

Supplementary information

Regulatory roles of *PURPLE FRUIT 1* and *OsPa* in pigmentation of rice grain

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Table S1. Genes of *Oryza sativa* investigated in this study.

GeneID	Gene name	Reference	Synonym	Reference	Allele involved
Os01g50110	<i>OsPF1</i>	This study	<i>MYB4</i>	Suzuki et al., 1997	<i>OsPF1_b</i> (9311 allele)
Os03g29614	<i>OsMYB3</i>	Zheng <i>et al.</i> , 2021	<i>OsKala3</i>	Maeda et al., 2014	<i>OsMYB3_p</i>
Os06g10350	<i>OsC1</i>			Reddy et al. 1998	<i>OsC1</i> (Nipponbare allele)
Os04g47080	<i>OsB1</i>		<i>Ra</i>	Hu et al., 1996	<i>OsB1_b</i> (9311 allele)
Os04g47059	<i>OsB2</i>		<i>B2</i>	Sakamoto et al. 2001	<i>OsB2_p</i>
			<i>Kala4</i>	Oikawa et al. 2015	
			<i>S1</i>	Sun et al. 2018	
Os04g47040	<i>OsPa</i>	Yang <i>et al.</i> , 2021	<i>Pa (OsPa)</i>	Meng et al. 2021	<i>OsPa_a</i> (Nipponbare allele)
Os02g45810	<i>OsTTG1</i>		<i>OsPAC1</i>	Zheng et al. 2019	<i>OsTTG1_b</i> (9311 allele)
			OsWD40-50	Ouyang et al. 2012	
Os11g32640	<i>OsCHS1</i>			Shih et al. 2008	<i>OsCHS1_a</i> (Nipponbare allele)
Os03g60509	<i>OsCHI</i>			Shih et al. 2008	<i>OsCHI_c</i> (Malugu allele)
Os04g56700	<i>OsF3H</i>			Shih et al. 2008	<i>OsF3H_a</i> (Nipponbare allele)
Os10g17260	<i>OsF3'H</i>			Shih et al. 2008	<i>OsF3'H_b</i> (9311 allele)
Os01g44260	<i>OsDFR</i>		<i>A, Rd</i>	Furukawa et al. 2006	<i>OsDFR_a</i> (Nipponbare allele)
Os01g27490	<i>OsANS1</i>			Shih et al. 2008	<i>OsANS1_a</i> (Nipponbare allele)
Os06g09240	<i>Os3GT</i>	This study			<i>Os3GT_a</i> (Nipponbare allele)
Os03g15360	<i>OsLAR</i>	This study			<i>OsLAR_a</i> (Nipponbare allele)

Table S2. Primers used in this study.

