New Phytologist

The ROP2-RIC7 pathway negatively regulates light-induced stomatal opening by inhibiting exocyst subunit Exo70B1 in Arabidopsis

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Received: *11 June 2015*
Accepted: *30 July 2015*

New Phytologist (2016) **209:** 624–635
doi: 10.1111/nph.13625

Key words: Exocyst subunit Exo70 family protein B1 (Exo70B1), guard cell, ROP-interactive CRIB motif-containing protein 7 (RIC7), Plant Rho-type (ROP) GTPase 2, stomatal movement, vesicle trafficking.

Summary

- Stomata are the tiny valves on the plant surface that mediate gas exchange between the plant and its environment. Stomatal opening needs to be tightly regulated to facilitate CO_2 uptake and prevent excess water loss. Plant Rho-type (ROP) GTPase 2 (ROP2) is a molecular component of the system that negatively regulates light-induced stomatal opening. Previously, ROP-interactive Cdc42- and Rac-interactive binding motif-containing protein 7 (RIC7) was suggested to function downstream of ROP2. However, the underlying molecular mechanism remains unknown.
- To understand the mechanism by which RIC7 regulates light-induced stomatal opening, we analyzed the stomatal responses of *ric7* mutant Arabidopsis plants and identified the target protein of RIC7 using a yeast two-hybrid screen.
- Light-induced stomatal opening was promoted by *ric7* knockout, whereas it was inhibited by RIC7 overexpression, indicating that RIC7 negatively regulates stomatal opening in Arabidopsis. RIC7 interacted with exocyst subunit Exo70 family protein B1 (Exo70B1), a component of the vesicle trafficking machinery. RIC7 and Exo70B1 localized to the plasma membrane region under light or constitutively active ROP2 conditions. The knockout mutant of Exo70B1 and *ric7/exo70b1* exhibited retarded light-induced stomatal opening.
- Our results suggest that ROP2 and RIC7 suppress excess stomatal opening by inhibiting Exo70B1, which most likely participates in the vesicle trafficking required for light-induced stomatal opening.

Introduction

Stomatal movements regulate gas exchange between plants and the environment, and are thus critical for plant growth and development. Stomatal movement is affected by environmental and internal conditions, such as light and circadian clock signals, atmospheric CO_2 concentrations, humidity, temperature, the plant hormone abscisic acid (ABA), and pathogens. Stomatal opening and closure depend on changes in guard cell volume, which involve ionic transport, net water flux, and reorganization of the actin cytoskeleton. Important signaling molecules that regulate these cellular responses include cytosolic Ca^{2+}, protein kinases and phosphatases, reactive oxygen species, heterotrimeric G proteins, and Plant Rho-type (ROP) small G proteins (Assmann & Wang, 2001; Schroeder *et al.*, 2001).

ROP GTPases, which form a distinct subfamily in the RHO GTPase superfamily, negatively regulate stomatal movements. The model plant Arabidopsis possesses 11 ROP GTPases, which are categorized into four phylogenetic subgroups (Craddock *et al.*, 2012). ROP6 and ROP10 are negative regulators of the stomatal response to ABA (Lemichez *et al.*, 2001; Zheng *et al.*, 2002). Plant Rho-type (ROP) GTPase 2 (ROP2) was reported to function as a negative regulator of stomatal movements, because it inhibited both light-induced stomatal opening and ABA-induced stomatal closure (Jeon *et al.*, 2008; Hwang *et al.*, 2011). ROP GTPases mediate diverse plant responses by interacting with a number of partners. ROP-interactive Cdc42- and Rac-interactive binding motif-containing proteins (RICs) bind activated ROP GTPases and mediate downstream processes (Wu *et al.*, 2001; Fu *et al.*, 2005; Gu *et al.*, 2005). Arabidopsis has eleven structurally divergent RICs, which may have evolved to control various ROP GTPase-dependent pathways. RIC3 and RIC4 regulate cortical actin filament organization in growing pollen tubes (Gu *et al.*, 2005; Cardenas *et al.*, 2008), and RIC1 regulates microtubule-mediated cell morphogenesis (Fu *et al.*, 2005). However, the function of many RICs remains to be determined.

During stomatal movements, guard cells undergo vast changes in surface area and volume (Meckel *et al.*, 2007), and physiological studies suggest that endocytosis and exocytosis are involved in

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these changes (Homann & Thiel, 1999; Blatt, 2000). Recently, the identities of some of the molecules involved in these trafficking systems have been revealed. Syntaxin of plants 121 (Syp 121), which is a member of the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) family and is important for membrane fusion of vesicles, was reported to promote stomatal opening by delivering K^+ channel KAT1 to the plasma membrane (Sutter *et al.*, 2006; Eisenach *et al.*, 2012). PATROL1, a protein harboring the mammalian uncoordinated (MUN) domain which is important for exocytosis in neuronal cells (Augustin *et al.*, 1999), regulates translocation of an Arabidopsis H^+-ATPase (AHA1) to the plasma membrane in response to various stomatal opening stimuli (Hashimoto-Sugimoto *et al.*, 2013), thus facilitating stomatal opening. Other members of membrane trafficking systems are probably also involved in regulating stomatal movement.

In animals, yeast, and plants, the exocyst complex is required for vesicle trafficking. The exocyst complex consists of eight subunits, that is, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (Guo *et al.*, 1999; Matern *et al.*, 2001; Zarsky *et al.*, 2013), and mediates tethering and spatial targeting of post-Golgi vesicles to the plasma membrane before vesicle fusion (Hsu *et al.*, 2004; He & Guo, 2009). Exo70 is important for the physical interaction between the exocyst complex and the plasma membrane (He *et al.*, 2007). Both animal and yeast genomes harbor a single *Exo70* gene, but plant genomes contain many such genes. For example, Arabidopsis has 23 Exo70 family genes, and it is speculated that these genes are differentially expressed in different cell types and play different functions in growth and development, based on their cell type- or cargo-specific roles (Li *et al.*, 2010).

Only a few plant Exo70s have been characterized to date. Exo70A1 plays a crucial role in pollen tube growth, fertility, and tracheary element development (Synek *et al.*, 2006; Li *et al.*, 2013b). Exo70C1 is important for pollen tube growth, and Exo70E2 is associated with exocyst-positive organelle (exosome)-mediated exocytosis (EXPO), which does not involve the conventional exocytic pathway (Li *et al.*, 2010; Wang *et al.*, 2010). Exo70B2 and Exo70H1 are related to plant defense against pathogen attack (Stegmann *et al.*, 2012). Exocyst subunit Exo70 family protein B1 (Exo70B1) is an important regulator of autophagosome formation and autophagy-related Golgi-independent import into the vacuole (Kulich *et al.*, 2013) and is involved in the immune response to pathogens (Stegmann *et al.*, 2013; Zhao *et al.*, 2015).

We previously reported that RIC7 functions in ROP2-mediated light-induced stomatal opening. We found that RIC7 interacts with active ROP2 *in vitro* and that RIC7 overexpression, similar to active ROP2 overexpression, inhibits light-induced stomatal opening in *Vicia faba* guard cells (Jeon *et al.*, 2008). However, the mechanism by which ROP2 and RIC7 regulate light-induced stomatal opening remained unclear. Here, we confirm that RIC7 is a negative regulator of light-induced stomatal opening and identify Exo70B1 as a downstream target of RIC7. We provide several lines of evidence that RIC7 interacts with Exo70B1 and, furthermore, that Exo70B1 is a positive component of light-induced stomatal opening. Thus, this work reveals a novel component of the small G protein signaling pathway that modulates the response of guard cells.

Materials and Methods

Plant material

Wild-type, mutant, and transgenic *Arabidopsis thaliana* lines (ecotype Columbia (Col-0)) were grown in a growth chamber under 90 $\mu mol\ m^{-2}\ s^{-1}$ white light at 22°C with a 16 h : 8 h, light : dark photoperiod. For stomatal measurements, fully expanded young leaves from 3- to 4-wk-old plants were used. *Vicia faba* var. minor cv. Geongang (Jeilseed, Korea) plants were grown in a glasshouse at 25°C. The fully expanded leaves of 4- to 5-wk-old *V. faba* plants were used for transient transformation using particle bombardment.

