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

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

42 Researchers have access to numerous options for RGN-mediated mutagenesis. To start with,
43 nuclease systems can be delivered into plant cells as DNA constructs, mRNA and gRNA or
44 ribonucleoprotein complexes (Woo et al., 2015; Svitashev et al., 2016; Zhang et al., 2016; Liang et al.,
45 2017; Lin et al., 2018), but *Agrobacterium*-mediated delivery of RGN-encoding T-DNAs remains the
46 most common strategy. Numerous nucleases have been utilized for plant genome editing, such as
47 Cas9 from *Staphylococcus aureus* and *Streptococcus pyogenes* (*Sa*Cas9*, Sp*Cas9) and modified
48 versions thereof (xCas9, Cas9-VQR) and Cas12 (Cpf1; *Fn*Cpf1, *Lb*Cpf1) (Li et al., 2013; Nekrasov et al.,
49 2013; Steinert et al., 2015; Tang et al., 2017; Hu et al., 2018; Ge et al., 2019; Zhong et al., 2019).
50 Multiple promoter systems are also available for guide RNA (sgRNA) expression, i.e. RNA polymerase
51 II (Pol II) or III (Pol III) promoters. In the former case, primary transcripts require processing, which
52 can be achieved by ribozymes, endogenous tRNA processing enzymes, or the ribonuclease Csy4 (Gao
53 and Zhao, 2014; Nissim et al., 2014; Xie et al., 2015; Cermak et al., 2017). The same strategies can
54 also be used in multiplexing applications for processing of multiple sgRNAs from a polycistronic
55 primary transcript. Alternatively, multiple sgRNAs can be expressed as individual transcriptional units
56 (TUs) by Pol III promoters, such as U6/U3.

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

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

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

84

85 # Results

86

87 ***Description of the vector system***

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

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

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

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

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

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

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

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

139

140 ***Assembly of plant transformation constructs containing up to 32 sgRNA transcriptional units***
141 An sgRNA TU consists of a U3/U6 promoter fragment, the variable section of the sgRNA and the
142 invariable sgRNA scaffold, followed by a polyT-stretch for termination of Pol III transcription. Shuttle
143 vectors are missing the variable section of the sgRNA, and hold a *ccdB* cassette at this position. In a
144 GoldenGate reaction using *Bpi*I, the *ccdB* cassette is replaced by two hybridized oligonucleotides (23-
145 24 nt in length) to obtain the full sgRNA TU of user-defined specificity (Figure 2a). Since empty
146 shuttle vectors contain the *ccdB* cassette for negative selection, these GoldenGate reactions can be
147 processed as polyclonal plasmid preparations after transformation (Appendix 1; Ordon et al., 2017).
148 The use of polyclonal plasmid preparations at this stage holds two advantages. On the one hand, the
149 time required to obtain the sgRNA TUs is reduced by one day. On the other hand, the risk of selecting
150 a single clone containing an erroneous sgRNA variable section (from inaccuracies during
151 oligonucleotide synthesis) is mitigated. Therefore, sequence verification of sgRNA TUs is not required
152 at this stage.

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

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

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

169

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

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

181 We first tested constructs containing the FAST cassette in *N. benthamiana*, but failed to detect seed
182 fluorescence. Subsequently, fusions of an *At2S3* seed storage protein promoter fragment (Kroj et al.,
183 2003) with red fluorophore-coding genes were incorporated as secondary marker cassettes. In
184 different transformations, the *2S3* promoter fragment was fused to mCherry, tagRFP-T or 2xtagRFP-
185 T, in combination with *A. tumefaciens g7* (Engler et al., 2014) or *N. benthamiana* extensin
186 transcriptional terminators (Diamos and Mason, 2018). Vectors provided in our toolkit (Appendix 1)
187 contain a p2S3:2xtagRFP-t*Nb*EU cassette. The marker was characterized with transformants
188 containing a p2S3:mCherry-tAtug7 cassette (Figure 3).

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

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

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

208 We tested performance of the seed fluorescence and *Bs3* cell death marker in a sequential selection
209 strategy for the isolation of mutants defective in *NRG1* (N requirement gene 1; Peart et al., 2005) and
210 assumed orthologs of *NPR1* (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1; Cao et al., 1997).
211 First, approximately 80 plants from three different T_1 families were grown from seeds pre-selected
212 for absence of seed fluorescence (5 – 6 h). After 3 - 4 weeks, plants were challenged with AvrBs3-
213 translocating *Pseudomonas fluorescens* bacteria (< 1 h), and plants that developed cell death (4/80,
214 5 %) were discarded. Remaining plants were genotyped with target gene-specific primers and
215 homozygous *nrg1* single or *npr1a/b* double mutants selected (Figure 4). When genotyped for
216 presence of the T-DNA, five out of six plants tested were negative (Figure 4). Thus, several
217 independent single or double mutant lines could be selected from genotyping only 80 T_1 segregants,
218 demonstrating high efficiency of the dual counter-selection strategy.

