Plant Molecular Biology (2019) 101:355–371
https://doi.org/10.1007/s11103-019-00907-w

Application of Cas12a and nCas9-activation-induced cytidine deaminase for genome editing and as a non-sexual strategy to generate homozygous/multiplex edited plants in the allotetraploid genome of tobacco

Chen-Tran Hsu[1] · Yu-Jung Cheng[1] · Yu-Hsua Yuan[1] · Wei-Fon Hung[1] · Qiao-Wei Cheng[1] · Fu-Hui Wu[1] · Lan-Ying Lee[2] · Stanton B. Gelvin[2] · Choun-Sea Lin[1]

Received: 22 February 2019 / Accepted: 2 August 2019 / Published online: 10 August 2019

Key message **Protoplasts can be used for genome editing using several different CRISPR systems, either separately or simultaneously, and that the resulting mutations can be recovered in regenerated non-chimaeric plants.**

Abstract Protoplast transfection and regeneration systems are useful platforms for CRISPR/Cas mutagenesis and genome editing. In this study, we demonstrate the use of Cpf1 (Cas12a) and nCas9-activation-induced cytidine deaminase (nCas9-Target-AID) systems to mutagenize *Nicotiana tabacum* protoplasts and to regenerate plants harboring the resulting mutations. We analyzed 20 progeny plants of Cas12a-mediated *phytoene desaturase* (*PDS*) mutagenized regenerants, as well as regenerants from wild-type protoplasts, and confirmed that their genotypes were inherited in a Mendelian manner. We used a Cas9 nickase (nCas9)-cytidine deaminase to conduct C to T editing of the *Ethylene receptor 1* (*ETR1*) gene in tobacco protoplasts and obtained edited regenerates. It is difficult to obtain homozygous edits of polyploid genomes when the editing efficiency is low. A second round of mutagenesis of partially edited regenerants (a two-step transfection protocol) allowed us to derive *ETR1* fully edited regenerants without the need for sexual reproduction. We applied three different Cas systems (SaCas9, Cas12a, and nCas9-Traget AID) using either a one-step or a two-step transfection platform to obtain triply mutated and/or edited tobacco regenerants. Our results indicate that these three Cas systems can function simultaneously within a single cell.

Keywords CRISPR/Cas · Chimeric plants · Cas12a · Cytidine deaminase · Polyploid genome editing

Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) are DNA sequences that play a key role in the immune system of prokaryotic organisms. The CRISPR associated endonuclease (Cas) can be guided by CRISPR RNA (crRNA) to specific DNA sequences (the protospacer) and can cleave DNA at or near these sites, generating double strand breaks (DSB). The repair of these DSB can result in mutations. This system has been widely used in many organisms, including plants (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013). Currently, several nucleases from different bacterial species are used for genome editing, including those from *Streptococcus pyogenes* (SpCas9; Jinek et al. 2012), *Staphylococcus aureus* (SaCas9; Ran et al. 2015), and *Francisella novicida* (FnCpf1: FnCas12a; Zetsche et al. 2015). Each nuclease recognizes its specific protospacer adjacent motif (PAM) sequence and thus provides various choices for the cleavage sites. Mutation of one or both nuclease domains of the Cas9 protein generates a Cas nickase (nCas) or a catalytically inactive (dead) Cas9 (dCas9), respectively (Qi et al. 2013). These modified Cas proteins can be further linked with the catalytic domain of other enzymes, such as a methyltransferase (Vojta et al. 2016) or cytidine deaminase (Komor et al. 2016; Nishida et al. 2016), to function in DNA methylation or base editing, respectively.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11103-019-00907-w) contains supplementary material, which is available to authorized users.

✉ Choun-Sea Lin
cslin99@gate.sinica.edu.tw

[1] Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan

[2] Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA

Genetic crosses between two lines in order to incorporate elite alleles is often used in crop breeding. However, it is difficult to combine two loci of interest which are closely linked. A targeted multiplex genome editing/mutation method may offer a solution to speed up the breeding process. Some successes have been reported by using multiple sgRNAs combined with Cas9 for genome editing (Xing et al. 2014; Xie et al. 2015; Ma et al. 2015). Although a variety of Cas proteins provides more choices of PAM sequences (Kleinstiver et al. 2015; Hu et al. 2018; Zhong et al. 2018), each PAM requires its specific Cas protein to function. Therefore, there is a need for multiplex nuclease tools for simultaneous mutation and/or editing of multiple loci.

Protoplast transfection is widely used in plant research (Marx 2016). Co-transfection of multiple plasmids and expression of multiple proteins in an individual protoplast are easily achieved (Lee et al. 2008, 2012; Lin et al. 2018). Protoplasts have also been transfected with CRISPR/Cas9 genome editing reagents to test the efficiencies of sgRNAs and to regenerate mutated/edited plants (Woo et al. 2015; Andersson et al. 2017, 2018; Lin et al. 2018; Zong et al. 2018). It should also be possible to use a protoplast transfection and regeneration strategy with multiple different Cas nucleases to edit multiple genes at the same time. In this study, we used Cas12a to target the *phytoene desaturase* (*PDS*) gene because mutation of all copies of this gene results in plants that exhibit an albino phenotype (Li et al. 2013; Shan et al. 2013; Nekrasov et al. 2013), a visible marker for our CRISPR experiments. We also used a nCas9-activation-induced cytidine deaminase (nCas9-Target-AID) to target the tobacco *Ethylene receptor 1* (*ETR1*) gene, a redundant negative regulator of ethylene signal transduction (O'Malley et al. 2005). We subsequently regenerated the mutated and/or edited plants. In order to mutate/edit three specific tobacco DNA regions, we applied various combinations of Cas12a, SaCas9, and nCas9-Target-AID in a single protoplast transfection. When the efficiency of mutagenesis and editing was low, we conducted a two-step transfection process using sequential transfection of protoplasts from a previously mutated/edited plant. This strategy achieved mutation and/or editing of three different DNA regions in an allotetraploid plant within one reproductive generation.

Materials and methods

Plant materials

Plantlets of tobacco (*Nicotiana tabacum* c.v. Wisconsin 38), potato (*Solanum tuberosum* cv. Kennebec), and wild tomato (*Lycopersicon peruvianum*) were grown in 1/2 MS medium (Murashige and Skoog, 1962) containing 30 g/L sucrose and 7 g/L agar (HI-AGAR, Hispanagar, Spain), pH 5.7. Plants were grown in a growth chamber at 26 °C with a light regime of (12 h light/12 h dark). Shoots were subcultured every month.

Protoplast isolation, transfection, and regeneration

Protocols for protoplast isolation, transfection, and regeneration were the same as in Lin et al. (2018) with some minor modifications as follows: To make protoplasts, leaves of in vitro grown explants were incubated in digestion solution (1/4 MS medium supplemented with 0.4 M mannitol, 1.0% cellulase R10, 0.5% macerozyme R10, and 3% sucrose, pH 5.7) overnight. Protoplasts were collected by centrifugation at 360×*g*, washed with W5 medium (Medgyesy et al. 1980), then resuspended in 1/2 MS medium supplemented with 0.4 M mannitol, 3% sucrose, pH 5.7. A total of 10^5 protoplasts were transfected with 20 µg plasmid DNA using a polyethylene glycol (PEG) method (Lin et al. 2018). The plasmid pE3170 expressing mRFP-NLS was co-transfected to evaluate the transfection efficiency (Lee et al. 2008).

For regeneration, the transfected protoplasts were washed in W5 solution and resuspended in 2 mL liquid callus medium (1/2 MS medium supplemented with 0.4 M mannitol, 30 g/L sucrose, 1 mg/L NAA, and 0.3 mg/L kinetin, pH 5.7), and incubated in a 5 cm diameter petri dish without shaking in the dark. After 2–3 weeks, the minicalli from proliferating protoplasts were subcultured in three plates containing 10 ml fresh liquid shooting medium (1/2 MS medium supplemented with 0.1 mg/L Thidiazorun, 0.4 M mannitol, and 30 g/L sucrose, pH 5.7) for shoot induction. The calli were incubated in light/dark cycling conditions (light/dark: 16/8 h). After 3–4 weeks, 100–500 shoot clusters with leaves were transferred to solidified rooting medium (1/2 MS medium supplemented and 30 g/L sucrose, pH 5.7) for rooting.

Plasmids

The plasmids used for transfection are described in Table 1. Three plasmids encoding three different Cas nucleases were used in this study: FnCas12a (Endo et al. 2016), SaCas9 (Kaya et al. 2016), and nCas9-Target-AID (Shimatani et al. 2017). The plasmids cr*NtPDS-1* and cr*NtPDS-2* contained full-length *Arabidopsis* codon-optimized *FnCas12a* driven by a PcUbi promotor and a crRNA driven by the AtU6 promotor. In addition, cr*NtPDS-1* encodes a crRNA Targeting 1 site and cr*NtPDS-2* encodes a crRNA Targeting 2 site. The target site sequences are TCATCCAGTCCTTAACACTTA AAC for cr*NtPDS-1* and ACATGGCAATGAACACCTCAT CTG for cr*NtPDS-2*. Plasmid gPDS_Sa encodes full-length *SaCas9* fused with 3XFLAG and 3XNLS tags driven by a CaMV 35S promotor, and an sgRNA driven by the AtU6 promotor. The target site sequence of gPDS_Sa is TTG

Table 1 The three Cas plasmids and the crRNA target sites used in this study

Plasmid name	Cas promoter	Cas	crRNA promoter	Gene target	Target site	References
crNtPDS-1	PcUbi	FnCas12a	AtU6	*PDS*	TCATCCAGTCCTTAACACTTAAAC	Endo et al. (2016)
crNtPDS-2	PcUbi	FnCas12a	AtU6	*PDS*	ACATGGCAATGAACACCTCATCTG	Endo et al. (2016)
gPDS_Sa	CaMV 35S	SaCas9	AtU6	*PDS*	TTGCATGCCTAACAAGCCAG	Kaya et al. (2016)
ETR^{site3}	PcUbi	nCas9	AtU6	*ETR1*	TGCACAAGAACCCATCTATA	Shimatani et al. (2017)

CATGCCTAACAAGCCAG. Plasmid *ETR*^{site3} encodes an *Arabidopsis* codon-optimized nCas9, PmCDA1, a *2A* peptide and NPTII fusion protein driven by a PcUbi promoter, and an sgRNA driven by the AtU6 promotor. The target site sequence for the *ETR1* gene is TGCACAAGAACC CATCTATA (*ETR1*^{site3}; Shimatani et al. 2017; Addgene clone ID: 91695), which is identical to those of wild tomato (*L. peruvianum*; pSolyc12g011330.2.1; solgenomics.net), potato (*S. tuberosum*; XM_006349935), and tobacco (*N. tabacum*; XM_016651146) as confirmed by genomic PCR and Sanger sequencing. *E. coli* cells containing these plasmids were grown in LB medium with 50 mg/L kanamycin. Plasmid DNA was isolated using plasmid midi-preparation kits (NucleoBond Xtra Midi, Macherey–Nagel, Dueren, Germany).

