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Efficient gene targeting in *Nicotiana tabacum* using CRISPR/SaCas9 and temperature tolerant LbCas12a

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Summary

Nicotiana tabacum is a non-food herb that has the potential to be utilized as bio-factory for generating medicines, vaccines or valuable small metabolites. To achieve these goals, the improvement of genetic tools for pre-designed genome modifications is indispensable. The development of CRISPR/Cas nucleases allows the induction of site-specific double-strand breaks to enhance homologous recombination-mediated gene targeting (GT). However, the efficiency of GT is still a challenging obstacle for many crops including tobacco. Recently, studies in several plant species indicated that by replacing SpCas9 with other CRISPR/Cas-based nucleases, GT efficiencies might be enhanced considerably. Therefore, we tested SaCas9 as well as a temperature-insensitive version of LbCas12a (ttLbCas12a) for targeting the tobacco *SuRB* gene. At the same time, we also optimized the protocol for Agrobacterium-mediated tobacco transformation and tissue culture. In this way, we could improve GT efficiencies to up to a third of the inoculated cotyledons when using ttLbCas12a, which outperformed SaCas9 considerably. In addition, we could show that the conversion tract length of the GT reaction can be up to 606 bp long and in the majority of cases, it is longer than 250 bp. We obtained multiple heritable GT events, mostly heterozygous, but also biallelic GT events and some without T-DNA integration. Thus, we were not only able to obtain CRISPR/Cas-based heritable GT events in allotetraploid *Nicotiana tabacum* for the first time, but our results also indicate that ttLbCas12a might be a superior alternative for gene editing and GT in tobacco as well as in other crops.

Introduction

Gene targeting (GT) is based on a homologous recombination (HR) reaction between a genomic target locus and an exogenously applied DNA template. Thus, pre-designed modifications can be introduced into a single specific site of the genome. In 1988, GT was achieved in plants for the first time (Paszkowski *et al*., 1988). This first experiment was performed in tobacco, but the frequencies were too low for practical applications. Despite its long history, further progress in the development of a GT technology for plants was slow. One fundamental reason for this is that the non-homologous end joining (NHEJ) pathway is the major DNA repair mechanism in the somatic cells of plants (Puchta, 2005). A major breakthrough, also performed in tobacco, was the demonstration that the induction of site-specific DNA double-strand breaks (DSBs) can enhance GT frequencies by up to several orders of magnitude (Puchta, 2016; Puchta *et al*., 1996). Later on, synthetic site-specific nucleases (SSNs) were developed. Again, the first studies on targeting natural genes in plants by the use of zinc finger nucleases (ZFNs) (Townsend *et al*., 2009) as well as transcription activator-like effector nucleases (TALENs) were performed in tobacco (Zhang *et al*., 2013).

More recently, SSNs of the CRISPR/Cas class, which originate from the bacterial immune system against foreign DNA (Barrangou and Marraffini, 2014), were adopted for plants. Cas nucleases use only a short protospacer sequence within their CRISPR RNA (crRNA) for the determination of target specificity. Due to their simple cloning requirements, CRISPR/Cas systems became the most popular tool for DSB induction in biology and breeding (Atkins and Voytas, 2020; Schindele *et al*., 2020; Zhu *et al*., 2020). Many different CRISPR/Cas orthologs and variants were already applied in plants for the induction of DSBs [see review (Zhang *et al*., 2019)]. The requirement of different protospacer adjacent motifs (PAMs) among CRISPR/Cas orthologs enables the development of diverse CRISPR/Cas tools to target almost any genomic locus. The most frequently applied CRISPR/Cas nuclease in plants is Cas9 from *Streptococcus pyogenes* (SpCas9). Recently, also Cas9 from *Staphylococcus aureus* (SaCas9) and Cas12a from *Lachnospiraceae bacterium ND2006* (LbCas12a) are used in plants more often. Both Cas9 nucleases generate DSBs with blunt ends, only 3 nucleotides upstream of the NGG PAM of SpCas9 or the NNGRRT PAM of SaCas9. Comparisons between SpCas9 and SaCas9 in some reports suggest that SaCas9 has a higher DSB induction efficiency than SpCas9 (Raitskin *et al*., 2019; Steinert *et al*., 2015). Different from Cas9, the LbCas12a endonuclease generates staggered DNA ends, and the cleavage site is distal from its TTTV PAM sequence. Recent evidence in many different organisms including plants indicates that DSBs induced by Cas12a might result in higher GT efficiencies than those induced by Cas9 (Li *et al*., 2018; Li *et al*., 2020; van Vu *et al*., 2020; Wolter and Puchta, 2019). However, LbCas12a was

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reported to have more variability in activity depending on the target locus or protospacer sequences (Bernabé-Orts *et al*., 2019). Moreover, it is less active at lower temperatures (Malzahn *et al*., 2019), hindering its application in plants. Recently, an engineered temperature-insensitive LbCas12a (ttLbCas12a) with a D156R mutation was shown to be more efficient than the wild-type enzyme for both mutagenesis and GT in Arabidopsis (Merker *et al*., 2020; Schindele and Puchta, 2020), making ttLbCas12a an excellent candidate for DSB induction in crops as well.

Instead of its use for smoking, cultivated tobacco, *Nicotiana tabacum,* as well as *Nicotiana benthamiana* hold great potential to be used as bio-factories for the production of medicines, vaccines or other valuable small metabolites (Capell *et al*., 2020; Ma *et al*., 2005; Nogueira *et al*., 2018; Stoger *et al*., 2014; Tschofen *et al*., 2016). To approach this goal, molecular tools for genome editing are necessary for building this 'molecular farm'. In recent years, many CRISPR/Cas-mediated GT applications in various plant species could obtain heritable events, as shown in rice, Arabidopsis, tomato and maize (Barone *et al*., 2020; Čermák *et al*., 2015; Dahan-Meir *et al*., 2018; Danilo *et al*., 2019; Endo *et al*., 2016; Hahn *et al*., 2018; Li *et al*., 2019; Li *et al*., 2020; Merker *et al*., 2020; Miki *et al*., 2018; Pater *et al*., 2018; Peng *et al*., 2020; van Vu *et al*., 2020; Wolter *et al*., 2018; Wolter and Puchta, 2019). However, GT applications performed in crops with complex genomes, such as barley or wheat, were relatively scarce and often lack heritable events (Baltes *et al*., 2014; Gil-Humanes *et al*., 2017; Watanabe *et al*., 2016). Despite its huge potential as bio-factory, only one study has been undertaken to establish a SpCas9-based GT approach in tobacco (Hirohata *et al*., 2019), but the efficiency was not very promising. Surprisingly, until now no report has been published on the production of CRISPR/Cas-based heritable GT events in *N. tabacum* or *N. benthamiana*.

