Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis

Zhengyan Fenga,b,c,1, Yanfei Maona,1, Nanfei Xu, Botao Zhanga, Pengliang Weia,b,c, Dong-Lei Yanga, Zhen Wanga,b,c, Zhengjing Zhanga,b,c, Rui Zhenga,b,c, Lan Yang, Liang Zenga, Xiaodong Liua, and Jian-Kang Zhu,a,d,2

aShanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai 200032, People’s Republic of China; bInstitute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, People’s Republic of China; cUniversity of Chinese Academy of Sciences, Shanghai 200032, People’s Republic of China; and dDepartment of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907

Contributed by Jian-Kang Zhu, January 18, 2014 (sent for review January 6, 2014)

The CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) system has emerged as a powerful tool for targeted gene editing in many organisms, including plants. However, all of the reported studies in plants focused on either transient systems or the first generation after the CRISPR/Cas system was stably transformed into plants. In this study we examined several plant generations with seven genes at 12 different target sites to determine the patterns, efficiency, specificity, and heritability of CRISPR/Cas-induced gene modifications or corrections in Arabidopsis. The proportion of plants bearing any mutations (chimeric, heterozygous, biallelic, or homozygous) was 71.2% at T1, 58.3% at T2, and 79.4% at T3 generations. CRISPR/Cas-induced mutations were predominantly 1 bp insertion and short deletions. Gene modifications detected in T1 plants occurred mostly in somatic cells, and consequently there were no T1 plants that were homozygous for a gene modification event. In contrast, ~22% of T2 plants were found to be homozygous for a modified gene. All homozygotes were stable to the next generation, without any new modifications at the target sites. There was no indication of any off-target mutations by examining the target sites and sequences highly homologous to the target sites and by in-depth whole-genome sequencing. Together our results show that the CRISPR/Cas system is a useful tool for generating versatile and heritable modifications specifically at target genes in plants.

Significance

The CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) system has been used to generate targeted gene editing in plants. However, it is not known whether CRISPR/Cas-induced gene modifications in plants occur in somatic cells only or whether some or all of the modifications can enter the germ line to become heritable. Through systematic and multigenerational analysis, this study demonstrates that although the majority of gene modifications detected in the first generation CRISPR/Cas transgenic Arabidopsis plants were somatic mutations only, heritable mutations could be found in subsequent generations. In addition, deep sequencing of CRISPR/Cas-modified Arabidopsis genomes did not detect any off-targets. The work demonstrates that the CRISPR/Cas method can effectively create specific gene modifications in planta that are stably transmitted through the germ line to future generations.


The authors declare no conflict of interest.

See Commentary on page 4357.

1Z.F. and Y.M. contributed equally to this work.

2To whom correspondence should be addressed. E-mail: jkzhu@purdue.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1400822111/-/DCSupplemental.

PNAS | March 25, 2014 | vol. 111 | no. 12 | 4632–4637
heterozygous, or biallelic for any mutation or modification (6, 7). In the present study, to investigate how the CRISPR/Cas system causes modifications of target genes and how the modified genes flow through subsequent generations, we analyzed the T2 and in some cases T3 progenies of some of these plants. In addition, we analyzed T1 and T2 transgenic plants for the APETALA 1 (AP1) target gene. Details on the AP1 gene and a few representative results from other genes are in SI Appendix, Fig. S1, and Table S1.

Arabidopsis plants were transformed with a construct containing the Cas9 gene and a single-stranded guide RNA (sgRNA) targeting the GAI gene, and many hygromycin-resistant T1 plants were recovered (6). Examination of a few leaves from early T1 seedlings revealed three types of NHEJ mutations: r 1:1 bp replacement, d3 3-bp deletion, and d4 4-bp deletion (Fig. 1). This analysis only demonstrated that targeted gene mutations happened at T1 or earlier but was not intended to find all mutations that could occur at various stages of T1 plant development.

Detailed studies were conducted at T2 and T3 generations to detect various possible mutations and mutation patterns. At the T2 generation, the mutations can be classified into five types (Fig. 1). Some T2 plants were found with both copies of the GAI gene mutated, either the same mutation [i.e., homozygous (line 55-3)] or different mutations [i.e., biallelic (line 55-21)]. All 34 T3 plants examined from homozygous T2 plant 55-3 were homozygous for the same d3 mutation, showing that the d3 mutation caused through NHEJ by the CRISPR/Cas system passed from T2 to T3 generation without any additional mutation, even earlier but was not intended to find all mutations that could occur at various stages of T1 plant development.

Some T2 lines were found with both copies of the GAI gene mutated, either the same mutation [i.e., homozygous (line 55-3)] or different mutations [i.e., biallelic (line 55-21)]. All 34 T3 plants examined from homozygous T2 plant 55-3 were homozygous for the same d3 mutation, showing that the d3 mutation caused through NHEJ by the CRISPR/Cas system passed from T2 to T3 generation without any additional mutation, even when Cas9 enzyme was present in those plants (Fig. 1, Table 1, and SI Appendix, Fig. S1, and Table S1). All of the many homozygous T2 lines from BRI1 gene editing also produced homozygous T3 progenies (SI Appendix, Fig. S1 and Table S1). Similarly, both d3 and d5 mutations from Line 55-21 passed from T2 to T3 generation, with a ratio of 6d3d3:14d3d5:9d5d5. A similar test showed that there is no significant difference between this ratio and the expected 1:2:1 ratio if the mutations were inherited following the classic Mendel’s law. The Cas9 construct was already segregated out in this line by the T2 generation. Another genotype detected at T2 was heterozygous, with one mutation, for example, d1 mutation in line 46-26. The 8d1d1:12d1WT:2WTWT ratio in T3 was also consistent with a Mendelian inheritance of the d1 mutation. Four chimeric T3 plants from this line are likely from continued CRISPR/Cas activity on the WT GAI gene. The Cas9 construct in this line also segregated by the Mendelian pattern. T2 chimera showed more complex patterns in transmitting mutations to the T3 generation. Whereas homozygous, chimeric, and WT plants were found in the T3 of chimeric T2 line 46-39 of the GAI target gene (Fig. 1 and Table 1), heterozygous, chimeric, and WT T3 plants were the progenies of two chimeric T2 lines of the BRII gene (SI Appendix, Fig. S1 and Table S1). All T2 lines that were WT for the target genes but positive for the CRISPR/Cas construct showed mutations in the T3 plants (Fig. 1, Table 1, and SI Appendix, Fig. S1 and Table S1).

Stability of Mutations. We determined the target site genotypes of many T2 and T3 lines for different target genes and multiple target sites (Table 1 and SI Appendix, Table S1). All possible genotypes (i.e., WT, homozygous, heterozygous, biallelic, and chimeric) were found at the T2 generation. When 19 T2 homozygotes were examined at T3 generation, all of them faithfully passed the same mutation from T2 to T3, without any further mutation or revision (Table 1 and SI Appendix, Table S1). This pattern of stable inheritance was seen in the absence of Cas9 (e.g., BRII sgRNA line 5-6) or presence of Cas9 (e.g., GAI sgRNA line 46-1) (Table 1 and SI Appendix, Table S1). Similarly, the four biallelic T2 lines passed their mutations to the T3 generation following an expected pattern of x(homozygous for mutation 1):y(biallelic):z (homozygous for mutation 2) (Table 1). A χ² test showed that the segregation in all these four lines conforms to the Mendelian 1:2:1 ratio. Again, there was no new mutation or revision, in the presence or absence of Cas9.

If no new mutation (n) occurred in a heterozygous mutant, the expected segregation pattern would be 1mm:2mWT:1WTWT. If new mutation occurred, then some of the mWT and WTWT genotypes could be biallelic, and even chimeras could appear. We followed five T2 heterozygous lines to the T3 generation. All of them exhibited the expected 1:2:1 segregation, and new mutations were detected in T3 plants in the form of chimeras.

We defined chimera as a plant with at least three different sequences (including WT) detected at the target site. A chimera must have different mutations at the target site in different parts of the plant. One, two, or none of the detected mutations may be passed to a progeny in the next generation. Six chimeric T2 lines for two different target genes were examined at T3 generation (Table 1 and SI Appendix, Table S1). One of the lines, S-14 for the JAZ1 target, showed a quite interesting pattern of inheritance. Although more than half of the T3 plants were WT, homozygotes were found for three of the different mutations detected at T2. JAZ1 T2 line 4-7 showed a similar segregation pattern, indicating that different mutations can be passed to the next generation through different flowers on the same plant. The third chimeric JAZ1 T2 line, line 24-5, also had homozygous T3 plants. The two chimeric BRI1 lines had heterozygous T3 plants for one of the mutations from T2 (SI Appendix, Table S1).

