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TECHNICAL ADVANCE

The CRISPR/Cas system can be used as nuclease for *in planta* gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny

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SUMMARY

The CRISPR/Cas nuclease is becoming a major tool for targeted mutagenesis in eukaryotes by inducing double-strand breaks (DSBs) at pre-selected genomic sites that are repaired by non-homologous end joining (NHEJ) in an error-prone way. In plants, it could be demonstrated that the Cas9 nuclease is able to induce heritable mutations in *Arabidopsis thaliana* and rice. Gene targeting (GT) by homologous recombination (HR) can also be induced by DSBs. Using a natural nuclease and marker genes, we previously developed an *in planta* GT strategy in which both a targeting vector and targeting locus are activated simultaneously via DSB induction during plant development. Here, we demonstrate that this strategy can be used for natural genes by CRISPR/Cas-mediated DSB induction. We were able to integrate a resistance cassette into the *ADH1* locus of *A. thaliana* via HR. Heritable events were identified using a PCR-based genotyping approach, characterised by Southern blotting and confirmed on the sequence level. A major concern is the specificity of the CRISPR/Cas nucleases. Off-target effects might be avoided using two adjacent sgRNA target sequences to guide the Cas9 nickase to each of the two DNA strands, resulting in the formation of a DSB. By amplicon deep sequencing, we demonstrate that this Cas9 paired nickase strategy has a mutagenic potential comparable with that of the nuclease, while the resulting mutations are mostly deletions. We also demonstrate the stable inheritance of such mutations in *A. thaliana.*

Keywords: genome editing, targeted mutagenesis, engineered nucleases, double-strand break repair, homologous recombination, technical advance.

INTRODUCTION

Engineered nucleases have become major tools for the introduction of mutations and more complex alterations into the genome of most organisms. The available toolbox consists of meganucleases, such as I-*Sce*I, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Mutations or gene targeting (GT) events are achieved by the site-specific introduction of double-strand breaks (DSBs) and the subsequent repair of these breaks via error-prone non-homologous end joining (NHEJ) or homologous recombination (HR). For plants, numerous studies have previously demonstrated the successful use of meganucleases, ZFNs and TALENs for different applications in various species (Puchta *et al.*, 1993, 1996; Salomon and Puchta, 1998; Kirik *et al.*, 2000; Siebert and Puchta, 2002; Lloyd *et al.*, 2005; Wright *et al.*, 2005; Shukla *et al.*, 2009; Townsend *et al.*, 2009; Gao *et al.*, 2010; Osakabe *et al.*, 2010; Zhang *et al.*, 2010, 2013; Curtin *et al.*, 2011; Mahfouz *et al.*, 2011; Antunes *et al.*, 2012; Li *et al.*, 2012; D'Halluin *et al.*, 2013; de Pater *et al.*, 2013; Shan *et al.*, 2013a; Wendt *et al.*, 2013; Gurushidze *et al.*, 2014; Liang *et al.*, 2014; Wang *et al.*, 2014).

Recently, the use of the RNA-guided endonuclease (RGN) CRISPR/Cas9 (for 'clustered regularly interspaced short palindromic repeats' and 'CRISPR-associated', respectively) has revolutionised the field of genome engineering. CRISPR loci are found in most bacteria and archaea and serve as an adaptive immune system against foreign DNA (for a review, see Wiedenheft *et al.*, 2012).

The molecular characterisation of the CRISPR/Cas system from *Streptococcus pyogenes* has revealed a simple and efficient mechanism of sequence-specific DSB induction (Jinek *et al.*, 2012). A short CRISPR RNA (crRNA) directly binds to a 20-nt recognition site (RS) on the DNA, the so-called protospacer. The sequence motif 'NGG' downstream of the protospacer (termed the protospacer-adjacent motif, PAM) is crucial for binding. A second RNA, termed *trans*-activating crRNA (tracrRNA), binds the crRNA, and the protein Cas9 is recruited to the complex. Cas9 is an endonuclease that contains two nuclease domains, the RuvC-like domain and the HNH motif, each cleaving one of the two DNA strands three base pairs upstream of the PAM. By mutating one of the catalytic amino acids in one of the nuclease domains (D10 or H840), Cas9 no longer acts as a nuclease but as a nickase, creating single-strand breaks (Jinek *et al.*, 2012).

We recently demonstrated that a codon-optimised version of Cas9 can be applied to efficiently induce NHEJ-mediated heritable mutations in *Arabidopsis thaliana* (Fauser *et al.*, 2014). Furthermore, we demonstrated that the Cas9 nickase does not produce a detectable amount of NHEJ events but is at least as efficient as the Cas9 nuclease or the homing endonuclease I-*Sce*I in inducing HR.

A further important step for genome engineering in plants is to demonstrate that the CRISPR/Cas nuclease is also useful for GT in plants. DSB-induced GT requires a site-specific nuclease as well as a template for HR-mediated DSB repair which must be supplied simultaneously (Puchta *et al.*, 1996). Therefore, a reasonable transformation frequency must be achieved. Unfortunately, many crop plants are minimally transformable, and the regeneration of transgenic material into fertile plants presents an additional challenge (for a recent review of the history of GT in plants; see Puchta and Fauser, 2013). In 2012, we developed the so-called *in planta* GT technology, which should be applicable to all transformable plant species, even if the transformation or regeneration efficiencies are extremely low (Fauser *et al.*, 2012). Indeed, a recent study in maize demonstrated in a similar experimental setup that an ectopic copy of the donor sequence could be used as a template after DSB induction in the target locus (Ayar *et al.*, 2013). *In planta* GT relies on a chromosomal donor molecule, e.g., a stably integrated T-DNA, as well as the simultaneous induction of at least three DSBs. The donor molecule harbours the GT cassette, consisting of the sequence that should be integrated into the target site via its homologous regions. These sequences are flanked by the same RS, which is also found within the original target. As soon as the respective nuclease is expressed, the GT vector is released from the donor molecule, and the target site is activated for somatic HR. So far, *in planta* GT has only been demonstrated for artificial reporter systems and the natural occurring meganuclease I-*Sce*I. Therefore, it was of upmost importance to test whether foreign sequences may also be integrated into naturally occurring target sites by *in planta* GT using engineered nucleases, such as the CRISPR/Cas system. Here, we demonstrate that the *in planta* GT technology can be used to integrate a resistance cassette into an endogenous locus by CRISPR/Cas-mediated DSB induction. Heritable events were identified by a PCR-based genotyping approach, characterised by Southern blotting and confirmed at the sequence level.