In this study, we successfully generated GT events in *N. tabacum* using SaCas9 as well as ttLbCas12a. We chose the acetolactate synthase (*ALS*) gene as target to introduce an herbicide-resistant phenotype for examining GT efficiencies. ALS is a key enzyme for the biosynthesis of branched-chain amino acids and can be suppressed by imidazolinone herbicides. In allotetraploid tobacco, ALS is encoded by two genes, *SuRA* and *SuRB*. Previous studies identified the corresponding resistance mutations from naturally occurring resistances [see review: (Tranel and Wright, 2002)]. Our results show that two herbicide resistance modifications, W568L and S647T, can be simultaneously incorporated into the *SuRB* gene with high efficiencies. Multiple GT events were obtained and inheritance to the next generation could be documented. These events were inherited in a Mendelian fashion, most as single alleles, some biallelic, some T-DNA-free. Analysis of mutagenesis by next-generation sequencing (NGS) indicated that ttLbCas12a possessed a two- to threefold higher editing efficiency than wild-type LbCas12a, suggesting ttLbCas12a is not only a suitable nuclease for GT induction but also a promising molecular tool for inducing mutations in general in various crop plants.

Results

Mutagenesis efficiency of LbCas12a and ttLbCas12a

Recent results of several groups indicate that the use of LbCas12a is an attractive alternative nuclease for GT in Arabidopsis, tomato and rice. Recently, we optimized the enzyme for use in Arabidopsis by exchanging a single amino acid resulting in ttLbCas12a, which showed a two- to sevenfold increase in editing efficiency compared to LbCas12a at ambient temperature (Schindele and Puchta, 2020). To compare the nuclease activity of LbCas12a and ttLbCas12a in a pilot experiment in tobacco, we performed the tobacco transformation using the sgRNA P3 for targeting the *SuRB* gene and cultured the resulting callus tissue at 22°C or 28°C (Figure 1a-c). After 6 and 10 weeks, genomic DNA was extracted from surviving calli originating from individual cotyledons and the target locus was amplified. Sequencing results were analysed by TIDE analysis for quantification of the mutation induction (Brinkman *et al*., 2014). For both the 6-week and 10-week cultured calli, we found that ttLbCas12a has a higher insertion–deletion (Indel) frequency than LbCas12a at both temperatures (Figure 1a, b). However, raising the temperature from 22°C to 28°C alone did not enhance mutation efficiency, neither in 6-week nor 10-week cultured samples. To obtain more insight into ttLbCas12a- and LbCas12a-mediated mutagenesis, using SaCas9 as a control, we performed NGS analysis of four targets: P1 and P2 sgRNAs for SaCas9, and P3 and P4 crRNAs for LbCas12a (Figure 1c, d), using 3-week samples cultured at 22°C. Eight transformed cotyledons were pooled for genomic DNA extraction. Because the PCR primers used cannot specifically recognize only the *SuRB* gene, we amplified a fragment representing not only the *SuRB* but also the *SuRA* gene (Data S1 and S2). As shown in Figure 1d, we obtained mutated sequences in about 5% of the reads for SaCas9 using either P1 or P2. For LbCas12a, we obtained a similar frequency for P3 and around 13% for P4. Most importantly, for both P3 and P4 targets, the Indel efficiencies of ttLbCas12a are increased 2.5-fold for P3 and almost threefold for P4 compared to the original LbCas12a (Figure 1d). In the best case, more than a third of the reads showed mutations. Among all Indels, both protospacers for SaCas9 show around 80% insertions, while mutagenesis using LbCas12a and ttLbCas12a is composed only of deletions (in over 99% of cases, see Figure 1e). The distributions of deletion sizes are different between P3 and P4. Whereas with P3 shorter deletions between 1 to 5 bp were achieved, P4 produced more larger deletions around 10 bp (Figure 1f). However, the differences between ttLbCas12a and LbCas12a are not obvious. Thus, we found that ttLbCas12a is clearly more efficient than the wild-type enzyme for generating DSB induction in tobacco. Therefore, we decided to use ttLbCas12a for our GT experiments in tobacco.

GT designs and culture conditions

To test the usefulness of ttLbCas12a and SaCas9 for GT in tobacco, we chose the *ALS* gene as target, which has also been used by others in various plant species including tobacco (Hirohata *et al*., 2019; Townsend *et al*., 2009; Zhang *et al*., 2013). Acetolactate synthase in *N. tabacum* is encoded by two genes, the *SuRA* open reading frame (ORF) which consists of 667 amino acids, and SuRB which consists of 664 amino acids. Their sequence identity on the amino acid level is 97.4% (649/666) and the identity on the nucleotide level is 96.1% (1924/2001) in the coding regions. We used the *SuRB* sequence for the design of the GT donor, similar to the previous report (Hirohata *et al*., 2019). To perform GT experiments, we designed three different GT vectors. We utilized the protospacers P1 and P2 for SaCas9 and protospacer P4 for ttLbCas12a for DSB induction in GT experiments. As shown in Figure 2a, vectors contain the Cas nuclease expression cassette, the GT donor with two flanking cleavage sites and gRNAs for simultaneous excision of the homologous donor out of the T-DNA and cleavage of the target *SuRB* to induce the HR-mediated GT reaction.

