Title: One-shot generation of duodecuple (12x) mutant Arabidopsis: Highly efficient routine editing in model species

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Summary (223 words)

Genome editing by RNA-guided nucleases in model species is still hampered by low efficiencies, and isolation of transgene-free individuals often requires tedious PCR screening. Here, we present a toolkit that mitigates these drawbacks for *Nicotiana benthamiana* and *Arabidopsis thaliana*. The toolkit is based on an intron-optimized *Sp*Cas9-coding gene (zCas9i), which conveys dramatically enhanced editing efficiencies. The zCas9i gene is combined with remaining components of the genome editing system in recipient vectors, which lack only the user-defined guide RNA transcriptional units. Up to 32 guide RNA transcriptional units can be introduced to these recipients by a simple and PCR-free cloning strategy, with the choice of three different RNA polymerase III promoters for guide RNA expression. We developed new markers to aid transgene counter-selection in *N. benthamiana*, and demonstrate their efficacy for isolation of several genome-edited *N. benthamiana* lines. In Arabidopsis, we explore the limits of multiplexing by simultaneously targeting 12 genes by 24 sgRNAs. Perhaps surprisingly, the limiting factor in such higher order multiplexing applications is Cas9 availability, rather than recombination or silencing of repetitive sgRNA TU arrays. Through a combination of phenotypic screening and pooled amplicon sequencing, we identify transgene-free duodecuple mutant Arabidopsis plants directly in the T_2 generation. This demonstrates high efficiency of the zCas9i gene, and reveals new perspectives for multiplexing to target gene families and to generate higher order mutants.

Introduction

RNA-guided nucleases (RGNs) derived from bacterial CRISPR/Cas systems have been re-purposed as programmable DNA-binding scaffolds with functions as diverse as base-editing, transcriptional regulation, chromatin modification, or fluorescence-labelling of specific DNA sequences in plant and animal systems (reviewed in Pickar-Oliver and Gersbach, 2019). The most prominent application, however, remains induction of double-strand breaks (DSBs) at user-defined positions for gene targeting or mutagenesis. Gene targeting depends on repair of the DSB by homology-directed repair (HDR), and requires co-delivery of an HDR template. Substantial improvements were achieved in gene targeting in the past few years, but it remains technically challenging in plants due to low efficiencies (e.g. Cermak et al., 2015; Gil-Humanes et al., 2017; Yin et al., 2017; Miki et al., 2018; Huang and Puchta, 2019; Wolter and Puchta, 2019). One reason for low gene targeting efficiencies is that DSBs are predominantly repaired by non-homologous end joining (NHEJ) in plant somatic cells (Knoll et al., 2014; Que et al., 2019). Although reducing the frequency of HDR, DSB repair by NHEJ is

sufficient for mutagenesis, as it can result in sequence alterations at junction sites. Mainly small insertions or deletions (commonly a single nucleotide) are induced (Bortesi et al., 2016), which, when they occur within coding sequences, disrupt gene function. NHEJ may be less error-prone than commonly assumed under natural conditions (Betermier et al., 2014; Rodgers and McVey, 2016), but precise repair of DSBs reconstitutes the target site, which may be then be cleaved again by the nuclease, thus enriching erroneous repair products in genome editing. Accordingly, induction of DSBs *via* programmable nucleases has been shown to work efficiently for targeted mutagenesis in many different plant species (see Chen et al., 2019; Xu et al., 2019 for recent lists of modifications in crop and non-crop species).

Researchers have access to numerous options for RGN-mediated mutagenesis. To start with, nuclease systems can be delivered into plant cells as DNA constructs, mRNA and gRNA or ribonucleoprotein complexes (Woo et al., 2015; Svitashev et al., 2016; Zhang et al., 2016; Liang et al., 2017; Lin et al., 2018), but *Agrobacterium*-mediated delivery of RGN-encoding T-DNAs remains the most common strategy. Numerous nucleases have been utilized for plant genome editing, such as Cas9 from *Staphylococcus aureus* and *Streptococcus pyogenes* (*Sa*Cas9*, Sp*Cas9) and modified versions thereof (xCas9, Cas9-VQR) and Cas12 (Cpf1; *Fn*Cpf1, *Lb*Cpf1) (Li et al., 2013; Nekrasov et al., 2013; Steinert et al., 2015; Tang et al., 2017; Hu et al., 2018; Ge et al., 2019; Zhong et al., 2019). Multiple promoter systems are also available for guide RNA (sgRNA) expression, i.e. RNA polymerase II (Pol II) or III (Pol III) promoters. In the former case, primary transcripts require processing, which can be achieved by ribozymes, endogenous tRNA processing enzymes, or the ribonuclease Csy4 (Gao and Zhao, 2014; Nissim et al., 2014; Xie et al., 2015; Cermak et al., 2017). The same strategies can also be used in multiplexing applications for processing of multiple sgRNAs from a polycistronic primary transcript. Alternatively, multiple sgRNAs can be expressed as individual transcriptional units (TUs) by Pol III promoters, such as U6/U3.

Many plant genome editing toolkits, which feature some or most of the cited options in respect to nuclease and sgRNA expressions systems, are available within the community (e.g. Xing et al., 2014; Lowder et al., 2015; Cermak et al., 2017; Wu et al., 2018; Hahn et al., 2019). It is difficult to make a well-informed decision as to which components or toolkit will perform best in a given situation, as efficiencies cannot be compared across studies. However, we and others have made an effort to systematically evaluate and improve the molecular components required for *Sp*Cas9-based genome editing in *Arabidopsis thaliana* (Arabidopsis; Castel et al., 2019; Ordon et al., 2019). Studies are not in full agreement, but several common principles have emerged: *i)* A promoter fragment of *RIBOSOMAL PROTEIN S5a* (*RPS5a*) is highly suitable for expression of Cas9 (Tsutsui and Higashiyama, 2017), and use of a strong transcriptional terminator is advised. *ii)* Improved sgRNA scaffolds (with a potential

termination signal removed and an extended hairpin loop) may enhance editing efficiencies (Chen et al., 2013; Dang et al., 2015). *iii)* Overall T-DNA architecture, *i.e.* orientation and order of TUs, influences editing efficiencies, showing that the whole can be different from the sum of its parts. *iv)* Codon usage, gene structure and nuclear localization signals (NLSs) may influence Cas9 editing efficiencies. *v)* Lastly, not only selection of transgenic individuals in the first generation, but also counter-selection in the following generation(s) is critical for rapid and streamlined production of final lines.

With the aforementioned considerations in mind, we have developed an improved set of Dicot Genome Editing (pDGE) vectors (Ordon et al., 2017) for routine editing in Arabidopsis and *Nicotiana benthamiana*. The system contains a highly efficient, intron-optimized Cas9 gene (zCas9i; characterized in detail in a companion manuscript (Gruetzner et al.)), which is combined with previously established or newly developed counter-selection markers. For editing in *N. benthamiana*, a counter-selection strategy for isolation of non-transgenic lines is presented. In Arabidopsis, we explore the potential of massive multiplexing, and generate a duodecuple (12x) Arabidopsis mutant line in a single step by multiplexing with 24 sgRNAs in Arabidopsis. This will facilitate reverse genetics in *N. benthamiana*, and reveals the potential of highly efficient editing systems for the generation of higher order mutants.

Results

Description of the vector system

The vector system described here is designed for rapid and simple generation of nuclease constructs for reverse genetics in Arabidopsis and *Nicotiana benthamiana*. It consists of preassembled recipient vectors (Figure 1a), containing all functional components except sgRNAs, and shuttle vectors for generation of sgRNA TUs (Figure 1b). Briefly, sgRNA TUs are generated by ligation of hybridized oligonucleotides into shuttle vectors in a first GoldenGate reaction, and are subsequently mobilized into recipient vectors in a second GoldenGate reaction (see Appendix 1 for detailed protocols). Final plant transformation constructs containing up to eight sgRNA TUs can be generated within four days with minimal effort and high fidelity (Figure 1c).

Shuttle vectors contain an improved sgRNA scaffold (Dang et al., 2015), and either a U6-26 promoter fragment from *A. thaliana* (e.g., Fauser et al., 2014; Ordon et al., 2017) or U6 or U3 promoter fragments from tomato (*Solanum lycopersicum; Sl*). The *Sl*U6/U3 promoter fragments were not

previously described (see also methods), and functionality was confirmed in reporter-based nuclease activity assays (Figure S1a). Similar editing efficiencies were measured upon expression of sgRNAs by *At*U6 or *Sl*U6/U3 promoter fragments. The *Sl*U3 promoter fragment was also included in an editing construct stably transformed into *N. benthamiana.* All tested T_0 lines contained mutations at the targeted loci, fully approving the *Sl*U3 regulatory element for sgRNA expression. Thus, the newly characterized U6/U3 promoter fragments most likely mediate levels of sgRNA expression similar to *At*U6-26, at least in *N. benthamiana*, and can be used in mixed sgRNA TU arrays or as an alternative to the *At*U6 promoter fragment.

All recipient vectors hold an intron-containing Cas9 gene, zCas9i, coding for Cas9 in fusion with two nuclear localization signals, which mediates enhanced expression and nuclear import of the Cas9 nuclease, and thus superior genome editing efficiencies (Gruetzner et al., companion manuscript). For each system, Arabidopsis and *N. benthamiana*, vectors for selection of primary transformants by resistance to Kanamycin, Hygromycin and Phosphinotricin (BASTA) were assembled. Furthermore, vectors contain cassettes either for expression of untagged or GFP-tagged Cas9 (as ^{NLS}GFP-Cas9^{NLS}), creating a total of 12 different recipient vectors (see Appendix 1). Nuclease activity was confirmed for all vectors in *N. benthamiana* reporter assays, and no obvious differences were observed for untagged or GFP-tagged Cas9 (Figure S1b). The GFP-tagged Cas9 demonstrated strong fluorescence mainly confined to nuclei, and may allow tracing of nuclease expression (Figure S1c). The vectors pDGE347 (BASTA) and pDGE463 (Kanamycin) were functionally confirmed by generation of multiple mutant lines in Arabidopsis and *N. benthamiana*, respectively.

In vectors designed for use in Arabidopsis (Appendix 1), Cas9 expression is controlled by a promoter fragment of *RIBOSOMAL PROTEIN S5a* (*RPS5a*), which provides high genome editing efficiencies in the T_1 generation (Tsutsui and Higashiyama, 2017; Ordon et al., 2019). In addition to marker genes for the selection of primary transformants, Arabidopsis vectors contain the FAST (fluorescence-accumulating seed techonology) cassette (Shimada et al., 2010). The FAST marker can be used for positive selection of transformants by seed fluorescence in the T_1 generation, and, conveniently, can also be used for the selection of non-transgenic seeds in later generations to separate desired genome modifications from the Cas9-encoding transgene by segregation (Castel et al., 2019; Ordon et al., 2019).

Vectors designed for genome editing in *N. benthamiana* (Appendix 1) contain Cas9 under control of the 35S promoter. Furthermore, *N. benthamiana* vectors contain a 2xtagRFP-coding gene under control of the *At*2S3 promoter fragment (Kroj et al., 2003) and the pepper (*Capsicum annuum*) *Bs3* gene under control of its own promoter (Römer et al., 2007). Similar to the FAST marker in

Arabidopsis vectors, these additional cassettes were incorporated to facilitate transgene counter-selection.

Due to the size of the intron-containing Cas9 and presence of additional expression cassettes, T-DNA regions of vectors presented here are relatively large (up to ~ 12 kb). It is a common concern that T-DNA size may reduce transformation efficiencies. However, we consistently observed high transformation efficiencies with our constructs in Arabidopsis and *N. benthamiana* (Figure S2), and it should be noted that natural T-DNAs are commonly 10 – 30 kb in size (Gelvin, 2003).