Isolation of the knockout mutants and quantification of *RIC7* and *Exo70B1* gene expression

Seeds of *ric7* (*ric7-1*; GK062G02) and *exo70b1* (*exo70b1-1*; GK114C03 and *exo70b1-2*; GK156G02) were obtained from the Arabidopsis Stock Center. Reverse transcriptase (RT)-PCR was performed to quantify the expression levels of *RIC7* and *Exo70B1*. Total RNA was extracted from the rosette leaves of 4-wk-old plants and then reverse transcribed into cDNA using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA). PCR was performed using gene-specific primers with 40 cycles each with denaturation at 95°C for 30 s, annealing at 56°C for 30 s for *Exo70B1* or 48°C for 30 s for *RIC7*, and extension at 72°C for 45 s. Gene-specific primers were *Tubulin8*-forward (5′-CTCACAGTCCCGGAGCTGAC AC-3′); *Tubulin8*-reverse (5′-GCTTCAGTGAAC TCCATC T-CGT-3′); *RIC7*-forward (5′-TACATATCTCAAGTTTTTG CAATAGAAGG-3′); *RIC7*-reverse (5′-CGTCTTCAAATTGA GGCATAGATCTATCG-3′); *Exo70B1*-forward (5′-TTCGTT TAT-GGAGGTTTGTCG-3′); and *Exo70B1*-reverse (5′-TGG TCATTTAGCAGGTG GTTC-3′). Representative results of two independent experiments are presented. Quantitative real-time PCR (Q-PCR) was carried out using a Takara TP800 thermal cycler and Takara SYBR RT-PCR Kit (Takara Bio, Kyoto, Japan), following the manufacturer's instructions. Expression levels of *RIC7* and *Exo70B1* were normalized against that of *Tubulin8*.

Stomatal movement assay

Stomatal movement was measured as described by Jeon *et al.* (2008). For stomatal opening, the intact rosette leaves of Arabidopsis and transformed *V. faba* leaves were floated on assay buffer containing 30 mM KCl and 10 mM MES, pH 6.1, under 180 $\mu mol\ m^{-2}\ s^{-1}$ white light at 23°C. For the stomatal closure assay, the intact rosette leaves of Arabidopsis were first floated on assay buffer containing 30 mM KCl and 10 mM MES, pH 6.1, for 3 h under 180 $\mu mol\ m^{-2}\ s^{-1}$ white light at 23°C to open

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stomata, and were then transferred to 10 mM KCl and 10 mM MES (pH 6.1) buffer containing 3 μM ABA. Abaxial epidermal strips peeled immediately before observation and stomatal apertures (pore width/length) were measured using a Zeiss Axiskop2 microscope (Carl Ziess Inc.; http://www.zeiss.com/micro) and Interactive Measurement software (Axio Vision, Jena, Germany).

Plants were grown in a controlled environment. However, there were seasonal variations in the extent and rate of stomatal opening. Stomatal opening tended to be more rapid in summer, while it was slower in winter. For the analysis of stomatal movement, we used multiple leaves from multiple plants (one leaf per plant, of similar size and at the same position) for individual experiments. In addition, we combined the results of more than three independent experiments performed in a single season.

Transient expression in *V. faba* guard cells

For transient expression of Arabidopsis genes and constructs in *V. faba*, the coding regions of *CA-rop2* (G15V), *DN-rop2* (D121A), *RIC7*, and *Exo70B1* were fused to the C terminus of CFP, YFP, or RFP under the control of the 35S promoter in the 326 vector. *Vicia faba* guard cells were biolistically transformed following the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, DNA-coated gold particles (diameter 1 μm) were fired into the abaxial surface of *V. faba* leaves at a helium pressure of 1350 psi and under a vacuum of 28 inches of mercury. Bombarded leaves were kept at 22°C in darkness. After 12 h to 4 d, epidermal fragments were peeled from the bombarded sites and fluorescence distribution was observed.

Yeast two-hybrid assay

Exo70B1 was inserted into pYEStrp2 and *RIC7* into pPC62LexA, and the resulting plasmids were introduced into the L40 yeast strain using a Frozen-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA, USA). Yeast transformants were selected on synthetic defined (SD)-Leu-Trp medium. An overnight culture of transformed yeast cells was diluted with sterile distilled water to $OD_{660} = 0.5$, and then serially diluted to 1 : 30, 1 : 900, and 1 : 27 000 with sterile distilled water. Ten microliters of each dilution was spotted onto SD–His–Leu–Trp plates supplemented with 10 mM 3-aminotriazole and plates were incubated at 30°C for 2 d to test the interaction between Exo70B1 and RIC7.

In vitro Exo70B1-RIC7 binding assay

The maltose-binding protein (MBP)-RIC7 fusion protein, glutathione S-transferase (GST), and GST-Exo70B1 were expressed in *Escherichia coli* and purified (Wu *et al.*, 2001). GST (50 μg) or GST-Exo70B1 (50 μg) was preincubated with 500 μl of binding buffer (20 mM Tris, pH 7.9, 20% glycerol, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 M NaCl, and 1X protease inhibitor) and 100 μl of agarose-GSH beads for 30 min at room temperature and purified MBP-RIC7 (200 μg) was added. After 2 h of incubation at 4°C, agarose beads were pelleted and rinsed four times with binding buffer. Proteins in the pellet were separated by SDS-PAGE, and western blotting was performed using anti-GST antibody conjugated with horseradish peroxidase (1 : 5000 dilution; Amersham Bioscience, Little Chalfont, UK) or anti-MBP antibody (1 : 10 000 dilution; New England Biolabs, Ipswich, MA, USA). Anti-mouse antibodies conjugated to alkaline phosphatase (1 : 5000 dilution; Promega) were used to detect anti-MBP.

Bimolecular fluorescence complementation (BiFC) assay

RIC7 and *ROP2* were cloned into pVYNE(R) (nYFP) and *Exo70B1* was cloned into pVYCE(R) (cYFP; Gehl *et al.*, 2009) using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen, Waltham, MA, USA). Gene constructs were transiently expressed in the leaves of 4- to 6-wk-old *Nicotiana benthamiana* plants grown in soil under glasshouse conditions using *Agrobacterium*-mediated infiltration and the plants were then incubated for 3–4 d. Fluorescence of the lower epidermis of leaf discs was visualized using a Zeiss LSM 510 Meta Laser scanning microscope (Zeiss; http://www.zeiss.com/). Fluorescence was detected using the 530 to 600 nm spectral settings for emission and 514 nm for excitation. Calnexin6 (Cnx6) is a subunit of molybdopterin synthase, which has the least physiological relevance to Exo70, and forms a homodimer (Gehl *et al.*, 2009). Cnx6 was used as a control in the BiFC assay. Leaves were co-transformed with pVYNE(R)-Cnx6 and pVYCE(R)-Exo70B1 as the negative control and with pVYNE(R)-Cnx6 and pVYCE(R)-Cnx6 as the positive control.

ABA measurement

Rosette leaves (100 mg) of 4-wk-old wild-type and *exo70b1-1* plants were collected and frozen immediately in liquid nitrogen, ground to powder, and extracted with cold 80% methanol. The samples were then centrifuged at 13 800 ***g*** for 2 min at 4°C, and the supernatant was filtered through a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) to remove pigments. The supernatant was completely dried in a SpeedVac and then Tris-buffered saline buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, and 3 mM NaN_3) was added to a final volume of 300 μl. Endogenous ABA concentrations were quantified using a Phytodetekt ABA Enzyme Immunoassay Test Kit (Agdia, Elkhart, IN, USA) in an enzyme-linked immunosorbent assay (ELISA) reader, according to the manufacturer's instructions.