T2 WT plants could have resulted from malfunction of the CRISPR/Cas system or segregration of T1 heterozygous lines. T3 progeny of 13 T2 WT lines with the Cas9 construct were tested for the presence of mutations; all of them harbored mutations (Table 1 and SI Appendix, Table S1), indicating that the CRISPR/Cas system was still functional at T2 and/or T3 generation.

Homologous Recombination Aided by CRISPR/Cas. When the CRISPR/Cas machinery makes a DSB, even in the presence of a suitable HR template, the DSB may be repaired by NHEJ or HR. Expression of a functional GUS gene can be easily detected by staining the plants for blue color (16); we took advantage of this and designed a GUS reporter gene to test HR in planta (7) (Fig. 24).

When T1 plants were stained for GUS expression at the cotyledon stage, 5 of 44 plants showed GUS staining in the cotyledons, suggesting that correct HR occurred in approximately 11% of the T1 plants at the cotyledon stage (7). As reported previously, the staining pattern of these five plants was mosaic, indicating that correct HR occurred in some but not all cells of the cotyledons at the T1 generation (7). Sixteen of the 44 T1...
lines, including 4 cotyledon-GUS positive lines, were further tested for GUS staining at T2 generation. Among these 16 T2 populations, only 2 populations had plants that were homozygous for the corrected GUS gene. This gave an overall germ-line HR success rate of 13% (2 of 16). In contrast to T1 (7) and some T2 plants (Fig. 2B), GUS staining of homozygous T2 seedlings was uniform (Fig. 2C), demonstrating that some of the correct HR events in the T1 generation was passed to T2.

When Cas9 makes a DSB, some of the breaks may be repaired by HR because an HR template was provided in the construct. However, even in the presence of an HR template, some DSB might be repaired by NHEJ. To determine the extent of HR vs. NHEJ, we identified plants with homozygous modifications (mutations or corrections) from five transgenic T2 populations (Fig. 2D). The proportion of homozygotes carrying modifications by HR ranged from 0 to 80%, giving an average of 35% ± 37%, whereas the proportion of modifications by NHEJ was 65% ± 37%.

**Varieties and Inheritance of Mutations.** We used CRISPR/Cas to edit several target genes, and in some cases several target sites in the same gene. With this variety in gene editing, we are interested in knowing how many types of NHEJ mutations usually occur at each of the examined generations in the presence of the CRISPR/Cas system, whether these mutations are equally transmitted to progenies, and whether the pattern of modifications and their inheritance depend on the target gene or target site. Related data are summarized in Fig. 3 and Table 2, and a detailed listing of detected mutation genotypes and their frequencies is in SI Appendix, Table S2.

After DSB, many types of mutations caused by NHEJ repair were detected (Fig. 3). The majority of the mutations were deletions, closely followed by insertions (Left Inset, Fig. 3). Base replacement and combined mutations (at least two mutation types) also occurred, albeit at much lower frequencies. The length of the mutations was predominantly 1 bp change (Right Inset, Fig. 3). There was a 150-bp distance between the two sgRNA target sites in the AP1 gene and a 229-bp gap between the two target sites in the TT4 gene; and the close distance between the two sites correlated with a significant percentage (25.8% in Right Inset, Fig. 3) of deletions between the two target sites.

### Table 1. Lineage of CRISPR/Cas-induced mutations in GA1 target site

<table>
<thead>
<tr>
<th>Line</th>
<th>Zygosity</th>
<th>Target genotype</th>
<th>Cas</th>
<th>Mutation segregation</th>
<th>Cas</th>
</tr>
</thead>
<tbody>
<tr>
<td>46-8</td>
<td>Biallele</td>
<td>d1i1</td>
<td>+</td>
<td>9d1d1:17d1:1:5i1i1</td>
<td>All+</td>
</tr>
<tr>
<td>55-1</td>
<td>Biallele</td>
<td>d5r1</td>
<td>+</td>
<td>14d5d5:13d5r1:9r1r1</td>
<td>29+:7−</td>
</tr>
<tr>
<td>55-17</td>
<td>Biallele</td>
<td>d5d3b</td>
<td>+</td>
<td>7d5d5:19d5d3b:9d3d3b</td>
<td>29+:6−</td>
</tr>
<tr>
<td>55-21</td>
<td>Biallele</td>
<td>d5d3b</td>
<td>−</td>
<td>6d5d5:14d5d3b:9d3d3b</td>
<td>All−</td>
</tr>
<tr>
<td>46-26</td>
<td>Heterozygote</td>
<td>d1+c1</td>
<td>+</td>
<td>8d1d1:12d1Wt:2Wt+4c1</td>
<td>22+:14−</td>
</tr>
<tr>
<td>46-1</td>
<td>Homozygote</td>
<td>d1d1</td>
<td>+</td>
<td>9d1d1</td>
<td>21+:3−</td>
</tr>
<tr>
<td>55-3</td>
<td>Homozygote</td>
<td>d3d3b</td>
<td>+</td>
<td>3d3d3b</td>
<td>34+:2−</td>
</tr>
<tr>
<td>46-39</td>
<td>Chimera</td>
<td>d1,c6 chi</td>
<td>+</td>
<td>12d1d1:14c1:10WT</td>
<td>All+</td>
</tr>
<tr>
<td>46-40</td>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>2c1:33WT</td>
<td>23+:13−</td>
</tr>
</tbody>
</table>

WT, wild-type sequence with no mutation detected; d#, number of bases deleted from target site; d#a, the same number of deletion as in d# but different position was deleted; i#, number of bases inserted at target site, r#, number of bases replaced at target site; chi, chimeric; +, Cas9 detected; −, Cas9 not detected.

---

**Fig. 2.** Modifications by HR and NHEJ of a transformed nonfunctional GUS reporter gene triggered by CRISPR/Cas in Arabidopsis. (A) Schematics to show vector construction and possible outcomes after a DSB in the GUUS reporter gene. (B) A T2 plant with mosaic GUS staining indicating that parts of the plant had correct HR events of the GUUS genes. (C) A T2 plant with uniform GUS staining suggesting that all cells on the plant contain a functional copy of the GUS gene as a result of correct HR. (D) Ratios of various GUS staining patterns and genotypes in progenies from five different T1 transgenic lines.
sites. There were some mutations that caused changes in 2 or 3 or even 10–100 bp, but longer mutations were rare. Nearly all of the insertions were 1 bp addition (Fig. 3); and base substitution also mostly involved 1 bp. In contrast, a considerable number of deletions involved relatively longer sequence segments.

Mutations were detected in T1 plants for all target sites in all target genes (Table 2). The number of different mutation types at a given target site detected at the T1 generation varied from 4 to 37 (Table 2). For most targets the mutation types ranged between 10 and 20. Target sites in the BRI1 gene produced large variations in the number of mutation types: five at the sgRNA2 target site and 37 at the sgRNA1 target site. For most gene targets, similar numbers of mutation types were found at T1 and T2 generations. Fewer mutation types of BRI1 gene targets were found at the T2 generation. At T3 the mutation type of the BRI1 gene was dominated by one type (Table 2), a 1-bp insertion (SI Appendix, Table S1). This loss of mutation types through generations might have resulted from the fact that we only had capacity to carry a limited number of progenies to the T3 generation. In addition, at least in the case of BRI1 gene, some mutations may be more detrimental than others and may not produce viable seeds.

All of the selected T1 plants were hygromycin resistant, and most, if not all, of them should have the functional CRISPR/Cas system because it was in the same transfer DNA (T-DNA) with the hygromycin resistance gene. Of all of the T1 plants, 71.2% were found to have mutated target genes (Table 2). Many homozygous mutants, nearly one-fourth, were found at the T2 generation (Table 2). Together with heterozygotes and chimeras, more than half (58.3%) of the T2 plants bore mutated versions of the target genes. At T3 nearly half of the lines tested (47.3%) were homozygous for a mutation, and the majority (79.4%) of T3 plants harbored mutated target genes.

There was a significant percentage of WT copy of the target genes at T1 and early stages of T2. The presence of these WT targets allowed CRISPR/Cas system to make DSB on them to produce new mutations; this is evident in Table 2, which shows that more than half (53%) of the mutations detected in T2 plants were not found at T1. In comparison, most T3 plants did not have new mutations, except for the JAZ1 target that did not have a homozygous mutant line at T2.

