Received: 27 April 2021 | Revised: 10 August 2021 | Accepted: 26 August 2021

DOI: 10.1111/dgd.12752

ORIGINAL ARTICLE

A small Rho GTPase OsRacB is required for pollen germination in rice

Yangfan Xu | Wenguo Cai | Xiaofei Chen | Mingjiao Chen | Wanqi Liang

Joint International Research Laboratory of Metabolic and Developmental Sciences, State Key Laboratory of Hybrid Rice, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China

Correspondence
Wanqi Liang, Joint International Research Laboratory of Metabolic and Developmental Sciences, State Key Laboratory of Hybrid Rice, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China.
Email: wqliang@sjtu.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: U19A2031

Communicating Editors: Shinichiro Sawa and Guojun Sheng

[Correction added on 24 February 2022, after first online publication: The copyright line was changed.]

Abstract

Plant Rho small GTPases (Rop/Rac) are versatile molecular switches regulating many plant developmental processes. Particularly, their important functions in regulating pollen development have been demonstrated in *Arabidopsis*. A group of conserved Rop/Rac activators RopGEFs were recently reported to regulate rice (*Oryza sativa*) pollen tube germination, indicating that rice and *Arabidopsis* may have a conserved Rop/Rac mediated signaling pathway in regulating pollen tube growth. However, the Rop/Rac activated by the rice pollen specific RopGEFs remains to be identified. Here we demonstrated a Rop/Rac gene, *OsRacB*, co-expressed with the mature pollen expressed Os*RopGEF2/3/6/8*. The knockout mutants were normal in anther and pollen development but defective in the pollen grain germination, suggesting a specific and non-redundant role of OsRacB in the mature pollen. We further demonstrated that OsRacB is directly activated by the pollen specific expressing OsRopGEFs in vitro. Together with the previous study, we establish a RopGEF-Rop/Rac regulon which plays essential roles in rice pollen grain germination. Our data encourage further identification of the upstream and downstream players of RopGEF-Rop/Rac signaling in pollen germination and have agricultural implications for breeding robust seed yielding cultivars.

KEYWORDS
pollen germination, Rho small GTPases, rice

1 | INTRODUCTION

Double fertilization is a crucial process in flowering plant sexual reproduction, which consists of multiple sophisticated steps. Pollen grains hydrate and germinate shortly after the mature pollen grains adhere to and is recognized by the compatible stigma; and thereafter pollen tubes penetrate through the pistil to deliver two immotile sperm cells into the ovules where the double fertilization occurs. Therefore, robust pollen germination and pollen tube growth are essential for successful fertilization and thus seed production in flowering plants. In recent decades, our understanding of the molecular mechanisms implicated in the regulation of the above process has greatly advanced with the genetic and biochemical studies in the dicot model species *Arabidopsis*. It is established that coordinated vesicular transport, highly dynamic cytoskeleton, cell wall deposition and remodeling, ROP GTPase (Rho-like small GTPase proteins)-based signaling, reactive oxygen species (ROS) and calcium signaling are crucial for pollen germination and pollen tube growth (Guan et al., 2013). Nevertheless, the molecular mechanisms underlying pollen tube germination and growth in monocot crops such as rice (*Oryza sativa*) are yet largely unknown.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2021 The Authors. *Development, Growth & Differentiation* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Society of Developmental Biologists

Rho-type GTPases of plants, commonly nominated as Racs in rice and ROPs in *Arabidopsis*, belong to the Rho/Ras small guanosine triphosphatases (GTPases) superfamily. ROP/Racs serve as molecular switches to regulate multiple fundamental cellular processes in plants, including defense, cell polarity establishment, cell wall patterning and hormone response (Feiguelman et al., 2018). Like the heterotrimeric G proteins (large G proteins), the monomeric ROP/Rac proteins switch their on-or-off states by cycling between a GTP bound (active) state and a GDP bound (inactive) state (Nielsen, 2020). Guanine nucleotide exchange factors (GEFs) activate ROP/Rac by catalyzing the exchange of GDP and GTP. Upon activation, the GTP-bound ROP/Racs interact with their downstream effectors to signal cellular responses, such as calcium oscillation, actin remodeling and reactive oxygen species burst (Uhrig & Hülskamp, 2006; Zhou et al., 2015). Active ROP/Rac returns to its resting state through its weak intrinsic GTPase activity for GTP hydrolysis or an efficient deactivation by GTPase-activating protein (GAP).

Fourteen *ROP* genes are found in the *Arabidopsis* genome (Christensen et al., 2003; Li et al., 1998). Among them, three closely related *Arabidopsis ROP* genes (*ROP1*, *ROP3*, and *ROP5*) are highly expressed in the mature pollen and play important roles in pollen tube growth. GTP-bound active ROP proteins are specifically localized to the apical plasma membrane of pollen tube to define its apical growing domain and regulate polar exocytosis (Li et al., 2018a; 2018b; Luo et al., 2017). In a feedback, exocytosis regulates the maintenance of ROP proteins on pollen tube apexes. Therefore, ROP proteins, their regulators and associated cellular processes maintain a self-organizing region for the pollen tube tip growth (Li, Luo, et al., 2018; Luo et al., 2017). Oriented pollen tube growth is dependent on the regulation of this self-organizing center by the female gametophyte derived attracting peptides. In *Arabidopsis*, pollen-preferentially expressed ROP activator RopGEF8/9/12/13 modify the ROP activity in response to the ovule-derived small peptides (Gu et al., 2006; Takeuchi & Higashiyama, 2016; Zhang & McCormick, 2007). These studies demonstrated that the ROP activity interchanges in *Arabidopsis* pollen tube is required for the pollen tube tip growth as well as pollen tube guidance.

Nevertheless, not until very recently the ROP/Rac activity cycle was shown to be involved in rice pollen tube growth. Kim et al. demonstrated that two highly conserved *OsRopGEF* genes play specific roles in regulating pollen germination and pollen tube growth (Kim et al., 2020). Their results suggested a conserved role of rice ROP/Racs in mediated signaling as in *Arabidopsis*, though more detailed studies of that in rice were required to support this assumption.

Here, we report that a rice mature pollen specifically expressing *Rac* gene, *OsRacB*, has a specific role in pollen germination. The loss-of-function mutants displayed normal anther and pollen development but defective pollen germination, leading to complete male sterility. In alignment with the previous study on rice pollen germination regulating OsRopGEF, we identified *OsRacB* as a direct effector of *OsRopGEF2/3/6*. Our results together with previous findings provide essential information on how the rice pollen germination and pollen tube growth is regulated at a molecular level.

