Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2020) **18**, pp. 313–315 doi: 10.1111/pbi.13217

Brief Communication

A new rice breeding method: CRISPR/Cas9 system editing of the *Xa13* promoter to cultivate transgene-free bacterial blight-resistant rice

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Received 21 April 2019;
revised 10 July 2019;
accepted 22 July 2019.
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Keywords: CRISPR/Cas9, *Xa13* promoter, bacterial blight-induced element, rice breeding, transgene-free.

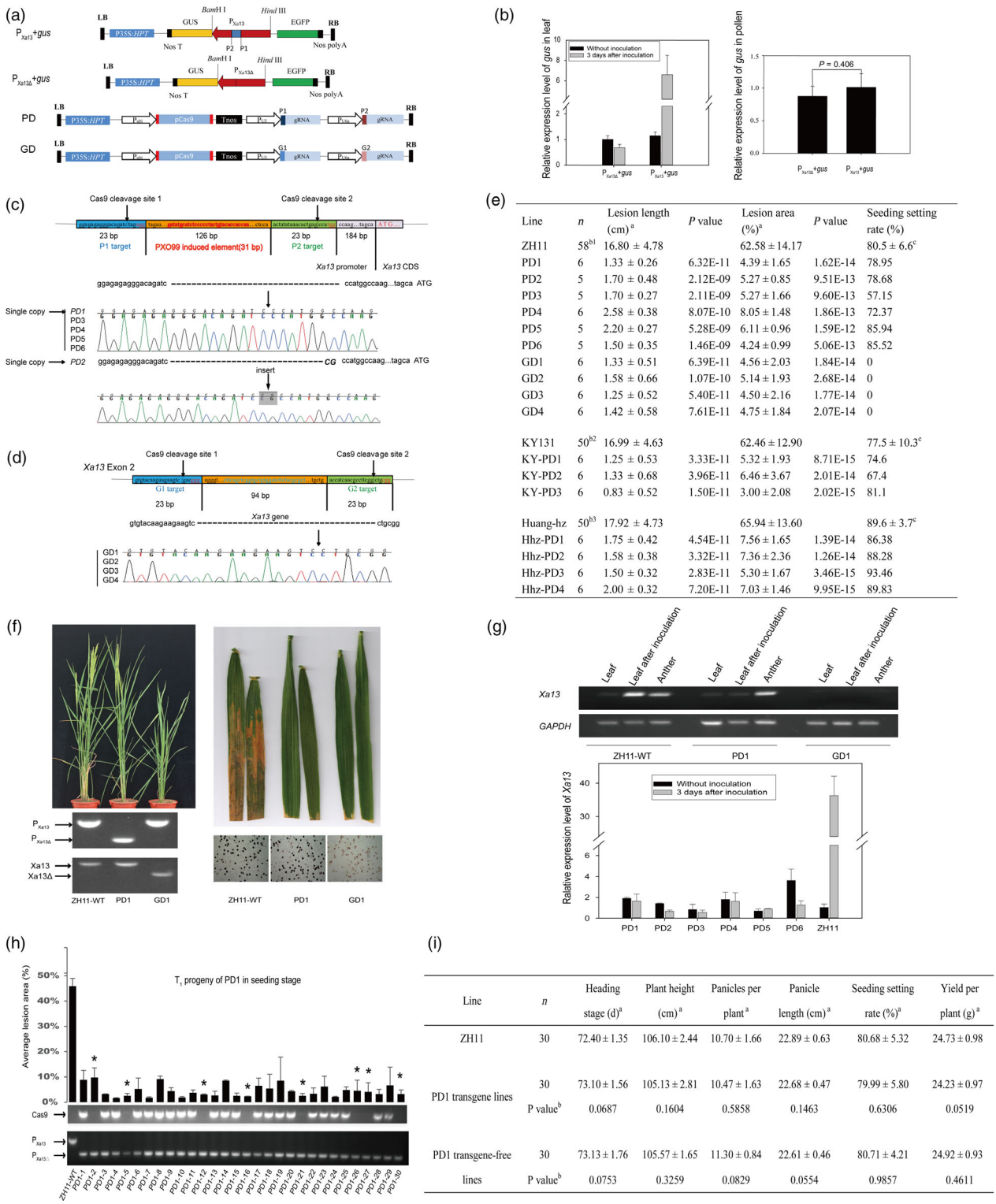
Dear Editor,

Since the first successful completion of genome-based editing in the model plant Arabidopsis using CRISPR/Cas9 in 2013 (Jiang *et al*., 2013), a large number of agronomic trait-related genes have been successfully edited in rice, including leaf colour-related genes *OsYSA*, *CAO1* and *OsPDS*; rice quality-related genes *OsWaxy* and *OsBADH2*; fertility-related genes *OsPMS3*; and plant type-related genes *LZAY1* and *OsIPA1* (Liu *et al*., 2017). However, most of the above reports are of one sgRNA mutation to a specific gene, and most of the mutation sites are selected in the gene coding region. In addition, some genes cannot adopt this strategy for editing CDS regions because of their specific functions. For example, the *Xa13* gene is a pluripotent gene for recessive resistance to bacterial blight that regulates rice bacterial blight resistance and participates in anther development. Constitutive interference or knockdown of *Xa13* improves disease resistance but reduces pollen fertility and the seed setting rate, which seriously restricting its application in rice breeding (Chu *et al*., 2006). However, due to the unique function and disease resistance mechanism of the *xa13*, this gene is increasingly favoured in practical breeding (Hajira *et al*., 2016).

Therefore, we evaluated editing efficiency through the website (http://crispr.hzau.edu.cn/cgi-bin/CRISPR/CRISPR) and designed two gRNAs targeting the promoter of the *xa13* gene (Figure 1a,c). A 149 bp deletion between the two gRNAs contains the 31 bp pathogen-induced element reported by Romer and Yuan, respectively (Romer *et al*., 2010; Yuan *et