Highly efficient generation of bacteria leaf blight resistance and transgene-free rice using genome editing and multiplexed selection system

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Abstract

Background Rice leaf blight is a worldwide devastating disease caused by bacteria *Xanthomonas oryzae pv. Oryzae* (*Xoo*). The UPT (up-regulated by transcription activator-like 1 effector) box in promoter region of the rice *Xa13* gene played a key role in *Xoo* pathogenicity. Mutation of key bacterial protein binding site in UPT box of *Xa13* to abolish PXO99-induced *Xa13* expression is a way to improve rice resistant to bacterial.

Highly efficient generation and selection transgene-free, edited plants helpful to shorten and simple the gene editing breeding process. Selective elimination of transgenic pollen of E0 plants can enrich proportion of E1 transgene-free offspring and expression of the color mark gene in seeds makes the selection of E2 plants is very convenient and efficient. In this study, a genome editing and multiplexed selection system was used to generate bacteria leaf blight resistance and transgene-free rice plants.

Results We introduced site specific mutations into the UPT box using CRISPR/Cas12a technology to hamper TAL (Transcription-Activator Like effectors) protein binding and gene activation, and generated genome edited rice with improved bacteria blight resistance. Transgenic pollens of E0 plants were eliminated by pollen specific expression of α-amylase gene *Zmaa1*, the proportion of transgene-free plants were enriched from 25% to 50% in single T-DNA insertion events in E1 generation. Transgenic seeds were visually identified and discarded by specific aleuronic expression of DsRed, which reduced 50% cost and achieved up to 98.64% of accuracy for selection of transgene-free edited plants.

Conclusion We demonstrated core nucleotide deletion in the UPT box of *Xa13* promoter conferred resistance to rice blight and selection of transgene-free plants were boosted by introducing multiplexed selection. The combination of genome editing and transgene-free selection is an efficient strategy to accelerate functional genomic research and plant breeding.

Background

New breeding techniques such as genome editing has extended beyond genetically-modified (GM) input-trait products and expanded into the commercialization of genome edited output-trait products[1]. Genome editing has numerous advantages over earlier technologies, most significantly in that it allows for targeted, single gene mutation across the entire plant genome[1]. The alteration of a specific DNA locus, without leaving behind heterologous genetic elements, offers significant advantage for this system over traditional genetic modification (GM) approaches.

Rapidly and efficiently generating transgene-free, edited plants is critical for breeder to shorten the gene editing breeding process. Presence of editing machinery component increases the difficulty of assessment of heritability and phenotypic stability of target-edited plants. Generation of new mutations by residual editing machinery makes it difficult to interpret the inheritance of mutant genotype(s). Meanwhile, the chance of off-target mutation is also increased [2] and may cause regulatory concerns related to genetically modified organisms[3]. Transgene-free plants can be obtained by segregation of

progeny in the subsequent generations and identified based on molecular detection. However, these methods are expensive, laborious and time consuming. Several strategies have been reported for screening or enriching transgene-free progeny. Fluorescence protein mCherry specifically expressed in *Arabidopsis* seeds was used for visual selection of edited progeny [4]. Another strategy for transgene-free plants was to incorporate editing vector with an RNAi expression cassette which silences the herbicide resistance gene CYP81A6 encoding a P450 cytochrome protein. Such strategy enables bentazon resistant transgene-free plants to be isolated from susceptible transgene plants by herbicide [5]. Although these strategies greatly accelerated the selection of transgene-free plans, they did not increase the portion of desired transgene-free plants in the population. Recently, He et al. reported a new strategy for transgene-free isolation via programmed self-elimination system (TKC, Transgene Killer CRISPR) which actively and automatically eliminates any plants that contain the T-DNA insertion [2]. Bacterial BARNASE gene driven by an embryo preferred promoter REG2 and rice ORFH79 gene driven by CaMV 35S promoter were used to kill any embryos and male gametophytes respectively that contain the transgenes. This method is effective and thorough; on the other hand it also brings the high risk of recovering low number of edited events. Desirable edited transgenic plants may also lose the chance of being selected when transgene component can be removed by segregation of progeny. In addition, using rice ORFH79 to kill male gametes limits the application of TKC among different crops. It is preferable to kill only transgenic male gametophytes, which can reduce the risk mentioned above and increase the proportion of transgene-free offspring. Another advantage of pollen-specific expression is that it can prevent the spread of transgenic pollen. Choosing components that are common to monocotyledons or dicotyledons can improve the efficiency of the system. The α-amylase gene *Zmaa1* is one potential candidate which has been applied successfully in Seed Production Technology (SPT) to kill transgenic pollen by disrupt starch accumulation during transgenic pollen maturation that deprived the necessary energy source for fertilization [6]. Other pollen inactivation genes such as *Barnase* and *DAM* have been used to deliver male sterility in diverse plants [7-11]. Therefore, these genes can be used as potential components for construction of gene editing vectors to kill transgenic pollen in diverse plant species and increase the proportion of non-transgenic offspring. Furthermore, a visual marker, such as *DsRed* and *mCherry* can also be used together with pollen-killer cassette so the non-transgenic progeny can be distinguished from the transgenic progeny by simple visual inspection.

Rice Bacterial leaf blight disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) resulted in severe yield losses, especially in Asia and Africa[12, 13]. About 42 genes conferring resistance against various races of *Xoo* have been identified in both cultivated and wild relatives of rice[14]. The *xa13* gene encoding a plasma membrane protein is fully recessive and confers resistance to *Philippine Xoo* race 6 (strain PXO99)[15]. Resistant allele of *xa13* gene differ from its dominant (susceptible) allele in coding region by only one amino acid residue- the 238th residue alanine of dominant XA13 protein is replaced by threonine in the recessive xa13 mutant protein[16]. However, disease resistance was not conferred by the amino acid residue variation, but due to alteration in the promoter regions which caused expression difference between the resistant and susceptible alleles [15, 16]. Compared with the dominant alleles, all recessive alleles had deletions, mutations and substitutions in the corresponding regions of promoter sequence

positions -86 to -69, suggesting that promoter mutations may result in xa13-mediated disease resistance[15]. By analyzing gene expression level driven by truncated and mutated *xa13* promoters in rice, it has been proved that promoter of *Xa13* harbors a UPT box which is the only PXO99 responsive *cis*-regulating elements in the activation of *Xa13* expression and the 5'-terminal second, third and fourth nucleotides of the box are the bind site for the bacterial protein [17, 18]. Li et al reported that 149 bp deletion in UPT of *Xa13* promoter deprived bacterial blight of its ability to induce *Xa13* gene expression and conferred race-specific resistance[19]. The identification of UPT box promoted application of genome editing technology to knock out key regions and confer resistance.