Genotype analysis

Genomic DNA from pooled transfected protoplasts was extracted using a Mini GenoPlus Genomic DNA Extraction Kit (Viogene, New Taipei City, Taiwan). DNA from the regenerants was isolated using a urea method (Sheu et al. 1996). Two pairs of primers were designed to amplify the sgRNA-targeted DNA region for each target gene (Table S1). PCR conditions were 94 °C for 5 min, 35 cycles of denaturing (94 °C for 30 s), annealing (55–63 °C for 30 s; detailed information is shown in Table S1), and polymerization (72 °C for 30 s), followed by an extension reaction at 72 °C for 3 min. The PCR product was digested with the appropriate restriction endonuclease (*PDS* gPDS_Sa, *Bst*N1; *PDS* cr*NtPDS-2*, *Bsr*D1) or subjected to T7E1 analysis (*PDS*, cr*NtPDS-1* and *ETR*, *ETR*^{site3}) and analyzed by electrophoresis through a 2% agarose gel. An aliquot of mutagenized PCR products from pooled protoplast DNA was subcloned into a T/A cloning vector (T&A Cloning Kit, Yestern Biotech, Taiwan). PCR products from pooled T/A cloning colonies and from genomic DNA regenerated plants were digested with the appropriate restriction enzyme or subjected to T7E1 analysis and further analyzed by electrophoresis. Putative mutated PCR products were sequenced by the Sanger method. Multiple sequences were informatically separated using Poly Peak Parser (http://yosttools.genetics.utah.edu/PolyPeakParser/; Hill et al. 2014) or further confirmed by sequential T/A cloning and sequencing.

Results

Mutagenesis of the tobacco *PDS* gene using *Francisella novicida* Cas12a

Assessing targeted mutations in protoplasts

We used two plasmids (*crNtPDS-1* and *crNtPDS-2*; Endo et al. 2016), separately or in combination, to target the *PDS* gene in transfected tobacco protoplasts. Both plasmids encode *F. novicida* Cas12a (FnCas12a) but different crRNAs (with Target 1 site and Target 2 site, repectively). These crRNAs target sites exist in both the *N. sylvestris* (S form) and *N. tomentosiformis* (T form) subgenomes of the allotetraploid *N. tabacum* genome. Four days after transfection, DNA from pooled protoplasts was isolated and used as templates to perform PCR. The PCR products were cloned and sequenced. We identified mutations at the corresponding target sites at various frequencies using either *crNtPDS-1* or *crNtPDS-2* alone, or co-transfection with both plasmids (Tables 2, S2). *crNtPDS-1* effected a higher mutagenesis efficiency than did *crNtPDS-2* (18.6% vs. 14.3%, Tables 2, S2). When we used both *crNtPDS-1* and *crNtPDS-2* for co-transfection, both large deletions (between the cut sites targeted by the two crRNAs) and smaller deletions at the cut sites of either of the crRNAs were observed (Table S2). In the co-transfection treatment, the mutation efficiency at the Target 1 site was higher than that at the Target 2 site. Most mutations were deletions, with only one clone showing an insertion (and a 2 bp deletion) at Target 2 site (Table S2). Deletions most frequently occurred at the 17th bp after the PAM, as expected. When both crRNAs were used, 7 out of 10 mutations occurred as large deletions of various length between the two target sites. Sequence information for all of the mutants is shown in Table S2.

Analysis of tobacco plants regenerated from *crNtPDS-1* and *crNtPDS-2* transfected protoplasts

Cas12a mutated protoplasts regenerated either green or albino plants. Figure 1 shows the positions of deletions in green regenerants following mutagenesis by Cas12a treatment using the *crNtPDS-1* or *crNtPDS-2* crRNA. Most deletions were located 16–23 bps from the PAM. The mutation

Table 2 Analysis of the CRISPR/Cas12a-mutagenized *PDS* gene by T/A cloning

Genome[a]	No crRNA		*crNtPDS-1*		*crNtPDS-2*		*crNtPDS-1* and *crNtPDS-2*	
	Mutated/total clones	%	Mutated/total clones	%	Mutated/total clones	%	Mutated/total clones	%
S	0/24	0	5/19	26.3	2/22	9.1	7/21	33.3
T	0/24	0	3/24	12.5	4/20	8.0	5/20	25.5
Average	0/48[b]	0	8/43	18.6	6/42	14.3	12/41	29.3

Sequence information is shown in Table S2

[a]S, *N. sylvestris*; T, *N. tomentosiformis*. The transfected pooled protoplast DNA was used as the template for PCR and different forms of the *NtPDS* gene were amplified by the specific primers shown in Table S1

[b]The average was calculated as the sum of the mutated clones/total clones in the S and T forms

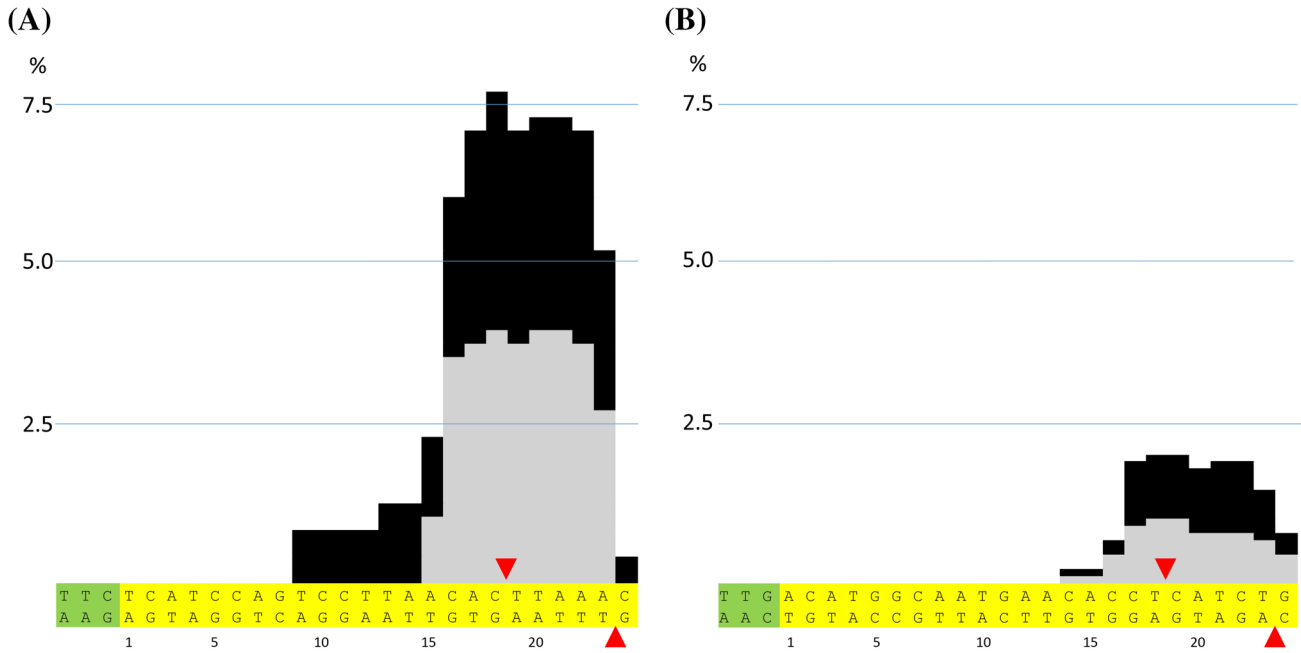


Fig. 1 Deletion positions in green plants regenerated from CRISPR/Cas12a-mutagenized tobacco protoplasts. Grey: mutagenesis of the S form gene. Black: mutagenesis of the T form gene. **a** *crNtPDS-1* plasmid transfection. **b** *crNtPDS-2* plasmid transfection. The sequence below the graph indicates the DNA sequence near the PAM (shown in green). Red triangles: Cas12a cleavage sites

efficiency of the S form gene was higher than that of the T form gene following transfection with the *crNtPDS-1* construct. In green regenerant plants, the *crNtPDS-1* construct effected a higher mutation efficiency than did the *crNtPDS-2* construct (18.3% vs. 7.5%; Table 3). Albino mutants were obtained (*crNtPDS-1*, 15; *crNtPDS-2*, 5; *crNtPDS-1* and *crNtPDS-2* co-transfected, 27) from all transfection treatments. Sequencing of PCR products revealed all four *PDS* alleles were mutated at the expected positions in these plants (Fig. 2; Table S3). In the *crNtPDS-1* and *crNtPDS-2* co-transfected albino plants, most of the plantlets contained mutations at Target 1 site (Table S3). Deletions indicated that both Target 1 and Target 2 sites were mutated (Fig. 2). There were also plants that contained mutations in both of the target sites but lacked a ~ 220 bp deletion between them (Fig. 2; Table S3). This result, lacking the ~ 220 bp deletion, suggests that the cleavage of these two targets did not occur at the same time but rather that one target site was cleaved first and ligated, then the other target site was cleaved and ligated. When cleavage of these two target sites occurred simultaneously, a ~ 220-bp deletion occurred between target sites 1 and 2.

