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Creation of fragrant rice by targeted knockout of the *OsBADH2* gene using TALEN technology

Qiwei Shan[1], Yi Zhang[1], Kunling Chen[1], Kang Zhang[1,2] and Caixia Gao[1,*]

[1]*State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China*
[2]*Beijing Genovo Biotechnology Co. Ltd, Beijing, China*

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*Correspondence
(Tel/fax 0086 10 64807727;
email cxgao@genetics.ac.cn)

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Summary

Fragrant rice is favoured worldwide because of its agreeable scent. The presence of a defective *badh2* allele encoding betaine aldehyde dehydrogenase (BADH2) results in the synthesis of 2-acetyl-1-pyrroline (2AP), which is a major fragrance compound. Here, transcription activator-like effector nucleases (TALENs) were engineered to target and disrupt the *OsBADH2* gene. Six heterozygous mutants (30%) were recovered from 20 transgenic hygromycin-resistant lines. Sanger sequencing confirmed that these lines had various indel mutations at the TALEN target site. All six transmitted the *BADH2* mutations to the T1 generation; and four T1 mutant lines tested also efficiently transmitted the mutations to the T2 generation. Mutant plants carrying only the desired DNA sequence change but not the TALEN transgene were obtained by segregation in the T1 and T2 generations. The 2AP content of rice grains of the T1 lines with homozygous mutations increased from 0 to 0.35–0.75 mg/kg, which was similar to the content of a positive control variety harbouring the *badh2-E7* mutation. We also simultaneously introduced three different pairs of TALENs targeting three separate rice genes into rice cells by bombardment and obtained lines with mutations in one, two and all three genes. These results indicate that targeted mutagenesis using TALENs is a useful approach to creating important agronomic traits.

Introduction

Fragrant rice (*Oryza sativa*) is gaining popularity worldwide for the characteristic fragrance of its grains. Its market price, particularly that of the Indian Basmati and the Thai Jasmine types, is much higher than that of the conventional nonfragrant rice (Bhattacharjee *et al.*, 2002). More than one hundred volatile compounds were detected in the flavour of cooked fragrant rice. Of these, 2-acetyl-1-pyrroline (2AP) is the most abundant and is considered responsible for the fragrance. No apparent 2AP (at least about two orders of magnitude less) is detected in nonfragrant rice (Jezussek *et al.*, 2002; Lorieux *et al.*, 1996).

Genetic analysis of the fragrant trait has shown that fragrance is controlled by a single recessive gene (initially referred to as *fgr*) on chromosome 8. Subsequently, positional cloning suggested that *fgr* encodes betaine aldehyde dehydrogenase 2 (BADH2) (Bradbury *et al.*, 2005). The corresponding full-length gene *OsBADH2* comprises 15 exons and 14 introns, encoding 503 amino acids. Sequence alignment of this gene between fragrant and nonfragrant rice varieties identified multiple types of mutation in fragrant rice, such as a 7-bp deletion in exon 2 (designated *badh2-E2*), an 8-bp deletion together with a 3-bp SNP in exon 7 (designated *badh2-E7*) and an 803-bp deletion between exons 4 and 5 (designated *badh2-E4/5*) (Bradbury *et al.*, 2005; Kovach *et al.*, 2009; Shao *et al.*, 2011; Shi *et al.*, 2008). Further confirmation that mutations in *OsBADH2* are responsible for the fragrant phenotype came from transgene complementation (Chen *et al.*, 2008) and RNA-induced down-regulation experiments (Chen *et al.*, 2012; Niu *et al.*, 2008). It is not yet clear how *OsBADH2* influences 2AP biosynthesis. One likely hypothesis is that BADH2 inhibits 2AP synthesis by diverting GABald, an upstream precursor of 2AP, to GABA; when BADH2 is dysfunctional, GABald accumulates and is converted to 2AP (Figure 1a) (Bradbury *et al.*, 2008; Chen *et al.*, 2008).

Currently, the main rice breeding technique is conventional hybrid breeding. In conventional breeding of fragrant rice, it takes several generations to transfer a natural *badh2* mutant gene into elite rice varieties by crossing and backcrossing. The process is laborious, time-consuming and expensive. Transgenic breeding using RNAi-based down-regulation of *OsBADH2* expression is an alternative approach (Chen *et al.*, 2012; Niu *et al.*, 2008). However, RNAi-mediated inhibition of *OsBADH2* expression is often incomplete (Chen *et al.*, 2012); transgene expression varies in different lines, so it is necessary to screen a large number of transgenic plants to identify candidate lines in which the transgene is stable expressed over generations; besides, rice lines derived by this method are regarded as transgenic and are subject to costly regulatory processes.

Genome editing technologies using sequence-specific nucleases (SSNs), including meganucleases, ZFNs, TALENs and the CRISPR/Cas9 system, have been developed to create targeted DNA double-strand breaks (DSBs) in various model and crop plant species (Voytas and Gao, 2014). The DSBs are mainly repaired by error-prone nonhomologous end joining (NHEJ) or by high-fidelity homologous recombination (HR). NHEJ often causes small insertions or deletions (indels) at the sites of breaks, frequently generating knockout mutations. Here, we report the creation of fragrant rice from a nonfragrant variety via targeted knockout of *OsBADH2* using the TALEN method. We previously described one pair of TALENs engineered to cleave *OsBADH2* (T-OsBADH2b)

