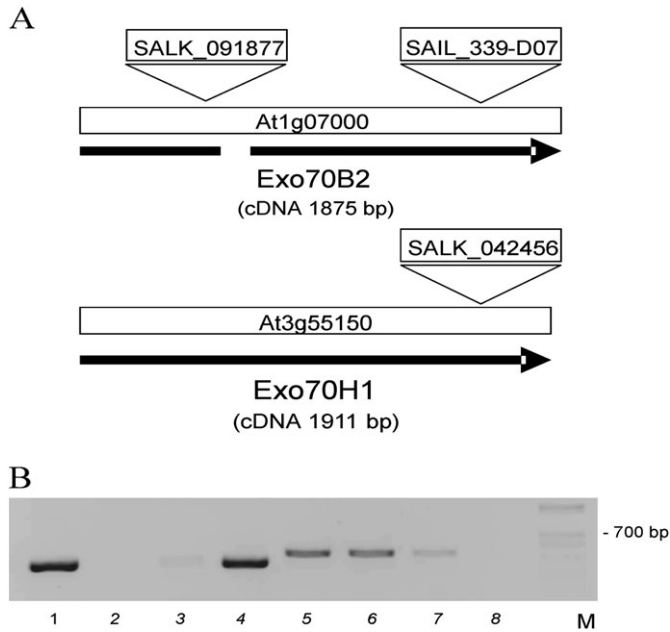
*Characterization of Arabidopsis exo70B2 and exo70H1 T-DNA mutant lines*

Based on the results of RT-PCR it was decided to continue work with Exo70B2 and Exo70H1, the two Exo70s that were highly up-regulated after elf18 treatment. Three *Arabidopsis* T-DNA insertional mutant lines for the genes SALK\_091877 (*exo70B2-1*), SAIL\_339-D07 (*exo70B2-2*), and SALK\_042456 (*exo70H1*) were obtained. The position of the insertions is shown in Fig. 2. The same figure shows the result of RT-PCR performed on 10-d-old seedlings, showing that these lines are knock-out mutants, since no cDNA fragments could be amplified for either of Exo70s. For both mutant lines, no phenotype deviation was observed; their appearance is indistinguishable from WT when cultivated *in vitro* on normal 0.5 strength MS medium, as well as when grown on medium containing 50 mM NaCl or in soil (data not shown). Borders of T-DNA



**Fig. 1.** The RT-PCR analysis of nine different *Arabidopsis* Exo70 mRNAs after treatment with elicitor peptide elf18 (c, control; e, elf18 treatment; M, marker).





**Fig. 2.** The position of T-DNA insertions in three *Arabidopsis* mutant lines. (A) SALK\_091877 (*exo70B2-1*), SAIL\_339-D07 (*exo70B2-2*), and SALK\_042456 (*exo70H1*). The RT-PCR (B) confirms that these mutants are knock-out lines, since no DNA fragments were obtained by RT-PCR (with the exception of a weak band in *exo70B2-1*; lanes 1–4, WT, *exo70B2-2*, *exo70B2-1*, *exo70H1*, respectively, with Exo70B2 primers; lanes 5–8, the same arrangement of cDNAs was used to amplify the Exo70H1 fragment).

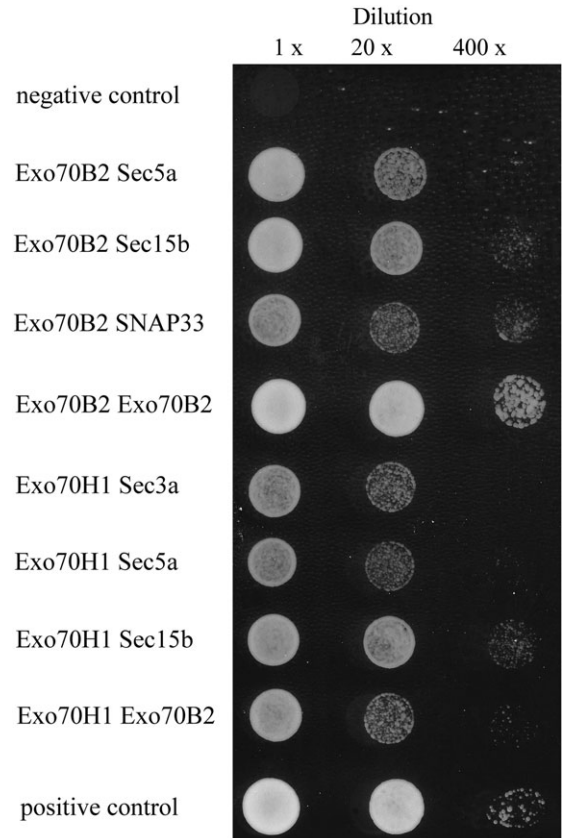
insertion were verified by sequencing genomic DNA of mutants (data not shown).

