DOI: 10.32615/bp.2019.017 BIOLOGIA PLANTARUM 63: 144-152, 2019

Characterisation of *Arabidopsis* flotillins in response to stresses

K. KROUMANOVÁ[1], D. KOCOURKOVÁ[1], M. DANĚK[1,2], L. LAMPAROVÁ[1,3], R. POSPÍCHALOVÁ[1], K. MALÍNSKÁ[1], Z. KRČKOVÁ[1], L. BURKETOVÁ[1], O. VALENTOVÁ[3], J. MARTINEC[1], and M. JANDA[1,3]*

Institute of Experimental Botany of the Czech Academy of Sciences, CZ-16502, Prague, Czech Republic[1]
Department of Experimental Plant Biology, Faculty of Science, Charles University, CZ-12844 Prague, Czech Republic[2]
Department of Biochemistry and Microbiology, University of Chemistry and Technology, Prague, CZ-16628 Prague, Czech Republic[3]

Abstract

Plant flotillins, a subgroup of the SPFH domain protein superfamily, consist of three proteins, *At*FLOT1, *At*FLOT2, and *At*FLOT3 in *Arabidopsis thaliana*. The exact functions of flotillins in plant cell has not been established yet. In this study we focused on the role of flotillins in response to both abiotic and biotic stresses and on the response to phytohormones abscisic acid and 1-naphthalene acetic acid (NAA) in *A. thaliana*. We observed transcriptomic changes of *AtFLOT* genes in response to high salinity and cold, treatment with 22-amino acid peptide from N-terminal part of flagellin (flg22), and after infection with *Botrytis cinerea*. Transcription of *AtFLOT2* increased up to 60 times after flg22 treatment. Also, treatment with *B. cinerea* increased transcription of *AtFLOT1* 10 times and of *AtFLOT3* 14 times. Furthermore, we used T-DNA knock-out single mutants for all three *A. thaliana* flotillins and we measured root growth in response to high salinity, cold, phosphate starvation, nitrogen starvation, and abscisic acid and NAA treatments. Subsequently, we measured the reactive oxygen species production and callose accumulation after the treatment with flg22. Next, we performed resistance assays to *Pseudomonas syringae* pv. *tomato* DC3000 and *B. cinerea*. In contrast to transcriptomic changes, knocking-out of only single *FLOT* gene did not lead to significant changes in response to all tested stresses.

Additional key words: abscisic acid, auxin, *Botrytis cinerea*, callose, cold, nutrient starvation, *Pseudomonas syringae*, ROS, salinity.

Introduction

Plants evolved sophisticated, efficient, and complex responses to both biotic and abiotic stresses. Plasma membrane (PM) serves as a highly exposed platform for responses to stress factors. Receptors responsible for recognition of threats are often present on PM (Ott 2017). Within PM, the crucial role has its compartmentalization to macro, micro, and nanodomains (Sekeres *et al.* 2015). It was shown that membrane microdomains are important for membrane trafficking, signal transduction, and response to pathogen attack (Lefebvre *et al.* 2007, Liu *et al.* 2009, Wang *et al.* 2015, Bucherl *et al.* 2017).

Plant flotillins along with prohibitins (PHB) belong to the stomatin/prohibitin/flotillin/HflK/C (SPFH) domain (also known as Band_7 domain) protein superfamily. Comparative genome analysis of this superfamily reveals deep evolutionary origin and diverse gene functions (Di *et al.* 2010). Flotillins are associated with membrane microdomains and are commonly used as markers of membrane microdomains in both mammalian and plant cells. Flotillins occur not only on the PM but also were

Submitted 10 May 2018, *last revision* 26 June 2018, *accepted* 30 June 2018.

Abbreviations:ABA - abscisic acid, Col-0 - Columbia-0, dpi - days post inoculation, elf18 - acetylated 18-amino acid fragment from N-terminal of elongation factor Tu (acetyl-MSKEKFERTKPHVNVGT), flg22 - 22-amino acid peptide from N-terminal part of flagellin (QRLSTGSRINSAKDDAAGLQIA), FLS2 - flagellin-sensitive 2, FLOT - flotillin, hpi - hours post inoculation, JA - jasmonic acid, MAMP - microbe-associated molecular pattern, MS - Murashige and Skoog, NAA - 1-naphthalene acetic acid, PM - plasma membrane, *Pst* - *Pseudomonas syringae* pv. *tomato* strain DC3000, ROS - reactive oxygen species, SA - salicylic acid, Ws-4 - Wassilievska-4, WT - wild-type.

Acknowledgments: This research was supported by the Czech Science Foundation (grant No. 14-09685S) and by the Ministry of Education, Youth, and Sport of the Czech Republic (MSMT No 21-SVV/2017).

* Author of correspondence; e-mail: martin.janda@vscht.cz

detected in endosomes (Glebov *et al.* 2006, Haney *et al.* 2010, Li *et al.* 2012, Jarsch *et al.* 2014, Yu *et al.* 2017). Three members of flotillin protein family were identified in *A. thaliana*, *At*FLOT1 (At5g25250), *At*FLOT2 (At5g52560), and *At*FLOT3 (At5g64870). It should be noted that in some literature the *At*FLOT1 and *At*FLOT2 are affiliated as *At*FLOT1a and *At*FLOT1b, respectively (Jarsch *et al.* 2014). Yu *et al.* (2017) showed that *At*FLOT1 and *At*FLOT2 share 94 % similarity of amino acid sequence and *At*FLOT1 share 85 % similarity with *At*FLOT3.

Flotillin functions were broadly studied and described in yeasts and mammals; while the proper role of flotillins in plants is still very barely understood. In plants flotillins were shown to play important role in plant-microbe interaction. In *Medicago truncatula,* seven genes encoding flotillin-like proteins were identified (Haney *et al.* 2010). From those *MtFLOT2* and *MtFLOT4* were significantly upregulated during early symbiotic events and play crucial role in establishing the relationship between *M. truncatula* and symbiotic nitrogen-fixing rhizobium *Sinorhizobium meliloti*. Additionally, co-localization of *Mt*FLOT4-mCherry with lysin motif receptor-like kinase 3 (LYK3) was observed in inoculated roots (Haney *et al.* 2010). Yu *et al.* (2017) showed that treatment of plants with flg22 leads to the increased degradation of *At*FLOT1. Moreover, accumulation of callose decreased in *Atflot1* amiRNAi plants in response to flg22 (Yu *et al.* 2017). By contrast, to our best knowledge, no data are available for the role of flotillins in plant responses to abiotic stresses. *In sillico* transcription analysis performed using *Genevestigator*®. It was shown that gene transcription of *AtFLOT*s is increased under various abiotic and biotic stresses (Daněk *et al.* 2016).

