the plant journal

SEB Society for Experimental Biology

The Plant Journal (2019) doi: 10.1111/tpj.14488

TECHNICAL ADVANCE

In planta gene targeting can be enhanced by the use of CRISPR/Cas12a

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Received 26 May 2019; revised 8 July 2019; accepted 29 July 2019.
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SUMMARY

The controlled change of plant genomes by homologous recombination (HR) is still difficult to achieve. We previously developed the *in planta* gene targeting (ipGT) technology which depends on the simultaneous activation of the target locus by a double-strand break and the excision of the target vector. Whereas the use of SpCas9 resulted in low ipGT frequencies in Arabidopsis, we were recently able to improve the efficiency by using egg cell-specific expression of the potent but less broadly applicable SaCas9 nuclease. In this study, we now tested whether we could improve ipGT further, by either performing it in cells with enhanced intrachromosomal HR efficiencies or by the use of Cas12a, a different kind of CRISPR/Cas nuclease with an alternative cutting mechanism. We could show before that plants possess three kinds of DNA ATPase complexes, which all lead to instabilities of homologous genomic repeats if lost by mutation. As these proteins act in independent pathways, we tested ipGT in double mutants in which intrachromosomal HR is enhanced 20–80-fold. However, we were not able to obtain higher ipGT frequencies, indicating that mechanisms for gene targeting (GT) and chromosomal repeat-induced HR differ. However, using LbCas12a, the GT frequencies were higher than with SaCas9, despite a lower non-homologous end-joining (NHEJ) induction efficiency, demonstrating the particular suitability of Cas12a to induce HR. As SaCas9 has substantial restrictions due to its longer GC rich PAM sequence, the use of LbCas12a with its AT-rich PAM broadens the range of ipGT drastically, particularly when targeting in CG-deserts like promoters and introns.

Keywords: CRISPR/Cas, genome editing, genome instability, double-strand break repair, homologous recombination, RTEL-1, Fanconi anaemia, blooms syndrome, *Arabidopsis thaliana*, technical advance.

INTRODUCTION

The emergence of the CRISPR/Cas technology enabled targeted double-strand break (DSB) induction at high efficiency in a simple way (Jinek *et al.*, 2012; Le Cong *et al.*, 2013). Since then, a broad range of new applications of the technology is currently transforming plant biology (Baltes *et al.*, 2017; Malzahn *et al.*, 2017; Puchta, 2017; Kumlehn *et al.*, 2018; Langner *et al.*, 2018; Chen *et al.*, 2019; Sedeek *et al.*, 2019; Wolter *et al.*, 2019). The stimulatory potential of targeted DSB induction for non-homologous end-joining (NHEJ)-mediated gene knockout and homologous recombination (HR)-mediated gene targeting (GT) in plants was already demonstrated long before the development of CRISPR/Cas (Puchta *et al.*, 1996; Salomon and Puchta, 1998). Whereas NHEJ-based targeted mutagenesis is now routine in plants, directed repair of DSBs via HR GT still poses a considerable challenge, as it requires the efficient simultaneous delivery of the nuclease for induction of a DSB in the target site as well as a repair template for HR. The first problem is that even when extrachromosomal DNA containing homologous sequences is available, NHEJ is by far the predominant pathway of DSB repair in plants (Puchta, 2005; Knoll *et al.*, 2014a,b). The second problem is that many crops still lack efficient transformation and/or regeneration procedures (Altpeter *et al.*, 2016). Isolated protoplasts can be transformed at high efficiency and GT is efficient in protoplasts (Townsend *et al.*, 2009; Zhang *et al.*, 2010), but to date, efficient regeneration of plants from protoplast is not possible for most crops (Puchta and Fauser, 2013). An exception is tobacco: GT in tobacco protoplasts with subsequent plant regeneration was already demonstrated with ZFNs and TALENs (Townsend *et al.*, 2009; Zhang *et al.*, 2013).

A possible way to favour HR as opposed to NHEJ-mediated DSB repair in plants is increasing repair template copy number by employing the geminiviral replication mechanism (Gutierrez, 1999; Gutierrez *et al.*, 2004; Baltes *et al.*, 2014). Using this approach, developed by the Voytas group, efficient GT via *Agrobacterium*-mediated transformation of cotyledons or leaf explants and subsequent plant regeneration in tomato and potato was reported until now (Baltes *et al.*, 2014; Cermak *et al.*, 2015; Butler *et al.*, 2016; Dahan-Meir *et al.*, 2018). Similar approaches have been successful in rice and wheat, albeit without subsequent plant regeneration (Gil-Humanes *et al.*, 2017; Wang *et al.*, 2017a). However, in Arabidopsis viral replication of the repair template was inefficient and, even though it could be detected, it failed to improve GT efficiency (Hahn *et al.*, 2018; de Pater *et al.*, 2018; Shan *et al.*, 2018). Biolistic transformation is another method that enables delivery of high amounts of repair template compared with *Agrobacterium*-mediated transformation (Altpeter *et al.*, 2016). Using biolistic delivery of Cas9 as well as a single-stranded repair template and subsequent regeneration of immature embryos, precise gene modifications and insertions were achieved in maize (Svitashev *et al.*, 2015), rice (Sun *et al.*, 2016), and soybean (Li *et al.*, 2015). Other attempts to shift the balance between NHEJ and HR include repression or knockout of NHEJ factors (Jia *et al.*, 2012; Qi *et al.*, 2013; Endo *et al.*, 2015; Nishizawa-Yokoi *et al.*, 2016), or employment of NHEJ inhibiting or HR promoting small molecules (van Chu *et al.*, 2015; Maruyama *et al.*, 2015; Yu *et al.*, 2015; Song *et al.*, 2016; Shao *et al.*, 2017). However, no study has been published to date on performing GT in plant cells and showing enhanced HR efficiency due to mutations in factors suppressing genomic instability.