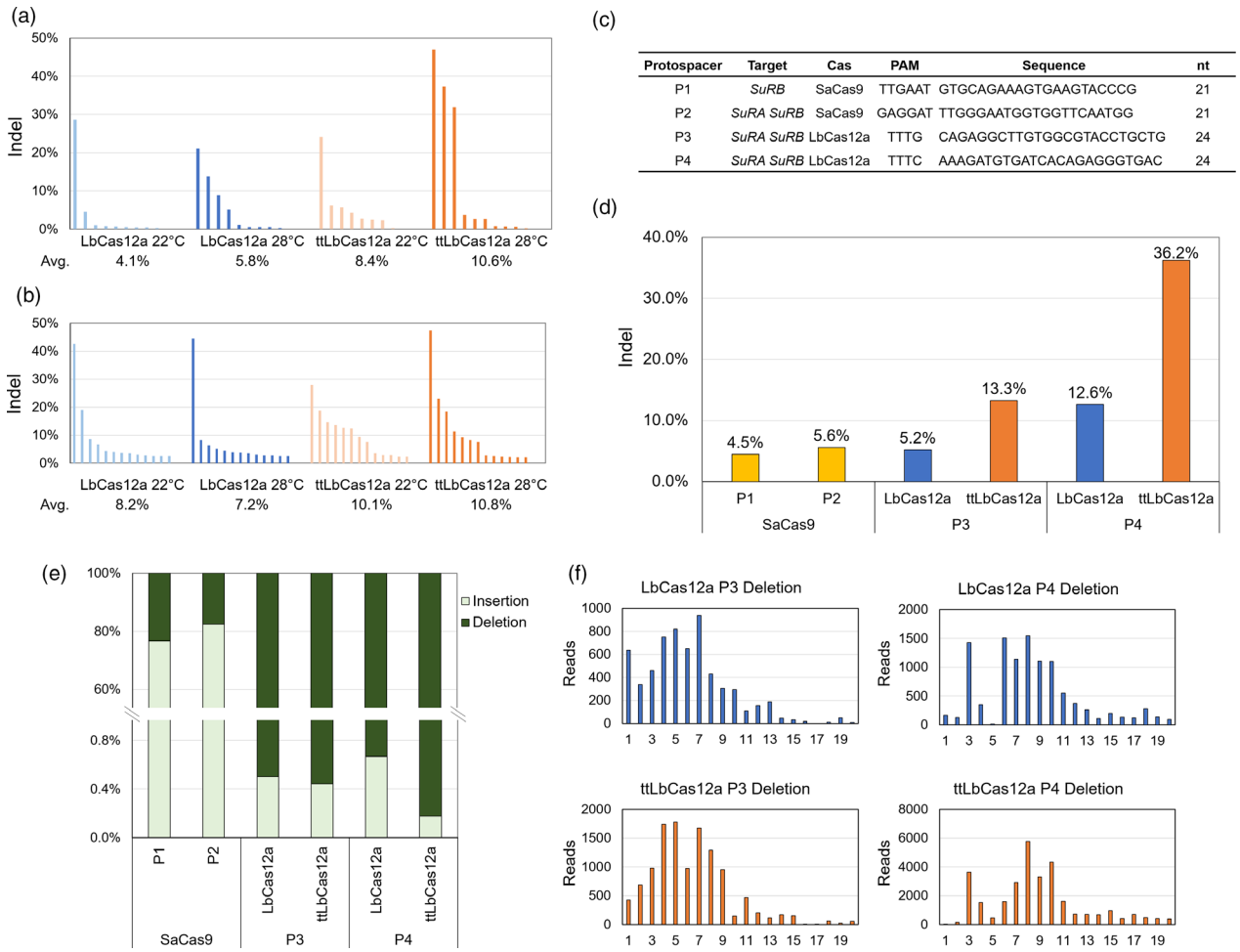


Figure 1 Mutagenesis efficiency of the SaCas9, LbCas12a, and ttLbCas12a nucleases. (a,b) The Indel efficiencies of transgenic calli after 6- (a) and 10-week (b) incubation at 22°C and 28°C were analysed by TIDE, using protospacer P3. The bars represent the percentage of reads with Indels from individual calli. (c) Sequences of the protospacers used to generate DSBs at the *SuRB* and *SuRA* loci. (d) Indel analysis by next-generation sequencing. (e) Comparison of the ratio of insertion and deletion formation after cutting by SaCas9, LbCas12a and ttLbCas12a as determined by NGS. (f) Comparison of distributions of deletion sizes obtained from LbCas12a and ttLbCas12a.

All three GT donor sequences contain several modifications. These include two imazapyr resistance mutations, W568L and S647T, a mutation that removes a genomic BglII site and a mutation that introduces a novel XhoI site into the genomic *SuRB* gene (Figure 2b-d). Furthermore, vector ttLb contains silent mutations in the donor sequence to block Cas12a cleavage within the homologous region, as well as the protospacer P4 and the ttLbCas12a ORF under the control of the ubiquitin promotor from *Petroselinum crispum* and the rubisco terminator (Figure 2b). Vector Sa-3sg equivalently contains the protospacer P1 and the SaCas9 ORF (Figure 2c). In addition, the homologous donor sequence contains a synonymous change at position Leu447 and two additional sgRNA target sites for the excision of the GT donor (Table S1). Vector Sa-at contains the protospacer P2 for SaCas9 to cut the genomic *SuRB* locus as well as excise the homologous sequence from the T-DNA donor (Figure 2d). The homologous donor sequence within Sa-at contains two synonymous changes at positions Leu447 and Thr477 on the site proximal to the DSB. The sequence distal to the DSB contains the W568L and S647T modifications. Between these, a 240 bp region from Arabidopsis *ALS* ORF (AT3G48560) is located, reducing homology in this region to only 72.5% identity (Figure S1). As the three constructs use different crRNAs to induce DSBs in the *SuRB* gene, different silent mutations had to be introduced in the homologous vector sequence to prevent cleavage of the donor. All silent mutations are synonymous changes (Figure 2e).

As only a tiny fraction of the transformation events will result in regenerated plants carrying a heritable GT event, one way to enhance GT efficiency is to improve the transformation and regeneration protocols of the respective plant species. Therefore, we optimized several steps of our transformation and in vitro cultivation protocol of tobacco, which is described below and shown in Figure 2f.

Agrobacterium-mediated tobacco cotyledon transformation was performed in germination plates. To reduce the variation between different experiments, we always adjusted agrobacterial cell density to $OD_{560} = 1$ in the infiltration solution. This agrobacteria solution was directly poured into the plates with 2-week-old tobacco seedlings. These seedlings, immersed within the agrobacteria solution, were placed into a desiccator for vacuum infiltration. Afterwards, the agrobacteria solution was