Assembly of plant transformation constructs containing up to 32 sgRNA transcriptional units

An sgRNA TU consists of a U3/U6 promoter fragment, the variable section of the sgRNA and the invariable sgRNA scaffold, followed by a polyT-stretch for termination of Pol III transcription. Shuttle vectors are missing the variable section of the sgRNA, and hold a *ccdB* cassette at this position. In a GoldenGate reaction using *Bpi*I, the *ccdB* cassette is replaced by two hybridized oligonucleotides (23-24 nt in length) to obtain the full sgRNA TU of user-defined specificity (Figure 2a). Since empty shuttle vectors contain the *ccdB* cassette for negative selection, these GoldenGate reactions can be processed as polyclonal plasmid preparations after transformation (Appendix 1; Ordon et al., 2017). The use of polyclonal plasmid preparations at this stage holds two advantages. On the one hand, the time required to obtain the sgRNA TUs is reduced by one day. On the other hand, the risk of selecting a single clone containing an erroneous sgRNA variable section (from inaccuracies during oligonucleotide synthesis) is mitigated. Therefore, sequence verification of sgRNA TUs is not required at this stage.

sgRNA TUs are subsequently mobilized into recipient vectors in a second GoldenGate reaction using *Bsa*I (Figure 2b). Depending on the set of shuttle vectors used in the assembly reaction, arrays of one, two, four, six or eight sgRNA TUs can be assembled in any recipient vector. Assembly of sgRNA arrays containing up to eight TUs was described previously, and takes place with high efficiency and accuracy (Ordon et al., 2017; see also Figure 2d).

To assemble sgRNA arrays exceeding eight TUs, we added an additional intermediate level called "multi-multi" (MM) to our cloning system. sgRNA arrays are first assembled in MM vectors as described above for recipient vectors (Figure 2c). In MM vectors, sgRNA arrays are flanked by *Bpi*I sites for release of the insert. The overhangs created by *Bpi*I release differ for MM vectors, and allow sequential assembly of the arrays in a recipient vector (Figure 2c). MM assemblies require an end-linker (Weber et al., 2011) for connection of the terminal overhang of the respective MM module with the overhang of the recipient vector (see Appendix 1). As recipient vectors are opened *via Bsa*I

and sgRNA arrays are released from MM vectors *via BpiI*, both enzymes are required in the GoldenGate reaction for assembly of sgRNA arrays. MM vectors allow assembly of up to 32 sgRNA TUs in a recipient vector. About 50 % of clones from assembly of up to 32 sgRNAs in recipients were correct when tested (Figure 2d).

A dual counter-selection strategy for isolation of transgene-free genome edited N. benthamiana

In the past few years we employed five different T-DNA architectures for genome editing in *N. benthamiana*. We generally observed high efficiencies with 35S promoter-driven Cas9 and *At*U6-driven sgRNA expression, as most T_0 plants did not possess wild type alleles at target loci. However, we found that the bottleneck in the generation of the mutants was to select T_1 plants containing desired edits, but not the Cas9-coding transgene. In several cases, isolation of a single transgene-free T_1 plant required screening of > 100 individuals, suggesting that primary transformants contained multiple T-DNA insertions.

In Arabidopsis, transgene counter-selection is largely facilitated by integration of the FAST marker in transformation constructs (Shimada et al., 2010; Castel et al., 2019; Ordon et al., 2019). We therefore aimed to develop a similar counter-selection strategy for *N. benthamiana*.

We first tested constructs containing the FAST cassette in *N. benthamiana*, but failed to detect seed fluorescence. Subsequently, fusions of an *At2S3* seed storage protein promoter fragment (Kroj et al., 2003) with red fluorophore-coding genes were incorporated as secondary marker cassettes. In different transformations, the *2S3* promoter fragment was fused to mCherry, tagRFP-T or 2xtagRFP-T, in combination with *A. tumefaciens g7* (Engler et al., 2014) or *N. benthamiana* extensin transcriptional terminators (Diamos and Mason, 2018). Vectors provided in our toolkit (Appendix 1) contain a p2S3:2xtagRFP-t*Nb*EU cassette. The marker was characterized with transformants containing a p2S3:mCherry-tAtug7 cassette (Figure 3).

T_1 seeds containing the p2S3:mCherry-tAtug7 cassette emitted weak seed fluorescence that could be detected *via* a camera using extended exposure times, but not by direct observation through the eyepiece (Figure 3). To assess the discriminative power of this weak marker, T_1 segregants were genotyped (using two different T-DNA specific primer pairs) with and without prior selection by seed fluorescence (Figure 3a,b). Without selection, most plants (20/22) contained the T-DNA. With fluorescence-based pre-selection, the number of T-DNA positive plants was strongly reduced: For three out of five families tested, all selected T_1 segregants were negative for the T-DNA (Figure 2b). In the remaining two families, pre-selection did not appear to have any positive effect; 11/12 plants still

contained the T-DNA (in total, 11/24). Conclusively, the seed fluorescence marker is an efficient means for the selection of non-transgenic segregants, but not in all T_1 families.

To further aid selection of non-transgenic *N. benthamiana* segregants, we also introduced the *Bs3* resistance gene from pepper (*Capsicum annuum*) under control of its own promoter in plant transformation constructs. *Bs3* is normally not expressed, but provokes cell death when induced by the transcription activator-like effector AvrBs3 (Römer et al., 2007; Boch et al., 2014). We assumed that *Bs3* could be used as an additional marker for presence of the T-DNA. Accordingly, the same plants used for PCR-genotyping (Figure 3b) were infiltrated with a bacterial strain translocating AvrBs3 into plant cells, and induction/presence of the *Bs3* gene was scored by appearance of cell death. Indeed, all plants genotyped positive for presence of the T-DNA developed robust cell death in response to AvrBs3 (Figure 3c).

We tested performance of the seed fluorescence and *Bs3* cell death marker in a sequential selection strategy for the isolation of mutants defective in *NRG1* (N requirement gene 1; Peart et al., 2005) and assumed orthologs of *NPR1* (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1; Cao et al., 1997). First, approximately 80 plants from three different T_1 families were grown from seeds pre-selected for absence of seed fluorescence (5 – 6 h). After 3 - 4 weeks, plants were challenged with AvrBs3-translocating *Pseudomonas fluorescens* bacteria (< 1 h), and plants that developed cell death (4/80, 5 %) were discarded. Remaining plants were genotyped with target gene-specific primers and homozygous *nrg1* single or *npr1a/b* double mutants selected (Figure 4). When genotyped for presence of the T-DNA, five out of six plants tested were negative (Figure 4). Thus, several independent single or double mutant lines could be selected from genotyping only 80 T_1 segregants, demonstrating high efficiency of the dual counter-selection strategy.

Editing 12 genes by multiplexing 24 sgRNAs in Arabidopsis thaliana: Analysis of primary transformants

The intron-optimized Cas9 gene expressed under control of the *RPS5a* promoter present in recipient vectors for Arabidopsis generates single and double mutant lines with high efficiencies in the T_1 generation (Gruetzner et al., companion manuscript). This encouraged us to test performance of our constructs in an ambitious multiplexing application, using an sgRNA array containing 24 TUs to target 12 different genes (Figure 5a). Selected target genes included several NLR-type resistance genes (*e.g. RPP2*, *RPP4*, *RPS5*) and developmental regulators (e.g. *ERECTA*, TOO MANY MOUTHS, *GLABROUS1*). Target genes and sites are listed in Table S1.

Two major problems were expected. On the one hand, efficiencies may decrease due to competition of many different sgRNAs for the limiting Cas9 nuclease core. On the other hand, repetitive sequences – the 24 sgRNA TUs represent successive blocks of ~ 200 nt differing only by the 20 nt variable section of the sgRNA – may result in frequent recombination events in *E. coli*, in *Agrobacterium*, during T-DNA transfer, or after T-DNA integration into the plant genome.

To control for recombination events, the construct containing the 24 sgRNA TUs was transformed into Columbia-0 (Col) wild type plants. Primary transformants were selected by resistance to BASTA, and DNA extracted for PCR-genotyping (Figure 5b). Different primer pairs reveal the presence of the Cas9 gene and three different blocks each encompassing four sgRNA TUs spread along the sgRNA array (Figure 5a). Furthermore, a primer pair for an amplicon of the endogenous *RPP2a* locus, which was also targeted by paired nucleases, was included for genotyping. In total, 36 randomly chosen primary transformants were analyzed (Figures 5b; S3). From these, a single transformant did apparently not contain the T-DNA (Figure 5b, lane1). For another transformant, only two of the three fragments covering the sgRNA array could be PCR-amplified (Figure S3), suggesting a partial integration of the T-DNA or a recombination event. All remaining transformants were positive for the tested amplicons, and signals indicative of deletions at the *RPP2* locus were detected for some transformants (Figure 5b, PCR 5, lanes 11, 14; Figure S3). Thus, direct repeats of the sgRNA array did not induce frequent recombination events, and an intact T-DNA region was likely transferred to most transformants (34/36, 94 %).

The sgRNA array was constructed in such way that it was flanked by sgRNAs for targeting of *ERECTA* (*ER*) and *GLABROUS1* (*GL1*). Inactivation of these genes leads to altered shoot morphology and absence of trichomes respectively (Oppenheimer et al., 1991; Torii et al., 1996); phenotypes that can easily be scored by visual inspection (see insets in Figure 5). We used appearance of *er* and *gl1* phenotypes to evaluate genome editing efficiency of the 24 sgRNA construct in the T_1 generation. As controls, T_1 plants from transformation of constructs containing sgRNAs targeting only *ER* or *GL1* or both of these loci (pDGE579, pDGE580 and pDGE581, respectively; Figure 5c) were analyzed. More than 80% of primary transformants were scored as *er* or *gl* when only these loci were targeted. Similar efficiencies were observed when both loci were targeted simultaneously (Figure 5c). Efficiencies dropped by approximately 30-40 % in transformants that had received the 24 sgRNA construct. Also, the frequency of chimeric plants, which can easily be recognized for *gl1*, appeared to increase. These observations suggest that, indeed, the availability of Cas9 becomes a limiting factor upon co-expression of numerous sgRNAs, which compete for integration into the nuclease core. Nonetheless, it should be noted that T_1 efficiencies remained at roughly 50 %, and thus considerably high.

Editing 12 genes: Analysis of T_2 segregants, and isolation of transgene-free dodadecuple mutant plants

We further analyzed the occurrence of mutations from multiplexing in the T_2 generation, with the additional goal of isolating a transgene-free duodecuple mutant directly in the T_2 generation. We first analyzed functionality of six different genes (*RPP2*, *FKD1*, *RPS2*, *RPP4*, *RPS5* and *TMM*) in phenotypic assays, which involved infection assays with several different strains of the plant pathogenic bacterium *Pseudomonas syringae* or the oomycete *Hyaloperonospora arabidopsidis* and microscopic analyses of destained leaves (Figure 6A). Mainly T_2 families from primary transformants scored as *er gl1* double mutants or that had at least one of these mutations were included in analyses (see Table S2 for details). Plants were grown from seeds selected for absence of fluorescence to avoid confounding effects due to presence of the T-DNA and occurrence of novel somatic mutations. On average, 13 plants from 28 independent T_2 families were analyzed for each phenotype; ~ 2500 phenotypes were scored. These analyses revealed that mutations at all six loci were present within the population, but occurred at different frequencies. *E.g.,* the *rps2* phenotype, which was most frequent, was observed in 89 % of the families analyzed, and occurred in 60 % of T_2 plants. The *rpp4* phenotype was present in only 29 % of families and 7 % of T_2 plants. Different editing efficiencies were expected due to variable on-target efficacy of sgRNAs, even though we attempted to mitigate this effect by targeting each locus with two sgRNAs.

