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Supplementary Information for

The Rho-family GTPase *OsRac1* controls rice grain size and yield by regulating cell division

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Materials and Methods

Plasmid construction and rice transformation. For construct expressing OsRac1 in rice, the full-length coding region of *OsRac1* was amplified from wild type (*Oryza sativa* Nipponbare) and cloned into binary vector pCAMBIA1301 driven by the maize Ubiquitin (UBI) promoter. The gene editing constructs for *OsRac1* and *OsMAPK6* via CRISPR-Cas9 were designed as previously described (1). A 2-kb DNA fragment upstream of the *OsRac1* start codon was amplified from BAC clone and cloned into the pCAMBIA1300-GUSPlus vector to generate construct *pOsRac1::GUS*. Resultant constructs *pUBI::OsRac1*, *pUBI::OsRac1-Cas9* and *pOsRac1::GUS* were transformed into Nipponbare by *Agrobacterium*-mediated transformation (2). The complementation construct *pUBI::OsMAPK6-Cas9* was transformed into transgenic line overexpressing *OsRac1* by *Agrobacterium*-mediated transformation. All constructs were confirmed by sequencing. The PCR primer sets are listed in *SI Appendix*, Table S1.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis. To analyze the *OsRac1* transcript levels, total RNA was extracted from different rice tissues at different developmental stages of WT, *OsRac1* transgenic lines by Trizol method (Invitrogen). To analyze the expression of cell cycle-related genes, RNA of 4-cm panicles was used for qRT-PCR analysis. First-strand cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). qRT-PCR analysis was performed on the ABI 7300 Real Time PCR System using the UNICON qPCR SYBR Green method (YEASEN). The *UBQ5* gene was used as reference gene to normalize the expression level of examined genes. The primers used in this study are listed in *SI Appendix*, Table S2.

RNA-seq and GO term analysis. For RNA-seq analysis, total RNA was extracted from 0.5-cm and 6-cm panicles of WT, *OsRac1* transgenic lines using the RNeasy Plant Mini Kit (Qiagen) and Illumina sequencing libraries were constructed according to the manufacturer's instructions. The libraries were sequenced with Hiseq2500. Reads were aligned to the rice genome (MSU7.0) using Tophat. Differentially expressed genes were indicated as false discovery rate (FDR) <0.05 with Cuffdiff.

Gene ontology (GO) enrichment was performed using BiNGO. GO information is from

the gene ontology website (http://www.geneontology.org/) or Rice Genome Annotation (http://rice.plantbiology.msu.edu/).

GUS staining. GUS staining of *pOsRac1::GUS* rice transgenic lines was performed as described previously (3). The samples were stained in GUS staining buffer for 48 h at 37°C, then cleared in 75% ethanol to remove the chlorophyll, and observed under the microscope.

Rice traits measurement. The grain width, length, 1,000-grain weight, yield per plant and yield per acre were measured when the rice plants were completely matured. For the traits measurement, we used grains from the middle part of the largest panicle. For the grain width and length measurement, 200 grains were measured once and the measurement was repeated for more than 3 times. For the 1,000-grain weight measurement, 500 grains were weighed for once and the measurement had more than 10 repeats. For yield per plant measurement, all full grains of one plant were weighed. more than 15 plants were measured. For the plot yield tests, rice was planted under 20 cm x 20 cm density, 400 plants for one repeat and the measurement had 4 repeats in shanghai Songjiang farm. All plants in the plots were harvested and threshed. After removing all the impurities, the grains were dried and weighed. For full grain number per plant and seed setting rate per plant measurement, more than 10 plants were measured. For productive tillers number per plant, plant height, panicle length, primary branches and secondary branches per panicle measurement, more than 10 plants were measured. The statistical analysis was performed by using the student's *t*-test.

Nucleus isolation and assessment of ploidy. After the panicle length was measured, the spikelet hulls at three different developmental stages were chopped with a sharp blade soaking in the nucleus isolation mixture (Beckman). Isolation of cell nuclei and assessment of ploidy were performed as described previously (4, 5). After filtering through a 40 μm nylon riddle, the nucleus suspension was loaded into the Flow cytometer to assess the nucleus ploidy. For each detection, the ploidy of 10000 to 20000 nucleuses was recorded. The numbers of diploid, isoploid and tetraploid nuclei were recorded, and the percentage of cells with 2C and 4C DNA content was calculated using FCS Express 4 software, respectively.

