Rice

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OsRopGEF10 Attenuates Cytokinin Signaling to Regulate Panicle Development and Grain Yield in Rice

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Abstract

Cytokinins, which play crucial roles in shoot development, substantially affect grain yield. In rice, the OsRopGEF10-OsRAC3 module is associated with cytokinin signaling and crown root development. However, the effects of RopGEF-mediated cytokinin signaling on rice shoot development and grain yield remain unclear. In this study, we investigated the role of OsRopGEF10 in SAM development and the underlying mechanism. We showed that overexpression of OsRopGEF10 inhibited SAM and panicle development, leading to decreased grain yield. Intriguingly, the overexpression of a specific amino acid mutant of OsRopGEF10, designated gef10-W260S, was found to promote panicle development and grain yield. Further analysis using the BiFC assay revealed that the gef10-W260S mutation disrupted the recruitment of rice histidine phosphotransfer proteins (OsAHP1/2) to the plasma membrane (PM), thereby promoting cytokinin signaling. This effect was corroborated by a dark-induced leaf senescence assay, which revealed an increased cytokinin response in the *gef10-W260S* ectopic expression lines, whereas the overexpression lines presented a suppressed cytokinin response. Moreover, we revealed that the enhanced panicle development in the *gef10-W260S* lines was attributable to the upregulated expression of several type-B response regulators (*RRs*) that are crucial for panicle development. Collectively, these findings revealed the negative regulatory function of OsRopGEF10 in the development of the shoot apical meristem (SAM) via interference with cytokinin signaling. Our study highlights the promising role of *OsRopGEF10* as a potential target for regulating SAM and panicle development in rice, revealing a valuable breeding strategy for increasing crop yield.

Keywords OsRopGEF10, Cytokinin, OsAHP1/2, SAM, Panicle, Grain yield

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Introduction

Rice has a long history of cultivation and consumption and is the staple food for more than half of the world's population (Wang et al., 2008). A high and stable yield of rice is essential for food security and a long-term goal of breeding. The Rho/RAC of plants (ROPs) of guanosine triphosphatases (GTPases) are crucial molecular switches that regulate various growth and developmental processes, including cell division, differentiation, cell polarity, tissue morphogenesis, organ development, and responses to phytohormones and abiotic stressors (Zhang et al., 2007; Duan et al. 2010; Chen et al. 2011; Wu et al. 2011; Akamatsu et al. 2015; Feiguelman et al. 2018; Zhang et al. 2019). Most studies on *Arabidopsis* and rice have revealed that ROP signaling pathways play a critical role in the regulation of plant growth and development, but little is known about the effect of ROP signaling on crop yield.

The activation of ROP/RAC GTPases is regulated by guanine nucleotide exchange factors (GEFs), which are flanked by variable N- and C-terminal regions and contain a highly conserved plant-specific ROP nucleotide exchanger (PRONE) domain that is essential for their activity (Berken et al., 2008). GEFs catalyze the activity of small GTPases by facilitating the release of GDP and the subsequent binding of GTP to RAC/ROP (Berken et al. 2005). In plants, PRONE GEFs aid in RAC/ROP-associated developmental processes, such as pollen tube and root hair development, plant immunity, stress responses, and hormonal regulation (Duan et al. 2010; Chen et al. 2011; Oda et al., 2012; Wang et al. 2017). The rice genome contains 11 *OsRopGEF* and 7 *OsRAC* genes (Miki et al., 2005; Kim et al. 2020). Phylogenetic analysis has categorized *OsRopGEF* members into two subfamilies (Kim et al. 2020). Among these, one subfamily comprises *OsRopGEF2*, *3*, *4*, *6*, and *8*, which are predominantly expressed in the pollen and facilitate pollen germination (Kim et al. 2020). *OsRAC6* (*OsRacB*), which is highly coexpressed with *OsRopGEF2/3/6/8* during late anther development stages, acts as a direct effector of *OsRopGEF2/3/6* in pollen germination (Xu et al. 2021). Moreover, OsRopGEF3 of this subfamily activates OsRac3 to regulate root hair growth (Kim et al. 2020). The second *OsRopGEF* subfamily comprises six members, namely, *OsRopGEF1*, *5*, *7*, *9*, *10*, and *11*. In this subfamily, the OsRopGEF1-OsRAC1 module aids in chitin-driven immune responses (Akamatsu et al. 2013). *OsRopGEF7B* (*OsRopGEF5*) has been linked to flower organ development (Huang et al. 2018). Recently, we reported that the OsRopGEF10-OsRAC3 module plays a role in crown root initiation in rice via its inhibitory effect on cytokinin signaling (Liu et al. 2023). Although OsRAC1 has been implicated in promoting rice grain size (Zhang et al. 2019), the functions of RopGEF members in terms of crop yield have yet to be determined.

Cytokinins positively affect the activity of the shoot apical meristem (SAM), which is responsible for the growth of new tissues and organs (Jameson et al., 2015). The plant cytokinin signaling pathway comprises three key components: a 'hybrid' histidine kinase (HK) receptor that contains both histidine kinase and receiver domains, histidine phosphotransfer (AHPs/HPs/HPTs) proteins and separate response regulators (RRs) (Schaller et al., 2007; Hwang et al. 2012; Kieber and Schaller, 2018). AHPs, which act downstream of HK receptors in cytokinin signaling, mediate the transfer of a phosphoryl group from the receiver domain of an activated HK receptor to the receiver domain of an RR in the multistep phosphorelay (Hwang et al. 2012; Kieber et al., 2014). The RRs involved in cytokinin signaling are categorized into two distinct types: type-B RRs and type-A RRs. Type-B RRs, which are activated by AHPs, are essential for the initial transcriptional response to cytokinin (Argyros et al., 2008; Ishida et al., 2008). Type-A RRs are primary response genes that function as negative regulators of cytokinin signaling (Kieber et al., 2014). Genetic studies have revealed that the expression of genes involved in cytokinin signaling or biosynthesis governs the development of SAMs and panicles (Burr et al. 2020). Suppression of these genes leads to decreased yield. A noteworthy example is the loss-of-function mutation in *OsHK4*, which disrupts cytokinin signaling, resulting in a reduced rice panicle size and spikelet number (Chun et al. 2023). This phenotype is reminiscent of mutations affecting the *OsHK5* and *OsHK6* genes (Burr et al. 2020; Chun et al. 2023). Moreover, rice *rr22* mutants resulting from the knockout of a type-B *RR* exhibit smaller panicles, highlighting the significance of these regulators in rice yield and development (Yamburenko et al. 2020). Mutants exhibiting defective cytokinin biosynthesis, particularly those involving the *LOG* (*Lonely Guy*) gene, exhibit a reduced panicle size and grain yield (Kurakawa et al. 2007). Conversely, in another study, moderate enhancement of cytokinin levels throughout the plant was found to potentially lead to increased yield (Zhao et al. 2015). Specifically, loss of function of *OsCKX2* causes cytokinin accumulation in rice inflorescence meristems (IMs), resulting in larger panicles and an increased grain number (Ashikari et al. 2005; Tu et al. 2021). Taken together, these results indicate that high cytokinin levels increase SAM activity, resulting in large panicles, whereas low cytokinin levels inhibit SAM development, resulting in smaller panicles in rice.

Recently, we elucidated the interaction between the OsRopGEF10-OsRAC3 module and OsAHP1/2, demonstrating their role in inhibiting cytokinin signaling to facilitate crown root initiation in rice (Liu et al. 2023). In

this study, the high expression of *OsRopGEF10* observed in the SAM and IM prompted us to investigate its potential function within the shoot apex. Our findings revealed that in addition to its role in crown root development, OsRopGEF10 also plays a key role in panicle development by impairing cytokinin responses. Appropriate modulation of the activity of cytokinin can substantially contribute to increasing the yield of cereal crops (Burr et al. 2020). We speculate that the modified OsRopGEF10-OsRAC3 module may contribute to rice yield. Here, we present evidence that manipulating OsRopGEF10-mediated cytokinin signaling through ectopic expression of a site-altered mutant, gef10-W260S, can enhance panicle development and significantly increase grain yield.

