

Supplemental Data

Altered flavonoid and lignin profiles in rice mutants deficient in *CHS*, *CHI*, and *CHIL* genes

Pui Ying Lam,^{1†} Lanxiang Wang,^{2†§} Andy C. W. Lui,² Hongjia Liu,³
Yuri Takeda-Kimura,^{1¶} Mo-Xian Chen,⁴ Fu-Yuan Zhu,⁵ Jianhua Zhang,⁶
Toshiaki Umezawa,^{1,7} Yuki Tobimatsu,^{1‡} Clive Lo^{2‡}

¹Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan; ²School of Biological Sciences, The University of Hong Kong, Pokfulam, Hong Kong, China; ³State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control, Institute of Crops and Nuclear Technology Utilization, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China; ⁴Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen, China; ⁵Co-Innovation Center for Sustainable Forestry in Southern China, College of Biology and the Environment, Nanjing Forestry University, Nanjing 210037, Jiangsu Province, China; ⁶Department of Biology, Hong Kong Baptist University, Hong Kong, China and State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Hong Kong, China; ⁷Realization of Sustainable Society, Kyoto University, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan.; [†]Co-first author; [‡]Senior authors. [§]Present address: Shenzhen Institute of Synthetic Biology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, China; [¶]Present address: Department of Botany, University of Wisconsin-Madison, 430 Lincoln Drive, Madison, WI 53706, USA.

[‡]Senior Authors:

Yuki Tobimatsu (ytobimatsu@rish.kyoto-u.ac.jp; +81-774-38-3626), and
Clive Lo (clivelo@hku.hk; +852-2299-0337)

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>OsCHI			
WT	MAAVSEVEVDGVVFPVVARPPGSGHAHFLAGAGLFFVFCVPCQWRSGNAVQANLLAGLI	60	
<i>OsCHI</i> -CRISPR-a	MAAVSEVEVDGVVFPVVARPPGSGHAHFLAGAGLFFVFCVPCQWRSGNAVQANLLAGLI	60	
<i>OsCHI</i> -CRISPR-b	MAAVSEVEVDGVVFPVVARPPGSGHAHFLAGAGLFFVFCVPCQWRSGNAVQANLLAGLI	60	
WT	DPSCRCEGSGDRRQLHQVHGHRVPGGGRGRAGAGQEVGRQVRRRARRRRRLLPRRRHRR	120	
<i>OsCHI</i> -CRISPR-a	DPSCRCEGSGDRRQLHQVHGHRVPGGGRGRAGAGQEVGRQVRRRARRRRRLLPRRRHRR	120	
<i>OsCHI</i> -CRISPR-b	DPSCRCEGSGDRRQLHQVHGHRVPGGGRGRAGAGQEVGRQVRRRARRRRRLLPRRRHRR	120	
WT	FREVHEGDDDDPAHRRAVLGQGDGELRRGVEGRRRVHGRRGRGRGQVQGGQLQAPQLPSGR	180	
<i>OsCHI</i> -CRISPR-a	FREVHEGDDDDPAHRRAVLGT TR * RRTASR-RGRPPACTRTTPRARP -- RTSSRRPSSPTAS	176	
<i>OsCHI</i> -CRISPR-b	FREVHEGDDDDPAHRRAVLGT TR * RRTASR-RGRPPACTRTTPRARP -- RTSSRRPSSPTAS	176	
WT	V--H---PLHPLPARR---PHRRVLQGLVGARGRRGGGGDREQCALRGGAGFHRRARG	231	
<i>OsCHI</i> -CRISPR-a	LRARPSSSPTPRPASSPSRSPTRTRRCQRAPWRRRRSRTGRSARRCWI PSSASTGSRRRS	236	
<i>OsCHI</i> -CRISPR-b	LRARPSSSPTPRPASSPSRSPTRTRRCQRAPWRRRRSRTGRSARRCWI PSSASTGSRRRS	236	
WT	LAGGEAEHSGPRLAEPGGIHRRRGGGGARAGVVRVKIELRINRASKSSSYTSTLLLPSC	291	
<i>OsCHI</i> -CRISPR-a	GA * R PASRS S * RRN -PPATW-RRRSPRRC P RENRAAN* PRI * E LLHFLYT S TALVFDNE	290	
<i>OsCHI</i> -CRISPR-b	GA * R PASRS S * RRN -PPATW-RRRSPRRC P RENRAAN* PRI * E LLHFLYT S TALVFDNE	290	
WT	LTMK*	295	
<i>OsCHI</i> -CRISPR-a	I----	291	
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Supplemental Fig. S1. Predicted effects of mutations on OsCHI in *OsCHI*-CRISPR mutant lines.

