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SHORT COMMUNICATION

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WD40-domain protein GORI is an integrative scaffold that is required for pollen tube growth in rice

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ABSTRACT

The pollen tube plays a critical role in angiosperm plants by delivering sperm gametes for double fertilization. Although the molecular mechanisms underlying pollen tube germination and growth are crucial to crop plants, they are poorly understood. Here, we describe recent advancements in the understanding of the role of the WD40-domain protein in regulating pollen germination and discuss future directions to investigate its role in rice. *GORI* encodes a seven-WD40-motif protein that interacts with an AP180 N-terminal homology (ANTH)-domain protein, which modulates clathrin-mediated endocytosis (CME), and regulates Rac6 activity in the apical plasma membrane of elongating pollen tubes. Loss of function of GORI or Rac6 reduces pollen germination and tube growth, thereby resulting in male sterility in rice. In contrast, overexpression of Rac6 increases pollen tube elongation, with this effect being rescued by GORI overexpression. In the absence of ANTH, pollen germination was reduced, similar to the results observed after inhibitor treatment, indicating that pollen germination partially requires CME. Our findings demonstrated that the GORI protein is a positive regulator of pollen germination and tube growth, serving as a link between Rac6 activity regulation and ANTH-mediated endocytosis.

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In higher plants, the pollen tube delivers immobile male gametes to female gametophytes for double fertilization. The identification of the genes involved in rice male gamete development is important for the regulation of fertility.[1–3] Mature pollen grains that are released from anthers hydrate on the stigma and germinate the pollen tube. The pollen tube subsequently penetrates the stigma, pistil, and ovary until one tube enters the micropyle. During this rapid and polar tip growth of pollen tube, it maintains signaling for continuous and unidirectional cell wall expansion. The molecular mechanisms underlying pollen tube growth have been mainly studied in *Arabidopsis*. The central regulator Rac GTPase (ROP) defines the expanding region at the plasma membrane (PM) via calcium ion concentration and actin dynamics.[4]

WD40-domain proteins make up a scaffold that facilitates protein–protein interactions from bacteria to mammals.[5] A protein containing seven WD40 motifs, JINGUBANG (JGB) is expressed in the vegetative nucleus and cytosol of pollen grains and functions as a negative regulator of pollen tube germination.[6] In another study, JGB was identified as ROP1 enhance 4 (REN4), functioning to modulate tip growth rate and direction in the PM of growing pollen tubes through ROP1 removal via clathrin-mediated endocytosis (CME).[7] In addition, REN4 was shown to interact with AP180 N-terminal homology (ANTH)-domain proteins.[7] In turn, ANTH-domain proteins mediate CME by binding to phosphoinositides, clathrin, and cargo proteins in mammals, whereas plant ANTH-domain proteins have been identified less frequently. The molecular modules linking endocytosis and pollen germination are widely unexplored in other plants.

In our recent work, we provided evidence that a WD40-motif protein in rice, Germinating modulator of rice pollen (GORI) (LOC_Os03g52870), is required for pollen germination.[8] GORI encodes a seven-WD40-motif protein that is homologous to JGB/RGB4 (AT2G26490) of *Arabidopsis*. Homozygous gene-edited transgenic rice (*Oryza sativa* ssp. japonica cv. Dongjin) generated via CRISPR-Cas9 showed low pollen germination and tube elongation, resulting in male sterility. A transcriptomics analysis, yeast two-hybrid screening, and co-immunoprecipitation study revealed that GORI interacts with endocytic proteins and ROP in rice.[8] Although the sequence homology and interacting partner are quite conserved, a clear distinction is the phenotype of JGB/REN4 and ROP in rice and *Arabidopsis*. JGB/REN4 and GORI function negatively and positively in pollen germination, respectively.[6,8] Overexpression of JGB/REN4 inhibits pollen germination and tube elongation,[6,7] whereas overexpression of GORI did not yield significant differences in rice.[8]