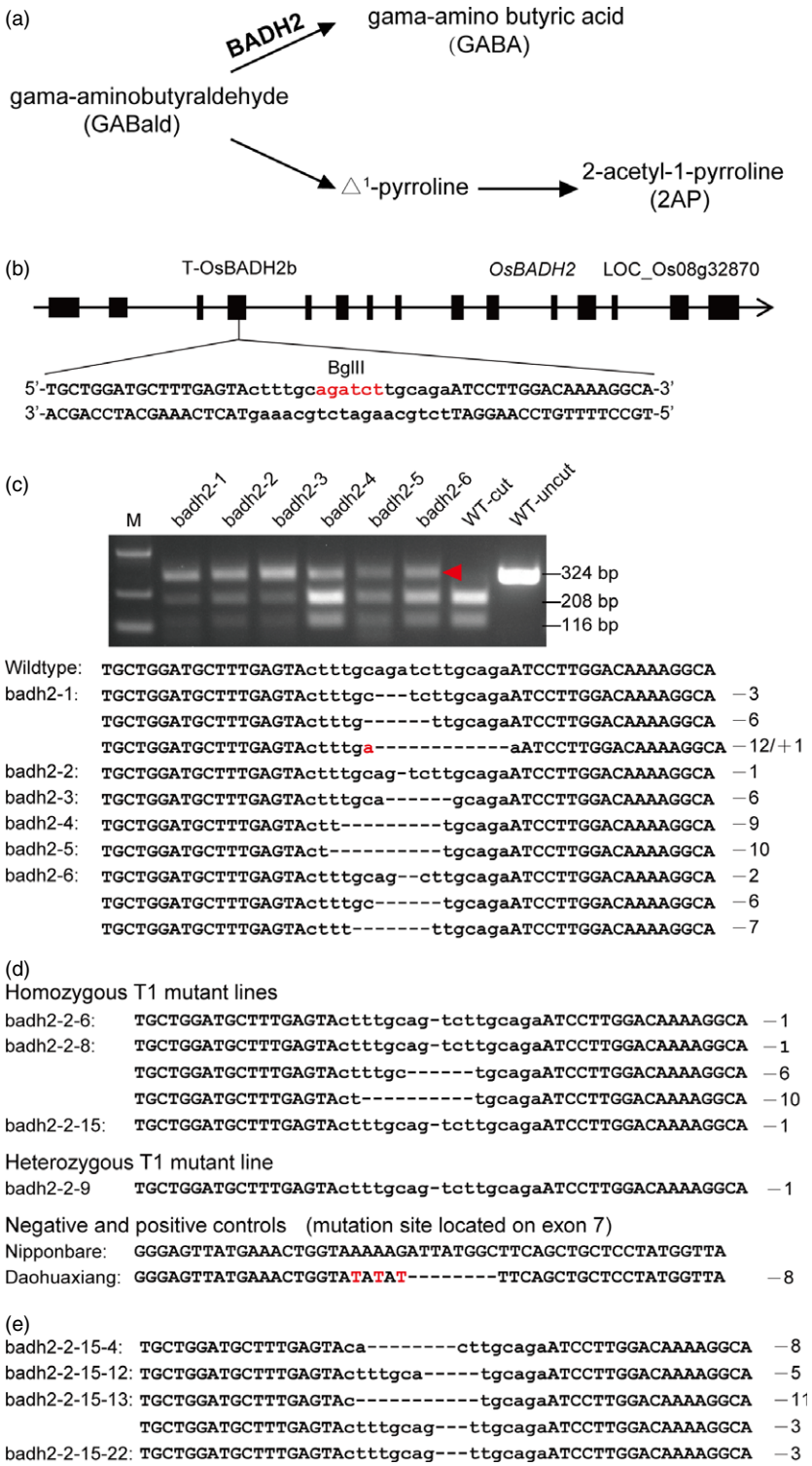

Figure 1 Transcription activator-like effector nuclease (TALEN)-induced mutations in the *OsBADH2* gene. (a) The 2AP pathway in rice. *OsBADH2* is responsible for the conversion of GABald into GABA; when BADH2 is inactive, GABald accumulates and 2AP is formed. (b) Schematic of *OsBADH2* gene structure and TALEN binding sites. *OsBADH2* contains 15 exons, indicated by black rectangles. The target site of T-OsBADH2b is shown beneath, with the TALEN binding sites in upper case and the spacer in lower case; the restriction enzyme site BglII is highlighted in red. (c) Detection of NHEJ-introduced indel mutations in six heterozygous T0 transgenic plants by PCR/RE assay and DNA sequencing. Mutations in independent plants were identified with *OsBADH2*-specific primers, and the PCR amplicons were digested with BglII. The red arrowhead indicates bands with mutations. The mutation sequences of six T0 plants are aligned under the gel. Deletions and insertions are indicated by dashes and red letters, respectively, and the numbers on the right side give the indel sizes. M, DNA molecular weight marker. (d) DNA sequences of three homozygous T1 lines, one heterozygous T1 line, a positive control (cv. Daohuaxiang, *badh2-E7*) and a negative control (cv. Nipponbare) used for measuring 2AP content. (e) New indel mutation sequences detected in four T2 seedlings of the *badh2-2* plant.

(Shan *et al.*, 2013). We have now used the same TALEN pair to create several heritable homozygous mutant rice lines. In these lines, there was substantial 2AP content, whereas no 2AP was detected in the wild-type control plant. We also attempted to knockout three genes (*OsBADH2*, *OsCKX2* and *OsDEP1*) simultaneously by particle bombardment and obtained a series of transgenic rice plants with single, double and triple knockouts, thus demonstrating the ability of TALENs to create multiplex gene knockouts.