Previously, we developed the *in planta* GT strategy, aimed at addressing the lack of efficient transformation and regeneration procedures for many crops. Here, the nuclease, gRNA, and repair template are stably integrated into the plant genome and the nuclease not only induces a DSB in the target, but also excises the repair template, activating both for HR (Fauser *et al.*, 2012). Consequently, GT events can happen anytime during the life cycle of the plant and when entering the germ-line, heritable GT events can be harvested as seeds in the next generation. Accordingly, in principle a single successful transformation event is sufficient to generate heritable GT events. This should make GT-mediated precise genome modifications amenable even to such crops that are still recalcitrant to efficient transformation and regeneration. As a proof-of-concept, site-specific insertions were achieved in Arabidopsis using a single T-DNA carrying both the sequence-specific nuclease and the HR donor (Schiml et al., 2014). However, the efficiency in these proof-of-concept experiments was not satisfactory. Recently, the technology was further improved. By combining *in planta* GT with viral replication of the repair template, the efficiency could be strongly increased in tomato (Dahan-Meir *et al.*, 2018). While Arabidopsis proved to be recalcitrant to geminiviral donor replication (Hahn *et al.*, 2018; de Pater *et al.*, 2018), we achieved efficiency improvements in Arabidopsis by using *Staphylococcus aureus* Cas9 (SaCas9) as the nuclease and expressing it from an egg cell-specific promoter (Wolter *et al.*, 2018).

Recent studies have identified three different ATPase containing protein complexes as suppressors of HR, as mutants lacking these factors showed a strong hyper-recombination phenotype (Hartung *et al.*, 2007, 2008; Knoll *et al.*, 2012; Recker *et al.*, 2014; Röhrig *et al.*, 2016). This suggests that, in the absence of these factors, the efficiency of *in planta* GT might be further enhanced.

Therefore, in this report, we tested whether the efficiency of the system can be further improved by the absence of HR suppressors. Eukaryotes possess a number of DNA helicases/ATPases that suppress HR between chromosomal sequences, by dissolving DNA recombination intermediates (Knoll and Puchta, 2011). This area is of specific importance for organisms with large, highly repetitive genomes like plants. In the last decade, our group was able to identify the respective factors for plants (Hartung *et al.*, 2007, 2008; Bonnet *et al.*, 2013; Schröpfer *et al.*, 2014; Röhrig *et al.*, 2016; Dorn *et al.*, 2018). The helicase AtRECQ4A forms the RTR complex together with the proteins AtTOP3α, AtRMI1, and AtRMI2 (Xu *et al.*, 2008). The knockout of any member of this complex leads to enhanced recombination frequencies between chromosomal repeats as shown with transgenic markers that are restored by HR (Puchta and Hohn, 2012).

The DNA translocase FANCM (Fanconi anaemia complementation group M) acts together with many other factors in the Fanconi anaemia (FA) core complex, which is involved in interstrand crosslink repair to ensure replication progression (Gari *et al.*, 2008). In plants, FANCM is involved in interstrand crosslink repair in a parallel pathway to RECQ4A (Dangel *et al.*, 2014) and suppresses HR (Knoll *et al.*, 2012). Interestingly, the group of Raphael Mercier could show that both helicases also play important but independent roles in meiotic HR, where they are involved in the suppression of meiotic crossovers (Crismani *et al.*, 2012; Serra *et al.*, 2018).

The iron-sulfur cluster-containing helicase RTEL1 (Regulator of Telomere Elongation Helicase1) has the ability to disassemble D-loop-like recombination intermediates. In addition to its crucial role in telomere maintenance (Vannier *et al.*, 2012), RTEL1 uses its D-loop disrupting activity to reverse HR processes (Barber *et al.*, 2008; Youds *et al.*, 2010) and therefore acts as an HR suppressor. The Arabidopsis homologue of RTEL1 also contributes to telomere maintenance, rDNA stability, and is involved in intrastrand

and interstrand crosslink repair (Recker *et al.*, 2014; Hu *et al.*, 2015; Röhrig *et al.*, 2016).

Importantly, RTEL1 acts in a pathway independent of the RTR complex as a suppressor of somatic HR. Dual loss of both HR suppressors RMI2 and RTEL1 leads to strong hyperrecombination, with 80-fold elevated HR-rate, resulting in a high degree of genomic instability associated with considerable growth and fertility defects and loss of 45S rDNA repeats (Röhrig *et al.*, 2016). Furthermore, the HR suppression activity of RTEL1 is also independent from that of FANCM, as the double mutant shows an increase of chromosomal HR of about 20 times (Recker *et al.*, 2014).

We speculated that such an increase in chromosomal HR efficiency might also be beneficial for GT, which is also based on HR. Therefore, we investigated whether the *rtel1 rmi2* and *rtel1 fancm* double mutants also show elevated GT rates.

Unfortunately, the long guanine rich PAM required for efficient SaCas9 cleavage (NNGRRT with R being G or A) constrains the flexibility of the system. Guanine-poor regions like promoters and introns are especially difficult to target with SaCas9. Therefore, we tested whether LbCas12a (Zetsche *et al.*, 2015), with its thymine rich PAM (TTTV), which occurs also more frequent, can serve as an efficient alternative nuclease for *in planta* GT. In addition, testing Cas12a variant was especially interesting as other reports, unfortunately based on very small numbers of GT events, claimed that the use of Cas12a as a nuclease might result in higher GT frequencies than the use of Cas9 (Begemann *et al.*, 2017; Ferenczi *et al.*, 2017; Moreno-Mateos *et al.*, 2017).

In general, two properties render Cas12a highly attractive for the *in planta* GT system and for HR based applications. On the one hand, Cas12a has different PAM requirements. The AT-rich PAM of Cas12a is an ideal complement to the CG-rich PAM of Cas9, and occurs more frequently within the genome. On the other hand, Cas12a cleaves the target DNA on the PAM distal side. In contrast with Cas9, this is far away from the seed sequence, which serves as nucleation site for base pairing between the crRNA and the target DNA (Fonfara *et al.*, 2016). Therefore, mismatches between crRNA and target DNA arising from erroneous NHEJ repair of cutting events will immediately disrupt further cleavage in the case of Cas9, whereas further cleavage reactions might still occur in the case of Cas12a. As after each cutting HR is again competing with NHEJ for DSB repair, the overall chances for HR rise over time as long as the re-cutting of the NHEJ repaired target site is possible. Therefore, we decided to analyse the potential of Cas12a for application as nuclease in the *in planta* GT system.