2 | RESULTS

2.1 | Co-expression of *OsRacB* with *OsRopGEF2/3/6/8* in late anther development stages

Genes preferentially expressed in the mature anther and pollen are presumed to be functionally associated with pollen tube germination and growth (Ling et al., 2015; Moon et al., 2018). At the late anther developmental stages, pollen grains progressively mature with accumulation of reserve materials and energy prepared for pollen grain germination and pollen tube growth. Recently, Kim et al. (2020) have illustrated that the pollen tube regulators *OsRopGEF2/3/6/8* are specifically expressed at the late anther development stages. We speculate that at least one of the targets of these RopGEFs, *i.e*. Rop/Rac, may have a similar expression pattern. Noticeably, previous in silico expression study indicated that *OsRac6*, more commonly known as *OsRacB*, is the only member that is highly expressed in mature pollen (Kim et al., 2020), implicating *OsRacB* as the potential downstream target of *OsRopGEF2/3/6/8*.

To test this hypothesis, we first confirmed the gene expression of the *OsRopGEF2/3/6/8* and *OsRacB* genes in our experimental conditions, and an *OsRacB* paralog, *OsRacD*, was used as a nonspecific expression gene control. Consistent with the previous report, the expression of *OsRopGEF2/3/6/8* was not detectable or at a low level during early anther development stages (Kim et al., 2020). Subsequently, their expression increased dramatically at stage 13 (Figure 1). A very similar expression pattern was also observed for *OsRacB*. In contrast, *OsRacD* had nonspecific and low expression at all anther developmental stages (Figure 1). These results prompted us to further explore the role of *OsRacB* in pollen germination and pollen tube growth.

2.2 | *OsRacB* is essential for rice reproduction

Previous study has shown that *Osropgef2/8* double mutant displayed reduced pollen germination and partial male sterility. To verify whether the mutation in *OsRacB* causes similar phenotype, two target sites in the first and second exon of *OsRacB* gene coding sequences were edited by CRISPR/Cas9 genome editing tool (Figure S1; Xie et al., 2015). The chosen targets were aimed at disrupting the conserved N-terminal domain of Rho GTPase in OsRacB (Christensen et al., 2003). A catalytic G-domain, which contained five G-box motifs (G1–G5), was on the N-terminal of OsRacB, and it was necessary for nucleotide binding, GTP hydrolysis, and Mg^{2+} binding (Feiguelman et al., 2018). Five homozygous or biallelic mutants were obtained (Figure S1). These mutants contained mutations that caused frame-shift and introduced stop codon before the G-domain in *Osracb-1/-3/-4*, while *Osracb-2/-5* only contained the G-box motifs G1 and G2, and thus represented knock-out alleles of *OsRacB*.

Osracb mutants were indistinguishable from wild-type plants at vegetative growth stage (Figure 2a, data not shown), indicating that *OsRacB* may be not essential for the vegetative growth. Nevertheless, *OsRacB* is clearly important for rice reproduction as almost no seeds were obtained from the *Osracb* mutants (Figure 2b and S2). This coincides

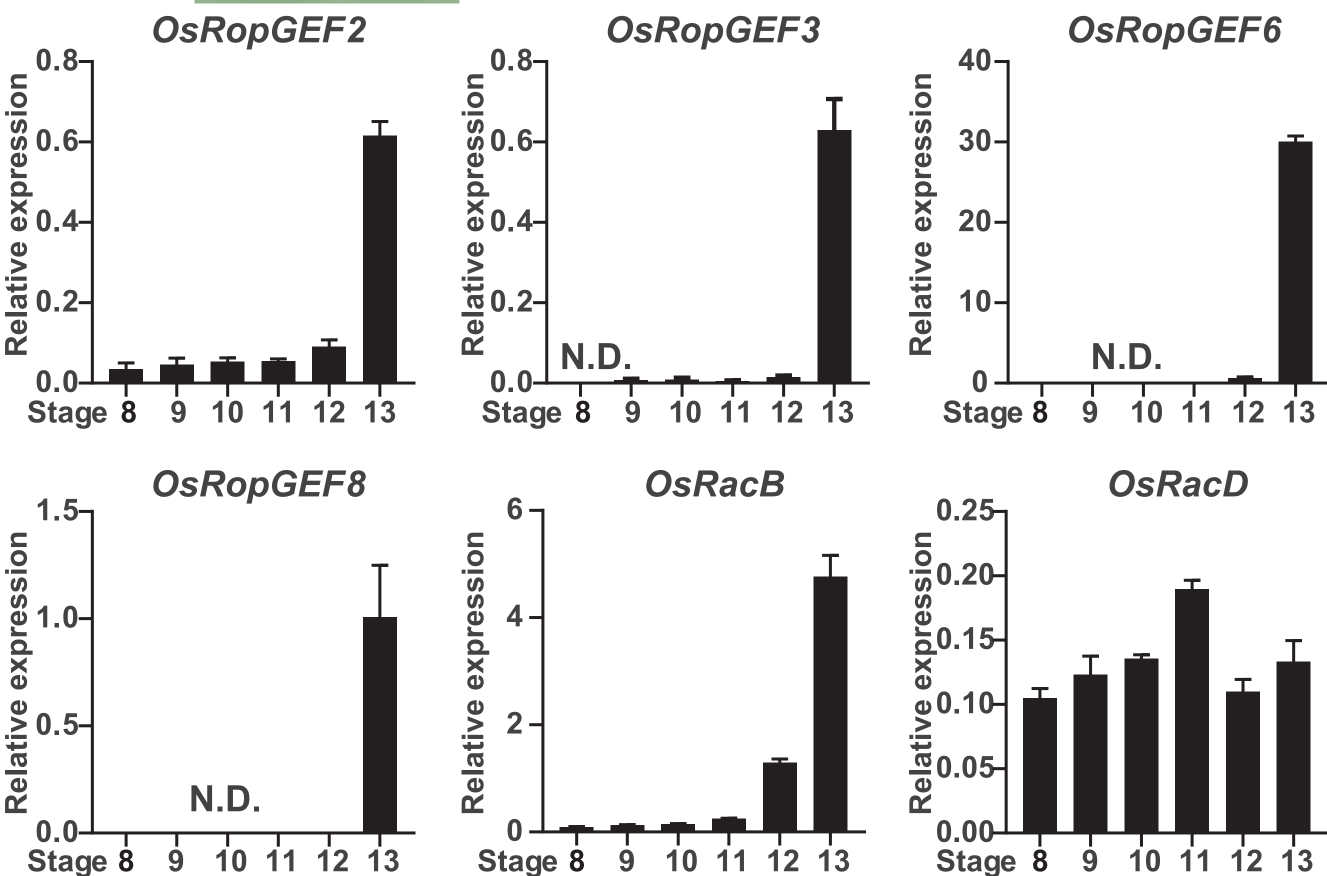

FIGURE 1 *OsRacB* co-expresses with *OsGEF2/3/6/8* at late anther developmental stage. qRT-PCR analysis of the expression of *OsRacB*, *OsRacD* and pollen specific *OsGEFs* in anthers at various developmental stages. Rice *ubiquitin* gene (*OsUBQ*, LOC_Os03g13170) was used as an internal control. X-axis represents different anther developmental stages. Each gene expression was normalized in relative with *OsUBQ*; N.D., not detectable, neglectable expression. Error bars indicate SD of three biological replicates

with the mature pollen preferred expression pattern of *OsRacB*. Our detailed phenotype observations suggest that the *Osracb* mutants had normal panicle, spikelet, anther development as wild-type (Figure 2c–g). Additionally, no difference of pollen grain morphology and pollen viability between *Osracb* mutants and wild-type was seen (Figure 2h–k). Taken together, the *Osracb* mutants did not exhibit defects before seed setting, which is similar to the *Osropgef2/8* double mutant.