Results

TALEN design and recovery of rice plants with *BADH2* mutations

The construction and validation of TALEN pairs has been described previously (Shan *et al.*, 2013). T-OsBADH2b targeting to the fourth exon of *OsBADH2* was used to create stable transgenic plants using *Agrobacterium*-mediated transformation. The TALEN pair recognizes two 17-bp sequences of contiguous DNA separated by an 18-bp spacer DNA that contains a BglII restriction site in its centre for identifying mutations by PCR restriction enzyme (PCR/RE) digestion assays (Figure 1b). A total of six T0 heterozygous mutant *BADH2* rice plants (badh2-1 to badh2-6) were recovered from 20 transgenic hygromycin-resistant plants (30%) from one *Agrobacterium*-mediated transformation experiment in rice variety Nipponbare (Figure 1c). Sequencing of the mutations showed that most were small deletions of 1–10 bp in the spacer region (Figure 1c and Table 1). Mutants badh2-3 and badh2-4 had 6-bp and 9-bp deletions, respectively, which did not result in frame shifts mutation. Plants badh2-2 and badh2-5 with 1-bp and 10-bp deletions, respectively, caused frameshifts and could have inactivated the gene. Hence, homozygous progeny of badh2-2 and badh2-5 were chosen for phenotypic analysis. In addition, we detected multiple changes at a single target site in plants badh2-1 and badh2-6, probably because these mutations occurred in different somatic cells. To demonstrate the wide application, the same TALEN pair was also transformed into an elite rice cultivar LPK, and 10 heterozygous mutant *BADH2* rice plants (19.2%) were regenerated from 52 transgenic T0 plants (Figure S1).

Transmission of TALEN-induced mutations to T1 and T2 generations

To see whether the mutations induced by T-OsBADH2b were transmitted to the next generation, all 6 T0 mutant plants were self-pollinated and individually genotyped, and a total of 171 T1 plants from the 6 T0 mutants were genotyped by PCR/RE to test for transmission of the mutations (Table 1). We found that all six mutations were transmitted to the T1 generation, the proportion ranging from 28.6% to 90.6%. Most of the T1 plants carrying the mutations were heterozygotes, except for the offspring of badh2-2. Among 32 T1 progeny of badh2-2, we identified four homozygous and 25 heterozygous mutant plants. No homozygous mutants were detected in the T1 generation of the other five T0 plants, probably because of the small number of T1 seeds (badh2-3, badh2-4 and badh2-5) or the mutations occurring in somatic cells that could not participate in the production of gametes (badh2-1, badh2-6). The progeny of three homozygous (badh2-2-6, badh2-2-8 and badh2-2-15) and one heterozygous (badh2-2-9) T1 mutant plants were investigated further (Figure 1d). All three homozygous mutations were faithfully transmitted, and the mutation in the heterozygous plant was transmitted in a Mendelian fashion (homozygote: heterozygote: wild type = 1 : 2 : 1) to the T2 generation (Table 2). Interestingly, some new indel mutations were detected in the T1 and T2 progeny of badh2-2. For example, whereas the mutation in badh2-2 was a 1-bp deletion, we identified additional 6-bp and 10-bp deletions in one of its offspring (badh2-2-8) (Table 2), and new indel mutation types were also identified in the progeny of line badh2-2-15. A detailed list of the new mutations detected and their sequences is given in Figure 1d and e. This suggests that the 1-bp deletion in the spacer region of badh2-2 did not prevent T-OsBADH2b from recognizing and further cleaving the target.

Generation of TALEN-free mutant rice lines

To obtain rice lines harbouring the desired *OsBADH2* mutations but not the selective marker or the TALEN construct, the PCR-based assay was used: primer pair F1/R1 to amplify the maize *Ubiquitin 1* promoter and N-terminus of TALEN-L, F2/R2 to amplify the C-terminus of TALEN-R and the *Nos* terminator and F3/R3 to amplify the hygromycin resistance gene (Figure 2a). A primer set amplifying the endogenous *BADH2* gene was used as an internal control in all three PCRs. Although all four T1 plants (badh2-2-6, badh2-2-8, badh2-2-9 and badh2-2-15) used for analysing mutation transmission from T1 to T2 carried TALEN constructs, the PCR assay failed to detect any T-DNA construct genes in 16 of 113 (14.2%) T1 plants derived from the 6 T0 lines and 7 of 96 (7.3%) T2 plants derived from the four T1 lines that contained the desired genetic modifications (Figure 2b, Tables 1

Table 1 TALEN-induced mutations in *OsBADH2* and their transmission to the T1 generation

			Mutation segregation in T1					
T0 plant ID	T0 genotype	T0 mutation type (bp)	Total	Homo	Hetero	WT	Transmission ratio (%)*	TALEN-free (%)†
badh2-1	Bb	−3, −6, −12/+1	10	0	5	5	50	0
badh2-2	Bb	−1	32	4	25	3	90.6	20.7 (6/29)
badh2-3	Bb	−6	5	0	2	3	40	0
badh2-4	Bb	−9	12	0	6	6	50	0
badh2-5	Bb	−10	14	0	4	10	28.6	0
badh2-6	Bb	−2, −6, −7	98	0	67	31	68.4	14.9 (10/67)

−n, nucleotide deletion of the indicated number; −n/+n, simultaneous nucleotide deletion and insertion of the indicated number at the same site.

*Transmission ratio was calculated based on the number of plants carrying the mutations over the total number of plants tested.

†TALEN-free ratio was calculated based on the number of mutant plants not harbouring the T-DNA construct over the total number of plants tested.

Table 2 Genetic analysis of mutations in *OsBADH2* and their transmission to the T2 generation

			Mutation segregation in T2					
T1 plant ID	T1 genotype	T1 mutation type (bp)	Total	Homo	Hetero	WT	Transmission ratio (%)*	TALEN-free (%)†
badh2-2-6	bb	−1	24	24	0	0	100	0
badh2-2-8	bb	−1, −6, −10	24	24	0	0	100	0
badh2-2-15	bb	−1	24	24	0	0	100	29.2 (7/24)
badh2-2-9	Bb	−1	24	5	13	6	75‡	0

‡Indicated that the segregation of the heterozygous lines (badh2-2-9) confirms to the Mendelian ratio (1 : 2 : 1) according to the χ^2 test ($P > 0.5$).

*Transmission ratio was calculated based on the number of plants carrying the mutations over the total number of plants tested.