Yeast two-hybrid screening and assay. The full length of *OsRac1* coding region was

cloned into the pGADT7 (AD) vector, and a rice cDNA library from seed of Zhonghua11 was constructed into pGBKT7 (BD) vector. A BD Matchmaker library construction and screening kit (Clontech Laboratories, Inc.) was used for yeast two-hybrid assays according to the manufacturer's user manual. The yeast strain Y187 transformed with pGADT7-OsRac1 was mated with the yeast strain AH109 transformed with the BD fusion library and the resulting progeny was cultured on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade/X-gal plates for positive colonies selection. The candidate proteins that interact with OsRac1 were characterized by sequencing the positive colonies. The full length coding region of target genes was cloned into the pGBKT7 (BD) vector and co-transformed with pGADT7-OsRac1 into the yeast strain AH109 to confirm the interaction. The primers used in this study are listed in *SI Appendix*, Table S3.

Co-Immunoprecipitation analysis. Rice protoplasts were isolated from two-week-old seedlings (grown in a rice growth chamber at 28°C, 40% humidity, and 80 $\mu mol \cdot m^2 \cdot s^{-1}$ light intensity under a 14-h light/10-h dark photoperiod) using the method described by Jen Sheen' lab. Rice protoplasts were co-transformed with p35S::FLAG-OsRac1 and p35S::OsMAPK6-GFP. The transformed protoplasts were cultured at 23°C under dark for 6-8 hours. After cultured, the protoplasts were collected and lysed in 250 µl of co-immunoprecipitation buffer [40 mM HEPES (PH 7.4), 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerol phosphate, 0.2% CHAPS, 1 x mixture inhibitors (Roche)], For immunoprecipitation, the protein extracts were incubated with anti-GFP antibody at 4°C for 2 hours, and then added 15 µl of protein G magnetic beads (Invitrogen) to incubate for another 2 hours. The immunoprecipitated proteins were washed three times with washing buffer [40 mM HEPES (PH 7.4), 15 mM NaCl, 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerol phosphate, 0.2% CHAPS], added same volume of 2 x SDS loading buffer and incubated at 95°C for 5 mins, then done SDS/PAGE running and western blot analyses. The primers used in this study are listed in *SI Appendix*, Table S4. The antibodies used in this study are listed in *SI Appendix*, Table S5.

Analysis of OsMAPK6 phosphorylation level. Two-week-old rice seedlings (1g) or young panicles (0.5 g) were grinded in liquid nitrogen and the ground tissues were suspected in 1ml protein extraction buffer [100 mM HEPES (PH 7.5), 5 mM EDTA, 10 mM Na_3VO_4, 10 mM NaF, 50 mM glycerol phosphate, 10 mM DTT, 1 pill/50ml EDTA free cocktail (Roche)], violent oscillated, and rotated in 4°C for 30 mins. The sample was then centrifuged (14000

rpm) for 10 mins, and same volume of 2 x SDS protein loading buffer was added and incubated at 95°C for 5 mins. The denatured sample was used for western blot assay using anti Phospho-p44/42 MAPK antibody (Cell Signaling Technology) at 1:1000 dilution for OsMAPK6 phosphorylation level detection and anti AtMPK6 antibody (Sigma) at 1:5000 dilution for OsMAPK6 protein level detection. Data were generated from at least three independent experiments. The antibodies used for western blot are listed in *SI Appendix*, Table S5.

References

1. Miao J, et al. (2013) Targeted mutagenesis in rice using CRISPR-Cas system. *Cell Res* 23(10):1233-1236.
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3. Duan P, et al. (2014) SMALL GRAIN 1, which encodes a mitogen-activated protein kinase kinase 4, influences grain size in rice. *Plant J* 77(4):547-557.
4. Qi P, et al. (2012) The novel quantitative trait locus GL3.1 controls rice grain size and yield by regulating Cyclin-T1;3. *Cell Res* 22(12):1666-1680.
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Supplementary Figures