2.3 | *OsRacB* mutations affect pollen germination and pollen tube growth

To distinguish whether the seed abortion in *Osracb* mutants was a result of the impairment of stamens or pistils, the male and female fertility of the *Osracb* mutant was tested by reciprocal crossings between *Osracb* and wild-type (WT, *O. sativa subsp. Japonica* cv. *9522*). In parallel, a previously characterized male sterile mutant *thermo-sensitive sterile 10* (*tms10*) was used as a maternal parent as a control for crossing efficiency (Yu et al., 2017). As all five alleles displayed similar phenotypes, we used *Osracb-1* in the following assays. *Osracb-1* and *tms10* displayed comparable seed setting rates when they were pollinated with wild-type pollen grains, respectively. On the contrary, wild-type plants set no seed when pollinated with *Osracb-1* pollen grains (Figure 3a,b), indicating that loss function of *OsRacB* affects male but not female reproduction.

Given the normal pollen grain viability of *Osracb-1*, we assume that the mutants are defective in the fertilization process. We therefore compared the pollen germination efficiency of *Osracb-1* and WT. Under our in vitro pollen germination condition, the germination rate of wild-type pollen grains was 78.8%. On the contrary, only 3.9% of *Osracb-1* pollen grains germinated even though the pollen hydration was not affected (Figure 3c,d). Consistently, no germination of *Osracb-1* pollen grains on the stigma was observed (Figure 3e). The *Osracb-1* single mutant phenocopies *Osropgef2/8* double mutants suggests that *OsRacB* might work in the same pathway with *OsRopGEF2/8*.

2.4 | OsRacB is directly activated by OsRopGEF2/3/6 in vitro

Generally, RopGEF proteins physically interact with ROP/Rac proteins and catalyze the otherwise slow GDP dissociation for subsequent GTP binding (Berken et al., 2005). To identify which

FIGURE 2 *Osracb-1* exhibits normal pollen maturation but is completely sterile. (a) Whole plants at the heading stage. Scale bar: 10 cm. (b) Panicles at the mature stage. Scale bar: 2 cm. (c) Panicles at the flowering stage. Scale bar: 2 cm. (d, e) Mature spikelets before anthesis. Scale bars: 1 mm. (f, g) Mature spikelets after removing the palea and lemma. Scale bars: 1 mm. (h, i) Scanning electron microscopy image of pollen grains at anther development stage 13. Scale bars: 5 μm. (j, k) Iodine potassium iodide (I_2-KI) staining of pollen grains at anther development stage 13. Scale bars: 50 μm

OsRopGEFs may interact with OsRacB, we performed a yeast-two-hybrid assay. Furthermore, a constitutively active form of OsRacB (CA-OsRacB) containing a G15V mutation and a dominant-negative form of OsRacB (DN-OsRacB) containing a T20N mutation were also tested, as previous studies have shown that GEFs may preferentially interact with different forms of GTPases (Gu et al., 2006; Shinohara et al., 2002; Yamaguchi et al., 2012). Our results indicated that wild-type OsRacB interacted with OsRopGEF3/8 but not OsRopGEF2/6 in yeast, while CA-/DN-OsRacB only associated to OsRopGEF8 (Figure 4a). We further detected the interaction through a pull-down assay. Using GST-fused CA-/DN-/wild-type OsRacB as baits, 6×His-tagged OsRopGEF2/3/6/8 were detected in the precipitated protein complexes by His antibody. As shown in Figure 4b, the recombinant GST fusion proteins OsRacB-GST, including CA-/DN-/wild-type forms, precipitated with RopGEF2/3/6/8-6xHis. Interestingly, OsRopGEF8 was more inclined to bind with DN-OsRacB, while OsRopGEF2/3/6 did not show any difference in the interaction with CA-/DN-/wild-type-OsRacB. Subsequently, the guanine exchange activity of OsRopGEF2/3/6/8 towards OsRacB was tested using a fluorescence spectroscopy–based assay (Eshraghi et al., 2020; Singh et al., 2017). This assay is based on the spectroscopic difference between bound and unbound N-MAR-GTP, a fluorescent analog to the guanine nucleotide. In our experiment, once bound to OsRacB, the emission intensity of the fluorophore would increase dramatically. The results showed that the intrinsic guanine nucleotide exchange rate of OsRacB increased with elevated OsRacB concentration (Figure S3a). The guanine nucleotide exchange rates can be dramatically enhanced by the presence of OsRopGEF2/3/6/8 in a concentration dependent manner (Figure 4c–f). Our experiments also showed that OsRopGEF3 has a stronger activation activity towards OsRacB comparing with the other tested OsRopGEF.

Taken together, our results demonstrated that OsRacB acts downstream of OsRopGEFs as a specific regulator of pollen germination.

3 | DISCUSSION

Our present work provides evidence that OsRacB functions downstream of the previously reported rice pollen specific RopGEFs to regulate pollen germination. Our results reveal that *OsRacB* co-expressed with *OsRopGEF2/3/6/8* in the mature anther. The knock-out *Osracb* mutants phenocopy the *Osropgef2/8* double mutant, exhibiting reduced pollen germination and seed setting. Further study through Y2H, pull-down and activation assay showed OsRacB is activated by OsRopGEF2/3/6/8 in vitro. It seems that the function of RopGEF-Racs regulon in pollen germination and growth is conserved in monocot and dicots. In *Arabidopsis*, different RopGEF and ROP/Racs have been shown to regulate pollen hydration, pollen germination, pollen tube growth and directed growth towards to ovules (Chen et al., 2013; Liu et al., 2021; Luo et al., 2017). A paradigm of Rop GTPase activity cycle mediated pollen-pistil