†TALEN-free ratio was calculated based on the number of mutant plants not harbouring the T-DNA construct over the total number of plants tested.

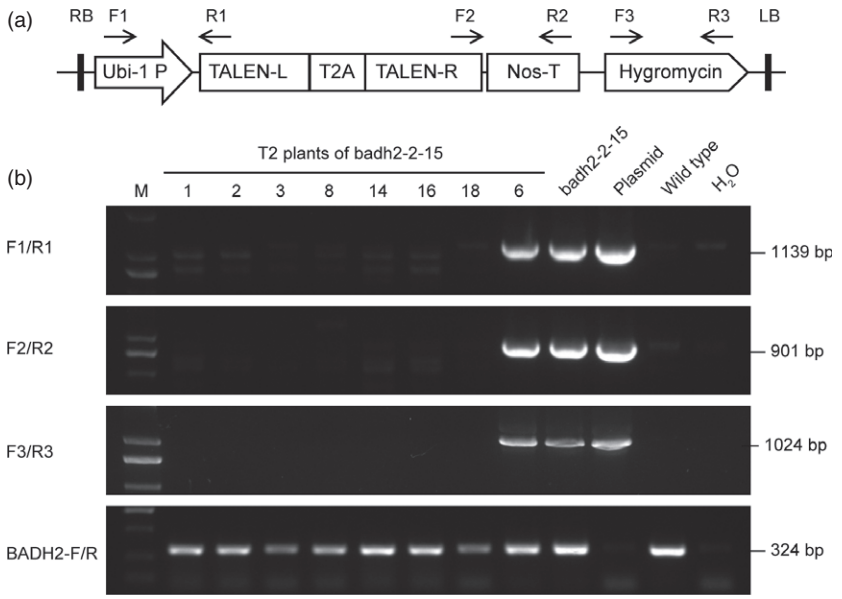

Figure 2 Segregation of the TALEN transgene in *badh2* mutants. (a) Schematic of the TALEN T-DNA construct showing the position of the three pairs of PCR primers used to survey different region of the TALEN transgene in the progeny of *badh2* mutants. F1/R1, for the *Ubiquitin 1* promoter and N-terminus of TALEN-L; F2/R2, for the C-terminus of TALEN-R and the *Nos* terminator; F3/R3, for the hygromycin resistance gene. (b) Gel images of the PCR products obtained with the three pairs of PCR primers and primers for the *OsBADH2* gene as an internal control in each reaction. M, DNA molecular weight marker. Numbers above the gel image refer to representative individual T2 plants of badh2-2-15. badh2-2-15, T1 plants; Plasmid, T-DNA expression plasmid containing T-OsBADH2b; Wild type, DNA from a nontransgenic wild-type rice plant; H_2O, negative control without any DNA. Numbers to the right of each gel indicate the sizes of amplicons.

and 2), suggesting that the T-DNA construct had been eliminated from these plants by segregation as a single genetic locus. This indicates that TALEN-free rice plant with the desired mutations can be relatively easily obtained by segregation among the progeny.

2AP content of mutant rice grains

Rice grains (T2 generation) gathered from three homozygous T1 lines (badh2-2-6, badh2-2-8 and badh2-2-15) and 1 heterozygous T1 line (badh2-2-9) were assayed for 2AP content by gas chromatography–mass spectrometry (GC-MS). It should be noted that the T2 grains of three homozygous T1 lines were all homozygote and the T2 grain of heterozygous T1 line was a mixture of homozygote, heterozygote and wild type. Grains of fragrant rice cultivar Daohuaxiang with the *badh2-E7* genotype were used as a positive control, and the nonfragrant rice cultivar Nipponbare served as a negative control (Figure 1d). 2, 4, 6- trimethyl pyridine (TMP) was used as internal standard because of its similar molecular weight and chemical characteristics to 2AP in GC-MS. As shown in Figure 3a–f, a 2AP peak was detected from all the homozygous, heterozygous and positive control lines, but none from the negative control line. After normalizing 2AP content based on the TMP yields, the grains from 3 homozygous lines had as high or slightly higher 2AP levels than the positive control Daohuaxiang (0.5–0.75 mg/kg versus 0.5 mg/kg); in the grains from the heterozygous line, the 2AP level was lower than in the positive control (0.35 versus 0.5 mg/kg). However, 2AP level was clearly produced in all the homozygous and heterozygous mutant lines created by TALENs (Figure 3g).

Multiplex gene knockout by bombardment with three pairs of TALENs

The development of multiplex genome engineering, in which several genes are altered simultaneously, will facilitate the generation of multiple agronomically important traits and enable the manipulation of complex traits. In diploid rice, a trait is often