219

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

222 The intron-optimized Cas9 gene expressed under control of the *RPS5a* promoter present in recipient
223 vectors for Arabidopsis generates single and double mutant lines with high efficiencies in the T_1
224 generation (Gruetzner et al., companion manuscript). This encouraged us to test performance of our
225 constructs in an ambitious multiplexing application, using an sgRNA array containing 24 TUs to target
226 12 different genes (Figure 5a). Selected target genes included several NLR-type resistance genes (*e.g.*
227 *RPP2*, *RPP4*, *RPS5*) and developmental regulators (e.g. *ERECTA*, TOO MANY MOUTHS, *GLABROUS1*).
228 Target genes and sites are listed in Table S1.

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

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

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

263

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

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

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

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

297 S2), and also line #1688 was highly mutagenic. Remaining lines were selected randomly. All lines we
298 analyzed contained mutations within at least seven loci that were targeted by the 24 sgRNAs (Figure
299 6c). As observed for sequencing of pools before, most plants contained wild type alleles at the *RPS5*
300 and *RPP4* loci, again confirming low efficiency of respective sgRNAs. However, the *rpp4* phenotype
301 was detected by phenotypic analyses within family #1681 (Table S2), and one of the two segregants
302 we analyzed indeed carried disruptive mutations at all 12 loci with edits at 40/48 target sites (Figures
303 6c, S4). Thus, enhanced efficiencies achieved by intron-optimization of the Cas9 sequence (Gruetzner
304 et al., companion manuscript) coupled with a high degree of multiplexing enabled us to isolate a
305 duodecuple Arabidopsis mutant within a single generation.

306 ## Discussion

307

308 ***A simple toolkit for routine use***

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

326

327

328 ***Transgene counter-selection strategies***

329 Strong seed fluorescence suitable as a non-destructive transformation marker has been obtained in
330 Arabidopsis by several different systems (Stuitje et al., 2003; Bensmihen et al., 2004; Shimada et al.,
331 2010). We have used both the FAST marker and the p*At2S3*:mCherry marker (not shown) for
332 selection in Arabidopsis transformations, and obtained easily discernable seed fluorescence with
333 both promoter/marker systems. Rare occurrence of T-DNA positive plants following negative
334 selection (in T_2) likely arises from accidental carry-over of fluorescence-positive seeds rather than
335 mis-phenotyping on the basis of seed fluorescence. In addition to a fluorescent marker, a
336 herbicide/antibiotic resistance gene was included in pDGE vectors for the convenient positive
337 selection of T-DNA-harboring Arabidopsis in the T_1 generation (Figure 1a, Appendix 1).

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

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

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

365

366 ***Multiplexing – limits and opportunities***

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

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

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

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

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

411

412 Materials and Methods

413

414 ***Plant growth conditions and transformation***

415 *N. benthamiana* wild-type plants were cultivated in a greenhouse with a 16-h light period (sunlight
416 and/or IP65 lamps (Philips) equipped with Agro 400 W bulbs (SON-T); 130–150 µE/m^2*s; switchpoint
417 ;100 µE/m^2*s), 60% relative humidity at 24/20°C (day/night). *N. benthamiana* plants were
418 transformed as previously described (Gantner et al., 2019); a detailed protocol is provided online
419 (dx.doi.org/10.17504/protocols.io.sbaeaie). Arabidopsis wild type accession Col was used, and plants
420 were cultivated under short day conditions (8h light, 23/21°C day/night, 60 % relative humidity) or in
421 a greenhouse under long day conditions (16h light) for seed set. Arabidopsis was transformed by
422 floral dipping as previously described (Logemann et al., 2006).