The present study was focused on the role of *At*FLOTs in response to following treatments: high salinity, cold, nitrogen and phosphate starvation, abscisic acid (ABA), 1-naphthalene acetic acid (NAA), *Pseudomonas syringae* (*Pst*) and *Botrytis cinerea* infection, and elicitors flg22 or elf18. We analysed transcription of *AtFLOTs* in WT plants together with knock-out T-DNA single mutants of flotillin genes.

Materials and methods

Plants and cultivation: In this study we used *Arabidopsis thaliana* wild type (WT) genotypes: Columbia-0 (Col-0) and Wassilievska-4 (Ws-4) and mutants *Atflot1* (SALK_203966C) and *Atflot3* (SALK_143325C) with Col-0 background, and *Atflot2* (FLAG_352D08) with Ws-4 background.

Surface-sterilized seeds were sown in *Jiffy 7* peat pellets and plants were grown for four weeks in soil, under a 10-h photoperiod, an irradiance of 90 - 120 µmol m^{-2} s^{-1}, a temperature of 22 °C and a 70 % relative humidity. They were watered with distilled water as necessary. Plants grown in these conditions were used for reactive oxygen species (ROS) determination and *Pst* DC3000 and *Botrytis cinerea* treatments.

Further, *A. thaliana* seedlings were grown in liquid Murashige and Skoog (MS) medium or on solid ½ MS medium. The liquid MS medium contained 4.41 g dm^{-3} MS vitamins (*Duchefa*, Haarlem, The Netherlands), 5 g dm^{-3} sucrose, and 5 g dm^{-3} (N-morpholino) ethanesulfonic acid (MES) monohydrate (*Duchefa*). The solid ½ MS medium contained 2.2 g dm^{-3} MS basal salts (*Duchefa*) and 10 g dm^{-3} agar (*Sigma-Aldrich,* St. Louis, USA). Both media were adjusted to pH 5.8 using 1 M KOH. For cultivation in the liquid, surface-sterilized seeds were sown in 24-well plates containing 0.4 cm^3 of liquid MS medium per well and 3 - 5 seeds. Plants were cultivated for 11 d under a 10-h photoperiod, an irradiance of 100 - 130 µmol m^{-2} s^{-1} and a temperature of 22 °C. On the 7th day, the medium in the wells was exchanged for a fresh one. Seedlings from liquid media were used for callose analysis.

For the root length analysis, the seedlings were grown on solid medium in square plates (12 cm side). The plates with seeds were placed to 4 °C for 48 h. Then, the seedlings were grown under a 16-h photoperiod, an irradiance of 100 µmol m^{-2} s^{-1}, and a temperature of 22 °C. At 5th day, seedlings of similar size were transferred to new plates and the length of root was marked. Experiments were designed so that WT seedlings and the particular mutant line were on the same plate. In one biological replicate 20 seedlings for WT and 17 - 20 seedlings for particular mutant line were used. At 7th day after transfer, the root length increase was marked. For the 1-naphthalene acetic acid (NAA) treatment, seedlings of similar size were transferred to new plates at 4th day and at 4th day after transfer, the root length increase was marked. The experiments were performed in three biological repeats.

Transcriptomic analysis: For transcriptomic analysis, seedlings were grown on solid ½ MS media in round plates (6 cm in diameter) lying horizontally in a 16-h photoperiod, an irradiance of 100 µmol m^{-2} s^{-1}, and a temperature of 22 °C. The seedlings (10 - 12 from each plate) were harvested at day 11 and the tissue was stored in liquid nitrogen. For one biological replicate 3 - 4 independent samples were used.

Total RNA was isolated from frozen plant tissue using the *Spectrum Plant Total* RNA kit (*Sigma-Aldrich*) and treated with a DNA-free kit (*Ambion*, Austin, TX, USA). Then 1 µg of RNA was used for reverse transcription to cDNA with *M-MLV RNase H⁻Point Mutant* reverse transcriptase (*Promega*, Fitchburg, WI, USA) and anchored oligo dT21 primer (*Metabion*, Martinsried, Germany). Gene transcription was quantified by q-PCR using *LightCycler 480 SYBR Green I Master* kit and *LightCycler 480* (*Roche*, Basel, Switzerland). The PCR conditions were: 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s, followed by a melting curve analysis. Relative transcription was calculated with normalization to the housekeeping gene *TIP41-like (At4g34270)*. The list of primers is in Table 1 Suppl.

Abiotic stresses: Under following abiotic stress conditions root length was measured. Seedlings were scanned at day 7 after transfer. Root length was analysed using software *JMicroVision®*. In selected cases transcription of *AtFLOT* genes was analysed as well.

For high salinity, the seedlings were grown on ½ MS solid medium containing 100 mM NaCl. For gene transcription analysis 11-d-old seedlings were flooded with 150 mM NaCl dissolved in liquid ½ MS medium for 3 h. As a control seedlings were flooded with ½ MS liquid medium.

For cold treatment, the seedlings were grown on ½ MS solid medium at 14 °C and control seedlings at 22 °C. For gene transcription analysis, 11-d-old seedlings were treated for 3 h by cultivation at 6 °C in darkness. Control seedlings were put into darkness for 3 h at 22 °C.

For phosphate starvation experiment, the control seedlings were grown on modified half-strength Hoagland's medium (Hoagland *et al.* 1950) with 1 % agar and the treated seedlings were grown on half-strength Hoagland's medium in which $NH_4H_2PO_4$ was replaced with NH_4Cl. The medium was adjusted to pH 6.2 with NaOH.

For nitrogen starvation experiment, the control seedlings were grown on medium containing 1 mM KH_2PO_4, 25 µM H_3BO_3, 2 µM $ZnSO_4$, 2 µM $MnSO_4$, 0.5 mM $MgSO_4$, 20 µM ferric citrate, 0.5 µM $CuSO_4$, 0.5 µM Na_2MoO_4, 1 mM NH_4NO_3, 0.25 mM CaSO4, and 1 % agar and the treated seedlings on the medium without NH_4NO_3.