Experiment	Target gene	Primer name	Sequence (5'→ 3')*
Y2H	OsMYB3	OsMYB3-Y2H-AD-F	<u>CCGGAATT</u> CATGGGGAGGAAGCCGTGC
		OsMYB3-Y2H-AD-R	ACGCGTCGACTCTACTGGTGGTGGTGGTGGT
	OsPF1	OsPF1-Y2H-AD-F	<u>TCCTCTGCTAGCAGAGAATTCATGAAGCGGAAGCGGCC</u>
		OsPF1-Y2H- AD-R	<u>CTAGAGTCGACTAATACTCTCGAGTTACTGTTTGGATTCCATGACGTCT</u>
	OsB1	OsB1-Y2H-AD-F	<u>GAATT</u> CATGGAAGAGACCCCTCTGCCATC
		OsB1-Y2H-AD-R	<u>TCTAGAC</u> CTAGCTTGCTATAGCTTTCGGAGAG
		OsB1-Y2H-BD-R	<u>CATATG</u> TCATCCCAGGTTCGACGGCAG
	OsB2	OsB2-Y2H-AD-F	<u>GAATT</u> CATGGCATCTGCTCCTCCAGTT
		OsB2-Y2H-AD-R	<u>ATACCGCTCGAGT</u> TACGGCGCCTTCCCCTGT
		OsB2-Y2H-BD-R	<u>TCCTCTGCTAGCTT</u> ACGGCGCCTTCCCCTGT
	OsPa	OsPa-Y2H-AD-F	<u>GCTAG</u> CATGGCATCTGCTCCTCCAGTTCA
		OsPa-Y2H-AD-R	<u>TCTAGAT</u> CTAGCCACCGATAGCTTTCTGGA
		OsPa-Y2H-BD-F	<u>ATCGAT</u> ATGGCATCTGCTCCTCCAGTTCA
		OsPa-Y2H-BD-R	<u>CATATG</u> CTAGCCACCGATAGCTTTCTGGA
	OsTTG1	OsPAC1-Y2H-AD-F	<u>GAATT</u> CATGGAGCAGCCCAAGCCG
		OsPAC1-Y2H-AD-R	<u>CTGCAGAT</u> CAGACCCTGAGAAGCTGGA
BiFC	OsMYB3	OsMYB3-BiFC-F	<u>CGCCACTAGTGGATCC</u> ATGGGGAGGAAGCCGTGC
		OsMYB3-BiFC-NR	<u>TCCATCCCCGGGAGCGGTACC</u> CTGGTGGTGGTGGTGGT
		OsMYB3-BiFC-CR	<u>TACATCCCCGGGAGCGGTACC</u> CTGGTGGTGGTGGTGGT
	OsPF1	OsPF1-BiFC-F	<u>CGCCACTAGTGGATCC</u> ATGAAGCGGAAGCGGCC
		OsPF1-BiFC-CR	<u>TACATCCCCGGGAGCGGTACC</u> CTGTTTGGATTCCATGACGTCT
		OsPF1-BiFC-NR	<u>TCCATCCCCGGGAGCGGTACC</u> CTGTTTGGATTCCATGACGTCT
	OsB2	OsB2-BiFC-F	<u>CGCCACTAGTGGATCC</u> ATGGCATCTGCTCCTCCAGTT
		OsB2-BiFC-NR	<u>TCCATCCCCGGGAGCGGTACC</u> CGGCGCCTTCCCCTGT
		OsB2-BiFC-CR	<u>TACATCCCCGGGAGCGGTACC</u> CGGCGCCTTCCCCTGT
	OsPa	OsPa-BiFC-F	<u>CGCCACTAGTGGATCC</u> ATGGCATCTGCTCCTCCAGTTCA
		OsPa-BiFC-NR	<u>TCCATCCCCGGGAGCGGTACC</u> GCCACCGATAGCTTTCTGGA
		OsPa-BiFC-CR	<u>TACATCCCCGGGAGCGGTACC</u> GCCACCGATAGCTTTCTGGA
	OsPAC1(TTG1)	OsPAC1-BiFC-F	<u>CGCCACTAGTGGATCC</u> ATGGAGCAGCCCAAGCCG
		OsPAC1-BiFC-NR	<u>TCCATCCCCGGGAGCGGTACC</u> GACCCTGAGAAGCTGGA
		OsPAC1-BiFC-CR	<u>TACATCCCCGGGAGCGGTACC</u> GACCCTGAGAAGCTGGA
qPCR	OsMYB3	OsMYB3-e1f	GACGACATCCTGGTGTCTCATAT
		OsMYB3-e2r2	AGTGTGGAGCCTGAGGATGAG
	OsPF1	OsPF1-e1f	GACGACGAGGAGGACCTCAT
		OsPF1-e2r	GAGCTTCTTGCTGAGATGCGAGTT
	OsB1	OsB1head	ATGGAAGAGACCCCTCTGCC
		OsB1e2r	CGTTGTAGAAGCCGTCCTTCC
	OsB2	OsB2-head	GCATCTGCTCCTCCAGTTCAG
		OsB2-e2r	TCGTCTTTACCTCGCGTTGTAG
	OsPa	OsPa-head	ATGGCATCTGCTCCTCCAGTTCA
		OsPa-e2r	CGTTGTAGAACCCATCATTTCCAAGTC
	OsPAC1(TTG1)	OsPAC1-head	ATGGAGCAGCCCAAGCCG
		OsPAC1-154r	GAGGTCCTCGAGGAAGCTG
	OsC1	OsC1-head	ATGGGGAGGAGAGCTTGCTGC
		OsC1-e2r	CGGAGCCTGCAGCTCTTGC
	OsDFR	OsDFR-e1f	CCTCCAGGCCGGCTACA
		OsDFR-e2r	CACAGCGTCAGCCTCTCCTT
	OsLARpro	OsLAR-e3f	CGTACACCTTCATCTGCTGCAACT
		OsLAR-e4r	GTCGCTTCCAGCCACGAAGAA
	OsCHS	OsCHS-e1f	CGAGCACATGGTCGAGCTCAA
		OsCHS-e2r	CTCCTCCGTCAGGTGCATGT