423

424

425 ***Molecular cloning and selection of tomato U6/U3 promoter fragments***

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

431 *Sl*U6/U3 promoter fragments were selected from multiple sequence alignments of U6/U3 genes and
432 upstream regions from Arabidopsis and tomato. Promoter fragments encompassing approximately
433 250 nt and 120 nt upstream of the predicted transcription start site were cloned, and functionally
434 verified by expression of sgRNAs in reporter-based assays. No functional differences were observed,
435 and shorter promoter fragments were used to generate data presented in this study. Shuttle vectors
436 containing promoter fragments were cloned as previously described (Ordon et al., 2017). Briefly, the
437 promoter fragment and a second fragment encompassing a *ccdB* cassette and the sgRNA scaffold
438 were PCR-amplified (*e.g.*, using oligonucleotides JS1744/1745 and STS24/JS1746 in case of the *Sl*U3
439 promoter), and subsequently fused by SOE-PCR. The SOE-PCR product was cloned into pUC57-BsaI in
440 a cut/ligation reaction using *Eco*RV to yield the M1E module. This served as PCR template to amplify
441 fragments for further shuttle vectors, which were (after *Dpn*I digestion) cloned as before. Plasmids
442 and oligonucleotides are summarized in Table S1. Additional plasmids are listed in Appendix 1 as part
443 of the cloning manual of our toolkit. Vector maps (GenBank) are provided in Appendix 2.

444

445 ***Agroinfiltration, reporter-based nuclease activity assays and localization studies***

446 For transient expression of proteins in *N. benthamiana* leaf tissues (agroinfiltration), respective T-
447 DNA constructs were transformed into *Agrobacterium* strain GV3101 pMP90. Plate-grown bacteria
448 were resuspended in Agrobacterium Infiltration Medium (AIM; 10 mM MES pH 5.8, 10 mM $MgCl_2$),
449 and infiltrated with a needleless syringe at an OD_{600} = 0.3 per strain. For qualitative determination of
450 GUS activity, leaf discs were taken 3 dpi, stained with GUS staining solution (10 mM phosphate buffer
451 pH 7, 10 mM EDTA, 1 mM potassium ferricyanate, 1 mM potassium ferrocyanate, 0,1 % Triton X-100,
452 0,1% X-Gluc) for 3 – 5h, destained with ethanol and dried in cellophane. Quantitative determination
453 of GUS activity was done as previously described (Ordon et al., 2017). Briefly, leaf material was
454 ground in liquid nitrogen, powder resuspended in extraction buffer and cleared lysates prepared by
455 centrifugation. Protein extracts were incubated with 4-methylumbelliferyl-beta-D-glucuronide
456 (4MUG; 5 mM) for 1 – 2h, and MU production measured on a Tecan plate reader. Live-cell imaging
457 was done using a Zeiss LSM780 confocal laser scanning microscope. GFP was excited using the 488
458 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

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

501

502 ***Amplicon sequencing and data analysis***

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

514

515 Accession Numbers

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

519

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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