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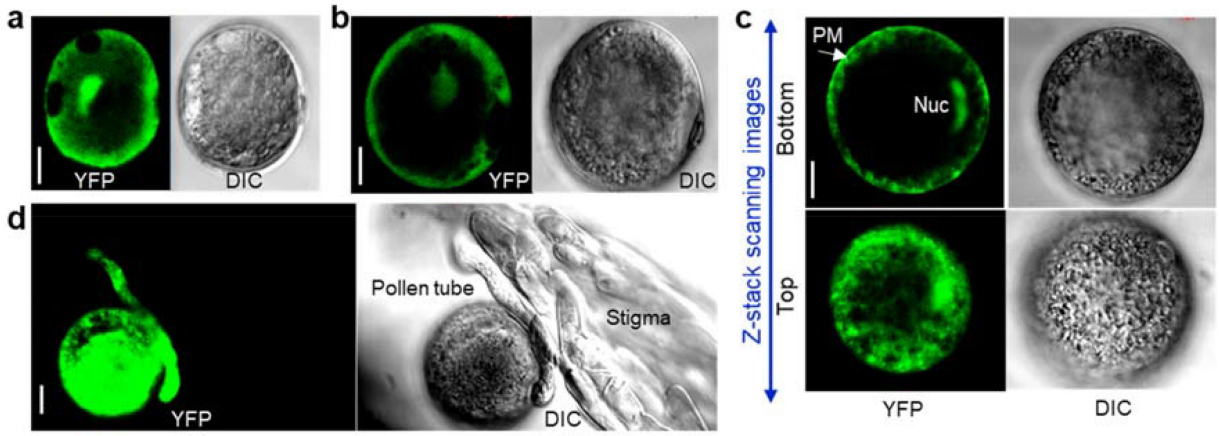

Figure 1. Subcellular localization of the GORI protein. Confocal microscopy images of rice pollen grains at bicellular stage (a), tricellular stage (b), and mature pollen stage (c) from the transgenic line expressing GORI fused with YFP driven by the native promoter (*pGORI:GORI-YFP*). (d) In vivo germinated pollen tubes are shown with stigma. Bars = 10 μm.

We generated *GORI-YFP* line in rice (*pGORI:GORI-YFP* introducing line) and observed its pollen-specific expression by a fluorescence microscope (Olympus BX61) or a confocal scanning laser microscope (CSLM) (LSM 510 META, Carl Zeiss). GORI is localized in the vegetative nucleus, cytoplasm, and PM of pollen grains at bicellular (Figure 1a), tricellular (Figure 1b), and mature (Figure 1c) pollen grains and pollen tubes (Figure 1d). During pollen tube growth, GORI is more concentrated in the PM region during pollen tube elongation.[8] This localization pattern is not altered in response to 5 μM brefeldin A (BFA) treatment (Figure 2b), which inhibits vesicular secretion. Interestingly, this apical tip localization of GORI is reduced by 2 nM Latrunculin B (LatB) treatment (Figure 2c), which promotes the depolymerization of actin filaments. This suggests that the apical tip localization of GORI depends on actin filaments, rather than vesicle-derived exocytosis. Thus, GORI may function as a key link between endocytosis and actin filaments.

Similar to the results observed for GORI, REN4 participates in the spatiotemporal control of pollen tube tip growth by interacting with an actin-dependent pathway to regulate ROP signaling.[7] *Arabidopsis* encodes three pollen-specific *ROP* genes, and we found that rice expresses one *ROP* gene in pollen, *Rac6* (*LOC_Os02g02840*).[9] We confirmed the physical interaction of GORI and Rac6 by co-immunoprecipitation.[8] When we made homozygous mutant of *Rac6* by CRISPR-Cas9, the plants showed male sterility because of the defective pollen germination, similar to the *gori* mutant.[8] We generated its overexpressing line by introducing *pRac6:Rac6-mRFP* into wild-type plants. In contrast with the reduced tube elongation conferred by ROP1 overexpression in *Arabidopsis*,[7] rice pollen tube elongation was enhanced by the increased *Rac6*. To understand the genetic relationship between *Rac6* and *GORI*, we made crossing of the Rac6-mRFP line and GORI-YFP line by their endogenous promoters (Figure 2a). Crossing lines showed that GORI-YFP and Rac6-mRFP are co-localized in the cytoplasm, showing strong apical PM distribution (Figure 2b) during later slow growth of the pollen tube.[9] Interestingly, we found that extra expression of *GORI* reduces the effect of pollen tube elongation by *Rac6* expression in the crossing line of Rac6-RFP and GORI-YFP (Figure 2a). When both signals exist together in the pollen tube, the length of pollen tube is reduced compared with the pollen tube with only *Rac6* expression. These results suggest that GORI functions in the regulation of Rac6 activity.