Results

OsRopGEF10 is Expressed in the SAM and IM

To determine the role of RopGEF10 in shoot development, we comprehensively examined the transcript levels of the *OsRopGEF*, *OsRAC* and *OsAHP* genes in the shoot apex via quantitative reverse transcription PCR (qRT–PCR). On the basis of previous reports (Kim et al. 2020; Xu et al. 2021), we excluded genes expressed specifically in pollen, including *OsRopGEF2*, *3*, *4*, *6*, 8 and *OsRac6*, from the analysis. Notably, among the *OsRopGEFs*, *OsRopGEF10* presented highest expression in the shoot, SAM and IM tissues (Figure S1A). The results revealed that two *RAC* members, *OsRAC3* and *OsRAC5*, presented relatively high expression in the shoot, SAM and IM tissues (Figure S1B). Two *OsAHP* genes, *OsAHP1* and *OsAHP2*, were relatively highly expressed in the SAM and IM tissues (Figure S1C). The expression of the remaining genes, including pseudo *OsPHP1* and *OsPHP2*, was negligible in the SAM and Ims (Figure S1C). The data indicated that the RopGEF10-OsRAC3 module and OsAHP1/2 were coexpressed in the SAM and IM. By analyzing transgenic plants harboring the *OsRopGEF10promoter*: β-glucuronidase (*GUS*) reporter gene, we observed high expression of *OsRopGEF10* in the developing embryo, floral meristems, spikelets, and floral organs, including the glumes and pistils (Fig. 1A–D), and relatively low expression in the lateral root primordia and leaf veins (Fig. 1E–H). Based on these results, combined with previous observations of the SAM-specific expression of *OsRopGEF10* (Liu et al. 2023), we inferred that *OsRopGEF10*-mediated signaling might be pivotal to development of the SAM. Thus, we considered *OsRopGEF10* a candidate gene for further investigations.

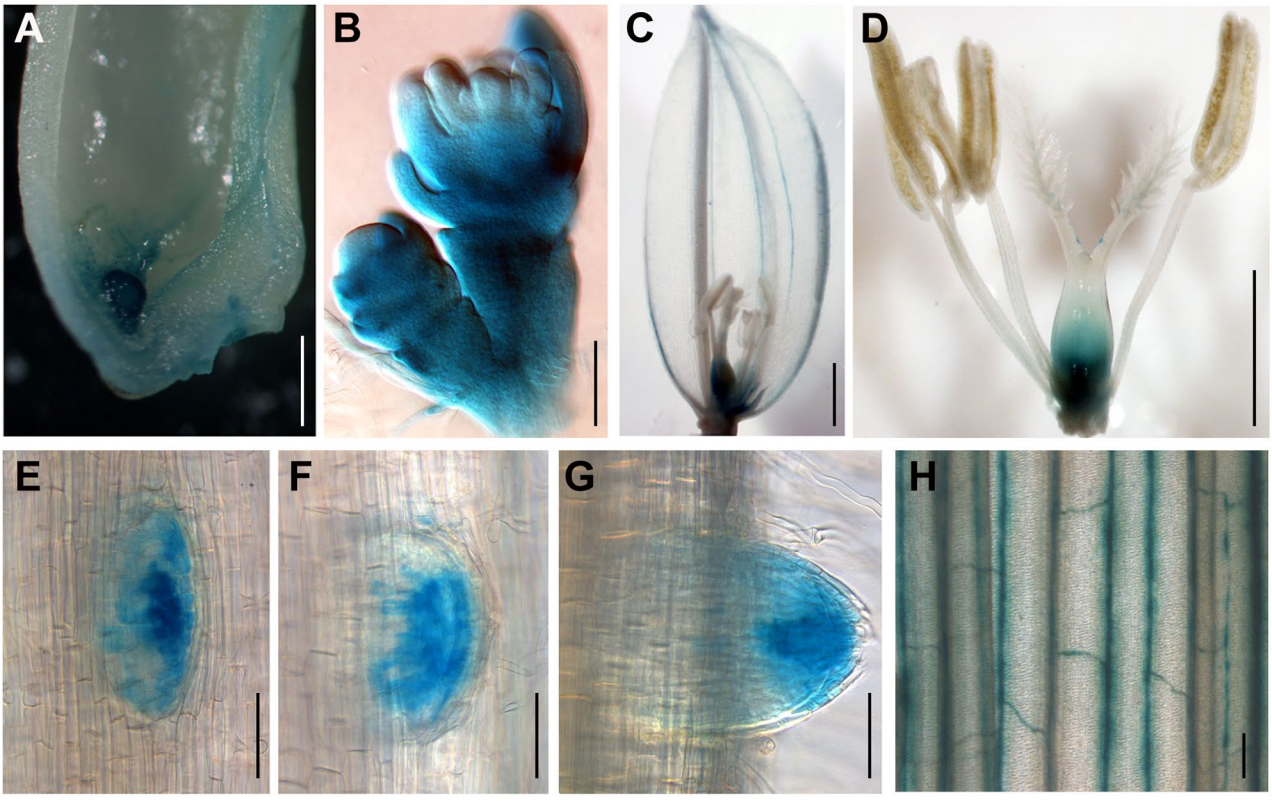

Fig. 1 Expression of *OsRopGEF10* in the developing embryo (**A**), floral meristem (**B**), glume (**C**), pistil (**D**), lateral root primordia at different developmental stages of seven-day-old seedlings (**E**-**G**), and leaf veins (**H**), as revealed through β-glucuronidase histochemical staining. Scale bars: (**A**, **C**, and **D**) 20 mm; (**B**) 20 μm; (**E**-**H**) 1 mm

OsRopGEF10 Negatively Regulates SAM Development

The PRONE domains of RopGEFs are responsible for GEF activity toward ROP GTPase in *Arabidopsis* (Berken et al., 2008). Mutation of a conserved site in the PRONE domain, leading to the substitution of tryptophan (W) with serine (S) (W160S), results in the complete attenuation of GEF8 activity (Thomas et al. 2007). This amino acid residue is conserved in rice RopGEF members. Therefore, the OsRopGEF10 mutant was generated by substituting tryptophan (W) with serine (S) at position 260 in the conserved PRONE domain (Figure S2). To evaluate the role of OsRopGEF10 in SAM development, we produced transgenic plants by overexpressing OsRopGEF10 (designated OE) and the OsRopGEF10-W260S mutant (designated *gef10-W260S*) in ZH11. The western blot results confirmed that OsRopGEF10 and OsRopGEF10-W260S were successfully overexpressed in multiple transgenic lines (Figure S3). Two lines with high expression levels of OsRopGEF10 or OsRopGEF10-W260S were selected for further studies (Figure S3). T1 seedlings were germinated and grown for 7 days. In a high proportion of OE lines (nearly 20%, $n=20$), twin or triplet seedlings emerged from a single seed, indicating abnormal embryonic development (Fig. 2A). Double SAMs developed occasionally in a single embryonic section of the OE lines (Fig. 2B, C). Observations of longitudinal sections of the SAM revealed that the OE meristems were smaller than those of the WT; the SAM height and width of the OE lines were also smaller than those of the WT (Fig. 2E-K, Fig. S3A-C). In contrast, SAM size was significantly larger in *gef10-W260S* lines compared with the WT (Fig. 2E–K and S3D, E). These results suggested that OsRopGEF10 played a negative role in SAM development.