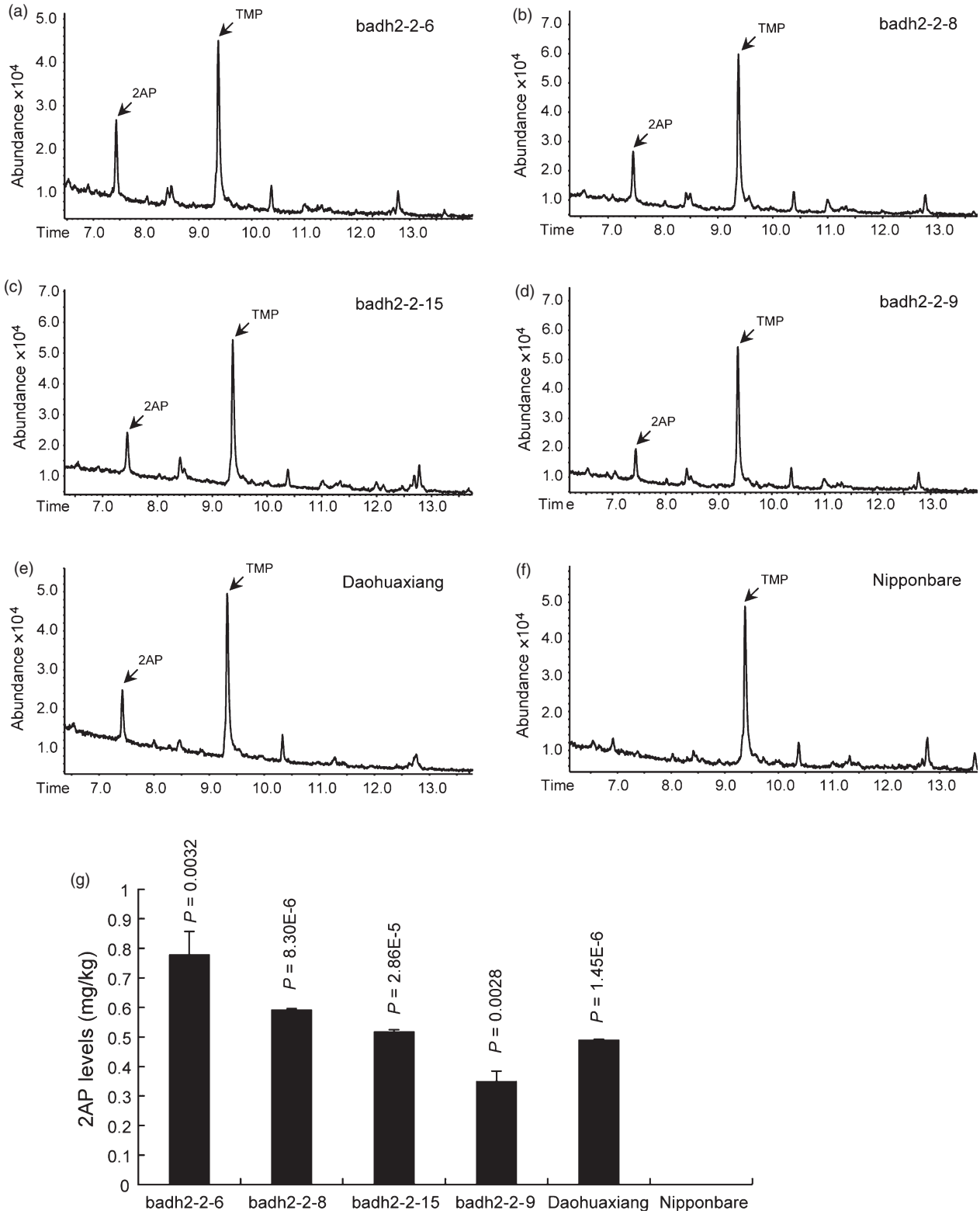

Figure 3 2AP contents of *badh2* mutant grains as measured by GC-MS. (a-f) Total ion chromatograms (TIC) of 2AP and TMP (as internal standard) in the TALEN-induced *badh2* mutant lines and control lines: (a) Grains of the homozygous T1 line, badh2-2-6. (b) Grains of the homozygous T1 line, badh2-2-8. (c) Grains of the homozygous T1 line, badh2-2-15. (d) Grains of the heterozygous T1 line, badh2-2-9. (e) Grains of the positive control, cv. Daohuaxiang. (f) Grains of the negative control, cv. Nipponbare. (g) 2AP levels of the *badh2* and control lines. Values are means ± SD of three replications. A Student's *t*-test was applied to generate *P*-values.

controlled by several genes, and a single gene mutation may not produce phenotypic change. To examine whether TALENs can disrupt multiple target genes, three TALEN pairs targeting *OsBADH2*, *OsCKX2* and *OsDEP1*, respectively, were introduced into rice calli by bombardment (Figures 1b and 4a). Mutation of *OsCKX2* and *OsDEP1* had been reported to increase the yield of rice grain (Ashikari *et al.*, 2005; Huang *et al.*, 2009). A total of 207 transgenic rice lines were regenerated from hygromycin-tolerant calli after 3-month selection, and PCR/RE assays were used to detect indel mutations. Among these transgenic lines were 20 (9.7%) *OsBADH2* mutants, 53 (25.6%) *OsCKX2* mutants and 19 (9.2%) *OsDEP1* mutants. Most were heterozygotes (Table 3). We examined all the mutants and found 4 plants (4/207, 1.9%) (C10, E5, F4, F9) containing mutations of all three target genes (Figure 4b). Sequencing of the PCR products spanning the TALEN target sites revealed that all these triply mutated rice plants contained site-specific indel mutations in the gene coding regions, and plants F4 and F9 were homozygous for *OsCKX2* and *OsDEP1* (Figure 4c). In addition to the triple-gene mutation, we also found a series of single and double mutants at various frequencies (Table 4, Figure S2). Previous studies have reported a low frequency of chromosome translocations between pairs of targeted DSBs on different mammalian chromosomes (Brunet *et al.*, 2009; Piganeau *et al.*, 2013). However, we did not detect any chromosome translocation among all the double and triple mutants (data not shown). These results demonstrate that simultaneous multiple gene mutations can be generated in rice by co-transformation of multipairs of TALENs.