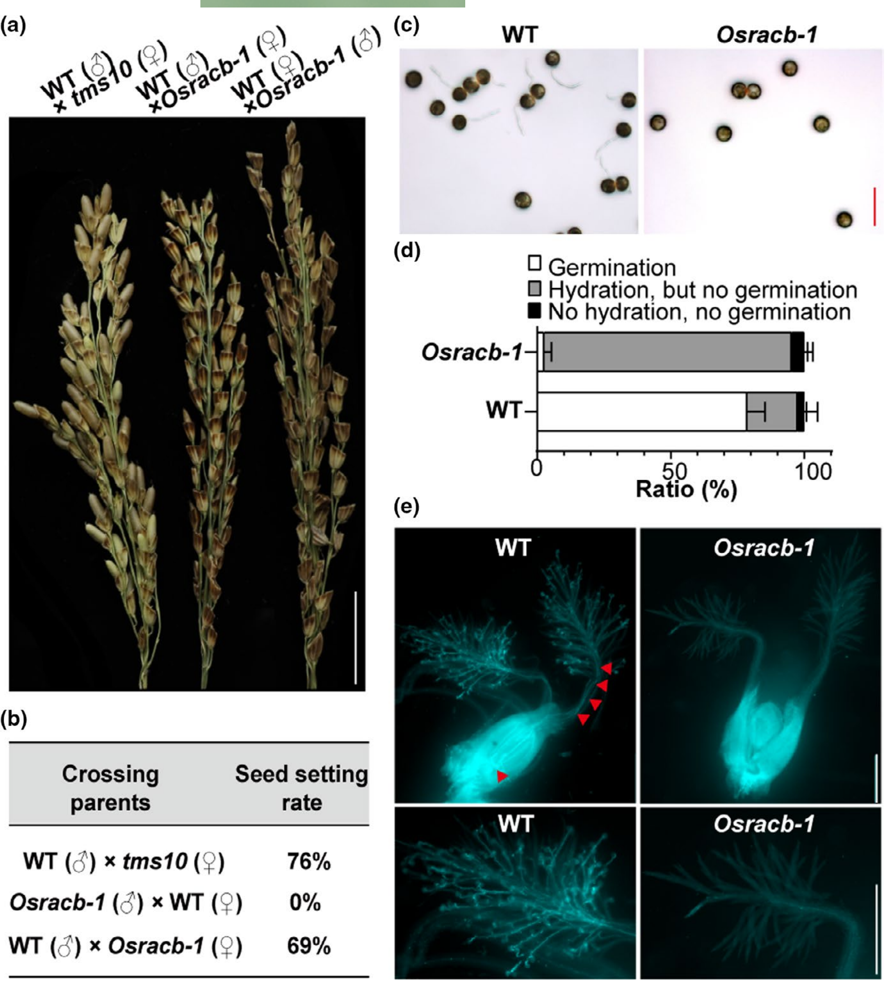

FIGURE 3 *OsRacB* mutation affects pollen tube germination. (a, b) Panicles (a) and seed setting rates (b) of the female parents of crosses between *tms10* (♀) × WT (♂), WT (♀) ×*Osracb-1*(♂) and *Osracb-1* (♀) × WT (♂). Scale bar: 2 cm. (c, d) In vitro pollen germination assay (c) and the germination rates (d) of WT and *Osracb-1* in the solid pollen media. Scale bar: 100 μm. (e) In vivo pollen-germination assays of WT and *Osracb-1*. Arrow heads indicate the pollen tube. Scale bar: 500 μm. Phenotypes in (a–e) were observed at least three times independently with similar results

communication was established in *Arabidopsis*. The ovule derived cysteine-rich polypeptides AtLUREs act as attractants for pollen growth (Okuda et al., 2009). They directly trigger Pollen-specific Receptor-like Kinases (PRKs), which subsequently activate RopGEFs (Chang et al., 2013; Takeuchi & Higashiyama, 2016; Zhang & McCormick, 2007). The gated ROP GTPase activity by RopGEFs is important for the pollen tube tip growth rate and direction. Our data instead suggested that the activation of OsRacB by RopGEFs is specifically required for pollen germination upon rice pollen grain hydration. Unlike the pollen tube growth guidance, very little is known about how RopGEF-Racs are involved in the pollen germination (Chen et al., 2013). The establishment of this RopGEFs-RacB axis would facilitate further investigating its upstream regulators and downstream effectors in this process. As pollen tube growth is essential for rice seeds production, revealing these regulators together with knowledge of the abundant rice genetic variations such as the 3K rice project (Rice Genome Project, 2014; Zhao et al., 2018) would enhance our opportunity to breed new rice cultivars with improved pollen vigor and robustness.

So far, the function of the seven rice *ROP/Racs* genes remains largely unrevealed. Besides *OsRacB*, *OsRac3* and *OsRac5* are also expressed in anther albeit not limited to the later stages (Kim et al., 2020). The complete sterility of *Osracb* suggests that *OsRacB* plays an essential and non-redundant role in pollen germination. *OsRac3* and *OsRac5* may be important for the post-germination growth as their *Arabidopsis* orthologs. Previous work demonstrated that *OsRacB* also participated in regulating plant pathogen resistance and salt tolerance (Jung et al., 2006; Luo et al., 2006). Particularly, the barley (*Hordeum vulgare*) orthologue *HvRacB* was shown to negatively regulate barley abiotic stress tolerance (McCollum et al., 2020; Schultheiss et al., 2003, 2005). Similarly, *OsRac1* a renowned rice disease resistance regulating gene was recently shown to moonlight as a grain size regulator (Ono et al., 2001; Suharsono et al., 2002; Zhang et al., 2019). In these various biological processes, the functional diversity of ROP/Racs may simply attribute to the same underling molecular mechanism such as ROS production and cytoskeleton organization, etc. The elucidation of the functional specificity of ROP/Rac requires thorough comparisons of their spatiotemporal expression patterns and associations with the activators and effectors.

In an earlier study, it has been shown that ROPs were directly activated by RopGEFs, and the first RopGEF in *Arabidopsis* was identified by a yeast two-hybrid screen using DN-ROP4 (D121N) as the bait (Berken et al., 2005). Our study also demonstrated that OsRopGEF2/3/6/8 directly interacts with OsRacB in the pull-down assay, and the guanine exchange reaction suggested their function in