Progeny of mutant regenerants from *crNtPDS-1* and *crNtPDS-2* transfected protoplasts

Plants regenerated from mutagenized protoplasts should be clonal, and the mutations should follow the rules of

Table 3 Analysis of mutations in green shoots regenerated from CRISPR/Cas12a-mutagenized tobacco protoplasts

Genome[a]	WT		*crNtPDS-1*		*crNtPDS-2*		*crNtPDS-1* and *crNtPDS-2*	
	Mutated/total regenerants	%	Mutated/total regenerants[b]	%	Mutated/total regenerants	%	Mutated/total regenerants	%
S	0/24	0	18/120	15.0	9/240	3.8	11/162	6.8
T	0/24	0	9/120	7.5	13/240	5.4	13/162	8.0
Total	0/24	0	22/120	18.3	18/240	7.5	18/162	11.1

Sequence information is shown in Table S3

[a]S, *N. sylvestris*; T, *N. tomentosiformis.* Transfected pooled protoplast DNA was used as the template for PCR and different forms of the *PDS* gene were amplified using specific primers listed in Table S1

[b]Mutated indicates that the regenerants contain at least one mutated allele of S form gene (S row) or the T form gene (T row). Total indicates that the regenerant contains at least one mutated allele in its genome

Mendelian inheritance. To verify this, we analyzed the DNA of albino and green regenerants by two different strategies. Because albino plants are sterile, we could not perform progeny analysis on them. We thus extracted genomic DNA directly from these plants and used it as a template for PCR amplification of the *PDS* genes, followed by cloning into a T/A cloning vector and Sanger sequencing.

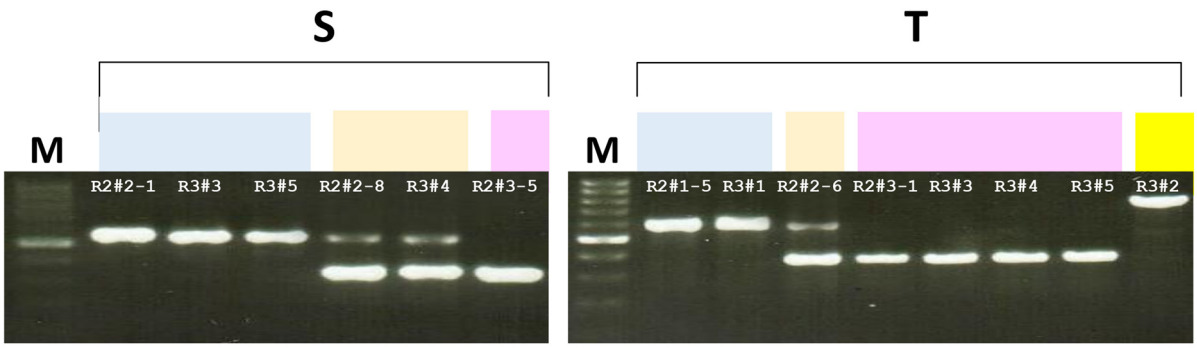
We obtained multiple different allelic combinations from the *crNtPDS-1* and *crNTPDS-2* co-transfected albino regenerants. One regenerant (T1+2_R2#3-5) had a homozygous mutant S form allele, whereas the other regenerants were biallelic mutants for the S form allele. Several regenerants (T1+2R2#3-1, T1+2R3#2, T1+2R3#3, T1+2R3#4, and T1+2R3#5) were homozygous for different mutant T form alleles, whereas other regenerants were biallelic for the mutant T form allele (Fig. 2). As expected for an albino regenerant, no wild-type alleles were found. Our analysis revealed two mutants containing more than two alleles of one of the subgenomes (T1+2R2#3-1 S form and T1+2R3#1 S form; Table S3). These results suggest that chimaeric plants were regenerated from multiple mutant protoplasts. However, the sequences of the *PDS* alleles of most albino regenerants indicated that the plants were clonal.

Because green mutant regenerants produced seeds, we were able to analyze the genomes of their progeny. From the progeny of 20 mutated regenerants, we obtained wild-type plants, plants with S form only mutations, plants with T form only mutations, and plants with mutations in both the S and T form genes. In all instances the mutant alleles segregated in a Mendelian fashion. No *PDS* mutations were observed in five wild-type progeny that were derived from different transfections (Fig. S1; Table S4, T1R1#42WT, T2R2#25WT, T2R2#26WT, T2R2#28WT, and T1+2R2#53WT). In mutant green regenerants derived using the *crNtPDS-1* guide only, mutations occurred in the S form gene but not in the T form gene (T1R1#13S, T1R1#48S, and T1R1#55S). Similarly, mutations occurred in the T form gene in green regenerant T1R1#69T. These mutated alleles could be inherited by the progeny of these plants (Fig. S1; Table S4). Similar mutation of either the S form or T form gene, but not both, also occurred in two other transfections (*crNtPDS-2*, *crNtPDS-1* and *crNTPDS-2* co-transfection). In regenerants with mutations in both subgenomes (S+T), the mutated alleles also were transferred to progeny in a Mendelian fashion (Fig. S1; Table S4).

Albino plants were identified amongst the progeny of these regenerants from different transfection treatments (T1R1#16S+T; T2R1#7S+T; T1+2R2#59S+T; Figs. 3, S1). For example, among the progeny of a plant containing the T2R1#7S+T genotype (derived from mutations at Target 2 site and containing mutated alleles of both the S and T form genomes), only the *SP, sp*, and *tp* alleles from the parent were found in the progeny (Fig. 3). Although *tp* is a three nucleotide deletion which caused a glutamic acid deletion of the 278th amino acid, this glutamic acid is conserved in the PLN02612 domain. This truncated protein is non-functional, and the *spsp tptp* progeny had an albino phenotype. Theoretically, 25% of the progeny should be albino, which was the result we obtained (Fig. 3). We analyzed all 30 seedlings in plate 1 (24 green and 6 albino regenerants). The theoretical ratio of (*SPSP tptp*):(*SPsp tptp*):(*spsp tptp*) alleles should be 1:2:1. Our result 6:18:6 fit this ratio ($x^2 = 0.779$; Fig. 3). We obtained similar results when analyzing the progeny of other mutagenizied and regenerated green plants (Fig. S1; Table S4).

Using nCas9-Target-AID to edit the genomes of potato, tobacco, and wild tomato protoplasts

The plasmid *nCas9*^{At}-*PmCDA1*^{At}-2A expresses the crRNA that targets the *ETR1* target sequence (*ETR1*^{site3}: TGCACA AGAACCCATCTATA), which is identical in wild tomato (*L. peruvianum*), potato (*S. tuberosum*), and tobacco (*N. tabacum*). We used this plasmid to edit the *ETR1* gene in protoplasts of these three species. Following transfection, the *ETR1* sequence was amplified from genomic DNA from



Sample No.	Genome Type	Mutation Type	Target site 2	Indel	Target site 1	Indel	Clone
Wild-type	S	None	TTGACATGGCAATGAACACCTCATCTG	0	TTCTCATCCAGTCCTTAACACTTAAAC	0	
T1+2R2#2-1	S	D	TTGACATGGCAATGAACAC---ATCTG	-3	TTCTCATCCAGTCCTTAA--------C	-8	12/24
			TTGACATGGCAATGAACACCTCATCTG	0	TTCTCATCCAGTCCTTA---------C	-9	12/24
T1+2R3#3	S	D	TTGACATGGCAATGAACAC---ATCTG	-3	TTCTCATCCAGTC----------C	-13	11/16
			TTGACATGGCAATGAACACCTCATCTG	0	TTCTCATCCAGTCCTTAAC-------C	-7	5/16
T1+2R3#5	S	D	TTGACATGGCAATGAACACCT-----G	-5	TTCTCATCCAGTCCTTA---------C	-9	9/14
			TTGACATGGCAATGAACAC---(-227)-----(+207)----------C	-20			5/14
T1+2R2#2-8	S	D	TTGACATGGCAATGAACAC-------G	-7	TTCTCATCCAGTCCTTAA--------C	-8	11/24
			TTGACATGGCAATGAACACC----------(-222)----------C	-222			13/24
T1+2R3#4	S	D	TTGACATGGCAATGA--------(-14)	-14	TTCTCATCCAGTCCTTAA-------AC	-7	7/12
			TTGACATGGCAATGAACACCT----------(-217)----------TAAAC	-217			5/12
T1+2R2#3-5	S	D	TTGACATGGCAATGAACA----------(-224)----------C	-224			16/16

Sample No.	Genome Type	Mutation Type	Target site 2	Indel	Target site 1	Indel	Clone
Wild-type	T	None	TTGACATGGCAATGAACACCTCATCTG	0	TTCTCATCCAGTCCTTAACACTTAAAC	0	
T1+2R2#1-5	T	D	TTGACATGGCAATGAACACCTCATCTG	0	TTCTCATCCAGTCCTTAA-------AC	-7	10/21
			TTGACATGGCAATGAACACCTCATCTG	0	TTCTCATCCAGTCCTTA--ACTTAAAC	-2	11/21
T1+2R3#1	T	D	TTGACATGGCAATGAACA-----(-16)	-16	TTCTCATCCAGTCCTTAAC----(-10)	-10	6/15
			TTGACATGGCAATGAACACC--ATCTG	-2	TTCTCATCCAGTCCTTAAC----(-10)	-10	9/15
T1+2R2#2-6	T	D	TTGACATGGCAATGAACAC------TG	-6	TTCTCATCCAGTCCTTAA--------C	-8	9/23
			TTGACATGGCAATGAACAC----------(-222)----------C	-222			14/23
T1+2R2#3-1	T	D	TTGACATGGCAATGAACACC----------(-220)----------AC	-220			24/24
T1+2R3#3	T	D	TTGACATGGCAATGAA----------(-225)----------C	-225			21/21
T1+2R3#4	T	D	TTGACATGGCAATGAACA----------(-222)----------AC	-222			24/24
T1+2R3#5	T	D	TTGACATGGCAATGAACACCTCA----------(-217)----------AC	-217			24/24
T1+2R3#2	T	D+I	----------(-255)----------(+434)	+179			17/17

pooled transfected protoplasts. T7E1 digestion of PCR products suggested that in all three species genome editing was successful (Fig. 4).