Dual luciferase assays	OsC1	OsC1-163-HindIII-F	<u>ATACCCAAGCTT</u> ATGGGGAGGAGAGCTTGCTGC
		OsC1-163-EcoRI-R	<u>GATCCGGAATT</u> CTACGCACACAAGTTCCAGGC
	OsMYB3	OsMYB3-163-Sall-F	<u>ACGTCGAC</u> GATGGGGAGGAAGCCGTGC
		OsMYB3-163-EcoRI-R	<u>ACGAATT</u> CTCTACTGGTGGTGGTGGTGGT
	OsPF1	OsPF1-163-F	<u>TGGAGAGGACAGCCCAAGCTT</u> ATGAAGCGGAAGCGGCC
		OsPF1-163-R	<u>TGATTTCAGCGTACCGAATT</u> CTTACTGTTTGGATTCCATGACGTCT
	OsB2	OsB2-163-HindIII-F	<u>ATACCCAAGCTT</u> ATGGCATCTGCTCCTCCAGTT
		OsB2-163-EcoRI-R	<u>GATCCGGAATT</u> CTTACGGCGCCTTCCCCTGT
	OsPa	OsLc-163-HindIII-F	<u>ATACCCAAGCTT</u> ATGGCATCTGCTCCTCCAGTTCA
		OsLc-163-XbaI-R	<u>AACTAGT</u> CTAGACTAGCCACCGATAGCTTTCTGGA
	OsPAC1(TTG1)	OsPAC1-163-Sall-F	<u>ACGTCGAC</u> GATGGAGCAGCCCAAGCCG
		OsPAC1-163-EcoRI-R	<u>ACGAATT</u> CTCAGACCCTGAGAAGCTGGA
	OsC1	OsC1-163-HindIII-F	<u>ATACCCAAGCTT</u> ATGGGGAGGAGAGCTTGCTGC
		OsC1-163-EcoRI-R	<u>GATCCGGAATT</u> CTACGCACACAAGTTCCAGGC
	OsCHIpro	OsCHIpro-1Kfb	<u>CAAGCTAATTCGAGCTCGGTACC</u> CTCGTCATTTCCACAAGCCTA
		OsCHIpro-R	<u>GTCTTCCATGGATCCGTCGAC</u> GGATACAGGATCGGGTGAGC
	OsCHSpro	OsCHSpro_5	<u>CAAGCTAATTCGAGCTCGGTACC</u> GTGCATATATACCAACTAAATAGTCATC
		OsCHSpro_3	<u>GTCTTCCATGGATCCGTCGAC</u> TCTCTCGACTAATTCACCAG
	OsDFRpro	OsDFRpro0921-F	<u>CAAGCTAATTCGAGCTCGGTACC</u> GACAGGACTTCTATAGATTATAAA
		OsDFRpro0921-R	<u>GTCTTCCATGGATCCGTCGAC</u> GGCGTACCGTGCGTGATC
	OsF3Hpro	OsF3Hpro-KpnI-F	<u>GAGCTCGGTACC</u> CAACCTACGATCTGATCACAC
		OsF3Hpro-NcoI-R	<u>CGTCTTCCATGGCTCGATCGAT</u> CGACCCGATGC
	OsF3'Hpro	OsF3pHpro-left	<u>CAAGCTAATTCGAGCTCGGTACC</u> GATTATACGTGAGCCGAATATGCATG
		OsF3pHpro-right	<u>CGTCTTCCATGGATCCGTCGACAAGCTT</u> GACCGTATGATCCGCTCGCT
	OsANSpro	OsANSpro-o6_5	<u>CAAGCTAATTCGAGCTCGGTACC</u> TAGGATGGCAATCTGTGGATGAT
		OsANSpro-o6_3	<u>GTCTTCCATGGATCCGTCGAC</u> GTGCGCCTCCCGAAGTA
	Os3GTpro	Os3GTpro-o6_5	<u>CAAGCTAATTCGAGCTCGGTACC</u> CCAACGGAATTCATTAGTC
		Os3GTpro-o6_3	<u>GTCTTCCATGGATCCGTCGAC</u> CGCCGGAGTGCGTGTGTGTGT
	OsLARpro	OsLARpro-1K	<u>CAAGCTAATTCGAGCTCGGTACC</u> GAGAGGGTTACCTTCCTTG
		OsLARpro-R	<u>GTCTTCCATGGATCCGTCGAC</u> GGATCGATCGATCGACCGAT
EMSA	OsMYB3	OsMYB3-Domain-F	<u>GGCATATGGAGCTCGGTACC</u> ATGGGGAGGAAGCCGTGC
		OsMYB3-Domain-R	<u>AGGTCGACAAGCTTGAATTCC</u> CGTTGCTGAGGGTGCT
	OsPF1	OsPF1-Domain-F	<u>GGCATATGGAGCTCGGTACC</u> ATGAAGCGGAAGCGGCC
		OsPF1-Domain-R	<u>AGGTCGACAAGCTTGAATTCC</u> TTCTTGCTGAGATGCGA
	OsB2	OsB2-Domain-F	<u>AATCCGGAATTCAATATTGATGAT</u> CGTGGTAGCGTGGC
		OsB2-Domain-R	<u>ATACGCGTCGAC</u> CGATGGCTCCCTGCTGCTG
	OsPa	OsPa-Domain-F	<u>AATCCGGAATTCACTGGT</u> CGTGGTAGTAGAGCA
		OsPa-Domain-R	<u>ATACGCGTCGAC</u> TGATGGCTCGCTGCTGGACT
Knockout	OsPF1	OsPF1-sgDNA	<u>GACGATCACGAGCATGGCAAT</u> TGG
	OsPa	OsPa-sgDNA	<u>GACTCAGCAGACCTCACGGC</u> TGG