To test these questions, we made use of our previously established herbicide assay system (Wolter *et al.*, 2018), where GT efficiency can be determined in a fast and efficient manner by introducing a herbicide resistance conferring point mutation via GT into the Arabidopsis genome and quantifying the number of resistant seedlings on respective herbicide containing medium. The system is based on the S653N point mutation in the acetolactate synthase gene (*ALS*, AT3G48560), which encodes for an essential enzyme required for the synthesis of branched chain amino acids. ALS activity is inhibited by sulfonylurea and imidazolin on herbicides (Chang and Duggleby, 1998; Yu and Powles, 2014). However, several point mutations rendering the enzyme resistant to specific herbicides are known (Lee *et al.*, 1988), the S653N mutation being one of them. This mutation confers resistance to the herbicide Imazapyr (IM) by prohibiting IM binding without incurring a major reduction in ALS activity (Garcia *et al.*, 2017).

RESULTS

Absence of HR suppressors RTEL1, RMI2 and FANCM1 does not enhance GT efficiency

In order to test whether the absence of HR suppressors can improve our *in planta* GT system, our previous GT approach using egg cell-specific SaCas9 expression was applied to these two double mutants by transforming the same GT construct used previously into the respective double mutants. The HR donor on this construct contains the S653N point mutation (mediating Imazapyr (IM) resistance) and homology arms (800 and 742 bp) on both sides of the break, which is induced 120 bp from the S653N point mutation (see Figure 1). Additionally, the donor contains *S. aureus* Cas9 recognition sequences at the flanks to enable excision from the genome and silent point mutations spanning the gRNA and PAM to prevent cleavage following successful HR.

In total, 66 transgenic lines for the *rtel1 fancm* background (the *rtel1-1 fancm-1* mutant were used), and 31 for the *rtel1 rmi2* background (the *rtel1-1 rmi2-2* mutant was used) were analyzed. The seeds of the T1 lines were harvested separately and sown on herbicide (IM)-containing medium to quantify the amount of herbicide-resistant T2 progeny. The results showed that GT efficiency was not enhanced in the absence of these factors (see Figure 2 and Table 1). Instead, a decrease was apparent, both in the rate of GT competent lines and average GT efficiency. Due to the reduced vitality of the double mutants, the germination rate was determined and was found to be around 90% in both double mutants, compared with 100% in wild-type. Therefore, the slightly reduced germination rate also lowered the obtained GT rate to some extent. However, even correcting the obtained GT efficiencies by this germination factor did not elevate the efficiencies above WT. Interestingly, for the *rtel1 fancm* background, a high amount of T1 lines that generated almost exclusively resistant seedlings was found (not included in the 'resistant plants (T2)'

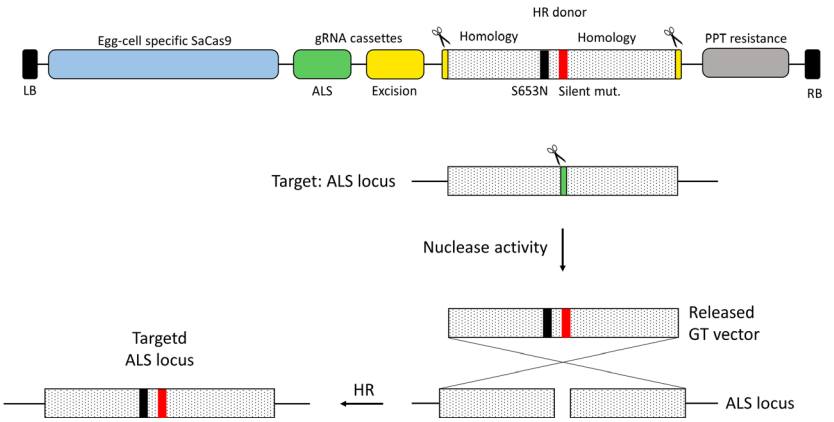

Figure 1. Design of the *in planta* GT assay. Arabidopsis plants were transformed with a T-DNA containing an egg cell-specific SaCas9 expression cassette, two sgRNA expression cassettes and the donor for HR. One sgRNA was programmed to cleave in *ALS* (in green), the other targeted a sequence flanking the HR donor (in yellow), which harbours a 1542-bp homology containing the S653N point mutation and silent mutations at the cleavage site. Three DSBs were induced, leading to simultaneous activation of the target site and excision of the HR donor, which can then can be used as template for repair of the target site by HR.

column but referred to as 'GT T1 lines' in Table 1): from 5 of 66 lines, all T2 progeny was resistant, indicating a GT event happening in the T1 egg cell immediately after transformation. However, the low amount of five events prohibits a safe statistical conclusion on this anomaly.

We took a representative sample of herbicide-resistant seedlings and analyzed the GT events at the molecular level. To prevent amplification of unintegrated donor, we used a primer combination binding outside the homology region (FW183 + FW58), as described previously (Wolter *et al.*, 2018). We found correct integration of the S653N point mutation, silent mutations, and correct junctions in 36% of herbicide-resistant plants (17 of 47 analyzed) for the *rtel1 fancm* background and 40% of resistant plants for the *rtel1 rmi2* background (20 of 50 analyzed). With only one exception, the S653N and silent mutations were changed by HR in only one allele, resulting in a heterozygous plant. We have found before with using the SaCas9 nuclease or the SpCas9 paired nickase approach that between 50 and 80% of the resistant seedlings arose due to ectopic targeting (Wolter *et al.*, 2018). Here, HR takes place also between target locus and targeting vector. However, in this reaction, the S653N point mutation is not corrected in the target locus but instead the truncated *ALS* gene of the vector is restored by the use of the information of the target locus. Integration then occurs elsewhere in the genome at an ectopic site and the target locus is not changed. Therefore, seedlings whose resistance was not caused by the introduction of the S653N mutation in the target locus were analyzed for ectopic GT events using the primer combination FW183 + FW57 as described previously (Wolter *et al.*, 2018) (see Figure S1). Indeed, we found that this was the case for 93% of resistant plants carrying no S653N mutation in the target locus in the *rtel1 fancm* background, and for 81% of resistant plants without S653N mutation in the target locus in the *rtel1 rmi2* background.