A major concern of the CRISPR/Cas system is whether the specificity defined by the 23-bp target sequence of the *Sp*Cas9 complex is sufficient to prevent off-target cleavage by the nuclease. Off-target activity has been assessed using different methods, including the application of varying sgRNAs to a reporter gene (Fu *et al.*, 2013), deep sequencing of potential off-target sites (Cho *et al.*, 2014), ChIP-seq with inactive Cas9 (Kuscu *et al.*, 2014) and whole-genome re-sequencing (Smith *et al.*, 2014; Veres *et al.*, 2014). However, the reported results vary strongly depending on the method, organism and target sequence; therefore, the off-target activity of CRISPR/Cas is still a major concern. This is especially the case for organisms with large genomes and many duplications, such as crop plants. To reduce the risk of off-target activity, it is possible to use paired nickases instead of the single nuclease, as has already been shown for human cell culture (Mali *et al.*, 2013; Ran *et al.*, 2013). Using two adjacent sgRNA target sequences to guide the Cas9 nickase to each of the two DNA strands results in the formation of a DSB. Because the off-target activity of the nickase only leads to SSBs that are re-ligated without inducing mutations, this approach doubles the length of the required target sequence to 46-nt and therefore strongly enhances the specificity of the system.

Here, we demonstrate the application of Cas9 paired nickases to plants. We have extended our existing Cas9 cloning system to rapidly generate paired nickase constructs for *Agrobacterium*-mediated transformation. By amplicon deep sequencing, we demonstrate that the mutagenic potential is in a comparable frequency to that of the Cas9 nuclease, while the resulting mutations are mostly deletions. We also demonstrate the stable inheritance of the obtained mutations via Sanger sequencing.

RESULTS

Engineering CRISPR/Cas-based constructs for *in planta* GT and paired nickases

As previously described, we cloned the *S. pyogenes* Cas9 (SpCas) open reading frame (ORF) that was codon-optimised for *A. thaliana* into a binary vector for *Agrobacterium*-mediated transformation (Fauser *et al.*, 2014). We also cloned a Cas9 variant containing an inactivating point mutation in the catalytic residue of the RuvC-like domain

(D10A), which acts as a nickase (Jinek *et al.*, 2012). Both of the constructs are driven by the constitutive ubiquitin 4-2 promoter from *Petroselinum crispum* (PcUbi4-2). The sgRNA chimera is under the control of the Arabidopsis U6-26 promoter and can easily be customised for any target site of interest as described (Fauser *et al.*, 2014).

In this study, we used this CRISPR/Cas system to demonstrate the feasibility of endogenous *in planta* GT at a naturally occurring target site (*ADH1*, alcohol dehydrogenase 1, AT1G77120). Therefore, the GT vector was supplied on the same T-DNA construct as the Cas9 expression cassette. In addition, we implemented paired nickases with our CRISPR/Cas cloning system by adding two independent sgRNA expression cassettes, each driven by the same AtU6-26 promoter (Figure 3a). Their activity was evaluated at an endogenous target site (*RTEL1*, regulator of telomere length 1, AT1G79950) by amplicon deep sequencing.

Generating lines for CRISPR/Cas-mediated *in planta* GT

To determine the feasibility of endogenous *in planta* GT, an RGN recognising *ADH1* was transformed via floral dipping. In contrast to the I-*Sce*I-based *proof of concept* experiment (Fauser *et al.*, 2012), the number of necessary constructs was reduced from three (artificial target, donor and I–*Sce*I expression construct) to a single T-DNA. Therefore, the GT cassette is located on the same T-DNA as the RGN (Figure 1). Primary transformants (T1 generation) were selected for further propagation in the greenhouse. The progeny of the T1 plants (T2 generation) were tested for Mendelian segregation on selection media to identify single-locus T-DNA integration events. Figure S1 summarises all lines used in the *in planta* GT approach.

Heritable CRISPR/Cas-mediated *in planta* GT events

In the T1 plants, the RGN is expressed and may induce three independent DSBs: one in the *ADH1* gene and two within the stably integrated T-DNA. While the DSB in the *ADH1* gene activates the target site for HR-mediated DSB repair, the GT vector is released as a linear double-stranded DNA molecule from the chromosomal T-DNA. The homology between the activated target site and the GT vector sequence is 674-bp on one end (upstream) and