To corroborate phenotypic analyses and to identify alleles present at target sites we conducted short read sequencing of PCR amplicons. Prior to sequencing, absence of the T-DNA/Cas9 was verified by PCR. The rare occurrence of Cas9-positive plants confirmed that the FAST marker is highly discriminative (Shimada et al., 2010), and these were excluded from sequence analysis. Ten T_2 segregants per family were pooled and respective DNAs used for PCR amplification of target sites. PCR amplicons were then subjected to short read sequencing. Eight different families were analyzed, and the editing frequency at each target site calculated (Figure 6b). Results from amplicon sequencing revealed differences in sgRNA efficiency at target site resolution (in contrast to phenotypic analyses, which provided target locus resolution), and correlated well with our phenotypic analyses. Low mutation efficiencies were detected at both target sites within *RPP4* and *RPS5*, and respective mutant phenotypes were rare within the population (Figure 6a,b). Similarly, the *rps2* mutant phenotype was most frequent, and high mutation efficiencies were detected for both sgRNAs targeting this locus.

Next, we used short read sequencing of target loci in individual plants. Two lines were selected based on phenotypic analysis: Most plants of family #1681 appeared to be mutant at all tested loci (Table

S2), and also line #1688 was highly mutagenic. Remaining lines were selected randomly. All lines we analyzed contained mutations within at least seven loci that were targeted by the 24 sgRNAs (Figure 6c). As observed for sequencing of pools before, most plants contained wild type alleles at the *RPS5* and *RPP4* loci, again confirming low efficiency of respective sgRNAs. However, the *rpp4* phenotype was detected by phenotypic analyses within family #1681 (Table S2), and one of the two segregants we analyzed indeed carried disruptive mutations at all 12 loci with edits at 40/48 target sites (Figures 6c, S4). Thus, enhanced efficiencies achieved by intron-optimization of the Cas9 sequence (Gruetzner et al., companion manuscript) coupled with a high degree of multiplexing enabled us to isolate a duodecuple Arabidopsis mutant within a single generation.

Discussion

A simple toolkit for routine use

Here we present a toolkit designed for routine mutagenesis in Arabidopsis and *Nicotiana benthamiana*. The unique features of our toolkit are: *i)* High efficiencies resulting from the employment of components optimized from benchmarking analyses; *ii)* Preassembled recipient vectors ensuring predictable performance and simple assembly; *iii)* Extensive multiplexing capacities extending up to 32 sgRNAs; *iv)* Incorporation of previously established or newly developed markers to facilitate isolation of stable and transgene-free lines from segregating populations. Assembly of final plant transformation vectors follows a simple nomenclature and workflow. The first step being the generation of sgRNA TUs, followed by assembly of arrays of sgRNA TUs into recipient vectors or intermediate multi-multi (MM) vectors (Figure 2). For assembly of sgRNA arrays exceeding eight sgRNA TUs, arrays are mobilized from MM vectors to recipient vectors in a third cloning step. The assembly process can be accomplished in as little as four (up to eight sgRNAs) to six (up to 32 sgRNAs) days without any PCR (Figures 1,2). All assembly steps rely on highly efficient GoldenGate cloning (using either *Bsa*I or/and *Bpi*I), and simple step-by-step protocols are provided in Appendix 1. The vectors from this kit are provided through Addgene together with a set of MoClo compatible vectors described in the accompanying manuscript (Gruetzner et al.). Therefore, this joint kit of vectors and parts will provide users a choice of parts, vectors and assembly strategies for construction of zCas9i constructs.

Transgene counter-selection strategies

Strong seed fluorescence suitable as a non-destructive transformation marker has been obtained in Arabidopsis by several different systems (Stuitje et al., 2003; Bensmihen et al., 2004; Shimada et al., 2010). We have used both the FAST marker and the p*At2S3*:mCherry marker (not shown) for selection in Arabidopsis transformations, and obtained easily discernable seed fluorescence with both promoter/marker systems. Rare occurrence of T-DNA positive plants following negative selection (in T_2) likely arises from accidental carry-over of fluorescence-positive seeds rather than mis-phenotyping on the basis of seed fluorescence. In addition to a fluorescent marker, a herbicide/antibiotic resistance gene was included in pDGE vectors for the convenient positive selection of T-DNA-harboring Arabidopsis in the T_1 generation (Figure 1a, Appendix 1).

In contrast to Arabidopsis systems, negative selection markers for *N. benthamiana* were, as far as we are aware, not previously described. Utilizing the FAST marker we did not detect any seed fluorescence. However, the p*At2S3*:mCherry cassette described here (Figures 3,4) and also the p*At2S3*:2xtagRFP-T cassette contained in pDGE *N. benthamiana* vectors allow pre-selection of non-transgenic seeds with a relatively low false-negative rate (5 – 10 %). This is a considerable improvement in comparison to direct PCR-based screening, but weak fluorescence emitted by transgenic seeds remains difficult to detect (Figure 3). To supplement counter-selection by seed fluorescence we have developed a marker based on *Bs3*-mediated inducible cell death which is very reliable and time efficient (Figure 3). *Bs3* selection requires plants to be cultivated for approximately three weeks prior to testing. This dual counter-selection strategy considerably reduces the work load for isolation of non-transgenic mutants (Figure 4), but improved negative selection systems could further simplify RGN-mediated mutagenesis in *N. benthamiana*.

An obvious strategy could include the optimization of the fluorescence-based marker by use of a strong seed-specific promoter from *N. benthamiana* or a closely related Solanaceae, as previously done for adaptation of the FAST marker for use in rice (Shimada et al., 2011). In another approach, *PRODUCTION OF ANTHOCYANIN PIGMENTS 1* (*AtPAP1*) was integrated in transformation constructs used in *N. tabaccum* to mark transgenic lines by accumulation of anthocyanins (Liu et al., 2019). Strong anthocyanin accumulation has also been artificially induced in tomato (Mathews et al., 2003; Cermak et al., 2015; Du et al., 2020), but we failed to induce a visible phenotype by expression of *At*PAP1 in *N. benthamiana*. Indeed, provision of an appropriate bHLH transcription factor (TF) together with a MYB-type TF such as PAP1 may be required for anthocyanin induction in *N. benthamiana* (Outchkourov et al., 2014; Fresquet-Corrales et al., 2017), but may also have dominant-negative effects (Fatihah et al., 2019). The most promising approach for efficient selection of transgene-free segregants in *N. benthamiana* might therefore be adaption of a previously reported

system based on expression of a bacterial *BARNASE* and a male gametophyte lethal gene, which efficiently eliminated transgene-positive segregants in rice without any negative effects on performance of primary transformants (He et al., 2018).

Multiplexing – limits and opportunities

Here we generated stable Arabidopsis transgenics expressing 24 sgRNAs from a repetitive array (Figure 5a). Each sgRNA TU within the array encompassed approximately 200 nt, and TUs differed only by the 19-20 nt representing the crRNA segment of the sgRNAs. Although direct repeats (also within sgRNA arrays) were previously reported to induce frequent recombination events (e.g. Vidigal and Ventura, 2015; Ding et al., 2019, and references therein), we did not observe instability of plasmids in *E. coli* or Agrobacterium, and most primary Arabidopsis transformants (34/36) appeared to contain an intact T-DNA insertion (Figure 5a). Thus, repetitive sgRNA arrays are not prone to recombination during steps required for plasmid assembly and plant transformation. This is in agreement with the notion that recombination events mainly occur upon use of lentiviral vectors, or may be selected for upon introduction of selective phenotypes (Najm et al., 2018; Reis et al., 2019, and references therein). Further, the final size of the sgRNA array and also initiation of transcription at a specific G/T nucleotide have been raised as common concerns against Pol III promoters for sgRNA expression (e.g. Cermak et al., 2017). It should be noted that sgRNA TU arrays could be assembled with high efficiencies using our vector system, and high transformation rates were consistently observed (Figure S2). We are not aware of any negative effects of the initiating first nucleotide of U6/U3-transcribed sgRNAs, which may be non-complementary to the target site.

Previous comparisons detected higher editing efficiencies upon expression of sgRNAs by Pol II in comparison to Pol III promoters (Cermak et al., 2017). While Pol II promoters may provide a means to further enhance editing efficiencies and also to gain enhanced spatial and temporal control of sgRNA expression, editing efficiencies exceeded 80 % for single and double mutants among T_1 transformants with the intron-optimized Cas9 (Figure 5c; Gruetzner et al., companion manuscript). In higher order multiplexing applications, availability of the Cas9 nuclease core rather than sgRNAs became the limiting factor.

We used multiplexing with 24 sgRNAs to isolate transgene-free duodecuple mutant Arabidopsis plants directly in the T_2 generation (Figure 6). In this proof-of-concept study, we selected highly mutagenic families by phenotypic analyses, and a single family with mutations in all targeted loci was identified by amplicon sequencing. It is noteworthy that overall mutation frequencies were increased in families that were readily scored as *er* and *gl1* mutants in T_1 in comparison to those that contained

only one of these mutations (Table S2). We excluded families descending from *ER GL1* T_1 plants from analyses, but they assumedly exhibited further reduced editing efficiencies. We also observed pronounced differences in sgRNA efficiency (Figure 6). Thus, a low efficiency sgRNA could be incorporated in higher level multiplexing construct in future applications (e.g. producing an easy-to-score phenotype as *gl1*, or targeting non-coding DNA), and mutations at the respective target site could be used as a proxy to identify highly mutagenic lines. As another means to identify lines strongly expressing the Cas9 nuclease, additional vectors coding for GFP-Cas9 fusions (Appendix 1) were created to potentially allow evaluation of Cas9 abundance by GFP fluorescence.

We see two potential applications for massive multiplexing in mutagenesis applications. On the one hand, higher order mutants may be generated, although our proof-of-concept study shows that this may require considerable screening. On the other hand, small to intermediate gene families may be targeted by multiple sgRNAs to generate different combinations of mutant alleles in individual lines. Distribution of mutations within targeted genes will not be random due to different cleavage efficiencies of sgRNAs. However, this extends the opportunity to use RGNs as a directed forward genetics tool via the analysis of transgene-free T_2 segregants for a phenotype of interest without prior knowledge of the genotype.

Materials and Methods

Plant growth conditions and transformation

N. benthamiana wild-type plants were cultivated in a greenhouse with a 16-h light period (sunlight and/or IP65 lamps (Philips) equipped with Agro 400 W bulbs (SON-T); 130–150 µE/m^2*s; switchpoint ;100 µE/m^2*s), 60% relative humidity at 24/20°C (day/night). *N. benthamiana* plants were transformed as previously described (Gantner et al., 2019); a detailed protocol is provided online (dx.doi.org/10.17504/protocols.io.sbaeaie). Arabidopsis wild type accession Col was used, and plants were cultivated under short day conditions (8h light, 23/21°C day/night, 60 % relative humidity) or in a greenhouse under long day conditions (16h light) for seed set. Arabidopsis was transformed by floral dipping as previously described (Logemann et al., 2006).

Molecular cloning and selection of tomato U6/U3 promoter fragments

The GoldenGate technique following the Modular Cloning syntax for hierarchical DNA assembly was used for most clonings (Engler et al., 2008; Weber et al., 2011). Previously reported plasmids belonging to the Modular Cloning Toolkit and the MoClo Plant Parts I and II collections were used (Engler et al., 2014; Gantner et al., 2018). For domestication of new DNA modules, respective fragments were amplified using Polymerase X (Roboklon), and ligated into Level 0 vectors.