Cas12a is an efficient alternative nuclease for *in planta* GT

We decided to test the Cas12a orthologue from *Lachnospiraceae bacterium ND2006* due to its comparably high

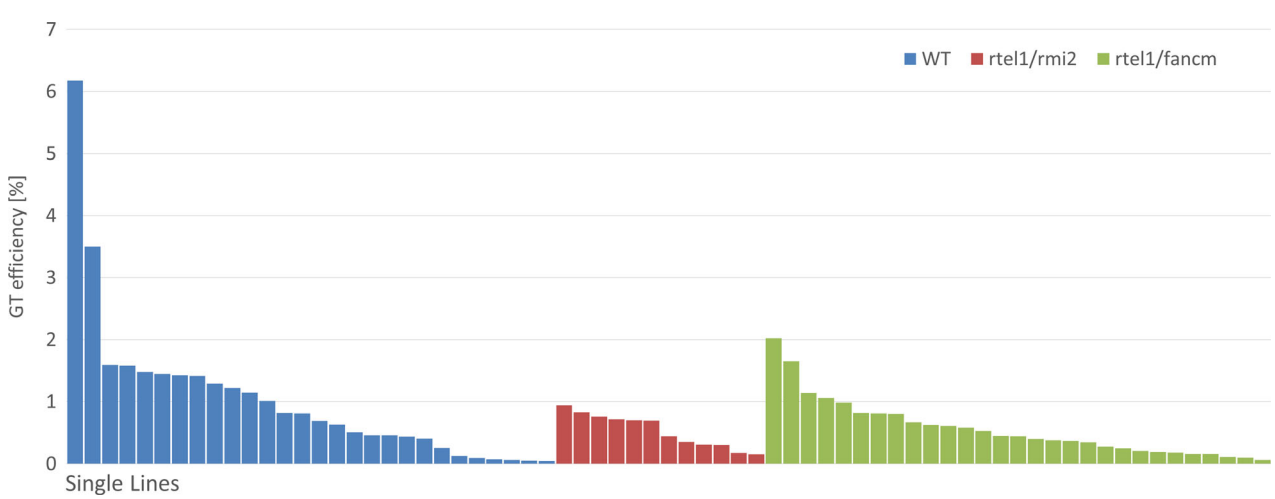

Figure 2. ipGT efficiency in *rtel1-1 fancm-1* and *rtel1-1 rmi2-2* double mutant backgrounds. GT efficiencies of single lines are shown in descending order (each bar represents the GT efficiency of a single line). Only positive lines generating GT events in the T2 generation are shown. For the double mutants, the real data are shown, without an adjustment for the lowered germination rate (see text for details).

Table 1 ipGT efficiency in *rtel1-1 fancm-1* and *rtel1-1 rmi2-2* double mutant backgrounds

Experiment	Number of T1 lines	GT competent T1 lines	Competent line rate	Inherited GT T1 lines	Amount of seeds (T2, estimate)	IM-resistant plants (T2)	Mean GT rate (%) (T1 → T2)
WT	34	27	79	0	~50 k	353	0.98
Repetition of WT	40	28	70	1	~90 k	599	0.95
rtel1/fancm1	66	34	52	5	~40 k	116	0.45
rmi2/rtel1	31	13	42	0	~20 k	53	0.53

The column 'GT-positive lines' indicates the amount of lines generating heritable GT events in the T2 generation. The column 'GT T1 lines' shows the number of T1 lines giving rise to almost completely IM-resistant T2 plants indicative of a GT event occurring in T0 egg cell or early T1 zygote. The mean GT rate was calculated as follows: first, the GT efficiency for each line was determined individually by dividing the number of resistant T2 plants by the total number of T2 seeds. Next, the mean GT rate was determined by calculating the average GT efficiency of all GT-positive lines. The resistant plants from the 'GT T1 lines' were excluded from the calculation, as their high numbers (almost all progeny resistant) would distort the statistics. The GT rates given are based on IM-resistant seedlings, therefore they contain perfect as well as ectopic GT events.

efficiency in plants, which was reported to be superior to alternative Cas12a variants (Hu *et al.*, 2017; Kim *et al.*, 2017; Tang *et al.*, 2017; Wang *et al.*, 2017b). Furthermore, for expression of the crRNA, we used the ribozyme-based system described before by Tang *et al.* (2017) (see Figure S2). We chose a 23-nt target sequence mediating cleavage at position 1944 of the *ALS* CDS, which is located closer to the S653N point mutation site (position 1958) than the SaCas9 cleavage site used previously (position 1835) (see Figure 3). In addition, in this GT design a silent PAM mutation was introduced into the HR donor. This way, cleavage of the repair template can be blocked by a single nucleotide exchange, whereas in the previous GT design multiple silent mutations in the protospacer sequence were necessary.

Two constructs were generated for the Cas12a GT approach. Firstly, a GT construct containing Cas12a driven by the previously used egg cell-specific promoter, in addition to a HR donor which was flanked by the target sequence used for HR induction in *ALS*. Therefore, only a single crRNA cassette was required. In addition, a construct for constitutive Cas12a expression (PcUBI4-2 promoter (Ubiquitin) from, Fauser *et al.*, 2012) using the same crRNA was generated in order to check somatic mutagenesis efficiency at the target site. To compare the cutting efficiency between the LbCas12a and SaCas9 GT approaches, a construct for constitutive SaCas9 expression (same UBI promoter), together with the SaCas9 gRNA used previously, was generated as a control.