* The underline nucleotides indicate sites for enzyme cut (and linker) except in the knockout experiment, where the sites are target sequence with PAM (bold).

Table S3. Genomes consulted in this study .

Species	Subgroup	Variety	Project ID	Date of access
<i>O. sativa</i>	<i>temperate japonica</i>	Nipponbare	PRJNA12269	4/29/2021
		Kitaake	PRJNA448171	4/29/2021
	<i>indica (sensu stricto)</i>	9311	PRJNA427873	4/29/2021
		Shuhui498	PRJNA318714	4/29/2021
		Minghui63	PRJNA30254	4/29/2021
	<i>aus</i>	Netal Boro	PRJNA565483	9/29/2021
		N22	PRJNA315689	2/27/2022
	<i>tropical japonica</i>	Ketan Nangka	PRJNA564615	2/27/2022
		Chao Meo	PRJNA565484	9/29/2021
	<i>aromatic</i>	IRGC 12485-1	PRJNA565479	9/29/2021
<i>O. nivara</i>		IRGC 100897	PRJNA48107	9/28/2021
<i>O. rufipogon</i>		W1943	PRJEB4137	9/28/2021

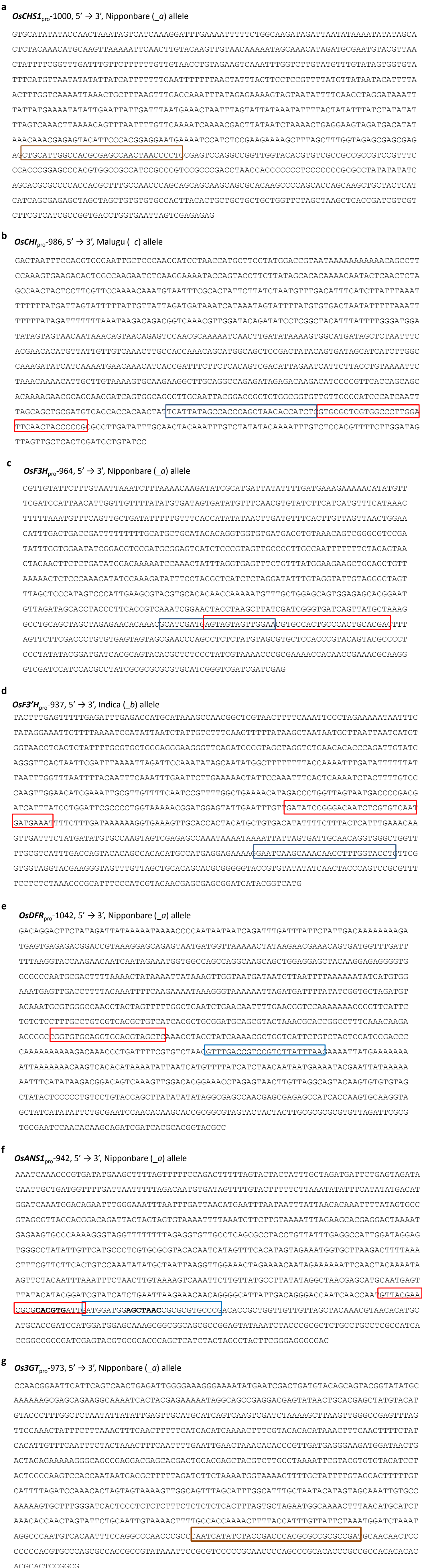


Fig. S1. Details of 5' sequences examined in this study. (a)-(g) 5' sequences of the structural genes of the anthocyanin pathway of *O. sativa*. The region contain at least partial promoter (_{pro}) with the length shown by the number in bp. The sequence fragments for probe making and containing *cis* elements for MYB are in blue rectangle, those containing *cis* elements for bHLH in red rectangle, and ones having both in brown rectangle. Label of allele follows *_a* from Nipponbare , *_b* from 9311, and *_c* from elsewhere.

a1: OsANS1_{pro}-pHIS2 + pAD2: OsANS1_{pro}-pHIS2 + pAD-OsPa**Background****Interaction****b**3: OsANS1_{pro}-pHIS2 + pAD4: OsANS1_{pro}-pHIS2 + pAD-OsMYB3 +
pAD-OsPa + pAD-OsTTG1**Background****Interaction****c**Os3GT_{pro}-pHIS2 + pADOs3GT_{pro}-pHIS2 + pAD-OsMYB3
+ pAD-OsPa + pAD-OsTTG1**Background****Interaction**

Fig. S2. Activation of *OsANS1* and *Os3GT* by OsPa in Y1H. (a) Single activations of OsPa on *OsANS1*_{pro}. The reference controls are shown in duplicates by colored bars. The background expression of *HIS2* is shown (1) against the reporter's expression when OsPa interacts with the promoter of *OsANS1* (2) in three biological replicates. A concentration of 1 mM 3-AT was applied to all. (b) Combined activations of OsPa on *OsANS1*_{pro}. The references were the same as (a). The treatments (3:background and 4: interaction) were in four replicates. All were under the condition of 5 mM 3-AT. (c) Combined activations of OsPa on *Os3GT*_{pro}. The references and the tests were in three biological replicates and all were under the condition of 4 mM 3-AT.