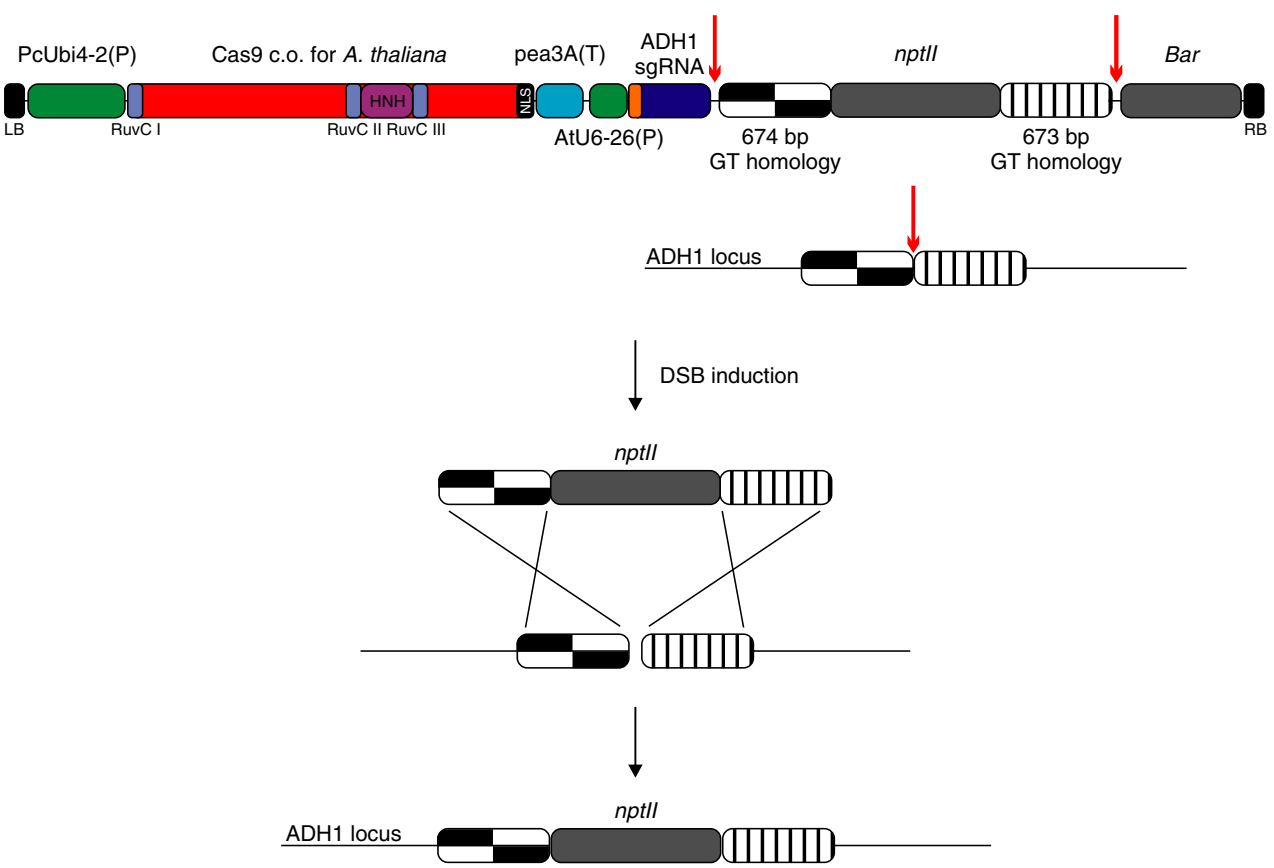


Figure 1. Outline of the CRISPR/Cas-based endogenous *in planta* GT system.
The T-DNA construct harbours the CRISPR/Cas expression system (Cas9, sgRNA) and a GT cassette. The GT cassette encodes a kanamycin (*nptII*) resistance cassette that is flanked by the homologous sequence information (674 bp upstream, 673 bp downstream) and two recognition sites for the Cas9 nuclease (red arrows). The same recognition site is also present in the natural target sequence *ADH1*. Therefore, DSB induction simultaneously leads to the activation of the target site for HR-mediated repair and to the release of the GT vector, which can then integrate into the *ADH1* locus using homologous sequences.

673-bp on the other (downstream). *In planta* GT events may occur anytime during the development of T1 plants due to the constitutive expression of the RGN. To test whether *in planta* GT events in *ADH1* could be transferred to the germline, we selected progeny of four primary transformants for a PCR-based genotyping approach. Thus, approximately 350 seedlings per line were analysed for GT-specific PCR products of the upstream or the downstream region. In total, 51 plants tested positive for either one of these regions were further cultivated in the greenhouse.

To confirm that the induced *in planta* GT events were inherited in a Mendelian fashion, we examined the segregation of these events in the T3 generation. Therefore, the progeny of the T2 plants (52 seeds per plant) were treated with allyl alcohol. T2 plants that are heterozygous for an *in planta* GT event produce a quarter viable seeds, while homozygously mutated T2 plants produce fully viable progeny. In total, two stable GT lines were identified out of approximately 1400 seedlings. For GT_ADH1 #2, one line was fully viable (GT_ADH1 #2-1), whereas the other line had a 3:1 segregation ratio (GT_ADH1 #2-2). The GT-specific PCR products of these individuals were Sanger sequenced to confirm the HR-mediated integration of the GT vector at the sequence level (Figure S2). Therefore, HR-mediated integration could be confirmed at both homologous regions for GT_ADH1 #2-1 but only at the downstream region for GT_ADH1 #2-2, indicating a one-sided GT event. Additionally, we PCR-amplified the original *ADH1* locus to determine whether additional targeted mutagenesis events are present within the RGN RS. GT_ADH1 #2-2 carries a wildtype allele, while GT_ADH1 #2-1 carries, in addition to the targeted allele, an additional NHEJ-mediated deletion event as second allele (Δ236/+4-bp). Therefore, the observed segregation ratio after allyl alcohol treatment can be explained by a transheterozygous genotype, represented by one GT and one targeted mutagenesis event.

The endogenous *in planta* GT events that were detected by PCR-based genotyping were also confirmed by Southern blot characterisation of the GT_ADH1 #2-1 and #2-2 progeny. The Southern blot analysis of the *in planta* GT line GT_ADH1 #2-1 is shown in Figure 2. To confirm that the GT vector was integrated via HR at the upstream junction, a Southern blot analysis using probe A on *Nco*l-digested DNA was performed (Figure 2b). While a 4.3-kb fragment indicates the native *ADH1* locus, the HR-mediated integration of the GT vector would result in a 3.8-kb fragment. Additionally, a 4.1-kb fragment is expected due to the observed NHEJ-mediated deletion of 232-bp in total. For all eight of the progeny lines of GT_ADH1 #2-1, the 3.8-kb fragment could be detected. Moreover, in four of the lines [#2-1 (1), #2-1 (2), #2-1 (4) and #2-1 (7)] the 4.1-kb fragment indicative of the mutated *ADH1* locus was also present. Therefore, half of the tested lines were heterozygous for the *in planta* GT event, and the other half were homozygous in the T2 generation. To confirm these results at the sequence level, the recombined upstream junction was PCR-amplified. A Sanger sequence analysis revealed the presence of the restored upstream junction without any mutation, demonstrating that the GT vector was integrated via HR. To confirm that the GT vector was also integrated via HR at the downstream junction, a Southern blot analysis using probe B on *Nco*l-digested DNA was performed (Figure 2c). Here, a 2.3-kb fragment is expected after the HR-mediated integration of the GT vector. For probe A, the native *ADH1* locus is represented by a 4.3-kb fragment, which is shifted to a 4.1-kb fragment if the deletion is present. In all eight of the lines, the GT-specific fragment could be detected, and the same lines [#2-1 (1), #2-1 (2), #2-1 (4) and #2-1 (7)] contained the fragment indicative of the mutated *ADH1* locus, thereby confirming the respective genotype. To confirm these results at the sequence level, the recombined downstream junction was PCR-amplified. In every case, a sequence analysis verified the HR-mediated integration without any mutation.