In rice, SAM size can affect phyllotaxy, plastochron, plant height, tillering number, and other important agronomic traits (Tsuda et al. 2011; Ikeda-Kawakatsu et al. 2015). The OE lines displayed delayed development in the early stage but were indistinguishable from the WT in the mature stage (Fig. 2D and S4A). In contrast, *gef10-W260S* lines were significantly taller than the WT lines in both the vegetative and mature stages (Fig. 2D and S4B, C). Similarly, the average fresh weight per seedling decreased in the OE lines but increased in the *gef10-W260S* lines (Figure S5D). Although the SAM size in the *OsRopGEF10* OE and *gef10-W260S* lines was strongly affected, no phyllotaxy or plastochron defects were observed in the vegetative phase (Fig. 2D). The total tiller and panicle numbers in both transgenic lines were also comparable to those in the WT (Figure S5E, F).

Panicle and Grain Sizes are Altered in *OsRopGEF10* Transgenic Lines

In rice, the phenotypic defects in panicles can be traced back to the size of the vegetative meristems and IMs (Kurakawa et al. 2007). To examine the effect of *OsRopGEF10* expression on panicle development, we investigated the panicle phenotype of the *OsRopGEF10* OE lines. These lines produced significantly smaller panicles and fewer primary branches and spikelets than did the WT (Fig. 3A-D). In contrast, the *gef10-W260S* lines formed larger panicles and a significantly greater number of primary branches and spikelets (Fig. 3A-D). The *gef10-W260S* lines presented a significant increase in grain yield per plant, which substantially decreased in the OE lines (Fig. 3E). These panicle phenotypes were consistent with their vegetative SAM phenotypes (Fig. 2D-I), suggesting a key role of OsRopGEF10 in inhibiting SAM and panicle development.

Although the OE lines produced fewer seeds, their grain size was significantly larger than that of the WT (Fig. 4A, B); they also presented a 12% increase in grain length and an 11% increase in thousand-grain weight compared with those of the WT (Fig. 4C-E). In contrast, the grain size and thousand-grain weight of the *gef10-W260S* lines were comparable to those of the WT (Fig. 4C-E). Given that the *gef10-W260S* lines had a significant increase in grain number per panicle without affecting grain size, the gef10-W260S mutation contributed to improved crop yield.

The gef10-W260S Mutation Disrupts the Interactions with OsRAC3 and OsAHP1/2

Generally, RopGEFs physically interact with ROP/RAC proteins to catalyze their dissociation from GDP for subsequent GTP binding (Berken et al. 2005). We confirmed the interaction between OsRAC3 and OsRopGEF10 using a yeast two-hybrid assay (Figure S6). OsRopGEF10 interacted preferentially with a constitutively active form of OsRAC3 (CA-OsRAC3) containing the G15V mutation but interacted weakly with a dominant-negative form of OsRAC3 (DN-OsRAC3) carrying the T20N mutation (Figure S6), indicating that OsRopGEF10 directly activated OsRAC3. In our previous study, we demonstrated that OsRopGEF10, OsRAC3, and OsAHPs interact physically in vivo and that OsRAC3 activation is required to recruit OsAHPs to the PM (Liu et al. 2023). Thus, we examined whether the OsRopGEF10-W260S mutation disrupted the OsRopGEF10-OsRAC3-OsAHP1/2 interaction. When expressed in rice protoplasts, mCherry-labeled gef10-W260S presented a markedly diminished signal compared with the signal of OsRopGEF10 in the cell periphery (Fig. 5A, B). Bimolecular fluorescence complementation (BiFC) assays revealed that various control combinations generated only background signals

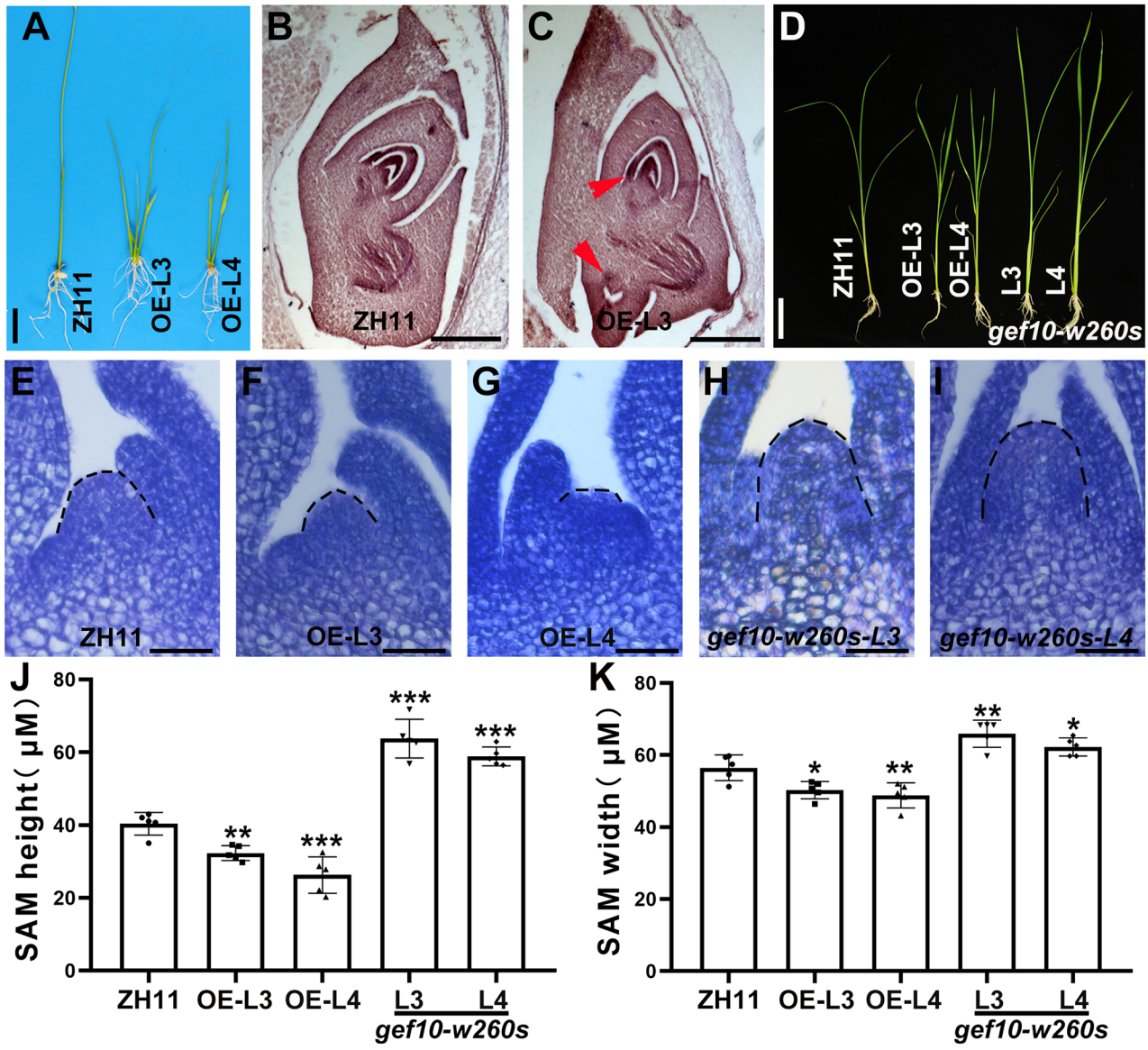

Fig. 2 *OsRopGEF10* expression affects SAM development. (**A**) Seven-day-old seedlings of ZH11 (WT) and two OsRopGEF10 OE lines. (**A** and **C**) Longitudinal sections of the embryonic SAM of ZH11 and OE-L3, respectively; red arrowheads indicate the SAM. (**D**) Phenotypes of 21-day-old ZH11 (WT), OE, and *gef10-W260S* lines. (**E**–**I**) Comparison of the SAM sizes of 21-day-old ZH11 (**E**), two OE lines (**F**, **G**) and two *gef10-W260S* lines (**H**, **I**) through longitudinal sections. (**J** and **K**) Quantitative analysis of the SAM height (**J**) and width (**K**) of 21-day-old ZH11, OE, and *gef10-W260S* lines. The data are presented as the means ± SDs ($n = 5$). Asterisks indicate significant differences determined using the Student's t test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bars: (**A**, **D**) 2 cm; (**B**–**I**) 40 μm