Protein sequence alignment of wild-type OsCHI and the mutated OsCHI in *OsCHI*-CRISPR-a and *OsCHI*-CRISPR-b. If translation starts using the native start codon, frame shift mutation occurs in *OsCHI*-CRISPR-a and *OsCHI*-CRISPR-b, leading to the formation of a premature stop codon. If translation starts using the in frame start codon after the mutation site, the region before the start codon is missing.

Asterisks, stop codons. In red, the first premature stop codon. In blue, protein sequence different with wild-type OsCHI if translation starts using the native start codon. In purple, in frame start codon after the mutation site.

A

>OsCHIL1

WT	MYCAVGTEIATVEVEGIPFPQEI TVSK----PLSL-LANGI-----TDIEIHFLQIK	47
<i>OsCHIL</i> -CRISPR-a	MYCAVGTEIATVEVEGIPF HSGDNRQ *AIVTAC*WHHRH*DPFPSDQIQRRHGV*S*KGQC	56
<i>OsCHIL</i> -CRISPR-b	MYCAVGTEIATVEVEGIPF LRR *PSVS----HCHCLLMASQ-----TLRSISFR-SN	46
WT	YNAIGVYLEKD-----NVLAHLESWKGKKA-----	73
<i>OsCHIL</i> -CRISPR-a	ASAFGELEGQEGRGACAGLMASFRP*FLLRWRNC*G*W*SRRSKDHNTACSWRVQCVTVS	112
<i>OsCHIL</i> -CRISPR-b	TTPSGCILKRT-----M---C*RIWRVGRARRPRSLCRRWLLSGPSF	84
WT	----ELVQDDGFFQ--ALVSAPVEKL--LRIVVIKEIKGSQYGVQLESSVRDRLVSVDK	124
<i>OsCHIL</i> -CRISPR-a	CQ*TS-----TRRMKKRLWRRSPNSFSP-----	134
<i>OsCHIL</i> -CRISPR-b	CSGGEIVEDSGDQDQRITIRRAAGEFSA*PSRVSRQVRG---G*RRSGEGHRILSVQV	139
WT	YEEDEEEALEKVTEFFQSKYFKPNSVITFHFPPTPGIAEISFVTEGKGE-----	173
<i>OsCHIL</i> -CRISPR-a	---SI---S-----SPTPSSPSTFLPPELQRYHL*QKARARRS*QWRTRMWPR*	175
<i>OsCHIL</i> -CRISPR-b	FQAQLRHH-----LPLSYHPWNCR--DIICDRRQGRGE-----	170
WT	AKLT--VEN-KNVA MI QKWYLGGESAVSPTTVKSLADQFAALLSA*	216
<i>OsCHIL</i> -CRISPR-a	FRSGTWENLQFLRRL*RAWQINSQHCSLH-----	204
<i>OsCHIL</i> -CRISPR-b	ADSGEQECG-RDDSEVVPGWIRICFSDDCEEPGRSIRSTALCM----	212