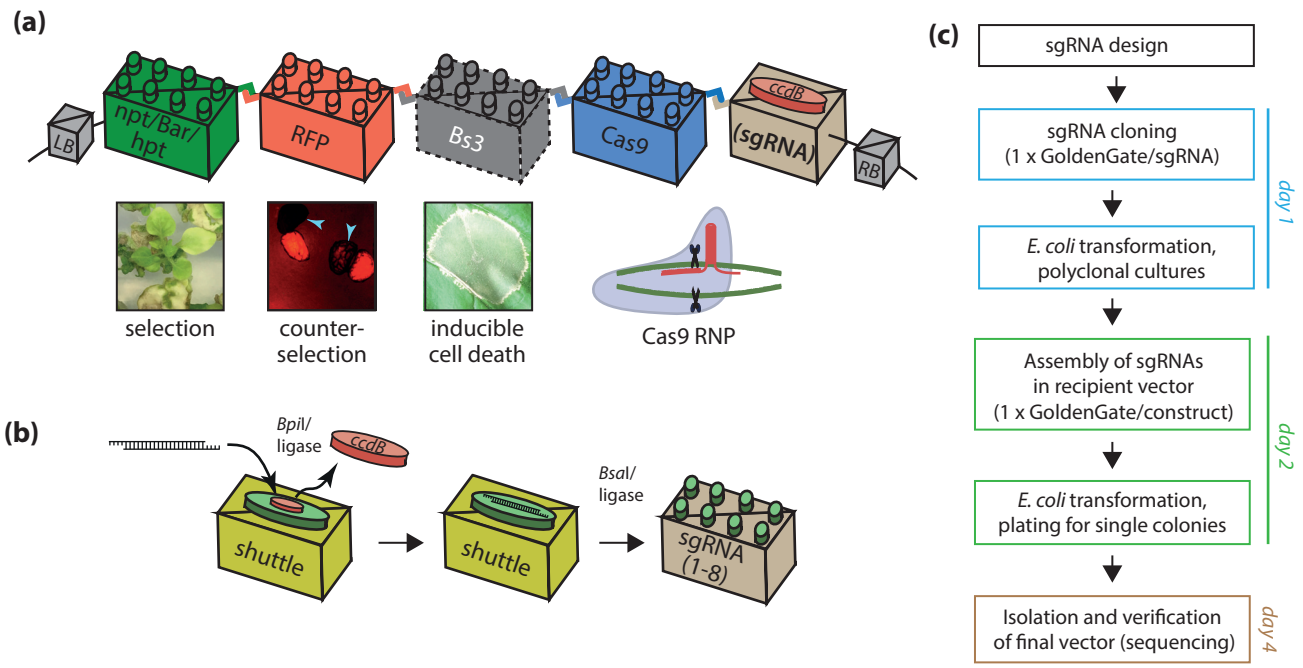


Figure 1: Schematic overview of the pDGE vector system.

(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

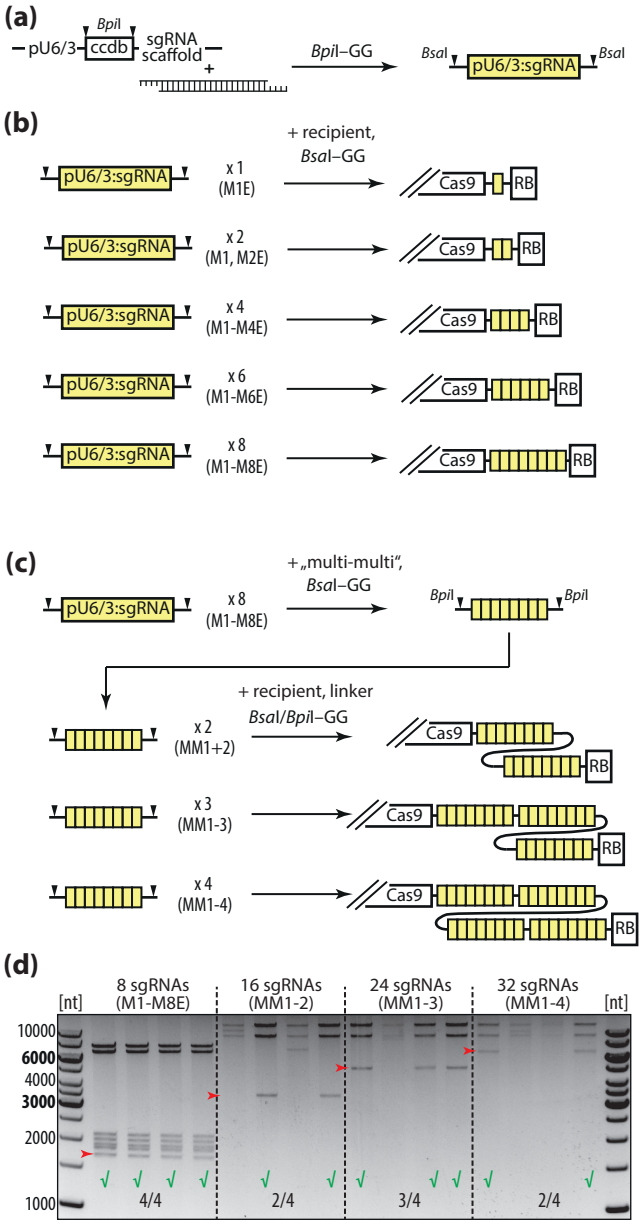
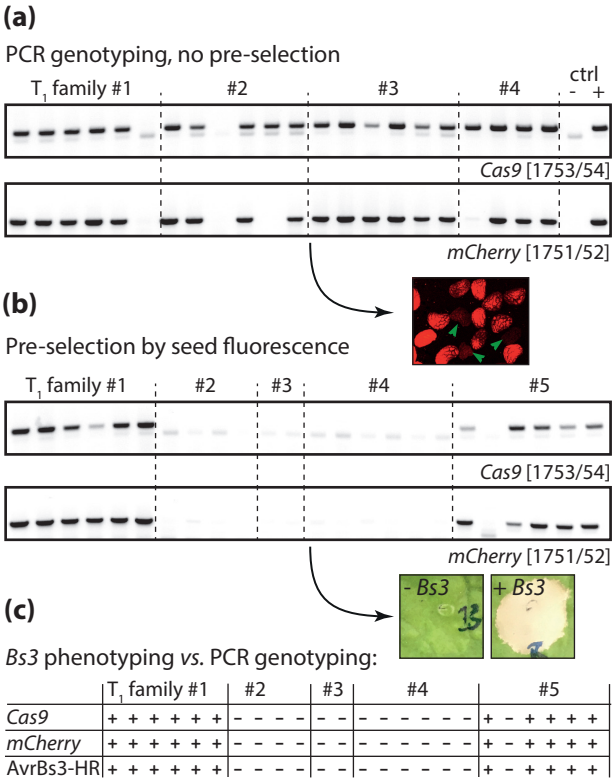