*Sl*U6/U3 promoter fragments were selected from multiple sequence alignments of U6/U3 genes and upstream regions from Arabidopsis and tomato. Promoter fragments encompassing approximately 250 nt and 120 nt upstream of the predicted transcription start site were cloned, and functionally verified by expression of sgRNAs in reporter-based assays. No functional differences were observed, and shorter promoter fragments were used to generate data presented in this study. Shuttle vectors containing promoter fragments were cloned as previously described (Ordon et al., 2017). Briefly, the promoter fragment and a second fragment encompassing a *ccdB* cassette and the sgRNA scaffold were PCR-amplified (*e.g.*, using oligonucleotides JS1744/1745 and STS24/JS1746 in case of the *Sl*U3 promoter), and subsequently fused by SOE-PCR. The SOE-PCR product was cloned into pUC57-BsaI in a cut/ligation reaction using *Eco*RV to yield the M1E module. This served as PCR template to amplify fragments for further shuttle vectors, which were (after *Dpn*I digestion) cloned as before. Plasmids and oligonucleotides are summarized in Table S1. Additional plasmids are listed in Appendix 1 as part of the cloning manual of our toolkit. Vector maps (GenBank) are provided in Appendix 2.

Agroinfiltration, reporter-based nuclease activity assays and localization studies

For transient expression of proteins in *N. benthamiana* leaf tissues (agroinfiltration), respective T-DNA constructs were transformed into *Agrobacterium* strain GV3101 pMP90. Plate-grown bacteria were resuspended in Agrobacterium Infiltration Medium (AIM; 10 mM MES pH 5.8, 10 mM $MgCl_2$), and infiltrated with a needleless syringe at an OD_{600} = 0.3 per strain. For qualitative determination of GUS activity, leaf discs were taken 3 dpi, stained with GUS staining solution (10 mM phosphate buffer pH 7, 10 mM EDTA, 1 mM potassium ferricyanate, 1 mM potassium ferrocyanate, 0,1 % Triton X-100, 0,1% X-Gluc) for 3 – 5h, destained with ethanol and dried in cellophane. Quantitative determination of GUS activity was done as previously described (Ordon et al., 2017). Briefly, leaf material was ground in liquid nitrogen, powder resuspended in extraction buffer and cleared lysates prepared by centrifugation. Protein extracts were incubated with 4-methylumbelliferyl-beta-D-glucuronide (4MUG; 5 mM) for 1 – 2h, and MU production measured on a Tecan plate reader. Live-cell imaging was done using a Zeiss LSM780 confocal laser scanning microscope. GFP was excited using the 488 nm laser, and the detector range was set to 493-532 nm.

459

460 ***Transgene counter-selection and genotyping***

461 A motorized SteREO Discovery.V12 microscope (Zeiss) with UV illumination and an RFP filter set
462 connected to an AxioCam MRc camera was used for selection of non-fluorescent seeds. Arabidopsis
463 seeds containing the FAST marker (Shimada et al., 2010; Engler et al., 2014) were sorted by direct
464 observation through the binocular. Weakly fluorescent *N. benthamiana* seeds containing the
465 p2S3:mCherry/RFP cassette were imaged using the camera with an exposure time of approximately
466 5 s. Seeds were aligned on a wet sheet of Whatman paper, imaged and sorted. Selected seeds were
467 directly sown into potting soil. After 3 – 4 weeks, segregants were infiltrated with a *Pseudomonas*
468 *fluorescens* strain containing a chromosomal integration of the *Pseudomonas syringae* type III
469 secretion system ("EtHAn"; Thomas et al., 2009) and a plasmid for expression and translocation of
470 the *Xanthomonas campestris* pv. *vesicatoria* transcription activator-like effector AvrBs3 (Gantner et
471 al., 2018). Plate-grown *P. fluorescens* bacteria were resuspended in 10 mM $MgCl_2$, and infiltrated at
472 an OD_{600} = 0.6. Cell death and development of the hypersensitive response was scored 2-3 dpi. DNA
473 was extracted from Arabidopsis and *N. benthamiana* by the CTAB method, and Taq polymerase or
474 Polymerase X (Roboklon) was used for genotyping.

475

476 ***Multiplex editing and phenotypic analyses in Arabidopsis***

477 All genome editing constructs were assembled as described in Appendix 1. sgRNA target sites were
478 selected using ChopChop (for Arabidopsis; Labun et al., 2016) or CRISPR-P (for N. benthamiana; Liu et
479 al., 2017). Target sites, oligonucleotides used for sgRNA construction and oligonucleotides used for
480 genotyping are listed in Table S1.

481 For phenotypic analyses in the T_1 generation, primary transformants were selected by resistance to
482 BASTA. Presence of trichomes (editing of *GLABROUS1)* was scored by visual inspection in the
483 vegetative phase, and editing of *ERECTA* upon bolting.

484 In the T_2 generation, non-transgenic seeds were selected by absence of seed fluorescence. Plants
485 were cultivated under short day conditions, and phenotypes associated with inactivation of genes
486 targeted by genome editing were accessed as following: Presence/absence of trichomes was
487 evaluated for editing of ***GLABROUS1*** at 2-3 weeks. The ***too many mouths*** and ***forked1*** phenotypes
488 were identified in de-stained first leaves. Destaining involved treatment of leaves with a 3:1 mix of
489 ethanol and acetic acid for 2 hours, overnight incubation in 95% ethanol and 1 hour in 5 % NaOH at
490 60°C (modified from Steynen and Schultz, 2003). Leaves were then mounted in 50% glycerol and
491 phenotyped *via* DIC microscopy utilizing an inverted AxioObserver with an AxiocamMRm-DDE

camera. ZenBlue software was used for capturing images and controlling the microscope. Three weeks-old seedlings were infected with *Hyaloperonospora arabidopsidis* isolates Cala2 and Emwa1 to analyze editing of the ***RPP2a*** (Sinapidou et al., 2004) and ***RPP4*** (van der Biezen et al., 2002) loci, respectively, and infection phenotypes were scored 6 dpi by Trypan Blue staining as previously described (Stuttmann et al., 2011). To evaluate functionality of ***RPS2*** (Debener et al., 1991) and ***RPS5*** (Warren et al., 1998), approximately 5 weeks-old plants were syringe-infiltrated with *Pseudomonas syringae* strain DC3000 derivatives containing plasmids for the expression of AvrRpt2 or AvrPphB, respectively, at an OD_{600}=0.05. Plants were left covered over-night, and development of the HR was scored 16-24 hpi. Results of phenotyping are presented in Table S2.

Amplicon sequencing and data analysis

Oligonucleotides for PCR-amplification (Table S1) were designed using the NCBI primer designing tool. DNAs from pooled plant material or individual plants were tested for absence of the T-DNA using zCas9i-specific oligonucleotides. Amplicons encompassing RGN-targeted regions were pooled, purified, quantified using a NanoDrop and sequenced by Genewiz (Amplicon-EZ). Paired-end raw reads were adapter- and quality- trimmed using Trim Galore with default parameters (https://github.com/FelixKrueger/TrimGalore, v0.4.0). Trimmed reads were merged (-minhsp 10 -fastq_minmergelen 20) and dereplicated (--strand both) using USEARCH (Edgar, 2010; v11.0.667_i86linux32). Merged, dereplicated reads were mapped to the sequences of the 15 amplicons using BWA mem with default parameters (Li and Durbin, 2010). Mappings were analyzed with samtools view (Li et al., 2009; v1.2, using htslib 1.2.1) allowing to extract statistics for individual amplicons.

Accession Numbers

Accession numbers for Arabidopsis genes are provided in Table S1. Sequence information for N. benthamiana genes can be accessed on solgenomics.net (*NRG1* - Niben101Scf02118g00018; *NPR1a* - Niben101Scf14780g01001; *NPR1b* - Niben101Scf11512g01004).

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Supporting Information

Supplemental Figure S1: Functional verification of genome editing vectors and U6/U3 promoters.

Supplemental Figure S2: Transformation efficiencies with pDGE vectors in *N. benthamiana*.

Supplemental Figure S3: Verification of T-DNA integrity in additional T_1 transformants.

Supplemental Figure S4: Alleles detected in T_2 segregants from multiplex editing.

Supplemental Table S1: Plasmids and oligonucleotides used in this study.

Supplemental Table S2: Phenotypic analysis of T_2 segregants from multiplex editing.

Appendix 1: Cloning manual for pDGE vector system.

Appendix 2: Annotated sequence files for pDGE vectors.

Author Contributions

KB, PM, JO, JE, JG, RH and CK performed experiments, analyzed data and contributed to preparation of figures. TB and JK analyzed amplicon sequencing data. SM provided the intron-optimized Cas9 module prior to publication, and discussed data. JS designed the study, supervised experimental work, prepared final figures, and wrote the manuscript with contributions from JE and all authors.

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754

Figure 1

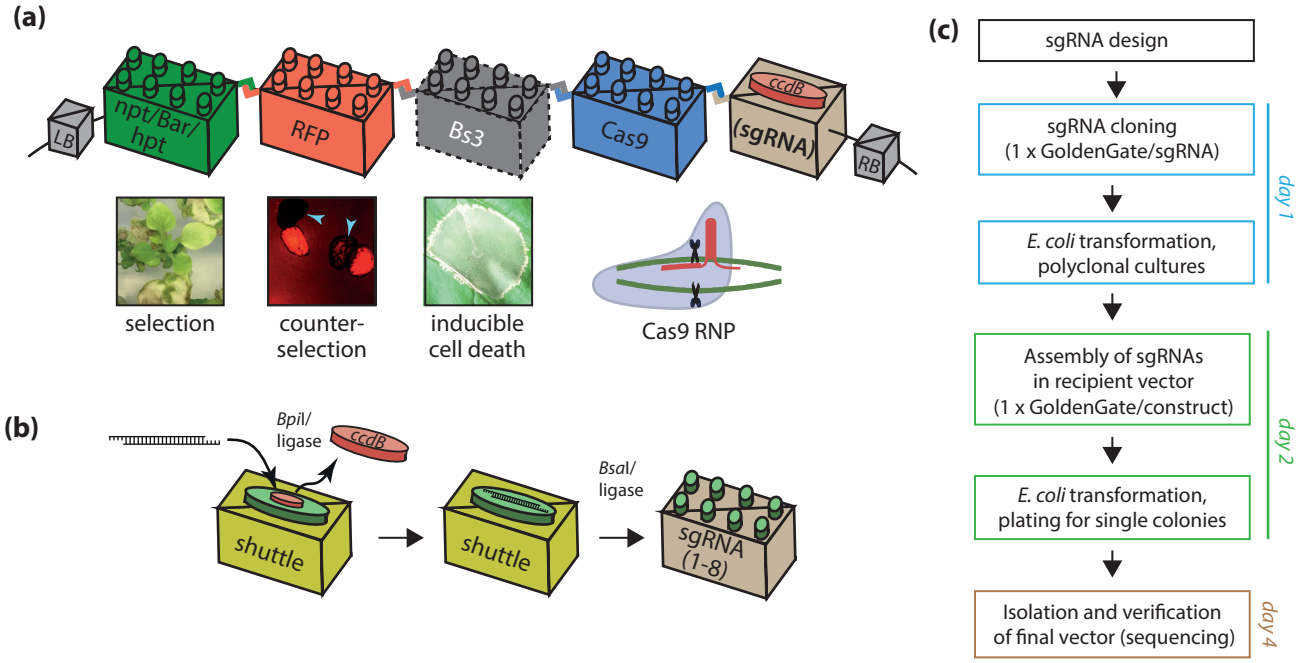


Figure 1: Schematic overview of the pDGE vector system.