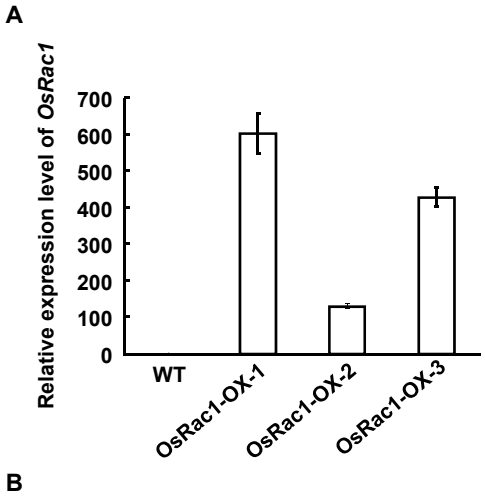

Fig. S1. Identification of OsRac1 rice transgenic lines. (*A*) OsRac1 expression levels in OsRac1-overexpressing transgenic lines. (*B*) The mutated sites of three OsRac1 CRISPR-Cas9 lines.

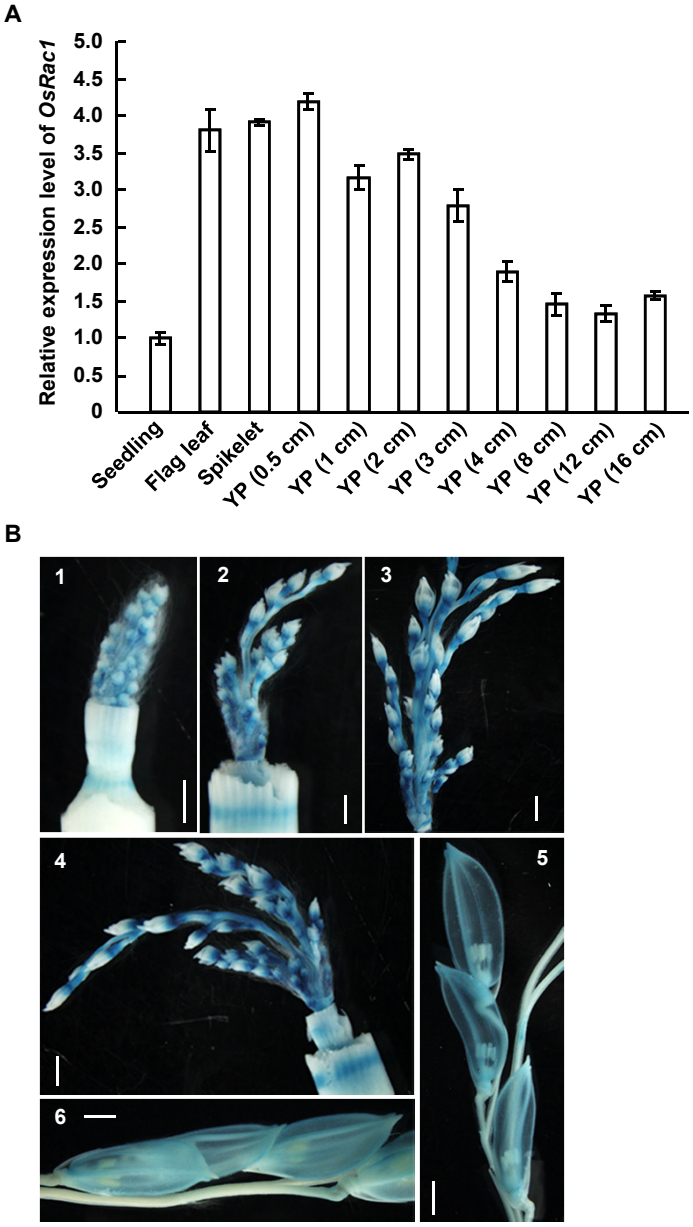

Fig. S2. Expression pattern of OsRac1 in various tissues analyzed by quantitative RT-PCR (qPCR) and GUS staining. (*A*) Expression levels of OsRac1 in various tissues. YP, young panicle. (*B*) Expression of OsRac1 in panicles revealed by GUS staining. *B1*, 2 mm young panicle; *B2*, 5 mm young panicle; *B3*, 1 cm young panicle; *B4*, 2 cm young panicle; *B5*, 6 cm young panicle; *B6*, 12 cm young panicle. Bar = 1 mm.