B

>OsCHIL2

WT	MATVEVEGIPFPQEI TVSKPLSLLAHGITDIEIHFLQIKY-----NAIGVYLEKDNVL	53
<i>OsCHIL</i> -CRISPR-a	MATVEVEGIPF LSGDHRQ *AVI-----TASFWHYRH*DSFPSDQIQRRHGV-S*KGQCT	50
<i>OsCHIL</i> -CRISPR-b	MATVE-----	5
WT	GHLESWKGKKAEEELVQDDGFFQALVSAPVEKLFRIVVIKEIKGSQYGVQLE-----SSVR	108
<i>OsCHIL</i> -CRISPR-a	RAFGELEGQEGRGACAGMMASF-----RP*FL-LLWRNYSGSW*SRRSKDHSTACSWR	100
<i>OsCHIL</i> -CRISPR-b	-----VEGIPFLMASF-----RP*FL-LLWRNYSGSW*SRRSKDHSTACSWR	44
WT	DRLVSVD-----KY---EDEEEESLEKVTEFFQSKYFKPNSVITFHFPNTPGI	153
<i>OsCHIL</i> -CRISPR-a	AQCATVSCQWTSTRMRKSLWRR*LNSFSPSISPTPSSPSTFLTPELQRYHL*QKARA	158
<i>OsCHIL</i> -CRISPR-b	AQCATVSCQWTSTRMRKSLWRR*LNSFSPSISPTPSSPSTFLTPELQRYHL*QKARA	102
WT	AEI-SFVTEG---KGEAKLTVEN-KNVA MI QKWYLGGESAVSPTTVKSLADQFAALLSA	208
<i>OsCHIL</i> -CRISPR-a	RRS*QWRTRMWPR*FRSGTWENLQFLRRL*RAWQINSQHCSLH-----	199
<i>OsCHIL</i> -CRISPR-b	RRS*QWRTRMWPR*FRSGTWENLQFLRRL*RAWQINSQHCSLH-----	143
WT	* 208	
<i>OsCHIL</i> -CRISPR-a	- 199	
<i>OsCHIL</i> -CRISPR-b	- 143	

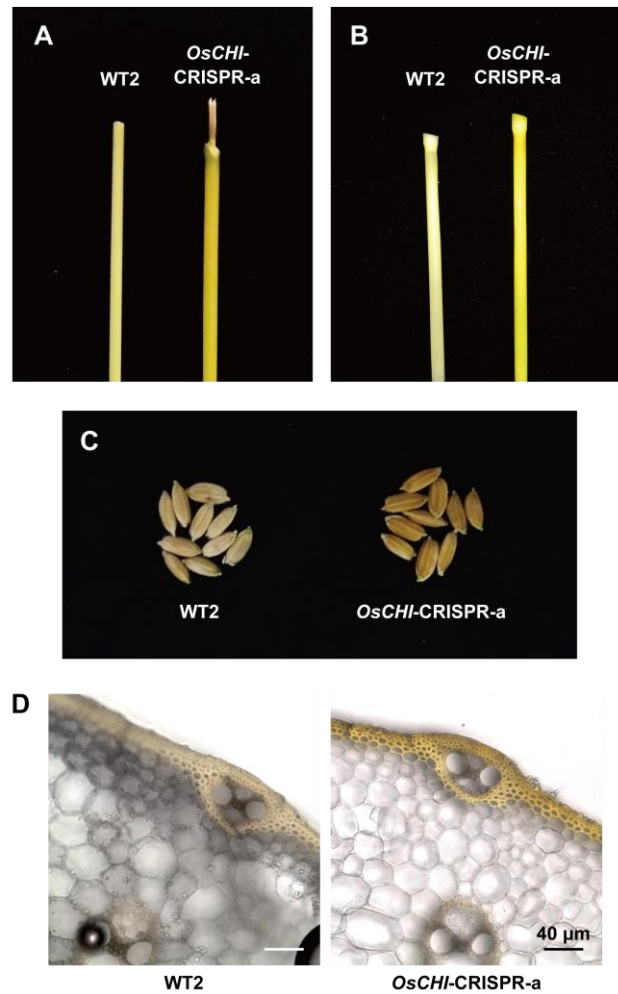
Supplemental Fig. S2. Predicted effects of mutations on OsCHIL1 and OsCHIL2 in *OsCHIL*-CRISPR mutant lines.