(a) Recipient vectors. Pre-assembled recipient vectors containing all components except sgRNA transcriptional units (TUs) build the core of the pDGE vector system. Recipients containing any of the indicated marker genes (green brick) for selection of primary transformants by resistance to Kanamycin, phosphinotricin (BASTA) or Hygromycin are available. Furthermore, vectors contain a secondary seed fluorescence marker (red brick) and optionally the pepper *Bs3* gene (grey brick), which confers AvrBs3- (an effector protein of plant pathogenic *Xanthomonas* bacteria) inducible cell death. All vectors contain a highly intron-optimized Cas9 expression cassette (blue brick), either coding for ^{NLS}Cas9^{NLS} or ^{NLS}GFP-Cas9^{NLS}. A *ccdB* cassette (light brown brick) functions as a placeholder, and is replaced by sgRNA TUs in a *Bsa*I GoldenGate reaction. LB, RB - left/right T-DNA border.
(b) Shuttle vectors. The variable fraction of sgRNAs is introduced into shuttle vectors as hybridized oligonucleotides (generally 23-24 nt in length) in a *Bpi*I GoldenGate reaction, and replaces a *ccdB* cassette. sgRNA TUs from up to eight shuttle vectors can subsequently be combined in a recipient vector in a *Bsa*I GoldenGate reaction. Shuttle vectors contain Arabidopsis U6-26 or tomato (*Solanum lycopersicum*, *Sl*) U6 or U3 promoter fragments. Each set of shuttle vectors (containing the different U6/U3 promoter fragments) encompasses 12 vectors differing by the *Bsa*I-generated GoldenGate overhangs.
(c) Timeline of vector assembly. After sgRNA design, oligonucleotides are cloned into shuttle vectors in a first GoldenGate reaction using *Bpi*I on day one. As shuttle vectors contain the *ccdB* negative selection cassette, polyclonal cultures can directly be prepared subsequent to transformation of GoldenGate reactions. On day two, plasmids are isolated from polyclonal cultures, and are used for a second GoldenGate reaction with *Bsa*I to mobilize sgRNA TUs from shuttle vectors into recipient vectors. On day four, final plant transformation vectors are prepared, and can be sent for sequence verification of the sgRNA array.

Figure 2

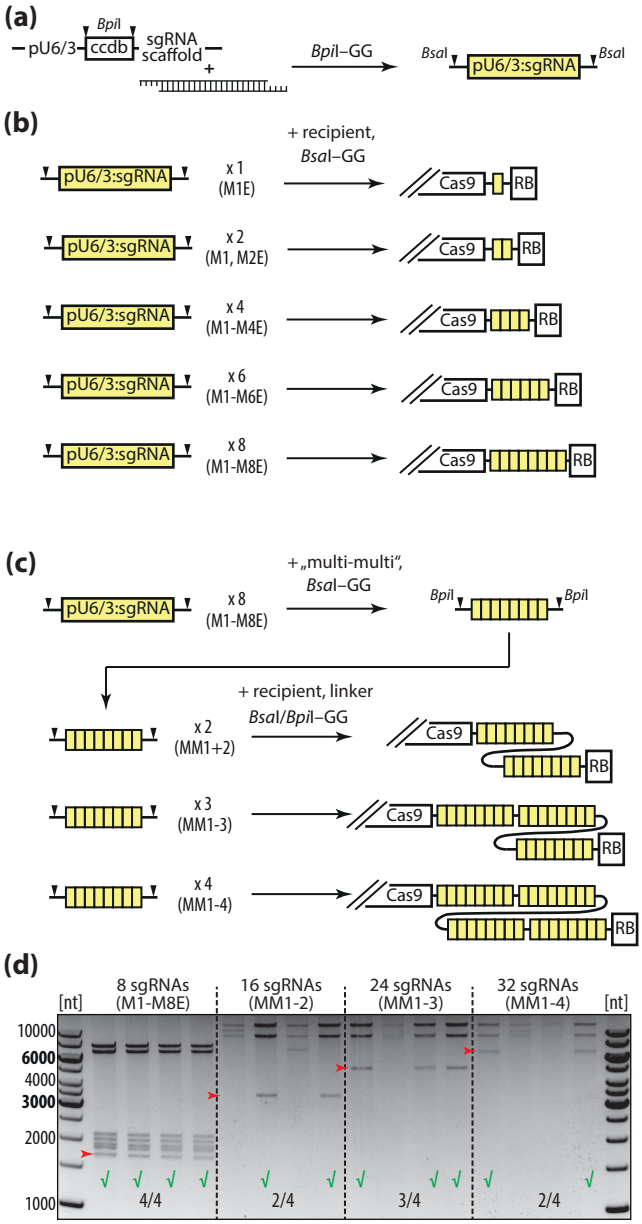


Figure 2: Assembly of arrays of sgRNA transcriptional units in recipient vectors.

(a) Loading of shuttle vectors with hybridized oligonucleotides in a *Bpi*I GoldenGate (GG) reaction yields sgRNA transcriptional units (TUs) flanked by *Bsa*I restriction sites. Shuttle vectors are denoted M1-M8 (for module 1-8) or, *e.g.*, M2E (E - end) for modules ligating with recipient vector overhangs and thus ending the array.
(b) Assembly of up to eight sgRNA TUs in a recipient vector. Loaded shuttle vectors are used in a *Bsa*I-GG reaction to mobilize the sgRNA TUs into a recipient vector. For a single sgRNA, only the loaded M1E shuttle vector is used. For arrays of two, four, six or eight sgRNA TUs in a recipient vector, the respective loaded shuttle vectors and an „end" module are used. As an example, assembly of four sgRNA TUs requires M1, M2, M3 and M4E modules.
(c) Assembly of up to 32 sgRNA TUs in two steps in a recipient vector. For the assembly of sgRNA arrays exceeding eight modules, an additional cloning step is required. sgRNA TUs are mobilized into intermediate „multi-multi" (MM) vectors in a *Bsa*I-GG reaction. MM modules are available for four different positions (MM1-4), and can be combined in a recipient vector in a further GG assembly. The assembly of MM modules requires the use of both *Bsa*I and *Bpi*I in the GG reaction, and end linkers are used to link overhangs of the MM modules to the vector. Using the MM modules, up to 32 sgRNA TUs can be assembled in a recipient vector within 6-7 days with high fidelity.
(d) Efficiency of cloning reactions. Four clones each from assembly of 8, 16, 24 or 32 sgRNAs in a recipient vector were randomly selected for plasmid isolation, and DNA was digested with *Pst*I/*Hind*III (8 sgRNAs) or *Hind*III (remaining assemblies). The band corresponding to the sgRNA array is marked with a red arrowhead.

Figure 3

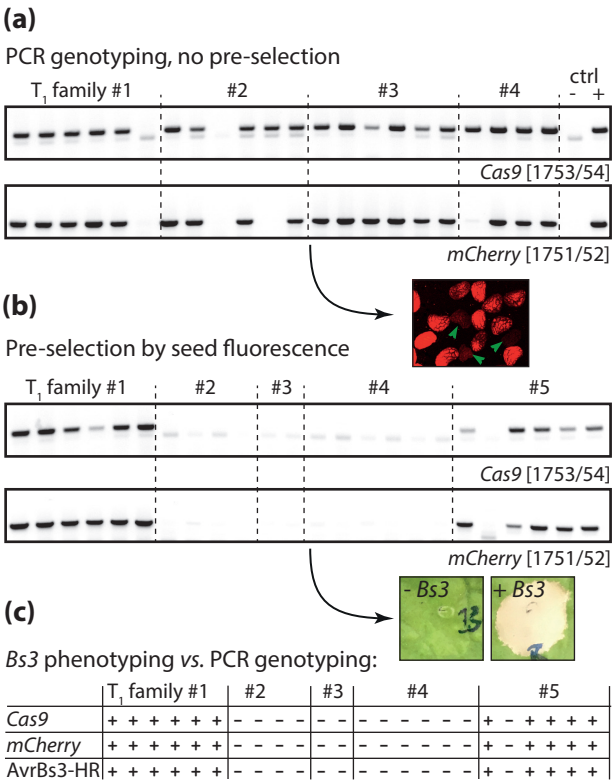


Figure 3: Transgene counter-selection in *N. benthamiana* by seed fluorescence and inducible cell death.

(a) PCR-genotyping of randomly selected *N. benthamiana* T_1 segregants with two different T-DNA-specific primer pairs. DNA of a wild type plant was used as negtive control (ctrl -), and DNA of a T_0 regenerated plant served as positive control (+).
(b) As in a), but seeds were pre-selected for absence of seed fluorescence.
(c) Phenotyping of T_1 segregants for presence of the *Bs3* marker gene. The same T_1 segregants analyzed by PCR in b) were infiltrated with a *Pseudomonas fluorescens* derivative for translocation of AvrBs3. Appearance of cell death (see inset) was evaluated 3 dpi, and results were summarized in a table in comparison to results from PCR genotyping.

Figure 4

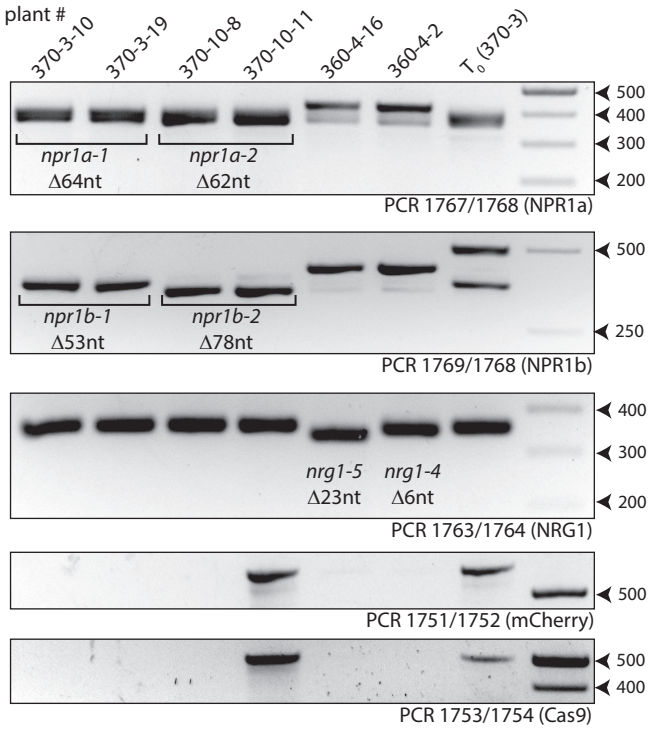
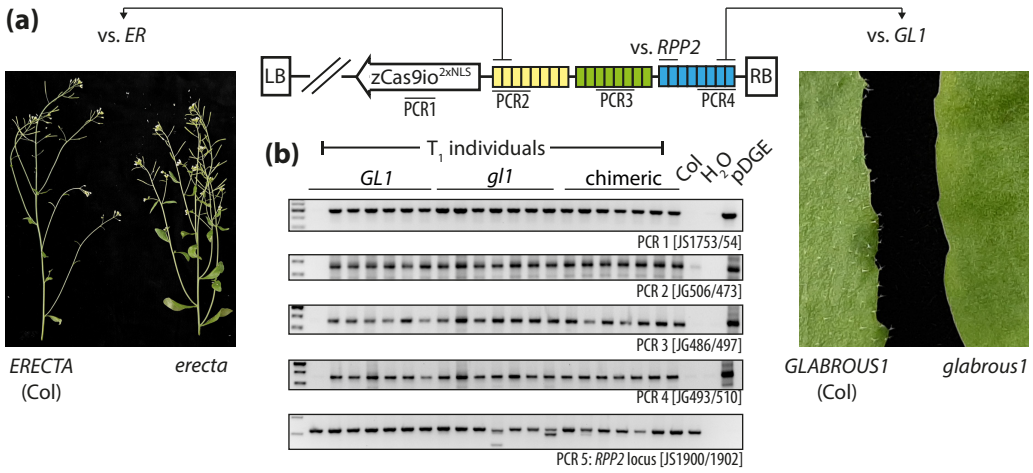


Figure 4: Efficient isolation of transgene-free genome-edited *N. benthamiana* lines by dual counter-selection.

In total 90 seeds lacking seed fluorescence were selected from three different T_1 families from two different transformations (5 -6 working hours). From these, approximately 80 T_1 plants were grown, and infiltrated with *P. fluorescens* bacteria translocating AvrBs3 (< 1h). 4/80 plants (5 %) showed cell death at 3 dpi, and were discarded. DNA was extracted from the remaining 76 plants, and used for genotyping with indicated primer pairs. For each family, transgene-free segregants homozygous for RGN-induced alleles at target loci were identified.