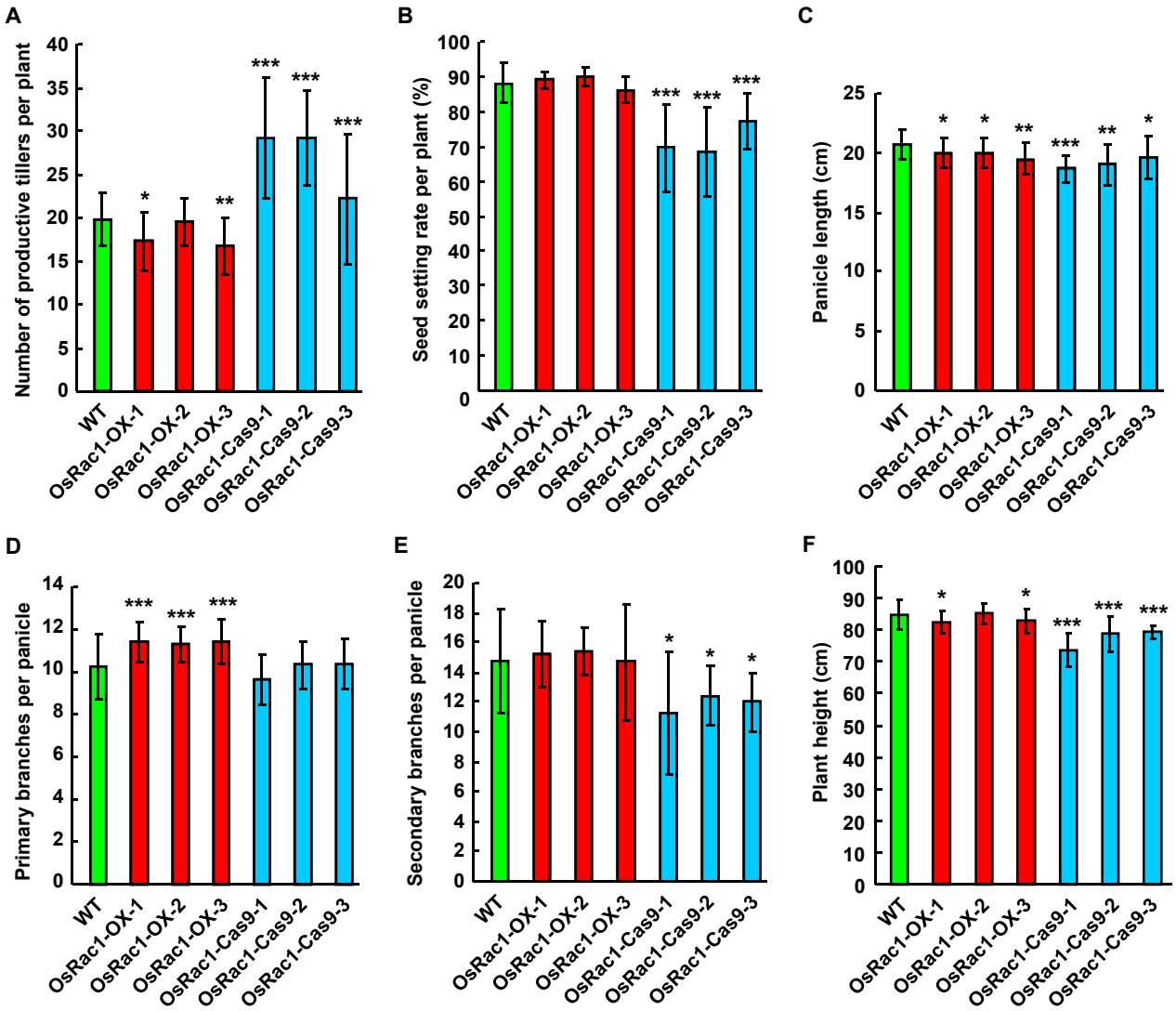

Fig. S3. The yield traits of WT and OsRac1 transgenic lines. Numbers of productive tillers per plant (*A*), seed setting rate per plant (*B*), panicle length (*C*), number of primary branches per panicle (*D*), number of secondary branches per panicle (*E*), and plant height *(F)* of WT and OsRac1 transgenic lines were measured and statistically analyzed. Data are shown as means ± SD in *A* ($n > 10$), *B* ($n > 20$), *C* ($n > 10$), *D* ($n > 18$), *E* ($n > 10$) and *F* (n > 10). * $P < 0.1$, ** $P < 0.01$, *** $P < 0.001$ (*t* test).