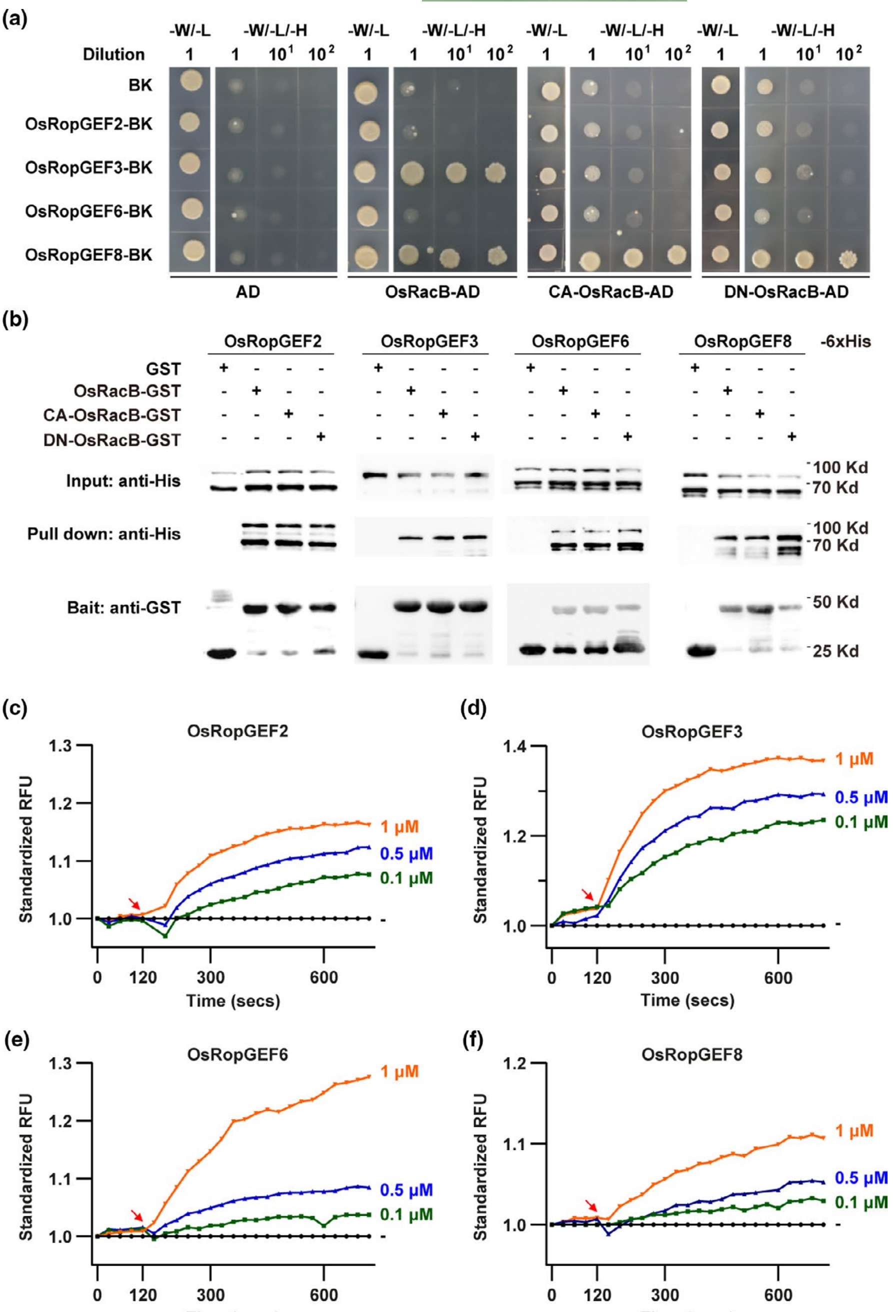

FIGURE 4 OsRacB is directly activated by OsRopGEF2/3/6 in vitro. (a) Yeast-two-hybrid with CA-/DN-/wild-type OsRacB and OsRopGEF2/3/6/8. *OsRacBs* was cloned into pGADT7, and *OsRopGEF2/3/6/8* were cloned into pGBKT7 (BK); Yeast medium: Yeast Synthetic Drop-out Medium without tryptophan and leucine (-W/-L) or without tryptophan, leucine, and histidine (-W/-L/-H). (b) OsRopGEFs-OsRacBs pull-down assay. (c-f) The guanine nucleotide exchange activity of OsRacB was enhanced by OsRopGEF2/3/6/8. 1 μM OsRacB was added to the exchange reaction buffer at 0 s, and multiple concentration (0.1/0.5/1 μM) of OsRopGEFs were added after five readings respectively (OsRacB alone as control groups). Standardized RFU: standardized Relative fluorescence was calculated in relative to its corresponding non-RopGEF mock and in relative to the florescence at the starting point (0 s)

activation of OsRacB. Nevertheless, our results from Y2H assay informed that OsRopGEF2/3/6/8 have different affinity to OsRacB. OsRopGEF8 interacted with CA, DN and the wild-type forms of OsRacB, and OsRopGEF3 only interacted with wild-type OsRacB. However, OsRopGEF2/6, which have been shown to activate OsRacB in guanine exchange reaction, did not interact with OsRacB in Y2H assay. It is speculated that the activities of OsRopGEF2/6 toward OsRacB may not rely on a strong and tight proteins association. Or the actual interactions between ROPs and GEFs would be hindered by the conditions in yeast, such as nuclear localization, and so on. Interestingly, OsRopGEF8 showed higher affinity to DN-OsRacB, which differs from other OsRopGEFs, indicating OsRopGEF8 may be easy to bind GDP-RacB and release the GDP in vivo. The recombinant OsRopGEF proteins showed multiple bands of different apparent molecular sizes on the SDS-PAGE gel except OsRopGEF3. Similar multiple bands were seen for *Arabidopsis* RopGEF proteins prepared from *E. coli* and infiltrated tobacco (Chang et al., 2013; Li et al., 2018a; 2018b; Zhao et al., 2015). Thus, the multiple bands are likely resulted from a yet unknown modification of the proteins rather than artifacts during proteins preparation. Our data showed that OsRacB interacted roughly equally to the two OsRopGEF2 bands of different sizes while it preferred the larger OsRopGEF8, suggesting a biological meaning of the unknown RopGEF modification. The nature of the modification and the significance of their association with RopGEF needs further investigation. The binding propensity may be the reason for the result that OsRopGEF8 showed lower activity in GEF activity assay compared with

OsRopGEF2/3/6 at the same concentration. In the previous study, OsRopGEF2/8 were shown to act redundantly to regulate pollen germination (Kim et al., 2020). Since *Osracb* mutants are nearly completely male sterile while the *Osropgef2/8* double mutants maintain low fertility. Other pollen specific OsRopGEFs, OsRopGEF3/6, may undertake the role as OsRacB activators to regulate pollen germination in vivo.

4 | MATERIALS AND METHODS

4.1 | Plant materials and growth conditions

Rice materials including wild-type (*Oryza sativa subsp. Japonica* cv. *9522*) and CRISPR/Cas9 edited lines were grown in the paddy field at Shanghai Jiao Tong University (Shanghai, China) during the natural growing season (from June to September).

For the targeted editing of *OsRacB*, CRISPR/Cas9 construction with sgRNA (5′-CATAAAGTGCGTCACCGTCG-3′; 5′-CAACTTCAGTG CCAACGTCG-3′) was used for targeting the first and second exon of the *OsRacB* (LOC_Os02g02840) according to Xie et al. (2015). Primers are listed in Table S1. Agrobacterium-mediated transformation of the rice callus and regeneration of rice plants were used for *OsRacB* gene edit lines.