#### *Exo70B2* and *H1* interact with several other exocyst subunits

To test if the two Exo70s, B2 and H1, are components of the exocyst complex, a two-hybrid system in yeast (Y2H) was used. Constructs already described by Hála *et al.* (2008) and newly prepared Exo70B2 and Exo70H1 were used.

For both Exo70s, no interaction was found either with Sec6 or with Sec8 (see Supplementary Fig. S2 at *JXB* online). However, positive interactions were found for Exo70B2 with Sec5a and Sec15b (Fig. 3). Exo70H1 interacts with Sec3a, Sec5a, and Sec15b. The most surprising was, however, the interaction of Exo70B2 with Exo70H1, and also of Exo70B2 with itself, pointing to the possibility that Exo70s dimerize, a situation that is so far undescribed for Exo70s of any other organism. The observed interactions were further verified by FRET analysis (see Supplementary Fig. S3 at *JXB* online).

To extend the Y2H analysis to a possible, non-exocyst interactor, SNAP33, a known participant of the response to pathogen attack in plants, was included (Kwon *et al.*, 2008). As a SNARE protein enabling membrane fusion, SNAP33 is expected to occur at least in the proximity of the exocyst complex. The Y2H revealed weak interaction of SNAP33 and Exo70B2. No interaction was found for SNAP33 and Exo70H1.



**Fig. 3.** Two hybrid assay in *S. cerevisiae*: interactions of Exo70B2 and Exo70H1 with several other exocyst subunits and SNARE protein AtSNAP33.

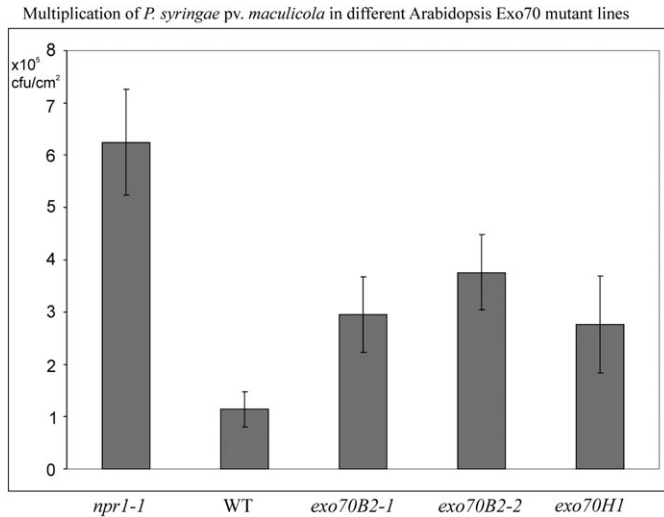
#### The susceptibility of Exo70s mutants to *Pseudomonas* infection

The three Exo70s mutant lines were subjected to *Pseudomonas syringae* pv. *maculicola* susceptibility assay, according to the protocol described by Katagiri *et al.* (2002). Leaves of *exo70B2-1*, *-2*, and *exo70H1* mutant lines, as well as of the wild-type and susceptible mutant *npr1-1* as a positive control (Cao *et al.*, 1994), were infiltrated with bacteria. The difference in the bacterial growth among these lines was monitored by counting the numbers of colonies obtained from homogenized leaf tissue. The enhanced susceptibility was revealed for both alleles of mutant *exo70B2* as well as for *exo70H1* (Fig. 4).