Discussion

Genome editing using SSNs provides good opportunities for crop improvement. To date, SSNs have been used to create gene knockout plants in a variety of important crops, such as rice, maize, wheat, barley and soya bean (Haun *et al.*, 2014; Li *et al.*, 2012; Liang *et al.*, 2014; Shukla *et al.*, 2009; Wang *et al.*, 2014; Wendt *et al.*, 2013). However, examples of real improvement of agronomic traits or the creation of excellent novel genotypes using SSNs are very limited. One of the first examples was the use of ZFNs to target the maize *IPK1* gene, which encodes an enzyme that catalyses the final step in phytate biosynthesis (Shukla *et al.*, 2009). Reducing the level of phytate in seeds is of value because phytate is an antinutritional component and also contributes to environment pollution. In rice, TALENs were used to create a mutation in the pathogen TAL effector binding site in the promoter of *OsSWEET14*, which contributes to pathogen survival and virulence, thereby eliminating the transcription of this gene and reducing the pathogen's virulence (Li *et al.*, 2012). Another good example occurred in bread wheat. By knocking out all six alleles encoding the MILDEW-RESISTANCE LOCUS (MLO) protein with one pair of TALENs, the authors generated a mutant line with broad-spectrum resistance to powdery mildew, a devastating fungal disease (Wang *et al.*, 2014). Haun *et al.* (2014) recently created soya bean lines that are low in polyunsaturated fats by introducing mutations in two fatty acid desaturase 2 genes (FAD2-1A and FAD2-1B). Here, we have provided another example of the use of SSN to disrupt biochemical pathways and create plants that accumulate valuable biosynthetic intermediates. We used TALEN technology to knockout *OsBADH2* and obtain homozygous mutants with significantly increased content of the fragrant chemical 2AP; the level produced is similar to or even higher than in the fragrant rice cultivar Daohuaxiang. This method provides rice breeders with a new way to breed fragrant rice. Precise molecular breeding using SSNs is superior to conventional or transgene-based breeding methods such as RNAi or genetic engineering. For example, (1) it can modify a target gene accurately; (2) there is no need for laborious crossing and backcrossing, so it is time-saving and convenient; and (3) 'clean' plants (with the selective marker and SSN transgene segregated away from the genome) can be obtained.

The mutations in the six T0 plants induced by TALENs were transmitted to the T1 generation, but not in a Mendelian ratio. For lines badh2-2, 2-3, 2-4 and 2-5, this may have been because of the small numbers of T1 seeds. In lines badh2-1 or 2-6, we detected multiple indel mutations at a single target site, and such chimeric mutations in the T0 plants may have resulted from delayed cleavage in the primary embryogenic cell. These mutations occurred in somatic cells that did not participate in the production of gametes. A similar phenomenon has been reported in rice, wheat, corn and *Arabidopsis* (Feng *et al.*, 2013, 2014; Liang *et al.*, 2014; Wang *et al.*, 2014).

It is noteworthy that sequencing revealed some new indel mutations among the T1 and T2 offspring of badh2-2. As only one nucleotide were deleted in the middle of the 18-bp spacer in T0 badh2-2, the deletion did not destroy either the left or the right TALEN binding sequence; hence, the FokI nuclease may still have been able to form active dimers and cleave the 17-bp spacer. The new indel could then have resulted from continued TALEN cleavage of the target. We identified several mutant lines that no longer contained the TALEN construct; these were generated in the progeny by segregation. No new indel mutations were detected in such progeny lines, for example badh2-2-15-1, 2-2-15-2, 2-2-15-3 and 2-2-15-8 (Figure 2). This suggests that it is important to segregate the TALEN transgene from the plant genome to stabilize the induced mutations as well as to satisfy biosafety concerns. Note that 'clean' fragrant lines of this kind differ from the wild type by only one nucleotide base pair.

Interestingly, we observed differences in the accumulation of 2AP in the grains of different homozygous T1 mutant lines derived from the same T0 plant, badh2-2: the 2AP content of badh2-2-6 was significantly higher than that of badh2-2-8 and badh2-2-15 (Figure 3g). The reason for this is currently unclear; however, it underlines the importance of selecting the mutant lines with the highest 2AP content for breeding programme. At the same time, the grains from the heterozygous line badh2-2-9 contained about half the 2AP content of homozygous badh2-2-6.

Table 3 Multiple gene knockouts in rice using TALENs, showing the frequencies of mutations in each of the three genes targeted

	Mutations in *OsBADH2* (%)			Mutations in *OsCKX2* (%)			Mutations in *OsDEP1* (%)		
No. of tested plants	Total	Homo	Hetero	Total	Homo	Hetero	Total	Homo	Hetero
207	20 (9.7)	2 (1.0)	18 (8.7)	53 (25.6)	19 (9.2)	34 (16.4)	19 (9.2)	6 (2.9)	13 (6.3)