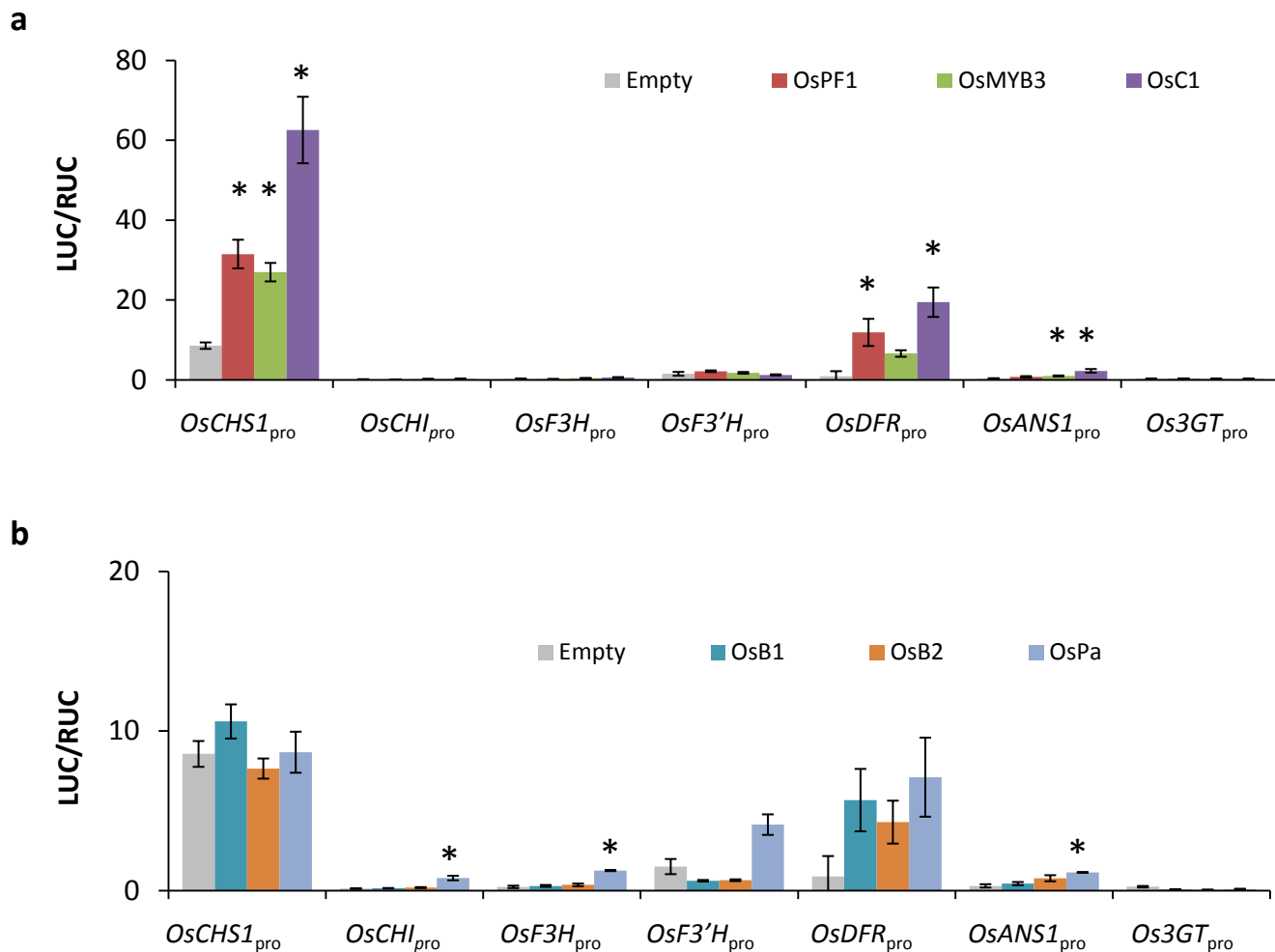
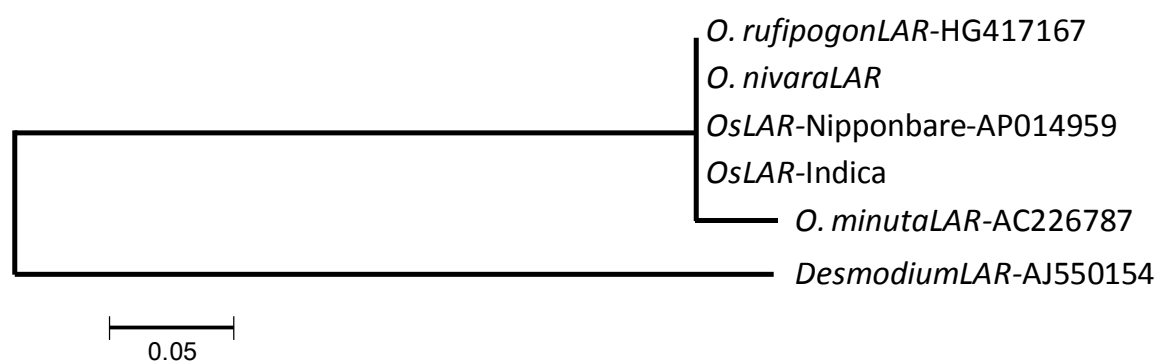