Co-localization analysis of Exo70B1 with organelle markers

The localization of Exo70B1 was compared with that of Golgi and trans-Golgi network/early endosome (TGN/EE) markers. Intact *V. faba* leaves were bombarded with RFP-Exo70B1 mixed with ST-GFP (a Golgi marker; Kim *et al.*, 2001), YFP-SYP61 (a TGN/EE marker; Uemura *et al.*, 2004) or Exo84b-DsRed (a marker that labels a compartment of the exocyst complex; Fendrych *et al.*, 2010). After incubation under darkness for

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2–4 d, the leaves were observed using a Zeiss LSM 510 Meta Laser scanning microscope (Zeiss; http://www.zeiss.com/).

Results

RIC7 suppresses both stomatal opening and closure in Arabidopsis

To analyze RIC7 function, we introduced a *RIC7 promoter::β-glucuronidase* reporter into Arabidopsis and examined expression of the reporter construct in 1- to 3-wk-old T2 transgenic plants. *RIC7* promoter activity was high in young leaves, the shoot apical meristem, and the root vasculature (Supporting Information Fig. S1a–c). *RIC7* expression was detected in most cell types of the leaf, and was highly expressed in stomatal guard cells (Fig. S1d,e).

We previously demonstrated that RIC7 functions downstream of ROP2 as a negative regulator of light-induced stomatal opening, by ectopically expressing Arabidopsis RIC7 in *V. faba* guard cells (Jeon *et al.*, 2008). In this study, we further examined the role of RIC7 in light-induced stomatal opening by using a series of Arabidopsis *RIC7* transgenic lines, including a *ric7* knockout mutant (*ric7-1*, GK_062G02) that harbors a T-DNA insertion in the second intron of *RIC7* (Fig. 1a,b); *RIC7* complementation lines (*ric7-1*/*RIC7promoter::RIC7-gDNA*, comp1, comp2 and comp4), in which genomic *RIC7* driven by its native promoter is transgenically expressed in the *ric7-1* background (Figs 1b, S2a); and *RIC7* overexpression (OX) lines (*OX1*, *OX3* and *OX8*), in which *RIC7* is constitutively expressed under the control of the 35S promoter (Figs 1c, S2b).

Light-induced stomatal opening was faster in *ric7-1* than in the wild-type: upon 1–2 h of light irradiation, *ric7-1* exhibited stomata that were *c.* 18% more widely open than those of the wild-type (Figs 1d, S3a). However, stomatal opening was similar between *ric7-1* and the wild-type observed after 3 h of light irradiation (data not shown). Light-induced stomatal opening was restored to wild-type rates in the *RIC7* complementation lines (comp1, comp2, and comp4), indicating that the rapid light-induced stomatal opening of *ric7-1* was caused by RIC7 loss of function (Figs 1d, S3a). By contrast, light-induced stomatal opening was slower in the *RIC7* overexpression lines, *OX1*, *OX3*, and *OX8*, than in the wild-type (Figs 1e, S3b). These results confirm that RIC7 plays a negative role in light-induced stomatal opening.

Next, we examined ABA-induced stomatal closure in the various *RIC7* mutant and transgenic plants. ABA-induced stomatal closure was faster in *ric7-1* than in the wild-type. Differences in ABA-induced stomatal closure between the wild-type and *ric7-1* were evident as early as 30 min after treatment. In the *RIC7* complementation lines, the rate of ABA-induced stomatal closure was similar to that in the wild-type (Fig. 1f). By contrast, *RIC7 OX* induced slower ABA-induced stomatal closure (Fig. 1g). These results indicate that RIC7 suppresses both light-induced and ABA-induced stomatal movements.

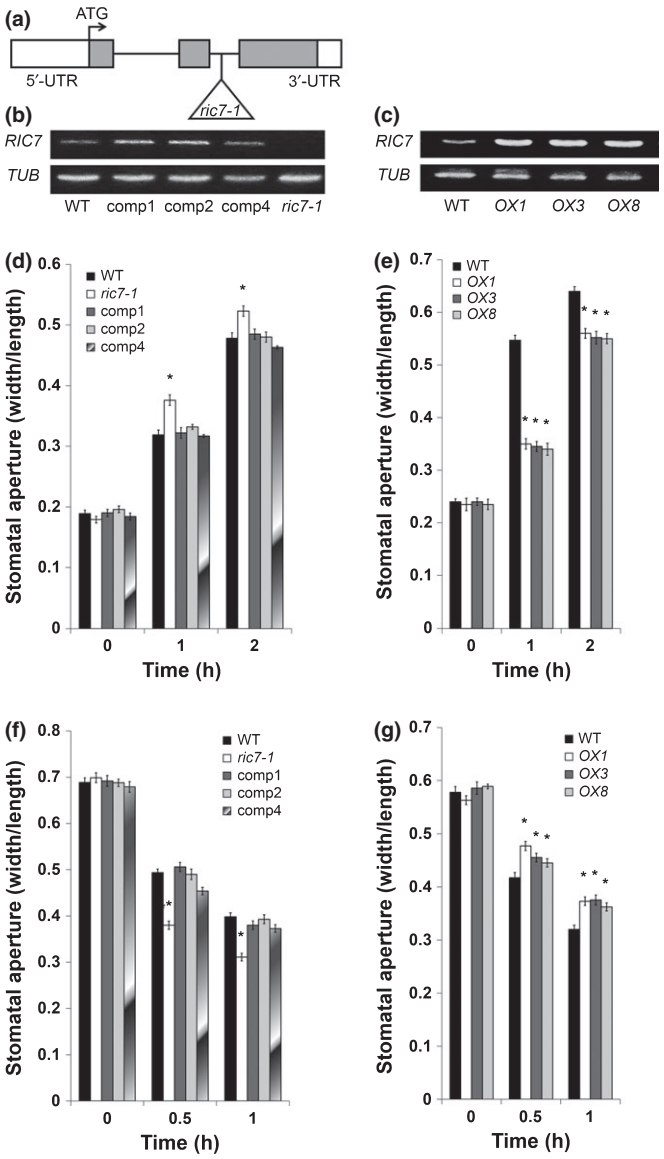

Fig. 1 Stomatal responses to light and abscisic acid (ABA) in Arabidopsis plants expressing various levels of *RIC7*. (a) T-DNA insertion locus in *ric7-1*. Unfilled boxes, untranslated regions (UTRs); gray boxes, exons; black lines, introns. (b, c) Transcript levels of *RIC7*. (b) *ric7-1*, the wild-type (WT; Col-0), and three independent RIC7 complementation lines (comp1, comp2, and comp4). (c) Expression levels of *RIC7* in *RIC7* overexpression (OX) (*OX1*, *OX3* and *OX8*) and wild-type (Col-0) lines. *Tubulin8* (*TUB*) is an internal control. (d, e) Light-induced stomatal opening of *RIC7* mutants and transgenics in comparison to the wild-type. (d) *ric7-1*, the wild-type, and *RIC7* complementation lines. (e) *RIC7 OX* lines and the wild-type. Plants were irradiated with white light of 180 μmol m^{-2} s^{-1} for the indicated periods of time before stomatal apertures were measured. Mean values (± SE) of the combined results of three independent experiments are presented ($n > 340$). Statistically significant differences from the wild-type control (Student's *t*-test): *, $P < 0.02$. The extent of stomatal movement varied depending on the season; in the summer, stomata exhibited larger initial apertures and faster light-induced opening (e) than in the winter (d). (f, g) ABA-induced stomatal closure in *RIC7* mutants and transgenics in comparison to the wild-type. (f) *ric7-1*, the wild-type, and *RIC7* complementation lines. (g) *RIC7 OX* lines and the wild-type. Plants were irradiated with white light of 180 μmol m^{-2} s^{-1} for 3 h before treatment with ABA (1 μM) and stomatal apertures were measured at the indicated time-points after ABA treatment. Mean values (± SE) of the combined results of three independent experiments are presented ($n > 340$; Student's *t*-test; *, $P < 0.02$).