We examined the phenotypes of some T3 plants bearing the same mutation of 1 bp insertion in the DSB site of the BRI1 target gene. All of these plants showed the same phenotype of small stature with bushy and wrinkled leaves (SI Appendix, Fig. S2). Even though the mutations were different in some lines for the TT4 targeted gene, the transparent testa phenotypes were indistinguishable (SI Appendix, Fig. S3).

### Specificity of the CRISPR/Cas-Induced Gene Editing

To evaluate the potential off-target effects of CRISPR/Cas, we carried out whole-genome sequencing on T1 or T2 Arabidopsis plants transformed with a CRISPR/Cas construct targeting GAI. The sequencing depth was approximately 60× (SI Appendix, Table S3). The Col-0 WT plants that the transgenic plants were derived from were also sequenced as a control. As shown in SI Appendix, Table S4, the number of SNPs and indels detected in the CRISPR/Cas transgenic plants was similar to the WT control plants compared with GenBank Arabidopsis reference sequence (SI Appendix, Table S4). Compared with one of the Col-0 WT controls, the numbers of SNPs and indels in the transgenic plants were smaller than compared with the GenBank reference genome 10, but the numbers were comparable to those in the other Col-0 WT control.

### Table 2. Frequency, number, and genotype of mutations at different generations and their inheritance to the next generation

<table>
<thead>
<tr>
<th>Target gene</th>
<th>sgRNA</th>
<th>No. of mutations detected at T1</th>
<th>% T1 plant bearing mutations</th>
<th>% Plant bearing mutations</th>
<th>No. of mutations</th>
<th>% From T1</th>
<th>% % New Homologous lines</th>
<th>% % New Homologous lines</th>
<th>% Plant bearing mutations</th>
<th>% From T1</th>
<th>% Homologous lines</th>
<th>% From T1</th>
<th>% Homologous lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAI</td>
<td>sgRNA1</td>
<td>4</td>
<td>47.10</td>
<td>53.80</td>
<td>10</td>
<td>20.00</td>
<td>80.00</td>
<td>18.80</td>
<td>99.00</td>
<td>7</td>
<td>100.00</td>
<td>92.10</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td>sgRNA1</td>
<td>37</td>
<td>84.20</td>
<td>34.60</td>
<td>4</td>
<td>25.00</td>
<td>75.00</td>
<td>7.70</td>
<td>100.00</td>
<td>1</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td>sgRNA2</td>
<td>5</td>
<td>33.30</td>
<td>32.60</td>
<td>1</td>
<td>100.00</td>
<td>0.00</td>
<td>25.60</td>
<td>56.40</td>
<td>3</td>
<td>100.00</td>
<td>49.50</td>
<td>21.80</td>
</tr>
<tr>
<td></td>
<td>sgRNA3</td>
<td>6</td>
<td>30.00</td>
<td>19.80</td>
<td>8</td>
<td>25.00</td>
<td>75.00</td>
<td>8.10</td>
<td>16.50</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>JAZ1</td>
<td>sgRNA1</td>
<td>19</td>
<td>81.70</td>
<td>21.90</td>
<td>19</td>
<td>15.80</td>
<td>84.20</td>
<td>0.00</td>
<td>49.50</td>
<td>9</td>
<td>44.40</td>
<td>29.30</td>
<td></td>
</tr>
<tr>
<td>CHLI1</td>
<td>sgRNA101</td>
<td>8</td>
<td>76.00</td>
<td>58.10</td>
<td>6</td>
<td>66.70</td>
<td>33.30</td>
<td>16.50</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>CHLI2</td>
<td>sgRNA280</td>
<td>10</td>
<td>89.00</td>
<td>72.50</td>
<td>8</td>
<td>62.50</td>
<td>37.50</td>
<td>24.30</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>TT4</td>
<td>sgRNA65</td>
<td>16</td>
<td>86.00</td>
<td>66.10</td>
<td>13</td>
<td>61.50</td>
<td>38.50</td>
<td>21.40</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>TT4</td>
<td>sgRNA296</td>
<td>12</td>
<td>81.00</td>
<td>72.90</td>
<td>8</td>
<td>50.00</td>
<td>50.00</td>
<td>20.80</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>AP1</td>
<td>sgRNA27</td>
<td>20</td>
<td>92.00</td>
<td>96.40</td>
<td>20</td>
<td>50.00</td>
<td>50.00</td>
<td>37.30</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>AP1</td>
<td>sgRNA194</td>
<td>15</td>
<td>74.00</td>
<td>89.20</td>
<td>16</td>
<td>37.50</td>
<td>62.50</td>
<td>42.20</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>GUUS</td>
<td>sgRNA1</td>
<td>8</td>
<td>80.00</td>
<td>81.80</td>
<td>8</td>
<td>50.00</td>
<td>50.00</td>
<td>43.40</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>13.3</td>
<td>71.20</td>
<td>58.30</td>
<td>10.1</td>
<td>47.00</td>
<td>53.00</td>
<td>22.20</td>
<td>79.40</td>
<td>4.2</td>
<td>88.90</td>
<td>11.10</td>
<td>47.30</td>
</tr>
</tbody>
</table>

NT, not tested.
Table 3. Summary of analyses of putative GAI-sgRNA1 off-target sites in Arabidopsis

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Predicted off-target sites</th>
<th>No. of mutation in target site</th>
<th>No. of mutation in putative off-target sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-1</td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Col-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T1-46</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T1-55</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T2-46</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Col-1 and Col-2 were 2 WT controls. Two of the transgenic lines were from the T1 generation, and the third line was from T2.

These data suggested that the WT Col-0 we used may have some small sequence differences with the Arabidopsis reference genome in the GenBank. The data further suggest that the SNPs and indels detected from the genome resequencing of the transgenic plants were likely due to sequencing errors and that the CRISPR/Cas did not cause large numbers of off-target mutations, if any.

We developed a biased workflow to identify the candidate off-target sites (SI Appendix, Fig. S4). According to previous reports, the off-target sites should have significant sequence similarities with the targets, and the last 12 bp of the 20-bp target sequence was most important (1, 12, 13, 17). Therefore, we generated two groups of putative target sites of CRISPR/Cas by the Blastn algorithm using the full 20-bp target sequence and the critical last 12-bp sequence (i.e., 9- to 20-bp fragment of the 20-bp target) as queries, respectively. These sites were used to search the corresponding SNPs and indels datasets to generate a shortlist of likely off-target sites. The sequencing reads of these putative off-target sites were then manually checked using Integrative Genome Viewer (IGV) to confirm the existence of a likely mutation caused by CRISPR/Cas. The presence of protospacer adjacent motif (PAM) was required for the site to be considered as a candidate site. In the end our analysis found no off-target site for all of the samples sequenced (Table 3). In contrast, the expected mutations in the intended target sites were easily identified for all samples (SI Appendix, Figs. S5–S8).

We also used PCR to directly test four putative off-targets of GAI sgRNA1 in the homologous genes of GAI followed by sequencing. No mutation was detected in any of the putative off-targets from approximately 60 T1 plants examined (Table 4). These analyses suggest that CRISPR/Cas-induced mutagenesis is highly specific in plants.

Another way to test for off-targets is to determine whether a mutated target site is subjected to continued CRISPR/Cas action, which would generate new mutations. Data in Table 1 and SI Appendix, Table S1 show that once the target site is fully mutated, in the cases of homozygous and biallelic plants, there was no new mutation in the next generation. This suggested that the CRISPR/Cas system was not able to make DSB on any of the altered target sites, even when the alteration was only 1 bp (Table 1 and SI Appendix, Table S1).

Table 4. Detection of possible mutations in genes highly homologous to the CRISPR/Cas target gene GAI

<table>
<thead>
<tr>
<th>Site name</th>
<th>Target sequence</th>
<th>No. of plants sequenced</th>
<th>No. of plants with mutation</th>
<th>Indel mutation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAI</td>
<td>GATGAGCTTTGACTGTTCTTG</td>
<td>60</td>
<td>24</td>
<td>40</td>
</tr>
<tr>
<td>RGL2</td>
<td>GAGGAGCTTCTGGCTGTTCGG</td>
<td>59</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RGA</td>
<td>GAGGAGCTTCTGGCTGTTCGG</td>
<td>58</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RGL3</td>
<td>GAGGAGCTTCTGGCTGTTCGG</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RGL1</td>
<td>GAGGAGCTTCTGGCTGTTCGG</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

PAM motifs are indicated in pink, mismatched nucleotides in bold, and mismatched nucleotides located in the last 12bp of the core sequence are bold and underlined.