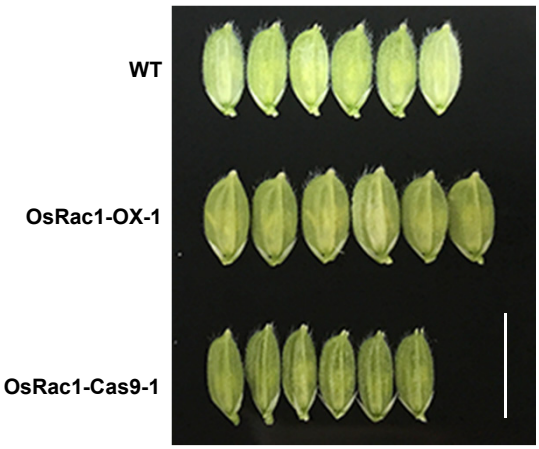

Fig. S4. OsRac1 controls spikelet size. Spikelets morphology of WT and OsRac1 transgenic lines are shown (bar = 1 cm).

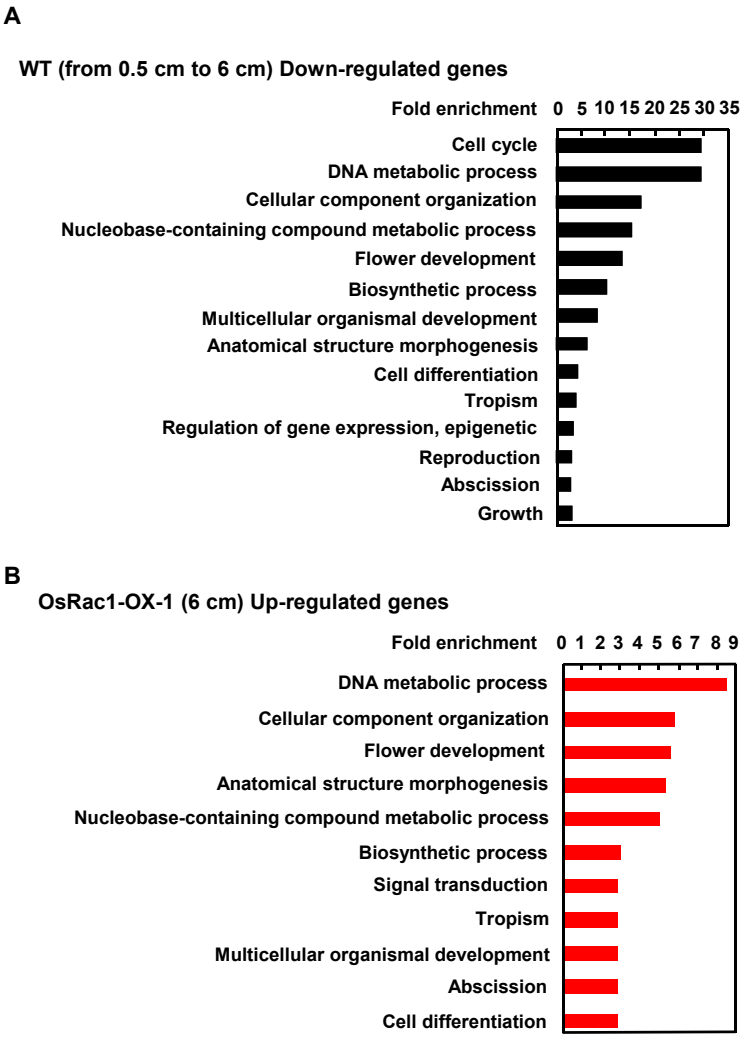

Fig. S5. RNA-seq transcripts analysis. (*A*) Functional category analysis for transcriptional response in WT from 0.5-cm young panicles to 6-cm young panicles. The compared sets of genes contained transcripts, which were significantly down-regulated ($p<0.05$) in 6-cm young panicles compared to 0.5-cm young panicles of WT. (*B*) Functional category analysis for transcriptional response in 6-cm young panicles of OsRac1-OX-1. The compared sets of genes contained transcripts, which were significantly up-regulated ($p<0.05$) in OsRac1-OX-1 compared to WT.