The molecular characterisation of the *in planta* GT line GT_ADH1 #2-2 confirmed the expected one-sided invasion event at the target locus (Figure S3). A Southern blot analysis using probe B on *Nco*l-digested DNA revealed HR-mediated integration at the downstream junction, which was also confirmed at the sequence level. However, all of the lines that were tested using probe A produced a fragment of approximately 6.9 kb. We successfully PCR-amplified the extended upstream junction for sequence analysis. Sequencing revealed an SDSA-mediated (synthesis-dependant strand annealing; Gorbunova and Levy, 1997; Rubin and Levy, 1997; Puchta, 1998) DSB repair reaction that was initiated using the downstream homology of the stably integrated T-DNA as a template. Thereby, the GT cassette, the sgRNA expression cassette, the pea3A terminator, and a quarter of the Cas9 ORF were copied and finally re-ligated to the upstream junction via NHEJ (Figure S3). Thus, the integration of a functional kanamycin resistance gene was mediated via HR on one end, while the other end was ligated by NHEJ.

Taken together, we were able to demonstrate that CRISPR/Cas-mediated *in planta* GT is feasible for natural sites in the *A. thaliana* genome. Heritable events can be selected by PCR-based genotyping on a reasonable-effort basis.

Amplicon deep sequencing of the mutations that are induced by Cas9 paired nickases

We cloned a Cas9 paired nickase construct targeting the natural *RTEL1* locus in *A. thaliana* using the previously characterised Cas9 D10A variant (Fauser *et al.*, 2014). In accordance with previous studies (Mali *et al.*, 2013; Ran *et al.*, 2013), we chose sgRNAs that bind their protospacers

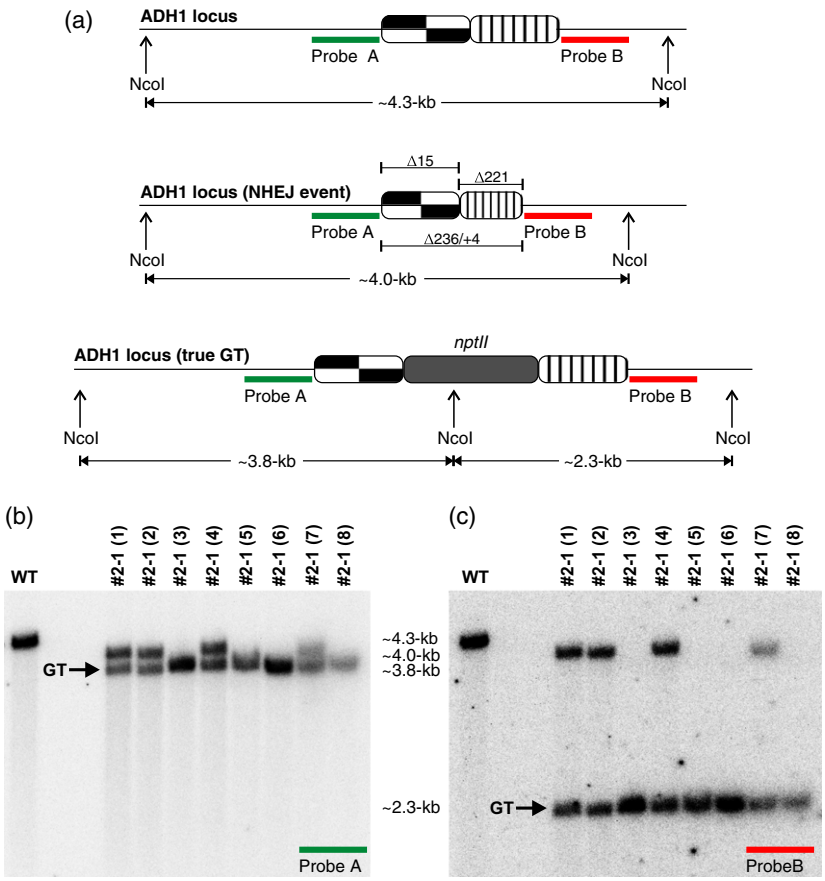


Figure 2. Southern blot analysis of a true GT event in GT_ADH1 #2-1.
(a) Depicted are the WT *ADH1* locus and two alleles in the line GT_ADH1 #2-1 (see Figure S1). While one allele shows a NHEJ-mediated deletion, the other arose by a two-sided true GT event.
(b) The HR-mediated integration of the GT vector into the upstream homology resulted in the 3.8-kb GT fragment in all eight of the tested progeny lines. Four of these lines [#2-1 (1), #2-1 (2), #2-1 (4) and #2-1 (7)] also resulted in the 4.0-kb fragment, which indicates the NHEJ-mediated targeted mutagenesis event. As GT_ADH1 #2-1 is a transheterozygous T2 plant, both events are segregating in the T3 generation.
(c) These results are confirmed by the 2.3-kb GT fragment, which is indicative of the HR-mediated integration of the GT vector into the downstream homology. Again, the same lines show the targeted mutagenesis event in *ADH1*.

with an offset of 18 bp, and the PAMs face outwards from the targeted sequence, thereby creating 5′ overhangs when cleavage occurs (Figure 3b). On the respective T-DNA both of the RNAs were expressed from independent transcription units each harbouring the same AtU6-26 promoter.

After *Agrobacterium*-mediated floral dip transformation, transgenic T1 plants were selected, and DNA was extracted after 2 weeks of growth from 30 plants. Somatic NHEJ-mediated mutagenesis events were analysed by amplicon deep sequencing on the Roche 454 system, as we previously did using the nuclease and the single nickase (Fauser *et al.*, 2014). Figure 3(c) depicts the results as read counts from the whole dataset by position, grouped into deletions, insertions and substitutions. The graph shows two broad peaks spanning the regions of the two protospacers with maxima of 17.3 and 12.7%. In contrast to the results for the Cas9 nuclease, the vast majority of the mutations obtained here is deletions.

To obtain a more in-depth impression of the mutation frequency and the resulting patterns, 250 reads were randomly selected from the whole dataset, and this reduced dataset was further analysed. Of the 250 reads, 107 showed a mutation in the relevant region between the two PAMs, indicating that 42.8% of the reads were mutated. Most of the mutations were deletions in the upstream protospacer (27.1%) or in the downstream protospacer (20.6%). In total, 15.9% of the mutations were deletions affecting both of the protospacers. Deletions strongly vary in size, ranging from a single to more than 100 nt. Insertions constitute 5.6% (upstream RS), 7.5% (between the two RSs) and 15% (downstream RS) of all of the observed mutations. Interestingly, the insertions sizes are considerably larger than for the Cas9 nuclease and increase to more than 80 nt (Fauser *et al.*, 2014). In most of the cases, the insertions originate directly from the sequence upstream or downstream of the insertion site, thereby creating direct repeats of different sizes. Combinations of insertions and deletions in the same read sum up to 8.3%. An alignment of all the individual mutations that are derived from the reduced dataset is shown in Figure 4.