#### Papillae formation in *Arabidopsis* exocyst mutants upon *B. graminis* f. sp. *hordei* inoculation

*Arabidopsis* T-DNA insertion mutants were also used to see if proper papillae formation upon the challenge of *A. thaliana* with pathogen might also be dependent on the function of Exo70B2 or Exo70H1 exocyst subunits.

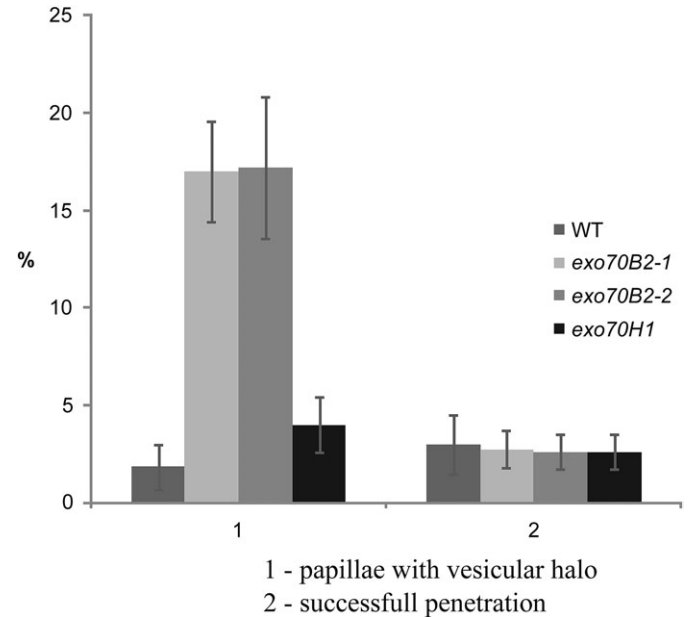
To inoculate wild-type and *exo70B2-1*, *exo70B2-2*, and *exo70H1* mutant plants with *B. graminis*, conidiospores obtained from infected barley were sprayed on to leaves. After 48 h, leaves were detached and stained with trypan blue for the visualization of the germinated spores and



**Fig. 4.** Test of susceptibility to *Pseudomonas* of three exocyst mutant lines: two *exo70B2s* and *exo70H1* compared to WT and sensitive mutant *npr1*. Relative number of *P. syringae* pv. *maculicola* colonies out of inoculated leaves for five different lines are presented as  $\times 10^5$  colony forming units per cm<sup>2</sup> (cfu cm<sup>-2</sup>).

development of appressoria and penetration pegs. Attention was paid to the epidermal cell wall response to these attempts – papillae formation. For each genotype, the distribution of the number of germinated spores having appressoria and a penetration peg, with and without a cell papillae reaction, were compared. However, no significant difference in the total number of papillae initiations was observed between mutants and WT (data not shown). Further on, two more stages of infection could be observed: (i) papillae having a strongly vesicular-like appearance on the intracellular side, and (ii) successful penetration of fungi through the plant cell wall, in the form of an elongated penetration peg, or the presence of haustorium and the growth of secondary hyphae. The remarkable difference in the proportion of papillae with a vesicular-like halo that significantly increased in *exo70B2-1* and *-2* mutants (Fig. 5; see Supplementary Fig. S4 at *JXB* online) could be seen.

Using the autofluorescence of the material deposited in papillae, compared with the bright-field observation, it was possible to focus on the details of their structures under the confocal microscope. It could be seen that papillae are differently formed in mutants with a less organized structure when compared with *Arabidopsis* wild-type papillae (Fig. 6A). The vesiculated structures of *exo70B2-1* and *-2* mutant papillae were even more obvious under the high magnification: the dense halo of vesicle-like structures filled with autofluorescing material surrounds the papillae. Two features of papilla structure were quantified and compared between WT and mutants: their diameters and the diameters of the vesicular halos (papilla including; Fig. 6B). The quantification confirmed that the vesicular halo around papillae is significantly bigger in the case of *exo70B2* mutants.