4.2 | Characterization of the mutant phenotype

Morphological images of the whole rice plants and the inflorescences after flowering and fruiting were photographed with a Canon digital camera. The spikelets, the opened flowers and the dehiscent anthers were captured by a Leica stereoscopy (M205A). For pollen viability assay, wild-type and *Osracb* anthers were soaked in iodine sodium iodine (I_2-KI) staining solution (8% KI, 1% I_2) and crushed with tweezers to release pollen grains, the staining was immediately imaged with the microscopy (Nikon ECLIPSE 80i with Nikon DS-Ri1 digital camera).

To observe the pollen morphology, wild-type and *Osracb* flowers were firstly fixed in FAA solution (5% formaldehyde [originally in 37% solution provided], 5% acetic acid, 63% ethanol, 27% H_2O) for 2 h, and then flowers were successively dehydrated with 70%–100% ethanol with 10% increase in ethanol each step. Subsequently, the samples were dried with an automated critical point dryer (Leica EM CPD300) and glued to a copper table with conductive adhesive with anthers crushed with tweezers to release the pollen grains. The released pollen grains were coated with gold using a vacuum coater (Leica EM SCD050) later. Finally, the pollen grains were photographed with a scanning electron microscope (HITACHI S-3400N II).

4.3 | In vitro pollen-germination assays

To observe the in vitro pollen germination, on the day of pollen dispersal and filaments elongation, wild-type and *Osracb* pollen grains were collected by gently shaking panicles into the solid germination medium (0.8%–1% Agar, 4% PEG-4000, 18% Sucrose, 1 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 0.01% H_3BO_3, pH = 6.4), and cultured in the dark oven at 28°C for 1 h. The germinating was imaged with microscopy (Nikon ECLIPSE 80i). Pollen-germination assays were repeated three times independently, for each repeat at least three flowers and more than 200 pollen grains in five sights were counted.

4.4 | Aniline blue staining of pollen tubes in vivo

To observe the pollen-tube in vivo, Aniline blue staining of pollen tubes in vivo was conducted as previously reported (Zhang et al., 2020). Briefly, about 6 hours after pollination, the pistils of wild-type and *Osracb* were cut and fixed with Carnoy's solution (glacial acetic acid: ethanol = 1:3) for 4 h, and then pistils were washed with 70%, 60%, 40%, 20% ethanol and distilled water successively. Subsequently, the pistils were softened with 8 M NaOH at room temperature overnight, then washed with distilled water, and stained in 0.1% aniline blue dye (in 0.1 M K_2HPO4, pH 8.5) at room temperature for 4–6 h in the dark. Fluorescent images were captured by an Eclipse Ni-E microscope (Nikon) under UV light.

4.5 | Gene expression analysis

For gene expression analysis, rice anthers developmental stages were judged from the anther morphology according to Zhang et al. (2011). The anthers were collected and extracted RNA with a TRIzol RNA extraction kit (TIANGEN Biotech) according to manufacturer's instructions. Subsequently, approximately the same amount of RNA was used for cDNAs with FastKing RT Kit (TIANGEN Biotech). qPCR primers were as listed in Table S1. QuantiNova SYBR Green PCR Kit (TIANGEN Biotech) was used for quantitative PCR (qPCR). qPCR cycling conditions are as follows: 95°C for 30 s for pre-incubation, 95°C for 5 s and 60°C for 30 s were used for two-step amplification, and total 50 cycles. The highly stable expression gene rice *Ubiquitin* (UBQ, LOC_Os03g13170) was used as an internal control (Li et al., 2014; Pabuayon et al., 2016).

To determine the significant expression changes of four *RopGEF* genes and *OsRacB* in different stages, we performed qPCR analysis with three independent biological replicates. Relative transcript levels and fold change were calculated by the $2^{-\Delta\Delta Ct}$ methods, respectively.

4.6 | Protein expression and purification

The dominant-negative (T20N) and constitutively active (G15V) OsRacB were generated by site-directed mutagenesis, according to previous research (Li et al., 1999). *CA-/DN-/wild-type OsRacB* and *OsRopGEF2/3/6/8* were cloned into pGEX-4T-1 and pET-32a(+) with a one-step cloning kit (ClonExpress® II, Vazyme), and wild-type *OsRacB* was also cloned into pET28a(+) (primers are listed

in Table S1). The confirmed plasmids were then transformed into *E. coli* (*Escherichia coli*) cells Rosetta (DE3). The cells were then inoculated into 500 ml of LB with corresponding antibiotics at 37°C until OD600 = 0.7. The cell culture was then cooled to 18°C before isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM, and cells were incubated for an additional 12 h at 18°C to induce the expression of the tagged proteins: CA-/DN-/wild-type OsRacB-GST (Glutathione S-Transferase), OsRacB-6xHis (polyhistidine) and OsRopGEFs-6xHis (polyhistidine). Cells were harvested by centrifugation at 4000 *g* for 20 min and stored at −80°C. The CA-/DN-/wild-type OsRacB-GST, OsRacB-6xHis and OsRopGEFs-6xHis tagged protein was purified using Glutathione HiCap Matrix or nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen), respectively, under native conditions according to the manufacturer's instructions.

4.7 | Protein interaction assay

For the Y2H assay, the coding sequences of CA-/DN-/wild-type OsRacB protein were cloned into pGADT7 and OsRopGEF2/3/6/8 into pGBKT7, then transformed into the yeast (*Saccharomyces cerevisiae*) strain AH109. The Y2H assays were performed according to the manufacturer's user manual (Clontech). Briefly, the transformed yeast cells were selected on synthetic dropout (SD/-Leu/-Trp) nutrient media, and the interaction was tested on the synthetic dropout medium plate (SD/-Leu/-Trp/-His). Primers used in this assay are shown in Table S1.

The procedures for the pull-down assay were modified from a previous report (Li et al., 2018a; 2018b). Approximately 2 μg of purified recombinant bait proteins (CA-/DN-/wild-type OsRacB-GST or GST) were incubated with 2 μg of prey protein (OsRopGEF2/3/6/8-6xHis) in 2 ml binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.6% [v/v] Triton X-100) at 4°C for 2.5 h. Following the addition of glutathione sepharose 4B beads (GE Healthcare), the samples were further incubated for 1 h. After washing with binding buffer, the precipitated proteins were eluted by heating the beads in 2× SDS loading buffer at 95°C for 10 min and then separated on a 10% (w/v) SDS-PAGE gel by electrophoresis, and transferred to nitrocellulose membranes (BioTrace^{TM}, Pall Laboratory). The proteins were analyzed by immunoblotting with anti-GST (Abcam, ab19256) and anti-His (ab237339) primary antibodies. The chemiluminescence of the membrane was subsequently developed with Omni-ECL™ (Epizyme, SQ202L) and imaged with ChemiDoc^{TM} MP (BioRad).