Figure 5



(c)

[%]	*er/er*	*ER/--*	chimeric	*gl1/gl1*	*GL1/--*	chimeric	*n*
pDGE579 *(ERECTA)*	94	6	0	n/a	n/a	n/a	54
pDGE580 *(GLABROUS1)*	n/a	n/a	n/a	86	9	6	35
pDGE581 *(ER, GL1)*	88	10	2	85	10	5	41
pDGE585 *(24 sgRNAs)*	66	32	2	45	27	28	128

Figure 5: Analysis of primary (T_1) transformants from multiplexing applications

(a) Schematic drawing of the plant transformation construct (pDGE585) containing 24 sgRNA transcriptional units (TUs). Blocks consisting of 8 sgRNA TUs each from intermediate cloning steps (positions 1 - 3) are depicted in yellow, green and blue, respectively. PCR amplicons used for verification of T-DNA integrity are indicated. Insets show phenotypes resulting from editing of loci targeted by sgRNAs flanking the array.
(b) Verification of T-DNA integrity in primary transformants. Phenotypes (trichome development; *GLABROUS1*) of primary transformants (selected for resistance to BASTA) used for DNA extractions are indicated. Untransformed wild type (Col) and the transformation vector (pDGE) were included as controls. PCR 5 amplifies a fragment of the *RPP2* locus trageted by two nucleases encoded by the multiplexing constructs as shown in a).
(c) Frequency of plants with *erecta* and *glabrous* phenotypes in T_1 plants from transformation of indicated constructs. pDGE579 and 580 contained sgRNA TUs (two each) for targeting of *ERECTA* and *GLABROUS1*. pDGE581 contained the four sgRNA TUs from pDGE579/580 in a single construct. pDGE585 contained the four sgRNA TUs from pDGE581, and 20 additional sgRNA TUs. Values indicate the number of primary transfromants showing a respective phenotype in percent. *n* indicates the total number of primary transformants analyzed for each construct.

Figure 6

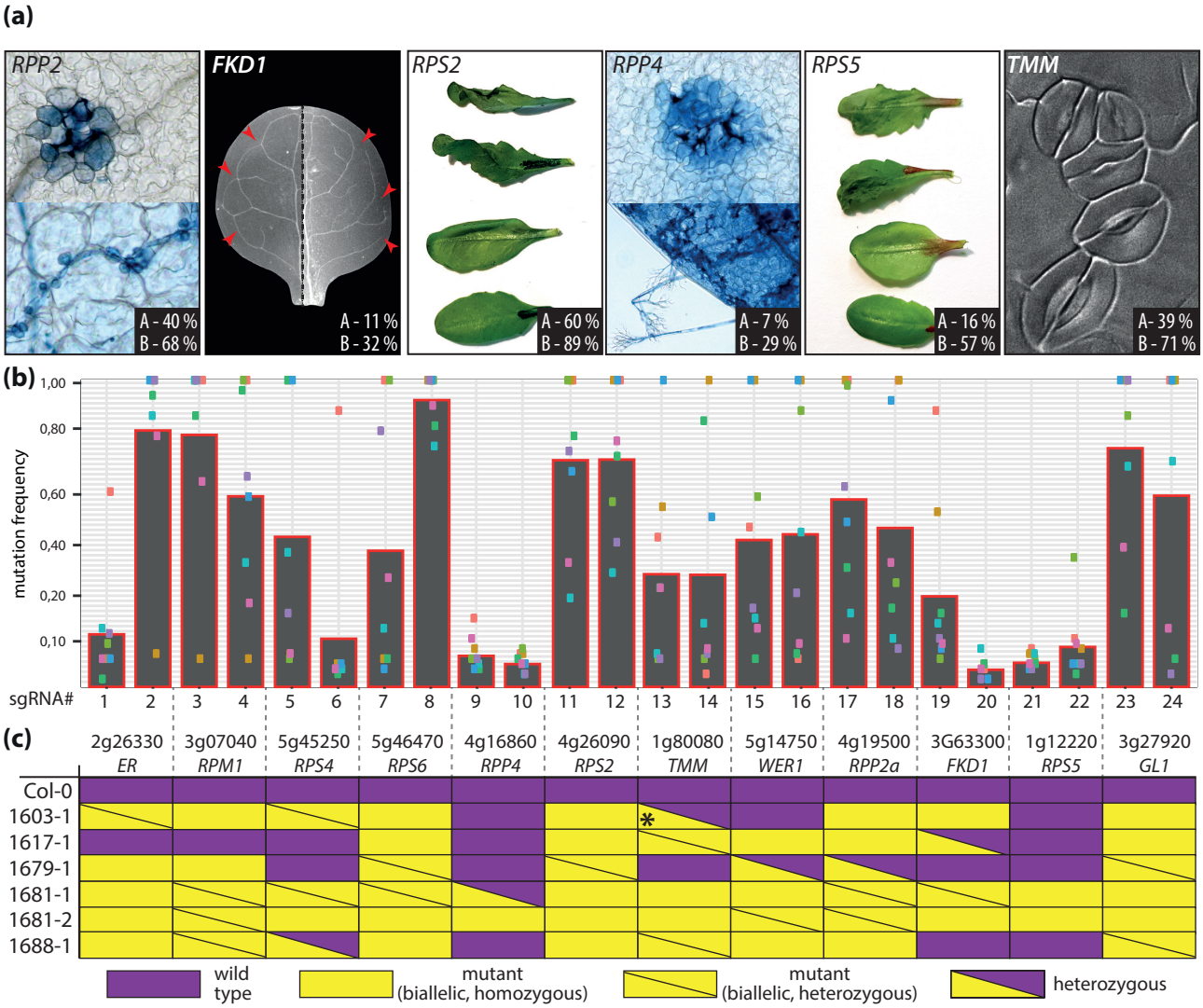
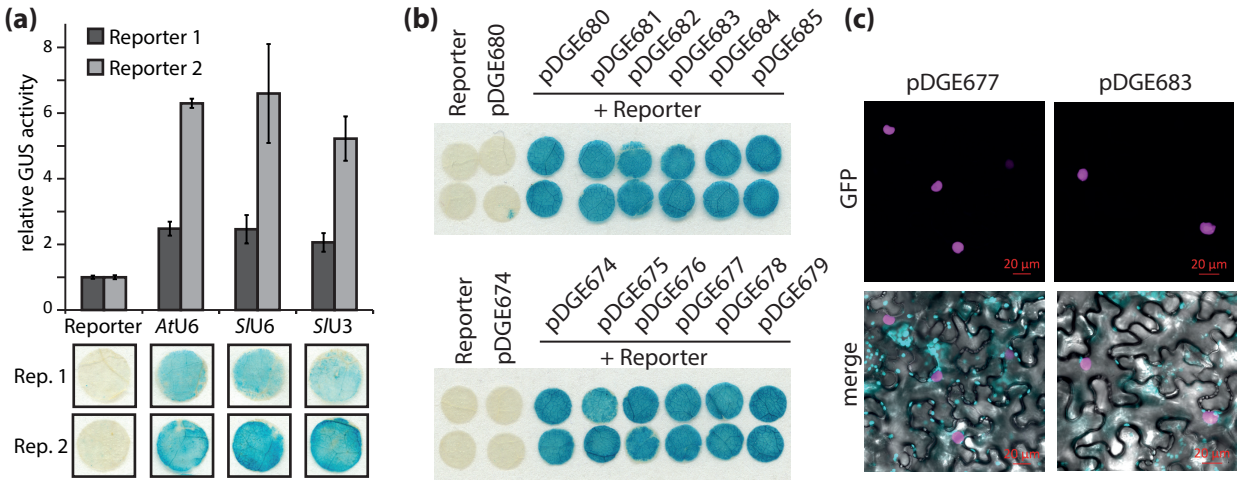


Figure 6: Analysis of transgene-free T_2 segregants from multiplex editing in Arabidopsis.

(a) Images of phenotypes that were assessed in T_2 segregants. The percentage of plants which were scored mutant for a respective phenotype among all tested segregants (A) and the percentage of T_2 families, in which the phenotype was detected (B), are indicated (see Table S2 for additional details). In average, 13 segregants from 28 independent T_2 families were analyzed for each phenotype.
(b) Mutation frequency at individual sgRNA target sites. DNA was extracted from 10 transgene-free segregants per T_2 family, and used as template for amplification of target loci. Pooled amplicons were sequenced by Illumina technology, and data was analyzed as described in methods to evaluate sgRNA efficiency. Mutation frequencies are color-coded for T_2 pools (#1603; #1606; #1611; #1613; #1673; #1679; #1681, #1696). Non-linear y-axis scaling for display of high and low values.
(c) Analysis of target locus integrity in single plants. Alleles at target loci from single transgene-free segregants were analyzed by amplicon sequencing. Each locus was targeted by two different sgRNAs, and a gene was scored as "mutant" if both detected alleles contained a mutation in at least one sgRNA target site. Details on detected alleles are provided in Figure S4. * - the mutant allele detected at the *TMM* locus in plant 1603-1 contained an in-frame deletion (6 nt), which might not disrupt TMM function.

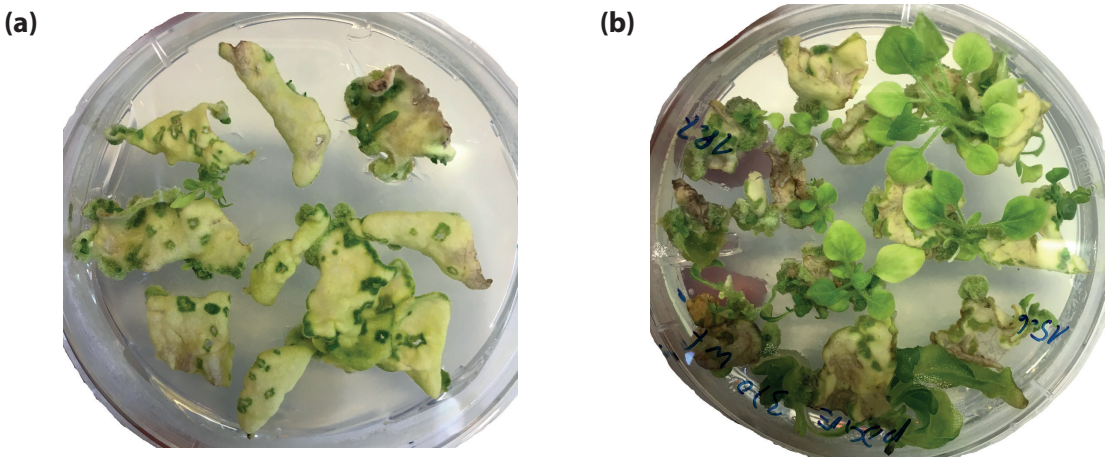
Supplemental Figure S1



Supplemental Figure S1: Transient agroinfiltration assays for functional verification of genome editing vectors.

(a) Comparison of nuclease efficiency upon sgRNA expression by *At*U6, *Sl*U6 or *Sl*U3 promoter fragments using ß-Glucuronidase (*GUS*) nuclease activity reporters. Reporters consist of a 35S promoter-controlled *GUS* gene, which is shifted out-of-frame by an insertion subsequent to the initiating ATG (Ordon et al., 2019). Nucleases target this insertion sequence, and repair of double-strand breaks leads to reconstitution of a functional *GUS* gene. *Agrobacterium* strains containing reporters with two different target sites (Reporter 1: ATCCCGGAATTATCAGCACGA**GGG**; Reporter 2: TATGCTGCATGTAATCTGAAA**GGG**; PAM in bold) were infiltrated into *N. benthamiana* leaves, alone or in combination with strains for nuclease expression. Nuclease constructs contained sgRNAs targeting the respective reporter construct under control of the indicated U3/U6 promoter fragments. GUS activity resulting from reporters alone was arbitrarily set to 1, and error bars represent standard deviations from four replicates. Leaf discs from qualitative GUS staining are shown below the graph. Leaf material was harvested 3 dpi.
(b) Verification of activity of nucleases encoded by the indicated constructs via *GUS*-based reporter assays. The sgRNA targeting Reporter 2 was cloned into the indicated recipient vectors, and Agrobacterium strains containing these constructs were used in reporter assays as in (a).
(c) Detection and localization of GFP-Cas9. Tissue samples from the same experiment as shown in (b) were analyzed by confocal laser scanning microscopy. GFP - magenta; chlorophyll A - cyan.