**Fig. 5.** Vesicular-like papillae distribution and penetration of *B. graminis* f. sp. *hordei* in WT, *exo70B2-1*, *exo70B2-2*, and *exo70H1* mutants. Abnormal papillae with vesicular halo and successful penetration of fungi (either as an elongated penetration peg or the presence of haustorium) are counted. For each genotype, 50–100 spores from 4–5 leaves were inspected.

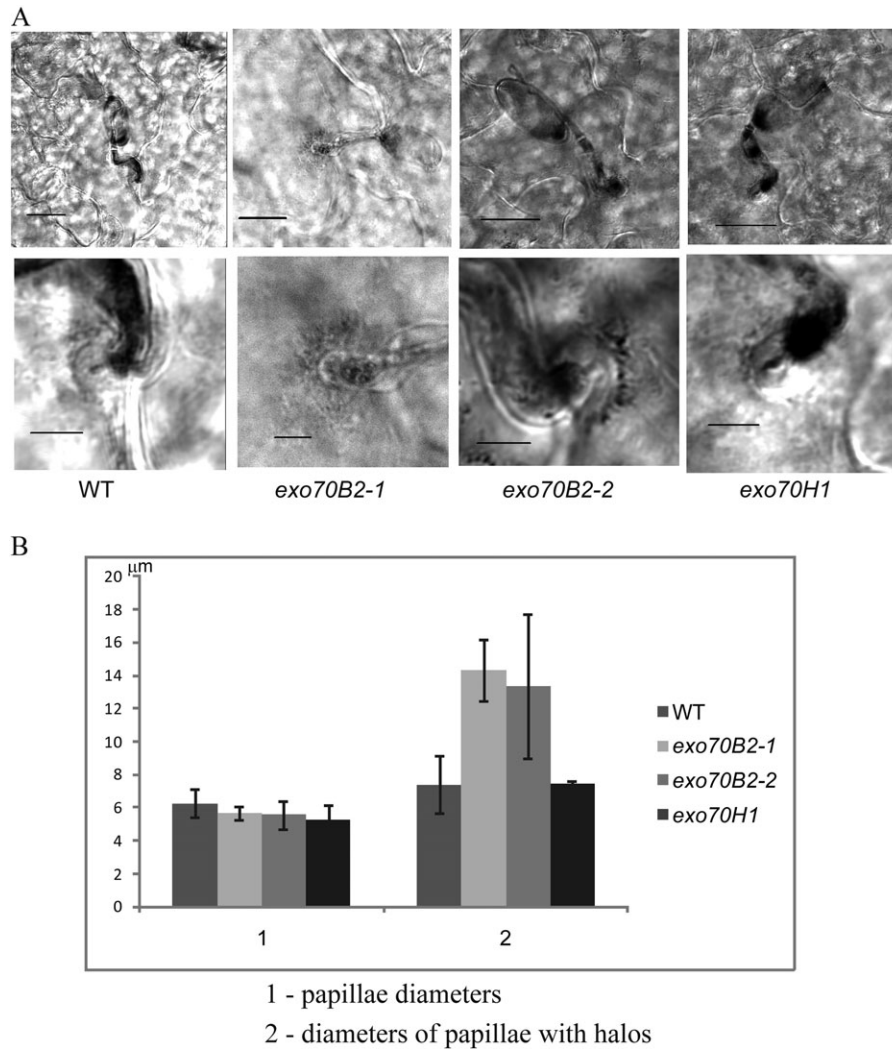
#### Transient expression and cellular localization of GFP tagged Exo70B2 and Exo70H1

Chimeric Exo70s carrying the green fluorescent protein (GFP) fused to Exo70B2 and Exo70H1 N- termini were constructed. The constructs were placed under the 35S promoter and used to transiently transform *N. benthamiana* leaves. Approximately 36 h after leaf agroinfiltration, the material was studied by confocal microscopy.