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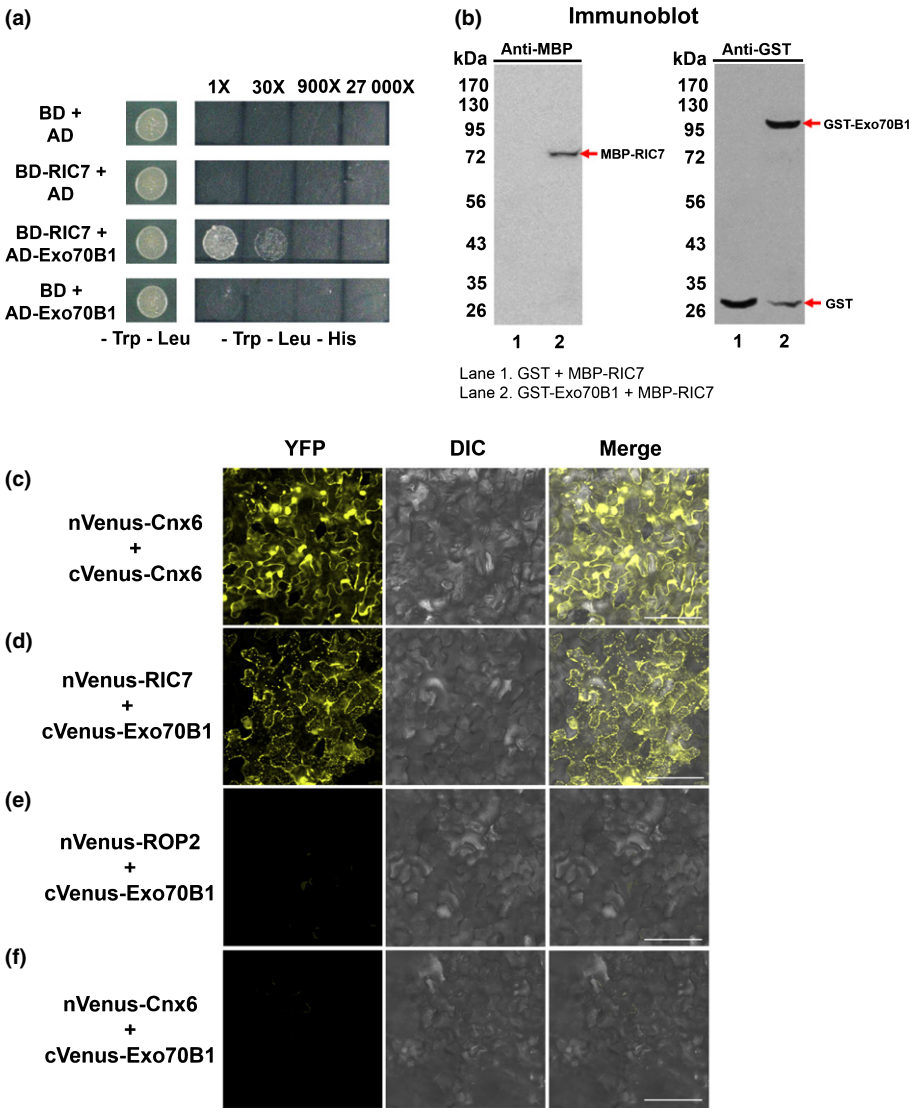

Fig. 2 Exo70B1 binds directly to RIC7, but not to ROP2. (a) Yeast two-hybrid assays showing interactions between RIC7 and Exo70B1. An overnight culture of transformed yeast cells was diluted with sterile distilled water to $OD_{660} = 0.5$, and then serially diluted to 1 : 30, 1 : 900, and 1 : 27 000 with sterile distilled water, and 10 μl of each dilution was spotted onto SD–His–Leu–Trp plates supplemented with 10 mM 3-aminotriazole and incubated at 30°C for 2 d to test the interaction. (b) *In vitro* interaction between maltose-binding protein (MBP)-RIC7 and glutathione S-transferase (GST)-Exo70B1 in a pull-down assay. MBP-RIC7 was mixed with GST-Exo70B1 or free GST. Exo70B1-bound RIC7 was then pulled down with glutathione-conjugated agarose beads and quantified using SDS-PAGE and immunoblot analysis. Immunoblots with anti-MBP (left) or -GST (right) antibodies. (c–f) *In vivo* interaction between RIC7 and Exo70B1 in a bimolecular fluorescence complementation (BiFC) assay. Representative confocal laser scanning microscopy images are presented showing BiFC signals from tobacco leaf epidermal cells expressing (c) Venus-N-Calnexin6 (Cnx6) and Venus-C-Cnx6 (positive control), (d) Venus-N-RIC7 and Venus-C-Exo70B1, (e) Venus-N-ROP2 and Venus-C-Exo70B1, or (f) Venus-N-Cnx6 and Venus-C-Exo70B1. VN-Cnx6 and VC-Cnx6 were co-expressed as a positive control, as Cnx6 is a subunit of molybdopterin synthase that forms homodimers. VN-Cnx6 and VC-Exo70B1 were co-expressed as a negative control, as Cnx6 has no physiological relationship with Exo70B1. Bars, 100 μm.

Exo70B1 interacts with RIC7

To elucidate the mechanism by which RIC7 negatively regulates stomatal movements, we searched for interaction partners of RIC7 in a yeast two-hybrid screen. Using RIC7 as bait, we identified multiple candidate binding partners of RIC7, including Exo70B1 (Fig. 2a). We then tested whether RIC7 directly interacts with these candidates using an *in vitro* pull-down assay. Only Exo70B1 directly interacted with RIC7 (Fig. 2b). GST-fused Exo70B1 pulled down MBP-fused RIC7, whereas free GST did not (Fig. 2b). We confirmed the direct interaction between Exo70B1 and RIC7 using a BiFC assay (Fig. 2c–f). Exo70B1 fused to the C-terminal fragment of Venus YFP (VC-Exo70B1) was co-expressed with either RIC7 or ROP2 fused to the N-terminal fragment of Venus YFP (VN-RIC7 or VN-ROP2, respectively) in tobacco (*Nicotiana benthamiana*) leaves using agro-infiltration. In tobacco leaves co-transformed with VN-RIC7 and VC-Exo70B1, strong YFP fluorescence was observed 72 h after infiltration (Fig. 2d). However, co-expression of VN-ROP2 and VC-Exo70B1 resulted in only very weak or no YFP fluorescence (Fig. 2e). These results support the notion that Exo70B1 directly interacts with RIC7 *in vivo*, but not with ROP2.

A web data search (Arabidopsis eFP browser; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) revealed that Exo70B1 has the highest level of expression in mature stomatal guard cells among the 23 Exo70 members present in Arabidopsis (Table S1), which supports its role in stomatal movements. Other members of the Exo70 family were expressed at levels that were less than half of that of Exo70B1 in guard cells.