Figure 2: Assembly of arrays of sgRNA transcriptional units in recipient vectors.

(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



	T_1 family #1						#2				#3		#4						#5					
Cas9	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+
mCherry	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+
AvrBs3-HR	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+

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

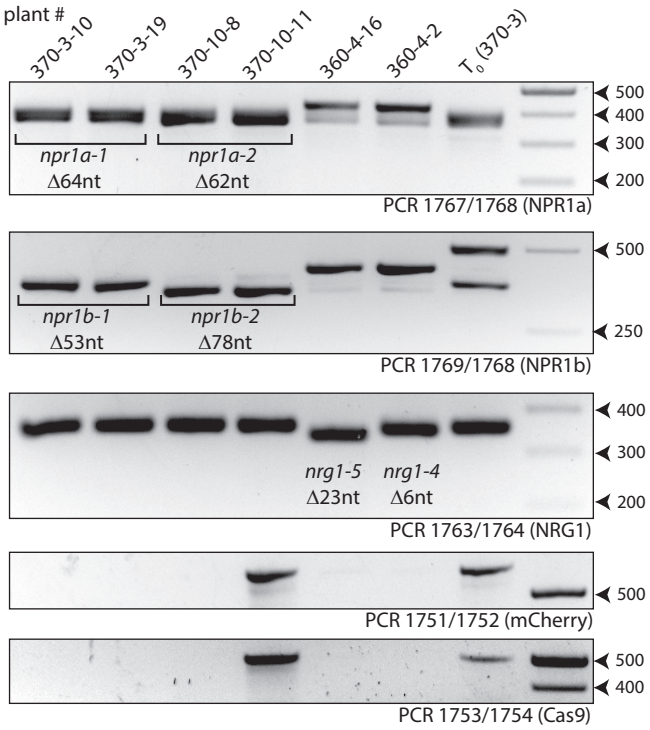
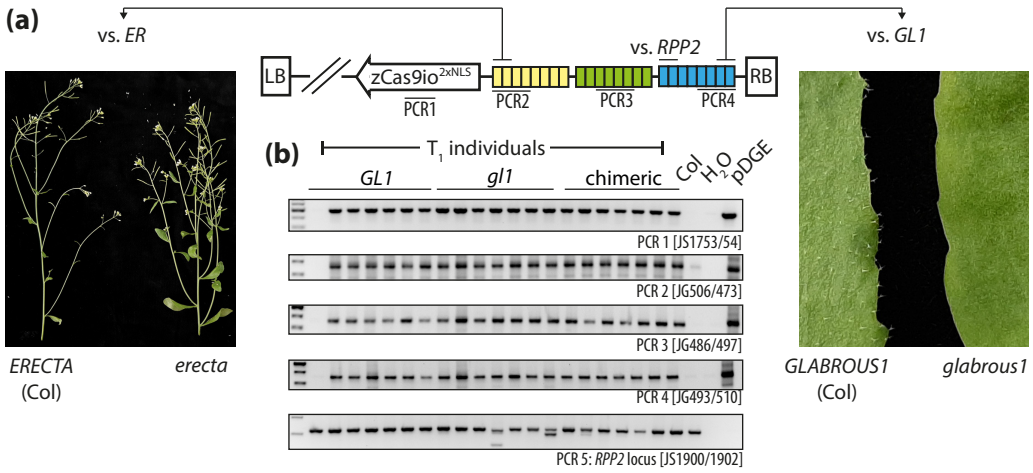


Figure 4: Efficient isolation of transgene-free genome-edited *N. benthamiana* lines by dual counter-selection.