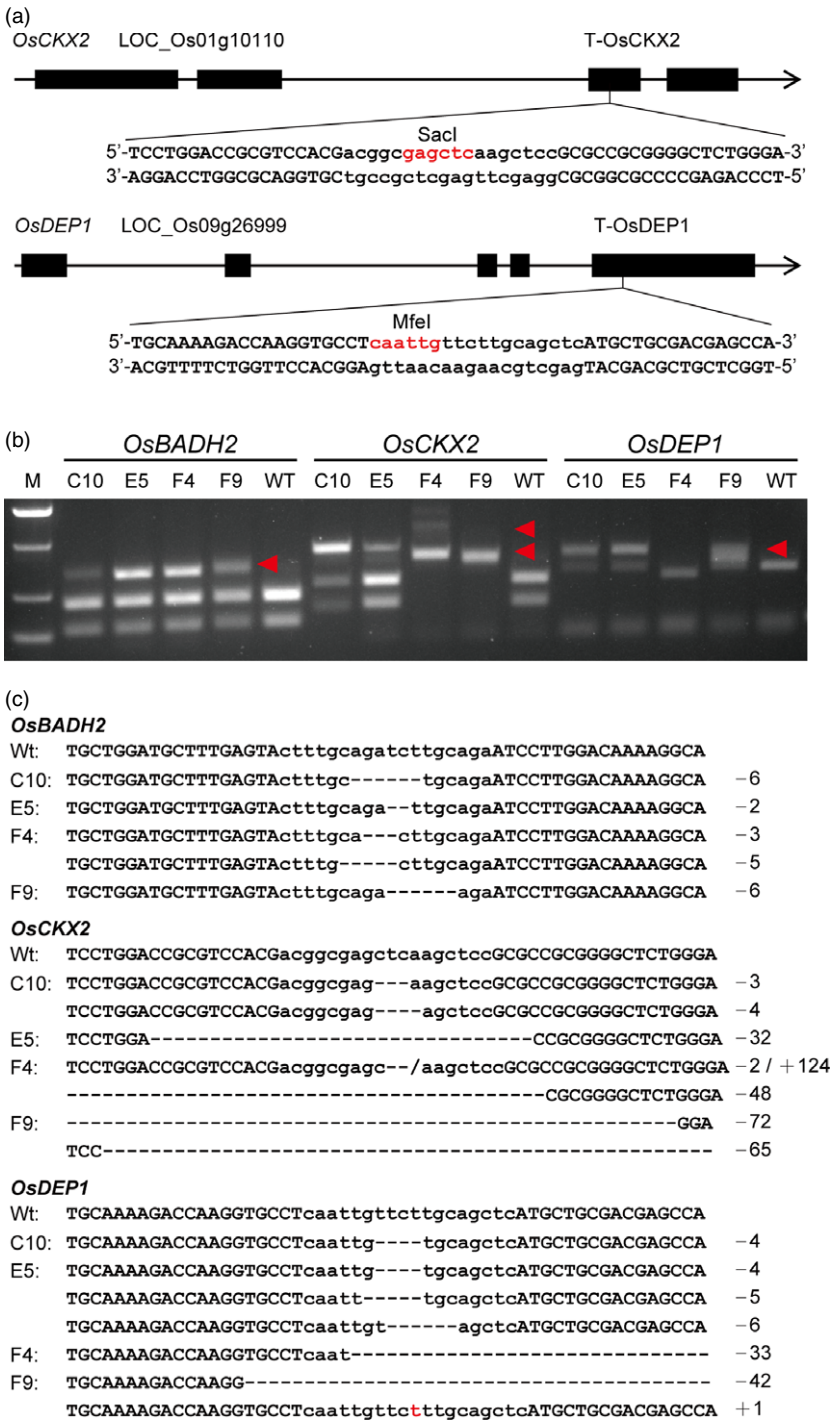

Figure 4 Multiple gene knockouts by co-transformation of three TALEN pairs. (a) Schematic of the *OsCKX2* and *OsDEP1* genes and the corresponding TALEN binding sites. *OsCKX2* contains 4 exons, and *OsDEP1*, 5 exons, indicated by black rectangles. The target sites of T-OsCKX2 and T-OsDEP1 are shown beneath, with the TALEN binding sites in upper case and spacer in lower case; the SacI and MfeI sites are highlighted in red. (b) Detection by PCR/RE assay of NHEJ-introduced indel mutations in four T0 plants mutated in all three genes (C10, E5, F4, F9). Mutations in independent plants were identified with gene-specific primers, and the PCR amplicons were digested with BglII, SacI and MfeI, respectively. Red arrowheads indicate mutant bands. (c) Sequencing of the TALEN-induced mutant alleles in each of the triple-gene mutated rice plants. Deletions and insertions are indicated by dashes and the '/' symbol, respectively, and the numbers on the right side show the sizes of the indels.

The grains of badh2-2-9 were in fact a mixture of homozygous mutant, heterozygote and wild type, and the reduced 2AP content was due to dilution of the homozygous mutant by the wild type and heterozygote.

TALENs are reported to have higher target specificity than ZFNs or the CRISPR/Cas9 system, as their binding sequences are longer (typically each TALEN monomer recognizes 15–20 bp) (Carroll, 2014). Although, almost all the SSNs available today can

Table 4 Multiple gene knockouts in rice using TALENs, showing the frequencies of mutations for all the combinations of single, double and triple genes targeted

	Single (%)			Double (%)			Triple (%)
No. of tested plants	*BADH2*	*CKX2*	*DEP1*	*BADH2/CKX2*	*BADH2/DEP1*	*CKX2/DEP1*	*BADH2/CKX2/DEP1*
207	6 (2.9)	30 (14.5)	2 (1.0)	8 (3.9)	2 (1.0)	11 (5.3)	4 (1.9)

accommodate one to several mutations within their target site, TALENs can only accommodate a relatively small number of position-dependent mismatches (Juillerat *et al.*, 2014). A study in human cells demonstrated that off-target effects were extremely rare even if there was only one nucleotide mismatch (Mussolino *et al.*, 2011). We identified several potential off-target sites for T-OsBADH2b using the PROGNOS program (Fine *et al.*, 2013) with less stringent criteria that allowed up to a 6-bp mismatch and 10- to 30-bp spacers (Table S1). We then examined three of the most likely off-target sites with 9-bp (OffT-1), 11-bp (OffT-5) and 10-bp (OffT-17) mismatches in their recognition sequences in the six T0 plants and found none. This suggests that this T-OsBADH2b TALEN pair induces mutations site specifically. It also suggests that it is worth using online tools to predict off-target TALEN sites.