Here we reported a method to generate *Xoo* resistance rice by CRISPR/Cas12a targeting the binding site of UPT box in *Xa13* promoter region and further demonstrated critical nucleotides (5' terminal second, third and fourth nucleotides) for TAL protein binding and *Xa13* activating. We improved transgene-free plant selection by applying a GM pollen elimination and dsRed marker selection system, which greatly enriched proportion and selection efficiency of transgene-free edited progeny (Fig. 1). The streamlined procedure demonstrated its utility in genome editing for plant breeding and basic research with improved process efficiency.

Results

Generation of LbCas12a transgenic rice plants and selection of events with edits in UPT of Xa13 promoter

To confer *Xoo* resistance to rice, we constructed two LbCas12a plasmid vectors that harbor 24 nt crRNA to target the UPT box of rice *Xa13* (DQ421395.1) promoter which is the binding site for the bacterial TAL effector protein. Core target site 'GCA' located at -79 position of the *Xa13* promoter is 24-bp away from the TTTV PAM motif on the opposite strand downstream (Fig 2D). A multiplexed selection system was used for increasing the proportion of and selecting for T-DNA-free plants in E1 generation. The multiplexed system consists of pPG47:: Bt1: Zm-aa1 and LTP2:: DsRed expression cassettes. To measure the effectiveness of the system, a construct, 27277 was created which contains both *Zmaa1* and *DsRed* expression cassettes; another construct 24259 was used as control (Fig 2A). Constructs were introduced into indica rice variety IR58025B via *Agrobacterium*-mediated transformation. T-DNA copy number was measured, and mutation identified by TaqMan assay and then confirmed by colony sequencing. Transformation frequency (TF, number of positive events/number of infected explants) and mutation rate (MR, number of mutant events/number of positive events) of construct 24277 and 24259 based on the results of three experiments were investigated (Table 1 and Fig. 2B). The TF and MF of the *Xa13* promoter targeted by LbCas12a were 46.1% and 39.6% for 24277, 68.3% and 56.4% for 24259. Both TF and MR of 24277 were lower than 24259. Range of deletion size derived by LbCas12a in 67 mutations were from 1 to 23 bp and the majority ranged from 8 to 10 bp in size (Fig. 2C). 44 single T-DNA insertion and backbone-free T0 events with three core nucleotides deletion in *Xa13* promoter from two constructs were chosen to produce T1 seeds for further evaluation (Fig. 2D).

Non-transgenic rate in progeny was increased by GM pollen elimination and non-transgenic seeds were identified visually through expression of color marker gene

T-DNA segregation in T1 offspring of 10 single copy T-DNA insertion T0 events from the two vectors was analyzed by TaqMan qPCR assay and RFP expression. As shown in Table 2, Most T1 plants from 24259 events harbored the T-DNA transgene which was no longer needed and the proportion of transgene-free progeny plants was from 19.68% to 29.65%. Compared with 24259, the proportion of transgene-free plants from 24277 was from 46.92% to 50% and the ratio between transgene and transgene-free plants was close to 1:1. The results indicated that expression of *Zmaa1* resulted in sterility of transgenic pollen, which led to the increase of the proportion of transgene-free offspring.

Normally, Transgene-free plants are identified by PCR or other molecular tests such as TaqMan assay. However, the traditional methods are laborious and inefficient and transgene-free plants are only identified after seed germination. For fast and efficient determination of transgene-free seeds, expression of RFP was placed under the control of an endosperm-preferred promoter, and transgene-free plants can be visually identified using a fluorescence detector (Fig. 3). *Zmaa1* and *DsRed* expression cassettes and other transgene components are located within the same T-DNA and thus are tightly linked and co-segregate. Therefore, T1 seeds of 24277 displaying strong red fluorescence were transgenic and easy to be distinguished from transgene-free seeds that do not show any fluorescence. In order to verify the accuracy of this visual detection method, these T1 seeds were germinated and confirmed by TaqMan assay. As shown in Table 2, average accuracy rate of visual detection reached 98.62%, confirming that the approach is highly effective for identification of transgene-free progeny seeds.

Core nucleotide deletion of UPTPthXo1 in Xa13 promoter results in resistance to PXO99

To characterize resistance to bacterial leaf blight, four homozygous mutant E1 lines L4, L5, L19 and L27 with deletion in one, two or all of the three core nucleotides of UPT box (Fig. 4A) were chosen for infection with the Philippine *Xoo* race 6 (strain PXO99) at the heading stages and relative expression level of *Xa13* gene determined by RT-PCR in the leaves of wild type and mutant lines 72 h after PXO99 infection. The mutant lines showed significant resistance to PXO99 strain (Fig. 4B), with an average lesion area of 2.4 ± 0.8% to 6.7 ± 3.1% compared with 60.0 ± 9.9% for the wild control IR58025B (Fig. 4C). Expression of *Xa13* gene in mutant lines was not detected which showed deletion of core nucleotides in UPT box resulted the *Xa13* gene lost its ability to be induced by PXO99 (Fig. 4D). Among mutant lines with various deletion of three core nucleotides, there was no obvious difference on lesion area ($P<0.001$, data was not shown). The result shows these three nucleotides of the UPT box are important for bacterial TAL effector protein binding and gene activation. The result that shows mutations in any one of these sites abolished PXO99-induced gene expression and conferred race specific resistance is consistent with the previous reports

[17, 19]. No negative agronomic phenotype such as decrease of fertility and seed setting rate was observed in the mutant lines with targeted edits in the *xa13* promoter (Fig. 4E).

Discussion

Genome editing has become a powerful tool for breeders to improve agronomic traits of crops in plant breeding[20]. The primary objective of genome editing is to obtain transgene-free edited plants or seeds with desired trait performance. In selfed progeny only about 25% of the E1 plants from a single copy T-DNA insertion T0 line are transgene-free, about 75% of the workload is wasted on analyzing the undesirable transgenic plants. Increased seed number of edited, transgene-free progeny in E1 generation can significantly increase the chance of selecting desirable edited lines and therefore shortening the breeding cycle. He et al. reported a highly efficient way to eliminate any T1 plants that containing the CRISPR/Cas9, TKC system would kill any embryos and male gametophytes that contain the transgenes; however, this system is like a double-edged sword since it brings risks while being efficient. CRISPR-mediated genome editing efficiency varies significantly among different species and even among different tissues of the same plant species[21]. Especially, low efficiency of precise gene editing in plants, including DNA fragment knock-in and gene replacement, remains a major challenge [22]. Precious plants with desired edits but still contains T-DNA component will be killed by TKC, instead of retaining it to be removed by segregation. Therefore, in our research, we increased the proportion of transgene-free offspring by only hampering the transmission of T-DNA in male gametophyte without interference in female gamete, and the additional benefit was to prevent the spread of transgenes through pollen.