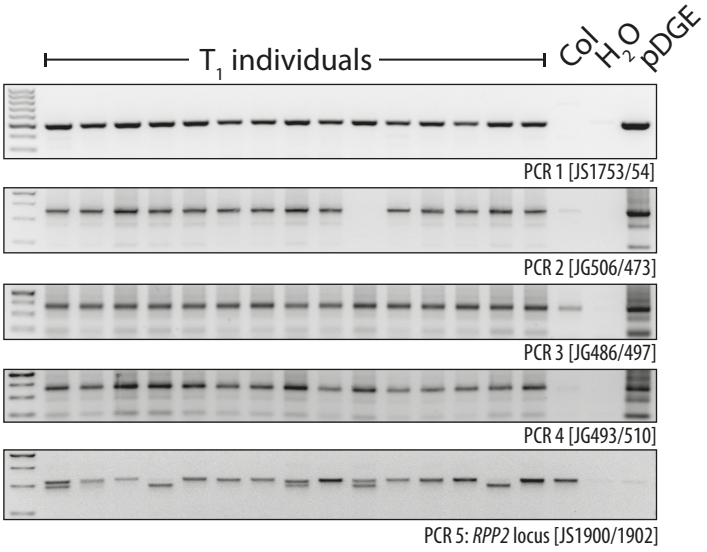
Supplemental Figure S2



Supplemental Figure S2: High efficiency of pDGE recipient vectors in *Agrobacterium*-mediated transformation of *Nicotiana benthamiana*

Greenhouse-grown *Nicotiana benthamiana* plants were transformed as described in Materials and Methods. Four to five weeks after transformation (a), the formation of multiple calli from individual leaf sections became apparent. After 6-7 weeks (b), shoots developed from most calli.

Supplemental Figure S3



Supplemental Figure S3: Verification of T-DNA integrity in additional T_1 transformants.

As in Figure 5b, but additional transformants were tested. PCR1 - Cas9; PCRs 2/3/4 - different amplicons within the array of 24 sgRNA TUs; PCR5 - *RPP2* locus, also targeted by sgRNAs.

Supplemental Figure S4

	Allele	At2g26330 *ER*		At3g07040 *RPM1*		At5g45250 *RPS4*		At5g46470 *RPS6*		At4g16860 *RPP4*		At4g26090 *RPS2*		At1g80080 *TMM*		At5g14750 *WER1*		At4g19500 *RPP2a*		At3G63300 *FKD1*		At1g12220 *RPS5*		At3g27920 *GL1*	
		sg_1	sg_2	sg_3	sg_4	sg_5	sg_6	sg_7	sg_8	sg_9	sg_10	sg_11	sg_12	sg_13	sg_14	sg_15	sg_16	sg_17	sg_18	sg_19	sg_20	sg_21	sg_22	sg_23	sg_24
Col-0	A	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
1603-1	A	wt		+1		+1	wt	-227		wt	wt	-11	-23	wt	wt	wt	wt	-5	+7	+1	wt	wt	wt	-3	-21
	B	+1	-19		-21	-140								-6											
1617-1	A	wt	wt	wt	wt	wt	wt	wt	+1	wt	wt	-1	+1	wt	+1	-27	-7	-65		wt	wt	wt	wt	-8	-7
	B													+1						+1					
1679-1	A	wt	-8	+1	-18	wt	wt	wt	+1	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	-24	wt
	B							wt	-15			-2	+1			-247		-1						-2	
1681-1	A	-49	-6	+1	+1	+1	wt	+1	+1	wt	wt	-1	-2	wt	-5	+1	-9	-26	-1	+1	+1	wt	+1	-148	
	B			-1	-1		+1		+2	+1								-75		-4					
1681-2	A	-49	-6	+1	+1	+1	wt	+1	+1	+1	wt	-1	-2	wt	-5	+1	-9	-26	-1	-4	+1	wt	+1	-148	
	B			-1	-1											-12		-75							
1688-1	A	wt	-14	+1	+1	+1	wt	+1	+1	wt	wt	-1	-30	+1	wt	-247		-66		wt	wt	wt	wt	-2	-7
	B			-149		wt									+1									-159	

Supplemental Figure S4: Details on alleles detected in single plants by amplicon sequencing.

Target loci from indicated transgene-free T_2 plants were PCR-amplified and analyzed by amplicon sequencing. Reads were quality filtered, merged and aligned to the Col reference genome to identify respective alleles. In most cases, analyzed PCR amplicons contained both target sites, thus allowing unambiguous assignment of alleles A and B. Target sites for loci marked by grey shading were PCR-amplified as two independent amplicons.

pDGE Dicot Genome Editing vectors: Cloning Manual v4

Shuttle vectors (Amp/Carb, Cm):

sgRNA shuttle vectors with *Arabidopsis thaliana* U6 promoter: guide sequence overhangs ATTG-GTTT
sgRNA shuttle vectors with *Solanum lycopersicum* U6 promoter: guide sequence overhangs ATTG-GTTT
sgRNA shuttle vectors with *Solanum lycopersicum* U3 promoter: guide sequence overhangs GTCA-GTTT

module type	*Arabidopsis thaliana* U6 promoter	*Solanum lycopersicum* U6 promoter	*Solanum lycopersicum* U3 promoter
M1E	pDGE331	pDGE654	pDGE473
M1	pDGE332	pDGE655	pDGE474
M2	pDGE333	pDGE656	pDGE475
M2E	pDGE334	pDGE657	pDGE476
M3	pDGE335	pDGE658	pDGE477
M4	pDGE336	pDGE659	pDGE478
M4E	pDGE337	pDGE660	pDGE479
M5	pDGE495	pDGE661	pDGE498
M6	pDGE496	pDGE662	pDGE499
M6E	pDGE497	pDGE663	pDGE500
M7	pDGE341	pDGE664	pDGE501
M8E	pDGE342	pDGE665	pDGE502

Recipient vectors for *Arabidopsis thaliana*:

Vector name	description	Cas9 cassette	Plant selection I	Plant selection II/ counter-selection	Bacterial selection
pDGE347	FAST_Bar_ pRPS5a:Cas9_ccdB	pRPS5a:Cas9	Bar [Phosphino-tricin (BASTA)]	FAST	Spec, Cm (ccdB)
pDGE651	FAST_hpt_ pRPS5a:Cas9_ccdB	pRPS5a:Cas9	hpt [Hygromycin]	FAST	Spec, Cm (ccdB)
pDGE652	FAST_nptII_ pRPS5a:Cas9_ccdB	pRPS5a:Cas9	nptII [Kanamycin]	FAST	Spec, Cm (ccdB)
pDGE666	FAST_Bar_pRPS5a:GFP-Cas9_ccdB	pRPS5a:GFP:Cas9	Bar [Phosphino-tricin (BASTA)]	FAST	Spec, Cm (ccdB)
pDGE667	FAST_hpt_pRPS5a: Cas9_ccdB	pRPS5a:GFP:Cas9	hpt [Hygromycin]	FAST	Spec, Cm (ccdB)
pDGE668	FAST_nptII_pRPS5a: GFP-Cas9_ccdB	pRPS5a:GFP:Cas9	nptII [Kanamycin]	FAST	Spec, Cm (ccdB)

Recipient vectors for *Nicotiana benthamiana*:

Vector name	description	Cas9 cassette	Plant selection I	Counter-selection	Bacterial selection
pDGE463	nptII_2xRFP_Bs3_ p2x35S:Cas9_ccdB	p35S:Cas9	nptII [Kanamycin]	RFP, Bs3	Spec, Cm (ccdB)
pDGE669	hpt_2xRFP_Bs3_ p2x35S:Cas9_ccdB	p35S:Cas9	hpt [Hygromycin]	RFP, Bs3	Spec, Cm (ccdB)
pDGE670	Bar_2xRFP_Bs3_ p2x35S:Cas9_ccdB	p35S:Cas9	Bar [Phosphino-tricin (BASTA)]	RFP, Bs3	Spec, Cm (ccdB)
pDGE671	nptII_2xRFP_Bs3_ p35S:GFP-Cas9_ccdB	p35S:GFP-Cas9	nptII [Kanamycin]	RFP, Bs3	Spec, Cm (ccdB)
pDGE672	hpt_2xRFP_Bs3_ p35S:GFP-Cas9_ccdB	p35S:GFP-Cas9	hpt [Hygromycin]	RFP, Bs3	Spec, Cm (ccdB)
pDGE673	Bar_2xRFP_Bs3_ p35S:GFP-Cas9_ccdB	p35S:GFP-Cas9	Bar [Phosphino-tricin (BASTA)]	RFP, Bs3	Spec, Cm (ccdB)

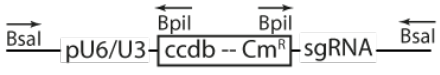
Recipient vectors and end-linkers for higher order multiplexing:

Vector name	description	overhangs [sgRNA TUs; *Bsa*I]	overhangs [array/linker release]	Bacterial selection
pDGE503	intermediate sgRNA array cloning vector position 1	TTGC-GCTT	TTGC-CCCT	Kan, Cm (ccdB)
pDGE504	intermediate sgRNA array cloning vector position 2	TTGC-GCTT	CCCT-CGGT	Kan, Cm (ccdB)
pDGE505	intermediate sgRNA array cloning vector position 3	TTGC-GCTT	CGGT-GACT	Kan, Cm (ccdB)
pDGE506	intermediate sgRNA array cloning vector position 4	TTGC-GCTT	GACT-CCAG	Kan, Cm (ccdB)
pDGE508	end-linker for cloning 2 sgRNA arrays into any recipient vector	n/a	CGGT-GCTT-	Kan
pDGE509	end-linker for cloning 3 sgRNA arrays into any recipient vector	n/a	GACT-GCTT	Kan
pDGE510	end-linker for cloning 4 sgRNA arrays into any recipient vector	n/a	CCAG-GCTT	Kan

- Most empty vectors contain a *ccdB* cassette and must be propagated in DB3.1 or *ccdB* survival cells (Invitrogen / Thermo Scientific).
- Amp – Ampicillin, Carb – Carbenicillin, Spec – Spectinomycin, Kan – Kanamycin, Cm - Chloramphenicol
- Annotated sequence files are provided as a multi-record genbank file, which can be opened with any DNA sequence analysis software (e.g. CLC, Lasergene, Sequencher, Geneious, Vector NTI).