(Fig. 5C–F). In contrast to the BiFC signal generated by OsRAC3 and OsRopGEF10 on the PM (Fig. 5G), no interaction signal between gef10-W260S and OsRAC3 was observed (Fig. 5H), indicating that the mutation at position 260 of OsRopGEF10 abolished the interaction of OsRopGEF10 with OsRAC3. Consistent with previous results, the coexpression of OsRopGEF10 and OsAHP1/2 generated discontinuous signal patches on the PM in protoplasts (Fig. 5I, K). In contrast, the interaction between gef10-W260S and OsAHP1/2 generated abnormally accumulated cytoplasmic signals. The interacting signals were partly colocalized with the nuclear marker IAA17 but were diminished on the PM (Fig. 5J, L). These results indicated that the mutated gef10-W260S protein failed to interact with and activate OsRAC3, which further interfered with the recruitment of OsAHP1/2 to the PM, eventually promoting cytokinin signaling. Since OsRAC5 exhibited an expression pattern similar to that of OsRAC3 in the SAM and IM (Figure S1B), we also examined the interaction between OsRAC5 and OsRopGEF10 or OsAHP1/2. The results showed that OsRAC5 also interacted with OsRopGEF10 and OsAHP1/2 but failed

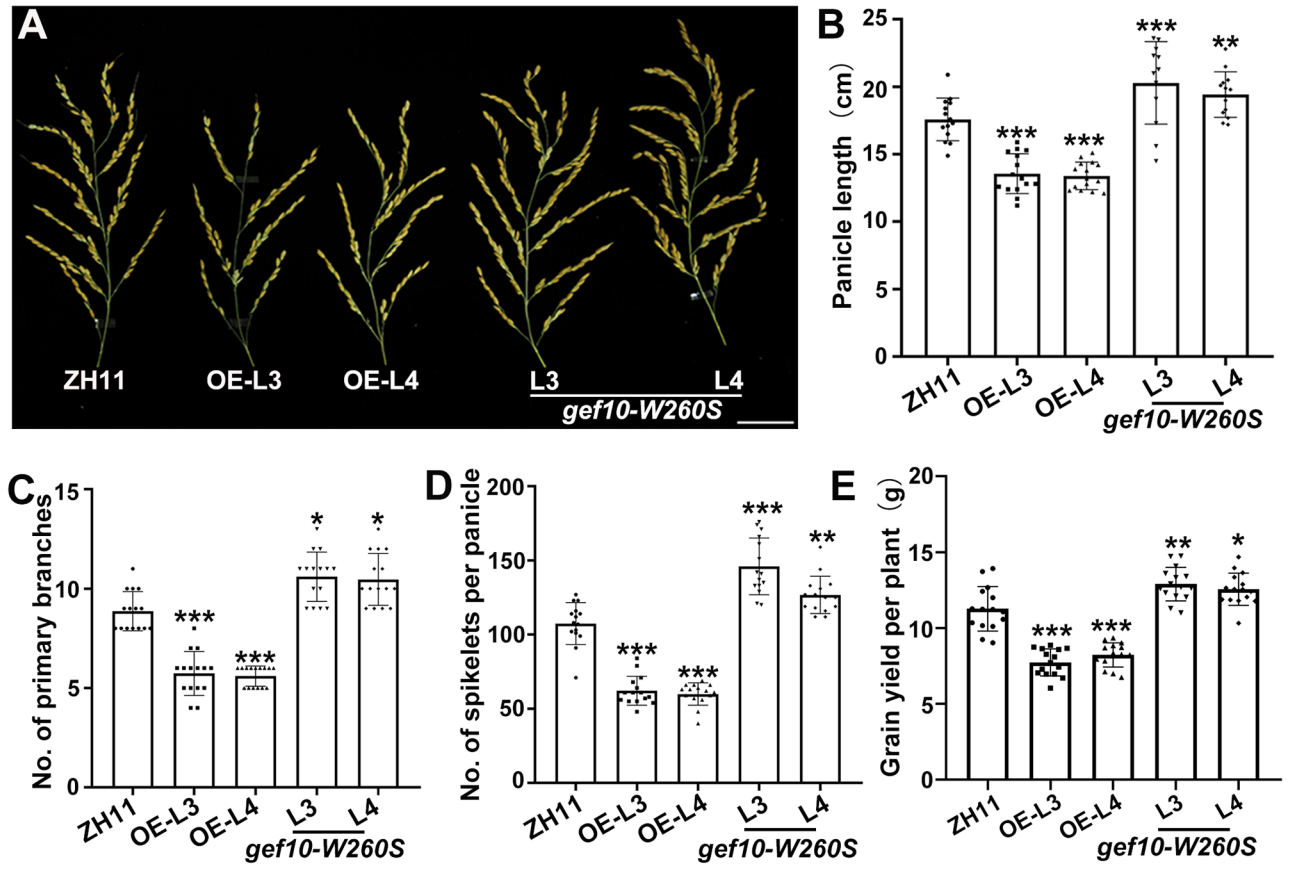

Fig. 3 Panicle phenotypes of the *OsRopGEF10* OE lines and *gef10-W260S* mutants. (**A**) Phenotypes of the main panicle from the ZH11, *OE*, and *gef10-W260S* lines. (**B**-**E**) Comparison of the length of the main panicle (**B**), primary branch number per main panicle (**C**), grain number per main panicle (**D**) and total grain yield per plant (**E**) in the WT, OE, and *gef10-W260S* lines. The data are presented as the means ± SDs ($n = 15$). The dots indicate the data points. At least 15 main panicles or plants were used to quantify the agronomic traits of the panicles. Three biological replicates were performed. Asterisks indicate significant differences determined using the Student's t test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Scale bars: (**A**-**B**) 10 cm; (**C**) 5 cm

to interact with gef10-W260S (Figure S7 A-F). Taken together, these data indicated that OsRAC3 together with OsRAC5 functioned in the same OsRopGEF10-OsRAC/ROP module to modulate cytokinin signaling.

Altered Cytokinin Response in *gef10-W260S* Mutants and OE Lines

To further examine whether *gef10-W260S* affects the cytokinin response, we compared cytokinin-induced leaf senescence of *gef10-W260S* lines with that of the WT. We observed that dark-induced leaf senescence under cytokinin treatment was more prominent in *gef10-W260S* than in ZH11, whereas OE lines presented hyposensitivity to cytokinin, as indicated by the residual chlorophyll content after incubation in the dark (Fig. 6A). Moreover, upon cytokinin (6-BA) treatment, the *gef10-W260S* lines accumulated a significantly greater chlorophyll content than did ZH11. In contrast, the chlorophyll content of the *OsRopGEF10* OE lines was lower (Fig. 6B). These results indicated that the cytokinin response was enhanced in *the gef10-W260S* lines but suppressed in the OE lines (Fig. 6A). Furthermore, we examined the expression of cytokinin-responsive genes (RRs) in developing panicles. Several type-A *RR* genes, which act as negative regulators of cytokinin signaling, were downregulated. Among these genes, *RR1*, a marker gene of cytokinin signaling in the SAM, was significantly upregulated (Fig. 6C). However, type-B *RRs*, including *RR21*, *RR23*, and *RR24*, which have been reported to function in panicle development, were more significantly upregulated in the panicles of the *gef10-W260S* mutant than in those of ZH11 (Fig. 6D), indicating elevated cytokinin signaling in the *gef10-W260S* mutant (Worthen et al. 2019). Overall, these results indicated that the gef10-W260S mutation promoted shoot development by enhancing cytokinin signaling.