In the case of Exo70B2, the signal was found in the cytoplasm, in patches adjacent to the plasma membrane, in cytoplasmic strands, and in the surrounding organelles and nucleus. A weak signal was also found inside the nuclei. The Exo70H1 demonstrates two types of localization within one cell: the punctuated, vesicle-like structures of approximately 0.5  $\mu$ m, located in the cortical cytoplasm adjacent to the membrane and around the nucleus, and a diffuse signal in cytoplasmic strands and the cytoplasm. Weaker intranuclear and strong peri-nuclear signals could also be seen in the case of Exo70H1. However, while the Exo70B2 signal is homogenously distributed inside the nucleus, the Exo70H1 demonstrates again a more punctuate pattern (Fig. 7).

## Discussion

The plant exocyst complex involvement in the regulation of cell polarity suggests that this complex might influence the capability of the plant cell to react defensively against pathogen attack. It has been demonstrated that Exo70H1,



**Fig. 6.** (A) Papilla structure in WT and in mutant lines. The fine papilla structure for each of the genotypes was observed by confocal microscopy combined with the bright field. The whole spore with cytoplasm surrounding are shown in the upper row (bar=20  $\mu\text{m}$ ); the lower row shows in more details papilla structures (bar=5  $\mu\text{m}$ ). The vesicular halo is visible in *exo70B2-1* and *exo70B2-2*. (B) The diameters of papillae and the diameters of the area of papillae with vesicular halos were measured for four different genotypes. The vesicular halo is significantly bigger for papillae of *exo70B2* mutants.

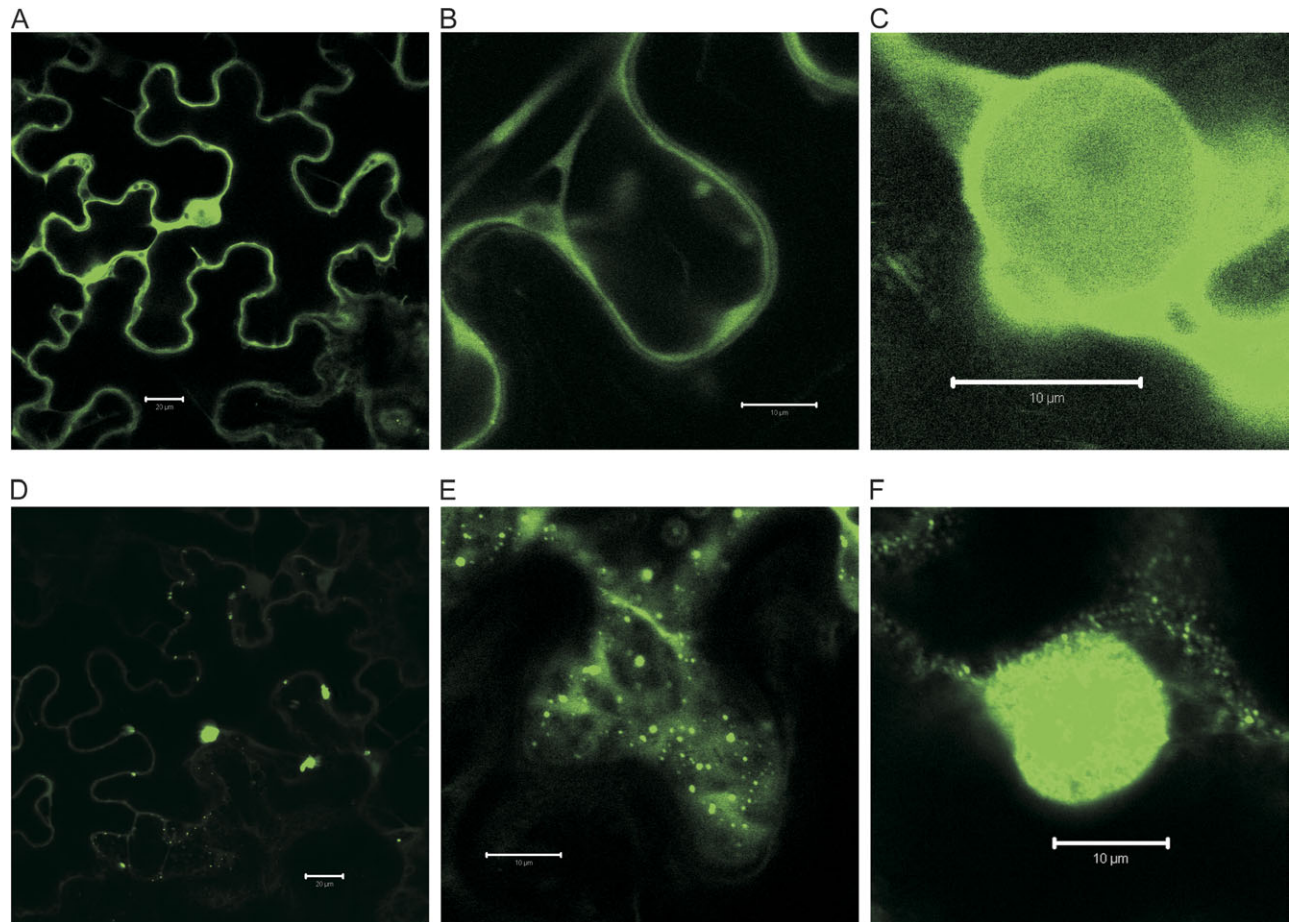
H2, H7, and B2 RNAs were strongly up-regulated by elf18 treatment. These results are in accordance with the works of Zipfel *et al.* (2004) and Thilmoney *et al.* (2006) that detected Exo70H1 among PAMP up-regulated genes.