Biotic stresses: The inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) was performed according to Katagiri *et al.* (2002) with slight modifications. In brief, bacteria were cultivated on the Luria-Bertani (LB) solid medium (with 1.2 %, m/v, agar) containing rifampicin (50 g dm^{-3}) overnight. Bacteria were resuspended in 10 mM $MgCl_2$ and a suspension was prepared to absorbance (A_{600}) = 0.001 for infiltration and A_{600} = 0.2 for dipping. For dipping inoculation suspension contained *Silwet Star* (0.02 %, v/v, *AgroBio*, Opava, Czech Republic). Four-week-old plants were infiltrated with needleless syringe or dipped for 30 s in bacterial suspension. Nine discs (6 mm diameter) from three plants were collected as one sample of one genotype at 0 dpi and 3 dpi. The leaf discs were grounded in 10 mM $MgCl_2$ and decimal dilution was performed. The colony forming units were counted. For gene transcription analysis, 4-week-old plants were infiltrated (using needleless syringe) with *Pst* DC3000 for 24 h, control plants were treated with 10 mM $MgCl_2$.

Four-week-old *A. thaliana* plants were treated with a 6-mm^3 drops containing *Botrytis cinerea BMM* spores (5×10^4 spores cm^{-3}) by applying one drop on one leaf, three leaves at similar developmental stage from one plant. The treated plants were transferred into the closed plastic box and were kept at low irradiance of 10 - 20 µmol m^{-2} s^{-1}), a 16-h photoperiod and a temperature of 21 °C for 96 h post inoculation (hpi). For gene transcription analysis, 4-week-old plants were treated with *Botrytis cinerea* BMM spores (5.10^4 spores cm^{-3}) diluted in potato dextrose broth (PDB) liquid medium for 48 h, control plants were treated with a drop of four times diluted PDB liquid medium.

Measurement of H_2O_2 production: H_2O_2 production was determined by the luminol-based assay as described in (Sasek *et al.* 2014). Discs, 3 mm in diameter, were cut from the fully developed leaves (two discs per leaf) of 4-week-old *A. thaliana* plants (three leaves per plant). Discs were incubated in white non-transparent 96-well plate (*NUNC*, *Thermo Fisher Scientific*, Waltham, MA, USA) in 0.15 cm^3 of distilled water for 16 h. Distilled water was replaced by 0.2 cm^3 of reaction solution containing 17 µg cm^{-3} of luminol, 10 µg cm^{-3} of horseradish peroxidase (*Sigma-Aldrich*) and 100 nM flg22 or 100 nM elf18. The measurement was performed immediately after adding the flg22 with a luminometer (*Tecan infinite F200*, Männedorf, Switzerland) for a period of 45 min.

Callose deposition in response to flg22: *A. thaliana* seedlings were treated with 1 µM flg22 for 24 h at day 11, the MS medium was replaced with fresh one with or without flg22. After 24 h the MS medium was replaced with ethanol:glacial acetic acid (3:1, v/v) until the seedlings were decolorized. The seedlings were then rehydrated in successive baths of 70 % ethanol (at least 1 h), 50 % ethanol (at least 1 h), 30 % ethanol (at least 1 h), and water (at least 2 h). Leaves were then incubated in 0.01 % (m/v) aniline blue in 150 mM K_2HPO_4, pH 9.5, for 4 - 6 h. Callose deposition was observed using fluorescence microscope *AxioImager ApoTome2* (*Carl Zeiss*, Oberkochen, Germany) and the number of callose spots per mm^2 were calculated using *Fiji* software (Schindelin *et al.* 2012). For gene transcription analysis, four-week-old *A. thaliana* plants were treated with 100 nM flg22 applied by needleless syringe infiltration for 1 and 4 h. Infiltration with distilled water was used as a control.

Treatments with phytohormones: The seedlings were grown on ½ MS medium containing 2 µM ABA dissolved in EtOH (0.01 %) and 1 % agar, control seedlings grew on ½ MS medium containing only EtOH. Medium was adjusted to pH 5.8 with KOH. Root length was analysed using software *JMicroVision®*. For gene transcription analysis seedlings at day 11 were flooded with 100 µM ABA in liquid ½ MS medium for 3 h as a control seedlings were flooded with 0.1 % EtOH in liquid ½ MS medium.

The 4-d-old seedlings grown on ½ MS were transferred on ½ MS medium containing 200 nM NAA and length of the root was marked on the plate. The seedlings were scanned at day 4 after transfer and the length of primary root was measured using software *Fiji* (Schindelin *et al.* 2012).

Statistical analysis: If not mentioned otherwise, two-tailed Student's *t*-test was used for statistical evaluation and statistical significance was assigned to difference with *P* values < 0.01.

Results

Transcriptions of all three flotillin genes in 11-d-old *A. thaliana* seedlings exposed to NaCl, cold, ABA, infection, and flg22 treatments were analysed. Flooding seedlings with 150 mM NaCl for 3 h significantly down regulated the transcription of *AtFLOT1* and *AtFLOT2* genes (Fig. 1*A*). Exposure of seedlings to 6 °C for 3 h increased the transcription of *AtFLOT3* gene (Fig. 1*B*). After infiltration of 4-week-old *A. thaliana* with 100 nM flg22, transcriptions of all *AtFLOT* genes increased 1 and 4 h after treatment (Fig. 1*C*) with strongly increased expressions of *AtFLOT1* and *AtFLOT3*. Upregulation of *AtFLOT3* was transient and after 4 h after returned to the basal level. The transcription of *AtFLOT1* and *AtFLOT2* genes further increased at 4 h after flg22 treatment (Fig. 1*C*). Interestingly, bacterial infection of plants with *Pst* DC3000 did not lead to the changes in transcription of *AtFLOT* genes (Fig. 1*D*), whereas infection with fungus *B. cinerea* induced transcription of *AtFLOT1* and *AtFLOT3* (Fig. 1*E*). Treatment with ABA altered the transription of all three *AtFLOTs*, however, the changes were not significant due to high variability of obtained data (Fig. 1*F*). Overall, transcription of at least one *AtFLOT* gene was significantly changed under 4 from 6 tested stress factors which strongly suggests involvement of *AtFLOTs* in response to stresses.