al*., 2011). To verify the effect of this 149 bp deletion on *Xa13* gene expression, we evaluated the feasibility of this experimental design in two promoter vectors (Figure 1a,b). Cas9 vectors with deletions in the promoter and CDS regions were edited according to Ma *et al*. (2015). The results showed that direct editing of the CDS region could improve the resistance of rice but cause changes in important agronomic traits and a sterile phenotype. However, the deletion of this promoter sequence would cause the *Xa13* gene to lose its ability to be induced by bacterial blight, thus making the rice lacking this fragment resistant to bacterial blight (Figure 1e,f). Since the *Xa13* gene plays a key role in pollen development, only a partial sequence of the *Xa13* gene promoter was edited to remove its ability to induce expression without affecting gene expression and function in the anthers (Figure 1 g). Therefore, this deletion strategy can improve rice disease resistance without affecting rice fertility. At present, there are few reports on the site-directed deletion of two targets and the selection of editing sites in noncoding promoter regions. This method of gene editing that does not mutate gene coding regions to alter gene expression patterns and instead simply edits promoter regions may be important for future genetic engineering and breeding (Jia *et al*., 2016; Peng *et al*., 2017).

A double-sgRNA site-directed mutation was directed to the DNA sequence deletion; it was easy to use PCR to identify whether the mutation site was a homozygous, heterozygous or no-deletion mutation; it greatly reduced the sequencing workload; and it improved the accuracy of selecting mutant plants (Figure 1f). The two sgRNA-mediated deletion mutations are relatively stable compared to the single-target-induced mutation, and the mutation result is predictable. Among the 17 homozygously mutated transgenic rice (PD1-6, GD1-4, KY-PD1-3 and Hhz-PD1-4) obtained in this study, all results were as expected, except for the PD2 insertion of two bases at the predicted cleavage site. This indicated that double-target sgRNA-mediated splicing mutations are more accurate than single-target sgRNA-mediated (Figure 1c,d).