For verifying cleavage activity at the chosen target sites, DNA was extracted from 10 primary transformants for each approach (whole seedlings), after 20 days of growth. The target sequence was PCR amplified using the primers FW56 + FW57 and the amplicons, including an untransformed control, were sequenced using primer FW56. The mutagenesis efficiency was determined using the TIDE software (Brinkman *et al.*, 2014), which identifies the major induced mutations in the projected editing site by analyzing the sequence traces by a decomposition algorithm. For the mutation analysis, it needs to be kept in mind, that an essential gene (*ALS*) is targeted. Accordingly, a selection against mutagenesis will occur on the cellular level as well as during primary transformant selection. Heavily mutated plants will be weaker, and mutated cells will proliferate less efficiently. For the LbCas12a target site, InDel rates above background could be detected in 7 of 10 T1

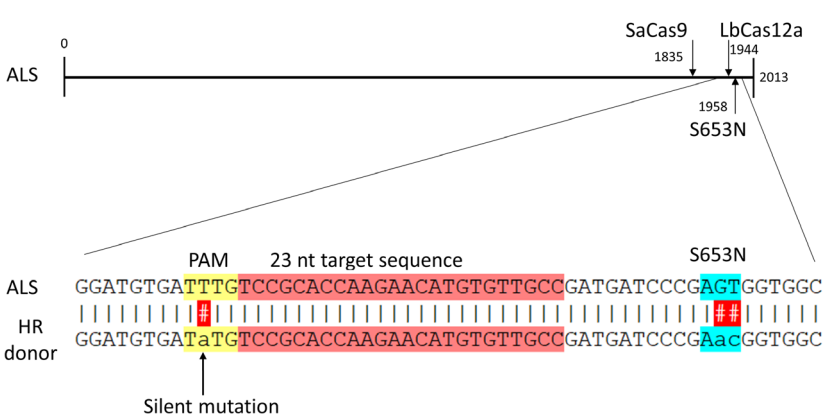

Figure 3. Cas12a GT design. On top, the *ALS* CDS is drawn to scale, with Cas9 and Cas12a target sequences and the point mutation site. Below is an alignment between the *ALS* and HR donor, showing the S653N point mutation and silent mutation as mismatches between the native *ALS* and HR donor sequence.

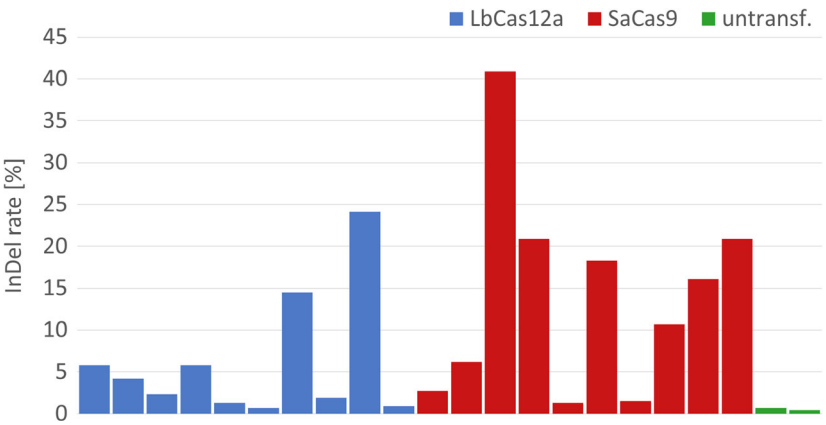

Figure 4. InDel rates of LbCas12a at the *ALS* locus. Mutagenesis is shown as the percentage of InDels. DNA was extracted from 20-day-old seedlings constitutively expressing LbCas12a and the respective crRNA. The mean InDel rates are 6.1% for LbCas12a and 13.9% for SaCas9.

seedlings (see Figure 4), whereas three lines did not show a clear difference to the untransformed control. The average InDel rate was 6.1%. The SaCas9 target site showed a higher cutting efficiency: 8 of 10 lines showed InDel rates that were unambiguously above the background level, with an average InDel rate of 13.9%. Therefore, SaCas9 was about twice as efficient as LbCas12a for initiating NHEJ-mediated mutations. This finding strongly indicates that SaCas9 is also about twice as efficient as LbCas12a in DSB induction at the target locus.

Following this, GT experiments were performed with LbCas12a. Primary transformants (T1 lines) were generated by floral dipping, grown to maturity and the seeds of each T1 line harvested individually. The seeds were then individually sown on herbicide containing medium to quantify the amount of resistant progeny obtained from each T1 line. In the two GT experiments, we performed with SaCas9 (Wolter *et al.*, 2018) we obtained very similar results resulting in an overall GT frequency of about 1% (0.98 and 0.95) across all T1 lines. More than two-thirds of all T1 lines generated heritable targeting events (79 and 70%). In the case of LbCas12a, a similar number of T1 lines were competent in generating GT events (71%); however, there was a 50% enhancement in the GT efficiency (1.47%) (Table 2). This is also reflected by the direct comparison of individual T1 lines. In the best case, a GT efficiency of about 6% could be achieved for both nucleases. For LbCas12a, 6 T1 lines (from 41) showed GT frequencies of more than 2%, whereas for SaCas9 there were only 2 (of 40) (see Figure 5). Therefore, in absolute numbers of GT events as well as in the proficiency of individual lines, the use of LbCas12a was superior over the use of SaCas9. This was especially surprising if we take into account that DSB induction at the target locus was twice as efficient for SaCas9 than for LbCas12a.

Finally, GT events were analyzed on the molecular level. Here, about half of the GT events (15 of 35 plants, 43%) were due to perfect homologous replacement of the targeted sequence by the vector at both DSB ends, similar to what we had previously found with SaCas9. In almost all other analyzed plants, the resistance was due to ectopic GT events, as described before (Wolter *et al.*, 2018) (see Table 3).