Following transcriptomic analysis, T-DNA knock-out single mutants available from public seeds depositories for each flotillin gene were used for phenotype analysis. These mutants do not transcribe particular *AtFLOT* genes (Fig. 1 Suppl.). We measured root growth of WT and mutant plants exposed to high salinity (100 mM NaCl; Fig. 2*A*), cold (14 °C; Fig. 2*B*), and phosphate (Fig. 2*C*) and nitrogen starvation (Fig. 2*D*). Seedlings were exposed to these stresses for 7 d. In contrast to transcriptomic analysis, comparison of flotillin mutant lines with their background genotype did not reveal any significant changes in root length and other noticeable morphological changes. These results indicate that any of single *AtFLOT* gene does not play particular role in acclimation to all tested abiotic stresses.

Furthermore, we focused on the role of the single flotillin gene in response to biotic stresses. Transcriptional analysis showed changes of *AtFLOT* gene expression in response to flg22 and *B. cinerea* (Fig. 1*C*,*E*). We measured H_2O_2 production upon microbe-associated molecular patterns (MAMP) treatment (Fig. 3*A*) since ROS burst is well described immediate and massive response to MAMPs (Smith *et al.* 2014). For *Atflot1* and *Atflot3* mutants we used flg22 as MAMP. Because *Atflot2* mutant has a genetic background Ws-4 which lacks the flagellin-

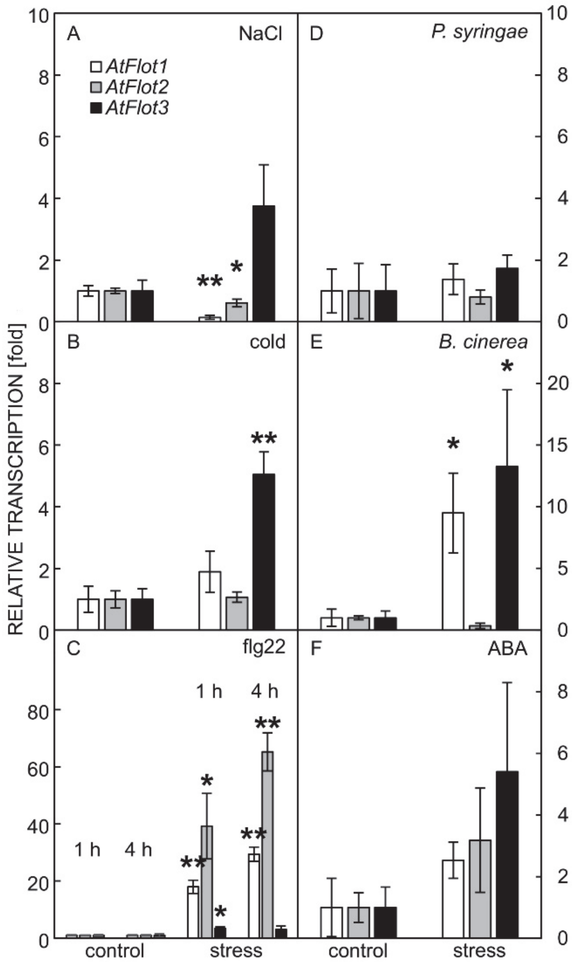

Fig. 1. Transcription analysis of *AtFLOT* genes in response to different stresses. *Arabidopsis thaliana* seedlings were treated at day 11 with 150 mM NaCl for 3 h (*A*), 6 °C for 3 h (*B*), 100 nM flg22 for 1 and 4 h (*C*), infiltration with *Pst* DC3000 for 24 h (*D*), inoculation with *Botrytis cinerea* BMM spores for 48 h (*E*), and 100 µM ABA for 3 h (*F*). Means ± SE, *n* = 3 to 4, *asterisks* indicate statistically significant differences compared to the corresponding control (* - P < 0.05, ** - P < 0.01, Student's *t*-test). Transcription was normalized to a reference gene *TIP41-like*.

sensitive 2 (FLS2) receptor for flg22 (Zipfel *et al.* 2004), we used elf18 (Lu *et al.* 2009). However, none of the mutants had affected ROS production in response to MAMPs (Fig. 3A). Furthermore we tested the resistance of *A. thaliana* WT and mutant plants toward the infection with *Pst* DC3000, which represent model pathosystem in the studies of plant-bacteria interactions (Xin *et al.* 2018).

Here we used two different experimental approaches: infiltration with needleless syringe (Fig. 3*B*) and flooding of plant rosettes in bacterial suspension (Fig. 2 Suppl.). In both setups, no differences in the number of bacteria were found in the mutant line in comparison to the controls. Not surprisingly, the genotypes with Ws-4 background were more susceptible to *Pst* DC3000 compared to Col-0 background genotypes (Figs. 3 and 2 Suppl.). As the *AtFLOT1* and *AtFLOT3* transcription was induced in response to *B. cinerea,* we tested if these mutants would have altered resistance to this necrotrophic fungus. However, we did not see any significant difference between infected mutant and control lines (Fig. 3*C*). Moreover, Ws-4 genotypes were more resistant to the infection in comparison with Col-0 genotypes (Fig. 3*C*).

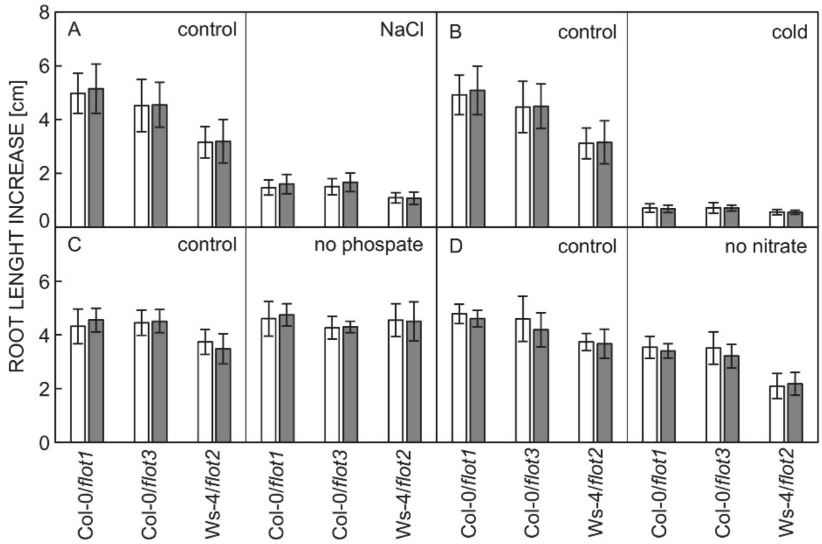

Fig. 2. Response of flotillin T-DNA *A. thaliana* mutants to abiotic stresses. Root growth of 12-d-old seedlings on the medium containing 100 mM NaCl for last 7 d (*A*). Root growth of 12-d-old seedlings 14 °C for last 7 d (*B*). Root growth of 12-d-old seedlings on the medium without phosphate for last 7 d (*C*). Root growth of 12-d-old seedlings medium without nitrogen for last 7 d (*D*). *White bars* represent WT, *grey bars* represent *Atflot* mutant. Means ± SDs, n = 17 to 20 (P < 0.01, Student's t-test)

Yu *et al.* (2017) showed decreased callose accumulation in response to flg22 in seedlings of their *Atflot1* knock-down mutant. Therefore, we measured callose accumulation in our *Atflot1* knock-out mutant, but we did not see the difference compared to the control line (Fig. 3*D*). Despite the transcriptional changes in response to biotic stress, we did not reveal the crucial role of particular *AtFLOT* gene under biotic stress conditions tested.