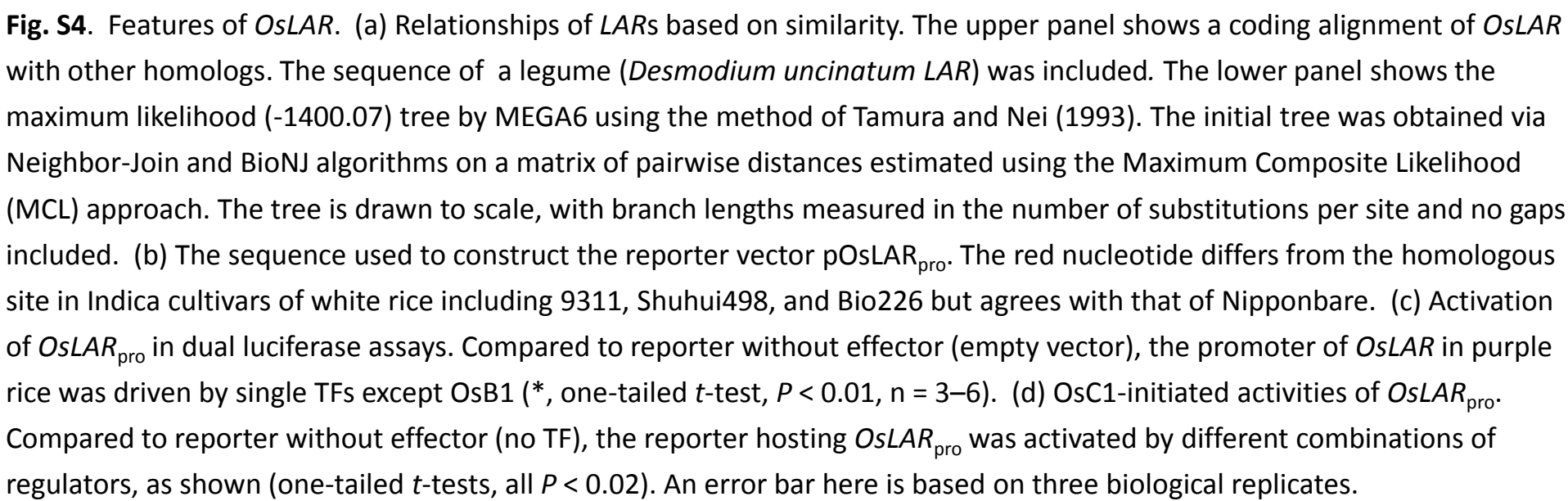