It will increase efficiency of editing pipeline if transgene-free selection system can be used as the common component of editing base vector. Rice *ORFH79* gene limits the application of TKC system in different crops. Devitalizing transgenic pollen by *Zmaa1* under promoter PG47 has been applied successfully in some monocot crops, such as corn, rice and wheat [6, 23, 24]. Therefore, it is an ideal candidate as a base component of a monocot crop editing vector. To extend application of transgene free selection system in dicotyledonous crops, we'll further test another anther tapetum specific promoter TA29 to drive expression of pollen lethal gene.

Since T-DNA transmission in female gametophytes is allowed in our system, a selection component was included to distinguish transgenic from non-transgenic progeny. Expression of the *DsRed* gene under an endosperm-preferred promoter LTP2 was used to select transgene-free seeds visually which is very convenient and efficient. This visual sorting cassette had been successfully tested in corn and rice seeds, but failed in soybeans seeds because of thick seed coat.

We generated transgene-free E1 plants resistant to bacterial pathogen PXO99 infection by mutating the target site of *xa13* gene promoter with CRISPR/Cas12a. Compared with Li's report in which bacterial resistance rice was conferred by a 149 bp deletion in *Xa13* promoter generated by a double-sgRNA site-directed mutation, we did it only by deleting two core nucleotides of UPTpthxo1 in the *Xa13* promoter (L27). We also verified that any absence of the 5'-terminal second, third, and fourth nucleotides of the

UPTPthXo1 box can confer race-specific resistance which provide a direct evidence to support the conclusion in Yuan's report [17]. Furthermore, the mutant plants with disease resistance did not show sterility or yield decline.

Conclusions

We developed a transgene free plant enrichment and selection method to generate bacterial pathogen resistant transgene-free rice. This method can be applied to accelerate breeding of genome edited materials in the breeding progress.

Materials And Methods

Vector construction

The rice codon optimized Cas12a from *Lachnospiraceae bacterium* ND2006 containing two nuclear localization signals (NLS) at its N- and C- terminals were from previous report except with 3bp changes to remove 2 Bsp119I and one RsrII sites[25]. This gene was driven by sugarcane Ubiquitin4 promoter (prSoUbi4) in two binary vectors. Tandem duplicate crRNA array (DR-S1-DR-S1) driven by OsU6 promoter was designed following Wang et al report[26]. A 9 bp poly (T) short sequence was used to terminate the crRNA (OsU6-DR-S1) expression cassette. Comparing to base vector 24259, two extra expression cassettes are present in test vector 24277.

Plant Transformation and plant nursery

IR58025B rice was used for transformation. *Agrobacterium*-mediated transformation was performed according to the protocol reported previously[27]. PMI positive plants were identified via selection medium containing mannose[28]. Survived plants went through a TaqMan assay to check the T-DNA copy number and target sequence mutation of *Xa13* promoter. Plants with single copy T-DNA insertion, backbone free and target site mutation of *Xa13* promoter were sent to the greenhouse. Plants were grown in 170 × 150 mm pots filled with turf, peat moss and nutrition soil at a ratio 3:2:1 plus 40g Osmocote 3-4 months controlled-release fertilizer (17-7-12) per pot in greenhouse. Watering was managed via drip irrigation. Growth conditions were 30 ± 2°C days, 25 ± 2°C night, and the photoperiod was set to 12 hours day and 12 hours night.

TaqMan assay and sequence analysis of targeted mutations

TaqMan quantitative PCR (qPCR) assay was performed to determine the T-DNA copy number and target sequence mutation. Genomic DNA was extracted from leaf following the protocol of Promega Magbeads Plant genome extraction kit. Gene specific primers/probes were designed using the software PrimerExpress3.0 and synthesized from Life Technology. Real-time (RT) qPCR was performed in an ABI

7900HT real-time PCR system. Each 10 μl Real-time PCR reaction contained 5 μl 2x Sigma JumpStart Master Mix (Sigma-Aldrich Corporation), 3 μl DNA, 0.2 μl 50x Taqman assay stock each (final concentration: 300 nM for primers and 100 nM for probe) and 1.6 μl water. Real-time PCR conditions were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 5 sec followed by 60 °C for 30 sec. The data were analyzed using the SDS 2.4 software.

The candidates screened by TaqMan assay were further confirmed by colony sequencing. The targeted regions were amplified with KOD-PLUS-Neo (Toyobo) and cloned into the pEASY vector (pEASY-Blunt Zero Cloning Kit, Transgen). Ten independent random clones were selected for Sanger sequencing (Life Technologies). The sequences were aligned to the wild-type in Vector NTI.

Seed color sorting

A fluorescence detector was used for seed color sorting. With the matching barrier filter glasses, the red fluorescent seeds could be easily sorted from the non-red fluorescent seeds. The number of red fluorescent and non-red fluorescent seeds in different E1 events was counted manually.

Pathogen inoculation

Xanthomonas oryzae pv. *Oryzae* (*Xoo*) strain PXO99 was cultivated on petri dish with YDC medium. Bacteria pathogen was scraped from petri dish to make suspension at 5 dpi with ddH2O and the suspension concentration was adjusted to OD 1.0 for inoculation. Rice plants were inoculated by leaf-clipping method at booting stage, and control planted were treated by ddH2O[29]. Inoculated plants were incubated in growth chamber with 25℃ darkness condition for 24 hours, then change to normal rice plant growth condition. Lesion length and area were measured at 14 days post inoculation, and calculated mean and standard deviation to analyze *Xoo* resistance.

Gene expression analyses

Total RNA from leaf, anther, and root of rice was extracted with RNAprep pure Plant Kit (Tiangen Biotech, www.tiangen.com); 1 μg of total RNA was used for cDNA synthesis using the Superscript III first-strand system (Invitrogen) and the oligo-dT primer. Semi-quantitative RT-PCR was conducted as described by Zhou et al[30]. PCR primers for *Xa13* were 5'- ATGGCAGGAGGTTTCTTGTCC-3' and 5'-AAGAAGCCGCCCACGTTC-3'; Primer sequences for *OsAction* (AY212324) control gene were 5'-GCAGAAGGATGCCTATGTTG-3' and 5'- GGACCCTCCTATCCAGACAC-3'.