In sillico transcriptomic analysis showed transcriptional changes of *AtFLOT* genes in response to phytohormones ABA and auxin (Daněk *et al.* 2016). Although our transcription analysis after treatment with ABA did not confirm microarray data, we performed the root growth assays with seedlings of all three knock-out mutants where we measured the root growth in presence of 100 µM ABA or 200 nM NAA. The root growth of *Atflot* mutants was similar to control lines (Fig. 4*A*,*B*).

Discussion

In our work we focused on the possible role of flotillins in response to different type of stresses in *A. thaliana*. The available transcriptomic microarray data indicated a possible involvement in response to abiotic and biotic stresses (Daněk *et al*. 2016). In terms of abiotic stress, here we show that transcription of *AtFLOT* genes is altered in early response (after 3 h) to high salinity leading to the inhibition of *AtFLOT1* and *AtFLOT2* (Fig. 1*A*) and exposure to cold leading to the induction of *AtFLOT3* transcription (Fig. 1*B*). In the case of biotic stress, the transcriptional changes were more robust compared to changes under abiotic stresses. In four-week-old *A. thaliana* the transcription of *AtFLOT1* and *AtFLOT2* was increased in response to flg22 (Fig. 1*C*) and transcription of *AtFLOT1* and *AtFLOT3* was increased in response to the infection by *B. cinerea* (Fig. 1*E*). The transcription profile upon *B. cinerea* treatment is interesting because under other tested conditions (high salinity, cold, flg22), mainly *AtFLOT1* and *AtFLOT2* share similar transcriptional pattern. This is not surprising with respect to the fact that they share 94 % sequence similarity and therefore they may function similarly as well. However, in response to *B. cinerea*, the transcription of *AtFLOT1* and *AtFLOT3* was induced as opposed to the transcription of *AtFLOT2* which remained stable (Fig. 1*E*). Hence, our results imply functional redundancy of *At*FLOT1 and *At*FLOT2 but only in some cases. Results from our transcriptomic analysis do not correspond in all cases with publicly available microarray data. *Pst* DC3000 infection did not affect transcription (Fig. 1*D*) and treatment with ABA did not show significant transcription changes due to very high variability of measurements (Fig. 1*F*). NaCl treatment had the opposite effect than it was revealed with microarray experiments, inhibition of transcription (Fig. 1*A*). However cold stress, flg22 treatment, and *B. cinerea* infection had similar effect on the *AtFLOTs* transcription as was found in database (Daněk *et al.* 2016). These discrepancies may be explained by slightly different conditions used in particular experiments. It must be noted as well that set up of microarray experiments does not allow discrimination between expressions of *AtFLOT1* and *AtFLOT2*.

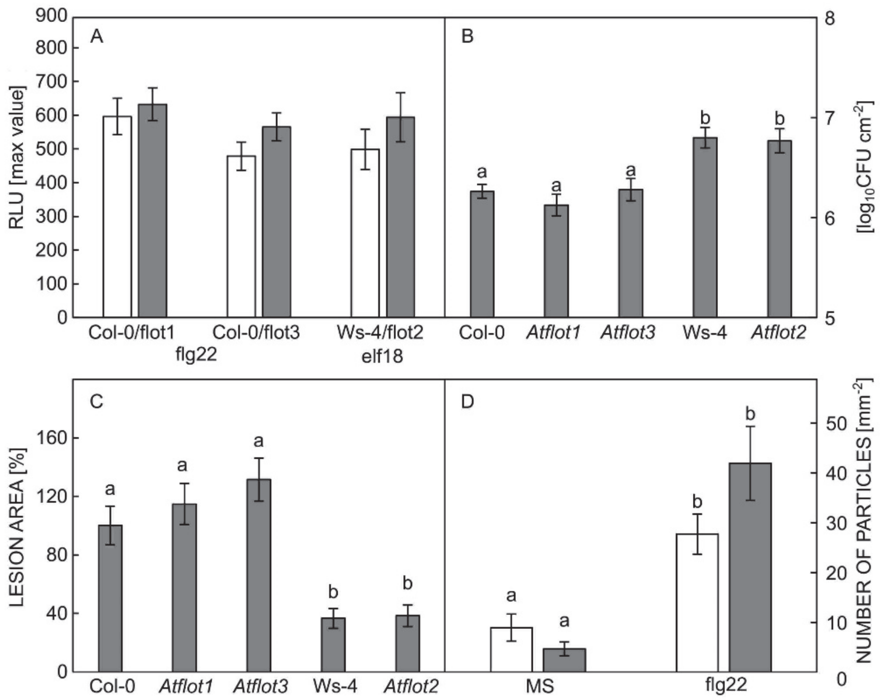

Fig. 3. Response of flotillin T-DNA *A. thaliana* mutants to biotic stresses. *A* - Maximum luminescence induced by 100 nM flg22 for *Atflot1* and *Atflot3* or 100 nM elf18 for *Atflot2* in leaf discs from 4-week-old plants (RLU - relative luminiscence unit). *B* - *Pst* DC3000 was infiltrated into leaves of 4-week-old plants. Values are demonstrated in $\log_{10}$ scale of colony forming units (CFU). *C* - Lesions on 4-week-old *A. thaliana* leaves infected with *Botrytis cinerea BMM* for 96 h. *D* - Callose deposition in 10-d-old seedlings of *A. thaliana* treated with 1 μM flg22 for 24 h (MS - control). In *A* and *D*, white bars represent WT, *grey bars* represent *Atflot* mutant. Means ± SEs, $n = 12$ for *A*,*C* and 6 for *B*,*D*). Different letters indicate significant differences between the samples ($P < 0.01$, Student's *t*-test).