We previously used a green tissue-specific promoter-driven *amiRNA* to specifically silence the *Xa13* gene in leaves, inhibiting the expression of *Xa13* in leaves without affecting its expression or function in anthers. This practice improves the disease resistance of rice, and it does not affect its fertility or seed setting rate. However, two problems remain: (1) the regulation of *Xa13* expression by *amiRNA* is at the posttranscriptional or translational level, meaning that this regulation is susceptible to environmental or other factors and that the resistance phenotype is not sufficiently stable. (2) Exogenous transgene fragments including *amiRNA* and the screening marker *hygromycin* will always exist in resistant rice (Li *et al*., 2012). The above problems have been solved in this study: (1) by editing the elements with specific functions in the promoter, the *Xa13* gene loses its ability to be induced by pathogens; this regulation is very stable at the transcriptional level. (2) In addition to eliminating the exogenous DNA transgene fragments, editing the promoter region simply



(e)

Line	*n*	Lesion length (cm)[a]	*P* value	Lesion area (%)[a]	*P* value	Seeding setting rate (%)
ZH11	58[b1]	16.80 ± 4.78		62.58 ± 14.17		80.5 ± 6.6[c]
PD1	6	1.33 ± 0.26	6.32E-11	4.39 ± 1.65	1.62E-14	78.95
PD2	5	1.70 ± 0.48	2.12E-09	5.27 ± 0.85	9.51E-13	78.68
PD3	5	1.70 ± 0.27	2.11E-09	5.27 ± 1.66	9.60E-13	57.15
PD4	6	2.58 ± 0.38	8.07E-10	8.05 ± 1.48	1.86E-13	72.37
PD5	5	2.20 ± 0.27	5.28E-09	6.11 ± 0.96	1.59E-12	85.94
PD6	5	1.50 ± 0.35	1.46E-09	4.24 ± 0.99	5.06E-13	85.52
GD1	6	1.33 ± 0.51	6.39E-11	4.56 ± 2.03	1.84E-14	0
GD2	6	1.58 ± 0.66	1.07E-10	5.14 ± 1.93	2.68E-14	0
GD3	6	1.25 ± 0.52	5.40E-11	4.50 ± 2.16	1.77E-14	0
GD4	6	1.42 ± 0.58	7.61E-11	4.75 ± 1.84	2.07E-14	0
KY131	50[b2]	16.99 ± 4.63		62.46 ± 12.90		77.5 ± 10.3[c]
KY-PD1	6	1.25 ± 0.53	3.33E-11	5.32 ± 1.93	8.71E-15	74.6
KY-PD2	6	1.33 ± 0.68	3.96E-11	6.46 ± 3.67	2.01E-14	67.4
KY-PD3	6	0.83 ± 0.52	1.50E-11	3.00 ± 2.08	2.02E-15	81.1
Huang-hz	50[b3]	17.92 ± 4.73		65.94 ± 13.60		89.6 ± 3.7[c]
Hhz-PD1	6	1.75 ± 0.42	4.54E-11	7.56 ± 1.65	1.39E-14	86.38
Hhz-PD2	6	1.58 ± 0.38	3.32E-11	7.36 ± 2.36	1.26E-14	88.28
Hhz-PD3	6	1.50 ± 0.32	2.83E-11	5.30 ± 1.67	3.46E-15	93.46
Hhz-PD4	6	2.00 ± 0.32	7.20E-11	7.03 ± 1.46	9.95E-15	89.83

(i)

Line	*n*	Heading stage (d)[a]	Plant height (cm)[a]	Panicles per plant[a]	Panicle length (cm)[a]	Seeding setting rate (%)[a]	Yield per plant (g)[a]
ZH11	30	72.40 ± 1.35	106.10 ± 2.44	10.70 ± 1.66	22.89 ± 0.63	80.68 ± 5.32	24.73 ± 0.98
PD1 transgene lines	30	73.10 ± 1.56	105.13 ± 2.81	10.47 ± 1.63	22.68 ± 0.47	79.99 ± 5.80	24.23 ± 0.97
	P value[b]	0.0687	0.1604	0.5858	0.1463	0.6306	0.0519
PD1 transgene-free lines	30	73.13 ± 1.76	105.57 ± 1.65	11.30 ± 0.84	22.61 ± 0.46	80.71 ± 4.21	24.92 ± 0.93
	P value[b]	0.0753	0.3259	0.0829	0.0554	0.9857	0.4611

changes the expression pattern of the gene without producing excess mRNA or protein, in contrast to the conventional editing of gene coding regions to generate frame-shifted or erroneous mRNA or protein. Editing the promoter region produces really transgene-free rice. In addition to the ZH11 varieties, we also verified two important popularized varieties, KY131 (japonica) and Huanghuazhan (indica) (Figure 1e). We obtained the same results, indicating that the method in this study is universal and can effectively promote the practical application of the recessive disease resistance pleiotropic gene *xa13*.