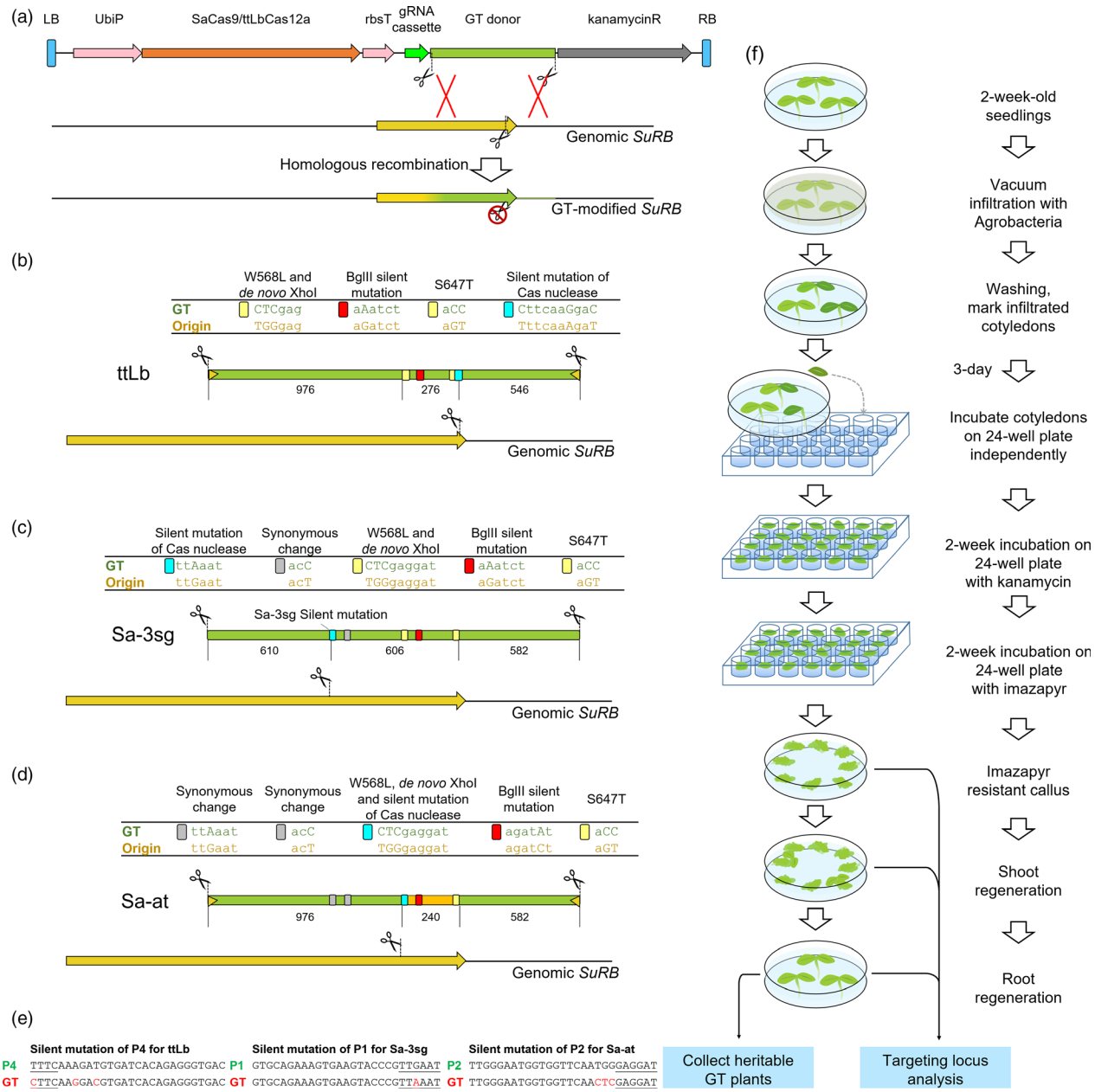


Figure 2 Setup of the GT experiments. (a-d) Design of the targeting vectors. Each construct contains a functional Cas nuclease and GT donor with silent mutation to prevent cleavage after HR. These differences between genomic *SuRB* and GT donors were listed. Both ttLb and Sa-at donors have their corresponding gRNA targets flanking the GT donor (yellow triangles). In Sa-at, the sequence between W568L and S647T comes from Arabidopsis *ALS* (orange). (e) The design of silent mutations to avoid cleavage within the homologous regions of the vectors. (f) Graphical sketch of the improved transformation/in vitro cultivation protocol used for GT.

discarded and the tobacco seedlings together with the germination medium were washed with sterilized H_2O. At this point, cotyledons that were efficiently infiltrated are slightly transparent and of darker green colour, similar to vitrification. Only these cotyledons were labelled, then cut from the seedlings three days later and transferred to a callus-inducing medium. To avoid interference between individual cotyledons, each cotyledon was cultured in an individual chamber of a 24-well plate for the first 4 weeks. We used 24-well plates as we found out in pilot experiments that the survival rate of arising calli was reduced on standard plates. Cotyledons were transferred to fresh medium every week. In the first 2 weeks, cotyledons were inoculated on medium containing kanamycin. Subsequently, the cotyledons were transferred to imazapyr-containing medium to start the selection of GT events from the third week on. After the first 4 weeks of culturing, cotyledons started to form callus tissue. The browning parts were removed to maintain the callus in a healthy state. In the meantime, we recorded the surviving cotyledons or calli every week (Figure S2). To ensure that each GT event we obtained was an independent event, every callus originating from the same cotyledon was labelled accordingly. After 12 weeks of culturing, the surviving samples reached a stationary phase. These

calli or regenerated shoots were then analysed for the molecular nature of the GT modifications.

As shown in Table 1, we performed all in all four independent experiments. In the first three experiments, we applied the ttLbCas12a as well as both SaCas9 vectors for GT. In the fourth experiment, we only applied the ttLbCas12a vector, but we compared two incubation temperatures. In the first experiment, we still applied standard plates for the first 4 weeks. As the resulting numbers of GT events turned out to be unsatisfactory, we started to use 24-well plates for all other experiments. With ttLbCas12a we obtained around threefold more imazapyr-resistant calli in the second experiment and sixfold more in the third experiment. The efficiency achieved using this enzyme was surprisingly high in both experiments. We were able to obtain imazapyr-resistant calli from almost every third cultivated cotyledon.

Molecular analysis of the gene-targeting events

Next, we set out to demonstrate by molecular analysis that the imazapyr resistance was indeed due to homologous recombination between target vector and the genomic *SuRB* locus. To characterize the molecular nature of the events in detail, we prepared genomic DNA and performed cleaved amplified polymorphic sequence (CAPS) analysis as well as sequence analysis of amplified PCR fragments.

A representative CAPS analysis for a number of ttLbCas12a induced GT events is shown in Figure 3b. The PCR fragments osG and pG were amplified for the analysis. For amplification of osG, one PCR primer with a binding site outside and the second with a binding site inside of the homologous region of the GT vector were used (Figure 3a). This PCR product is used for confirmation of HR at the left homologous arm of the GT donor by CAPS, and sequence analysis for the detection of the respective newly introduced mutations. The second PCR primer set has binding sites outside of the homologous region but not in the GT vector, resulting in the amplification of the fragment pG. The sequence of this PCR product is required to confirm that HR reactions took place at both the left and right homologous arms of the GT donor. To discriminate between the genomic sequence and amplified GT events, we used restriction digests of both fragments. Depending on the conversion tract length of the HR reaction, a BglII site should be removed and a *de novo* XhoI site introduced into the genomic *SuRB* site. Thus, we could not only confirm GT events but also define the conversion tract length more accurately. Both amplification products can be obtained from the wild type as well as the targeted allele. However, the WT fragments carry a BglII but no XhoI site. If targeting occurs in one allele, a mixture of both the GT and WT allele will be amplified. In this case, partial digestions should be visible for both enzymes after gel electrophoresis. In principle, imazapyr resistance can arise also by one-sided GT, such as ectopic GT, which has been found in other DSB induced GT studies before (Wolter *et al.*, 2018; Wolter and Puchta, 2019). In this case, the information is copied from the genomic *SuRB* locus into the vector, which later integrates elsewhere into the genome. In consequence, a third ectopic *ALS* copy is present beside the two unchanged WT alleles. We can discriminate between one-sided and perfect GT events if both the osG and the pG amplification products contain the desired mutations for the imazapyr resistance and, depending on conversion tract length, the mutation changing the restrictions site(s). This is clearly the case for sample ttLb-1. In this case, digestions of the osG as well as the pG fragment show that the BglII site was removed and the XhoI introduced in one of the two genomic alleles. Sample ttLb-2 represents an example of an ectopic GT event. Here, only the digestions of the osG amplicon reveal the desired changes in the restriction fragments. In contrast, pG reveals that both alleles of the target locus were not changed. However, as shown for sample ttLb-3, CAPS analysis might not be able to discriminate GT from WT if the conversion tract is too short for co-converting the restriction site mutations. However, we confirmed by sequence analysis of pG that ttLb-3 is a perfect GT event which introduced the herbicide resistance mutation S647T and the silent mutation to block cutting in the *SuRB* ORF. Sample ttLb-4 shows a biallelic GT event.

Table 1 Gene targeting efficiencies

	Construct	Inoculated cotyledons	Imazapyr-resistant calli	GT efficiency (%)	Perfect GT events	Ratio of perfect GT/all GT (%)
exp 1[†]	ttLb	16	2	13	2	100
	Sa-3sg	16	2	13	2	100
	Sa-at	16	1	6	0	0
exp 2	ttLb	42	12	29	6	50
	Sa-3sg	71	8	11	4	50
	Sa-at	72	7	10	5	71
exp 3	ttLb	31	10	32	7	70
	Sa-3sg	22	1	5	0	0
	Sa-at	22	1	5	1	100
exp 4	ttLb	39	4	10	3	75
	ttLb[‡]	23	3	13	2	67
sum	ttLb	151	31	21	20	66
	Sa-3sg	131	11	9	6	50
	Sa-at	135	9	7	6	67

[†]Traditional culture in plate at first 4 weeks.

[‡]Tissue-culture performed at 28 °C.