The second goal of the work was to investigate direct involvement of a particular *AtFLOT* gene in response to stresses. For that purpose, we used T-DNA knock-out mutants for every single *AtFLOT* gene. The proper characterisation of the mutants is the critical point. Li *et al.* (2012) showed that some of *AtFLOT1* T-DNA insertion mutant lines had similar *AtFLOT1* expression as WT or even over-expression. Here we used different T-DNA line of *Atflot1* than Li *et al.* (2012) and also T-DNA mutants of *Atflot2* and *Atflot3* (Fig. 1 Suppl.). Obtained T-DNA lines did not show any transcription of particular *AtFLOT* genes (Fig. 1 Suppl.). We used above mentioned mutants for the phenotypic analysis in response to abiotic and biotic stress and for the treatment with phytohormones ABA and NAA.

According to best of our knowledge, no screening study of the involvement of flotillins in abiotic stresses exists until now. *In silico AtFLOTs* transcriptional data, as well as our results, indicated involvement of flotillins in response to abiotic stresses. Moreover, in yeasts and mammals flotillins play a role in endocytosis (Otto *et al.* 2011). Similar role was suggested for *AtFLOT1*. In specific conditions, it was shown that endocytosis of several plasma membrane (PM) proteins such as NADPH/respiratory burst oxidase protein D (RbohD), plasma membrane intrinsic protein 2 (PIP2;1), brassinosteroid insensitive 1 (BRI1) and ammonium transporter 1 (AMT1-3) is mainly dependent on clathrin mediated endocytosis but the role of microdomains and

AtFLOT1 cannot be excluded (Hao *et al.* 2014, Li *et al.* 2012, Liu *et al.* 2009, Wang *et al.* 2013, Yu *et al.* 2017). The role of endocytosis in abiotic stresses was described as well. For example, salt stress increases PM endocytosis (Hamaji *et al.* 2009, Leshem *et al.* 2006) and cold stress inhibits intracellular trafficking (Shibasaki *et al.* 2009). We tested the root growth of mutant and WT plants under high salinity, cold, nitrogen starvation, and phosphate starvation. No differences between mutant and WT root growth under tested abiotic stresses were observed (Fig. 2). One of the possible explanations of this observation is gene redundancy of *A. thaliana* flotillins. This explanation is supported with our results and with results of Li *et al.* (2012) (for details see below) and it is reasonable especially in the case of *AtFLOT1* and *AtFLOT2*. To reveal the role of redundant genes it is necessary to prepare the multiple *Atflot* knock-out mutant lines. CRISPR-Cas9 methodology would be a method of choice because *AtFLOT1* and *AtFLOT2* are in linkage and therefore it is not possible to obtain double mutant by crossing. Also, another experimental design should be considered.

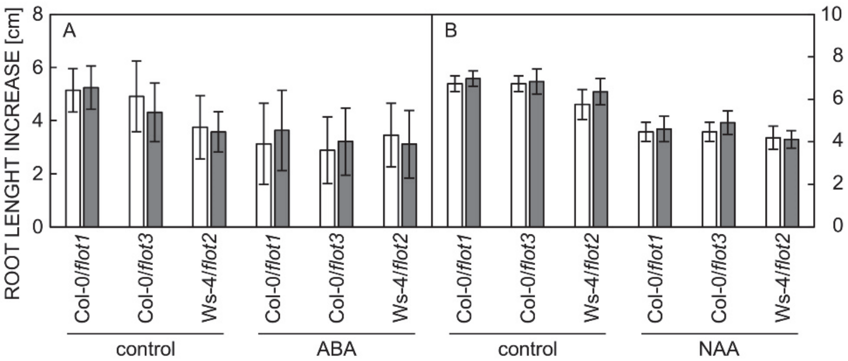

Fig. 4. Response of flotillin T-DNA *A. thaliana* mutants to phytohormones. *A* - The root growth of 12-d-old seedlings on the medium containing 2 µM ABA for last 7 d. *B* - The root growth of 8-d-old seedlings on the media containing 200 nM NAA for last 4 d. *White bars* represent WT, *grey bars* represent *Atflot* mutant. Means ± SD, *n* = 11 to 30.

In contrast to abiotic stresses, data showing the involvement of flotillins in biotic stresses already exist. The critical role of flotillins was shown in *Medicago truncatula* in response to symbiotic rhizobial infection. In *M. truncatula* seven flotillin-genes are recognised. Using silenced mutants in *MtFLOT2* and *MtFLOT4* it was approved that they are required for host derived infection threads and nodule formation (Haney *et al.* 2010). We tested T-DNA *Atflot* lines in response to *Pst* DC3000 and *B. cinerea*. However, we did not observe any difference between WT and T-DNA lines after *Pst* DC3000 and *B. cinerea* infection. The only differences we observed between the background genotypes Col-0 and Ws-4. Plants with Col-0 background were more resistant against *Pst* DC3000 and interestingly, more susceptible to *B. cinerea* in comparison to plants with Ws-4 background (Fig. 3*B*,*C*). However, a molecular background of this phenomenon is not known.

Upregulation of *AtFLOT* gene transcription after flg22 treatment was shown (Daněk *et al.* 2016, Millet *et al.* 2010). In agreement, we observed increased transcription of *AtFLOT* genes after treatment with flg22. It is known that flg22 treatment results in higher ROS production and callose deposition (Denoux *et al.* 2008). We studied transient ROS production in response to treatment with flg22 in our T-DNA mutant lines and we did not observe any difference between flotillin knock-out mutant and WT plants (unpublished results). Also, we studied callose deposition in our T-DNA mutant lines and we did not observe any difference between flotillin knock-out mutant and WT plants. In contrary, Yu *et al.* (2017) showed that knock-down mutant of *Atflot1* has decreased callose deposition in response to flg22. This contradiction may be explained by the downregulation of transcription of both *AtFLOT1* and *AtFLOT2*. Yu *et al.* (2017) used in their study amiRNAi lines. The same lines were used by Li *et al.* (2012). Besides down-regulation of *AtFLOT1,* three from their four amiRNAi lines exhibited also down-regulation of *AtFLOT2* (Li *et al.* 2012). Li *et al*. (2012) also described growth inhibition of *AmiRNAflot1* line, however, we did not observe root growth retardation of our T-DNA *AtFLOT1* line. These indicate that for observed decrease in callose deposition in response to flg22 and also the growth inhibition, both *AtFLOT1* and *AtFLOT2* are responsible.