PCR products containing the mutagenized region of the ETR1 gene were cloned into a T/A cloning vector and sequenced. DNA sequence analysis showed that in tobacco

◂**Fig. 2** Analysis of regenerated albino shoots from CRISPR/Cas12a *crNtPDS-1* and *crNtPDS-2* co-transfected tobacco protoplasts. In the gel images above each table, each lane presents the *PDS* PCR products from the designated regenerated albino plant. Primers used to amplify the S form *PDS* gene: forward, AGCCAATATGTCAGTCGATC; reverse, ACAGAGTGAAAAAGTTCAGAAA. Primers used to amplify the T form *PDS* gene: forward, TTGGGGCTTACCCAAATATGC; reverse, TTCTCCTGCAAATTGATAATTC (Table S1). Additional sequence information is shown in Table S3. *S* S form of the *PDS* gene, *T* T form of the *PDS* gene, *D* deletion, *I* insertion, *M* marker. Background lanes in blue: Only one major band in the PCR product that is similar to the wild-type amplicon size. Background lanes in orange: two major bands in the PCR product. Background lanes in pink: Only one major band in the PCR product with a size similar to that of a deletion formed by Target 1 site and Target 2 site cleavage. Background in yellow: Unexpected size PCR product. The underlined letters indicate the PAM sequence

protoplast experiments most of the editing was C to T transitions between the third and fifth nucleotides from the 5′ end of the target site (C_3 and C_5, Fig. 4a, b). In total, 35.4% (17/48) showed deletions and 12.5% (6/48) showed cytosine editing. The occurrence of deletions was surprising but has been reported before when using this Cas9-cytidine deaminase enzyme (Shimatani et al. 2017). The editing efficiency of the S form allele was 8.3% (2/24), whereas that of the T form allele was 16.7% (4/24). Although most edited cytosines became T, we observed one example of C to G editing. The edited positions were similar in potato and wild tomato (C_3 and C_5). However, both lower editing and mutation frequencies occurred in potato and wild tomato protoplasts compared to those of tobacco protoplasts (Fig. 4c, d).

Transfected tobacco protoplasts were cultured and regenerated into plantlets. The *ETR1* genes from 118 regenerants transfected by nCas9-Target-AID were analyzed by PCR and Sanger sequencing (Fig. 5; Table S5). A total of 15.3% of the regenerants contained insertion or deletion mutations, whereas 16.9% contained C to T or C to G editing changes. The editing efficiency of the S form was 8.4%, and that of the T form was 6.8%. Two regenerants contained edited alleles in both the S and T form genomes (Fig. 5; Table S5). The positions with the highest editing efficiency were C_3 and C_5, which had similar efficiencies. There were few edited events occurring further upstream of the target sequences (C_{-1}, C_{-5}, and C_{-6}) or nearer to the PAM sequence (C_{11} and C_{12}, Fig. 5).

To determine if the regenerated plants derived from a single cell, we isolated total DNA from three different single base edited or mutated regenerants and used them as templates for PCR of the *ETR1* gene. Sequence analysis of the PCR products revealed that all regenerants contained biallelic mutations, indicating that these regenerants were derived from single protoplasts (Table S6).

From these experiments we edited two C residues (C_3 and C_5). However, we did not know if these two edited bases occurred in the same chromosome. We therefore cloned the region of DNA encoding these two positions: the edited DNA from the S form only (R2_#1, R2_#41), the T form only (R2_#11, R2_#18), and double edited regenerants (R2_#8) (Fig. 6). Sequencing these clones indicated that the two editing events were in the same allele and that the other allele was wild-type (Fig. 6).

Progeny analysis of edited and/or mutated tobacco *ETR1* alleles

We analyzed the *ETR1* gene in the progeny of edited regenerants (Figs. 7, S2; Table S7). The 71st amino acid of the wild-type ETR1 protein is valine (GTG, complementary strand: C_3AC_5). In R2_#8, the SE^I (S form Edited to Isoleucine) and TE^I (T form Edited to Isoleucine), in which the 71st amino acid was edited to isoleucine (ATA, the complementary strand: T_3AT_5), the edited allele could be found in the progeny (Fig. 7). The editing at different positions and mutations also could be found in their respective progeny. The R2_#34 plant contained edited alleles in both the S form gene (SE^L, S form Edited to Leucine; TTA, complementary strand: T_3AA_5), and in the T form gene [*te**, T form Edited to stop codon (*)] by editing of cytosines C_{12} and C_{13}; TAA, complementary strand: $T_{12}T_{13}A$), and mutated alleles in both the S and T form genes [sm^{+10} (S form mutation, 10 bp insertion) and tm^{-19} (T form mutation, 19 bp deletion)]. All four of these alleles were found in progeny plants. These results indicate that the alleles edited and mutated by nCas9-Target-AID could be inherited. This phenomenon also was observed in R3_#9, a deletion mutation which created an out-of-frame mutation (sm^{-5}, S form mutation, 5 bp deletion; Fig. S2).

However, we did not detect mutations in the progeny of the edited regenerant TE^I (R3#20; Fig. S2) which also contained two other deletions [sm^{-14} (S form mutated, 14 bp deletion) and SM^{-18} (S form mutated, 18 bp deletion; Fig. S2). Gametes with these alleles could not produce viable seeds. Pollen from R3_#9 were used to fertilize the R3_#20. The sm^{-5} allele, but not the TE^I allele, was found in progeny plants (Fig. S2).

Two-step transfection and homozygous edited alleles in tobacco

Although homozygous ($SE^ISE^ITE^ITE^I$) edited tobacco plants could be obtained by crossing heterozygous parents, crossing would be an issue for those crops that are propagated by vegetative methods. In Figs. 2, 6, and in Tables S2 and S6, we demonstrate that the regenerants are derived from a single Cas12a-mediated mutated protoplast, and that cells from the regenerants have the same genetic background. However, protoplasts from these regenerants could

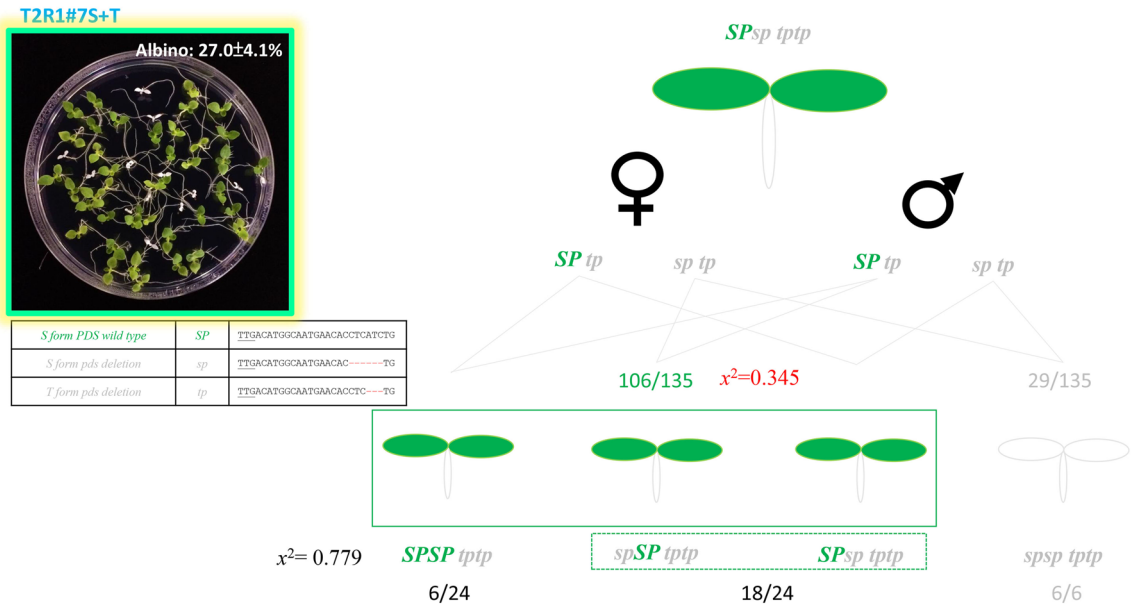


Fig. 3 Progeny analysis of CRISPR/Cas12a-mutagenized tobacco containing the T2R1_#7S+T genome. Seeds were sown on plates containing ½ MS medium and photographed. The number 27.0 ± 4.1% indicates the percentage of albino mutant seedlings. The table shows sequence information on different alleles of the parental plant. A total of 24 green and six albino (*) progeny were analyzed. The numerator indicates the progeny of this genotype. Figures summarizing the remaining regenerants are shown in Fig. S1, and sequence information of progeny plants is shown in Table S4. The results of a Chi square analysis (x^2) are shown for the phenotype (red) and genotype (black). ± standard deviation

provide a homozygous mutant genomic background for further genome editing. To obtain $SE^{I}SE^{I}TE^{I}TE^{I}$ tobacco plants without sexual propagation, we further edited protoplasts from the heterozygous edited regenerant R2_#8 (both S and T form alleles were edited and are heterozygous). We transfected protoplasts from these heterozygous plants with the plasmid *nCas9*^{At}-*PmCDA1*^{At}-*2A* and analyzed 24 non-transfected R2_#8 protoplast regenerants (#8#N1 to #8#N24); all of these were heterozygous for the S and T form *ETR1* genes, as was their progenitor plant R2_#8 (Table S8). This result indicates that protoplast regeneration itself does not induce detectable levels of target site gene editing or mutation. We sequenced 93 *nCas9*^{At}-*PmCDA1*^{At}-*2A* transfected regenerants (Fig. 8; Table S8). Ten regenerants (10.8%) had changes at the target site. Of these, six had edited cytosines. These regenerants could be divided into two classes: four alleles edited or three alleles edited. Among the regenerants with three edited alleles, only the S (#8#8 and #8#24) or the T (#8#36) form allele was homozygous; the other allele was heterozygous. Among the regenerants with four edited alleles, there were two genotypes. One genotype was biallelic for the T form allele (C_5 to G and C_5 to T editing) and the S form allele was homozygous (#8#75 and #8#95). The other genotype regenerant (#8#61) contained all four C_5 bases changed to a T (Fig. 8).

Multiplex Cas proteins

We simultaneously transfected tobacco protoplasts with several different Cas nuclease/crRNA plasmid combinations (Cas12a, nCas9-Target-AID, and SaCas9) that target different genes (SaCas9 and Cas12a target the *PDS* gene; nCas9-Target-AID targets the *ETR1* gene). PCR products of DNA extracted from pooled protoplasts after transfection indicated the expected edited and/or mutated DNA sequences (Fig. S3). These results suggest that all three editing reagents can simultaneously function during protoplast co-transfection. We regenerated 130 plants from this triple plasmid co-transfection; among these, 121 were green and nine were albino plants. PCR amplicons from 24 green and nine albino regenerants were sequenced (Table S9). The editing and mutation efficiencies of each nuclease in the green regenerants were 4.2% for Cas12a, 8.4% for nCas9-Target-AID, and 33.3% for SaCas9. There was one SaCas9 and Cas12a double mutant (Triple green-19) and two SaCas9 + nCas9-Target-AID mutated/editing mutant (Triple green-8 and Triple green-20) obtained among these green regenerants (Table S9). Sequence analysis from nine albino regenerants revealed two triple mutations (Triple albino-2 and Triple albino-4) and one triple edited/mutation (Triple albino-9, Table S9). These results suggest that, although the efficiency was low, three different CRISPR-Cas systems could simultaneously

function in the same protoplast, and that we could regenerate triply edited and/or mutated plants from these protoplasts.