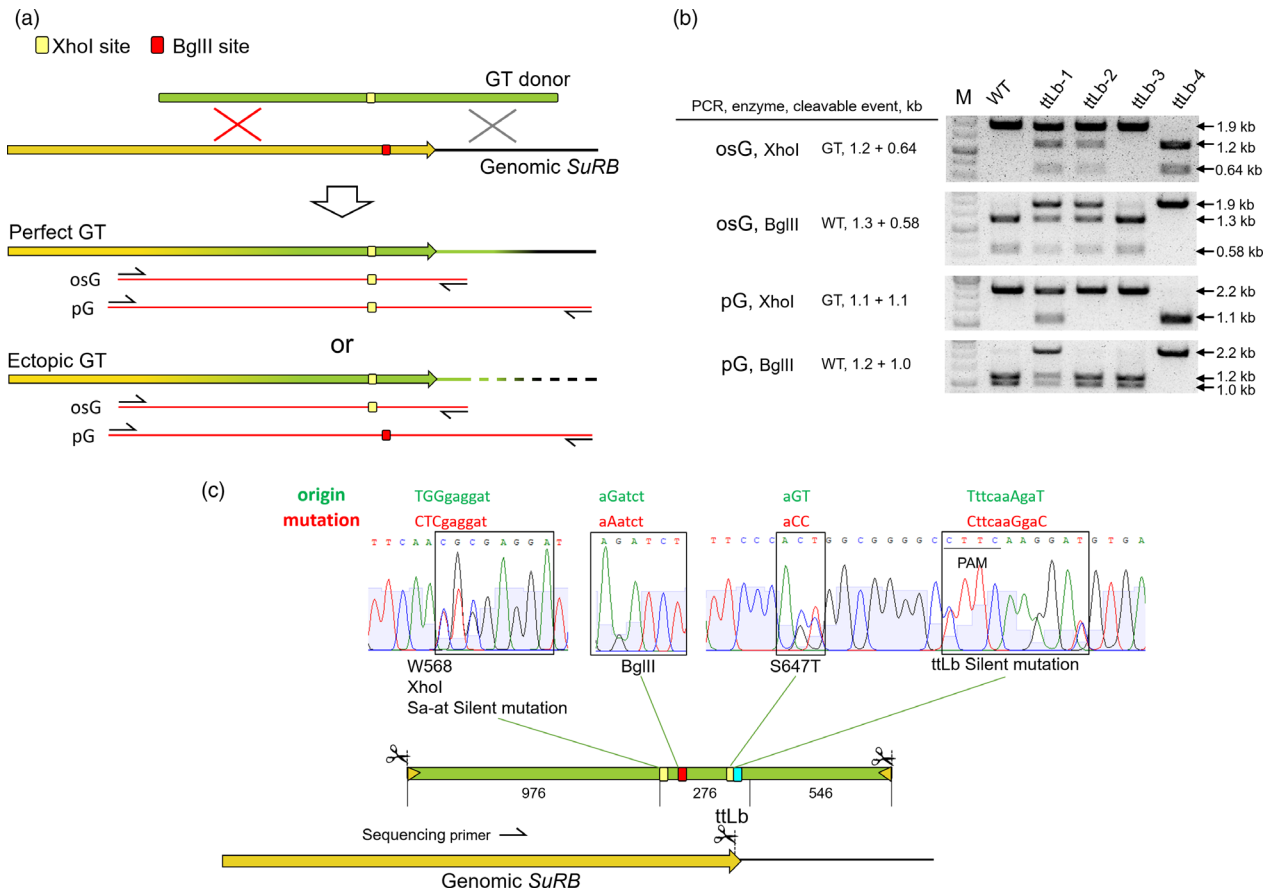


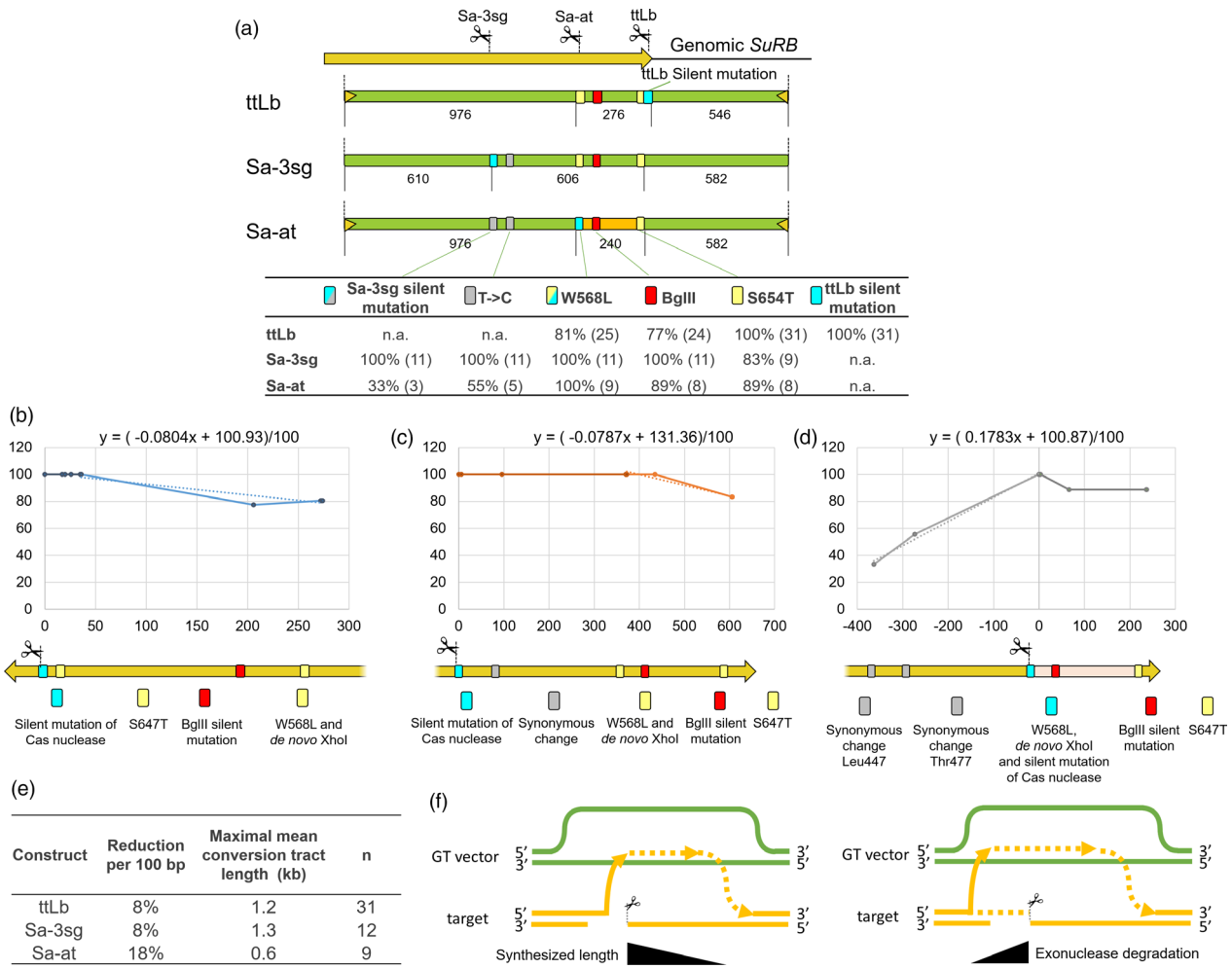
Figure 3 Analysis of GT events by CAPS assay and Sanger sequencing. (a) Two PCR reactions are used to confirm GT and discriminate between perfect GT or ectopic GT. The osG amplicon was used to confirm HR at the 5′ homology and the pG amplicon was used to confirm HR at both the 5′ and 3′ homologies. The BglII site only exists in genomic *SuRB* and the XhoI site only appears in the GT donor. (b) CAPS assay shows different types of GT events: ttLb-1, perfect GT; ttLb-2, ectopic GT; ttLb-3 perfect GT with short conversion length; ttLb-4, biallelic perfect GT. (c) A representative Sanger sequencing result of the amplicon pG showing heterozygous GT and wild-type sequences in the chromatogram of the ttLb GT event ttLb-9.