Assessment of germinal mutations by the paired nickases

The inheritance of targeted mutations is a crucial step for genome engineering in plants. To determine inherited mutations that are caused by the Cas9 paired nickases, the absence of the Cas9 expression construct was confirmed in T2 plants by PCR. From 20 single-locus lines, 20 plants each were checked for the presence of Cas9. A total number of 121 plants did not present the expected PCR band, indicating that the T-DNA was already lost due to segregation.

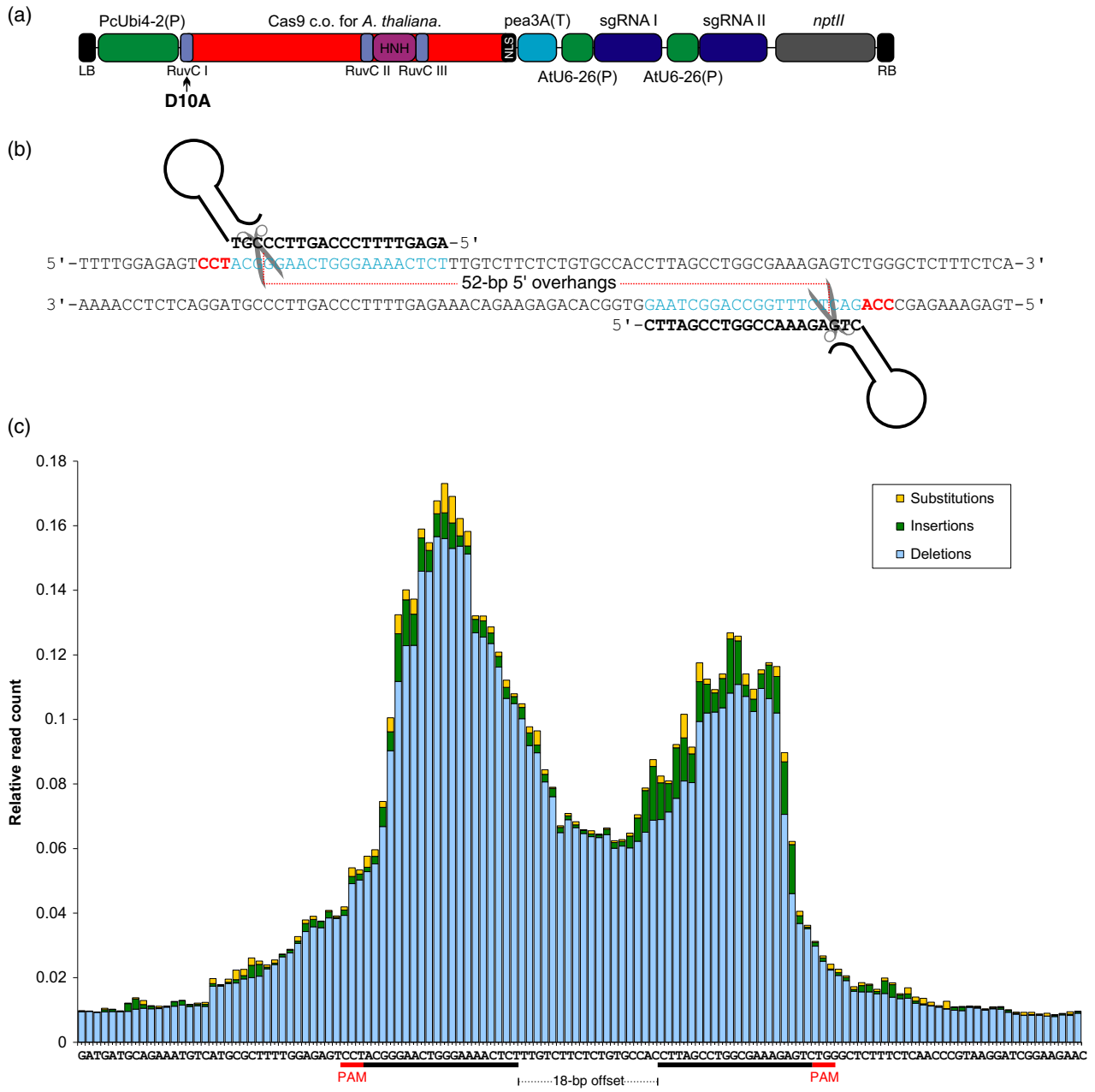
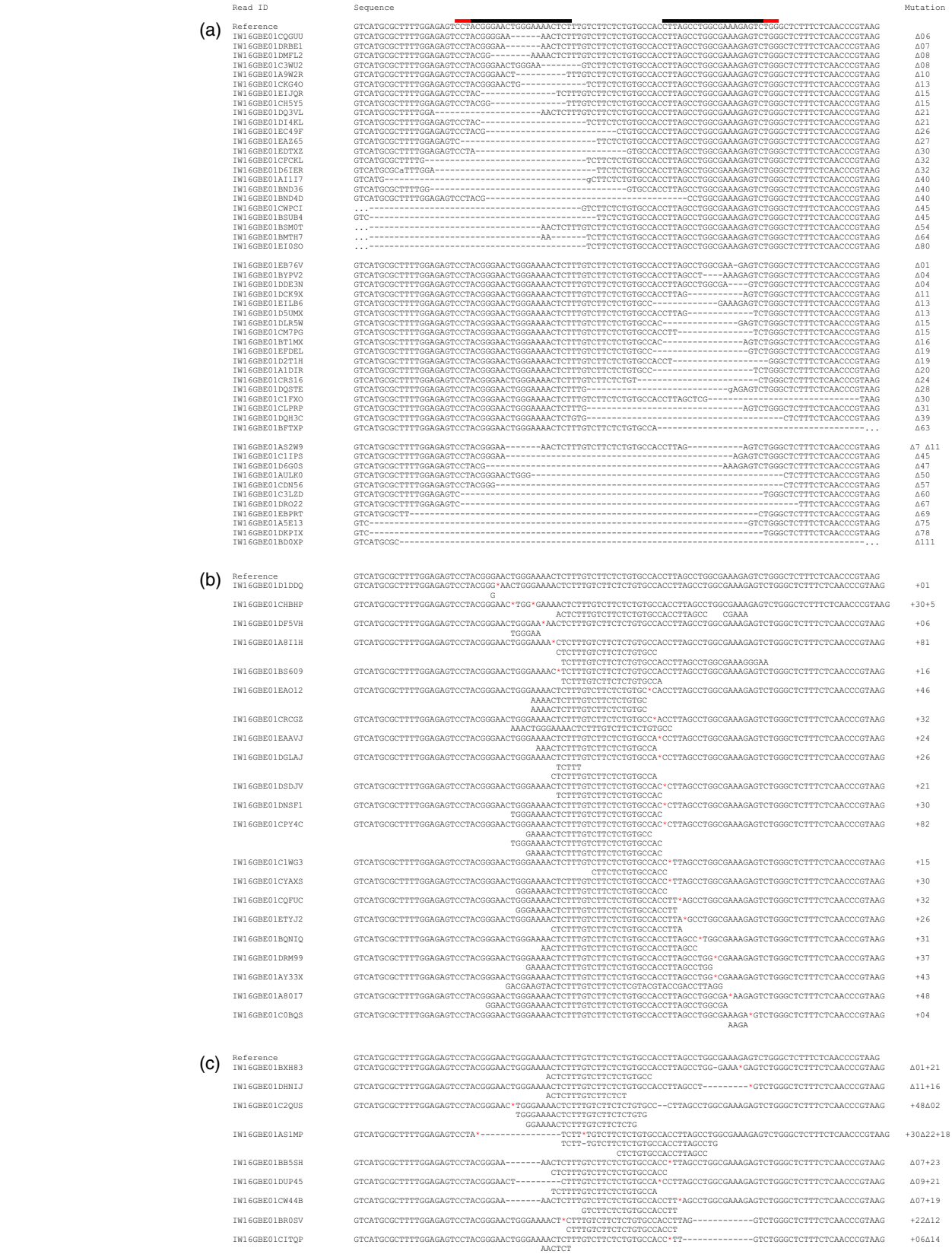