Exo70B1 is a positive regulator of light induced-stomatal opening in Arabodopsis

To understand the function of Exo70B1 in guard cells, we analyzed stomatal movements of two independent alleles of *exo70b1* knockout Arabidopsis mutants. Both *exo70b1-1* (GK_114C03) and *exo70b1-2* (GK_156G02) have T-DNA inserted in the single exon of *Exo70B1*, resulting in complete knocking out of *Exo70B1* expression (Kulich *et al.*, 2013). Light-induced stomatal opening was retarded in *exo70b1* mutants relative to the wild-

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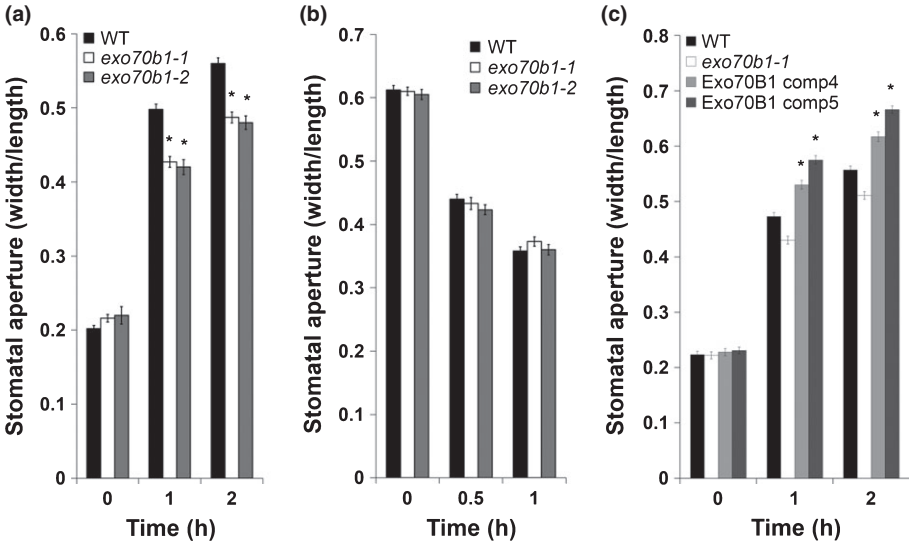

Fig. 3 Exo70B1 is a positive regulator of light-induced stomatal opening in Arabidopsis. (a) Light-induced stomatal opening in *exo70b1-1* and *exo70b1-2* in comparison to the wild-type (WT). Plants were irradiated with white light of 180 μmol m^{-2} s^{-1} for the indicated periods of time before stomatal apertures were measured. Mean values (± SE) of the combined results of three independent experiments are presented ($n > 435$; Student's *t*-test; *, $P < 0.02$). (b) Abscisic acid (ABA)-induced stomatal closure in *exo70b1-1* and *exo70b1-2*, in comparison to the wild-type. Plants were irradiated with white light of 180 μmol m^{-2} s^{-1} for 3 h before treatment with ABA (1 μM). Mean values (± SE) of the combined results of three independent experiments are presented ($n > 435$; Student's *t*-test; *, $P < 0.02$). (c) Light-induced stomatal opening in Exo70B1 comp4 and 5, the wild-type, and *exo70b1-1*. Plants were irradiated with white light of 180 μmol m^{-2} s^{-1} for the indicated periods of time before stomatal apertures were measured. Mean values (± SE) of the combined results of three independent experiments are presented ($n > 214$; Student's *t*-test; *, $P < 0.02$).

type: stomata of *exo70b1-1* and *exo70b1-2* opened less than those of the wild-type by *c.* 14% at 1 h (Fig. 3a). However, ABA-induced stomatal closure of *exo70b1* was similar to that of the wild-type (Fig. 3b). A previous report showed that the *exo70b1* mutant accumulated higher concentrations of ABA than did the wild-type (Kulich *et al.*, 2013). As ABA suppresses stomatal opening, we tested whether the *exo70b1* mutant accumulated high concentrations of ABA under our experimental conditions, but did not find a significant difference in ABA concentrations between 4-wk-old *exo70b1* and wild-type plants, which were the same age as those used for the stomatal movement assays (Fig. S4). Furthermore, the mutant did not exhibit the previously described cell death phenotype (Kulich *et al.*, 2013) at this age, but displayed accelerated cell death by 6 wk after germination (Fig. S5). This result excluded the possibility that the difference in stomatal opening movement observed in young plants was attributable to ABA concentrations or other defense-related processes.

Next, we generated *Exo70B1* complementation lines (*exo70b1-1/35Spromoter::Exo70B1-CDS-sGFP*, Exo70B1 comp4 and comp5) in which *Exo70B1* was constitutively expressed under the control of the 35S promoter in the *exo70b1-1* background (Fig. S6a). Exo70B1-sGFP protein was expressed in these lines (Fig. S6b). To determine whether the *Exo70B1* complementation lines recovered the retarded stomatal opening phenotype, we compared the light-induced stomatal opening of the complementation lines with that of the wild-type and *exo70b1-1* plants. Light-induced stomatal opening was faster in Exo70B1 comp4 and comp5 than in the wild-type: after 1–2 h of light irradiation, the stomatal apertures of Exo70B1 comp4 and comp5 were *c.* 12% larger than were those of the wild-type (Fig. 3c). These results confirm that Exo70B1 positively regulates light-induced stomatal opening.

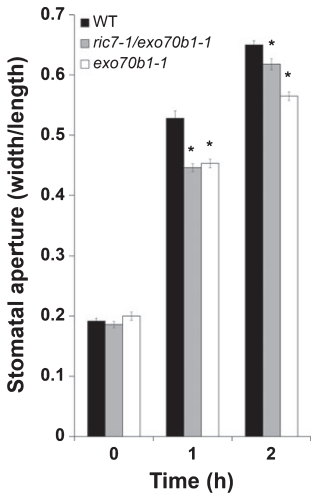

Fig. 4 Light-induced stomatal opening is retarded in *ric7-1* and *exo70b1-1* double knockout (*ric7-1/exo70b1-1*) Arabidopsis. Light-induced stomatal opening in *ric7-1/exo70b1-1* was examined in comparison to the wild-type (WT) and *exo70b1-1*. Plants were irradiated with white light of 180 μmol m^{-2} s^{-1} for the indicated periods of time before stomatal apertures were measured. Mean values (± SE) of the combined results of three independent experiments are presented ($n > 229$; Student's *t*-test; *, $P < 0.02$).

Exo70B1 acts downstream of RIC7

To establish whether Exo70B1 functions downstream of RIC7, we generated a *ric7-1/exo70b1-1* double knockout mutant (by crossing *ric7-1* with *exo70b1-1*) and examined its light-induced stomatal opening. Light-induced stomatal opening was slower in *ric7-1/exo70b1-1* than in the wild-type: after 1 h of irradiation,

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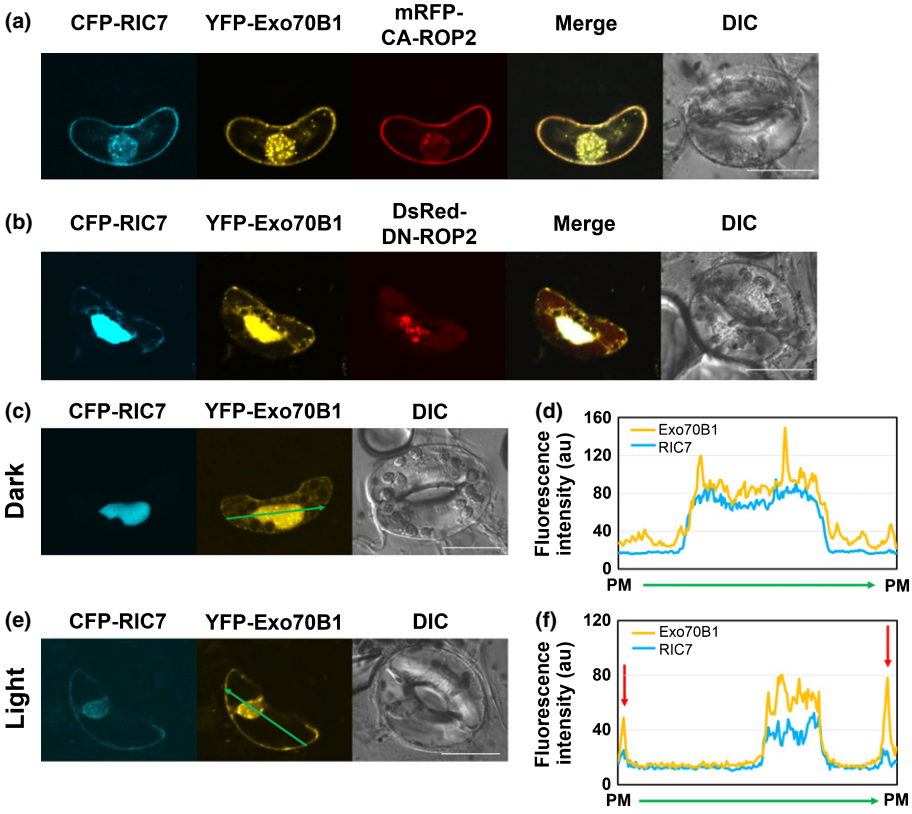