4.8 | Guanine nucleotide exchange assay

The guanine nucleotide exchange activity of different concentrations of OsRacB, and the effect of RopGEF2/3/6/8 on the activity of OsRacB were monitored using a RhoGEF Exchange Assay Biochem Kit (Cytoskeleton, Cat. # BK100) according to the manufacturer's protocol. The fluorescence intensity was detected by a multimode microplate reader (Tecan, Spark). OsRacB-6xHis and OsRopGEF2/3/6/8-6xHis were expressed in *E. coli* as described above, OsRopGEF2/3/6/8. Briefly, 2.5 μl OsRacB was added to 7.5 μl of 2×exchange reaction buffer [40 mM Tris pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$, 1.5 μM N-MAR-GTP (BK100, Cytoskeleton)] in black 384-well plates. Plates were then read immediately (excitation, 485 nm ± 20 nm; emission, 535 nm ± 20 nm; gain, 65). After five readings (1 reading per 30 s), 5 μl OsRopGEF2/3/6/8 or ddH_2O was added to the wells, with an immediate resumption of the reading (60 readings for a total of 30 min). Three independent assays were performed. Fold-change relative fluorescence was normalized to the fluorescence at the starting point (0 s); standardized relative fluorescence was calculated in relative to its corresponding non-RopGEF mock and in relative to the florescence at the starting point (0 s).

ACKNOWLEDGEMENTS

This work was supported by funds from National Natural Science Foundation of China (U19A2031). W. C. is supported by China Postdoctoral Science Foundation (Grant 2017M621451).

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Conceived by: W. C. and W. L. F. X. and W. C. did the major analysis and experiments. F. X and W. C. and W. L. wrote the manuscript. All authors read and approved the final manuscript.

ORCID

Wenguo Cai https://orcid.org/0000-0002-7197-5070
Wanqi Liang https://orcid.org/0000-0002-9938-5793

REFERENCES

Berken, A., Thomas, C., & Wittinghofer, A. (2005). A new family of RhoGEFs activates the Rop molecular switch in plants. *Nature*, *436*(7054), 1176–1180.

Chang, F., Gu, Y., Ma, H., & Yang, Z. (2013). AtPRK2 promotes ROP1 activation via RopGEFs in the control of polarized pollen tube growth. *Molecular Plant*, *6*(4), 1187–1201. https://doi.org/10.1093/mp/sss103

Chen, C. Y., Zheng, W. G., Cheung, A. Y., & Wu, H. M. (2013). Pollen germination activates the apical membrane-located RAC/ROP GTPase switch. *Molecular Plant*, *6*(4), 1358–1361. https://doi.org/10.1093/mp/sst074

Christensen, T. M., Vejlupkova, Z., Sharma, Y. K., Arthur, K. M., Spatafora, J. W., Albright, C. A., & Fowler, J. E. (2003). Conserved subgroups and developmental regulation in the monocot *Rop* gene family. *Plant Physiology*, *133*(4), 1791–1808.

Eshraghi, M., Ramírez-Jarquín, U. N., Shahani, N., Nuzzo, T., De Rosa, A., Swarnkar, S., Galli, N., Rivera, O., Tsaprailis, G., Scharager-Tapia, C., Crynen, G., Li, Q., Thiolat, M.-L., Bezard, E., Usiello, A., & Subramaniam, S. (2020). RasGRP1 is a causal factor in the development of l-DOPA–induced dyskinesia in Parkinson's disease. Science. *Advances*, *6*(18), eaaz7001. https://doi.org/10.1126/sciadv.aaz7001

Feiguelman, G., Fu, Y., & Yalovsky, S. (2018). ROP GTPases structure-function and signaling pathways. *Plant Physiology*, *176*(1), 57. https://doi.org/10.1104/pp.17.01415

Gu, Y., Li, S., Lord, E. M., & Yang, Z. (2006). Members of a novel class of *Arabidopsis* Rho Guanine Nucleotide Exchange Factors control Rho GTPase-dependent polar growth. *The Plant Cell*, *18*(2), 366–381.

Guan, Y., Guo, J., Li, H., & Yang, Z. (2013). Signaling in pollen tube growth: Crosstalk, feedback, and missing links. *Molecular Plant*, *6*(4), 1053–1064. https://doi.org/10.1093/mp/sst070

Jung, Y.-H., Agrawal, G. K., Rakwal, R., Kim, J.-A., Lee, M.-O., Choi, P. G., Kim, Y. J., Kim, M.-J., Shibato, J., Kim, S.-H., Iwahashi, H., & Jwa, N.-S. (2006). Functional characterization of OsRacB GTPase – a potentially negative regulator of basal disease resistance in rice. *Plant Physiology and Biochemistry*, *44*(1), 68–77. https://doi.org/10.1016/j.plaphy.2005.12.001

Kim, E.-J., Park, S.-W., Hong, W.-J., Silva, J., Liang, W., Zhang, D., Jung, K.-H., & Kim, Y.-J. (2020). Genome-wide analysis of RopGEF gene family to identify genes contributing to pollen tube growth in rice (Oryza sativa). *BMC Plant Biology*, *20*(1), 95. https://doi.org/10.1186/s12870-020-2298-5

Li, E., Cui, Y., Ge, F.-R., Chai, S., Zhang, W.-T., Feng, Q.-N., & Zhang, Y. (2018). AGC1.5 kinase phosphorylates RopGEFs to control pollen tube growth. *Molecular Plant*, *11*(9), 1198–1209.

Li, G., Liang, W., Zhang, X., Ren, H., Hu, J., Bennett, M. J., & Zhang, D. (2014). Rice actin-binding protein RMD is a key link in the auxin–actin regulatory loop that controls cell growth. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(28), 10377–10382.

Li, H., Lin, Y., Heath, R. M., Zhu, M. X., & Yang, Z. (1999). Control of pollen tube tip growth by a Rop GTPase-dependent pathway that leads to tip-localized calcium influx. *The Plant Cell*, *11*(9), 1731–1742.

Li, H., Luo, N., Wang, W., Liu, Z., Chen, J., Zhao, L., & Yang, Z. (2018). The REN4 rheostat dynamically coordinates the apical and lateral domains of *Arabidopsis* pollen tubes. *Nature Communications*, *9*(1), 2573.

Li, H., Wu, G., Ware, D., Davis, K. R., & Yang, Z. (1998). *Arabidopsis* Rho-Related GTPases: Differential gene expression in pollen and polar localization in fission yeast. *Plant Physiology*, *118*(2), 407–417.

Ling, S., Chen, C., Wang, Y., Sun, X., Lu, Z., Ouyang, Y., & Yao, J. (2015). The mature anther-preferentially expressed genes are associated with pollen fertility, pollen germination and anther dehiscence in rice. *BMC Genomics*, *16*, 101.