DISCUSSION

Enhancing GT efficiencies in plants has been a major effort for many years, as the nature of the plant cell renders this endeavour especially challenging (Puchta and Fauser,

Table 2 Overview of the ipGT experiment employing *Lb*Cas12a as the nuclease

Experiment	Number of T1 lines	GT competent T1 lines	Competent line rate	Inherited GT T1 lines	Amount of seeds (T2, estimate)	IM-resistant plants (T2)	Mean GT rate (%) (T1 → T2)
SaCas9	34	27	79	0	~50 k	353	0.98
SaCas9 repetition	40	28	70	1	~90 k	599	0.95
LbCas12a	41	29	71	0	~55 k	603	1.47

The column 'GT-positive lines' indicates the amount of lines generating heritable GT events in the T2 generation. The column 'GT T1 lines' shows the number of T1 lines giving rise to almost completely IM-resistant T2 plants indicative of a GT event occurring in T0 egg cell or early T1 zygote. The mean GT rate was calculated as follows: first, the GT efficiency for each line was determined individually by dividing the number of resistant T2 plants by the total number of T2 seeds. Next, the mean GT rate was determined by calculating the average GT efficiency of all GT-positive lines. The resistant plants from the 'GT T1 lines' were excluded from the calculation, as their high numbers (almost all progeny resistant) would distort the statistics. The GT rates given are based on IM-resistant seedlings, therefore they contain perfect as well as ectopic GT events.

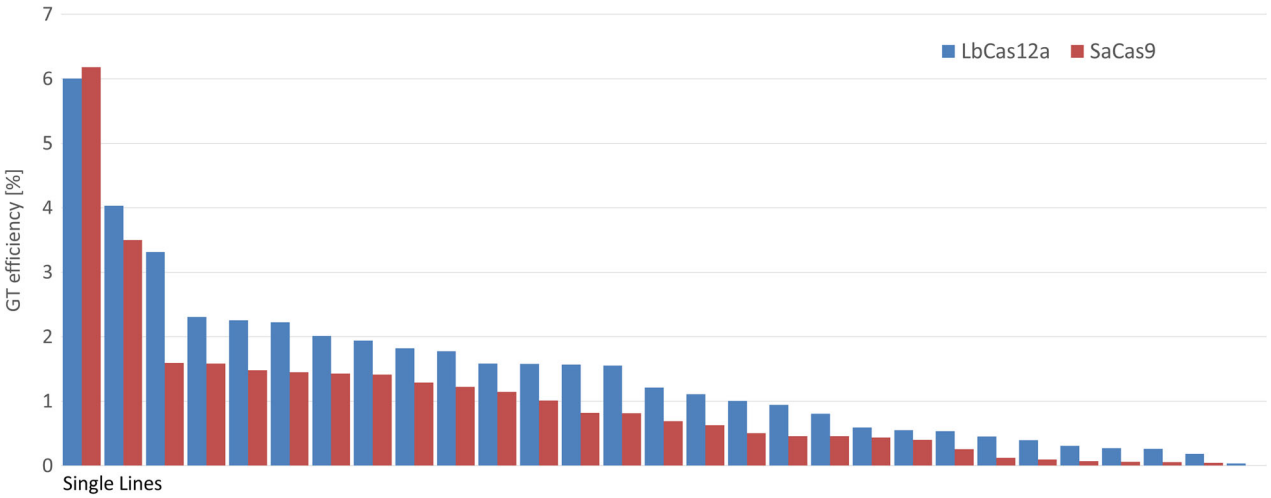

Figure 5. Direct comparison of ipGT efficiencies using either *Lb*Cas12a or *Sa*Cas9 as nuclease. Each bar represents the GT efficiency of a single line. The analyzed lines for both approaches are ordered in a descending manner. Only lines generating heritable GT events are shown.

2013). The presence of the cell wall impedes high transformation rates in natural plant cells, such as those obtained in mammalian tissue culture, and the balance between HR and NHEJ is greatly shifted towards the latter. Accordingly, diverse attempts have been undertaken to address the challenge of efficient GT in plants (see Steinert *et al.*, 2016; Huang and Puchta, 2019; for recent reviews). In this communication, we addressed the issue as to whether *in planta* GT can be improved by deactivating the suppression of chromosomal HR or by applying a different kind of CRISPR/Cas nuclease.

First, it was investigated whether the absence of specific HR suppressing factors in respective genetic backgrounds has a stimulatory effect on *in planta* GT. For this purpose, the two double mutants *rtel1 fancm* and *rtel1 rmi2* were transformed with our previously described egg cell-specific GT construct (DSB induction by SaCas9 expressed from egg cell-specific promoter), and the results were compared with the efficiencies obtained from WT plants transformed with the same construct. From the results, it could be concluded that both double mutant backgrounds do not have a stimulatory effect on GT efficiencies. This result came as a surprise, as these two mutants showed around 20-fold (*rtel1 fancm*) and around 80-fold (*rtel1 rmi2*) increased HR frequencies in HR assays when measuring somatic HR events between homologous chromosomes or the sister chromatid (Recker *et al.*, 2014; Röhrig *et al.*, 2016). This raised the question why this was the case. The RTR complex, as well as FANCM and RTEL1, are required for either avoiding or repairing DNA damage arising during DNA replication. It is assumed that chromosomal homologous sequences become instable in their absence due to the stalling of replication forks. This situation is in line with the finding that mild replication defects induced by weak alleles of the replicative polymerase delta catalytic subunit also show enhanced frequencies of HR between chromosomal repeats (Schuermann *et al.*, 2009).

Table 3 Molecular analysis of LbCas21a-mediated ipGT compared with SaCas9-mediated ipGT

	SaCas9	LbCas12a
Resistant plants analyzed	72	35
Resistance from perfect GT events	36 (50%)	15 (43%)
Resistance from ectopic GT events	35	17
No GT event	1	0
Amplification not possible	0	3

Indeed, when a DSB was induced by a site-specific endonuclease between repeats in the Arabidopsis genome in the mutant background of *recq4a*, another member of the RTR complex, no enhancement but a reduction of HR efficiency was observed (Mannuss *et al.*, 2010). Cas9 induced DSBs are two sided and replication-independent. Their repair using a chromosomal homology as template is best described by the synthesis-dependent strand annealing (SDSA) model of recombination (Puchta, 2005). Indeed, also for GT, it was shown early on that the reaction is in line with the SDSA model (Puchta, 1998), which was confirmed by various studies (for review see Huang and Puchta, 2019). However, replication associated HR is initiated by a single break end, and repair occurs by a number of other mechanisms (Knoll *et al.*, 2014a). Therefore, our result clearly showed that *in planta* GT cannot be enhanced by an experimental situation that promotes replicative HR.