Fig. 5 RIC7 and Exo70B1 are re-localized in response to ROP2 activation and a light stimulus. (a, b) Localization of YFP-Exo70B1 in *Vicia faba* guard cells, when expressed together with CFP-RIC7 and mRFP-CA-ROP2 (a) or with CFP-RIC7 and DsRed-DN-ROP2 (b), respectively. Differential interference contrast (DIC) images of guard cells are given on the right. (c–f) Localization of YFP-Exo70B1 and CFP-RIC7 in *V. faba* guard cells under dark (c, d) and light (e, f) conditions. (c, e) Represent active guard cell images. (d, f) Fluorescence intensity profiles of YFP-Exo70B1 and CFP-RIC7 in *V. faba* guard cells in (c, e). Fluorescence intensity was scanned along lines across the cell (green arrows, c, e). Red arrows indicate co-localization of YFP-Exo70B1 and CFP-RIC7 at the plasma membrane (PM) in guard cells irradiated with white light for 2 h (f). Confocal microscopy images of *V. faba* guard cells were obtained at the median plane. Bars, 20 μm.

the stomatal apertures of the *ric7-1/exo70b1-1* plants were *c.* 12% smaller than were those of the wild-type (Fig. 4). The retarded stomatal opening phenotype of *ric7-1/exo70b1-1* was similar to that of *exo70b1-1* (Fig. 4) and opposite to that of *ric7-1* (Fig. 1d), suggesting that Exo70B1 is a downstream target of RIC7.

RIC7 and Exo70B1 are re-localized by light irradiation and active ROP2

Because the localization of RIC7 changes depending on ROP2 activity (Jeon *et al.*, 2008), we examined whether the localization of Exo70B1 was also affected by the status of ROP2. To test this, we examined the localization of Exo70B1 in *V. faba* guard cells expressing either constitutively active ROP2 (CA-ROP2) or a dominant negative form of ROP2 (DN-ROP2) fused with RFP. When co-expressed with mRFP-CA-ROP2, CFP-RIC7 and YFP-Exo70B1 co-localized to the plasma membrane region, and also exhibited punctate localization in the cytoplasm and nucleus (Fig. 5a). By contrast, when expressed together with DsRed-DN-ROP2, CFP-RIC7 and YFP-Exo70B1 were not in the plasma membrane region; CFP-RIC7 was mainly localized to the nucleus, whereas YFP-Exo70B1 was mainly localized in a punctate pattern in the cytoplasm and the nucleus (Fig. 5b). Similar localization patterns were observed when different fluorescent proteins were fused to the test proteins (i.e. YFP-CA-ROP2 or YFP-DN-ROP2 and DsRed-Exo70B1), excluding the possible influence of the fluorescent fusion protein (Fig. S7a,b).

We then tested whether light, a natural stimulus that induces stomatal opening and activates ROP2, relocates RIC7 and Exo70B1. We bombarded *V. faba* guard cells with CFP-RIC7 and YFP-Exo7B1, and incubated the cells under darkness for 2 d. The guard cells were then observed either directly (dark control) or after 2 h of irradiation with white light, which opened stomata fully. Under dark conditions, RIC7 was localized mostly to the nucleus, and Exo70B1 was present in a punctate pattern in the cytosol and in the nucleus (Fig. 5c,d), similarly to when Exo70B1 was co-expressed with DN-ROP2 (Fig. 5b). After a 2-h light treatment, a subset of RIC7 and Exo70B1 proteins was localized to the plasma membrane region (Fig. 5e,f), similarly to when these proteins were co-expressed with CA-ROP2 (Fig. 5a). These results suggest that light-mediated ROP2 activation alters the localization of RIC7 and Exo70B1.

Exo70B1 is co-localized with Exo84B

If Exo70B1 functions as a component of the exocyst complex in guard cells, it should co-localize with Exo84B, an established subunit of the exocyst complex (Fendrych *et al.*, 2010). We expressed YFP-Exo70B1 and Exo84B-DsRed in *V. faba* guard cells, and found that Exo70B1 was partially co-localized with Exo84B both under darkness and light (Fig. 6). Under darkness, both Exo70B1 and Exo84B were uniformly distributed in punctate patterns throughout the cell (Fig. 6a), and most of the Exo70B1 puncta coincided with Exo84B puncta, except in the nucleus which lacked Exo84B (Fig. 6a). In cells irradiated with white light for

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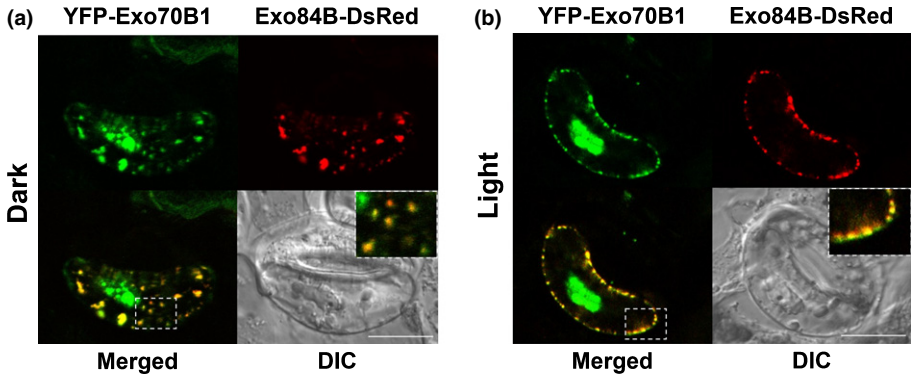

Fig. 6 Exo70B1 is co-localized with Exo84B in *Vicia faba* guard cells. Intact *V. faba* guard cells were co-transformed with YFP-Exo70B1 and Exo84B-DsRed (a component of the exocyst complex) under dark (a) and light (b) conditions. Differential interference contrast (DIC) images of guard cells are given at the bottom right. Insets are enlarged images of the boxed areas. Confocal microscopy images of *V. faba* guard cells were obtained at the median plane. Bars, 20 μm.

3 h, Exo70B1 was also in punctate patterns co-localized with Exo84B, except in the nucleus, but in this case, the two proteins were in the periphery of guard cells (Fig. 6b).

Exo70B1 is not localized to the Golgi or TGN/EEs

Next we examined whether the punctate fluorescence of Exo70B1 in the cytosol represents localization to the Golgi or TGN/EE, components of the conventional exocytosis pathway. For this purpose, we expressed ST-GFP (Golgi marker; Kim *et al.*, 2001) and YFP-syntaxin of plants 61 (SYP61; a TGN/EE marker; Uemura *et al.*, 2004) in *V. faba* guard cells together with DsRed-fused Exo70B1. We examined whether Exo70B1 localized to the Golgi or TGN/EE by counting the number of dots in which DsRed fluorescence overlapped with GFP or YFP fluorescence in cells co-expressing DsRed-Exo70B1 and ST-GFP or YFP-SYP61, respectively. Only 3–4% of the ST-GFP signal and 11–12% of the YFP-SYP61 signal co-localized with Exo70B1 in darkness (Figs 7a,c, S8a,c) or light (Figs 7b,d, S8b,d). The Pearson values of correlation for Golgi or TGN/EE and Exo70B1 were 0.02 and 0.06, respectively, indicating no correlation. Similar results were found when using mCherry-fused Exo70B1 instead of DsRed-fused Exo70B1 (Fig. S9). Thus, Exo70B1 does not seem to be localized to the Golgi or TGN/EE.