Acknowledgements

We thank Prof. Yaoguang Liu (South China Agricultural University) for providing the Cas9 vector. This research was supported by the National Natural Science Foundation of China (Grant No.

Figure 1 CRISPR/Cas9 system editing of the *Xa13* promoter to cultivate transgene-free bacterial blight-resistant rice. (a) Schematic diagram of two promoter expression vectors and two CRISPR/Cas9 editing vectors. Four expression vectors were constructed in this study: P_{Xa13}+*gus* is the *Xa13* promoter vector; $P_{Xa13\Delta}$+*gus* is the *Xa13* promoter truncation vector, which had a 149 bp deletion; PD is the promoter editing vector with two gRNAs (P1 and P2); PD is a two-gRNA-mediated *Xa13* promoter editing vector that deletes 149 bp as $P_{Xa13\Delta}$; and GD is a two-gRNA-mediated (G1 and G2) *Xa13* CDS editing vector. (b) The identification of *gus* expression patterns in two promoter expression vectors. The expression of *gus* was up-regulated 6.6 times after inoculation in P_{Xa13}+*gus* leaf but was not obviously up-regulated in transgenic $P_{Xa13\Delta}$+*gus* rice. Student's *t*-test showed that the expression of *gus* in the anther was not significantly different between P_{Xa13}+*gus* and $P_{Xa13\Delta}$+*gus* ($P = 0.406 > 0.05$). Error bars indicate ±SD of three biological repeats. (c) Results of *Xa13* promoter editing mediated by two gRNAs (P1 and P2) using the CRISPR/Cas9 system in ZH11. Five transgenic plants (PD1, 3, 4, 5, and 6) were perfectly connected and repaired after cutting at the two target sites without any base deletions or insertions. PD2 was connected and repaired after cutting at the two target sites with two base (CG) insertions. (d) Results of *Xa13* CDS editing mediated by two gRNAs (G1 and G2) using the CRISPR/Cas9 system in ZH11. (e) Disease resistance and the seeding setting rate of 17 homozygously mutated transgenic rice in T_0 generation. Both of them were resistant to PXO99, but GD1-4 did not seed. [a] Data are the mean ± SD. [b1] 58 leaf samples from 10 plants. [b2&b3] 50 leaf samples from 10 plants. [c] n = 10. (f) Agronomic traits, disease resistance and pollen fertility in transgenic rice. The transgenic rice GD displayed small plant architecture and infertile pollen and thus could not be stained with I_2-KI; however, PD was normal, as ZH11-WT. (g) The expression analysis of *Xa13* in PD and GD. The primer sequence for expression was deleted in GD, so the PCR products could not be amplified from GD. The expression of *Xa13* in PD was not induced by pathogens as in ZH11-WT, but the background expression in leaves and anthers was not significantly different from that in ZH11-WT. (h) Screen for transgene-free plants in the T_1 progeny of PD1. All T_1 lines exhibited good disease resistance to PXO99 at the seeding stage, and eight transgene-free lines (Asterisks) were selected from 30 T_1 plants. (i) Main agronomic traits of transgene/transgene-free PD1 in the T_2 generation. Both transgene PD1 and transgene-free PD1 showed no significant differences compared to ZH11-WT in the heading stage, plant height, number of panicles per plant, panicle length, or seeding setting rate. [a] Data are the mean ± SD of 30 plants. [b] Student's *t*-test between transgene/transgene-free PD1 and ZH11.

31701399) and the China Postdoctoral Science Foundation (Grant No. 2018M632885).

Conflict of interest

The authors have declared no conflict of interest.

Author contributions

Y, L., C, X. and C, L. designed the research; C, L., W, L. and Z, Z. performed the research; C, L. and H, C. the analysed data; and C, L., W, L. and Y, L. wrote the paper.

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