Due to the strong functional relatedness of exocyst and SNARE proteins, similarity was expected in the way that resistance is compromised in our mutants with that of the *pen1* mutant. Our results indeed revealed distinctly enhanced susceptibility to *P. syringae* pv. *maculicola* in *exo70B2* and, to a lesser extent, in the *exo70H1* mutant. The assay is based on the infiltration of bacteria into the intercellular spaces of the leaf tissue, where bacterial progress depends on the effectiveness of the plant apoplastic defence responses. It is speculated that the exocytosis defects in *exo70B2* and *exo70H1* mutants render the apoplastic environment more permissive for bacterial growth. So far, to the best of our knowledge, the only data on the involvement of exocytosis in the response to

*Pseudomonas* challenge were presented for AtSNAP33, which is up-regulated after inoculation with *Pseudomonas syringae* pv. *tomato* (Wick *et al.*, 2003); much more effort is dedicated to the elucidation of the gene-for-gene aspect of resistance to *Pseudomonas* (Dangl *et al.*, 1992; Kunkel *et al.*, 1993; Yu *et al.*, 1993).

Based on the fact that the SNARE protein PEN1 is required for the timely assembly of the papillae and focal secretion after *Blumeria* attack (Assaad *et al.*, 2004, Kwon *et al.*, 2008) and that, in yeasts and mammals, the SNARE proteins function just one step downstream from the exocyst complex in the execution of the secretory pathway function, it is probable that the malfunction of the exocyst complex might have an impact on papillae formation as well. Therefore, in the exocyst T-DNA *Arabidopsis* insertional mutants of *exo70B2* and *exo70H1* paralogues possible differences in papillae formation were analysed using an already established and well-described pathogen:





**Fig. 7.** The localization of N-GFP tagged Exo70B2 and Exo70H1 transiently expressed in *N. benthamiana* epidermal cells after agroinfiltration. Typical intracellular localization of GFP-Exo70B2 (A–C) and GFP-Exo70H1 (D–E) observed 24–36 h post-infiltration is shown. Details of peri-nuclear and intra-nuclear localization for Exo70B2 (C) and Exo70H1 (F) are shown on the right. (A, D) Bars 20 µm; (B, C, E, F) bars 10 µm).