List Of Abbreviations

XOO: Xanthomonas oryzae pv. Oryzae

UPT: Up-regulated by TAL effectors

TAL: Transcription-Activator Like effectors

GM: Genetic modification

TKC: Transgene Killer CRISPR

SPT: Seed Production Technology

PAM: Protospacer adjacent motif

TF: Transformation frequency

MR: Mutation rate

RFP: Red fluorescent protein

LTP: Lipid transfer protein

RT-PCR: Reverse transcription-polymerase chain reaction

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its additional files). Materials are not publicly available due to patent and licensing limitation.

Competing interests

The authors declared that they have no competing interests.

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Authors' contributions

KY and XC designed the experiments. KY wrote the manuscript and managed plant care in the greenhouse. ZQ performed pathogen inoculation and resistance evaluation experiment. HG performed the rice transformation. LG performed the vector design and construction. JW performed TaqMan assay and sequence analysis of mutant. JL performed the qRT-PCR of *Xa13*. DL, JX and XC provided technical support. All authors read and approved the final manuscript.

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Tables

Tables 1-2 are available in the Supplementary Files.

Figures

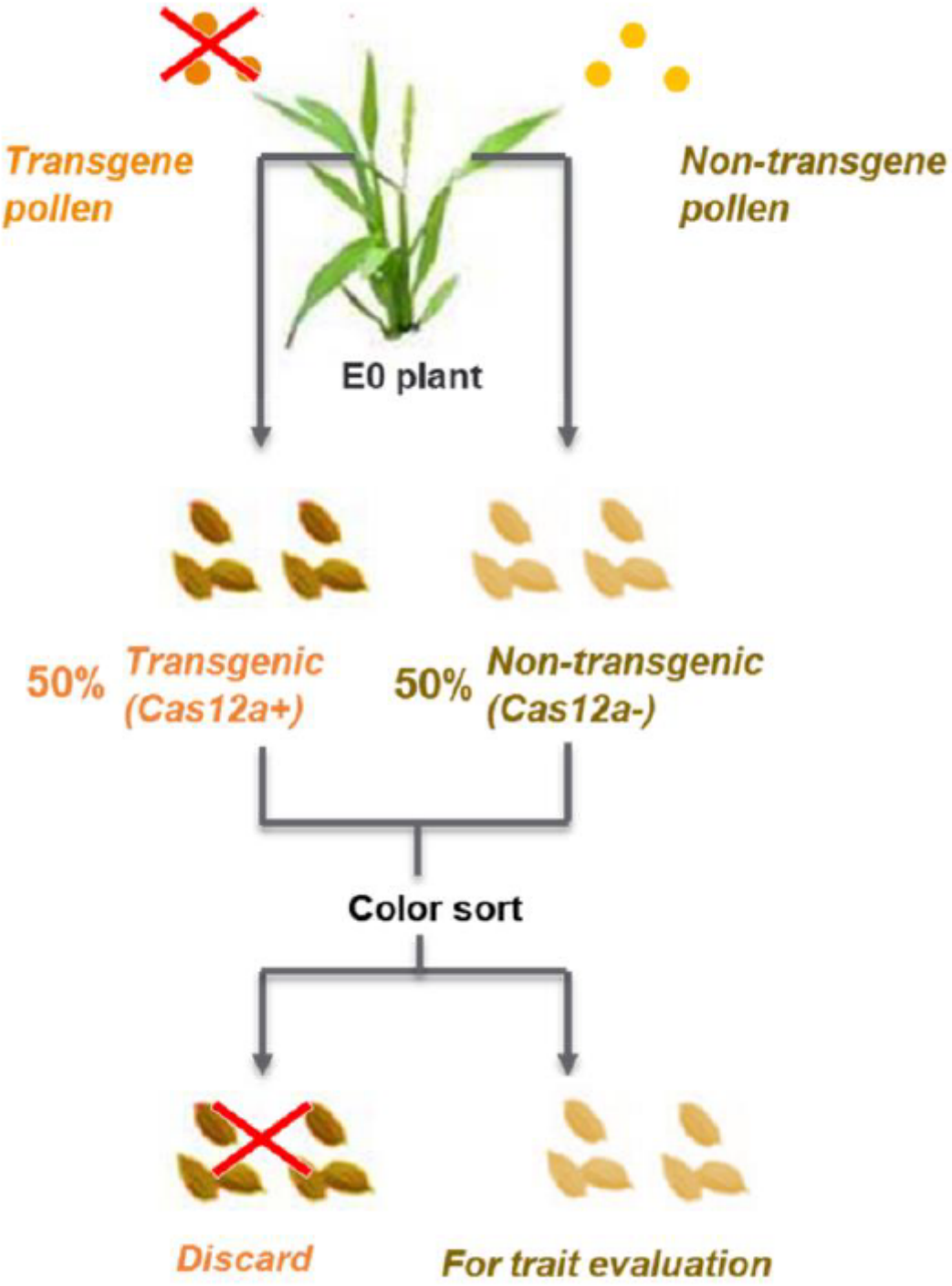
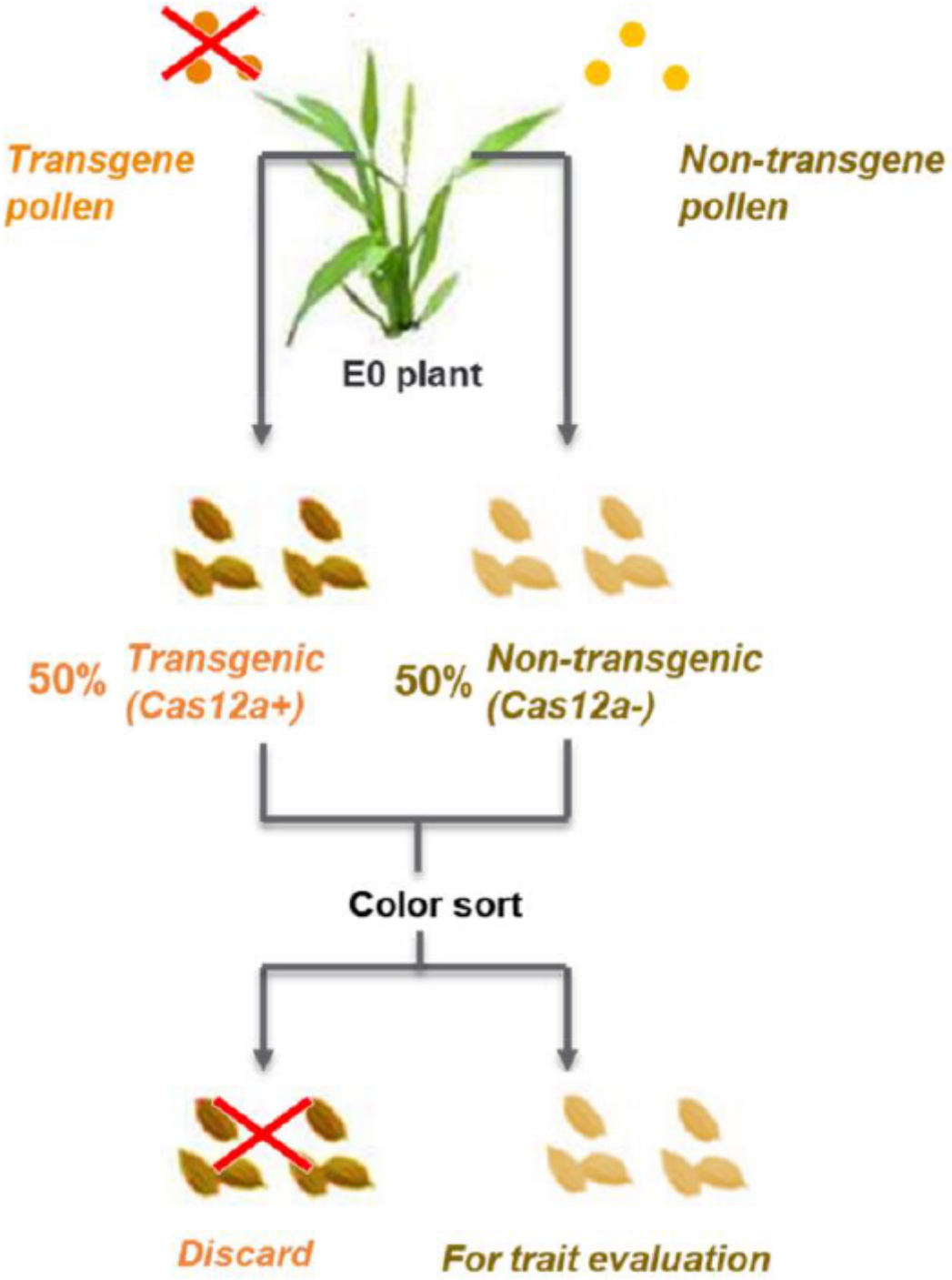