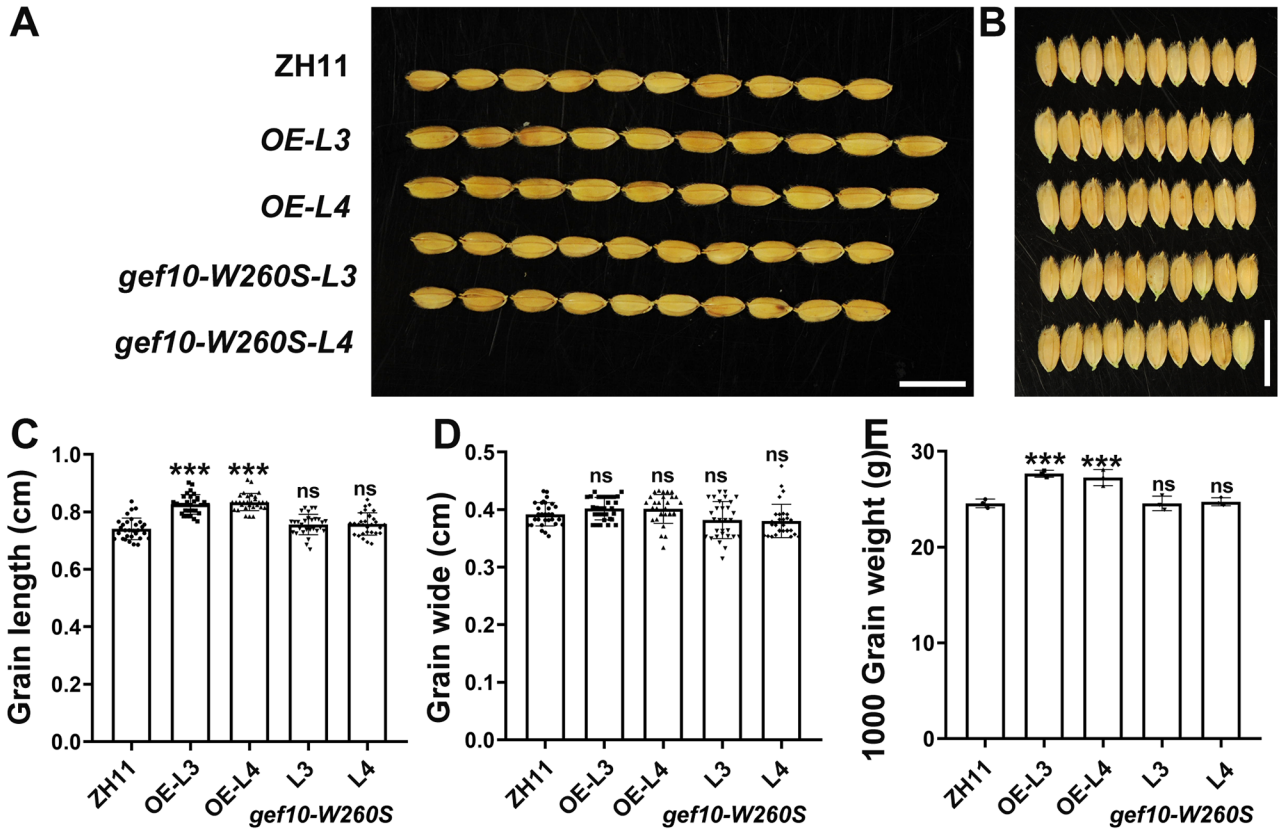

Fig. 4 Grain phenotype of the *gef10-W260S* mutant and OE lines. (**A**-**B**) Grain morphology of the *gef10-W260S* and OE lines. (**C**-**D**) Statistics of the grain length (**C**) and grain width (**D**) in the ZH11, OE and *gef10-W260S* lines. The data are presented as the means ± SDs, and the dots represent data points. At least 30 grains were used for the statistical analysis. Three biological replicates were used. (**E**) Comparison of 1000-grain weights of the ZH11, OE, and *gef10-W260S* lines. The data are presented as the means ± SDs from three replicates. Asterisks indicate statistically significant differences determined using the Student's t test. ns, not significant, $^{***}p < 0.001$. Scale bar: 1 cm

OsRopGEF10 has been Subjected to Artificial Selection During Japonica Rice Domestication

Given the key roles of *OsRopGEF10* in rice panicle and root development, we investigated its natural variation in rice germplasm. Using resequencing data from the ECOGEMS database (http://venyao.xyz/ECOGEMS), we identified single nucleotide polymorphisms (SNPs) in the coding region (1707 bp), promoter region (2000 bp) and untranslated region (UTR, 510 bp) of the gene (Fig. 7A) (Yao et al. 2019). Seven amino acid variations corresponding to SNPs in the CDS region were identified across the six haplotypes (Fig. 7A). Based on the analysis of sequences in different accessions, the SNP sites of *OsRopGEF10* were classified into six major haplotypes (Hap 1−6) (Fig. 7A, B). Notably, Hap1 and Hap2 accessions were identified mainly in *indica* and *wild Oryza species*, whereas Hap3 and Hap4 accessions were identified primarily in *japonica* (Fig. 7B). A comparison of the nucleotide diversity among *indica*, *japonica*, and *wild Oryza species* accessions revealed that nucleotide diversity, measured as Pi, was significantly lower in *japonica* accessions (Fig. 7C), suggesting that *OsRopGEF10* was subjected to artificial selection in *japonica* during rice domestication.

Discussion

In this study, we determined that *OsRopGEF10* was coexpressed with several *RACs* and *OsAHPs* in the SAM and developing IM (Figure S1), indicating an indispensable role of the OsRopGEF10-OsRACs module in SAM development. Given the poorly understood roles of *RopGEFs* in monocots, this study aimed to characterize the role of OsRopGEF10 in rice SAM and panicle development. Our results revealed that OsRopGEF10 played a pivotal role in regulating rice SAM and panicle development by modulating cytokinin signaling.

The development of inflorescence in rice is particularly sensitive to changes in cytokinin activity because of the prominent role played by cytokinin in regulating meristematic activity (Kieber et al., 2014). We showed that OsRopGEF10 regulated SAM and panicle development in rice. This process was associated with cytokinin signaling based on two key observations. First, OsRopGEF10 modulated cytokinin signaling by directly interacting