In addition to different Cas9 orthologues, we now tested a completely different CRISPR/Cas nuclease for *in planta* GT, namely Cas12a. Surprisingly, a GT efficiency was

achieved that was higher to that was obtained using SaCas9. This applies for the absolute number of GT events as well as for the number of T1 lines with higher recombination frequencies. Therefore, LbCas12a is a highly attractive alternative to SaCas9 as a nuclease for *in planta* GT. In addition to the 3.3 million sites of the SaCas9 GRRT PAM in the Arabidopsis genome, 6.5 million sites of the LbCas12a TTTV PAM are now accessible. For the more relaxed TTTN PAM of LbCas12a, there are even 10.5 million sites. In addition, specific genomic regions such as promoters and introns have a naturally very low GC content, which makes such genomic regions difficult to target by Cas9 variants with their CG-rich PAMs. However, such genomic regions are consequently very AT rich, this renders them easily targetable by LbCas12a with its AT-rich PAM. Therefore, the opportunity to use LbCas12a for *in planta* GT substantially broadened the range of targetable sequences and made the system much more flexible. It should be noted that Cas9 and Cas12a variants with engineered PAMs have been developed, for example xCas9 (Hu *et al.*, 2018) which recognizes a broad range of PAM sequences including NG, GAA and GAT, and enAsCas12a (Kleinstiver *et al.*, 2019) which not only exhibits substantially extended targeting range but also improved activity and specificity. It would be interesting to see whether ipGT can be further improved by testing these engineered nucleases.

In addition, an interesting finding from this study is the non-linear relationship when comparing the different nucleases in terms of cleavage efficiency and GT efficiency. Using constitutive nuclease expression, we obtained a higher cleavage efficiency at the SaCas9 target site than at the LbCas12a target site. In contrast, using the same target sites, LbCas12a was superior concerning GT efficiency. This finding suggests that nuclease properties other than cleavage efficiency influence GT efficiency. For LbCas12a, two properties are conceivable that might promote GT. First, LbCas12a does not generate blunt ends but staggered breaks with 5′ overhangs (Zetsche *et al.*, 2015). Second, LbCas12a cleaves the DNA distant from the seed region (Zetsche *et al.*, 2015) which might allow further cleavage even after NHEJ-mediated minor mutations at the cleavage site. Previously we have tested the effect of longer 5′ overhangs for *in planta* GT, which we induced by the use of the paired nickase approach (Schiml *et al.*, 2014, 2016). As this did not lead to higher GT frequencies compared with the use of the SaCas9 nuclease, we are favouring the latter hypothesis.

Other recent publications also demonstrated the high suitability of Cas12a as nuclease for GT applications. In zebrafish, LbCas12a achieved higher HR mediated DNA insertion rates than *Sp*Cas9 in direct comparison (Moreno-Mateos *et al.*, 2017). Although based on small numbers, in an experimental setup involving biolistic transformation of rice, *Fn*Cas12a achieved GT frequencies of up to 8% (Begemann *et al.*, 2017). When delivered as pre-assembled ribonucleoprotein complex into *Chlamydomonas*, *Lb*Cas12a was highly efficient at homology-directed DNA replacement (Ferenczi *et al.*, 2017). In an experiment involving single-stranded oligonucleotides as repair template, LbCas12a clearly outperformed SpCas9 for GT applications in human cells (Wang *et al.*, 2018). However, when plasmids were used as repair template, SpCas9 was more efficient. Recently, LbCas12a was also successfully applied for GT in rice using biolistic transformation of rice calli (Li *et al.*, 2018) and in tomato using *Agrobacterium*-mediated cotyledon transformation (van Vu *et al.*, 2019). In the latter report, LbCas12a showed stronger GT efficiency compared with SpCas9 in direct comparison, especially at higher temperatures.

Besides demonstrating that LbCas12a is especially suited for performing *in planta* GT experiments in Arabidopsis, our data clearly showed that, independently of which

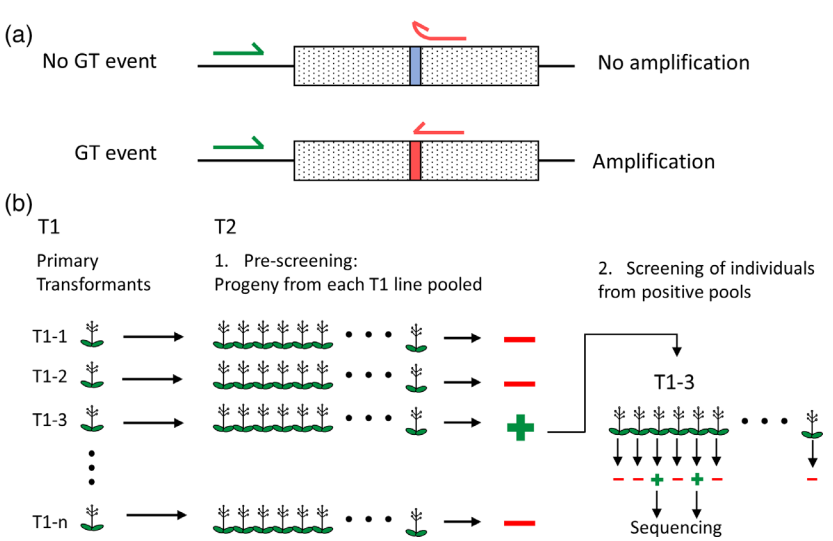

Figure 6. Two-step screening procedure for identification of correct ipGT-positive plants. (a) PCR primers can be designed in such a way that a product is only produced if the modified nucleotides at the sensitive 3′ end of the primer binding site are integrated into the target locus. This way, amplification is inhibited if the target site exhibits the unmodified sequence. (b) In a first step, pools of T2 progeny from different T1 lines are screened for positive signals. In a second step, individuals from pools identified as positive in the first step are screened for correct GT, which can be confirmed by sequencing.