Figure 1

Schematic diagram of strategy for transgene-free plant enrichment and selection. Increase the proportion of non-transgenic offspring by hampering T-DNA transmission in male gametophytes and sort non-transgenic seeds by visual selection marker. For single T-DNA insertion line, Self-pollination of the resulting E1 propagates 50% of transgenic seeds and 50% of non-transgenic seeds, which means the

portion of desired E1 seeds will be increased from 25% to 50%. For multi-copies T-DNA insertion line, T-DNA free progeny segregation rate will increase 2n (2 to the power n) times. N equal to copy number.



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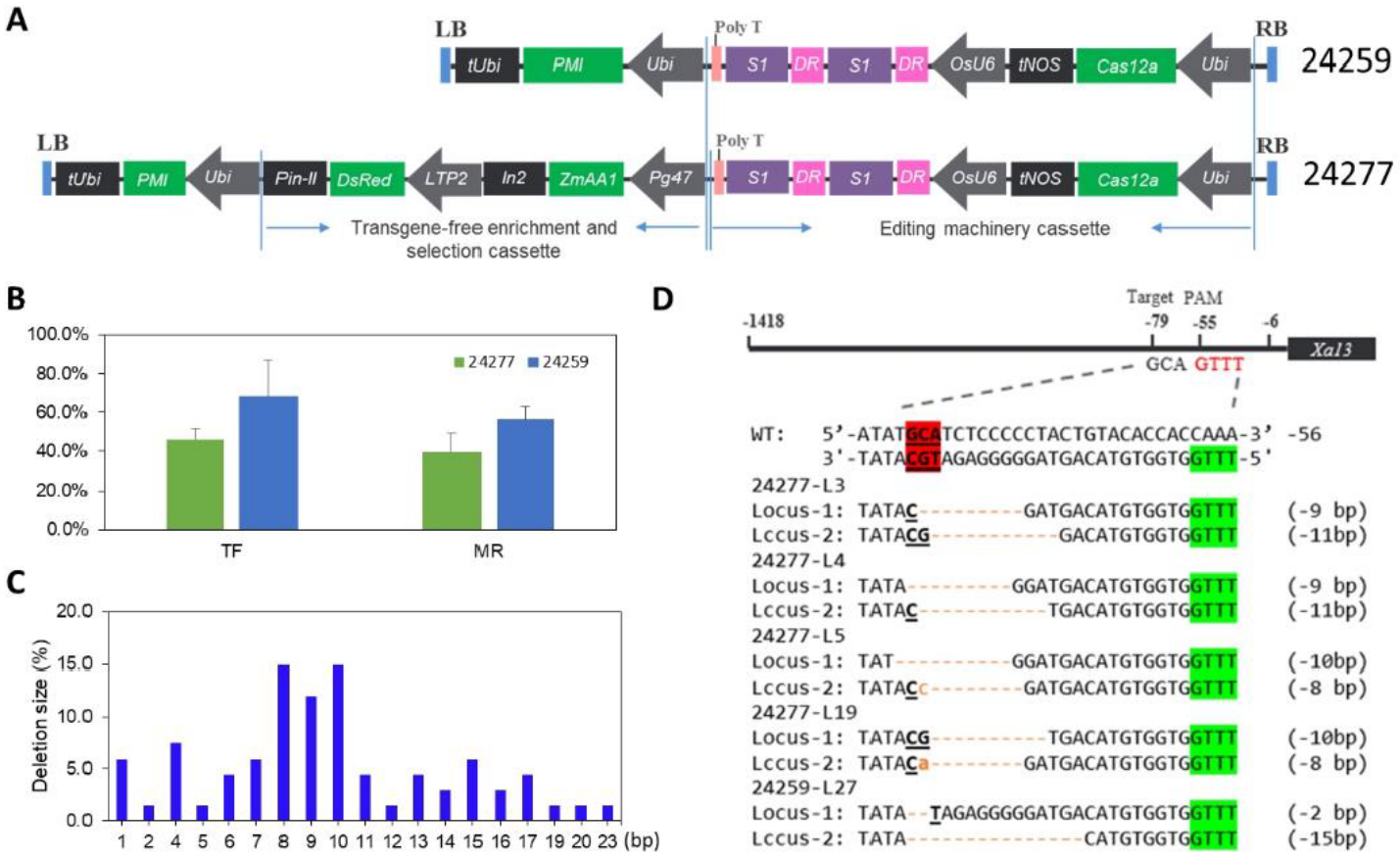
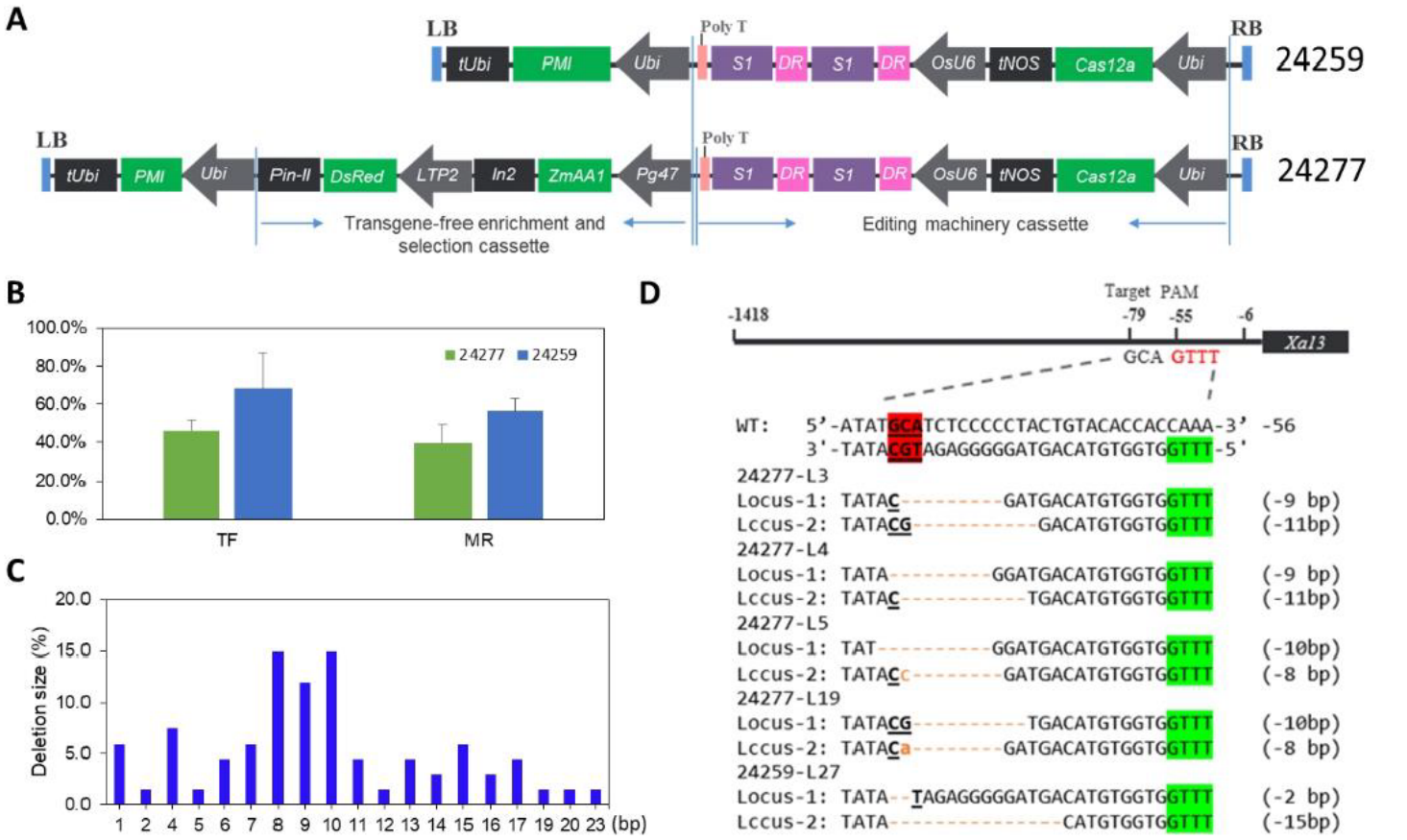