To increase the number of triply edited and/or mutated regenerants, we transfected protoplasts of the nCas9-Target-AID edited regenerant R2_#8 with plasmids encoding SaCas9 and Cas12a, and their cognate crRNAs. Within pooled protoplast DNA, we detected a truncated form of the *PDS* gene. This result indicates that SaCas9 and Cas12a cleavage of the same gene at their respective target sites can generate a deletion. We cloned and sequenced the PCR products of the *PDS* gene; 12.2% (6/49) of the products contained mutations at both the SaCas9 and the Cas12a target sites. Among 507 regenerants, 401 (79.1%) were green and 106 (20.9%) were albino. Among the green mutants, 56.1% (23/41) were mutated by SaCas9 only, none were mutated by Cas12a only, and 17.1% (7/41) were mutated by both SaCas9 and Cas12a (Table S10). Among the albino mutants, 50% were mutated by SaCas9 only, none were mutated by Cas12a-only, and 50% were mutated by both SaCas9 and Cas12a. These results indicate that 24.1% of the 507 regenerated plants were triply edited and/or mutated.

Discussion

We report that plant protoplasts can be used for genome editing using several different CRISPR systems, either separately or simultaneously, and that the resulting mutations can be recovered in regenerated non-chimaeric plants. These regenerants segregate the mutant alleles to progeny plants in a Mendelian fashion. Thus, it is possible to generate heterozygous, homozygous, or biallelic mutations simultaneously in several genes of both monoploid and polyploid species without the need for sexual propagation. The lack of need for sexual reproduction proved important for recovery of plants harboring certain homozygous *ETR1* mutations because such mutations caused gamete disfunction. In addition, the simultaneous use of two or three different genome editing systems (Cas9, Cas12a, and nCas9-target-AID) by protoplast co-transfection of plasmids encoding these reagents obviates the need for construction of large, complex T-DNAs for *Agrobacterium*-mediated transformation. Our results indicate that protoplasts can be a useful platform for the rapid generation of homozgous or biallelic mutations in genes of those species that can be regenerated from protoplasts.

Use of Cas12a for mutagenesis

Most reported Cas12a plant mutagenesis protocols used LbCas12a from *Lachnospiraceae bacterium* (Zhong et al. 2018; Tang et al. 2017; Hu et al. 2017; Begemann et al. 2017; Wang et al. 2017; Xu et al. 2017; Kim et al. 2017). However, Cas12a from *Francisella novicida* (FnCas12a) can also function in both monocot and dicot plants (Zhong et al. 2018; Wang et al. 2017; Endo et al. 2016), although LbCas12a was reported to have a higher mutation efficiency than either FnCas12a (Zhong et al. 2018) or Cas12a from *Acidaminococcus* sp. (AsCas12a; Kim et al. 2017). We used FnCas12a because its PAM sequence is simpler than that of LpCas12a (TTN vs. TTTV). An alternative Cas12a has even less PAM limitation (Gao et al. 2017), and was previously used to introduce mutations into rice genes (Zhong et al. 2018).

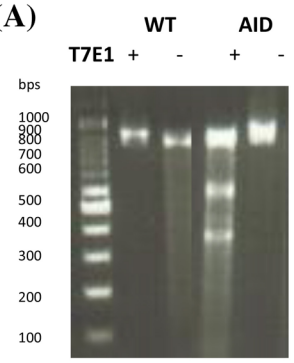
In addition to the type of Cas12a enzyme used for mutagenesis, the form of the corresponding crRNA also influences mutagenesis efficiency. In rice, an initial low Cas12a mutagenesis efficiency could be increased by altering the presentation of the pre-crRNA molecule (Tang et al. 2017; Xu et al. 2017). In this study, we demonstrated that the mutation frequency can be as high as 18.3% using a mature crRNA construct.

We have previously shown that SaCas9 can induce mutations in the tobacco *PDS* genes (Lin et al. 2018), and that we could regenerate albino mutant plants from mutagenized tobacco protoplasts. However, it is difficult to compare the mutagenesis efficiency of SaCas9 with that of FnCas12a because their target sites differ. Endo et al. (2016) were able to generate chimaeric but not albino tobacco *PDS* mutants using this same FnCas12a construct and *Agrobacterium*-mediated transformation. Similarly, Xu et al. (2017) could generate heterozygous or chimaeric, but not homozygous, rice mutants using an *Agrobacterium*–delivered *LbCas12a* gene. These results suggest that Cas12a can only induce DNA cleavage after the first division of the embryogenic cell and might therefore require a longer period to function than does Cas9 in vivo (Xu et al. 2017). However, Tang et al. (2017) reported that by using a double ribozyme system for precise processing of mature crRNAs, most LbCas12a-induced rice mutant plants were non-mosaic. Our results indicate that in protoplasts, transient expression of mature crRNA and FnCas12a can cause cleavage of the DNA before cell division, resulting in non-chimaeric *PDS* albino mutant plants lacking any green sectors. Similar results were shown for the albino *PDS* mutants derived from SaCas9 transfection (Lin et al. 2018).

Use of nCas9-Target-AID for genome editing

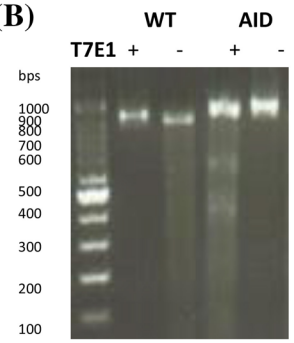
Because "knock-in" alterations of gene sequences by homology-dependent repair (HDR) is currently inefficient in plants, we used the alternative method of base editing of C to T using cytidine deaminase. Such an approach has previously been used in plants to change single nucleotides (Zong et al. 2017; Li et al. 2017; Lu and Zhu 2017; Ren et al. 2017; Shimatani et al. 2017). In plants, three cytidine

(A)



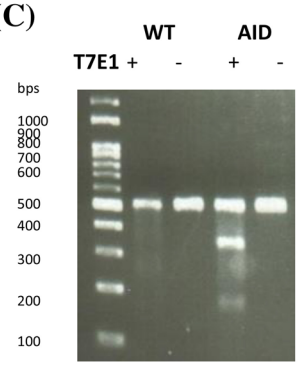
Sample	Genotype Type	*ETR1* AID target sequences	Number of clones
Tobacco S	WT	CTGCACAAGAACCCATCTATATGG	14
	E	CTGTATAAGAACCCATCTATATGG	1
		CTGTAGAAGAACCCATCTATATGG	1
	M	CTGCACAAGAACCCAT-----TATGG	1
		CTGCAC----------CTATATGG	1
		CTGCACA----------TATGG	3
		----------TGG	2
		CTG----------(+110)--------ATATGG	1

(B)



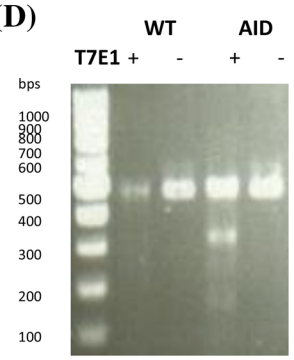
Sample	Genotype Type	*ETR1* AID target sequences	Number of clones
Tobacco T	WT	CTGCACAAGAACCCATCTATATGG	11
	E	CTGTATAAGAACCCATCTATATGG	3
		CTGTACAAGAACCCATCTATATGG	1
	M	CTGTAT--AGAACCCATCTATATGG	1
		-------ACAAGAACCCATCTATATGG	3
		CT----------G	2
		CTGCAC----------CTATATGG	1
		CTGC----------ATATGG	2

(C)



Sample	Genotype Type	*ETR1* AID target sequences	Number of clones
Tomato	WT	CTGCACAAGAACCCATCTATATGG	17
	E	CTGTATAAGAACCCATCTATATGG	7

(D)



Sample	Genotype Type	*ETR1* AID target sequences	Number of clones
Potato	WT	CTGCACAAGAACCCATCTATATGG	20
	E	CTGTATAAGAACCCATCTATATGG	1
		CTGTACA----------TATGG	1
	M	CTGCACA----------TCTATATGG	1
		CTGCACA----------TATATGG	1

◂**Fig. 4** T/A cloning *ETR1* sequences of tobacco [**a** S form; **b** T form], wild tomato (**c**) and potato (**d**) transfected protoplasts. T7E1 digestion patterns are shown in the gel photograph on the left of each panel. + T7E1 digestion, − undigested. The sequence of various T/A clones is shown on the right of each panel. Red colors indicate the edited base or mutation. Black, target site; black underline, PAM sequence; blue, sequences upstream of the target site; E, edited base; M, mutation; - in red, deleted base(s); WT, wild-type sequence. Numbers inside brackets, number of bases deleted or inserted

deaminase-based platforms have been developed: Target-AID in tomato and rice (Shimatani et al. 2017), hAID in rice (Ren et al. 2018), and Apolipoprotein B mRNA editing enzyme catalytic polypeptide 1 (APOBEC1) in *Arabidopsis* and rice (Zong et al. 2017, 2018; Li et al. 2017; Lu and Zhu 2017; Ren et al. 2017). In a rice study, 32.5% of the transformants contained a GC to GT edit in the 7th base from the target site in the *OsCDC48* gene (Zong et al. 2017). However, in all other studies either no (*OsNRT1.1B* gene; Lu and Zhu 2017; *BRT1* gene; Ren et al. 2017) or very low efficiency editing (Zong et al. 2017) was observed. In this report, we demonstrate that nCas9-Target-AID can work well in wild tomato, potato, and tobacco protoplasts, and that genome-edited tobacco protoplasts can be regenerated.

APOBEC1 and Target-AID enzymes were reported to generate G or A substitutions (Komor et al. 2016; Nishida et al. 2016), and we also observed this phenomenon (Figs. 5, 8). Using *Agrobacterium*-mediated transformation, Shimatani et al. (2017) showed that T1 generation rice and tomato transformants contained C to G edits 8–14% of the time, depending on the distance of the C residue from the PAM site. Our results indicated that 3.4% (4/118) of the C_5 residues in regenerants were edited to G and 0.8% (1/118) of the C_5 residues in regenerants were edited to A (Fig. 5). On the other hand, multiple C residues were often edited simultaneously. Therefore, it will be important to design sgRNAs carefully in order to edit specific C residues within a sequence containing multiple closely-spaced C residues.