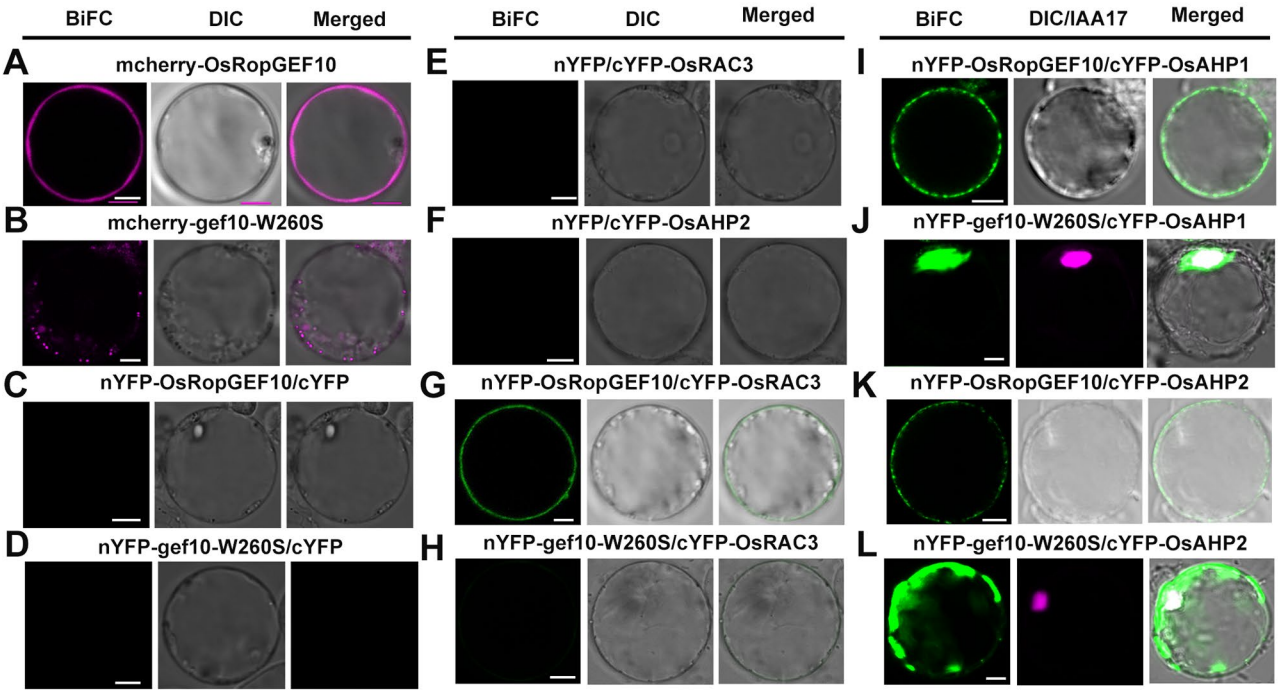

Fig. 5 Bimolecular fluorescence complementation (BiFC) assays of interactions between OsRopGEF10, OsRAC3, and OsAHP1/2 in rice protoplasts. (**A**) Localization of mCherry-OsRopGEF10 in rice protoplasts. (**B**) Localization of mCherry-gef10-W260S in rice protoplasts. (**C**-**F**) Coexpression of nYFP-OsRopGEF10 with cYFP (**C**), nYFP-gef10-W260S with cYFP (**D**), cYFP-OsRac3 with nYFP (**E**), and cYFP-OsAHP2 with nYFP (**F**) in rice protoplasts as negative controls. (**G**-**H**) nYFP-OsRopGEF10 interacted with cYFP-OsRAC3 at the PM (**G**), but nYFP-gef10-W260S failed to interact with cYFP-OsRAC3 (**H**). (**I**-**L**) The interaction between nYFP-OsRopGEF10 and cYFP-OsAHP1/2 generated BiFC signals on the PM (I, K), whereas the coexpression of nYFP-gef10-W260S and cYFP-OsAHP1/2 generated cytoplasmic BiFC signals (**J**, **L**). Scale bar: 5 μm

with the OsAHP proteins and disrupting their subcellular localization. As key positive components of cytokinin signaling, AHP proteins function by shuttling between the cytoplasm and nucleus to transfer the phosphate signal from the ER/PM-localized HK receptors to the nucleus-localized B-type ARRs (Kieber et al., 2014). Sequestering OsAHP1/2 on the PM via the OsRopGEF10-OsRAC3 module interferes with cytokinin signaling. However, mutation of the conserved 260th amino acid in OsRopGEF10 led to the retention of OsAHP1/2 in the cytoplasm and nucleus. On the other hand, gef10-W260S failed to activate OsRACs, which interfered with the recruitment of OsAHP1/2 to the PM; therefore, gef10-W260S facilitated cytokinin signaling. The enlarged SAM and panicles observed in the *gef10-W260S* lines are highly likely to reflect elevated cytokinin signaling. Second, both the *gef10-W260S* and OE lines presented altered cytokinin sensitivity in a dark-induced leaf senescence assay. The hypersensitive response to dark-induced chlorophyll degradation in the *gef10-W260S* mutant lines and the decreased cytokinin response in the OE lines consistently indicated that OsRopGEF10 negatively regulated cytokinin responses (Fig. 6). In addition, using RT–qPCR, we found that the expression of the type-A gene *RR1* and several type-B *RRs*, including *RR21/23/24* with a particular involvement in panicle and spikelet development, was significantly upregulated in *gef10-W260S*. Taken together, these data indicated a negative role of OsRopGEF10 in cytokinin signaling.

These results were consistent with the developmental phenotypes of SAMs and panicles in these *OsRopGEF10* OE and *gef10-W260S* lines stemming from a defect in cytokinin signaling. By suppressing cytokinin signaling, *OsRopGEF10* overexpression inhibited SAM and panicle development, resulting in reduced panicle and grain yields (Figs. 2 and 3). Most of these phenotypes were also observed in other rice lines with reduced cytokinin function, such as type-B *rr* multiple mutants, AHP RNAi lines or *hk5,6* mutants (Burr et al. 2020; Chun et al. 2023). Conversely, due to the enhanced cytokinin response, the *gef10-W260S* lines presented enlarged SAMs and panicles and thus increased grain yield per plant (Fig. 3). A previous study revealed that high-order type-B *RR* mutants, *rr21/22/23*, exhibit small panicles and fewer spikelets, and that *RR21* and *RR23* contribute most significantly to the panicle phenotypes (Worthen et al. 2019). Therefore, the panicle phenotype of the *gef10-W260S* mutant was attributable to the upregulated expression of *RR21/23/24*.

However, the phenomenon of polyembryo seedling production by the *OsRopGEF10* OE lines might