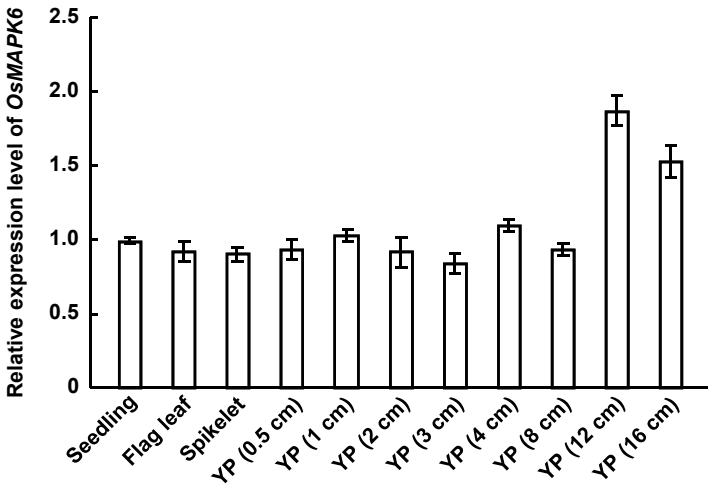

Fig. S6. Expression pattern of OsMAPK6 in various tissues analyzed by qPCR analysis. Seedling, flag leaf, spikelet, young panicles (YPs) at different developmental stages were analyzed.

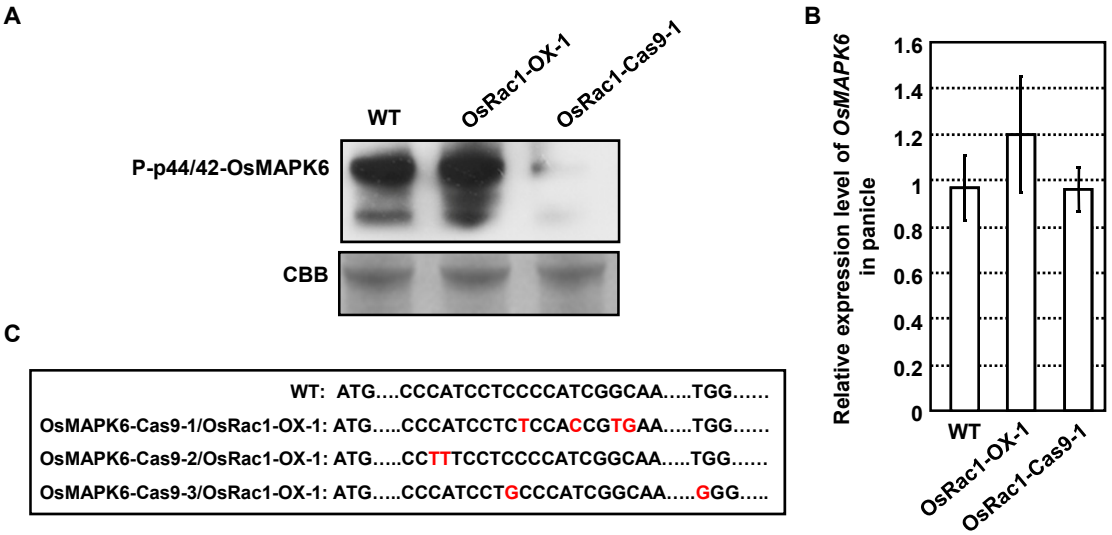

Fig. S7. OsRac1 regulates OsMAPK6 in rice panicles. (*A*) The phosphorylation level of OsMAPK6 in 5.5-cm young panicles of WT and OsRac1 transgenic lines. The loading control was total protein. CBB, Coomassie brilliant blue. (*B*) Expression level of OsMAPK6 in 6-cm young panicles of WT and OsRac1 transgenic lines by qPCR analysis. (*C*) The mutated sites of OsMAPK6 CRISPR-Cas9 transgenic lines under OsRac1 overexpression background.

Table S1. Primers used in constructs for rice transformation.