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

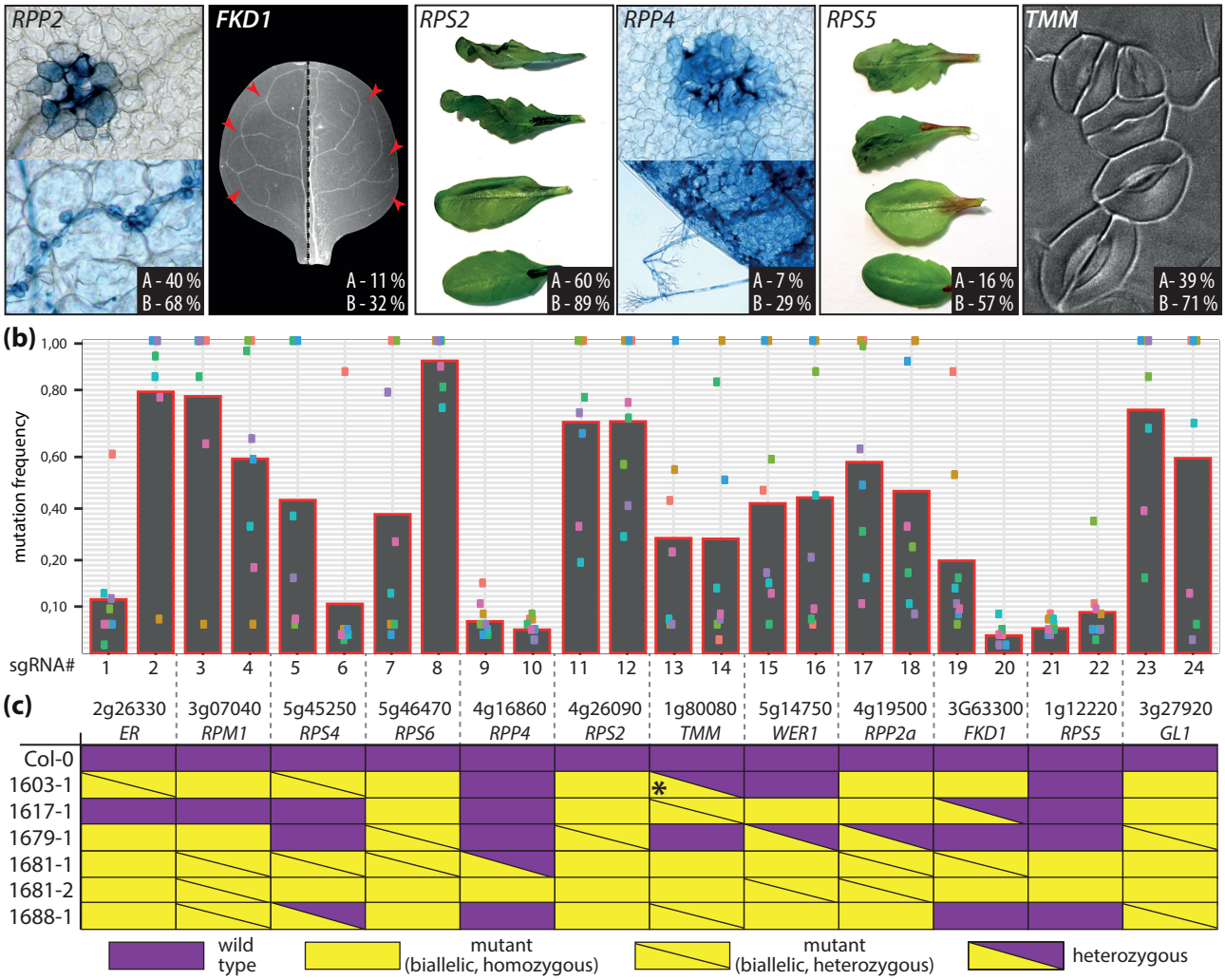
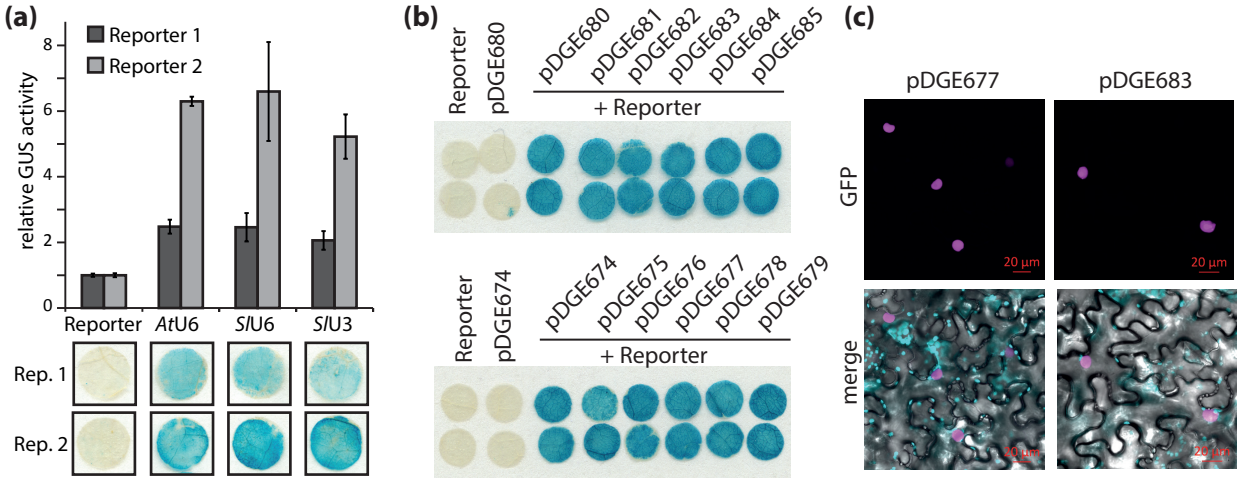