Figure 2

Generation of Xa13 promoter mutants using CRISPR/Cas12a editing vectors. (A) The schematic of the T-DNA region of the editing vector of 24259 and 24277. (B) Comparison of transformation frequency between two vectors. (C) Deletion sizes derived by LbCas12a in 67 E0 lines from two constructs; bp, base pairs. (D) Different deletion occurred at expected target site- 5'-terminal second, third, and fourth nucleotides of UPTPthXo1 box in Xa13 promoter in the 5 E0 events which were selected as candidates for further resistance evaluation.



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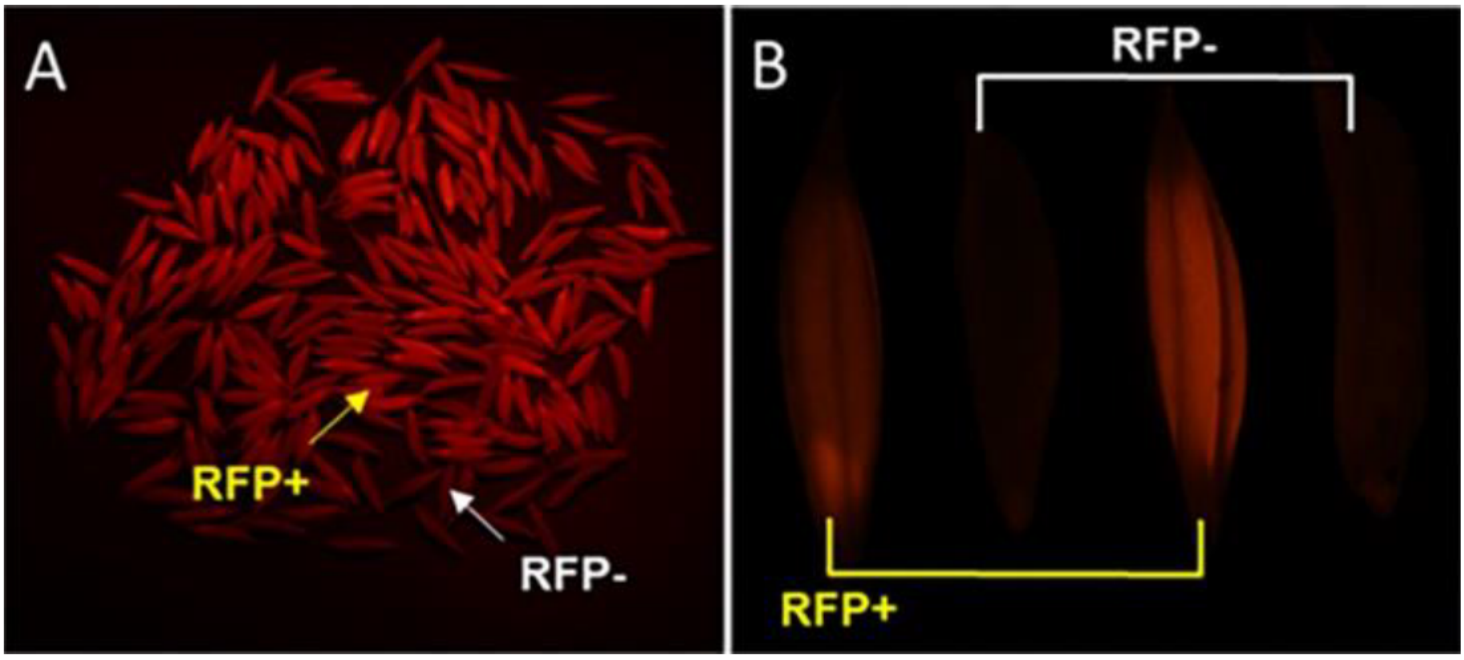
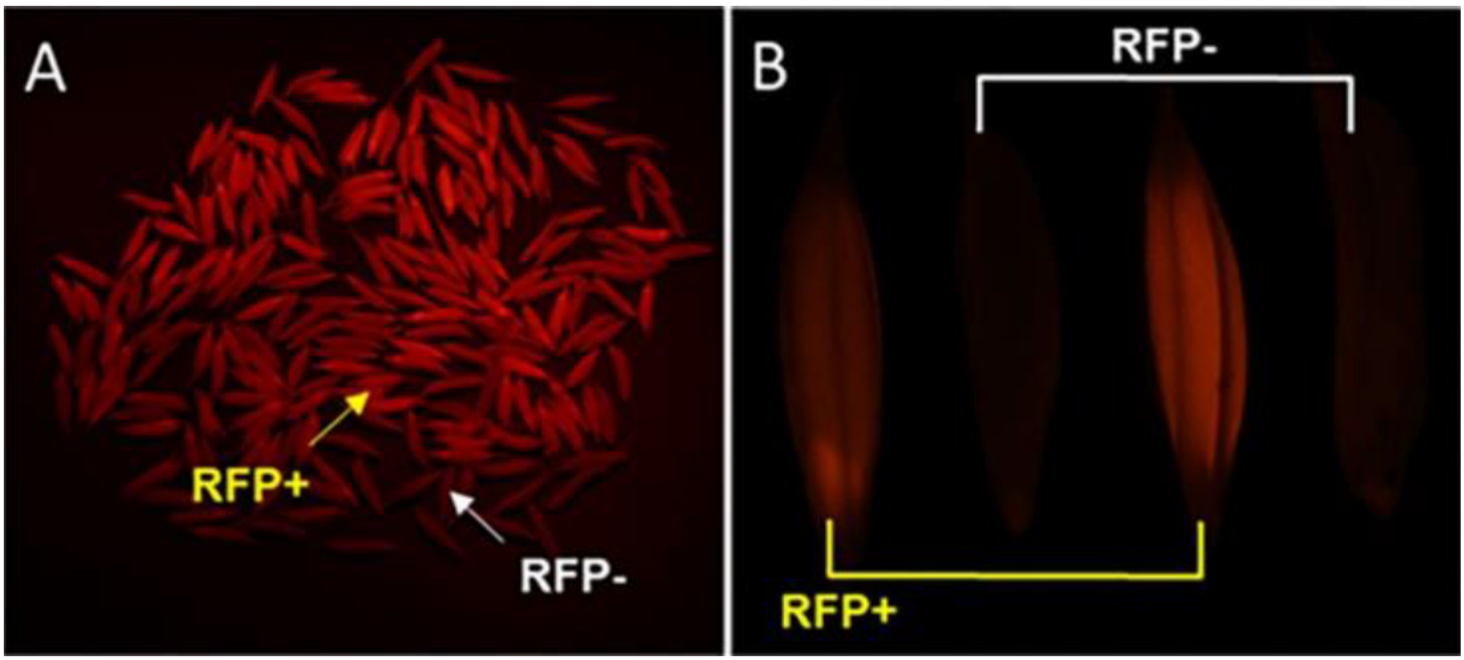


Figure 3

Visual sorting T1 seeds by fluorescence detector. The T-DNA free seeds do not produce red fluorescence. (A) Visual difference between T-DNA free population and T-DNA insertion population under fluorescence detector. (B) No red fluorescence was detected in T-DNA free seeds.