Figure 3. Targeted mutagenesis by Cas9 paired nickases.
(a) Cas9 paired nickases T-DNA construct.
(b) Paired nickases target site in the *RTEL1* locus. Nicking of the DNA strands results in 52-bp 5′ overhangs.
(c) Relative numbers of Cas9 paired nickases-induced mutations by position as determined by amplicon deep sequencing. The mutations are grouped into deletions, insertions and substitutions. The mutation frequency peaks in the region of the Cas9 target sites, while the majority of mutations are formed by deletions. The total number of reads is 72 187.

A 280-bp amplicon spanning the mutated region of the *RTEL1* locus was generated from these plants. The amplicon was obtained for 78 plants. Thirty six PCR products represented the wild type sequence only, while 42 plants indicate a heterozygous or transheterozygous genotype. Using subcloning, direct PCR analysis as well as heterozy-

Figure 4. Alignment of all of the individual mutations in the reduced deep sequencing dataset.
The mutations are grouped into deletions (a), further grouped into deletions affecting left RS, right RS or both, insertions (b), and combined deletions/insertions (c). In most cases, insertions originate from the targeted sequence region; therefore, the inserted sequences were aligned to the respective read sequence and are shown underneath each read.



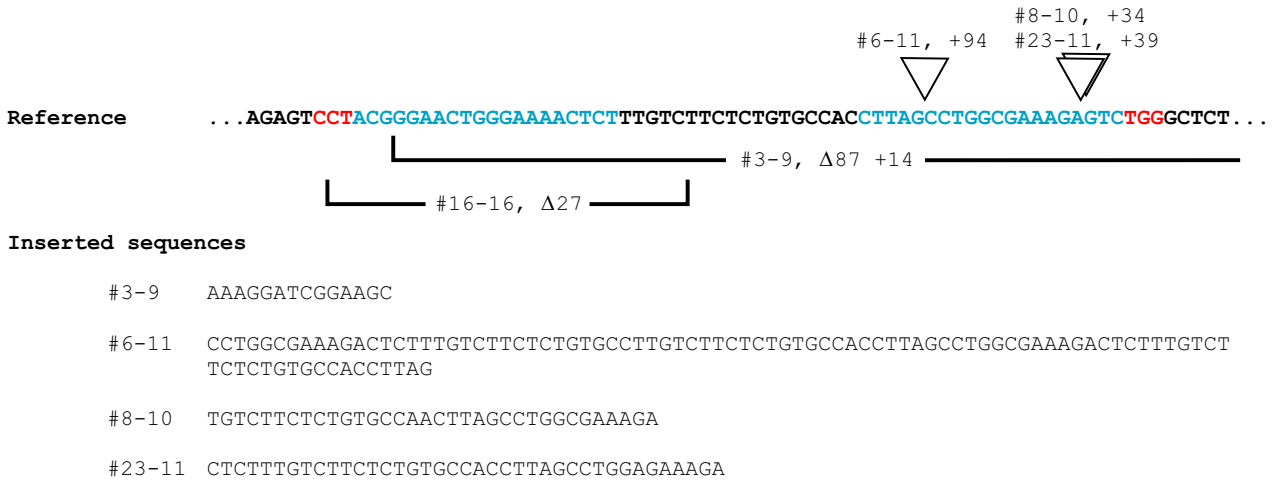


Figure 5. Deletions and insertions that were induced by Cas9 paired nickases can be transferred via the germline to the next generation in *A. thaliana.*
The mutated sequences were obtained by analysis of individual heritable heterozygous mutations via subcloning (#3-9 and #16-16), by independently sequencing of gel-separated PCR bands (#6-11) as well as by the use of the web-based program Poly Peak Parser after sequencing heterozygous PCR products (#8-10 and #23-11) (Hill *et al.*, 2014).

gous sequencing combined with analysis with the web-based program Poly Peak Parser (Hill *et al.*, 2014), we defined the molecular structure of representative mutations (Figure 5). It turns out that mutations resulting in deletion as well as insertion could be transferred through the germline to the next generation.

DISCUSSION

DSB-mediated genome engineering techniques, such as targeted mutagenesis and GT, are of tremendous interest for either basic research or plant breeding (Puchta and Fauser, 2014). The widespread use of engineered nucleases in plants was boosted by the development of a rapid and robust Golden Gate cloning procedure for TALENs (Cermak *et al.*, 2011). More recently, different variants of the CRISPR/Cas system were described for application in plants, achieving somatic (Li *et al.*, 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013b) and even heritable mutations (Fauser *et al.*, 2014; Feng *et al.*, 2014; Jiang *et al.*, 2014; Zhang *et al.*, 2014).