Discussion

Humans thrive when and only when food supply is sufficient. Most of our foods are from improved varieties of animals and plants. Crop improvement by breeding has been largely based on genetic diversity that came from a long history of natural mutations. As the demand on food accelerates and the pressure on crop breeding continues to build, the need to create gene diversity through rapid mutation becomes urgent. In the last few decades mutagenesis of plant genes by means of chemical treatment and radiation has been extensively used to create new traits in plants. Although these agents can greatly speed up the mutation process, the resulting changes are random in terms of which genes are mutated and the direction of the mutations. For these reasons, plant biologists have been searching for a way to specifically, precisely, and easily edit plant genes. Zinc finger nuclease (ZFN) and, more recently, transcription activator-like effector nuclease (TALEN) have been tested as tools for targeted plant gene editing. Several publications have reported successful modifications in native plant genes, but both methods are tedious, expensive, and with only a small number of genes modified so far (18–25). In sharp contrast, we report specific modifications of seven genes in this study in the span of just a few months, demonstrating the extremely easy-to-use feature of the CRISPR/Cas system. We tested both native and transgenes, and all of them were successfully edited, resulting in homozygotes of mutated or corrected genes. The success rate is 100%. Our results show that the efficiency of the CRISPR/Cas system is high enough that plants with edited genes can be easily found by molecular analysis; no phenotypic marker is necessary.

When Arabidopsis plants are infiltrated for the transformation, the CRISPR/Cas components are inserted into the genome of some cells of the infiltrated plants (T0). The Cas9 enzyme may then be expressed and hence cause DSB that is repaired through NHEJ, resulting in mutations. We did not test T0 plants to determine whether any mutations of target genes occurred in the T0 generation. If such events happened in the germ-line cells, mutant heterozygotes, or even homozygotes and biallelic plants, could be expected in the T1 generation. In the seven target genes and 12 target sites examined in this study, we did not find any of these genotypes at T1 (Table 2), indicating either that mutations did not occur in the germ-line cells at T0 or that such event was rare. By comparing the data at T1, T2, and T3 generations in Table 2, it is clear that editing of the target genes took place at high frequency at T1 and that some of the mutations passed to T2 and then T3 following the Mendelian model. In some Cas+ lines, even when no mutation was detected at the early seedling stage at T1, homozygous mutant plants were found at T2 (Table 1 and SI Appendix, Table S1), suggesting that the gene editing arising at late T1 stage can be transmitted to the next generation.

Mutations continued to occur in plants with a WT copy of the target gene segregated from heterozygotes, demonstrating that the CRISPR/Cas system was stable and fully functional in later generations. This provided ample opportunity for the target gene to be edited.
Clearly, many of the mutations detected in T1 plants were somatic mutations only because they were not found in the corresponding T2 populations. Thus, consistent with the fact that germine cells constitute only a very small proportion of the entire cell population of a plant, the frequency of mutations detected in T1 seedlings may not reflect the extent of somatic mutations but does not provide a good indication of germ-line mutation rate at T0 or T1. Our study suggests that screening of heritable mutations should be done in T2 or later generations. In the future, the use of germine-specific promoters to drive Cas9 expression is expected to increase the rate of germ-line mutations while avoiding or reducing the number of somatic mutations.

Genetic transformation of plants with foreign genes has been used extensively to create novel traits. Some of these traits, such as herbicide tolerance and insect resistance, have been successfully applied to large-scale commercialization and brought both economic and environmental benefits. However, the creation of transgenic crops is expensive; one of the reasons is that the insertion location of the foreign gene construct into the host genome by current technologies cannot be controlled. Therefore, expression level of the transgene varies from event to event and cannot be predicted. As a result, many transgenic events have to be generated and screened to find an event with a suitable insertion site and a desirable level and pattern of expression of the transgene. Because the CRISPR/Cas system facilitates HR, in the future this system could allow a gene, or a whole construct, to be precisely inserted into a specific location of a host plant genome. Besides simplified event screening and evaluation, this system could also reduce or even eliminate the risk of adventitious gene insertion. At present, delivery of the gene of interest to increase the rate of germ-line mutations while avoiding or reducing the number of somatic mutations.

CRISPR gene editing in plants. Another way to check for targeting specificity is to examine whether the mutated target gene is subjected to further modifications on the mutated gene. This result further supports that the specificity of the CRISPR/Cas gene editing system is very specific, such that no off-targeting was found for any the targets we tested in Arabidopsis, and even a single mutation at a target site rendered the site inaccessible to the CRISPR targeting. Our systematic study clearly showed that the CRISPR/Cas system is highly efficient and specific in editing Arabidopsis genes and that the edited target genes can be stably inherited.

Materials and Methods
Detailed information is provided in SI Appendix and our previous publications (6, 7). In brief, the target regions were amplified by PCR using primers listed in Table S3, and cloned into the pEASY-Blunt vector (Transgen Biotech, China) for analysis by Sanger sequencing. Whole genome sequencing was performed using the Illumina Hiseq 2500. Identification of SNPs and indels was done by using the SAMtools software, using TAIR 10 as reference. The candidate off-targets were identified by the Blastn algorithm and confirmed manually with the IGV software.

ACKNOWLEDGMENTS. We thank the Core Facility of Genomics and Bioinformatics in the Shanghai Center for Plant Stress Biology for whole-genome sequencing. The work was supported by the Chinese Academy of Sciences.

Supporting Appendix

SI Material and Methods

Growth of Arabidopsis

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used in all experiments. Seeds were sown on 1/2 MS plates and stratified for 3 days at 4°C, then grown under long-day conditions (16 h light/8 h dark) at 22 °C for 5 days before being transplanted to soil.

Screening of T2 transgenic plants with endogenous gene targets

Seeds from T1 transgenic Arabidopsis plants were collected and sowed in soil for phenotyping. DNA was extracted from leaves 4 weeks after planting. The existence of CRISPR-Cas T-DNA was confirmed by PCR using primers HFP3 and HFP4, cas9-2F and cas9-R. Furthermore, the target regions were amplified by PCR with corresponding primers and the PCR products were sequenced. The primers used in the PCR reactions are listed in Supplemental Table 3. The samples showing multi peaks in the target sites were further analyzed by Sanger sequencing of the PCR product after cloning into the *pEASY*-Blunt Cloning vector (Transgen Biotech, China). At least 12 clones were sequenced for each sample. The samples showing single mutation or wild type sequence peaks in the target sites were marked as homozygotes or wild type plants, respectively.

Genotyping of T3 transgenic plants

Seeds from different T2 transgenic lines were grown on 1/2 MS plate in controlled growth chambers. The ten-day old seedlings were prepared for extracting genomic DNA. The target region was sequenced to identify the genotype of T3 generation. The PCR products of bi-allele, heterozygote and chimera plants were then cloned into *pEASY*-Blunt Cloning vector and a number of clones were sequenced to confirm the genotype.

GUS staining

Whole plant of 10/15/20-day-old Arabidopsis plants were subjected to the histochemical GUS staining at 37°C for 12h as previously described(1) and decolorized in 70% EtOH before photographing with a BX51 microscope (BX51, Olympus, Japan).

Whole genome sequencing and off-target identification

DNA was extracted from 100 mg leaf samples of *Arabidopsis*. Library preparation and sequencing (Illumina Hiseq 2500) were performed following the manufacturer’s instructions. For data analysis, sequence reads were mapped to *Arabidopsis thaliana* TAIR 10 (10th release of the *Arabidopsis thaliana* genome sequence from the Arabidopsis Information Resource). Identification of SNPs and indels was conducted using the SAMtools software and use TAIR 10 as reference. The data analysis was then repeated using one of the wild-type Col-0 as reference for identifying SNPs and indels. For identifying the candidate off-target sites, two groups of putative target sites of CRISPR/Cas were searched for by the Blastn algorithm using the full 20 bp target sequence and the critical last 12 bp sequence (i.e. 9-20 bp fragment of the
20 bp target) as queries, respectively. The candidate sites were then used in searches in the corresponding SNPs and indels datasets to generate a shortlist of likely off-target sites. The sequencing reads of these putative off-target sites were then manually checked using IGV to confirm the existence of a likely mutation caused by CRISPR/Cas. The work flow is shown in Supplemental Fig. S4.