Through yeast two-hybrid experiments, we found that the C terminus of the ANTH-domain of OsANTH3 (LOC_Os02g07900) interacts with GORI.[10] To get the information of their subcellular localization, we co-expressed p35S:GORI-GFP and p35S:OsANTH3-mRFP in leaves of tobacco (*Nicotiana benthamiana*) by *Agrobacterium tumefaciens* GV3101. Two days after infiltration, the fluorescence was observed with a CSLM. Both proteins localize in the nucleus, cytoplasm, and plasma membrane (Figure 1b). BFA inhibits exocytosis but allows the first step of endocytosis, thus inhibits the endocytic recycling of PM proteins. When BFA is used to treat tobacco leaves expressing *OsANTH11* or *OsANTH3*, BFA-induced endosomal vesicles were observed by *OsANTH3*, but not by *OsANTH11* sharing similar sequence homology and expression pattern (Lee et al., 2021), indicating that OsANTH3 is recycled via endocytosis. To determine whether the interaction between OsANTH3 and GORI affects the endocytosis of GORI, we performed BFA treatment for an hour after the tobacco leaf was immersed in 10 uM BFA. BFA treatment of GORI and ANTH yielded endosome-like structures in the cytoplasm (Figure 1e), indicating that the proteins targeted by CME recycle to the PM.