Somewhat surprisingly, nCas9-Target-AID transfected protoplasts and the derived regenerants contained indel mutations. Such mutagenesis by a nCas9-based enzyme has previously been seen by others using an *Agrobacterium*-mediated transformation platform (Shimatani et al. 2017). These results suggest that the generation of indel mutations by a Cas9 nickase is not affected by the DNA delivery method. Such "off-editing" can be a disadvantage for genome manipulation in other systems using different cytidine deaminases versions (Komor et al. 2016). A new base editing system using the bacteriophage Mu protein Gam was developed by Komor et al. (2017). This Mu protein can bind DNA double strand breaks to reduce the frequency of indel formation. This base editing platform needs to be further tested in plants.

Genome editing using multiple CRISPR proteins

Most plant CRISPR mutagenesis studies have used *Agrobacterium*-mediated transformation to deliver genome editing reagents, such as Cas nucleases and single-guide RNAs (sgRNA), to plant cells. Because of the size of the genes encoding the various Cas endonucleases, the T-DNAs of these binary vectors, including the selection marker and sgRNA genes, are ~15 kbp. Although it is possible to infect plants with multiple *Agrobacterium* strains each containing a T-DNA with different genome editing reagents, one cannot guarantee that all reagents would be delivered to the same cell. Thus, the size of the T-DNA region would expand rapidly if additional genome editing reagents are added to a single T-DNA. Whereas recent advances have been made in assembling multiple genetic units within T-DNAs (e.g., Collier et al. 2018), such T-DNAs remain difficult to generate for the average laboratory. Co-transfection of protoplasts with multiple plasmids, each encoding a different genome editing reagent, is an attractive alternative method to *Agrobacterium*-mediated transformation, especially for those species that can readily be regenerated from protoplasts. Such an approach also provides flexibility in employing multiple different Cas nucleases simultaneously. Protoplast transfection and transient transgene expression may be especially useful for genome editing of those crops that are propagated by vegetative methods when the integration of transgenes encoding the genome editing reagents needs to be avoided for regulatory reasons.

In a previous study, we reported that expression of SpCas9 and two sgRNAs could generate deletions (Lin et al. 2018). In this report, we show that two different nucleases (SaCas9 and FnCas12a) and their cognate sg/crRNAs can also generate deletions in the tobacco genome, and that these deletions can be maintained in regenerated plants and their progeny. Because different Cas nucleases use different PAM sequences, this multiple nuclease strategy may provide more choice for suitable target sites. In addition, we show that we can also effect C to T editing, in addition to Cas9 and/or Cas12a mutagenesis, in protoplasts and their resulting regenerated plants and progeny.

We suggest two strategies to achieve triple editing and/or mutagenesis of a genome: (1) Simultaneously transfect all three sets of genome editing reagents into protoplasts, or (2) transfect the first reagent into protoplasts, regenerate plants, then transfect the second and third reagents into protoplasts of the resulting mutant plants. There are advantages and disadvantages to each of these approaches. It is obviously faster to transfect all three Cas nucleases simultaneously and attempt to regenerate plants that are triply edited with one cycle of transfection and regeneration. However, the efficiency of generating mutations at each target site is low, and it may be costly and labor-consuming to screen regenerants

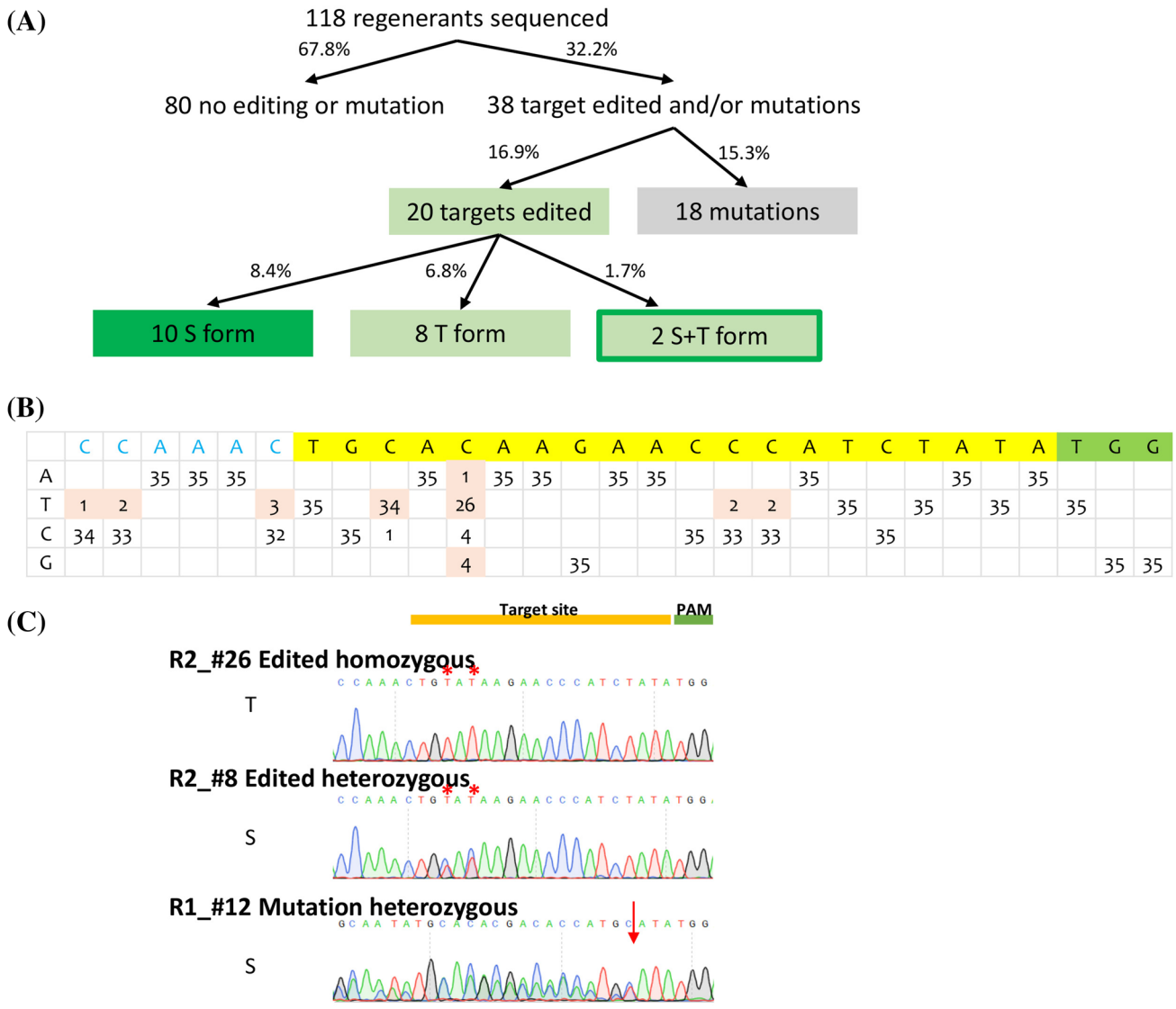


Fig. 5 Analysis of plants regenerated from nCas9-Target-AID transfected tobacco protoplasts. **a** Summary of the genotypes. Targets edited, no mutation; mutation, plants containing at least one mutated allele. **b** Edited chromosome sequences from target edited or mutated regenerants were analyzed. Letters with yellow background, target site; green background, PAM sequence; numbers in pink, the number of edited chromosomes; letters in blue, sequence 5′ upstream of the target site. **c** Sequencing traces of different editing types. *Edited in the predicted region; arrow, cleavage site. Complete sequence information can be found in Table S5

to find the rare triply mutated plant. Using two (or multiple) rounds of transfection and regeneration generally results in fewer regenerants to analyze at each step. However, the disadvantage of this strategy is the time required for regeneration. If this multi-step approach were used, we suggest that mutation of any gene that may alter the tissue culture behavior of the plants or cells be done last. For example, we tried to co-transfect SaCas9-generated albino mutants with Cas12a and nCas9-Target-AID. However, we could isolate only a few protoplasts from the albino plants, and these could not be regenerated (data not shown). Although a two-step transformation regime using *Agrobacterium*-mediated transformation could also achieve this goal, it often resulted in chimaeric plants (Endo et al. 2016; Shimatani et al. 2017). Because plants regenerated from individual protoplasts are generally not chimaeric, mutagenesis of protoplasts may more rapidly result in homozygous or biallelic mutations.

To obtain triply edited and/or mutated regenerants, optimization of the protocols is very important. For example, it is important to optimize the amount and concentration of DNA for transfection, the molar ratio of each plasmid used, the number of constructs transfected, etc. Alternatively, one can use CRISPR RNPs during transfection. These issues require further investigation.