A, Protein sequence alignment of wild-type OsCHIL1 and the mutated OsCHIL1 in *OsCHIL*-CRISPR-a and *OsCHIL*-CRISPR-b.

B, Protein sequence alignment of wild-type OsCHIL2 and the mutated OsCHIL2 in *OsCHIL*-CRISPR-a and *OsCHIL*-CRISPR-b.

If translation starts using the native start codon, frame shift mutation occurs in *OsCHIL*-CRISPR-a and *OsCHIL*-CRISPR-b, leading to the formation of a premature stop codon. If translation starts using the in frame start codon after the mutation site, the region before the start codon is missing.

Asterisks, stop codons. In blue, protein sequence different with wild-type OsCHIL1 (A) or OsCHIL2 (B) if translation starts using the native start codon. In red, the first premature stop codon. In purple, in frame start codon after the mutation site.

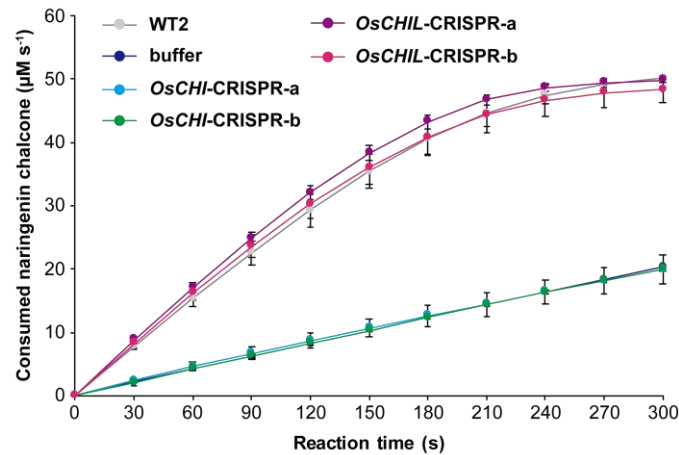


Supplemental Fig. S3. Phenotypes of *OsCHI*-CRISPR and wild-type control lines.

A and B, Mature culm tissues of WT2 and *OsCHI*-CRISPR-a without any treatment (A), and after fixation and solvent extraction (B). Culm of *OsCHI*-CRISPR-a appears to be yellow.

C, Rice grains of WT2 and *OsCHI*-CRISPR-a. Spikelets of *OsCHI*-CRISPR-a appear to be yellow.

D, Transverse sections of culm tissues of WT2 and *OsCHI*-CRISPR-a after fixation and solvent extraction and before subjecting to any staining. The secondary cell walls of the vascular bundles and cortical sclerenchyma fibers in *OsCHI*-CRISPR-a appear to be yellow.



Supplemental Fig. S4. CHI catalytic activities of crude proteins of *OsCHI*-CRISPR, *OsCHIL*-CRISPR and their isogenic wild type.

Crude proteins extracted from wild-type control (WT2), *OsCHI*-CRISPR and *OsCHIL*-CRISPR mutants were used to perform CHI enzyme assay with naringenin chalcone as a substrate. Crude proteins of WT2 and *OsCHIL*-CRISPR mutant lines could rapidly consume naringenin chalcone. Crude proteins of *OsCHI*-CRISPR mutant lines harbored similar CHI activities with that of spontaneous isomerization occurred in reaction mixtures that only contained extraction buffer. These results suggest that *OsCHIL*s unlikely harbor CHI catalytic activities nor serve as enhancers of CHI.

Values refer to means \pm standard deviations from biologically independent plants ($n = 3$).