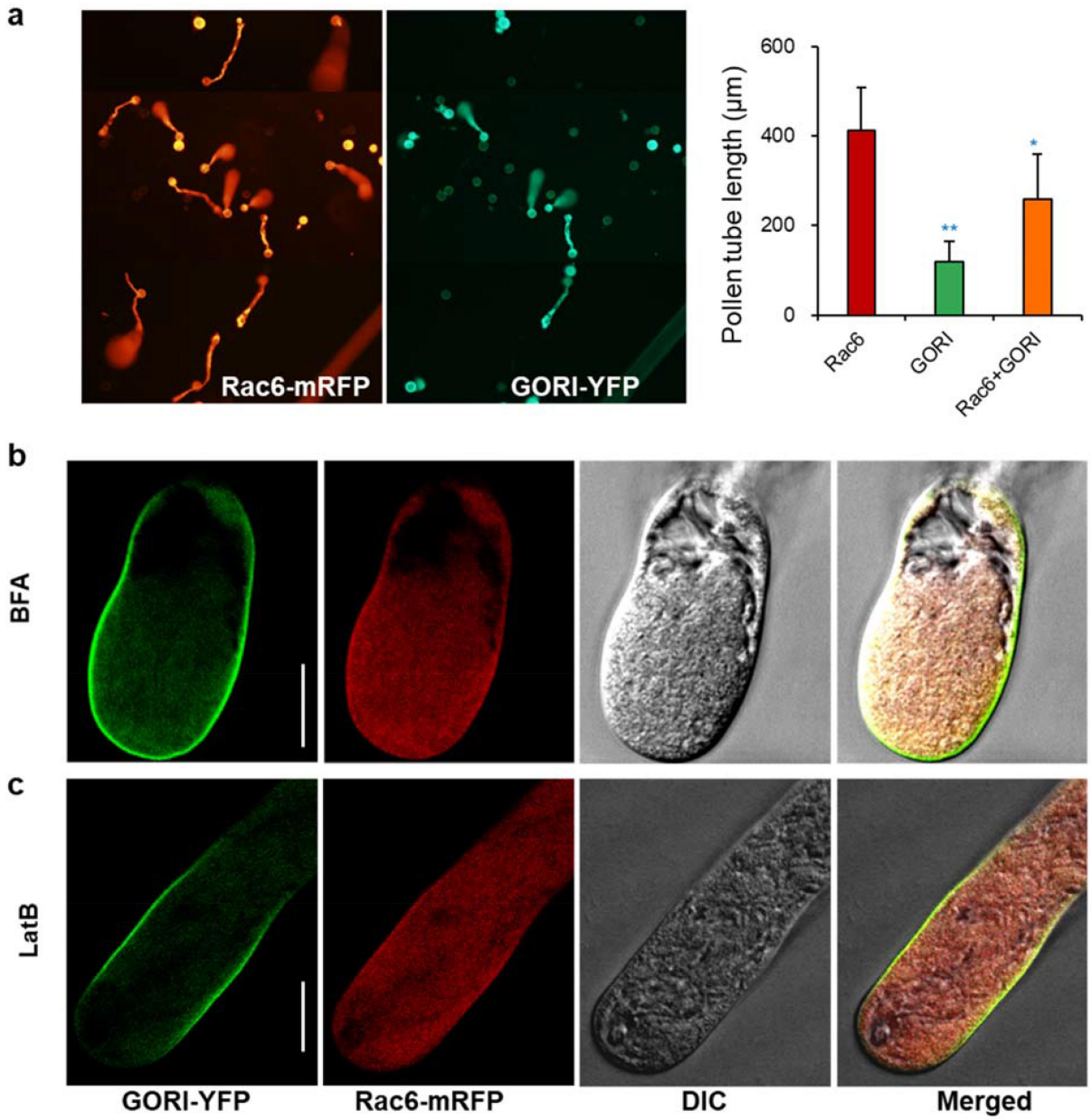

Figure 2. *In vitro* pollen germination of Rac6- and GORI-overexpressing lines. (a) The crossed line of GORI-overexpressing (GORI-YFP) and Rac6-overexpressing (Rac6-mRFP) lines was observed for pollen tube growth under fluorescence. Fresh pollen grains collected just after anthesis were shaken directly onto a solid or liquid pollen germination medium (PGM). The PGM was freshly prepared with 20% (w/v) sucrose, 10% polyethylene glycol (PEG) 4000, 3 mM calcium nitrate, 40 mg/L boric acid and 10 mg/L vitamin B1 (pH 6.8–7.0). The solidified PGM slide with 1% agarose was covered with a cover glass and incubated in a moist chamber at 28°C in the dark for 20 min. The representative pollen tubes for both signals were selected and combined with multiple focused photo images. Pollen tube length was compared with that of GORI-overexpressing (GORI-YFP) and Rac6-overexpressing (Rac6-mRFP) lines. Values represent the mean + SD. Statistical difference was analyzed compared with Rac6-GFP for five replicates ($n > 20$). * $p < .05$, ** $p < .001$. (b) GORI-YFP and Rac6-mRFP were monitored in the *in vitro* pollen tubes of transgenic rice using a confocal microscope. The pollen grains were germinated in PGM and immediately supplemented with 5 μM brefeldin A (BFA) treatment and 2 nM Latrunculin B (LatB), respectively, and observed during 15–20 min. BFA treatment did not change the apical localization of GORI (b), whereas (c) LatB did it. Bars = 10 μm.

We concluded that GORI could control ROP activity through ANTH3-mediated CME. To investigate the CME effect on rice pollen tube growth, we used a CME inhibitor, ikarugamycin (IKA) (Figure 3). The administration of IKA at different concentrations in pollen germination medium affected rice pollen germination but not tube elongation.[10] Our results confirmed that OsANTH3 and CME are required for rice pollen germination, whereas clathrin-independent endocytosis or other genes might affect elongation. It will be interesting to investigate other mechanisms of endocytosis and its recycling of cargo proteins during rice pollen tube growth.