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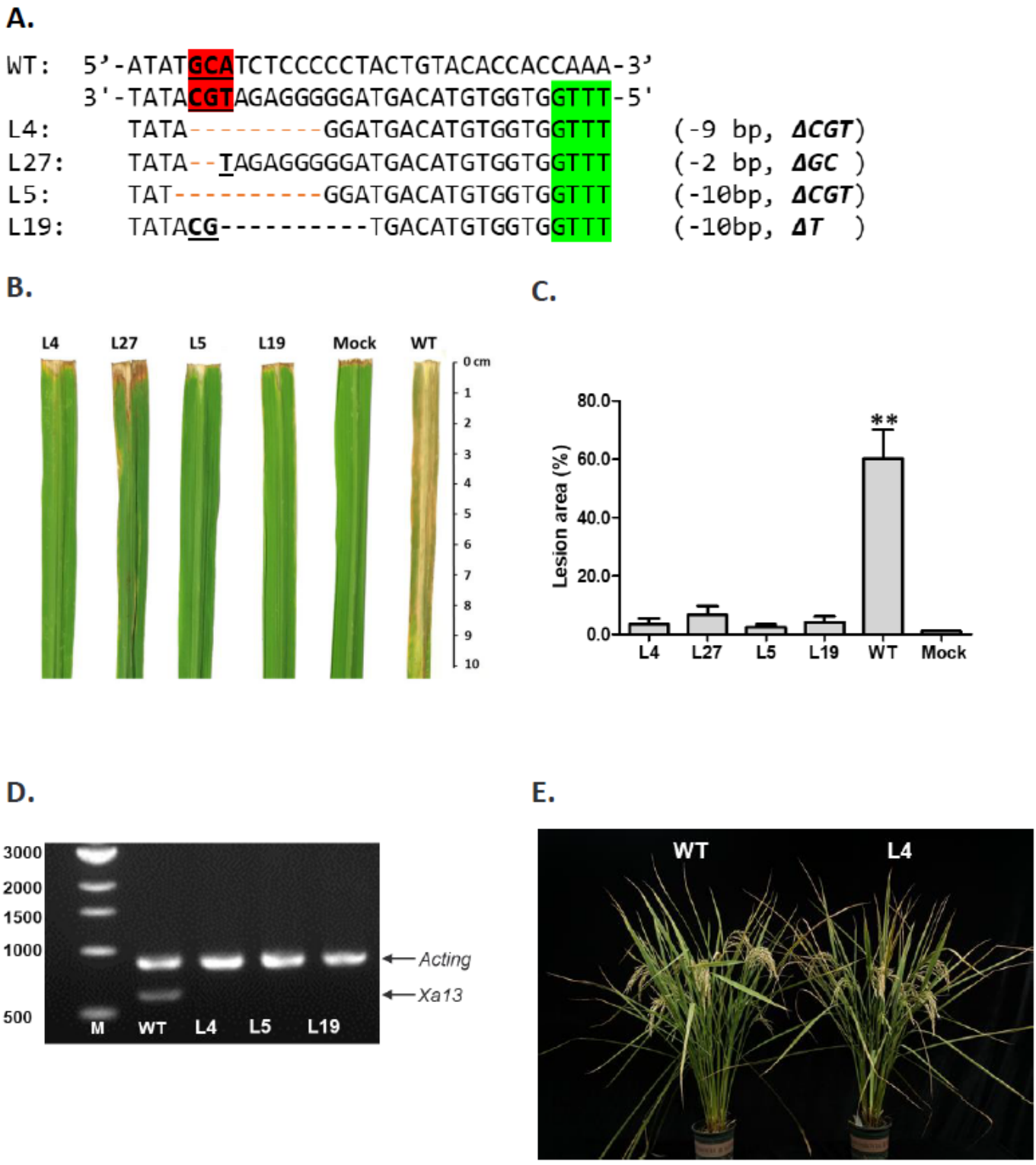
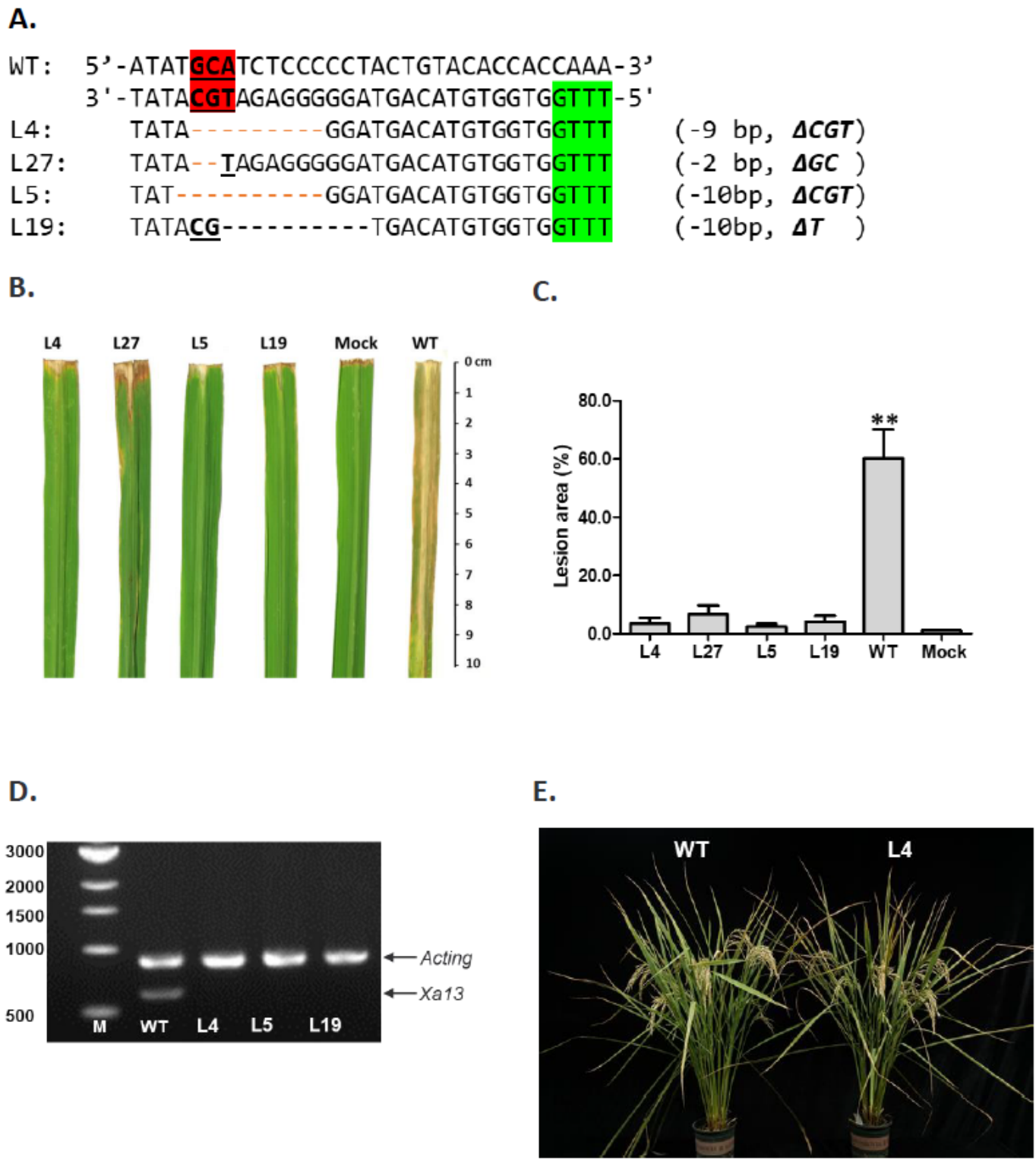


Figure 4

Targeted mutation of Xa13 promoter to enhance resistance to bacterial blight resistance. (A) Homozygous mutagenesis in Xa13 promoter of four E1 lines selected for resistance evaluation. (B) Leaves of WT and mutant lines at heading stage showing resistance or susceptibility to bacterial blight at heading stage. Leaves were detached from the inoculated plants at 14 dpi for photography. (C) Lesion area of wild type and mutant lines 14 days after inoculation. WT, wild type and Mock, without pathogen.

The asterisk (**) indicates that a significant difference (P< 0.01) was detected between mutant lines and wild type plants. (D) Expression of the Xa13(DQ421395.1) gene determined by RT-PCR in the leaves of wild type and mutant lines 72 h after PXO99 infection. The OsActin gene (AY212324) was used as an internal control. (E) Morphology of wild type and mutant line. The gel images in (d) were cropped; the original gel images are shown in Additional file 4.



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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Tables.xlsx
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- Additionfile1SequenceanalysisofmutationinXa13promoterofE0lines.xlsx
- Additionfile1SequenceanalysisofmutationinXa13promoterofE0lines.xlsx
- Additionfile2HomozygousmutagenesisinXa13promoterofE1lines.xlsx
- Additionfile2HomozygousmutagenesisinXa13promoterofE1lines.xlsx
- Additionfile3LesionlengthandareaofmutantlinesandWTafterinfection.xlsx
- Additionfile3LesionlengthandareaofmutantlinesandWTafterinfection.xlsx
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