Phytohormones play indispensable roles in plant growth and development and in response to both biotic and abiotic stresses (Santner *et al.* 2009, Denance *et al.* 2013, Janda and Ruelland 2015). Based on available transcriptomic data we were focused on the role of flotillins in response to ABA and NAA treatments. As for the biotic and abiotic stresses the role of endocytosis and microdomains in ABA and auxin mediated events are demonstrated. For example, auxin transporter PIN1 is present in microdomains (Titapiwatanakun *et al.* 2009) and polar distribution of auxin transporters is dependent on clathrin mediated endocytosis (Kitakura *et al.* 2011). ABA triggers the selective endocytosis of *A. thaliana* potassium channel KAT1 and its recycling to the PM in epidermal and guard cells (Sutter *et al.* 2007). As for the response to abiotic stress we analysed the effect of ABA and NAA to the root growth of *Atflot* T-DNA mutant lines. However, we did not observe any difference between T-DNA flotillin mutants and WT plants (Fig. 4).

We are aware that interpretation of the data obtained by the application of just one T-DNA mutated allele might be risky. Re-evaluation of function of *abp1-1* mutant could serve as an example of such situation (Dai *et al*. 2015, Enders *et al*. 2015, Michalko *et al*. 2015). Also the recent controversy dealing with commonly used *Syngenta Arabidopsis* insertion lines (SAIL) seeds stock with *qrt1* background serves as highly important warning (Nikoronova *et al.* 2018). However, we believe that our results are not misinterpreted. In our study we did not use seeds from SAIL stock. Moreover, unlike in the above mentioned studies, we do not show an effect of T-DNA insertion into flotillin genes and therefore it is not necessary to consider additional T-DNA insertions.

In conclusion our transcriptomic analyses showed altered transcription of *AtFLOT* genes in response to both biotic and abiotic stresses. We obtained set of T-DNA single mutants which do not transcribe particular *AtFLOT* genes and we used them to screen involvement of single flotillin genes in response to broad spectrum of stresses. Our data showed that single flotillin genes are not the crucial components of *A. thaliana* response reactions to all stress conditions tested. The explanation could be the functional redundancy between *At*FLOTs. Flotillins most probably act through the interaction with other proteins, thus their high sequence similarity may explain their redundancy. Creation of multiple knock-out lines will be necessary for further studies.

References

Bucherl, C. A., Jarsch, I. K., Schudoma, C., Segonzac, C., Mbengue, M., Robatzek, S., MacLean, D., Ott, T., Zipfel, C.: Plant immune and growth receptors share common signalling components but localise to distinct plasma membrane nanodomains. - eLife **6**: e25114, 2017.

Daněk, M., Valentová, O., Martinec, J.: Flotillins, erlins, and hirs: from animal base camp to plant new horizons. - Crit. Rev. Plant Sci. **35**: 191-214, 2016.

Denance, N., Sanchez-Vallet, A., Goffner, D., Molina, A.: Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. - Front. Plant Sci. **4**: 155, 2013.

Denoux, C., Galletti, R., Mammarella, N., Gopalan, S., Werck, D., De Lorenzo, G., Ferrari, S., Ausubel, F. M., Dewdney, J.: Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. - Mol. Plants **1**: 423-445, 2008.

Dai, X., Zhang, Y., Zhang, D., Chen, J., Gao, X., Estelle, M., Zhao, Y.: Embryonic lethality of *Arabidopsis* abp1-1 is caused by deletion of the adjacent *BSM* gene. - Nat. Plants **1**: pii: 15183, 2015.

Di, C., Xu, W., Su, Z., Yuan, J. S.: Comparative genome analysis of *PHB* gene family reveals deep evolutionary origins and diverse gene function. - BMC Bioinformatics **11**(Suppl 6): S22, 2010.

Enders, T. A., Oh, S., Yang, Z. B., Montgomery, B. L., Strader, L. C.: Genome sequencing of *Arabidopsis abp1-5* reveals second-site mutations that may affect phenotypes. - Plant Cell **27**: 1820-1826, 2015.

Glebov, O.O., Bright, N A., Nichols, B.J.: Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. - Nat. Cell Biol **8**: 46-54, 2006.

Hamaji, K., Nagira, M., Yoshida, K., Ohnishi, M., Oda, Y., Uemura, T., Goh, T., Sato, M.H., Morita, M.T., Tasaka, M., Hasezaw, S., Nakano, A., Hara-Nishimura, I., Maeshima, M., Fukaki, H., Mimura, T.: Dynamic aspects of ion accumulation by vesicle traffic under salt stress in *Arabidopsis*. - Plant Cell Physiol. **50**: 2023-2033, 2009.

Haney, C.H., Long, S.R.: Plant flotillins are required for infection by nitrogen-fixing bacteria. - Proc. nat. Acad. Sci. USA **107**: 478-483, 2010.

Hao, H., Fan, L., Chen, T., Li, R., Li, X., He, Q., Botella, M. A., Lin, J.: Clathrin and membrane microdomains cooperatively regulate RbohD dynamics and activity in *Arabidopsis*. - Plant Cell **26**: 1729-1745, 2014.

Hoagland, H., Arnon, D.I.: The water-culture method for growing plants without soil. - Calif. Agr. Exp. Sta **347**: 1-32, 1950.

Janda, M., Ruelland, E.: Magical mystery tour: salicylic acid signalling. - Environ.eExp. Bot. **114**: 117-128, 2015.

Jarsch, I.K., Konrad, S.S., Stratil, T.F., Urbanus, S.L., Szymanski, W., Braun, P., Braun, K.H., Ott, T.: Plasma membranes are subcompartmentalized into a plethora of coexisting and diverse microdomains in *Arabidopsis* and *Nicotiana benthamiana*. - Plant Cell **26**: 1698-1711, 2014.

Katagiri, F., Thilmony, R., He, S.Y.: The *Arabidopsis thaliana* - *Pseudomonas syringae* interaction. - Arabidopsis Book 1: e0039, 2002

Kitakura, S., Vanneste, S., Robert, S., Lofke, C., Teichmann, T., Tanaka, H., Friml, J.: Clathrin mediates endocytosis and polar distribution of PIN auxin transporters in *Arabidopsis*. - Plant Cell **23**: 1920-1931, 2011.