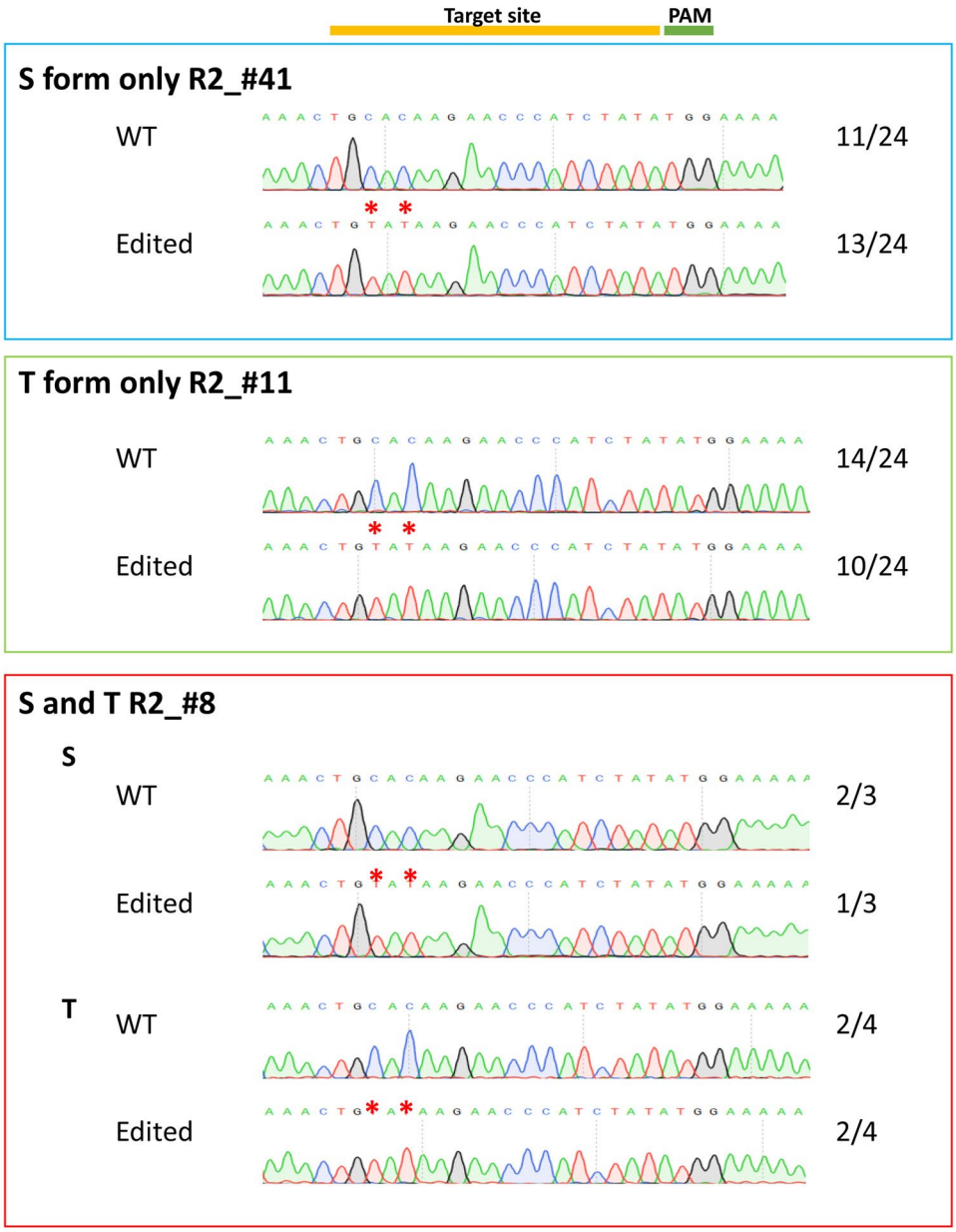


Fig. 6 Analysis of sequences from T/A clones of Target-AID transfected tobacco regenerants. The *ETR1* amplicons from different genome types (S form only; T form only; S and T form edited) were cloned and sequenced. The numbers shown at the right are the number of clones with this sequence/total number of sequenced clones. *W* wild-type, *DE* double edited in C_3 and C_5. *Edited in the predicted region

	S only				T only				S+T	
	R2_#1*		R2_#41		R2_#11		R2_#18		R2_#8	
	W	DE	W	DE	W	DE	W	DE	W	DE
S	11	13	3	7	-	-	-	-	2	1
T	-	-	-	-	14	10	8	5	2	2

Chimeric and edited/mutated allele transmission

Previous reports, using *Agrobacterium*-mediated transformation and Cas12a or nCas9-Target-AID, indicated that a high percentage of regenerants were chimaeric (Endo et al. 2016; Shimatani et al. 2017). It is likely that the edited or mutated allele was detected in somatic cells but not present in germline cells, and therefore could not be transmitted to progeny. In this report we demonstrated that, for those Cas12a and nCas9-Target-AID edited regenerants that could

(A)

ETR1_R2_#8

(B)

S form ETR1 wild type	SE	TGCACAAGAACCCATCTATATGG
S form ETR1 edited	SE^I	TGTATAAGAACCCATCTATATGG
T form ETR1 wild type	TE	CACCAAACTGCACAAGAACCCATCTATATGG
T form ETR1 edited	TE^I	CACTAAACTGTATAAGAACCCATCTATATGG

(C)

♀

♂	SETE	$SETE^I$	SE^ITE	SE^ITE^I
SETE	SESE TETE	SESE $TETE^I$	$SESE^I$ TETE	$SESE^I$ $TETE^I$
$SETE^I$	SESE $TETE^I$	SESE TE^ITE^I	$SESE^I$ $TETE^I$	$SESE^I$ TE^ITE^I
SE^ITE	$SESE^I$ TETE	$SESE^I$ $TETE^I$	SE^ISE^I TETE	SE^ISE^I $TETE^I$
SE^ITE^I	$SESE^I$ $TETE^I$	$SESE^I$ TE^ITE^I	sese $TETE^I$	SE^ISE^I TE^ITE^I

(D)

S	SE^ISE^I	SESE	SESE	$SESE^I$	$SESE^I$	$SESE^I$	SE^ISE^I	SE^ISE^I	SE^ISE^I
T	TE^ITE^I	TE^ITE^I	$TETE^I$	TETE	$TETE^I$	TE^ITE^I	$TETE^I$	TETE	TE^ITE^I
	3	1	2	2	6	2	5	1	2

Fig. 7 Progeny analysis of Target-AID-mutagenized/edited tobacco plant R2#34. **a** Seeds were sown on plates containing ½ MS medium and photographed. **b** The table shows sequence information for different *ETR1* alleles of the parental plant. (SE^L, S form Edited to Leucine; TTA, complementary strand: T_3AA_5), and in the T form gene [*te**, T form Edited to stop codon (*)] by editing of cytosines C_{12} and C_{13}; TAA, complementary strand: $T_{12}T_{13}A$), and mutated alleles in both the S and T form genes [sm^{+10} (S form mutation, 10 bp insertion) and tm^{-19} (T form mutation, 19 bp deletion)]; -, deletion. Numbers inside brackets, number of bases deleted or inserted. Letters in red, edited bases. **c** Gamete genotypes (x axis, female; y axis, male) and gene combinations of the progeny. **d** A total of 24 progeny plants were analyzed. The numerator indicates the number of progeny plants of this genotype. A summary of the other regenerants is shown in Fig. S2 and DNA sequence information of these progeny plants is shown in Table S5

produce viable gametes, these mutations were transmitted to the next generation. We obtained some unexpected results with nCas9-Target-AID edited/mutated *ETR1* alleles: A particular V to I amino acid change in the T form genome could not be sexually transmitted to progeny. However, Shimatani et al. (2017) showed that this same amino acid change could be sexually transmitted in tomato. Therefore, some edited *ETR1* alleles can be transmitted to the next generation. Aberrant transmission may result for the following reasons: First, ETR1 is related to reproductive growth (Hall and Bleecker 2003; Qu et al. 2007); therefore, the edited/mutated alleles may not be transmitted to progeny. However, this theory cannot explain why this phenomenon only occurred for some alleles. Second, *ETR1* is related to stress response (Cao et al. 2007). Different *Arabidopsis etr* mutant lines have different responses under stress. After treatment with 100 mM NaCl, *etr-1* showed growth reduction, but there was no growth difference among *etr-6*, *etr-8*, and wild-type plants (Cao et al. 2007). Plants harboring specific edited/mutated alleles, which make them more sensitive to stress, may not be able to transmit these alleles to progeny. Third, mutations in genes other than *ETR1* may have occurred during protoplast regeneration (Fossi et al. 2019). In potato, 15 protoplast regenerants were sequenced and different mutations were identified including insertion/deletions, chromosome rearrangements, and aneuploidy. However, somaclonal mutations were not identified in plantlets derived from (or by) cutting (Fossi et al. 2019). Such somaclonal mutations may affect meiosis; therefore, in our experiments, plants harboring the edited/mutated alleles may have incurred secondary mutations during protoplast regeneration that would not allow them to transmit the mutant *etr1* allele to progeny. The mechanism by which this particular mutation could not be passed on to progeny plants requires further investigation.

Conclusions

In this report, we demonstrate that three CRISPR nucleases, Cas9, Cas12a, and nCas9-Target-AID, can be used in tobacco protoplasts, and that the edited plants can be regenerated and the edited alleles can be transmitted to progeny

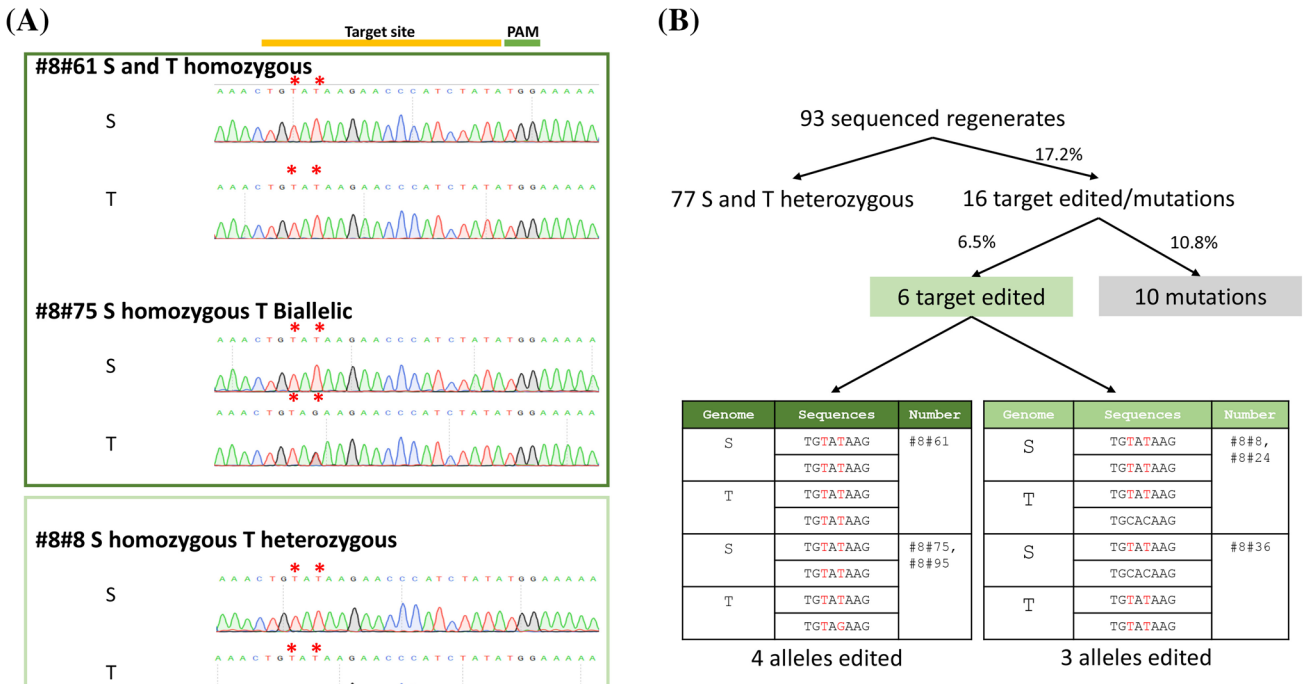


Fig. 8 Analysis of plants regenerated from Target-AID transfected heterozygous tobacco protoplasts from line R2#8. **a** Chromatograms of the DNA sequence from different types of editing in the mutated regenerates. S, *N. sylvestris*; T, *N. tomentosiformis.* *Edited in the predicted region. Dark green frame, four alleles edited including S and T homozygous (#8#61) and S homozygous T biallelic (#8#75). **b** Summary of the 93 transfected regenerants. The tables at the bottom give sequence information of four edited alleles (dark green) and three edited alleles (light green). Letters in red, edited sequences in the predicted region

plants. These results validate the use of genome engineering tools, initially developed in plants for *Agrobacterium*-mediated transformation, in a protoplast regeneration system. An advantage of protoplast-based mutagenesis is that plants regenerated from mutagenized protoplasts are not chimaeric, and all of the edited alleles can be transmitted to the progeny. We further show that we can simultaneously use multiple different Cas nucleases, either in one or two rounds of transfection, to mutagenize the genomes of protoplasts from polyploid plants and obtain plants containing homozygous or biallelic mutations without sexual propagation. The limitation of this technology is thus the ability to regenerate plants from protoplasts of important crop species.