To confirm the subcellular localization of Exo70B1 in Arabidopsis, we observed the spatial distribution of Exo70B1 in the guard cells, the pavement cells, root hairs, and root tip cells of plants expressing Exo70B1-sGFP (Figs 3c, S6). Exo70B1 was localized to the nucleus and cytosol, and in a punctate pattern (Fig. 7e), similar to observations in *V. faba* guard cells.

Discussion

Inhibition of stomatal opening by ROP2 and RIC7 as a pacing mechanism

Light induces stomatal opening, which facilitates the CO_2 uptake necessary for photosynthesis. Whereas light-induced stomatal opening is essential for efficient plant growth, prolonged stomatal opening may lead to excessive water loss, which might threaten a plant's survival. Therefore, plants require a pacing mechanism that regulates stomatal opening, and we reported previously that ROP2 serves such a function by negatively regulating light-induced stomatal opening (Jeon *et al.*, 2008). We now report that RIC7, a downstream factor of ROP2 (Jeon *et al.*, 2008), binds to a component of the exocyst complex, Exo70B1, thereby inhibiting stomatal opening.

RIC7 suppresses both stomatal opening and closing movements, similarly to active ROP2

In a previous study, we found that RIC7 overexpression, similar to active ROP2 overexpression, inhibits light-induced stomatal opening in *V. faba* guard cells (Jeon *et al.*, 2008). In this study, we used Arabidopsis *RIC7* mutant and transgenics to show that RIC7 is a negative regulator of both light-induced stomatal opening and ABA-induced stomatal closure. Both light-induced stomatal opening and ABA-induced stomatal closure were accelerated in a *ric7* mutant (Fig. 1d,f). By contrast, stomatal responses to light and ABA were retarded in *RIC7* overexpression plants (Fig. 1e,g). These results suggest that RIC7, like ROP2, negatively regulates both stomatal opening and closure (Jeon *et al.*, 2008; Hwang *et al.*, 2011). As RIC7 is a downstream effector of ROP2 (Jeon *et al.*, 2008), these results indicate that RIC7 mediates ROP2 signals to prevent excessive stomatal opening and closure in Arabidopsis. Interestingly, the extent of stomatal opening and closure was affected to a greater extent in the *ROP2* mutant and transgenics than in the *RIC7* mutant and transgenics (compare Fig. 5d in Jeon *et al.*, 2008 with Fig. 1d,e). Previous studies revealed that ROP2 regulates multiple signaling pathways by altering the activity of many downstream targets. Thus, it is possible that ROP2 regulates stomatal opening and closure not only via RIC7, but also by interacting with other downstream factors.

RIC7 acts as a linker between ROP2 and Exo70B1

In yeast and animal systems, Rho GTPases bind directly to Exo70 (Adamo *et al.*, 1999; Dong *et al.*, 2005; He *et al.*, 2007). However, our results suggest that RIC7 is a linker protein that connects ROP2 with Exo70B1. Exo70B1 directly interacted with RIC7 in an *in vitro* pull-down assay (Fig. 2b), as well as in a BiFC assay (Fig. 2d). However, Exo70B1 did not bind to ROP2 directly (Fig. 2e). This difference probably exists because plant

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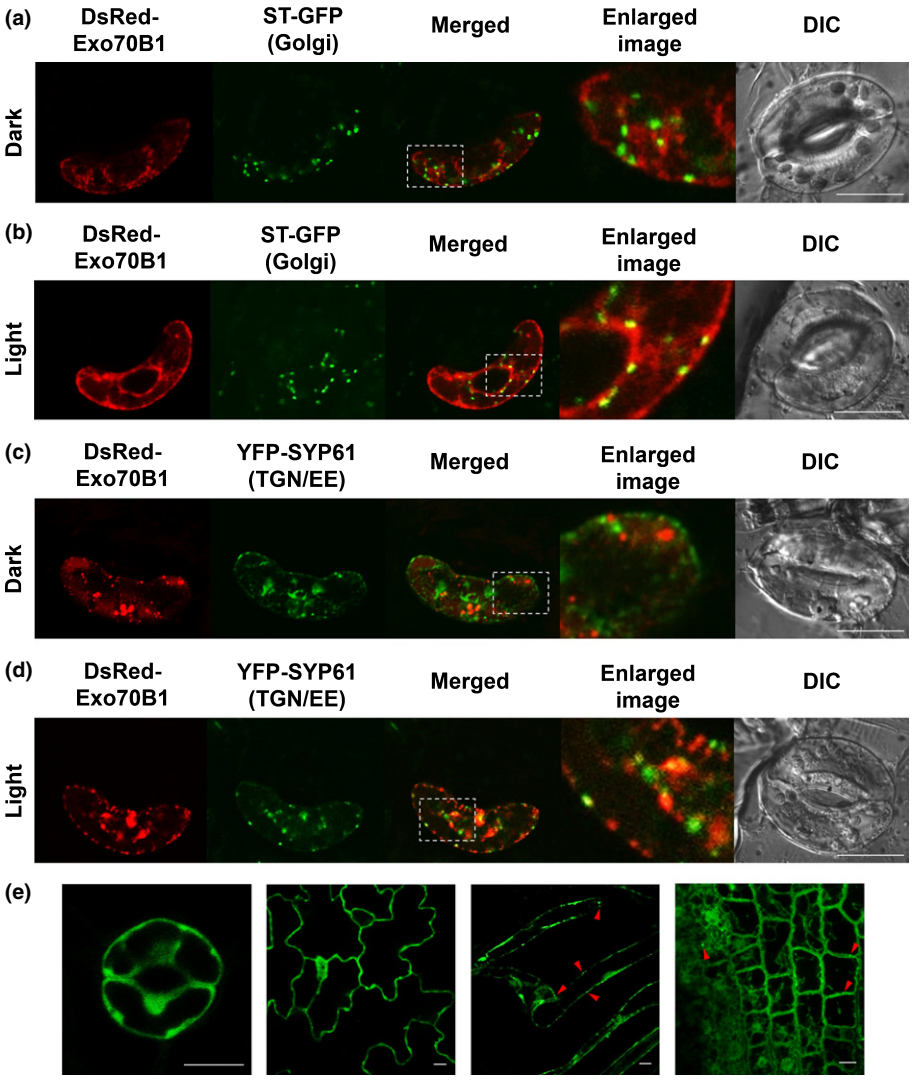

Fig. 7 Exo70B1 is not localized to the Golgi or trans-Golgi network (TGN)/early endosome (EE). (a, b) Images of intact *Vicia faba* guard cells co-transformed with DsRed-Exo70B1 and the Golgi marker ST-GFP. Representative images under dark (a) and light (b) conditions are presented. (c, d) Images of intact *V. faba* guard cells co-transformed with DsRed-Exo70B1 and the TGN/EE marker YFP-SYP61. Representative images under dark (c) and light (d) conditions are presented. Transformed leaves were kept under dark or irradiated with white light of 180 μmol m^{-2} s^{-1} for 3 h. Confocal microscopy images of *V. faba* guard cells were obtained at the median plane. Bars, 20 μm. Enlarged images of the boxed areas are presented. Differential interference contrast (DIC) images of guard cells are given on the right. (e) Localization of Exo70B1-sGFP in Arabidopsis guard cells, pavement cells, root hairs, and root tip cells. Red arrowheads indicate punctate localization of Exo70B1. Seven-day-old Exo70B1-sGFP seedlings were used. Bars, 10 μm.

Exo70 proteins do not have a conserved C-terminal-binding motif, which mediates the Rho-Exo70 interaction in animal and yeast cells (Zarsky *et al.*, 2009). This is not surprising, because Arabidopsis interactor of constitutive active ROPs 1 (ICR1) mediates the interaction of ROP GTPase with Sec3, another component of the exocyst complex (Lavy *et al.*, 2007), although in yeast, Sec3 binds directly to Cdc42 (Zhang *et al.*, 2001). Linker proteins may be commonly required for the interaction between plant ROPs and exocyst complexes. RIC7 interacted with Exo70B1 in the *in vitro* system (Fig. 2b), and the interaction did not require active ROP2. Thus, we speculate that active ROP2 does not mediate binding of RIC7 to Exo70B1, but recruits RIC7 and Exo70B1 to the plasma membrane region (Fig. 5).