Lefebvre, B., Furt, F., Hartmann, M. A., Michaelson, L. V., Carde, J. P., Sargueil-Boiron, F., Rossignol, M., Napier, J. A., Cullimore, J., Bessoule, J. J., Mongrand, S.: Characterization of lipid rafts from *Medicago truncatula* root plasma membranes: a proteomic study reveals the presence of a raft-associated redox system. - Plant Physio.l **144**: 402-418, 2007.

Leshem, Y., Melamed-Book, N., Cagnac, O., Ronen, G., Nishri, Y., Solomon, M., Cohen, G., Levine, A.: Suppression of *Arabidopsis* vesicle-SNARE expression inhibited fusion of H_2O_2-containing vesicles with tonoplast and increased salt tolerance. – Proc. nat. Acad. Sci. USA **103**: 18008-18013, 2006.

Li, R., Liu, P., Wan, Y., Chen, T., Wang, Q., Mettbach, U., Baluska, F., Samaj, J., Fang, X., Lucas, W. J., Lin, J.: A membrane microdomain-associated protein, *Arabidopsis* Flot1, is involved in a clathrin-independent endocytic pathway and is required for seedling development. - Plant Cell **24**: 2105-2122, 2012.

Liu, P., Li, R. L., Zhang, L., Wang, Q.L., Niehaus, K., Baluska, F., Samaj, J., Lin, J.X.: Lipid microdomain polarization is required for NADPH oxidase-dependent ROS signaling in *Picea meyeri* pollen tube tip growth. - Plant J. **60**: 303-313, 2009.

Lu, X., Tintor, N., Mentzel, T., Kombrink, E., Boller, T., Robatzek, S., Schulze-Lefert, P., Saijo, Y.: Uncoupling of sustained MAMP receptor signaling from early outputs in an *Arabidopsis* endoplasmic reticulum glucosidase II allele. – Proc. natl.Acad. Sci. USA **106**: 22522-22527, 2009.

Michalko, J., Dravecka, M., Bollenbach, T., Friml, J.: Embryo-lethal phenotypes in early *abp1* mutants are due to disruption of the neighboring *BSM* gene. - F1000Res **4**: 1104, 2015.

Millet, Y.A., Danna, C.H., Clay, N.K., Songnuan, W., Simon, M.D., Werck-Reichhart, D., Ausubel, F M.: Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. - Plant Cell **22**: 973-990, 2010.

Nikonorova, N., Yue, K., Beeckman, T., De Smet, I.: *Arabidopsis* research requires a critical re-evaluation of genetic tools. - J. exp. Bot. **15**: 3541-3544, 2018.

Ott, T.: Membrane nanodomains and microdomains in plant-microbe interactions. - Curr. Opin. Plant Biol. **40**: 82-88, 2017.

Otto, G.P., Nichols, B.J.: The roles of flotillin microdomains - endocytosis and beyond. - J. cell. Sci. **124**: 3933-3940, 2011.

Santner, A., Estelle, M.: Recent advances and emerging trends in plant hormone signalling. - Nature **459**: 1071-1078, 2009.

Sasek, V., Janda, M., Delage, E., Puyaubert, J., Guivarc'h, A., López Maseda, E., Dobrev, P.I., Caius, J., Bóka, K., Valentová, O., Burketová, L., Zachowski, A., Ruelland, E.: Constitutive salicylic acid accumulation in pi4kIIIbeta1beta2 *Arabidopsis* plants stunts rosette but not root growth. - New Phytol **203**: 805-816, 2014.

Sekeres, J., Pleskot, R., Pejchar, P., Zarsky, V., Potocky, M.: The song of lipids and proteins: dynamic lipid-protein interfaces in the regulation of plant cell polarity at different scales. - J. exp. Bot. **66**: 1587-1598, 2015.

Shibasaki, K., Uemura, M., Tsurumi, S., Rahman, A.: Auxin response in *Arabidopsis* under cold stress: underlying molecular mechanisms. - Plant Cell **21**: 3823-3838, 2009.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A.: Fiji: an open-source platform for biological-image analysis. - Nat. Methods **9**: 676-682, 2012.

Smith, J.M., Heese, A.: Rapid bioassay to measure early reactive oxygen species production in *Arabidopsis* leaf tissue in response to living *Pseudomonas syringae*. - Plant Methods **10**: 6, 2014.

Sutter, J. U., Sieben, C., Hartel, A., Eisenach, C., Thiel, G., Blatt, M.R.: Abscisic acid triggers the endocytosis of the *Arabidopsis* KAT1 K+ channel and its recycling to the plasma membrane. - Curr. Biol. **17**: 1396-1402, 2007.

Titapiwatanakun, B., Blakeslee, J.J., Bandyopadhyay, A., Yang, H., Mravec, J., Sauer, M., Cheng, Y., Adamec, J., Nagashima, A., Geisler, M., Sakai, T., Friml, J., Peer ,W.A., Murphy, A.S.: ABCB19/PGP19 stabilises PIN1 in membrane microdomains in *Arabidopsis*. - Plant J. **57**: 27-44, 2009.

Wang, L., Li, H., Lv, X.Q., Chen, T., Li, R.L., Xue, Y.Q., Jiang, J.J., Jin, B., Baluska, F., Samaj, J., Wang, X.L., Lin, J.X.: Spatio-temporal dynamics of the BRI1 receptor and its regulation by membrane microdomains in living *Arabidopsis* cells. - Mol. Plants **8**: 1334-1349, 2015.

Wang, Q., Zhao, Y., Luo, W., Li, R., He, Q., Fang, X., Michele, R. D., Ast, C., Von Wiren, N., Lin, J.: Single-particle analysis reveals shutoff control of the *Arabidopsis* ammonium transporter AMT1;3 by clustering and internalization. – Proc. nat. Acad. Sci. USA **110**: 13204-13209, 2013.

Xin, X.F., Kvitko, B., He, S.Y.: *Pseudomonas syringae*: what it takes to be a pathogen. - Nat. Rev. Microbiol. **16**: 316-328, 2018.

Yu, M., Liu, H., Dong, Z., Xiao, J., Su, B., Fan, L., Komis, G., Samaj, J., Lin, J., Li, R.: The dynamics and endocytosis of Flot1 protein in response to flg22 in *Arabidopsis*. - J. Plant Physio.l **215**: 73-84, 2017.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D., Felix, G., Boller, T.: Bacterial disease resistance in *Arabidopsis* through flagellin perception. - Nature **428**: 764-767, 2004.