Acknowledgements We acknowledge Drs. Masaki Endo and Seiichi Toki of the Plant Genome Engineering Research Unit, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, for Cas12a plasmids. This research was supported by Academia Sinica, Innovative Translational Agricultural Research Administrative Office (AS-KPQ-107-ITAR-10), and the Ministry of Science and Technology (105-2313-B-001 -007 -MY3), Taiwan.

Author contributions All authors conceived and designed the experiments. CSL, CTH, YJC, QWC, YHY, and WFH performed targeted mutagenesis analysis. CTH and CSL conducted protoplast regeneration. CTH, YJC, QWC, YHY, QWC, FHW, and WFH performed molecular biology experiments. CSL, LYL, and SBG interpreted the data. CSL, LYL, and SBG wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

Andersson M, Turesson H, Nicolia A, Fält AS, Samuelsson M, Hofvander P (2017) Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. Plant Cell Rep 36:117–128

Andersson M, Turesson H, Olsson N, Fält AS, Ohlsson P, Gonzalez MN, Samuelsson M, Hofvander P (2018) Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. Physiol Plant 164:378–384

Cao WH, Liu J, He XJ, Mu RL, Zhou HL, Chen SY, Zhang JS (2007) Modulation of ethylene responses affects plant salt-stress responses. Plant Physiol 143:707–719

Collier R, Thomson JG, Thilmony R (2018) A versatile and robust *Agrobacterium*-based gene stacking system generates high-quality transgenic *Arabidopsis* plants. Plant J 95:573–583

Endo A, Masafumi M, Kaya H, Toki S (2016) Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from *Francisella novicida*. Sci Rep 6:38169

Fossi M, Amundson K, Kuppu S, Britt A, Comai L (2019) Regeneration of *Solanum tuberosum* plants from protoplasts induces widespread genome instability. Plant Physiol 180:78–86

Gao L, Cox DBT, Yan WX, Manteiga JC, Schneider MW, Yamano T, Nishimasu H, Nureki O, Crosetto N, Zhang F (2017) Engineered Cpf1 variants with altered PAM specificities. Nat Biotechnol 35:789–792

Hall AE, Bleecker AB (2003) Analysis of combinatorial loss-of-function mutants in the *Arabidopsis* ethylene receptors reveals that the ers1 etr1 double mutant has severe developmental defects that are EIN2 dependent. Plant Cell 15:2032–2041

Hill JT, Demarest BL, Bisgrove BW, Su YC, Smith M, Yost HJ (2014) Poly peak parser: method and software for identification of unknown indels using Sanger sequencing of PCR products. Dev Dyn 243:1632–1636

Hu X, Wang C, Liu Q, Fu Y, Wang K (2017) Targeted mutagenesis in rice using CRISPR-Cpf1 system. J Genet Genom 44:71–73

Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z, Liu DR (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature 556:57–63

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821

Kaya H, Mikami M, Endo A, Endo M, Toki S (2016) Highly specific targeted mutagenesis in plants using *Staphylococcus aureus* Cas9. Sci Rep 6:26871

Kim H, Kim ST, Ryu J, Kang BC, Kim JS, Kim SG (2017) CRISPR/Cpf1-mediated DNA-free plant genome editing. Nat Commun 8:14406

Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales AP, Li Z, Peterson RT, Yeh JR, Aryee MJ, Joung JK (2015) Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 523:481–485

Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533:420–424

Komor AC, Zhao KT, Packer MS, Gaudelli NM, Waterbury AL, Koblan LW, Kim YB, Badran AH, Liu DR (2017) Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T: a base editors with higher efficiency and product purity. Sci Adv 3(8):eaao4774

Lee L-Y, Fang M-J, Kuang L-Y, Gelvin SB (2008) Vectors for multi-color bimolecular fluorescence complementation to investigate protein-protein interactions in living plant cells. Plant Meth 4:24

Lee L-Y, Wu F-H, Hsu C-T, Shen S-C, Yeh H-Y, Liao D-C, Fang M-J, Liu N-T, Yen Y-C, Dokládal L, Sykorová E, Gelvin SB, Lin CS (2012) Screening a cDNA library for protein-protein interactions directly in planta. Plant Cell 24:1746–1759

Li JF, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. Nat Biotechnol 31:688–691

Li J, Sun Y, Du J, Zhao Y, Xia L (2017) Generation of targeted point mutations in rice by a modified CRISPR/Cas9 system. Mol Plant 10:526–529

Lin C-S, Hsu C-T, Yang L-H, Lee L-Y, Fu J-Y, Cheng Q-W, Wu F-H, Hsiao H-C, Zhang Y, Zhang R, Chang W-J, Yu C-T, Wang W, Liao L-J, Gelvin SB, Shih MC (2018) Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. Plant Biotechnol J 16:1295–1310

Lu Y, Zhu JK (2017) Precise editing of a target base in the rice genome using a modified CRISPR/Cas9 system. Mol Plant 10:523–525

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y, Xie Y, Shen R, Chen S, Wang Z, Chen Y, Guo J, Chen L, Zhao X, Dong Z, Liu YG (2015) A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol Plant 8:1274–1284

Marx V (2016) Plants: a tool box of cell-based assays. Nat Meth 13:551–554

Medgyesy P, Menczel L, Maliga P (1980) The use of cytoplasmic streptomycin resistance: chloroplast transfer from *Nicotiana tabacum* into *Nicotiana sylvestris*, and isolation of their somatic hybrids. Mol Gen Genet 179:693–698

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. Nat Biotechnol 31:691–693

Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, Tabata M, Mochizuki M, Miyabe A, Araki M, Hara KY, Shimatani Z, Kondo A (2016) Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. Science 353:aaf8729

O'Malley RC, Rodriguez FI, Esch JJ, Binder BM, O'Donnell P, Klee HJ, Bleecker AB (2005) Ethylene-binding activity, gene expression levels, and receptor system output for ethylene receptor family members from *Arabidopsis* and tomato. Plant J 41:651–659

Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152:1173–1183

Qu X, Hall BP, Gao Z, Schaller GE (2007) A strong constitutive ethylene-response phenotype conferred on *Arabidopsis* plants containing null mutations in the ethylene receptors ETR1 and ERS1. BMC Plant Biol 7:3

Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F (2015) In vivo genome editing using *Staphylococcus aureus* Cas9. Nature 520:186–191

Ren B, Yan F, Kuang Y, Li N, Zhang D, Lin H, Zhou H (2017) A CRISPR/Cas9 toolkit for efficient targeted base editing to induce genetic variations in rice. Sci China Life Sci 60:516–519

Ren B, Yan F, Kuang Y, Li N, Zhang D, Zhou X, Lin H, Zhou H (2018) Improved base editor for efficiently inducing genetic variations in rice with CRISPR/Cas9-guided hyperactive hAID mutant. Mol Plant 11:623–626

Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol 31:686–688

Sheu JJ, Yu TS, Tong WF, Yu SM (1996) Carbohydrate starvation stimulates differential expression of rice α-amylase genes that is modulated through complicated transcriptional and postranscriptional process. J Biol Chem 27:26998–27004

Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, Ishii H, Teramura H, Yamamoto T, Komatsu H, Miura K, Ezura H, Nishida K, Ariizumi T, Kondo A (2017) Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. Nat Biotechnol 35:441–443

Tang X, Lowder LG, Zhang T, Malzahn AA, Zheng X, Voytas DF, Zhong Z, Chen Y, Ren Q, Li Q, Kirkland ER, Zhang Y, Qi Y (2017) A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. Nat Plants 3:17018

Vojta A, Dobrinić P, Tadić V, Bočkor L, Korać P, Julg B, Klasić M, Zoldoš V (2016) Repurposing the CRISPR-Cas9 system for targeted DNA methylation. Nucl Acids Res 44:5615–5628

Wang M, Mao Y, Lu Y, Tao X, Zhu JK (2017) Multiplex gene editing in rice using the CRISPR-Cpf1 system. Mol Plant 10:1011–1013

Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, Kim H, Kim SG, Kim ST, Choe S, Kim JS (2015) DNA-free genome editing in

plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol 33:1162–1164
Xie K, Minkenberg B, Yang Y (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci USA 112:3570–5357
Xing HL, Dong L, Wang ZP, Zhang HY, Han CY, Liu B, Wang XC, Chen QJ (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol 14:327
Xu R, Qin R, Li H, Li D, Li L, Wei P, Yang J (2017) Generation of targeted mutant rice using a CRISPR-Cpf1 system. Plant Biotechnol J 15:713–717
Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV, Zhang F (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163:759–771
Zhong Z, Zhang Y, You Q, Tang X, Ren Q, Liu S, Yang L, Wang Y, Liu X, Liu B, Zhang T, Zheng X, Le Y, Zhang Y, Qi Y (2018) Plant genome editing using FnCpf1 and LbCpf1 nucleases at redefined and altered PAM sites. Mol Plant 11:999–1002
Zong Y, Wang Y, Li C, Zhang R, Chen K, Ran Y, Qiu JL, Wang D, Gao C (2017) Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. Nat Biotechnol 35:438–440
Zong Y, Song Q, Li C, Jin S, Zhang D, Wang Y, Qiu JL, Gao C (2018) Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. Nat Biotechnol 36:950–953