Construct	Forward primer (5'-3')	Reverse primer (5'-3')
OsRac1-OX	CGGGATCCATGAGCTCGGCGGCGG CGGCG	CGGATATCCGCGAAACAAGCGCTTC
OsRac1-Cas9	GGCAAGCGCCAATGTCTCCGTGGA	AAACTCCACGGAGACATTGGCGCT
OsMAPK6-Cas9-A	GGCACCCATCCTCCCCATCGGCAA	AAACTTGCCGATGGGGAGGATGGG
OsMAPK6-Cas9-B	GGCACTGAAGGGTGATGAGCAGAC	AAACGTCTGCTCATCACCCTTCAG
Promoter-OsRac1	CACCGTAGGAGGAAGGCTTTCACC	CTCCCCCGCCCACTCCGGC

Table S2. Primers used in quantitative real-time PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
OsRac1	CAACGGAGCAGGGAGAAGAAC	CTTCCGCAAAAGTACCGCCT
OsMAPK6	AAGTACAAGCCCCCCATCCTC	AACGTCATTGAATGAATTCC
KRP3	ATGGGCAAGTACCTCAGGAG	GCACTCCACCGCCTCACACC
KRP4	CAGAAGAGGCAGGGCGGGGA	AGTGGCTGCGCCTTGTGGTA
KRP5	AAGGAGAAGGAGACCGCCAG	AACGACAAACAGAGGTCTCC
CYCA2.1	AGGCGCAACAGGCTGCAGCTC	CGCTTTTTGTCTTCCTGGCA
CYCA2.2	CAACCAAGACGTTCCTGAGG	ATGCCCTGTAGCCGGTCACC
CYCA2.3	GTTTCGGTTGACGAGACGATGT	CGCTGCAAGGAACCTAGAACTG
CYCB2.1	AAGTTTGGCCAGGAGTGAGCA	TCAAGAGCATCAGCGTCGAGA
CYCB2.2	CTCAAGGCTGCACAATCTGACA	GCATTGACGGCTGGAATTTG
CYCD3.1	ATGGCTTTCGCCACGCTCTT	CGCGGCCGTGAGCGCGGAG
CDT2	TACTTCAGTTGACTGGTGTG	GCAATTCACCATCTGCACTGG
E2F2	TGTTGGTGGCTGCCGATAT	CGCCAGGTGCACCCTTT
MAPK	ACAGAGCAGCCGAATTTTGAGA	TCAATGGGCAGGATGTCGCTC
CDC20	TCGAATCACCTGTTTGTTGGC	TGGAGACAATCCAACGCAAAG
CDC45	TCCTACGACTTCGACGTC	GGTGTTCTTCCGCAGCGC
MAD2	GAGCCATGCATATTCGACGTA	GGTGTCGAAGGAATGCAGCTT
H1	CGCCGGTGGCTCCTCCCACC	GAGTCCTTGGCGGCGTCGGAG

Table S3. Primers used in yeast two-hybrid screening.

Construct	Forward primer (5'-3')	Reverse primer (5'-3')
OsRac1-AD	CGGATCCATGAGCTCGGCGGCGG CGGCGA	ACGCGTCGACCGCGAAACAAGCGCT TC
CA-OsRac1-AD	CGGATCCATGAGCTCGGCGGCGG CGGCGA	CTTCCCCACCGCGACGTCCCCCACG GTGA
	CGTCACCGTGGGGGACGTCGCGG TGGGGA	ACGCGTCGACCGCGAAACAAGCGCT TC
DN-OsRac1-AD	CGGATCCATGAGCTCGGCGGCGG CGGCGA	CCTATCTTCACGGAGAGCCAACTTG GTTC
	GTTGGAACCAAGTTGGCTCTCCGT GAAGAT	ACGCGTCGACCGCGAAACAAGCGCT TC
OsMAPK6-BD	GGATCCATGGACGCCGGGGCGCA GC	GTCGACTTTGTCCTCGGACAATGCA TGCTG

Table S4. Primers used in Co-immunoprecipitation.

Construct	Forward primer (5'-3')	Reverse primer (5'-3')
OsRac1-Flag	CACCATGAGCTCGGCGGCGGCGGCG	CGCGAAACAAGCGCTTC
OsMAPK6-GFP	CACCATGGACGCCGGGGCGCAGCCGC	CTGGTAATCAGGGTTGAACG CAAG

Table S5. Antibodies used in western blot.

Name	Species	Company	Working concentration
anti-Flag	Mouse	Abmart	1:5000
anti-GFP	Mouse	Abmart	1:5000
anti-P-p44/42-MAPK6	Rabbit	Cell Signaling	1:1000
anti-AtMPK6	Rabbit	Sigma	1:5000