Fig. S3. Single effects of transcription factors on the anthocyanin pathway genes in dual luciferase assays. (a) Activations by OsPF1, OsMYB3, or OsC1. Each effector vector (2 μ g) was introduced along with a reporter vector (2 μ g) into the protoplasts of B16-44. The empty vector was included as the negative control (Empty). Level of an activation was reported by the ratio of LUC/RUC. Each treatment had biological replicates (2–7) shown by the error bar around the mean. A significant activation (one-sided Student's *t*-test, *, $P < 0.05$, $n \geq 3$) was inferred from the comparison between a treatment and the negative control. (b) Single effects of OsB1, OsB2, or OsPa. The tests and layout followed (a).



GAGCAGGGTTACCTTCCTTGTCTCTGGATCTTGGCCTTGACGTTGGCGACGGCGTCGCTGTTCTCCACCTCGAGGGTGATG
GTCTTCCCCGTCAGCGTCTTCACGAAGATCTGCATCCTCAACTCCGCTTGCTTTCTCTCTCCCTCGCGAGTCGCGGACCCC
TCTCTCCTCCGCTAATCTGGTGCTCTCGAGCGAGTGGAATGGCGGTGGTGTTGGTGGCGAGAGCGAACTGATGGCGGCG
CGGATTGTGCGGCCTTATATATCCTCCACTGACGTAACGCGCCCGTCAAGTCACCGATAAGCCAAAATACTACTACCCAT
TAACCGAGCGCTTTTACTGCAAAGATAGAGAAAGATCAGAGGGGAGAGAGCAACGCAACTGCGACGCTCAGGAGTGGTGG
TAAACACCGTGTGCTAATCAGATGAGGCCAGATCGCGGCCCGTACGAGATAGCCACTGGCCCAGACCGCACTGAGATCT
CAACAAATTTGCGGGCCCATATCAGTTGGGGGCAGATGAGATGAGACGAGAAGACGACCAGAGAGAGACACGAAGAACAG
AGGAAAGCGGTGGCGGAGGCAGAGCACGAGACATGTTTCTGTATCTGGAGTGGAATTTTGTGGATTTTAAGGAGCATTT
CATTCTGTAGAGGCATTTTTATGCATACAACGGGGGCCCTTGATTAACGCCTCGCTGCTGGTGCGCCGTCCTAGCTAGGT
AGATATCATCCAGGAAAATCTGGGATTATTCAACACCTAACTCTTGAGTTTTGGCCGTCTATACCCGTACGTCGCACGA
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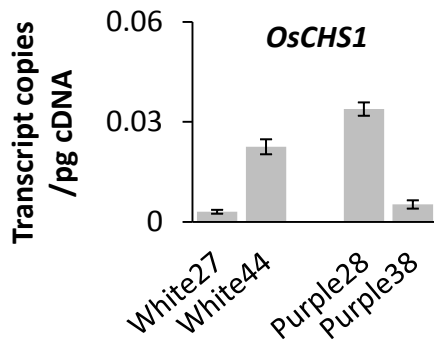
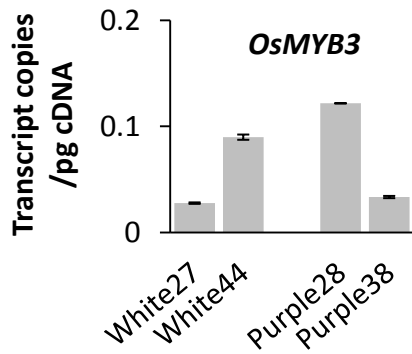


Fig. S5. Expressions of *OsMYB3* and *OsCHS1* in the paddy field. The samples (shown along the x-axis) are the same as ones in Figure 4. The error bars are based on two replicated measurements.