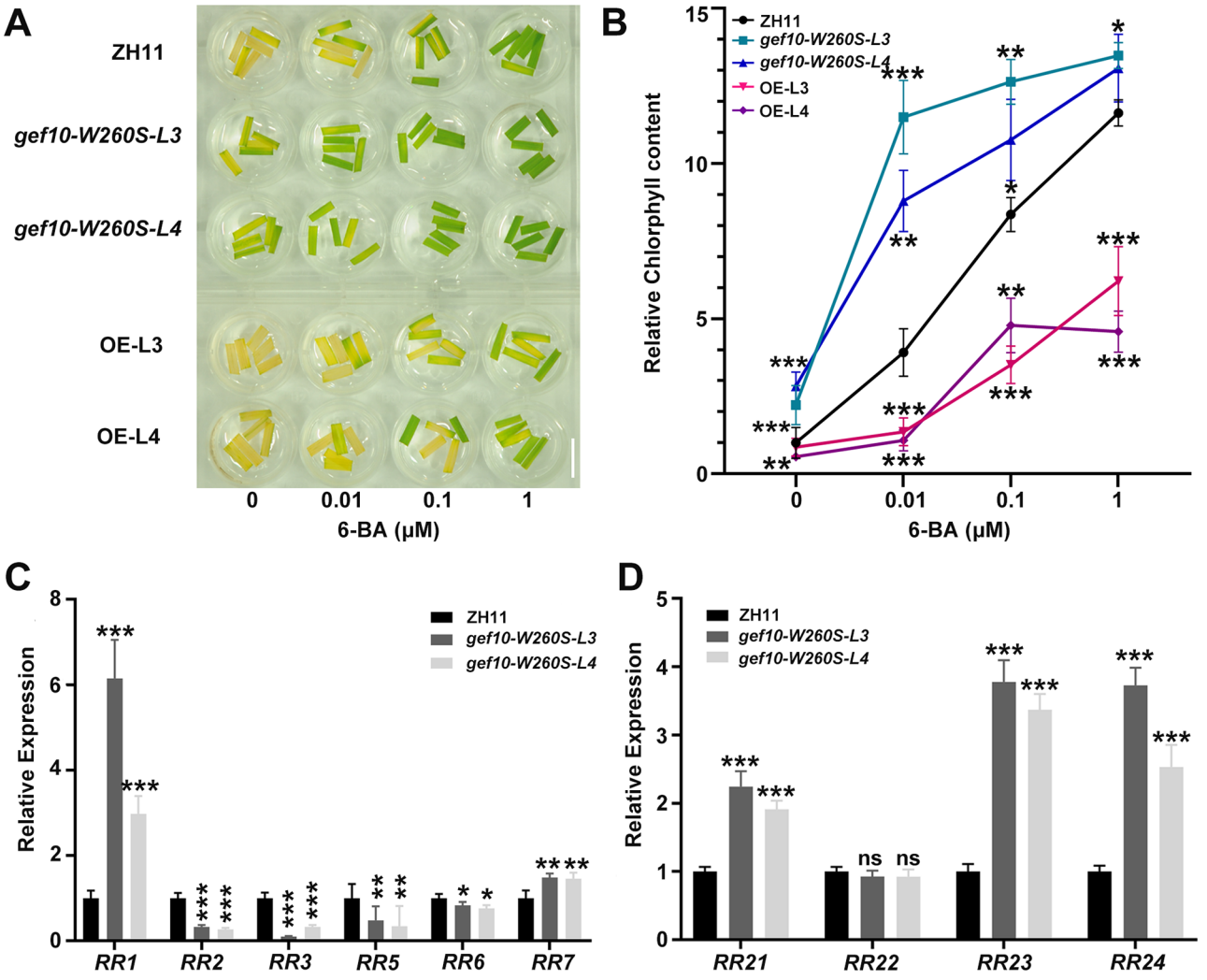

Fig. 6 Cytokinin response in the *gef10-W260S* and OE lines. (**A**) The leaves of ZH11, *gef10-W260S*, and OE lines were incubated in 1/2 MS liquid medium supplemented with various concentrations of 6-benzylamino purine (6-BA) for 48 h in the dark. (**B**) Quantitative analysis of the chlorophyll content after culture in the medium supplemented with different concentrations of 6-BA for 48 h in the dark. (**C**-**D**) qRT–PCR assays of the expression of type-A *RR* genes (**C**) and type-B *RR* genes (**D**) in 7-week-old panicles (< 0.5 cm in length) of the wild-type and *gef10-W260S* lines. The level of the WT mRNA was set at 1. Actin was used as an internal control. Significant differences were determined using a student's t-test. ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Error bars represent the three biological replicate. Scale bar: 1 cm

be independent of cytokinin signaling. In *Arabidopsis*, during early embryogenesis, suspensor cells can be reprogrammed to achieve the desired embryonic fate, leading to the formation of a second embryo and, eventually, twin-like seedlings (Radoeva et al. 2020). ROP signaling is essential in the auxin-mediated response and embryo development (Wu et al. 2011). Our previous study revealed that the perturbation of ROP signaling interferes with polar auxin transport, resulting in seedlings with two apical meristems (Huang et al. 2014). In rice, ROP signaling participates in auxin signaling because the expression of OsRopGEF10 and OsRAC3 is induced by auxin (Liu et al. 2023). Given the critical role of auxin in embryo development, the potential effect of the *OsRopGEF10-OsRAC3* module on the auxin pathway, which results in multiple embryonic seedlings, cannot be overlooked.

Rice grain yield is primarily determined by three components: the number of panicles per plant, the number of grains per panicle, and the grain weight. Among these factors, the grain number per panicle is a critical trait for determining rice yield (Li et al. 2013). Although the *OsRopGEF10* OE lines exhibited increased grain size and a greater thousand-grain weight, they produced significantly smaller panicles accompanied by a substantial reduction in the number of grains per panicle. Previous studies have reported that decreased cytokinin activity in the SAM leads to a reduced yield but an increase in seed size, which can be explained by the mechanism of compensation for the increase in seed size with a reduced

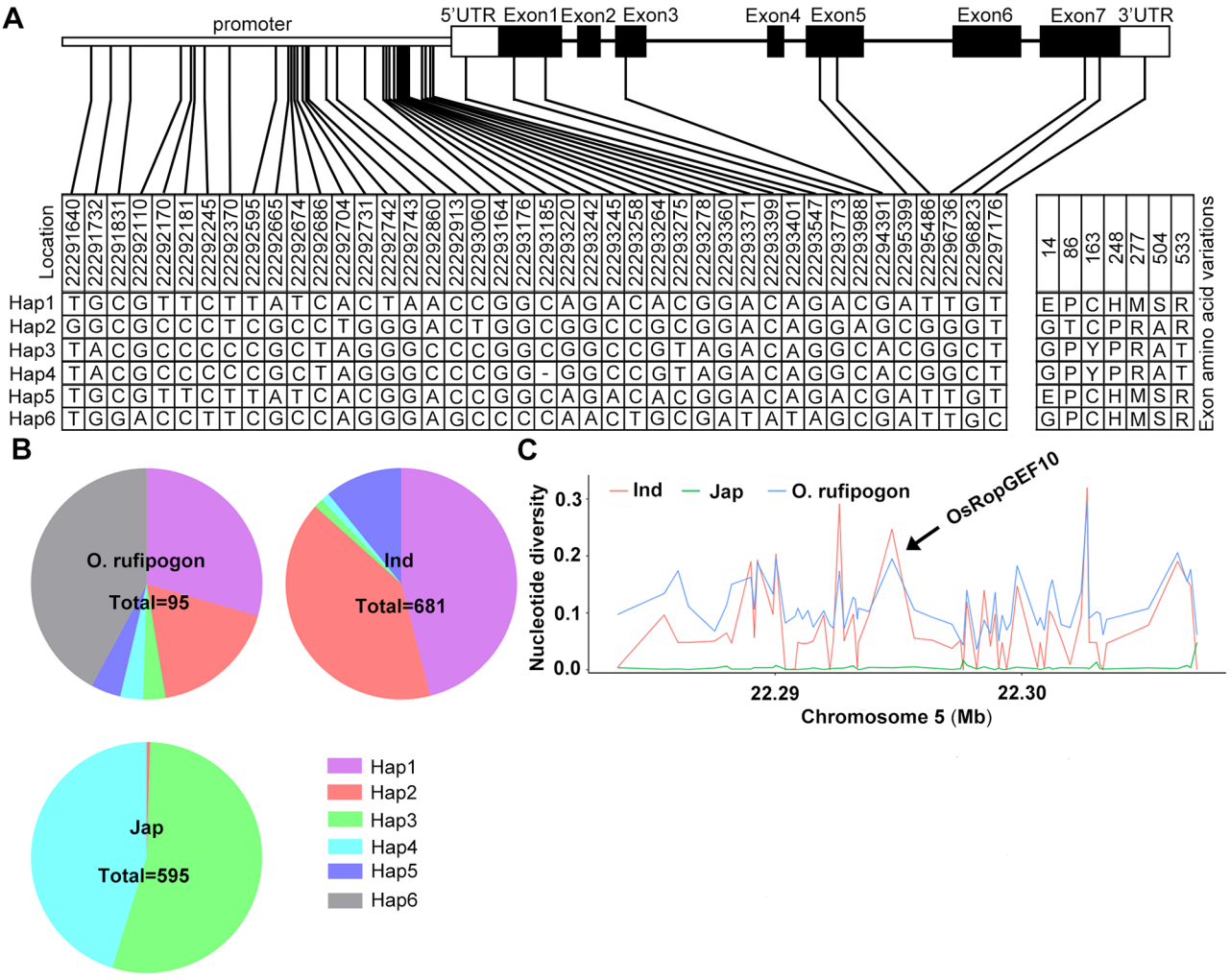

Fig. 7 Natural variations in *OsRopGEF10*. (**A**) Haplotype analysis of OsRopGEF10 based on genomic variation and haplotype frequency in rice germplasm. The amino acid variations corresponding to SNPs in the CDS region and their locations in the protein sequence are shown on the right. (**B**) Number of accessions and haplotype frequencies in different types of germplasms; different haplotypes are shown in different colors. Ind: *indica*; Jap: *japonica*. (**C**) Nucleotide diversity (*Pi*) for *O. rufipogon*, *indica*, and *japonica* rice groups in the 30-kb region spanning *OsRopGEF10*

seed number. Conversely, the *gef10-W260S* lines, surprisingly, produced larger panicles with a marked increase in the number of grains per panicle, without affecting the grain size. This finding presents a valuable breeding strategy for enhancing crop yield. Overall, our study provides insights into the molecular mechanisms of ROP signaling underlying the control of SAM development and grain yield, suggesting that *OsRopGEF10* may be a promising breeding tool for improving rice grain production.