Here, both the osG and the pG amplification products carry only the modification copied from the targeting vector and no wild-type *SuRB* allele is present anymore.

For all lines, both the osG and pG PCR products were Sanger sequenced. Figure 3c shows a representative example with overlapping spectrums resulting from a heterozygous plant with one wild-type *SuRB* and one GT-modified *SuRB* allele.

The results of the molecular analysis of all herbicide-resistant calli are summed up in Table 1. All resistant calli, independent of the length of the conversion tract, contained at least one herbicide-resistant mutation, either W568L or S647T. If at least one of these mutations was present at the target locus, as determined by sequencing of the pG amplification product, the respective callus was classified as a perfect GT event. If we sum up all events, more than half of the events obtained with SaCas9 as well as about two thirds obtained with ttLbCas12a turned out to be perfect GT events. This ratio is somewhat higher than for Arabidopsis, where perfect events could be identified in about half of the cases using SaCas9, LbCas12a or ttLbCas12a (Merker *et al.*, 2020; Wolter *et al.*, 2018; Wolter and Puchta, 2019). Others also reported low rates of perfect targeting events in Arabidopsis (Shan *et al.*, 2018).

Due to the mutations introduced in the *SuRB* gene, we could also estimate the conversion tract length of the DSB repair reaction. The data are shown in Figure 4. The herbicide conferring mutation closest to the DSB site, S647T in the case of the ttLb construct and W568L in the case of the Sa-3sg and Sa-at constructs, was found in all GT events. Our analysis shows that the second amino acid substitution was not co-converted in all samples (Figure 4a). For ttLb-mediated GT events, 6 of 31 events did not contain W568L. For Sa-3sg-mediated GT events, 2 of 11 events did not contain S647T and for the Sa-at 1 of 9 events. To get an estimate about the mean length of the conversion tract, we drew x/y plots, setting the DSB site as zero point, the distance to DSB site as x value for each nucleotide modification, the percentage of incorporation as y value, to draw the regression line (Figure 4b-d). For ttLb, the DSB site was defined to be between the 22nd and the 23rd nucleotide downstream of the PAM. In ttLb-mediated GT events, the S647T modification was converted in 100% of the events, but the mutations removing the BglII site and adding the W568L modifications were only present in about 80% of the GT events. Using the x/y values of S647T to W568L, we can therefore estimate that for ttLb there is a reduction of incorporation efficiency of about 8% per 100 bp (Figure 4e). For Sa-3sg, this value is also about 8% per 100 bp, estimated from W568L to S647T. This pattern can easily be explained by the synthesis-dependent strand annealing (SDSA) pathway model, as the probability of reinvasion of the newly



Construct	Reduction per 100 bp	Maximal mean conversion tract length (kb)	n
ttLb	8%	1.2	31
Sa-3sg	8%	1.3	12
Sa-at	18%	0.6	9

Figure 4 Detailed characterization of the GT reaction. (a) Schematic representation of the mutations present in the different vectors and their inclusion in the genomic *SuRB* locus due to targeting. (b-d) Plots to determine the respective maximal conversion tract length during the GT reactions. (e) Summary of the results shown in b to d. (f) Gene conversion tract length is not only influenced by the efficiency of DNA synthesis during SDSA-mediated repair but also by a putative exonuclease-mediated degradation of DSB ends before invasion.

extended 3′ end after copying from the target rises with length of the newly synthesized strand (Figure 4f).

The analysis of Sa-at-mediated GT events also indicates that heterologies close to the DSB might be tolerated and can be integrated into the target locus with reasonable efficiency. Although the 240 bp long Arabidopsis sequence located between W568L and S647T only has 72.5% identity between vector and target, in 8 out of 9 cases it was still copied into the target. Interestingly, one GT event only contains the W568L mutation, showing that in principle a 14 bp homology behind the mutation seems to be able to complete this perfect GT event. Sa-at contains two synonymous changes at positions Leu447 and Thr477 on the other proximal side of the DSB. Here, more than half of Sa-at-mediated GT events contain the synonymous change at Thr477, while the synonymous change at Leu447 is present only in a third of the events. Using the x/y value of W568L to Thr477, the reduction of incorporation rate per 100 bp is around 20% (Figure 4d,e). These values were further used to estimate the maximal mean conversion tract length, which is 0.6 kb in the region between W568L to Thr477 of Sa-at. In ttLb and Sa-3sg-mediated GT, the lengths are around 1.2 kb and 1.3 kb, respectively. The conversion tracts at both flanking regions of the DSB could be explained by exonucleolytic degradation of the genomic DNA ends between DSB induction and strand invasion (Figure 4f).

Heritable GT events and T-DNA integration-free GT events

In principle, in our setup, GT events could occur by transient expression of the nuclease as well as the kanamycin resistance gene on the T-DNA and without its later integration into the genome. To determine how many GT events we obtained which did not have any genomic T-DNA integration, we designed five primer pairs: one for the kanamycin resistance gene, two for ttLbCas12a, and two for SaCas9 (Table S1). Our positive control was an amplified genomic *SuRA* or *SuRB* sequence upstream of the GT homology. Each genomic DNA sample was confirmed by three independent rounds of PCR analysis to reduce false-negative results. Only if the amplification of both the Cas and kanamycin resistance genes were negative, these GT events were

Table 2 Segregation of resistance and PCR detection of T-DNA

Line	Imazapyr resistance		T-DNA
ttLb-3	70%	(141/201)	–
ttLb-4†	100%	(104/104)	+
ttLb-5	73%	(69/95)	+
ttLb-6	75%	(145/194)	–
ttLb-7	67%	(97/142)	+
ttLb-8	74%	(68/92)	+
Sa-3sg-1†	100%	(104/104)	+
Sa-3sg-2	69%	(60/87)	+
Sa-at-1	68%	(64/96)	+
Sa-at-2	75%	(62/83)	+

All lines are $P > 0.05$ by Chi-Square test of 3 to 1 segregation rate besides biallelic GT lines.

† biallelic GT at T0 generation.

considered to be free of T-DNA integration. We found 29% (7/24) of ttLb-mediated GT events that did not have any detectable T-DNA integration and one from Sa-at.