Liu, C., Shen, L., Xiao, Y., Vyshedsky, D., Peng, C., Sun, X., & Li, C. (2021). Pollen PCP-B peptides unlock a stigma peptide–receptor kinase gating mechanism for pollination. *Science*, *372*(6538), 171–175.

Luo, M., Gu, S. H., Zhao, S. H., Zhang, F., & Wu, N. H. (2006). Rice GTPase OsRacB: Potential accessory factor in plant salt-stress signaling. *Acta Biochimica Et Biophysica Sinica*, *38*(6), 393–402.

Luo, N., Yan, A., Liu, G., Guo, J., Rong, D., Kanaoka, M. M., & Yang, Z. (2017). Exocytosis-coordinated mechanisms for tip growth underlie pollen tube growth guidance. *Nature Communications*, *8*(1), 1687.

McCollum, C., Engelhardt, S., Weiss, L., & Hückelhoven, R. (2020). ROP INTERACTIVE PARTNER b interacts with RACB and supports fungal penetration into barley epidermal cells. *Plant Physiology*, *184*(2), 823–836.

Moon, S., Oo, M. M., Kim, B., Koh, H. J., Oh, S. A., Yi, G., & Jung, K. H. (2018). Genome-wide analyses of late pollen-preferred genes conserved in various rice cultivars and functional identification of a gene involved in the key processes of late pollen development. *Rice (New York. N.Y.)*, *11*(1), 28.

Nielsen, E. (2020). The Small GTPase superfamily in plants: A conserved regulatory module with novel functions. *Annual Review of Plant Biology*, *71*(1), 247–272.

Okuda, S., Tsutsui, H., Shiina, K., Sprunck, S., Takeuchi, H., Yui, R., & Higashiyama, T. (2009). Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. *Nature*, *458*(7236), 357–361.

Ono, E., Wong, H. L., Kawasaki, T., Hasegawa, M., Kodama, O., & Shimamoto, K. (2001). Essential role of the small GTPase Rac in disease resistance of rice. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(2), 759–764.

Pabuayon, I. M., Yamamoto, N., Trinidad, J. L., Longkumer, T., Raorane, M. L., & Kohli, A. (2016). Reference genes for accurate gene expression analyses across different tissues, developmental stages and genotypes in rice for drought tolerance. *Rice (New York, N.Y.)*, *9*(1), 32.

Rice Genome Project (2014). The 3,000 rice genomes project. *GigaScience*, *3*(1), 7.

Schultheiss, H., Dechert, C., Kogel, K. H., & Hückelhoven, R. (2003). Functional analysis of barley RAC/ROP G-protein family members in susceptibility to the powdery mildew fungus. *Plant Journal*, *36*(5), 589–601.

Schultheiss, H., Hensel, G., Imani, J., Broeders, S., Sonnewald, U., Kogel, K.-H., & Hückelhoven, R. (2005). Ectopic expression of constitutively activated *RacB* in barley enhances susceptibility to powdery mildew and abiotic stress. *Plant Physiology*, *139*(1), 353–362.

Shinohara, M., Terada, Y., Iwamatsu, A., Shinohara, A., Mochizuki, N., Higuchi, M., & Jessberger, R. (2002). SWAP-70 is a guanine-nucleotide-exchange factor that mediates signalling of membrane ruffling. *Nature*, *416*(6882), 759–763.

Singh, N. K., Janjanam, J., & Rao, G. N. (2017). p115 RhoGEF activates the Rac1 GTPase signaling cascade in MCP1-induced vascular smooth muscle cell migration and proliferation. *Journal of Biological Chemistry*, *292*(34), 14080–14091.

Suharsono, U., Fujisawa, Y., Kawasaki, T., Iwasaki, Y., Satoh, H., & Shimamoto, K. (2002). The heterotrimeric G protein alpha subunit acts upstream of the small GTPase Rac in disease resistance of rice. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(20), 13307–13312.

Takeuchi, H., & Higashiyama, T. (2016). Tip-localized receptors control pollen tube growth and LURE sensing in *Arabidopsis*. *Nature*, *531*(7593), 245–248.

Uhrig, J. F., & Hülskamp, M. (2006). Plant GTPases: Regulation of morphogenesis by ROPs and ROS. *Current Biology*, *16*(6), R211–R213.

Xie, K., Minkenberg, B., & Yang, Y. (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(11), 3570–3575.

Yamaguchi, K., Imai, K., Akamatsu, A., Mihashi, M., Hayashi, N., Shimamoto, K., & Kawasaki, T. (2012). SWAP70 functions as a Rac/Rop guanine nucleotide-exchange factor in rice. *Plant Journal*, *70*(3), 389–397.

Yu, J., Han, J., Kim, Y.-J., Song, M., Yang, Z., He, Y., & Zhang, D. (2017). Two rice receptor-like kinases maintain male fertility under changing temperatures. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(46), 12327–12332.

Zhang, D., Luo, X., & Zhu, L. (2011). Cytological analysis and genetic control of rice anther development. *Journal of Genetics and Genomics*, *38*(9), 379–390.

Zhang, X., Zhao, G., Tan, Q., Yuan, H., Betts, N., Zhu, L., & Liang, W. (2020). Rice pollen aperture formation is regulated by the interplay between OsINP1 and OsDAF1. *Nature Plants*, *6*(4), 394–403.

Zhang, Y., & McCormick, S. (2007). A distinct mechanism regulating a pollen-specific guanine nucleotide exchange factor for the small GTPase Rop in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(47), 18830–18835.

Zhang, Y., Xiong, Y., Liu, R., Xue, H. W., & Yang, Z. (2019). The Rho-family GTPase OsRac1 controls rice grain size and yield by regulating cell division. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(32), 16121–16126.

Zhao, Q., Feng, Q., Lu, H., Li, Y., Wang, A., Tian, Q., & Huang, X. (2018). Pan-genome analysis highlights the extent of genomic variation in cultivated and wild rice. *Nature Genetics*, *50*(2), 278–284.

Zhao, S., Wu, Y., He, Y., Wang, Y., Xiao, J., Li, L., & Wu, Y. (2015). RopGEF2 is involved in ABA-suppression of seed germination and post-germination growth of Arabidopsis. *The Plant Journal*, *84*(5), 886–899.

Zhou, Z., Shi, H., Chen, B., Zhang, R., Huang, S., & Fu, Y. (2015). *Arabidopsis* RIC1 severs actin filaments at the apex to regulate pollen tube growth. *The Plant Cell*, *27*(4), 1140–1161.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Xu, Y., Cai, W., Chen, X., Chen, M., & Liang, W. (2022). A small Rho GTPase OsRacB is required for pollen germination in rice. *Development, Growth & Differentiation*, 64, 88–97. https://doi.org/10.1111/dgd.12752