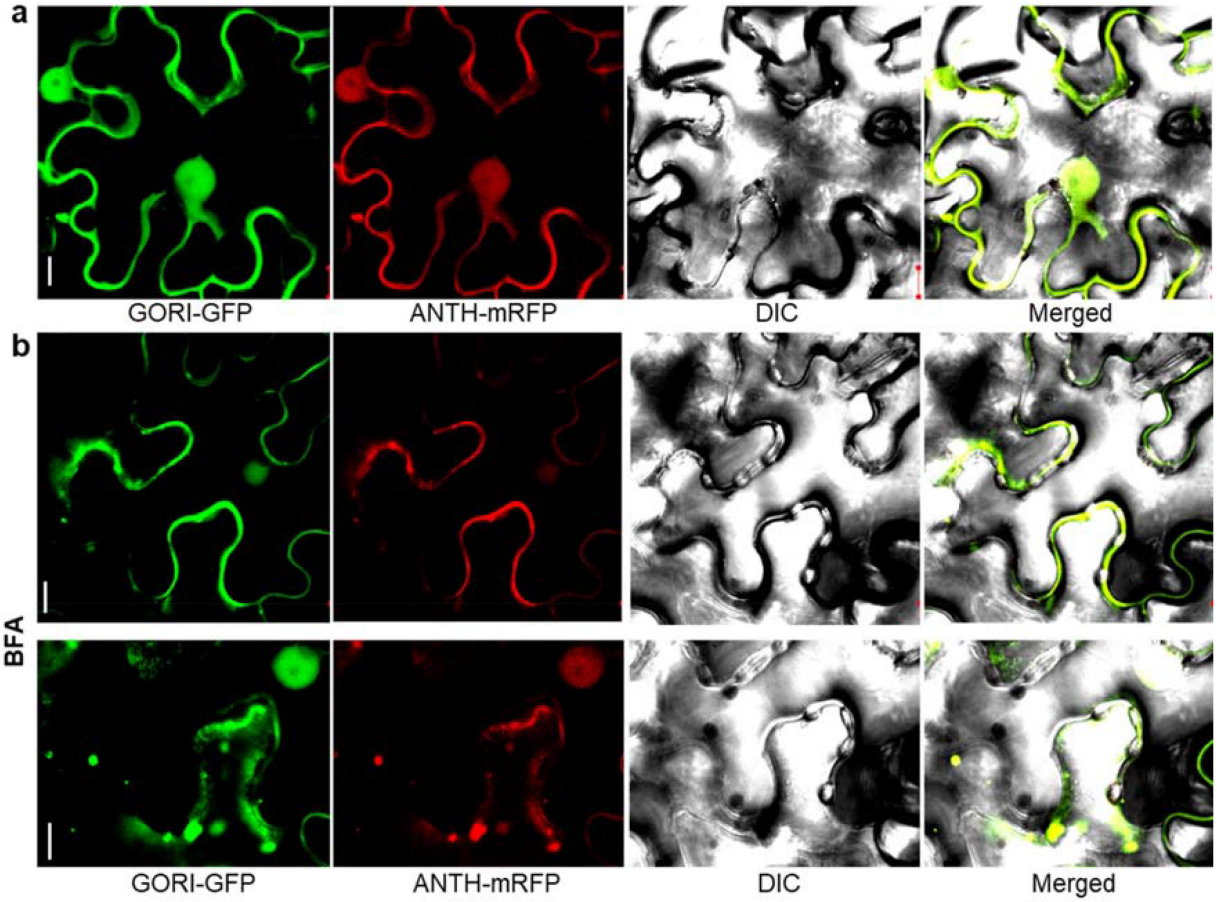

Figure 3. Subcellular localization of GORI and ANTH in tobacco epidermal cells. (a) Co-localization of GORI (*p35S:GORI-GFP*) and OsANTH3 (*p35S:ANTH-mRFP*) shows the signal in the PM, cytoplasm, and nucleus (b). Brefeldin A (BFA) treatment (10 μM) induces endosome-like structures containing GORI and ANTH signals in the cytoplasm (different focus image of top and bottom). Bars = 10 μm.

Recently, we identified the key players in pollen germination and tube elongation in rice, combined with transcriptomics data analysis and mutant generation using CRISPR-Cas9.[8–12] These discoveries extended our knowledge of conserved and diversified mechanisms in monocot and dicot plants. In addition, gamete-specific genes could be used to generate a pollen-specific promoter for an editing system,[13] if applicable.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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