Fig. S1. Mutations and segregation of the *BRI1* gene modified by CRISPR/Cas
Fig. S2. Phenotype of T3 plants from different CRISPR/Cas transgenic lines but with the same one base pair insertion in the BRI1 gene.
Fig. S3. Phenotype of T3 seeds from different lines with different mutations in the TT4 gene caused by CRISPR/Cas. A similar white-seeds phenotype was seen in all the mutants compared with normal seeds from WT (Col) plants. All pictures were taken from an Olympus SZX2-FOF microscope under the same lighting conditions. Mutants are labeled with line number followed by mutation type; d# for deletion and i# for insertion; # is the number of base pair changed.
Fig. S4. Analysis workflow of the whole genome re-sequencing data to identify candidate off-target sites in Arabidopsis.
Fig. S5. Whole genome sequence data for the GAI-sgRNA1 target region in Col-0 sample viewed by IGV. No mutation was detected. The target sequence is labelled with a red line.
Fig. S6. GAI-sgRNA1 target region in the GAI-sgRNA1 T1-46 sample viewed by IGV. Mutations (deletions) are found in some sequences in the target region. The target sequence is labelled with a red line.
Fig. S7. GAI-sgRNA1 target region in the GAI-sgRNA1 T2-46 sample viewed by IGV. One bp deletions are found in some sequences in the target region. The target sequence is labelled with a red line.
Fig. S8. GAI-sgRNA1 target region in the GAI-sgRNA1 T1-55 sample viewed by IGV. Deletions are seen in some sequences in, or near the target region. The target sequence is labelled with a red line.
<table>
<thead>
<tr>
<th>Target gene</th>
<th>sgRNA</th>
<th>Line</th>
<th>Zygosity</th>
<th>Target genotype</th>
<th>Cas</th>
<th>Mutation segregation</th>
<th>Cas</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRI1</td>
<td>sgRNA1</td>
<td>61-11</td>
<td>Heterozygote</td>
<td>d1WT</td>
<td>+</td>
<td>8d1d1:18d1WT:9chi</td>
<td>27+:9-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA1</td>
<td>61-36</td>
<td>Heterozygote</td>
<td>d1WT</td>
<td>+</td>
<td>8d1d1:9d1WT:(4WT+2chi)</td>
<td>18+:6-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA1</td>
<td>61-4</td>
<td>Heterozygote</td>
<td>i1WT</td>
<td>+</td>
<td>8i1i1:14i1WT:(6WT+4chi)</td>
<td>27+:9-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA1</td>
<td>61-9</td>
<td>Heterozygote</td>
<td>dxWT</td>
<td>+</td>
<td>9dxdx:15dxWT:12chi</td>
<td>All+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA1</td>
<td>10-1</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>6Mutation:35WT</td>
<td>48+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA1</td>
<td>10-1</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>17Mutation:27WT</td>
<td>44+:4-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA1</td>
<td>11-3</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>16Mutation:26WT</td>
<td>48+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA1</td>
<td>11-4</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>22Mutation:18WT</td>
<td>47+:11-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>4-m2</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>2i1i1</td>
<td>2+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>4-m4</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>5+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>4-m5</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>4+:1-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>4-m6</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>5+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m1</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>5+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m10</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>5+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m11</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>6i1i1</td>
<td>6+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m14</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>5-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m16</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>5+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m3</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>4+:1-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m4</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>-</td>
<td>2i1i1</td>
<td>2-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m5</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>5+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m6</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>-</td>
<td>5i1i1</td>
<td>5-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m8</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>5+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-m9</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>-</td>
<td>5i1i1</td>
<td>5-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>4-1</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>40Mutation</td>
<td>40+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>4-2</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>42Mutation:2WT</td>
<td>45+:3-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-1</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>39Mutation:7WT</td>
<td>45+:2-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA2</td>
<td>5-2</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>40Mutation:4WT</td>
<td>42+:5-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA3</td>
<td>48-53</td>
<td>Chimera</td>
<td>d1,d5,d32 chi</td>
<td>+</td>
<td>4d32WT:14WT</td>
<td>17+:1-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA3</td>
<td>48-54</td>
<td>Chimera</td>
<td>d1,d52 chi</td>
<td>+</td>
<td>11d1WT:4WT:11chi</td>
<td>31+:1-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA3</td>
<td>12-m2</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>5+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA3</td>
<td>69-m2</td>
<td>Homozygote</td>
<td>i1i1</td>
<td>+</td>
<td>5i1i1</td>
<td>5+</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA3</td>
<td>12-1</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>30Mutation:12WT</td>
<td>42+:6-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA3</td>
<td>12-6</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>23Mutation:19WT</td>
<td>46+:2-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA3</td>
<td>5-3</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>27Mutation:12WT</td>
<td>40+:8-</td>
</tr>
<tr>
<td>BRI1</td>
<td>sgRNA3</td>
<td>5-5</td>
<td>Wild-type</td>
<td>WT</td>
<td>+</td>
<td>33Mutation:8WT</td>
<td>45+:3-</td>
</tr>
<tr>
<td>JAZ1</td>
<td>sgRNA1</td>
<td>4-7</td>
<td>Chimera</td>
<td>i1,d16,d17,d22</td>
<td>+</td>
<td>12i1i1:1d16d17:1d17d16:1d16WT:12WTWT:5chi</td>
<td>All+</td>
</tr>
<tr>
<td>JAZ1</td>
<td>sgRNA1</td>
<td>24-5</td>
<td>Chimera</td>
<td>d1,d4</td>
<td>+</td>
<td>4d16d16:7d4WT:18WTWT:5chi</td>
<td>All+</td>
</tr>
<tr>
<td>JAZ1</td>
<td>sgRNA1</td>
<td>8-14</td>
<td>Chimera</td>
<td>dxax,dxb chi</td>
<td>+</td>
<td>4dxaxdx:5dxbdxb:4d123d123:19WT</td>
<td>24+:12-</td>
</tr>
</tbody>
</table>

WT, wild-type sequence with no mutation detected; d#, # of bases deleted from target site; d#a, the same number of deletion as in d# but different position was deleted; i#, # of bases inserted at target site, chi, chimeric; +, Cas9 detected; -, Cas9 not detected.
### Table S2. List of NHEJ mutations at T1 and T2 generation in each target site.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Mutation type</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GUUS-sgRNA1</strong>&lt;br&gt;WT sequence&lt;br&gt;ATAACAGGGTAATAGAGATAAAGGGAGGCCTG</td>
<td><strong>T1 mutants:</strong>&lt;br&gt;ATAACAGGGTAATAGAGA--GGGAGGCCTG</td>
<td>d1</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGATA--GGGAGGCCTG</td>
<td>d2</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGA-----GGGAGGCCTG</td>
<td>d6</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGATATAATGGGAGGCCTG</td>
<td>r1</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGATAGAGGGAAGGCCTG</td>
<td>i1a</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGATAGGGAAGGCCTG</td>
<td>i1b</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGATAGGGAAGGCCTG</td>
<td>i2</td>
</tr>
<tr>
<td></td>
<td><strong>T2 mutants:</strong>&lt;br&gt;ATAACAGGGTAATA---------AGGGAGGCCTG</td>
<td>d7</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGA--------GGGAGGCCTG</td>
<td>d5</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGA---------GGGAGGCCTG</td>
<td>d4a</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGA---------GGGAGGCCTG</td>
<td>i2</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGATATA-------GGGAGGCCTG</td>
<td>i1</td>
</tr>
<tr>
<td></td>
<td>ATAACAGGGTAATAGAGATATA-------GGGAGGCCTG</td>
<td>d1</td>
</tr>
<tr>
<td></td>
<td><strong>API-sgRNA27</strong>&lt;br&gt;WT sequence&lt;br&gt;AAGGGGTAGGGTTCAATTGAAGAGGATAGAGAACA</td>
<td><strong>T1 mutants:</strong>&lt;br&gt;AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d17</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d19</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d1a</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d1b</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d1c</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d2a</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d2b</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d3a</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d2c</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d3b</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d4</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d7</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d8a</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d8b</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>d9</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>dxa</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>dxb</td>
</tr>
<tr>
<td></td>
<td>AAGGGGTAGGGTTCAATTGAAAAAGAGGATAGAGAACA</td>
<td>dxc</td>
</tr>
</tbody>
</table>
AAGGGGTAGGGTTCAATTGAAAGAGGATAGAGAACA
AAGGGGTAGGGTTCAATTGCAAGAGGATAGAGAACA