Multiplex gene knockout is important for analysing polyploid organisms (such as bread wheat and *Brassica napus*) and diploid organisms with duplicated genomes (such as soya bean). In such cases, four or six alleles may need to be targeted simultaneously in order to introduce a gene function or phenotype change. One strategy for multiplex targeted mutagenesis using sequence-specific nucleases is to design a pair of SSNs targeting the conserved region in different genomes or duplicated genes and to select for organisms with all the targeted copies mutated (Li *et al.*, 2013a). Using this strategy, our group successfully disrupted the wheat *MLO* gene using a pair of TALENs and obtained a *mlo-aabbdd* mutant with high levels of resistance to powdery mildew (Wang *et al.*, 2014). Alternatively, if the corresponding genes in the different genomes have low homology, or one wishes to target unrelated genes, two or more SSN pairs targeting different gene-specific regions can be designed and introduced together into cells, followed by selection for lines with mutations in all the relevant genes. This strategy has been applied in human cells and model animals such as mice, rat, zebrafish, *Bombyx mori* and *Xenopus laevis* (Cong *et al.*, 2013; Jao *et al.*, 2013; Li *et al.*, 2013b, 2014; Ma *et al.*, 2014; Ota *et al.*, 2014; Sakane *et al.*, 2013; Sakuma *et al.*, 2014; Wang *et al.*, 2013). Only two of these examples used the TALEN technique (Li *et al.*, 2014; Sakane *et al.*, 2013), while the others employed the CRISPR/Cas9 system. We have described above the first successful example of multiplex gene knockout in crop plants; it involved co-transforming three pairs of TALENs targeting, respectively, the genes *OsBADH2*, *OsCKX2* and *OsDEP1* related to rice quality and yield. Although the CRISPR/Cas9 system is straightforward and effective for multiplex genome editing because an sgRNA of only 100-nt is required to guide Cas9 nuclease to the target sites, our results suggest that TALEN technology can also be used efficiently for multiple gene disruption in crop plants.

Experimental procedures

Plasmid construction

TALEN-coding plasmids were constructed as previously described (Shan *et al.*, 2013). Three pairs of TALENs were employed in this work: pGW3-T-OsBADH2b, pGW3-T-OsCKX2 and pGW3-T-OsDEP1 targeting *OsBADH2*, *OsCKX2* and *OsDEP1* gene, respectively. The T-DNA construct consisted of a TALEN expression cassette driven by the maize *Ubiquitin 1* promoter and a hygromycin selection marker gene driven by the 35S promoter. Pairs of TALEN monomers were linked by a T2A translational skipping sequence to form a complete open reading frame.

Agrobacterium-mediated rice transformation

The TALEN-encoding T-DNA binary vector (pGW3-T-OsBADH2b) was transformed into *Agrobacterium tumefaciens* strain AGL1 by electroporation. *Agrobacterium*-mediated transformation of embryogenic calli derived from rice cultivars Nipponbare and LPK was conducted according to Hiei *et al.* (1994). Hygromycin-containing medium were used for selection and regeneration of transgenic plants. After 3–4 months of cultivation, transgenic seedlings could be transferred to a paddy field during the rice-growing season or to a greenhouse (16-h light at 30 °C/8-h dark at 22 °C).

Biolistic rice transformation for multiplex gene knockout

Particle bombardments were performed using a PDS1000/He particle bombardment system (Bio-Rad, Hercules, CA, USA) with a target distance of 6.0 cm from the staying plate at helium pressure 1100 psi. Plasmid DNAs were mixed at 1 : 1 : 1 (pGW3-T-OsBADH2b: pGW3-T-OsCKX2: pGW3-T-OsDEP1) molar ratios prior to bombardment. One-month-old embryogenic calli (60–80 pieces) of rice cultivar Nipponbare were bombarded using a previously reported protocol (Li *et al.*, 1993). Hygromycin-containing medium was used for selection and regeneration of transgenic plants.

Gas chromatography–mass spectrometry (GC-MS) determination of 2AP content

The basic protocols have been described previously (Chen *et al.*, 2012). Dehulled rice grains (0.5 g) were placed in a 2-mL centrifuge tube and milled thoroughly in a high-speed shaker. The rice flour was transferred to a 5-mL jaw bottle to which was added 2 mL of extraction buffer (a 1 : 1 (v:v) solution of anhydrous ethanol and methylene chloride containing 0.5 mg/L of 2, 4, 6-trimethyl pyridine as an internal standard). The bottle was sealed and extracted at 80 °C for 3 h. It was then cooled to room temperature and centrifuged at 13 800 g for 5 min. The supernatant was pipetted into a sample bottle and 2AP measured with the GC-MS device (GC7890A-5975C MS; Agilent Technologies, Santa Clara, CA, USA). The initial temperature of the DB-5 MS capillary column (30 m × 0.25 mm × 0.25 μm) (J&W) was set to 50 °C; after 2 min at 50 °C, the temperature was first increased to 120 °C at a rate of 5 °C/min and then to 280 °C at a rate of 15 °C/min and maintained for 3 min. The mass spectrometer was operated in the electron impact (EI) mode with an ionization voltage of 70 eV and an ion

source temperature of 230 °C. 2AP content was calculated from the equation:

$$C2 = (A2 \times C1 \times V \times 0.001)/(A1 \times W),$$

where C2 is 2AP content (mg/kg); C1, TMP concentration (mg/L); V, volume of sample injected; A1, peak area of TMP; and A2, peak area of 2AP.

PCR/RE assays and PCR-based genotyping of the T-DNA construct

Rice genomic DNA from approximately 0.5–1.0 g of leaf tissue was extracted with a DNA Quick Plant System (Tiangen, Beijing, China). PCR amplification was performed using EASY Taq polymerase (TransGen Biotech, Beijing, China) and 50 ng of genomic DNA. PCR amplicons were then digested by the appropriate restriction enzymes to screen the mutated plants. Undigested bands or PCR products were cloned into the TA cloning vector pUC-T (CWBIO, Beijing, China), and about 10 positive colonies were sequenced. PCR primers for the PCR/RE assay and for testing the presence of the T-DNA construct are listed in Table S2.

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Conflict of interest statement

The authors declare competing financial interests: the authors' patent related to this work has been granted (Chinese patent number ZL 2012 1 0548714.7).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Detection of NHEJ-introduced *badh2* indel mutations in 10 heterozygous T0 transgenic plants.

Figure S2 PCR/RE assay to detect indel mutations in representative 82 T0 plants mutated in three genes.

Table S1 Potential off-target site of T-OsBADH2b identified with the PROGNOS program.

Table S2 PCR primers used in this research.