nuclease was used, T1 lines would be obtained that differ drastically in the number of GT events in the population of seedlings in the T2 generation. Therefore, it is important to apply a sophisticated PCR screening protocol for identifying GT events to minimize the work load in case no phenotypic change can be used for the identification of targeted events (see also Miki *et al.*, 2018). For this purpose, we propose a two-step PCR screening protocol: in a first round of screening, pools of about 100 T2 seedlings from around 20 lines can be screened for a positive GT signal taking only a leaf as sample. T2 pools of the three to five lines showing the strongest positive signals are then used in a second round of screening to identify the positive individual(s) within the respective pool (see Figure 6). Using this two-step screening procedure, the time and effort required for identification of individuals with the desired genomic modification can be kept to a minimum while still assuring success. We recently applied a similar protocol for identifying successfully SaCas9-induced heritable chromosomal inversions in Arabidopsis (Schmidt *et al.*, 2019).

EXPERIMENTAL PROCEDURES

T-DNA constructs used in this study

For transformation of the *rtel1-1 rmi2-2* and *rtel1-1 fancm-1* mutant backgrounds, we modified our previously described egg cell-specific *S. aureus* GT construct pDSB/DSB-EC (Wolter *et al.*, 2018) by exchanging the resistance cassette from Kanamycin to PPT via HindIII.

The LbCas12a coding sequence was synthesized with Arabidopsis codon optimization and fused with a C-terminal SV40-NLS from BioCat (https://www.biocat.com). It was inserted into pDe-Cas9 in place of SpCas9 by *Asc*I, forming pDe-LbCas12a. Furthermore, it was inserted via *Asc*I into pDe-EC-SaCas9 (a destination vector containing SaCas9 driven by the egg cell promoter and rbcs-E9 terminator), instead of SaCas9, yielding pDe-EC-LbCas12a. A Gateway compatible entry-vector containing the Lb-crRNA flanked by ribozymes and driven by the AtU6-26 promoter was also synthesized from BioCat. crRNA programming of the entry-vector was achieved using oligos FW243 + FW244, which were inserted into the entry-vector as annealed oligos. The programmed crRNAs were transferred from the entry-vector to pDe-LbCas12a and pDe-EC-LbCas12a via Gateway reaction, resulting in pDe-LbCas12a-ALS. The former (pDe-Lb-Cas12a-ALS) was the final construct used for the InDel efficiency analysis. The HR donor for the Cas12a-mediated GT experiments was generated by Gibson assembly using our previously described HR donor as template. The fragments for the Gibson assembly were generated using the primers FW251-FW256. The resulting plasmid pDe-EC-LbCas12a-ALS-GTVc12 was the final construct used for the LbCas12a GT experiments. All constructs are available on request.

Plant transformation, selection and handling

Arabidopsis plants used in this study were exclusively of Columbia-0 (WG) ecotype background. If sterile conditions were required, seeds were sown on agar plates containing germination medium [GM; 4.9 g L^{-1} Murashige and Skoog medium (Murashige and Skoog, 1962), 10 g L^{-1} saccharose, pH 5.7, 7.6 g L^{-1} plant-agar]. If sterile conditions were not required, seeds were sown on substrate containing 1:1 Floraton 3 (Floragard Vertriebs GmbH, www.floragard.de) and vermiculite (Deutsche Vermiculite Dämmstoff GmbH, www.vermiculite.de). The transformation of the Arabidopsis plants was performed as previously described (Clough and Bent, 1998), using the *Agrobacterium* strain GV3101.

Determination of GT efficiency

Imazapyr (IM) herbicide assays for determination of GT efficiency were performed as described previously (Wolter *et al.*, 2018). In brief, primary transformants were identified on the respective selection medium, verified by PCR and grown to maturity. The seeds were harvested for each primary transformant separately and sown for each primary transformant independently on 5 μM IM-containing medium. The number of IM-resistant seedlings was determined after 14 days of growth.

Molecular characterization of GT events

The molecular characterization of GT events was performed as described previously (Wolter *et al.*, 2018). In brief, DNA was purified from resistant seedlings and the endogenous target site was amplified using primers FW58/FW183, followed by Sanger sequencing using primers FW56 and FW42 to check for the junctions and desired point mutations. For detection of ectopic GT events, the recombined T-DNA site was amplified using primers FW57/FW183 and Sanger sequencing was performed using FW56 and FW42.

Data availability statement

All DNA constructs are freely available on request as well as all original (sequencing) data files (requests to HP).

ACKNOWLEDGEMENTS

We thank Luisa Telpl, Sakia Gabsi, Maren Scheidle and Waltraud Wehrle for excellent technical assistance. This work was funded by the German Federal Ministry of Education and Research (PLANT 2030_Soph-Gen-Tom Grant 031B0530) and the European Research Council (ERC) (grant number ERC-2016-AdG_741306 CRISBREED).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

HP conceived the research. FW designed and executed the experiments. Both authors wrote the article.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Molecular analysis of GT events.

Figure S2. crRNA expression system used for *Lb*Cas12a and *Fn*Cas12a application.

Table S1. Detailed results of LbCas12a mediated *in planta* GT for each single line.

Table S2. Oligos used in this study.

Sequence S1. HR donor (*S. aureus* GT approach).

Sequence S2. SaCas9 expression cassette with egg cell-specific promoter and RBCs E9 terminator.

Sequence S3. sgRNA expression system for *S. aureus*.

Sequence S4. LbCas12a sequence used in this study (Arabidopsis codon optimized, with C-terminal nucleoplasmin NLS).

Sequence S5. crRNA expression system for LbCas12a.

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