T2 mutants:
AAGGGGTAGGGATCAA--AAAGAGGATAGAGAACA
AAGGGGTAGGGATCAA-AAAGAGGATAGAGAACA
AAGGGGTAGGGTTCAATTGAAATTTGGATAGAGAACA
AAGGGGTAGGGTTCAATCAAAGAGGATAGAGAACA
AAGGGGTAGGGATCAAAGAGGATCAAAAGAGGATAGAGAACA
AAGGGGTAGGGTTCAATT---------GAGAACA
AAGGGGTAGGGTTCAATTGCAAGAGGATAGAGAACA
AAGGGG--------AAGAGGATAGAGAACA
AAGGGGTAGGGTTCAATT-GAAGAGGATAGAGAACA
AAGGGGTAGGGTTCAATT---AAGAGGATAGAGAACA
AAGGGGTAGGGTTCAATT-G--GAGGATAGAGAACA
AAGGGGTAGGGTTCAATTG--GAGGATAGAGAACA
AAGGGGTAGGGTTCAATTG---GAGGATAGAGAACA
AAGGGGTAGGGTTCAATTG------TAGAGAACA
AAGGGGTAGGGTTCAATTG------------------
AAGGGGTAGGGTTCAATTG---------------
AAGGGGTAGGGTTCAATTGTCAGTGGAGTATTCGA

AP1-sgRNA194
WT sequence
TCTTCGAATACTCCACTGATTCTTGGTAACTTCAACTAA

T1 Mutants:
------------------------TGGTAACTTC dxc 2
------------------------TTGGTAACTTC dxd 1
------------------------TTCTTGTAACCTTC dxf 2
------------------------ATCTTGTAACCTTC dxg 3
------------------------TATCTTGTAACCTTC dkx 1
------------------------AATCTTGTAACCTTC dxl 1
------------------------TGATTCTTGGTAACTTC dxm 2
------------------------GTAGTTCTTGGTAACTTC dxn 1
TCTTCGAATCT-----------TAACTTC d14 1
TCTTCGAATCTCCACTGAAAACTCTC d8 1
TCTTCGAATCTCCACT------------GTAACCTTC d7 1
TCTTCGAATCTCCAC--------TTGGTAACCTTC d6a 1
TCTTCGAATCTCCACT-------TCTTGGTAACCTC d3a 1
TCTTCGAATACTCCACTG--TCTTGGTAACTTC
d2  1
TCTTCGAATACTCCACT-ATTCTTGGTAACTTC
d1a  4
TCTTCGAATACTCCACTG-TTCTTGGTAACTTC
d1b  3
TCTTCGAATACTCCACTGATATTCTTGGTAACTTC
d1a  3
TCTTCGAATACTCCACTG-TTCTTGGTAACTTC
d1b  1
TCTTCGAATACTCCACTGATATTCTTGGTAACTTC
d1b  9
CCACTGGGAACTTCTTGGTAACTTCTTGGTAACTTC
d1b  3

T2 Mutants:
----------------------------------ACTAA
dxa  1
-------------------------------TCAACTAA
dxb  1
-----------------------TGGTAACTTCAACTAA
dxc  3
----------------------TTGGTAACTTCAACTAA
dxd  3
--------------------TCTTGGTAACTTCAACTAA
dxe  7
-------------------TTCTTGGTAACTTCAACTAA
dx1 10
------------------ATTCTTGGTAACTTCAACTAA
dxf  3
-----------------------------------------
dxg  3
-----------------------------------------
dxm  3
TCTTCGAATACTCCACTG---------------------A
d20a  1
TCTTCGAATACTCCACTG---------------------
d20b  3
TCTTCGAATACTCCACTG---------------------ACTTCAACTAA
d10  1
TCTTCGAATACTCCACTG---------------------
d6b  1
TCTTCGAATACTCCACTG---------------------
d5  1
TCTTCGAATACTCCACTG---------------------ACTTCAACTAA
d1a  3
TCTTCGAATACTCCACTG---------------------
d1b  1
CTTGTTCCTCTATCTTATTCTTGGTAACTTCAACTAA
r1  1
GTTTCTCGTCGTACTTAGTGTTCTTGGTAACTTCAACTAA
cxa  3
TCTTCGAATACTCCACTG---------------------
d1b  28
TCTTCGAATACTCCACTG---------------------
c2  2
TCTTGTTCCTATCTTATTCTTGGTAACTTCAACTAA
cxb  2
TCTTCGAATACTCCACTG---------------------
id2  2

TT4-sgRNA65
WT sequence
GCTGATGGACCTGCAGGCATCTTGGCTATTGGCAC
T1 Mutants:
GCTG--------------------
dxa  1
GCTGATGG------------------------
dxd  3
GCTGATGGA----------------------
dxe  1
GCTGATGGAC---------------------
dxf  4
GCTGATGGACCTGCTGATGGA--
dxg  1
GCTGATGGACCTGCTGATGGA--
d19  1
GCTGATGGACCTGCTGATGGA--
d15  1
GCTGATGGACCTGCTGATGGA--
d11  1
GCTGATGGACCTGCTGATGGA--
d3  1
GCTGATGGACCTGCTGATGGA--
d2a  1
GCTGATGGACCTGCTGATGGA--
d2b  1
GCTGATGGACCTGCTGATGGA--
d1a  12
GCTGATGGACAAGACTCACAACGCTTCGTCTA
cxa 2
CTCATCCAAAGAAGGCAAGGCGACCCACCATCATCATTATGATA
cxb 1
CTGAGGATACTCCGCAGATGCAGGCTCTTGGCTATTGGG
exc 1
GCTGATGGACCCTGAGGACACCTTGGCTATTGGG
i1a 10
GCTGATGGACCAATTGCAGGCGACCTTGGCTATTGGC
i3 1
GCTGATGGACCCCTGAGAACAGCAGGCGACCTTGGCTATTGGC
i8 1
T2 Mutants:
----------------------------------TTGGCAC
dxb 2
GCTGATG--------------------------
dxc 2
GCTGATGGACCTGGCTATTTGGC
dxg 3
GCTGATGGACCA-----------
dxh 2
GC------------------------TATTGGCAC
d24a 1
GCTGA-------------------------TGGCAC
d24b 1
GCTGATG--------CAGGCATCTTGGCTATTGGCAC
d6 2
GCTGATGGACCTGGGCAATTTGCGACACGTAC
d6 2
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d24a 1
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d24b 1
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d25a 1
T2 Mutants:
----------------------------------TTGGCAC
dxb 2
GCTGATG--------------------------
dxc 2
GCTGATGGACCTGGCTATTTGGC
dxg 3
GCTGATGGACCA-----------
dxh 2
GC------------------------TATTGGCAC
d24a 1
GCTGA-------------------------TGGCAC
d24b 1
GCTGATG--------CAGGCATCTTGGCTATTGGCAC
d6 2
GCTGATGGACCTGGGCAATTTGCGACACGTAC
d6 2
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d24a 1
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d24b 1
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d25a 1
T2 Mutants:
----------------------------------TTGGCAC
dxb 2
GCTGATG--------------------------
dxc 2
GCTGATGGACCTGGCTATTTGGC
dxg 3
GCTGATGGACCA-----------
dxh 2
GC------------------------TATTGGCAC
d24a 1
GCTGA-------------------------TGGCAC
d24b 1
GCTGATG--------CAGGCATCTTGGCTATTGGCAC
d6 2
GCTGATGGACCTGGGCAATTTGCGACACGTAC
d6 2
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d24a 1
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d24b 1
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d25a 1
T2 Mutants:
----------------------------------TTGGCAC
dxb 2
GCTGATG--------------------------
dxc 2
GCTGATGGACCTGGCTATTTGGC
dxg 3
GCTGATGGACCA-----------
dxh 2
GC------------------------TATTGGCAC
d24a 1
GCTGA-------------------------TGGCAC
d24b 1
GCTGATG--------CAGGCATCTTGGCTATTGGCAC
d6 2
GCTGATGGACCTGGGCAATTTGCGACACGTAC
d6 2
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d24a 1
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d24b 1
GCTGATGGACCTTGGCGACAGTGCTTCGACACGTAC
d25a 1

TT4-sgRNA296
WT sequence
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
T1 mutants:
----------------------------------ATTGGAAACGTCAC
dxc 4
----------------------------------ATTGGAAACGTCAC
dxb 2
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
dxd 2
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
d22 1
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
d12 1
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
d6 1
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
dxf 1
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
d2 1
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
d1 13
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
dia 4
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
ilb 5
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
ila 1
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
ilb 5
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
cxa 1
ACTATTTACAGCGCAAGTCAAGTCCGAAACGTCAC
i5 1
GTCGAATAGCCAGAACACGCTTGCAATTCGAAAACGTCAC
cxb 1
GTCGAATAGCCAGAACACGCTTGCAATTCGAAAACGTCAC
cxd 1
GTCGAATAGCCAGAACACGCTTGCAATTCGAAAACGTCAC
cxc 1
T2 mutants:
----------------------------------GGAAACGTCAC
dxa 1
----------------------------------GGAAACGTCAC
dxb 1
**WT sequence**