Overall vector architecture:

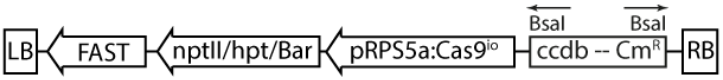
Shuttle vectors



*At*U6: pDGE331, 332, 333, 334, 335, 336, 337, 495, 496, 497, 341, 342
*Sl*U3: pDGE473, 474, 475, 476, 477, 478, 479, 498, 499, 500, 501, 502
*Sl*U6: pDGE654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665

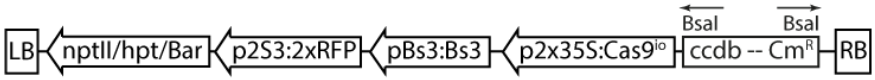
Recipient vectors

pDGE347, 651, 652
Arabidopsis, Cas9

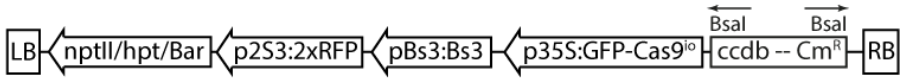


pDGE666, 667, 668
Arabidopsis, GFP-Cas9

pDGE463, 669, 670
N. benth., Cas9

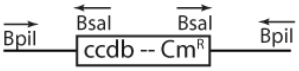


pDGE671, 672, 673
N. benth., GFP-Cas9

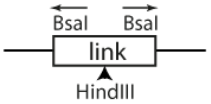


„multi-multi" intermediate cloning vectors

pDGE503 - 506
MM1-4



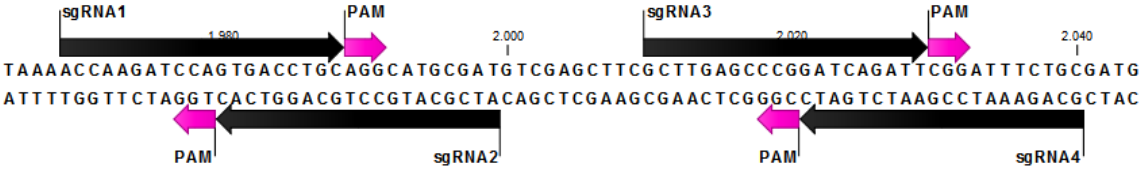
pDGE508 - 510
MM end-linkers



Note: The *Bsa*I fragment (prom-ccdB-sgRNA) of an M1E shuttle vector (pDGE331 (*At*U6), pDGE473 (*Sl*U3) or pDGE654 (*Sl*U6)) can be transferred into any recipient vector *via* a *Bsa*I GoldenGate reaction to obtain a novel recipient for rapid generation of single nuclease constructs. Such "one step, one nuclease" vectors (Ordon et al., 2017, Plant Journal) serve for direct cloning of hybridized oligonucleotides in the recipient, but do not offer multiplexing options. In the *Bsa*I GoldenGate reaction, both the empty recipient (*e.g.* pDGE347) and the assembly product (*e.g.* pDGE347 + *Bsa*I fragment from pDGE331) have the *ccdB* and *cat* (Chloramphenicol^R) genes. It is therefore critical to add the M1E module in excess to the GoldenGate reaction (e.g. 10 fmol recipient, 40 fmol M1E), and to re-digest the GoldenGate reaction with *Bsa*I after a denaturation cycle, prior to transformation into *ccdB* survival cells.

Selection and design of guide RNAs

sgRNA variable sequences are loaded into sgRNA shuttle vectors as 23-24 nt long, hybridized oligos in a *Bpi*I GoldenGate reaction. Phosphorylation of oligonucleotides is not required. Any online tool (such as *e.g.* CHOPCHOP or CRISPR-P) may be used for selection of sgRNAs, and cloning overhangs can be added manually or directly via the sgRNA design programs. Note that cloning overhangs differ for U3 and U6 promoter systems. Examples of target sites on plus and minus strands, and design of respective oligos to produce sgRNAs with the pDGE vector system, are provided below:



Primer design for U6 promoter system [ATTG – GTTT]:

sgRNA1:
Oligo 1 attgACCAAGATCCAGTGACCTGC
Oligo 2 aaacGCAGGTCACTGGATCTTGGT

sgRNA3:
Oligo 1 attGCTTGAGCCCGGATCAGATT
Oligo 2 aaacAATCTGATCCGGGCTCAAG

sgRNA2:
Oligo 1 attgATCGCATGCCTGCAGGTCAC
Oligo 2 aaacGTGACCTGCAGGCATGCGAT

sgRNA4:
Oligo 1 attGCAGAAATCCGAATCTGATC
Oligo 2 aaacGATCAGATTCGGATTTCTG

Primer design for U3 promoter system [GTCA-GTTT]:

sgRNA1:
Oligo 1 gtcACCAAGATCCAGTGACCTGC
Oligo 2 aaacGCAGGTCACTGGATCTTGG

sgRNA3:
Oligo 1 gtcaGCTTGAGCCCGGATCAGATT
Oligo 2 aaacAATCTGATCCGGGCTCAAGC

sgRNA2:
Oligo 1 gtcATCGCATGCCTGCAGGTCAC
Oligo 2 aaacGTGACCTGCAGGCATGCGA

sgRNA4:
Oligo 1 gtcaGCAGAAATCCGAATCTGATC
Oligo 2 aaacGATCAGATTCGGATTTCTGC

- The last nucleotide of the forward cloning overhang (marked in red) is the assumed transcription start site
- U6 promoter system:
  sgRNAs 1/2 will contain 20 bases complementary to the target strand and a single non-complementary nucleotide in position 1. Oligonucleotides are 24 nt in length.
  sgRNAs 3/4 will contain 20 bases complementary to the target strand. Oligonucleotides are 23 nt in length.
- U3 promoter system:
  sgRNAs 1/2 will contain 20 bases complementary to the target strand. Oligonucleotides are 23 nt in length.
  sgRNAs 3/4 will contain 20 bases complementary to the target strand and a single non-complementary nucleotide in position 1. Oligonucleotides are 24 nt in length.
- Avoid *Bsa*I sites [GGTCTC], *Bpi*I sites [GAAGAC] and polyT stretches [≥ 4 Ts; transcriptional termination] in sgRNA sequences.

Cloning of sgRNA TUs in shuttle vectors:

1. Hybridization of oligonucleotides
   - oligonucleotide stock concentration: 100 µM
   - mix oligos at 10 µM (for example 5 µl of each oligo + 40 µl H_2O)
   - denature oligos by heating to 98 °C for 5 min
   - let cool down slowly (leaving tube @ RT for several minutes is sufficient)
   - prepare a 1:200 dilution (50 fmol/µl) of the hybridized oligos

2. Loading of oligonucleotides into sgRNA shuttle vectors

cut/ligation reaction:

20 fmol ≈ 60 ng	shuttle vector	37 °C	2 min	10-30 cycles
50 fmol = 1 µl	hybridized oligos	16 °C	5 min	
1 µl	10 x Ligation buffer	50 °C	10 min	
1 µl	10 x BSA (1 mg/ml)	80 °C	10 min	
0,3 µl	*Bpi*I			
0,3 µl	T4 DNA Ligase (1 u/µl)			
	H_2O			
10 µl	Total			

- transform cut/ligation reaction into Dh10b/TopTen cells
- optional: plate a 50 µl aliquot of the transformation on an LB-Carb plate
- directly inoculate liquid cultures (3 ml) with the transformed cells in selective media (LB-Carb)
- use liquid cultures for polyclonal plasmid preparation
- optional: perform test digest of the polyclonal plasmid preparations, *e.g. Pvu*II

Assembly of plant transformation vectors containing up to eight sgRNA TUs

Derivatives of suitable shuttle vectors, loaded with sgRNA sequences, are used for assembly of one, two, four, six or eight sgRNA TUs in any recipient vector by a *Bsa*I cut/ligation reaction. This yields final constructs for plant transformation. Shuttle vectors from the different promoter systems are compatible. The following modules can be assembled:

1 sgRNA TU: M1E module
2 sgRNA TUs: M1 and M2E module
4 sgRNA TUs: M1, M2, M3 and M4E module
6 sgRNA TUs: M1, M2, M3, M4, M5, M6E module
8 sgRNA TUs: M1, M2, M3, M4, M5, M6, M7, M8E module

cut/ligation reaction:

20 fmol ≈ 300 ng	recipient	37 °C	2 min	20-50 cycles
20 fmol ≈ 40 ng	sgRNA TU shuttle vectors (40 ng of each module)	16 °C	5 min	
2 µl	10 x Ligation buffer	50 °C	10 min	
2 µl	10 x BSA (1 mg/ml)	80 °C	10 min	
1 µl	*Bsa*I			
1 µl	T4 DNA Ligase (1-5 u/µl)			
	H_2O			
20	Total			

- transform cut/ligation reaction into Dh10b/TopTen cells
- plate on LB-Spec media
- 1-2 sgRNA TUs: Start liquid cultures from 2 clones
- 4-6 sgRNA TUs: Start liquid cultures from 2-3 clones
- 8 sgRNA TUs: Start liquid cultures from 3-4 colonies
- isolate plasmid DNA and verify by restriction digest
- confirm sgRNAarray by DNA sequencing:
  1-4 sgRNA TU: oligonucleotide JS1132
  6-8 sgRNA TUs: oligonucleotides JS1132 and M13f
- transform plasmid into your favorite *Agrobacterium* strain

useful oligonucleotides:

name	sequence	location
M13f	GAATATCATCCGGTGCAGC	sgRNA TU 1, fwd
JS838	GCCAGCTTCTATGAGTACTGA	sgRNA TU 5, fwd
JS1132	AACGCTCTTTTCTCTTAGGT	vector, reverse

Assembly of plant transformation vectors containing up to 32 sgRNA TUs

- sgRNA TUs, mobilized from shuttle vectors, are first assembled in intermediate "multi-multi" vectors as described (section "Assembly of plant transformation vectors containing up to eight sgRNA TUs"). Multi-multi modules carry Kanamycin as selectable marker!

cut/ligation reaction:

20 fmol ≈ 50 ng	multi-multi (*e.g.* pDGE503)	37 °C	2 min	20-50 cycles
20 fmol ≈ 40 ng	sgRNA TU shuttle vectors (40 ng of each module)	16 °C	5 min	
2 µl	10 x Ligation buffer	50 °C	10 min	
2 µl	10 x BSA (1 mg/ml)	80 °C	10 min	
1 µl	*Bsa*I			
1 µl	T4 DNA Ligase (1-5 u/µl)			
	H_2O			
20	Total			

- Plasmid isolation, verification by restriction digest (*e.g. Bpi*I)
- sgRNAs arrays are verified by sequencing at this stage [oligonucleotides M13f, JS1692]
- sgRNA arrays are subsequently mobilized from multi-multi modules into any recipient vector in a GoldenGate reaction using both *Bsa*I and *Bpi*I (see next page).
- End-linkers are used to link the terminal overhang of a multi-multi module to the recipient vector.

The following assemblies are compatible:

up to 16 sgRNAs: derivatives of pDGE503 and pDGE504 together with pDGE508 (end-linker)
up to 24 sgRNAs: derivatives of pDGE503, 504 and 505 together with pDGE509 (end-linker)
up to 32 sgRNAs: derivatives of pDGE503, 504, 505 and 506 together with pDGE510 (end-linker)

cut/ligation reaction:

20 fmol ≈ 300 ng	recipient	37 °C	2 min	20-50 cycles
20 fmol ≈ 50 ng	loaded multi-multi (50 ng of each module)	16 °C	5 min	
20 fmol ≈ 30 ng	suitable end-linker	50 °C	10 min	
2 µl	10 x Ligation buffer	80 °C	10 min	
2 µl	10 x BSA (1 mg/ml)			
0.7 µl	*Bsa*I			
0.7 µl	*Bpi*I			
0.7 µl	T4 DNA Ligase (1-5 u/µl)			
	H_2O			
20	Total			

- transform cut/ligation reaction into Dh10b/TopTen cells
- plate on LB-Spec media
- start liquid cultures from 3-4 colonies
- isolate plasmid DNA and verify by restriction digest (e.g. *Hind*III)
- transform plasmid into your favorite *Agrobacterium* strain

useful oligonucleotides:

Name	sequence	location
M13f	GAATATCATCCGGTGCAGC	sgRNA TU 1, fwd
JS838	GCCAGCTTCTATGAGTACTGA	sgRNA TU 5, fwd
JS1692	CACCTGACGTCTAAGAAACC	vector, reverse