*B. graminis* f. sp. *hordei*. Under our experimental conditions local changes at the site of the pathogen penetration attempt were observed. Mutants in the exocyst subunit Exo70B2 showed most apparent deviations in the cell wall structure. Over-accumulation of vesicle-like structures around *B. graminis* f. sp. *hordei* attacking sites in *Arabidopsis* *exo70B2* mutants is one of the strongest deviations from the normal execution of the final steps in the polarized exocytosis linked to papillae formation. In the work of Assaad *et al.* (2004), the presence of the GFP-PEN1 in papillae, and also in an endomembrane compartment of roughly 1 µm diameter located in the vicinity of papillae, was found. The authors speculate that this may be the same type of vesicles described by Zeyen and Bushnell (1979) and suggested by Huckelhoven *et al.* (1999) to play a role in the targeting of reactive oxygen species toward infection sites. However, Collins *et al.* (2003) reported a decrease in this type of vesicle in SNARE *pen1* mutants at sites of failed penetration by the fungus, which seemed counter-intuitive, as an accumulation of vesicles having problems fusing with the plasma membrane is more to be expected (Novick *et al.*, 1980; Littleton *et al.*, 1998). Our observation of the increase

of a vesicle-like halo around papillae in *exo70B2* mutants suggests that docking of secretory vesicles might in this case be partially compromised.

The Exo70s in *Arabidopsis* and other land plants form a big multigene family (Synek *et al.*, 2006), therefore functional redundancy but also specialization could exist in the reaction to pathogen attack. Redundancy is expected in Exo70H1 and Exo70H2 as they are highly homologous and the expression of both is up-regulated in response to pathogen stimuli. It is assumed that the loss of Exo70H1 is partially compensated by the Exo70H2. The T-DNA insertional mutant for the Exo70H2 gene is not yet available and the way to solve this problem could be to design plant harbouring RNAi against both of these Exo70s.

The possibility that Exo70s could homo- or heterodimerize, as shown in our Y2H assay, opens interesting questions about the existence of different exocyst subcomplexes, down to the level of dimers or oligomers, that would lead to the fast and effective assembly of the whole complex, where and when needed. In yeasts, the existence of a Sec6p dimer capable of interacting with t-SNARE Sec9p was confirmed by Sivaram *et al.* (2005). Functionally different exocyst



subcomplexes, dependent and independent on the Sec15, Sec8, or Exo70 functions were recently proposed to exist in *Drosophila* (Mehta *et al.*, 2005) as well as in mammals, where they are either employed in trafficking toward the tight junction sites or desmosomes, and in *Schizosaccharomyces pombe* sexual reproduction (Andersen and Yeaman, 2009; Sharifmoghadam *et al.*, 2010).

The information on the subcellular localization of Exo70B2 and Exo70H1 was obtained from the transient assay system after agroinfiltration. It was expected that *Arabidopsis* plants stably transformed with the native promoter driven constructs, exposed to the pathogen attack, could show a localization pattern similar to the one found for PEN1-GFP. This protein is homogeneously localized on the plasma membrane, however, upon pathogen attack, it is actively recruited to the site of the pathogen penetration attempt (Assaad *et al.*, 2004).

Plant immunity is a complex phenomenon that features many parallels with the innate immunity of animals. It involves the basic cellular processes, including exocytosis. The tethering complex exocyst and its role in plant cell polarity might show us how the subtle intracellular events lead to whole-plant resistance or disease. Not only that the study of exocyst-controlled events in the polarized exocytosis and the formation of cell wall appositions may help us to understand these important details of plant immunity better, but also vice versa, the localized plant defence reaction may help us to understand better the regulation of the secretory vesicles targeting in plants.

## Supplementary data

Supplementary data are available at *JXB* online.

**Table S1.** Primers used for RT-PCR analysis and cloning procedures.

**Figure S1.** The Genevestigator expression data for several *Arabidopsis* Exo70s.

**Figure S2.** Non-interacting pairs in Y2H assays of Exo70B2 and H1.

**Figure S3.** FRET assessment of possible interactions found for Exo70B2 and H1.

**Figure S4.** The papillae with vesicles-like appearing halo in *exo70B2* mutants in comparison to typical WT papilla.

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