Previously, we described an *in planta* GT technique that uses an artificial I-*Sce*I-based GUS reporter system (Fauser *et al.*, 2012). The underlying mechanism of this technology relies on the simultaneous induction of at least three DSBs: one within the target site and two within the chromosomal donor. The chromosomal donor may be represented by a stable integrated T-DNA harbouring the actual GT vector within a GT cassette. Here, the GT vector is flanked by the same two RSs that are found within the target sequence. Upon the expression of the respective endonuclease, the GT vector is excised and may integrate into the simultaneously activated target site via HR. In contrast to classical GT experiments, where the GT vector is supplied directly as a T-DNA by *Agrobacterium*-mediated transformation (Puchta *et al.*, 1996), the GT vector is produced by the plant itself in a large number of cells throughout the entire life-cycle. Therefore, GT may occur in plant tissue that is more prone to HR and over a longer period compared to conventional methods in which the GT vector is only transiently present in transformable tissues. While in the proof of concept study, two DSBs within the target site resulted in a GT-mediated replacement of sequence information, we demonstrate here that sequence information may also be integrated by the induction of a single DSB. Much more crucial, however, is the demonstrated application of the *in planta* GT technology for a natural target site using the CRISPR/Cas system. Moreover, both the RGN expression construct and the GT vector were supplied on a single T-DNA, which makes the system extremely user friendly and permits detection of heritable GT events in only two generations. We previously demonstrated that although the obtained targeting efficiency might vary depending on the genomic loci of the target and donor, targeted clonal events could be found by screening a few hundred Arabidopsis seeds at most (Fauser *et al.*, 2012). Although the number of events that were detected in this study is too small for a sound statistical comparison, the obtained frequency was similar to that previously reported. We were able to identify heritable endogenous GT events by simple PCR analysis without positive or negative selection markers at a reasonable expense. Additionally, the original T-DNA can be segregated from the GT events. Therefore, the final plants will carry only the pre-defined changes within the target sequence but not the T-DNA.

Here, we show that the integration of a foreign gene at a pre-selected locus can be achieved by *in planta* GT using the CRISPR/Cas system. Thus, these techniques should be useful for application in crop plants for gene stacking to improve elite cultivars. Another important application of

the *in planta* GT technology would be the exchange of single bases within the coding sequence of a protein of interest (Fauser *et al.*, 2012). In future studies, it will be important to demonstrate that *in planta* GT can also be used for this purpose, which is also relevant for the public acceptance of genome engineering in plants in general, as no transgenes would be present in the respective plant. Nevertheless, at least in the European Union, such plants would still be classified as genetically modified organisms (GMOs) because the current classification is based on the applied techniques rather than on the resulting organism (Hartung and Schiemann, 2014; Voytas and Gao, 2014).

We also report here the application of Cas9 paired nickases for heritable targeted mutagenesis in plants. To create a DSB, the Cas9 nickase must be guided to two adjacent positions in the plant genome. The basic idea behind this strategy is to indirectly induce a mutagenic DSB. It is expected that if the induced SSBs lie in close proximity to each other on the two different DNA strands, a DSB with single-stranded overhangs will be formed. This event is very well characterised for restriction enzymes, where in most cases single-stranded ends of one to four bases are produced. The NHEJ-mediated repair of such DSBs might lead to the formation of insertions or deletions at that position (Salomon and Puchta, 1998; for a review, see Puchta, 2005; Knoll *et al.*, 2014). In our study, single-stranded DNA overhangs of 52 nt are produced by the paired nickases. The advantage of the paired nickases over the single Cas9 nuclease lies in the enhanced specificity. Although there have been numerous studies that demonstrated the limited off-target activity of Cas9, there are still doubts as to whether the 23-nt target sequence provides sufficient specificity (Fu *et al.*, 2013; Cho *et al.*, 2014; Kuscu *et al.*, 2014; Smith *et al.*, 2014; Veres *et al.*, 2014). This doubt is of particular interest for work on crop plants with very large genomes and many duplications. The application of Cas9 paired nickases overcomes this problem by doubling the RS length to 46-nt. We have previously demonstrated by deep sequencing that the application of a single Cas9 nickase, in contrast to the nuclease in *A. thaliana*, does not produce a detectable number of mutations at the target site using one of the sgRNAs from this study (Fauser *et al.*, 2014). Therefore, it is expected that the off-target binding of one of the sgRNAs does not produce mutations.

We have extended our existing Cas9 cloning procedure (Fauser *et al.*, 2014) to rapidly assemble paired nickase constructs that are ready for *Agrobacterium*-mediated transformation in three simple cloning steps. We demonstrated by amplicon deep sequencing that the efficiency of paired nickases to induce NHEJ is in the same range as that of a Cas9 nuclease. The resulting somatic mutations are mostly deletions covering either one of the two target sites. Although these findings might seem counter intuitive at first sight, they can be explained by the fact that mutations that do not affect the RSs do not abolish nickase activity and are consequently not stable. Therefore, deletions peak at the positions of the two RSs. Interestingly we also found insertions but at lower frequencies than for a Cas9 nuclease. While in case of the nuclease, the insertions were mainly only 1 bp in size, the insertions that were characterised here were considerably bigger, and their sequences originated mostly from the vicinity of the insertion site. It is possible that DSBs with overhangs of 52 nt are processed at least somewhat differently from DSBs without or short overhangs (e.g., 4 nt for I-*Sce*I). Several repair pathways might compete for longer single-stranded DNA ends so that more complex rearrangements, such as the copying of adjacent sequences, might occur more often. Recently, in a pioneering bioinformatic study, it was proposed that the origins of smaller tandem duplication events in rice are due to adjacently occurring SSBs (Vaughn and Bennetzen, 2014). Although in this regard the dataset that is presented here is too limited for final conclusions, our results agree with the proposed model. It is generally assumed that SSBs occur naturally in close proximity to each other in either the same or different strands during different DNA repair processes. Thus, having a tool for the induction of several SSBs at the same time is not only attractive for genome engineering, but might also be relevant for studies reconstructing genome evolution.