Supplemental Table S1. Off target analysis of T₃ generation of *OsCHI*-CRISPR and *OsCHIL*-CRISPR mutant lines.

sgRNA name	Off-target site name	Sequence (5' to 3') ^a	CRISPR-P off-target score	Location (Chromosome:start)	Gene locus	Mutation
sgRNA-a	Off-target-a1	CGGC AG GCAGTACTCGGAC G <u>TGG</u>	0.8	Chr3:+19289	Exon (LOC_Os03g01018)	n.d.
	Off-target-a2	CG T CG CG GAG C ACTCGGACAC <u>CGG</u>	0.5	Chr3:+8809743	Exon (LOC_Os03g15980)	n.d.
	Off-target-a3	CGGG CG GAGTACTCG T ACAT <u>TGG</u>	0.4	Chr3:+31598624	Exon (LOC_Os03g55530)	n.d.
sgRNA-b	Off-target-b1	GA ACTC C ATCCCATTT C CTCAAG	0.8	Chr6:+15503611	Exon (LOC_Os06g27380)	n.d.
	Off-target-b2	GCAGGG A ATCCCATTT C AGC <u>AGG</u>	0.1	Chr1:+4971979	Exon (LOC_Os01g09649)	n.d.
	Off-target-b3	AG GGGG C ATCCCAT T GCCT G AAG	0.1	Chr3:-28010595	Exon (LOC_Os03g49170)	n.d.
sgRNA-c	Off-target-c1	G C T GTGCTTT T TGCAGGATGAGGG	1.3	Chr3:-563747	Exon (LOC_Os03g01915)	n.d.
	Off-target-c2	GAG C AGCT A GT G GAGGATGAAAG	0.6	Chr4:+218051	Exon (LOC_Os04g01280)	n.d.
	Off-target-c3	GAGG C GCTTGT G GAG T TGAT <u>TGG</u>	0.4	Chr6:-12412691	Exon (LOC_Os06g21480)	n.d.

Top 3 off target sites predicted by CRISPR-P 2.0 (Liu et al., 2017) were sequenced.

^aBold: mismatch nucleotides compared with the sequence of sgRNA. Underlined: PAM sites.

n.d.: not detected.

Reference

Liu H, Ding Y, Zhou Y, Jun W, Xie K, Chen L-L (2017) CRISPR-P 2.0: an improved CRISPR-Cas9 tool for genome editing in plants. *Mol Plant* **10**: 530-532

Supplemental Table S2. Klason lignin assay of *OsCHI*-CRISPR-b, *OsCHIL*-CRISPR-b and wild-type control line.

	WT2	<i>OsCHI</i> -CRISPR-b	<i>OsCHIL</i> -CRISPR-b
Klason lignin (mg/g CWR)	120.4 ± 6.3	112.0 ± 9.8	126.0 ± 3.2

Values refer to means ± standard deviations from biologically independent plants (Student's *t*-test, *n* = 3).

Supplemental Table S3. Cell wall polysaccharide analysis of *OsCHS1*-TDNA, *OsCHI*-CRISPR-a, *OsCHIL*-CRISPR-a and their isogenic wild types.

	WT1 (cv. Zhonghua 11)	<i>OsCHS1</i> -TDNA (cv. Zhonghua 11)	WT2 (cv. Nipponbare)	<i>OsCHI</i> -CRISPR-a (cv. Nipponbare)	<i>OsCHIL</i> -CRISPR-a (cv. Nipponbare)
Crystalline glucan (mg/g CWR)	391.2 ± 5.6	381.3 ± 3.9	376.9 ± 2.7	342.0 ± 11.7**	381.1 ± 6.5
Amorphous glucan (mg/g CWR)	44.8 ± 6.0	53.8 ± 5.2	46.7 ± 1.1	52.9 ± 4.6	48.9 ± 2.6
Arabinan (mg/g CWR)	38.1 ± 2.3	36.3 ± 2.5	36.0 ± 2.3	39.0 ± 0.3	32.8 ± 1.6
Xylan (mg/g CWR)	127.6 ± 15.6	129.5 ± 15.5	108.2 ± 3.4	124.0 ± 6.9*	112.6 ± 3.2
Mannan (mg/g CWR)	2.4 ± 0.2	2.3 ± 0.1	3.0 ± 0.2	3.3 ± 0.1	2.5 ± 0.1**
Galactan (mg/g CWR)	18.2 ± 2.2	17.0 ± 1.4	15.3 ± 1.6	16.5 ± 1.0	14.1 ± 0.9

Values refer to means ± standard deviations from biologically independent plants. Asterisks indicate significant differences between mutant line and its isogenic wild type (Student's *t*-test, *n* = 3, *: *p* < 0.05, **: *p* < 0.01). CWR, cell wall residue.