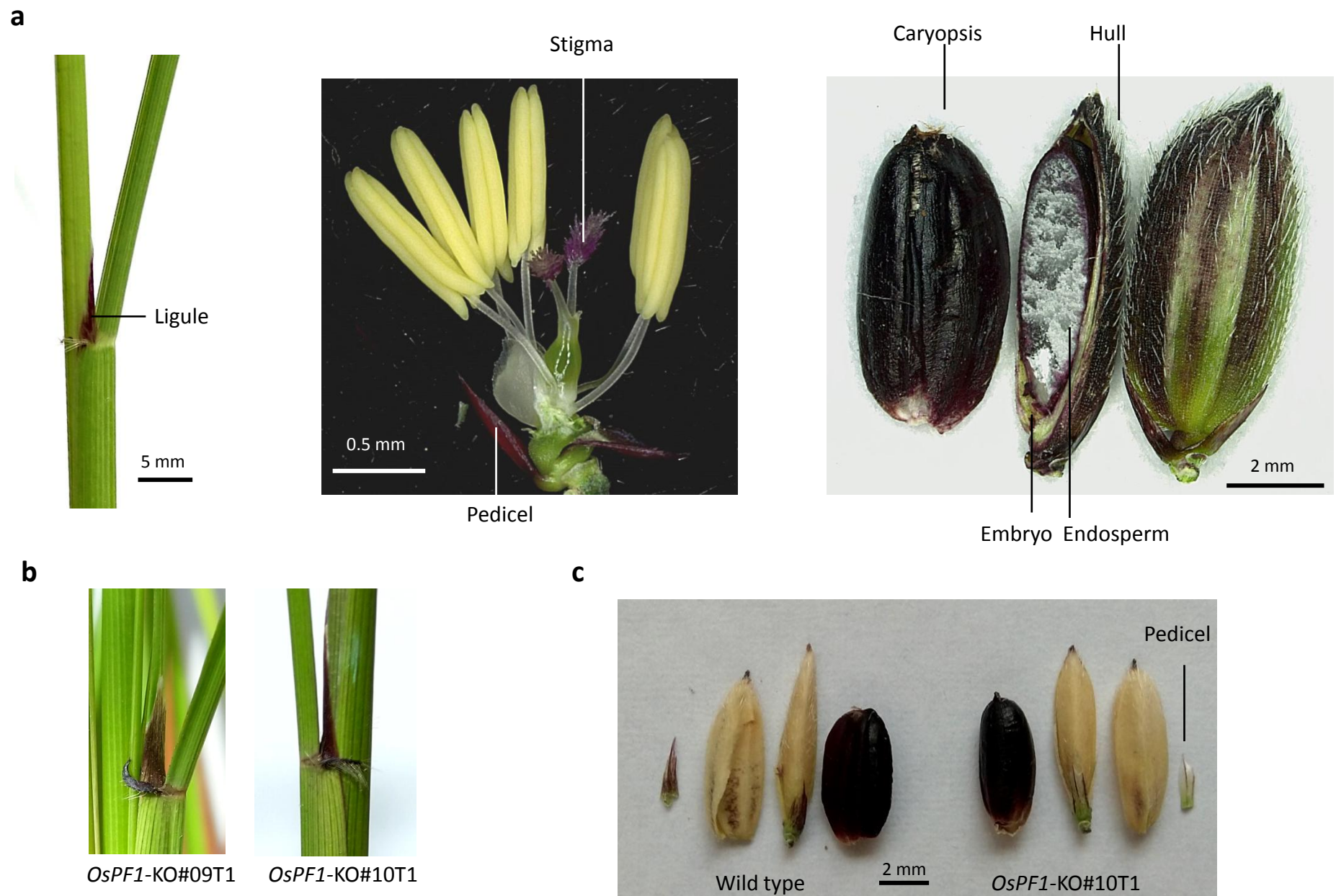


Fig. S6. Phenotypes of the wild type and *OsPF1* mutants.

(a) Pigmented parts of Heidao. The purple ligule (left), stigma (middle), caryopsis and hull of grains at ~24 DAP.

(b) The ligules of mutated *OsPF1* lines.

(c) Mature grains of a mutated *OsPF1* line. The fruit parts were separated for comparison between the wild type and the mutant grown under the same condition.