Multiple functions of Exo70B1 in Arabidopsis

The phenotypes of *exo70b1* mutant plants indicate that Exo70B1 is a positive regulator of light-induced stomatal opening in Arabidopsis (Fig. 3a,c); light-induced stomatal opening was slower in the *exo70b1* knockout mutant than in the wild-type (Fig. 3a) and faster in Exo70B1 complementation plants with higher levels of *Exo70B1* expression than the wild-type (Fig. 3c). However, Exo70B1 function is not limited to stomatal opening. Exo70B1 is important for autophagy-related vacuole transport. Exo70B1 is a positive component of autophagy, and is internalized into the vacuole (Kulich *et al.*, 2013). The *exo70b1* mutants exhibited increased nitrogen starvation susceptibility and anthocyanin accumulation defects (Kulich *et al.*, 2013). Exo70B1 is also important for the defense response, as *exo70b1* mutants exhibit enhanced disease resistance to *Golovinomyces cichoracearum* (Zhao *et al.*, 2015) and an ectopic hypersensitive response mediated by salicylic acid accumulation (Kulich *et al.*, 2013). Furthermore, our finding that Exo70B1 was also localized to the nucleus (Fig. 7e) suggests that it has entirely different additional functions like mammalian Exo70 (Dellago *et al.*, 2011).

How does Exo70B1 regulate stomatal opening?

We speculate that Exo70B1 promotes rapid stomatal opening by facilitating the trafficking of ion transporters to the plasma membrane and vacuoles in guard cells. Light, one of the most

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important inducers of stomatal opening, is perceived by phototropins and chlorophylls. Once activated, these photoreceptors stimulate the activity of multiple ion transporters, increasing ion fluxes into the cytosol and vacuoles of guard cells, and thereby driving water influx and stomatal opening (Kinoshita *et al.*, 2001; Pandey *et al.*, 2007; Shimazaki *et al.*, 2007). Rapid stomatal opening requires an increase in the surface area of the plasma membrane (Meckel *et al.*, 2007), dynamic reorganization of vacuolar membrane (Gao *et al.*, 2005), and increases in the activities of transporters (Pandey *et al.*, 2007). Transport activities can be increased by the delivery of new transporters to the plasma membrane and probably also to the vacuolar membrane (Pandey *et al.*, 2007). Some reports have emphasized the importance of exocytosis for the delivery of new transporters to the membranes of opening guard cells. For example, an increase in guard cell volume is accompanied by the incorporation of more KAT1 into the plasma membrane (Meckel *et al.*, 2004). Translocation of an H^+-ATPase AHA1 to the plasma membrane is regulated by PATROL1 (a putative exocytosis-related protein), and knockout of this protein suppresses stomatal opening (Augustin *et al.*, 1999; Hashimoto-Sugimoto *et al.*, 2013). However, the mechanisms and components involved in transporter trafficking during stomatal opening are only beginning to be revealed.

Based on the following lines of evidence, we hypothesize that Exo70B1 is involved in vesicle trafficking in guard cells. First, Exo70B1 is a well-known component of the exocyst complex (Guo *et al.*, 1999; Matern *et al.*, 2001; He *et al.*, 2007; Kulich *et al.*, 2013) and co-localized with Exo84B, another exocyst component in guard cells (Fig. 6). Second, Exo70B1 was shown to co-localize with Exo70E2, which is associated with EXPO, a marker protein for unconventional exocytosis (Ding *et al.*, 2014; Poulsen *et al.*, 2014). In guard cells, Exo70B1 is not co-localized with the Golgi or TGN/EE markers (Fig. 7a–d), suggesting that Exo70B1 mediates Golgi-independent trafficking of endoplasmic reticulum (ER) -derived vesicles in guard cells. Third, it was reported that Exo70B1 is involved in autophagy-related Golgi-independent transport to the vacuole, and is localized inside the vacuole (Kulich *et al.*, 2013). Vacuole fusion involving vesicle trafficking is reported to be necessary for stomatal opening (Gao *et al.*, 2005, 2009; Li *et al.*, 2013a; Andres *et al.*, 2014). Therefore, Exo70B1 may also be involved in trafficking of ER-derived vesicles to vacuoles in guard cells. Future studies are needed to clarify whether Exo70B1 indeed regulates vesicle trafficking and/or trafficking of ion transporters to the plasma membrane and vacuolar membrane in guard cells.

Exo70B1 binding to RIC7 probably inhibits Exo70B1 function

RIC7 and Exo70B1 re-localized to the same plasma membrane regions in response to ROP2 activity (Fig. 5a,b). Moreover, the two proteins bind to each other. However, RIC7 and Exo70B1 have opposing functions in stomatal movement; EXO70B1 is a positive regulator of stomatal opening, whereas RIC7 is a negative regulator. We speculate that these two proteins fine-tune the extent of stomatal opening. Stimuli that induce stomatal opening activate positive regulators that promote stomatal opening, thereby allowing for carbon dioxide absorption, but might also activate a negative mechanism that prevents excessive stomatal opening, which could cause excessive water loss. Thus, we propose that RIC7 interacts with and inhibits the function of Exo70B1 to optimize the extent of stomatal opening. This conclusion is supported by the observation that the rate of light-induced stomatal opening is almost identical in the *exo70b1 ric7* double knockout mutant (Fig. 4) and *exo70b1* (Fig. 3a). It is tempting to speculate that Exo70B1 tethers vesicles to the target membrane using the conserved phosphatidylinositol 4,5-bisphosphate binding site at its C-terminus (Zarsky *et al.*, 2009) during exocytosis. Binding to RIC7 might limit this function of Exo70B1, thereby inhibiting a critical step in vesicle trafficking, and thus also light-induced stomatal opening.

In summary, we have demonstrated that RIC7 is a regulator of stomatal movement, and have identified its interaction partner, Exo70B1, which positively regulates stomatal opening. When recruited by active ROP2 to the plasma membrane region, RIC7 inhibits Exo70B1 function. Exo70B1 is the first member of the Exo70 family shown to be involved in stomatal movement. Moreover, Exo70B1 is a novel target of the small G protein ROP2-RIC7 signaling pathway, which modulates the kinetics of stomatal movement.

Acknowledgements

This work was supported by a grant to Y.L. from the Global Research Laboratory program (grant no. K20607000006) funded by the Ministry of Science, Information and Communication Technology, and Future Planning (MSIP) and BK21 Plus (10Z20130012243) funded by the Ministry of Education, Korea, and by a grant to J-U.H. from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (grant no. 2015023602).

Author contributions

D.H., J-U.H. and Y.L. planned and designed the research. D.H., B.W.J. and S.Y.K. performed the experiments. D.H., J-U.H. and Y.L. analyzed the data and wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 RIC7 gene expression in Arabidopsis plants.

Fig. S2 Expression levels of *RIC7* as quantified by real-time PCR.

Fig. S3 Stomatal responses to light in Arabidopsis plants expressing various levels of *RIC7*.

Fig. S4 Endogenous levels of ABA are similar in *exo70b1-1* and wild-type plants.

Fig. S5 Cell death phenotype of *exo70b1-1* at 6 wk of age.

Fig. S6 Overexpression of *Exo70B1* in *exo70b1-1* (i.e. the comp4 and comp5 complementation lines).

Fig. S7 Localization of Exo70B1 and RIC7 is different under different ROP2 conditions.

Fig. S8 Exo70B1 is not localized to the Golgi or TGN/early endosome.

Fig. S9 Localization of mCherry-fused Exo70B1 and Exo84B.

Table S1 Expression levels of Exo70 family genes in Arabidopsis guard cells

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