Supplemental Table S4. Primers used in this study.

Primer name	Sequence (5' to 3')	Target gene(s)	Purpose
LL302-a-F	GGCAGGAGGGCATCCCATTTCCTC	<i>OsCHIL1</i> & <i>OsCHIL2</i>	Construction of sgRNA-b in CRISPR/Cas9 binary vector used in the generation of <i>OsCHIL</i> -CRISPR
LL302-a-R	AAACGAGGAAATGGGATGCCCTCC		
LL302-b-F	GCCGGAGGAGCTTGTGCAGGATGA		Construction of sgRNA-c in CRISPR/Cas9 binary vector used in the generation of <i>OsCHIL</i> -CRISPR
LL302-b-R	AAACTCATCCTGCACAAGCTCCTC		
CL2223	ATGGTCGAGCTCAAGGAGAA	<i>OsCHS1</i>	Left primer. Genotyping of <i>OsCHS1</i> -TDNA
CL2224	CAAACCGTTGTCCTCTTTCC		Right primer. Genotyping of <i>OsCHS1</i> -TDNA
CL2227	AATCCAGATCCCCGAATTA	T-DNA	Border primer. Genotyping of <i>OsCHS1</i> -TDNA
CL3988	CGGAGTAATCGGCAACAACG	<i>OsCHI</i>	Genotyping of <i>OsCHI</i> -CRISPR
CL3989	CATCAAGTTCACGGCCATCG		
CL4569	TGATTGTTGTAAGTGCAGTGGG	<i>OsCHIL1</i>	Genotyping of <i>OsCHIL</i> -CRISPR
CL4570	CCACACGTAAACATCATCAATCA		
CL4571	TGGTAGTAGCTGCACACACA	<i>OsCHIL2</i>	Genotyping of <i>OsCHIL</i> -CRISPR
CL4572	GAGCGGCAGACTGAACCTTA		

Supplemental Table S4. Primers used in this study (continued).

Primer name	Sequence (5' to 3')	Target gene(s)	Purpose
OsUBQ5-F	ACCACTTCGACCGCCACTACT	<i>OsUBQ5</i>	RT-qPCR of <i>OsUBQ5</i>
OsUBQ5-R	ACGCCTAAGCCTGCTGGTT		
OsCHS1-F	ACCCGGACTACTACTTCAGGAT	<i>OsCHS1</i>	RT-qPCR of <i>OsCHS1</i>
OsCHS1-R	TCTTCCTGATCTGCGACTTGT		
OsCHS2-F	CACCAACAGCGAGCACCTAA	<i>OsCHS2</i>	RT-qPCR of <i>OsCHS2</i>
OsCHS2-R	ACACGCACATGCTAGGGTTC		
OsCHI-F	CGCAGGTTTGTGGTGTGGTTC	<i>OsCHI</i>	RT-qPCR of <i>OsCHI</i>
OsCHI-R	CGGATCGATCAAACCAGCAAG		
OsCHIL1-F	GAGGATAGTGGTGATCAAGGAG	<i>OsCHIL1</i>	RT-qPCR of <i>OsCHIL1</i>
OsCHIL1-R	CATCCTCCTCGTACTTGTCTAC		
OsCHIL2-F	CCATGGCATTACAGACATTGAG	<i>OsCHIL2</i>	RT-qPCR of <i>OsCHIL2</i>
OsCHIL2-R	GATCACCACGATCCTGAATAGT		