The most important hurdle for genome engineering techniques in plants is to demonstrate that the change is not only inducible in somatic tissue but may also be transmitted to the germline and passed onto the next generation. Indeed, we demonstrated that mutations that were induced by Cas9 paired nickases and Cas9-mediated *in planta* GT were heritable. The techniques that are described in this article will widen our toolbox for plant genome engineering and be attractive for use in basic research as well as in plant breeding.

EXPERIMENTAL PROCEDURES

Strains

All used plant lines were on Columbia-0 background. The seeds were sown on agar plates containing germination medium [GM: 4.9 g L^{-1} Murashige & Skoog medium (Murashige and Skoog, 1962), 10 g L^{-1} saccharose, pH 5.7, 7.6 g L^{-1} plant-agar] or on substrate containing 1:1 Floraton 3 (Floragard Vertriebs GmbH, www.floragard.de) and Vermiculite (Deutsche Vermiculite Dämmstoff GmbH, www.vermiculite.de).

T-DNA constructs

The vectors that were used in this study are based on our previously described Cas9 cloning vectors (Fauser *et al.*, 2014). For the cloning of paired nickases, we re-designed pEn-chimera by adding restriction sites for *Bsu*36I and *Mlu*I on each side of the U6-26–sgRNA sequence. The new construct was produced by PCR using primers SS-135/SS-136, adding the restriction sites and attR sites for Gateway® cloning (Life Technologies Inc., www.lifetech.com).

The PCR product was then TA-cloned into the pGEM®-T-Easy vector (Promega Corp., www.promega.com), creating pEn-C1.1 (see Sequences S1 for sequence information on pEn-C1.1). Spacers can be introduced via *Bbs*I as previously described (Fauser *et al.*, 2014). The two chimera constructs are then cloned together into pDe-CAS9-D10A. The first chimera is transferred using *Bsu*36I, *Mlu*I or both, and the second chimera is added by Gateway® LR-reaction, resulting in the final Cas9 paired nickase expression vector. All of the plasmids (pEn-C1.1, pDe-CAS9 and pDe-CAS9-D10A) are available upon request.

The *in planta* GT construct is based on the vector that was previously used for targeted mutagenesis in *ADH1* (Fauser *et al.*, 2014). For CRISPR/Cas-mediated *in planta* GT, we added the GT cassette into the respective pCAS9-TPC backbone using B*su*36I and *Aat*II. Therefore, the GT cassette was synthesised by GeneArt® (Life Technologies Inc.) and contained the following elements from 5′ to 3′: *Bsu*36I RS, ADH1 protospacer, ADH1 upstream GT homology (674 bp), kanamycin resistance cassette, ADH1 downstream GT homology (673 bp), ADH1 protospacer and *Aat*II recognition site (see Sequences S1 for sequence information on the GT vector and Table S1 for all primers used in this study).

Plant transformation

Arabidopsis plants were transformed via *Agrobacterium*-mediated transformation as previously described (Clough and Bent, 1998; *Agrobacterium* strain GV3101).

Evaluation of *in planta* GT events

Genomic DNA (T1 to T4 generation) was analysed by PCR using primers FF-216/FF-219 (GT_ADH1 #2-1) and FF-239/Cas9_rev1 (GT_ADH1 #2-2), which were used to detect the upstream HR-mediated integration of the GT vector and subsequent sequencing. Primers FF-220/FF-221 were used to detect the downstream HR-mediated integration of the GT vector and subsequent sequencing. Primers FF-192/FF-199 (GT_ADH1 #2-1) and FF-227/FF-228 (GT_ADH1 #2-2), were used to amplify the original *ADH1* locus and for subsequent sequencing. Primers FF-240/FF-246 were used to amplify the one-sided integration event, whereby primers FF-82, FF-199, FF-240, FF-242, FF-245, FF-246, SS-42, SS-61, M13-FP, M13-RP, K1 and K5 were used for subsequent sequencing. Allyl alcohol treatment was performed as previously described (Jacobs *et al.*, 1988).

Plant DNA extraction and Southern analysis

DNA was extracted from batches of 20 T4 siblings representing the progeny of a T3 plant. The extraction was performed as previously described (Salomon and Puchta, 1998). Southern blotting of *Nco*I-digested genomic DNA using the membrane 'Hybond N+' (GE Healthcare, www.gehealthcare.com) was performed as previously described (Salomon and Puchta, 1998). The DNA probes were labelled as previously described (Pacher *et al.*, 2007). The probes were PCR-amplified from genomic DNA (*A. thaliana*, Col-0): probe A using primers FF-240 and FF-241 and probe B using primers FF-245 and FF-246.

Amplicon deep sequencing

Paired nickase T1 plants were grown on GM with 500 mg L^{-1} cefotaxime and 30 mg L^{-1} kanamycin. DNA was extracted from batches of 30 plants after 2 weeks of incubation as previously described (Salomon and Puchta, 1998). Amplicons containing Multiplex Identifiers (MIDs) for pooled sequencing were generated by using a proofreading polymerase with 100 ng of genomic DNA using primers SS-149/SS-150 and purified using the PEQLAB Cycle Pure Kit (PEQLAB Biotechnologie GmbH, www.peqlab.com). Roche® 454 Sequencing was performed by Eurofins Genomics (Eurofins Genomics GmbH, www.eurofinsgenomics.eu). The data analysis was performed using the Galaxy web server (Giardine *et al.*, 2005; Blankenberg *et al.*, 2010; Goecks *et al.*, 2010) and Integrative Genomics Viewer 2.3 (Robinson *et al.*, 2011).

Evaluation of the germinal mutations that were induced by paired nickases

Primary transformants (T1) were selected on agar plates containing GM, kanamycin and cefotaxime for further cultivation in the greenhouse. T2 progeny were sown onto selection media and checked for 3:1 segregation to identify single-locus lines. From each single-locus line, 20 T2 plants were grown on GM and subjected to molecular analysis: the absence of the Cas9-expressing T-DNA was verified by PCR using primers SS-48/SS-153. Then, the targeted *RTEL*-locus was amplified in every plant without the Cas9 construct using primers SS-184/SS-185, and the amplicons were Sanger sequenced.

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SUPPORTING INFORMATION

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

Figure S1. Summary of all lines used for endogenous *in planta* GT.

Figure S2. PCR-based genotyping of endogenous *in planta* GT events.

Figure S3. Southern blot analysis of a one-sided GT event in GT_ADH1 #2-2.

Table S1. Primers used in this study.

Sequences S1. *ADH1 in planta* GT vector and pEn-C1.1 entry construct.

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