Conclusions

This study highlights the key role of OsRopGEF10 in SAM and panicle development and contributes to cereal yield. The overexpression of *OsRopGEF10* led to smaller panicles and reduced grain yield, whereas the ectopic expression of a specific site-mutated *OsRopGEF10*, *gef10-W260S*, promoted panicle development and improved grain yield. We demonstrated that the modified OsRopGEF10-OsRAC module promoted panicle development and grain yield by enhancing cytokinin signaling, further indicating that OsRopGEF10 may be a promising target gene for breeding high-yield crops.

Materials and methods

Plant Materials and Growth Conditions

All the seeds were surface sterilized and germinated in 1/2 MS (Murashige and Skoog) medium containing 0.8% agar supplemented with 1% (w/v) sucrose at 25 °C under a 14-h light/10-h dark cycle, with a photon density of 300 μmol m^{-2} s^{-1}. Ten-day-old seedlings were transferred to a greenhouse and grown for two weeks. The plants were then transferred to a paddy field.

Vector Construction and Rice Transformation

To construct the *gef10-W260S* mutant vector, a point mutation for W260S was introduced by replacing the cDNA sequence TGG with TCG at position 779 bp following a method described previously (Thomas et al. 2007). The primers used for introduction of the mutations are listed in Table S1. The resulting full-length cDNA of *gef10-W260S* was amplified via the primer sets OE-OsRopGEF10-F and OE-OsRopGEF10-R and inserted into the *Hind*III and *Bam*HI sites of the pOx vector. The resulting fusion constructs were subsequently introduced into the *Agrobacterium tumefaciens* strain EHA105 to facilitate *Agrobacterium*-mediated transformation of rice (ZH11), as previously described (Zhao et al. 2009). Transgenic plants were selected on 1/2 MS medium containing 50 mg/L hygromycin at 25 °C. The primers used for vector construction are listed in Table S1.

GUS Staining and Histological Analysis

A 2464-bp promoter region from ZH11 was cloned and inserted into the pCAMBIA-1305 vector to create a *pOsRopGEF10*::GUS construct, and the resulting plasmid was subsequently transformed into the *Oryza sativa* japonica cultivar Zhonghua 11, as described previously (Liu et al. 2023). The collected tissue samples (developing embryo, floral meristem, spikelets, and pistil) were subjected to 8 h of incubation in GUS staining solution (comprising 0.1 M sodium phosphate buffer, pH 7.0; 10 mM sodium EDTA; 2 mM potassium ferrocyanide; 2 mM potassium ferricyanide; and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-glucuronic acid). The stained samples were then decolorized in 70% ethanol and photographed under a microscope (Olympus BX51). For the histological analysis of the SAM, 10-day-old seedlings were collected and fixed in formaldehyde-acetic acid-ethanol fixative (FAA) solution. Thin sections were prepared and photographed as described previously (Liu et al. 2023).

Phenotypic Analysis and Western Blotting Analysis

A total of 15 panicles from the field-grown plants at the mature stage were collected and photographed. Plant height was measured in the field. The length, number of primary branches, and number of main panicles were determined after harvest. The total grains of each plant were collected to measure the grain yield per plant. At least 15 independent plants were used for the analysis. The grain length and width were measured via ImageJ. To analyze the protein levels of OsRopGEF10 and gef10-W260S in rice, the leaves of 2-week-old transgenic plants (weighing 100 mg) were collected and ground into powder in liquid nitrogen, followed by cell lysis with RIPA buffer (Beyotime, Shanghai) for 30 min. The cell lysates were boiled in 5× SDS loading buffer and centrifuged at 12,000 ×g for 10 min, followed by separation of proteins via 12% SDS–PAGE. Immunoblotting with an anti-FLAG antibody (Abmart, cat no. M20008, 1:5000) was performed for protein detection.

Protein Interaction Assays in Yeast

Full-length OsRAC3, CA-OsRAC3, and DN-OsRAC3 were cloned and inserted into pGBKT7, and OsRopGEF10 cDNA was cloned and inserted into pGADT7, resulting in OsRAC3, CA-OsRAC3, DN-OsRAC3-BD, and OsRopGEF10-AD, respectively (see Supplemental Table S1 for primer information). The bait and prey vectors were cotransformed into the Y2H Gold strain (Clontech) to test their interactions, as described previously (Liu et al. 2023).

BiFC Assays in Rice Protoplasts

Rice seedlings cultured in the dark for 7–10 days were used to prepare protoplasts. Protoplast isolation and transformation were carried out as previously described, with some modifications. nYFP-gef10-W260S was cotransformed with cYFP-OsAHP1/2 or OsRAC3 into rice protoplasts (Yoo et al. 2007). The combinations of nYFP/cYFP with the NYFP-OsRopGEF10, gef10-W260S, OsRAC3, or CYFP-OSAHP1/2 vectors were used as controls. YFP expression in the protoplasts was visualized via confocal microscopy (Zeiss 780 & 7Live) 16 h after transformation. The fluorescent signals were visualized with excitation (Ex) and emission (Em) wavelengths (Ex/Em) of 488 nm/505–530 nm for GFP, 514 nm/530–560 nm for YFP, and 543 nm/560–620 nm for mCherry. The sequences of the primers used for plasmid construction are listed in Supplemental Table S1.

Chlorophyll Content Measurement

The second leaves from 35-day-old ZH11, *OsRopGEF10-OE* and *gef10-W260S* transgenic plants at the tillering stage were collected and cut into 5-mm segments. The chlorophyll was extracted using 80% (v/v) acetone for 24 h, and its content was determined through spectrophotometric measurements at 645 and 663 nm, as previously described (Mackinney 1941).

RNA Extraction and qRT–PCR

To determine the expression of the *RopGEF*, *OsRAC* and *OsAHP* genes in the SAM, we collected shoots from 10-day-old seedlings and vegetative SAM from 21-day-old seedlings. IMs from young panicles (early-stage plants with inflorescence lengths < 0.5 cm) were collected from 7-week-old plants. In total, three biological replicates for each sample were obtained from 20 to 40 plants. The samples were frozen in liquid nitrogen. Total RNA from three biological replicates was extracted using TRIzol reagent (Magen, Guangzhou, China) and then

reverse transcribed using the HiScript®II Q RT SuperMix for qPCR (+gDNA) wiper (Vazyme, Nanjing, China) following the manufacturer's protocol. qRT–PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and a real-time PCR detection system (Bio-Rad), with three biological replicates. The rice *ACTIN* gene was used as the internal control. The primers used for qRT–PCR are listed in Table S1.

Haplotype and Nucleotide Diversity Analysis

To determine the haplotypes of *OsRopGEF10*, we used 1,276 cultivated rice accessions and 95 wild rice (*Oryza rufipogon*) accessions across 8,584,244 SNP sites in the ECOGEMS database (http://venyao.xyz/ECOGEMS/).

Abbreviations

RAC/ROP	Rho-related GTPase from plants
GEF	Guanine nucleotide exchange factors
SAM	Shoot apical meristem
AHP	Histidine phosphotransfer proteins
PM	Plasma membrane
HK	Histidine kinase
RR	Response regulator
WT	Wild-type
CKX2	Cytokinin oxidase/dehydrogenase 2
OE	Overexpression
IAA	Indole-3-acetic acid
BiFC	Bimolecular fluorescence complementation
SNPs	Single nucleotide polymorphisms
UTR	Untranslated region
MS Medium	Murashige & Skoog Medium
cDNA	Complementary DNA
GUS	β-Glucuronidase
YFP	Yellow fluorescent protein
GFP	Green fluorescent protein
RNA	Inflorescence meristem
IM	Inflorescence meristem
qRT-PCR	Real-time quantitative PCR

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Author Contributions

L.Z. and H.L. designed the experiment. H.L. and M.L. wrote the manuscript. M.L., L.F. and J.J. performed experiments and analyzed the data. H.Y. and M.L. was responsible for field management. All authors have read and agreed to the published version of the manuscript.

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Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no competing interests.

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