To demonstrate that the GT events obtained in our study are heritable, the imazapyr-resistant seedlings were transferred to the greenhouse and grown for seed harvesting. The resulting T1 seeds were sown out onto imazapyr and kanamycin containing medium. As expected, imazapyr-resistant seedlings in the T1 generation were detected in all lines tested (Table 2). 8 of 10 lines revealed 3 to 1 Mendelian segregation of imazapyr resistant to non-resistant plants, indicating a single targeted allele. Two lines showed 100% imazapyr-resistant T1 plants, consistent with their sequencing results from T0, indicating biallelic GT events. Two of the tested lines did not carry any detectable Cas nuclease or kanamycin resistance gene sequences, indicating that they were transgene-free.

Discussion

Different cleavage outcomes by LbCas12a and SaCas9

Previous studies demonstrated that SaCas9 and LbCas12a are quite efficient SSNs for generating mutations in tobacco (Bernabé-Orts *et al*., 2019; Kaya *et al*., 2016). Our analysis using SaCas9, LbCas12a, and ttLbCas12a suggests that ttLbCas12a is an even more efficient tool for generating NHEJ-based mutations in the tobacco genome. So far, ttLbCas12a has only been applied in Arabidopsis and Drosophila for gene editing (Merker *et al*., 2020; Port *et al*., 2020; Schindele and Puchta, 2020). Here we show that compared to LbCas12a, mutagenesis efficiency by ttLbCas12a is increased around 2.5-fold in tobacco. The distribution of deletion length is similar between LbCas12 and ttLbCas12a, suggesting what we observed was mainly the increase of cleavage activity. Different from the ttLbCas12a results in Arabidopsis (Merker *et al*., 2020; Schindele and Puchta, 2020), we observed only a minor enhancement of mutagenesis (Figure 1) as well as GT efficiency at 28°C compared to 22°C (Table 1). Therefore, ttLbCas12a seems to be an excellent alternative to Cas9 under normal cultivation conditions for tobacco. The usefulness of ttLbCas12a applies most likely not only to other Solanaceae but other plants in general. Moreover, ttLbCas12a generates short deletions of several bps, identical to the wild-type LbCas12a, which are easier to be detected by PCR compared to single nucleotide Indels generated by Cas9.

In a previous report in Arabidopsis, LbCas12a-mediated GT showed similar GT efficiency but a lower mutagenesis efficiency, compared to SaCas9-mediated GT (Wolter and Puchta, 2019). Our results now show that both mutagenesis and GT efficiencies induced by ttLbCas12a outperform those of SaCas9 in tobacco. We suggest that the main reason for this surprising fact is that Cas12a, in comparison to Cas9, cleaves the DNA further away from the seed sequence. Therefore, repeated cleavage is possible after rejoining of the broken ends as arising minor mutations might not always completely block gRNA binding. Thus, although the probability of HR repair is low for each individual DSB repair event, consecutive DSB inductions at the same site enhance the overall probability for a successful GT event. An alternative explanation for the higher efficiency of Cas12a in GT is that Cas12a generates 5′ staggered DNA ends, in contrast to Cas9, which produces blunt ends. 5′ overhangs have been shown previously to induce efficient GT in green algae and human cells (Bothmer *et al*., 2017; Ferenczi *et al*., 2017). However, as 5′ overhangs produced by a paired nickase approach in Arabidopsis could not enhance GT (Wolter *et al*., 2018), we favour the former explanation.

Transformation and culture conditions

Surprisingly, only a single paper was published till now reporting on CRISPR/Cas-mediated GT in tobacco (Hirohata *et al*., 2019). In contrast to the previous study, we applied ttLbCas12a as well as SaCas9 instead of SpCas9 and thus obtained much higher efficiencies and a larger number of heritable events. However, this improvement is not only due to the use of more efficient nucleases but also due to the changes we introduced in the transformation protocol and tissue culture conditions. There are three major steps we regard as important for this optimization – selection of infiltrated cotyledons, short-term treatment with kanamycin in the first phase after transformation, as well as the culturing of each explant individually. We directly inoculate the agrobacteria in the tobacco germinating plates via vacuum infiltration. Only choosing fully infiltrated cotyledons by visual inspection for further cultivation reduces the amount of labour-intense tissue-culture work by enriching transformed samples and thus also GT events. We included antibiotic selection in the initial stage of cell culture, inspired by the previous GT screening in tomato (Danilo *et al*., 2019). We could not obtain GT events if we treated the cells with imazapyr after either two weeks of incubation without kanamycin or immediately after the 3-day transformation period. In contrast to antibiotic resistance genes that can be transiently expressed from the T-DNA without the need for integration, GT-derived imazapyr resistance requires time until HR is complete and modified ALS proteins are sufficiently accumulated to enable cell survival in presence of the herbicide. Furthermore, we assume that the application of kanamycin represents a further step of enriching transformed cells by killing cells which do not contain T-DNA. As we were also able to obtain GT events without detectable T-DNA integration, a genomic integration of the kanamycin resistance genes does not seem to be a requirement for survival during this short cultivation period under antibiotic selection.

The production of secondary metabolites, such as phenolic compounds as part of defence responses from stressed tissues or cells, is a general problem in plant tissue culture (Dias *et al*., 2016). These browning compounds can be observed within the callus itself. They are released into the medium as well, leading to the death of nearby tissue. We attempted to minimize the release

of such toxic compounds into the medium that would trigger the defence and stress responses of healthy tissue. Therefore, we singled every cotyledon out and incubated them individually in a 24-well plate for a total of four weeks, two on kanamycin medium and two on imazapyr medium. Whether this type of culture is beneficial for generating transgenic tobacco in general might be worth further examination.

GT events with diverse characteristics hint SDSA pathway for gene replacement

Our GT design partially follows the 'in planta gene targeting' (ipGT) approach, which induces breaks flanking the GT donor (Fauser *et al*., 2012). However, our method in this study differs from the classical ipGT approach in Arabidopsis using the egg-cell specific expression of the nuclease (Wolter *et al*., 2018), as we did not select T-DNA-integrated transgenic plants first. Thus, excision of the GT donor from the vector could occur before but also after integration of the T-DNA. A similar approach has also been used in rice before (Li *et al*., 2018; Li *et al*., 2020).

The molecular analysis of the GT events allowed us to better define mechanistic details. There are several different mechanisms describing subcategories of HR, namely single-strand annealing (SSA), SDSA and double-strand break repair (DSBR). Multiple lines of evidence have been accumulated over time that SDSA is the major HR mechanism for GT in plants (Huang and Puchta, 2019; Puchta, 1998). In this pathway, the DSB is repaired by copying the genetic information from the vector into the target locus by DNA synthesis. DSB repair is initiated by 5′ resection, 3′ single-stranded DNA (ssDNA) invasion of the one break end into the donor sequence and extension of the invading strand (Figure 4f). However, it is also possible that one 3′ ssDNA of the homologous vector can invade the donor DNA with the same mechanism. In our setup, this can lead to the restoration of a functional extrachromosomal *SuR* gene, which might integrate elsewhere in the genome by NHEJ. These kinds of events are classified as ectopic targeting events, as the respective cells carry the desired change at an ectopic position whereas the targeted genomic locus remains unaltered. Indeed, our experiments performed in Arabidopsis demonstrated that this class of events occurs in about half of the cases (Merker *et al*., 2020; Wolter *et al*., 2018; Wolter and Puchta, 2019). In our current experiments, the ratio of perfect GT is somewhat higher (Table 1). However, we are not sure if the difference is significant and whether, if at all, it might be due to the different GT methods, as the ipGT vector has to be chromosomally integrated before its activation by DSB induction. In any case, our current GT protocol, in contrast to ipGT does not require vector integration. Two lines, ttLb-3 and ttLb-6, carry heritable GT events but without the presence of a transgene. This is important when it comes to practical applications. Even if plants with an integrated transgene are not banned from the fields in a row of countries, they are required to go through a much more cost- and time-intensive regulation process before they can be used in agriculture.