GCTTCAGGCCAGGTAACACAGTCTCTCC

**T1 mutants:**

- CCTTCACTCTCTCTGCCAGGTAACACAGTCTCTCC
- GC----GGCAGCAGGTAACACAGTCTCTCC
- GCTTCAGGGCAGGTAACACAGTCTCTCC
- GCTTCAGCACAGGTAACACAGTCTCTCC
- GCTTCACTCTCTCTGCCAGGTAACACAGTCTCTCC
- GCTTCAGGGCAGGTAACACAGTCTCTCC
- GCTTCAGCACAGGTAACACAGTCTCTCC
- GCTTTCACTCTCTCTGCCAGGTAACACAGTCTCTCC
- GCTTCAGGGCAGGTAACACAGTCTCTCC
- GCTTCAGCACAGGTAACACAGTCTCTCC
- GCTTCAGGGCAGGTAACACAGTCTCTCC
- GCTTCAGCACAGGTAACACAGTCTCTCC
- GCTTCACTCTCTCTGCCAGGTAACACAGTCTCTCC

**T2 mutants:**

- GCTTCAGGACAGGTAACACAGTCTCTCC
- GCTTCAGCACAGGTAACACAGTCTCTCC
- GCTTCAGGACAGGTAACACAGTCTCTCC
- GCTTCAGGACAGGTAACACAGTCTCTCC
- GCTTCAGGACAGGTAACACAGTCTCTCC
- GCTTCAGGACAGGTAACACAGTCTCTCC
- GCTTCAGGACAGGTAACACAGTCTCTCC
- GCTTCAGGACAGGTAACACAGTCTCTCC

**CHLII-sgRNA101**

**WT sequence**

GCTTCAGGCCAGGTAACACAGTCTCTCC

**T1 mutants:**

- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA

**T2 mutants:**

- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA

**CHLII-sgRNA280**

**WT sequence**

GCTTCAGGCCAGGTAACACAGTCTCTCC

**T1 mutants:**

- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA

**T2 mutants:**

- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
- CAGATCTCGTTACCAGATGTCTCTGTTATGATGTCGCTACA
T2 mutants:
CAGATCTCGTTACCATGT--TCTGTTTATGAATGTGCTACA d1 2
CAGATCTCGTTACCATGT------------------CGCTACA d15 4
CAGATCTCGTTACC--CTCTGTTTATGAATGTGCTACA d4b 1
CAGATCTCGTTACCATGT------TTATGAATGTGCTACA d5 1
CAGATCTCGTTACCATGT--ATGAATGTGCTACA d6b 1
CAGATCTCGTTACCATG-----------------TATGAATGTGCTACA d7 1
----------------------------------CTCTGTTATGAATGTGCTACA dx 1
CAGATCTCGTTACCATGTACTCTGTTATGAATGTGCTACA i1a 6
CAGATCTCGTTACCATGTCCTCTGTTATGAATGTGCTACA i1b 22
CAGATCTCGTTACCATGTTCTCTGTTATGAATGTGCTACA i1c 6
CAGATCTCGTTACCATGTGCTCTGTTATGAATGTGCTACA i1d 1
GAI–sgRNA1
WT sequence
GATGAGCTTCTCTAGCTGTTCTTGG
T1 mutants:
GATGAGCTTCTAGC---TCTTGG d3a 1
GATGAGCTTCTAGC---TTGG d3b 1
GATGAGCTTCTAGCTGCTCTTGG r1 1
GATGAGCTTCTAGCTGCTCTTGG d1 1
GATGAGCTTCTAGCTGCTCTTGG d1 3
GATGAGCTTCTAGCTGCTCTTGG d1 20
GATGAGCTTCTAGCTGCTCTTGG d3b 1
GATGAGCTTCTAGCTGCTCTTGG d3c 13
GATGAGCTTCTAGCTGCTCTTGG d5 13
GATGAG--------------TGG d14 1
GATGAGCTTCTAGCTGCTCTTGG i1 1
GATGAGCTTCTAGCTGCTCTTGG cx a 1
GATGAGCTTCTAGCTGCTCTTGG cx b 1
BRI1–sgRNA1
WT sequence
TTGGGTCAAAGATATCTCTCTTG
T1 mutants:
TTGGGTCAAAGATATCTCTCTTTTC
TTGGGTCAAAGATATCTCTCTTTTC i1a 1
TTGGGTCAAAGATATCTCTCTTTTC i1b 1
TTGGGTCAAAGATATCTCTCTTTTC i5 1
TTGGGTCAAAGATATCTCTCTTTTC d1a 1
TTGGGTCAAAGATATCTCTCTTTTC d1b 1
TTGGGTCAAAGATATCTCTCTTTTC cx a 1
TTGGGTCAAAGATATCTCTCTTTTC cx b 1
TTGGGTCAAAGATATCTCTCTTTTC d2a 1
TTGGGTCAAAGATATCTCTCTTTTC d2b 1
TTGGGTCAAAGATATCTCTCTTTTC d3 1
TTGGGTCAAAGATATCTCTCTTTTC d4 1
TGGGTCATAAC------CTCTGGTTTCG
d5a 1
TGGGTCATAACGATA------GCTTTCG
d5b 1
tGGTCTAA------TCTCTGGTTTCG
d5c 1
TGGGTCTAATAACGAT------GCTTTCG
d6a 1
TGGGTCATAAGCTATA------GCTTTCG
d6b 1
TGGGTCATAATAACCTCTGGTTTCG
d6c 1
TGGGTCATAATAACCTCTGGTTCG
d7a 1
TGGGTCATATAACCTCTGGTTCG
d7b 1
TGGGTCATAATAACCTCTGGTTCG
cxc 1
TGGGTCATATAACCTCTGGTTCG
d9 1
TGGGTCATATAACCTCTGGTTCG
cxd 1
TGGGTCATATAACCTCTGGTTCG
d10 1
TGGGTCATATAACCTCTGGTTCG
d14 1
TGGGTCATATAACCTCTGGTTCG
d18 1
TGGGTCATATAACCTCTGGTTCG
d21 1
TGGGTCATATAACCTCTGGTTCG
d22 1
TGGGTCATATAACCTCTGGTTCG
cxe 1
TGGGTCATATAACCTCTGGTTCG
cxf 1
TGGGTCATATAACCTCTGGTTCG
d25 1
TGGGTCATATAACCTCTGGTTCG
cxg 1
TG------------------------
d34 1
TGGGTCATATAACCTCTGGTTCG
d35 1
TGGGTCATATAACCTCTGGTTCG
d37 1
TGGGTCATATAACCTCTGGTTCG
d51 1
TGGGTCATATAACCTCTGGTTCG
d89 1
T2 mutants:
--------------------CTGG
dx 2
TTGGGTCATAACGATATTCTCTGG
r1 17
TTGGGTCATAACGATATTCTCTGG
d1b 1
TTGGGTCATAACGATATTCTCTGG
d2c 1
BRI1-sgRNA2
WT sequence
GACATACATGAGCTCCTGAGG
t1 mutants:
GACATACATGAGCTC-TGAGG
d1 1
GACATACATGAGCTG-GAGG
d8 1
GACATACATGAGCTG-GAGG
dia 1
GACATACATGAGCTC-TGAGG
d5 1
GACATACATGAGCTG-GAGG
ilb 1
T2 mutants:
GACATACATGAGCTCCCTGAGG
ilb 50
BRI1-sgRNA3
WT sequence
GACATACATGAGCTG-GAGG
T1 mutants:
GAGCTGACATAG-CTGAGGAATCC  d1  1
GAGCTGACATAGCCTGAGGAATCC  i1a  1
GAGCTGACATAGCAGTGGAGGAATCC  i1b  1
GAGCTGACATAGCTGAGGAATCC  i1c  1
GAGCTGACA-----TGAGGAATCC  d5  1
T2 mutants:

-----------------------------------   d52  1
-----------------------------GAGGAATCC   d32  1
-------------TGAGGAATCCCT  d29  1
GAGCT---AT---CTGAGGAATCCCT  d5  1
GAGCTGACAT---CTGAGGAATCCCT  d3  1
GAGCTGACATAG-CTGAGGAATCCCT  d1  2
GAGCTGACATAGCCTGATGAATCCT  r1  1
GAGCTGACATAGCAGTGGAGGAATCCCT  i1b  7

JAZ1-sgRNA1
WT sequence
TAGCAATAGGAAGTTCTGTCAATGGTG

T1 mutants:

TAGCAATAGGAAGTTCTGCCCAATGGTGTTG  r1  1
TAGCAATAGGAAGTTCTC-TCAATGGTGTTG  d1a  1
TAGCAATAGGAAGTTCTGTAATGGTGTTG  d1b  1
TAGCAATAGGAAGTTCTGAATGGTGTTG  d2  1
TAGCAATAGGAAGTTCTGCAATGGTGTTG  i1  1
TAGCAATAGGAAGCTCCC--CAATGGTGTTG  cxa  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d4a  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d4b  1
TAGCAATAGGAAGCTCCC--CAATGGTGTTG  d7a  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d11  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d16  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d22a  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d51  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d53  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d84  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  im  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  i29  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  cxb  1
T2 mutants:

----------------------TTCAACAATGGTG  d125  1
-----------------------------------  d112  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d22b  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d16  2
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d13  1
dxa  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  dxb  1
TAGCAATAGGAAGTTCTGTCATGGTGTTG  d9  1
TAGCAATAGGAAGTTCTG-------TG          d7b          2
TAGCAATAGGAAGTTCTGATATCGGT-TT-------G          d6          1
TAGCAATAGGAAGTTCTGATATCGGT-TT-----G          d5          1
TAGCAATAGGAAGTTCTGATATCGGT-TT-----G          d4c          1
TAGCAATAGGAAGTT-----CAATGGTG          d4b          2
TAGCAATAGGAAGTTCTGT-----TGCTG          d3          1
TAGCAATAGGAAGTTCTGT-----TGCTG          d1c          2
TAGCAATAGGAAGTTCTGT-----TGCTG          d1b          2
TAGCAATAGGAAGTTCTGT-----TGCTG          d1a          1
TAGCAATAGGAAGTTCTGT-----TGCTG          i1          3
TAGCAATAGGAAGTTCTGT-----TGCTG          i7          1

d#, # of bases deleted from target site; d# a, the same number of deletion as in d# but different position was deleted; i#, # of bases inserted at target site, r#, # of bases replaced at target site; c#, combined mutation; x, mutation occurred, but number of mutated base could not be determined.
Table S3. Summary of the whole genome re-sequencing data

<table>
<thead>
<tr>
<th>SampleName</th>
<th>Reads Number</th>
<th>Data Size (bp)</th>
<th>Depth (X)</th>
<th>Mapped Reads</th>
<th>Map Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>94,783,100</td>
<td>9,573,093,100</td>
<td>63.82</td>
<td>92,068,265</td>
<td>97.1%</td>
</tr>
<tr>
<td>GAI-sgRNA1 T1-46</td>
<td>89,807,364</td>
<td>9,070,543,764</td>
<td>60.47</td>
<td>86,845,340</td>
<td>96.7%</td>
</tr>
<tr>
<td>GAI-sgRNA1 T1-55</td>
<td>98,180,740</td>
<td>9,916,254,740</td>
<td>66.11</td>
<td>94,728,656</td>
<td>96.5%</td>
</tr>
<tr>
<td>GAI-sgRNA1 T2-46</td>
<td>87,180,454</td>
<td>8,805,225,854</td>
<td>58.70</td>
<td>76,161,273</td>
<td>87.4%</td>
</tr>
</tbody>
</table>
Table S4. Summary of the whole-genome analysis of potential off-target effects of CRISPR/Cas in *Arabidopsis*.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Sample Name</th>
<th>SNP Number</th>
<th>Insertion Number</th>
<th>Deletion Number</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>To GenBank</td>
<td>Arabidopsis sequence</td>
<td>Col-1</td>
<td>4,844</td>
<td>1,534</td>
<td>2,032</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Col-2</td>
<td>4,664</td>
<td>1,507</td>
<td>1,953</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAI-sgRNA1 T1-46</td>
<td>4,633</td>
<td>1,500</td>
<td>1,995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAI-sgRNA1 T1-55</td>
<td>4,545</td>
<td>1,494</td>
<td>2,013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAI-sgRNA1 T2-46</td>
<td>4,664</td>
<td>1,462</td>
<td>1,922</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Col-2</td>
<td>3,833</td>
<td>1,373</td>
<td>1,238</td>
</tr>
<tr>
<td>To Col-1 WT</td>
<td>sequence</td>
<td>GAI-sgRNA1 T1-46</td>
<td>3,721</td>
<td>1,374</td>
<td>1,214</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAI-sgRNA1 T1-55</td>
<td>3,709</td>
<td>1,396</td>
<td>1,210</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAI-sgRNA1 T2-46</td>
<td>3,762</td>
<td>1,335</td>
<td>1,204</td>
</tr>
</tbody>
</table>

Col-1 and Col-2 were 2 WT controls. Two of the transgenic lines were from the T1 generation and the third line was from T2.
Table S5. List of the primers used in this paper

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFP3</td>
<td>TGAAAAAGCCTGAACCTCACCG</td>
</tr>
<tr>
<td>HFP4</td>
<td>TATTTCTTTGCCCCTCAGCG</td>
</tr>
<tr>
<td>AtBRI1 chop1 F</td>
<td>GAATCTCTGACGAATCTATCC</td>
</tr>
<tr>
<td>AtBRI1 chop1 R</td>
<td>CACTCTTTTCTTCATCCATCC</td>
</tr>
<tr>
<td>AtBRI1 chop2 F</td>
<td>GATGGGATGGAAGAAAGAGGT</td>
</tr>
<tr>
<td>AtBRI1 chop2 R</td>
<td>CTCATCTCTCAACCAAGAAG</td>
</tr>
<tr>
<td>GAI-F</td>
<td>TGTTATTAGAGTGGATGGTTGAGAGTG</td>
</tr>
<tr>
<td>GAI-R</td>
<td>AGCCGTCGCTGTAGTGTTG</td>
</tr>
<tr>
<td>JAZ-F</td>
<td>CAACCATGAGTTATTCCTTGT</td>
</tr>
<tr>
<td>JAZ-R</td>
<td>GGATTTAGACAGGCAGCAATAAC</td>
</tr>
<tr>
<td>Cas9-2F</td>
<td>CCAAGAGGACTACAGGTAAG</td>
</tr>
<tr>
<td>Cas9-R</td>
<td>ATGGGATCTTACTCTTTTTTTGGCTCGG</td>
</tr>
<tr>
<td>RGA -F</td>
<td>ATGAATGATGATTGAAAGTGAGGTAGTAG</td>
</tr>
<tr>
<td>RGA -R</td>
<td>CGAGCATGATTTCCACACG</td>
</tr>
<tr>
<td>RGL1-F</td>
<td>CTCCCCGGATCTTCTAATCTTTTC</td>
</tr>
<tr>
<td>RGL1-R</td>
<td>CGACTTTCTCATAGCACCAG</td>
</tr>
<tr>
<td>RGL2-F</td>
<td>AAACCTTACCCACCCCATGAGT</td>
</tr>
<tr>
<td>RGL2-R</td>
<td>GCCTGGACAGTCTTAAACTCC</td>
</tr>
<tr>
<td>RGL3-F</td>
<td>TCTCCAGGTCCTCCACTCTTC</td>
</tr>
<tr>
<td>RGL3-R</td>
<td>TCTCCCGATAAGCACCAG</td>
</tr>
<tr>
<td>GU-F1</td>
<td>GTGGCCATTCAGTCTTGATCGC</td>
</tr>
<tr>
<td>GU-F2</td>
<td>ATGCCGGAATCCATCGCAGCG</td>
</tr>
<tr>
<td>US-R2</td>
<td>CACCATTCAGCCGGGAGTCTAGTC</td>
</tr>
<tr>
<td>TT4-F-159</td>
<td>CTGCCCGTCCATCTAACCCTAC</td>
</tr>
<tr>
<td>TT4-407-R</td>
<td>GACTGCAGCCACCCAGATGT</td>
</tr>
<tr>
<td>CHLI1-3-F</td>
<td>GGCGTCTCTTTCTTGAGACCATC</td>
</tr>
<tr>
<td>CHLI1-262-R</td>
<td>CGGAAACATGGTTACAGGACC</td>
</tr>
<tr>
<td>CHLI2-3-F</td>
<td>GGGGCTCTTTTCTCGGAAAGAT</td>
</tr>
<tr>
<td>CHLI2-463-R</td>
<td>CGGATACACAGTGTTCCGCA</td>
</tr>
<tr>
<td>AP1-F-113</td>
<td>GGTTCATACCAAGATCTGAGC</td>
</tr>
<tr>
<td>AP1-271-R</td>
<td>TCAAGTAGTCAACTTAAGGGGG</td>
</tr>
</tbody>
</table>