Figure 6: Analysis of transgene-free T_2 segregants from multiplex editing in Arabidopsis.

(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.

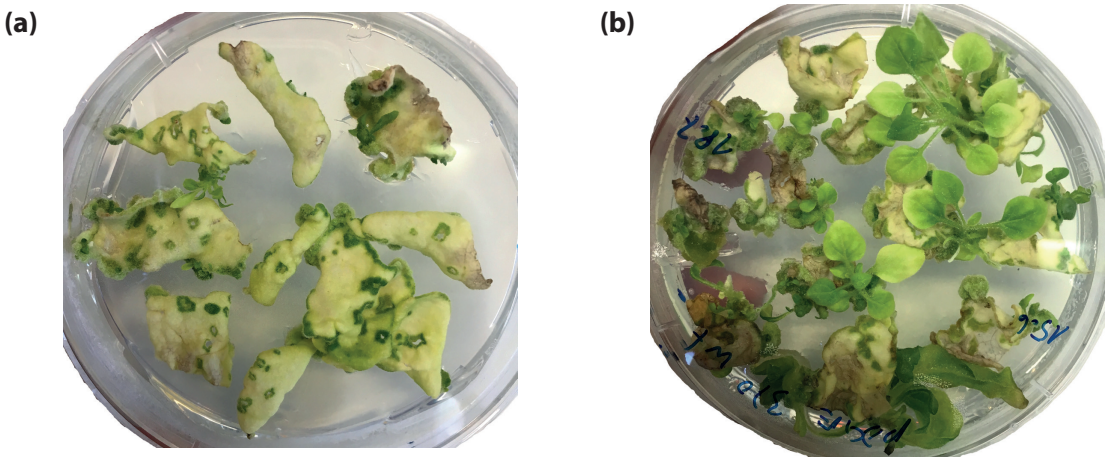
Supplemental Figure S1



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.

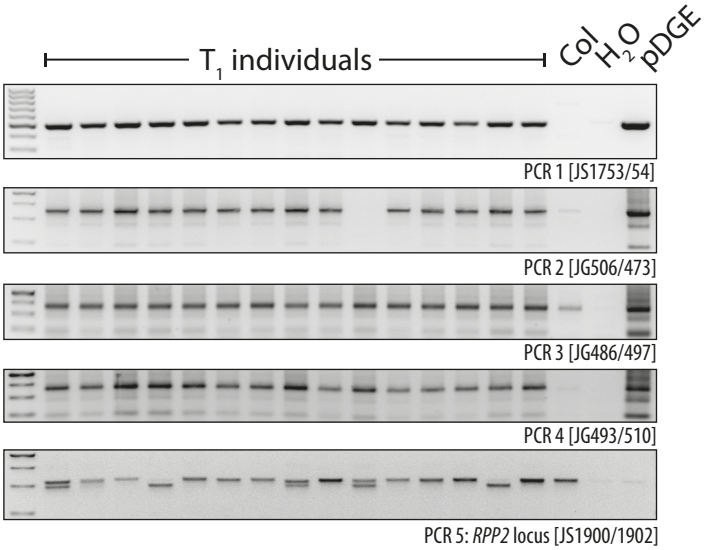
Supplemental Figure S2



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)

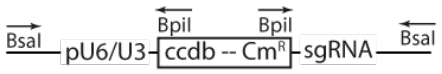
Recipient vectors and end-linkers for higher order multiplexing:

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:

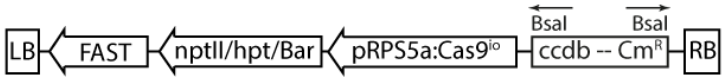
Shuttle vectors



*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

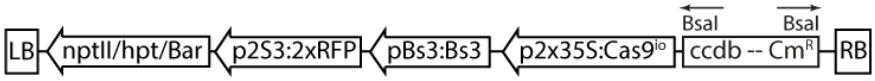
Recipient vectors

pDGE347, 651, 652
Arabidopsis, Cas9

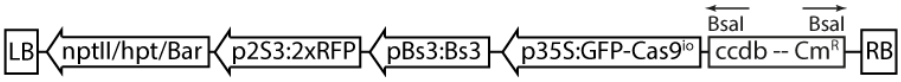


pDGE666, 667, 668
Arabidopsis, GFP-Cas9

pDGE463, 669, 670
N. benth., Cas9

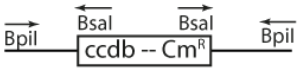


pDGE671, 672, 673
N. benth., GFP-Cas9

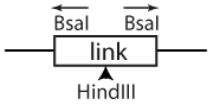


„multi-multi" intermediate cloning vectors

pDGE503 - 506
MM1-4



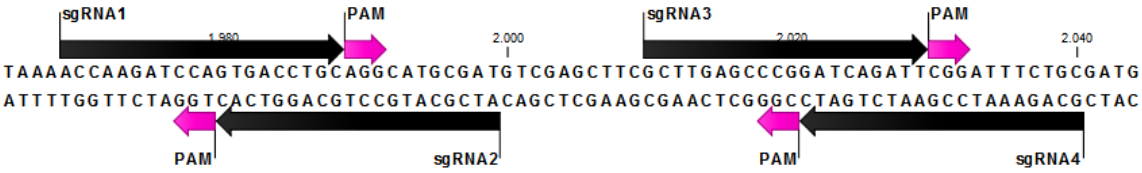
pDGE508 - 510
MM end-linkers



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

sgRNA2:
Oligo 1 attgATCGCATGCCTGCAGGTCAC
Oligo 2 aaacGTGACCTGCAGGCATGCGAT

sgRNA3:
Oligo 1 attGCTTGAGCCCGGATCAGATT
Oligo 2 aaacAATCTGATCCGGGCTCAAG

sgRNA4:
Oligo 1 attGCAGAAATCCGAATCTGATC
Oligo 2 aaacGATCAGATTCGGATTTCTG

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

sgRNA1:
Oligo 1 gtcACCAAGATCCAGTGACCTGC
Oligo 2 aaacGCAGGTCACTGGATCTTGG

sgRNA2:
Oligo 1 gtcATCGCATGCCTGCAGGTCAC
Oligo 2 aaacGTGACCTGCAGGCATGCGA

sgRNA3:
Oligo 1 gtcaGCTTGAGCCCGGATCAGATT
Oligo 2 aaacAATCTGATCCGGGCTCAAGC

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
50 fmol = 1 µl	hybridized oligos
1 µl	10 x Ligation buffer
1 µl	10 x BSA (1 mg/ml)
0,3 µl	*Bpi*I
0,3 µl	T4 DNA Ligase (1 u/µl)
	H_2O
10 µl	Total

37 °C	2 min	10-30 cycles
16 °C	5 min	
50 °C	10 min	
80 °C	10 min	

- 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
20 fmol ≈ 40 ng	sgRNA TU shuttle vectors (40 ng of each module)
2 µl	10 x Ligation buffer
2 µl	10 x BSA (1 mg/ml)
1 µl	*Bsa*I
1 µl	T4 DNA Ligase (1-5 u/µl)
	H_2O
20	Total

37 °C	2 min	20-50 cycles
16 °C	5 min	
50 °C	10 min	
80 °C	10 min	

- 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)
20 fmol ≈ 40 ng	sgRNA TU shuttle vectors (40 ng of each module)
2 µl	10 x Ligation buffer
2 µl	10 x BSA (1 mg/ml)
1 µl	*Bsa*I
1 µl	T4 DNA Ligase (1-5 u/µl)
	H_2O
20	Total

37 °C	2 min	20-50 cycles
16 °C	5 min	
50 °C	10 min	
80 °C	10 min	

- 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	
		50 °C	10 min	
20 fmol ≈ 30 ng	suitable end-linker	80 °C	10 min	
2 µl	10 x Ligation buffer			
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