To further characterize the SDSA pathway, we used the presence of mutations from the GT vector in the targeted genomic locus for estimating the maximal mean conversion tract length. Such analysis should also give a hint on the required length of the homologous arms for efficient GT (Figure 3e). Using the percentile probability of gene conversion length, we estimated the predicted maximal conversion length from our GT events. The required lengths differ between GT constructs, ranging from 0.6 kb to 1.3 kb. Interestingly, the two synonymous modifications, both of which reside on the left homologous arm of the Sa-at GT donor and cannot be selected by imazapyr, were incorporated into the genome with a lower chance indicating a selection bias. Similar results of GT with higher gene conversion rates at the selectable side were observed in human cells, too (Kan *et al*., 2014). All in all, our results suggest that a homology length of 0.5 to 1 kb for each end of the DSB should be used when designing GT vectors. The use of longer homologies might not be very helpful for increasing GT efficiencies further. On the other side, it might be very difficult to induce changes in the genome if the DSB is induced at distance of more than 0.5 kb away from pre-designed changes.

To summarize, we could demonstrate that with ttLbCas12a, efficient GT can be achieved in tobacco. Application of ttLbCas12a is highly likely to further enhance other established GT systems that used wild-type LbCas12a in rice or the geminivirus approach in tomato (Li *et al*., 2020; van Vu *et al*., 2020). Moreover, it might also be well suited for ipGT in maize with the help of heat shock expression (Barone *et al*., 2020), which itself might also improve ttLbCas12a activity. As well as for simple induction of mutations, ttLbCas12a seems to be an attractive alternative to other CRSPR/Cas nuclease used in plants by now.

Methods

Construction and cloning

Protospacers were cloned into entry vectors as previously described (Steinert *et al*., 2015) and primers used for cloning are listed in Table S1. GT donors were synthesized by BioCat (Data S3). Constructs for mutagenesis were recombined between entry vector and destination vector using LR Clonase II (Thermo). The GT donor and gRNA expression cassettes were assembled into the binary vectors with the SaCas9 or LbCas12a expression cassette by Instant Sticky-end Ligase Master Mix (New England Biolabs). The destination and entry vectors used, pDe-SaCas9 and pEn-Sa-Chimera, were described before (Steinert *et al*., 2015). For LbCas12a and ttLbCas12a, vectors were used as described previously (Schindele and Puchta, 2020), only that the rubisco terminator from pea was replaced by the rubisco terminator from chrysanthemum (Outchkourov *et al*., 2003).

Tobacco transformation and culture condition

Nicotiana tabacum L. cv. Petite Havana line SR1 plants were used. Seeds were germinated on MS medium (Murashige & Skoog, Serva) with 3% sucrose, 0.8% agar in Petri dishes of 2 cm height (Sarstedt AG & Co. KG), 16-hour day/night cycle. For GT, agrobacterium strain GV3101 was used for vacuum infiltration. The infiltration method was modified from a previous study (Puchta, 1999). Bacterial densities of OD_{560} were measured to determine the amount of agrobacterium equal to $OD_{560} = 1$ (an optical density of 1 at 560 nm) in 50 mL. The proper amount of bacteria was transferred to falcon tubes and centrifuged for 8 min of 5000***g*** at room temperature, then resuspended and washed twice with 15 mL of 10 mM $MgSO_4$. After centrifugation, bacteria were resuspended in 50 mL of 10 mM $MgSO_4$ solution with acetosyringone 100 mg/L. For each plate with two-week-old tobacco seedlings, 25 mL agrobacterium solution was poured into the plate. After 250 mbar vacuum for 15 min, agrobacterium solution was discarded, seedlings and plate were washed twice with 25 mL sterilized H_2O. Fully infiltrated cotyledons were marked, and seedlings were incubated at 22°C for 3 days. Afterwards, the marked cotyledons were transferred to 24-well plates with callus-inducing medium containing kanamycin

100 mg/L, cefotaxime 500 mg/L, and hormones, as described previously (Salomon and Puchta, 1998). The plant material was transferred to fresh medium every 7 days. After two weeks, plant samples were transferred to fresh callus-inducing medium with 0.5 μM imazapyr every week. After incubating in 24-well plates for four weeks, six to nine calli were transferred to fresh 0.5 μM imazapyr medium in Petri dishes each week. Dead areas and browning tissues were removed during each transfer. Shoots were transferred to MS medium without hormones for root regeneration. After 10–16 weeks, tissue samples from calli or T0 seedlings were taken for genomic DNA extraction.

For mutagenesis analysis, the same transformation method was used but GV3101 was replaced by EHA105, a kind gift from Dr. Bert van der Zaal's lab. After transformation for three days, marked cotyledons were transferred to callus-inducing medium containing kanamycin 100 mg/L. Dead areas and browning tissues were removed each week and trimmed samples were transferred to the fresh medium.

Mutagenesis and gene targeting analysis

Both Sanger sequencing and next-generation sequencing (NGS) reactions were performed by Eurofins. Tracking of Indels by Decomposition (TIDE) analysis was used to determine the efficiency of mutagenesis in each callus sample (Brinkman *et al*., 2014). The target *SuRB* and *SuRA* loci were amplified by PCR and the chromatograms of Sanger sequencing were analysed, using the sequencing trace files from wild-type as control.

We used Q5 High-Fidelity DNA Polymerase (NEB) to reduce PCR errors during amplification. Two amplicons were sequenced by NGSelect Amplicon 2nd PCR platform (Eurofins). The NGS results of fastq files were analysed by Cas-analyser (Park *et al*., 2017), using the common sequence from *SuRB* and *SuRA* (Data S2), and then analysed by Excel.

Primers and amplicons used for CAPS assay and GT analysis were listed (Table S1). After amplification, purification was performed by the peqGOLD Cycle-Pure Kit (Peqlab). Restriction enzymes for CAPS assay were purchased from NEB and digestion was performed overnight at 37°C.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

T.H. and H.P. designed the experiments and wrote the manuscript. T.H. and B.A. performed experiments and analysed data. T.H., B.A., P.S. and H.P. edited the manuscript. H.P. supervised the project.

Data availability statement

All DNA constructs as well as sequence files not listed in the supporting information are available upon request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Alignment of *SuRB* gene with modified *ALS* gene from Arabidopsis in Sa-at vector.
Figure S2 Survival trends of imazapyr-resistant calli with different GT vectors and culture conditions.
Table S1. Primers
Data S1 *SuRB* and *SuRA* sequence.
Data S2 *SuRB* and *SuRA* sequences for NGS analysis.
Data S3 Gene target donor template sequence.

