Targeted Mutagenesis, Precise Gene Editing, and Site-Specific Gene Insertion in Maize Using Cas9 and Guide RNA

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Targeted mutagenesis, editing of endogenous maize (*Zea mays*) genes, and site-specific insertion of a trait gene using clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas)-guide RNA technology are reported in maize. DNA vectors expressing maize codon-optimized *Streptococcus pyogenes* Cas9 endonuclease and single guide RNAs were cointroduced with or without DNA repair templates into maize immature embryos by biolistic transformation targeting five different genomic regions: upstream of the *liguleless1* (*LIG1*) gene, male fertility genes (*Ms26* and *Ms45*), and acetolactate synthase (*ALS*) genes (*ALS1* and *ALS2*). Mutations were subsequently identified at all sites targeted, and plants containing biallelic multiplex mutations at *LIG1*, *Ms26*, and *Ms45* were recovered. Biolistic delivery of guide RNAs (as RNA molecules) directly into immature embryo cells containing preintegrated Cas9 also resulted in targeted mutations. Editing the *ALS2* gene using either single-stranded oligonucleotides or double-stranded DNA vectors as repair templates yielded chlorsulfuron-resistant plants. Double-strand breaks generated by RNA-guided Cas9 endonuclease also stimulated insertion of a trait gene at a site near *LIG1* by homology-directed repair. Progeny showed expected Mendelian segregation of mutations, edits, and targeted gene insertions. The examples reported in this study demonstrate the utility of Cas9-guide RNA technology as a plant genome editing tool to enhance plant breeding and crop research needed to meet growing agriculture demands of the future.

The evolution of plant breeding, one of the first scientific activities known in the history of human kind, has allowed rapid progress of our civilization and continues to be important for the improvement of crops and sustainability of agriculture. Traditional breeding, however, is limited by the starting genetic diversity. With the advent of modern molecular biology, breeder’s options have been substantially broadened, allowing the purposeful transfer of genetic information from either related or diverse organisms into different plant species, thus specifically improving agricultural performance. The most common methods of DNA delivery, particle bombardment and *Agrobacterium*-mediated transformation, lead to random gene insertions throughout the genome. Integration of trait genes into a specific location is considerably more attractive and can be accomplished through homology-directed repair (HDR). Although a few publications before 2009 have reported successful direct application of HDR to modify endogenous plant genes, this occurs at impractically low frequencies (Halfter et al., 1992; Offringa et al., 1993; Miao and Lam, 1995; Kempin et al., 1997; Hanin et al., 2001; Terada et al., 2002, 2007). Studies in different organisms, including plants, have shown that coincident generation of targeted DNA double-strand breaks (DSBs) is central to improving the frequency (up to 1,000-fold or more) of gene editing and site-specific gene insertion by HDR (Puchta et al., 1993, 1996; Choulika et al., 1995; Smith et al., 1995). DSBs in eukaryotic cells can be repaired using two different pathways—nonhomologous end joining (NHEJ) and HDR (Roth and Wilson, 1986; Moore and Haber, 1996; Puchta et al., 1996; Jasin and Rothstein, 2013). The NHEJ pathway is most common in somatic cells and prone to imperfect repair, which results in mutations, such as deletions, insertions, inversions, or translocations. HDR, although less frequent, can be directed by the addition of DNA molecules homologous to the DSB region. By using repair DNA templates that contain sequence variation or promote the insertion of expression cassettes, gene editing, and site-specific gene integration can be achieved.

During the past decade, different nucleases capable of generating targeted DSBs were developed and have become important tools to improve gene editing and site-specific integration in different species, including plants (Puchta and Fauser, 2014; Voytas and Gao, 2014; Kumar and Jain, 2015). Although Zinc-finger nucleases (ZFNs), customized homing endonucleases ( mega-nucleases), and transcription activator-like effector
nucleases (TALENs) advanced genome editing, the recently discovered Cas9-guide RNA (gRNA) system has revolutionized the field (Doudna and Charpentier, 2014; Hsu et al., 2014). This technology emerged from a bacterial adaptive defense system that includes clustered palindromic repeats and Cas endonuclease, which protect the host from plasmid and viral infection (Barrangou et al., 2007). Three CRISPR-Cas systems (types I, II, and III) with different mechanisms have been described in bacteria and archea (Makarova et al., 2012; Jinek et al., 2012). To generate site-specific DSBs, the system minimally requires Cas9 protein and a duplex of CRISPR RNA (crRNA), a transencoded CRISPR RNA (tracrRNA), and the presence of a protospacer-adjacent motif (PAM), which flanks the 3’ end of the crRNA-targeted sequence (Gasiunas et al., 2012; Cong et al., 2013). It has been shown that fusion of the crRNA and tracrRNA into a single gRNA molecule not only simplified the system consisting now of two instead of three components but also, significantly improved the frequency of genomic DNA cleavage (Jinek et al., 2012; Mali et al., 2013). Thus, in contrast to ZFNs, mega-nucleases, and TALENs, which require sophisticated protein engineering to establish specific target site recognition (Smith et al., 2000, 2006; Arnould et al., 2006; Maeder et al., 2008; Boch et al., 2009; Weber et al., 2011), Cas9-gRNA reagents are simple to design. Targeting of genomic sequences by Cas9-gRNA relies on binding of PAM nucleotides (NGG for Streptococcus pyogenes) by Cas9 and base pairing of the gRNA to the adjacent target site sequence.

The Cas9-gRNA system has been shown to function in many different organisms, including plants. To date, it has been used to generate targeted gene modifications in multiple plant species: Arabidopsis (Arabidopsis thaliana), orange (Citrus sinensis), tobacco (Nicotiana tabaccum), rice (Oryza sativa), tomato (Solanum lyco-persicum), sorghum (Sorghum bicolor), wheat (Triticum aestivum; for review, see Kumar and Jain, 2015), maize (Zea mays; Liang et al., 2014), and soybean (Glycine max; Jacobs et al., 2015). The majority of these studies, however, report genome modifications in plants by NHEJ to generate mutations and gene knockouts. Although knockouts are very valuable and allow gene function analysis, their application is limited. HDR brings other opportunities in genome editing but has been more challenging. As a result, reports describing successful gene editing or site-specific trait gene integration through HDR in plants are limited (Cai et al., 2009; Shukla et al., 2009; Townsend et al., 2009; Ainley et al., 2013; D’Halluin et al., 2013; Li et al., 2013; Shan et al., 2013; Zhang et al., 2013).

The data presented in this report show that the Cas9-gRNA system can efficiently facilitate simultaneous multiple gene knockouts, native gene editing, and site-specific gene integration in maize. Analysis of T1 plants established that the gene knockouts, edits, and insertions were heritable and exhibited expected Mendelian segregation. This is the first report, to our knowledge, of Cas9-gRNA technology application in maize showing gene mutagenesis upon direct delivery of gRNA in the form of RNA molecules, native gene editing, and site-specific gene integration.

RESULTS

Targeted Mutations in Maize

To test whether maize-optimized RNA-guided Cas9 endonuclease can generate DSBs and alter maize sequences through error-prone NHEJ repair, 12 different sites at five genomic locations were targeted for cleavage (Fig. 1; Supplemental Table S1). These target sequences were selected first by identifying the NGG PAM sequence required for S. pyogenes Cas9 and then, capturing the 17 to 24 nucleotides immediately upstream of the PAM sequence for use as the spacer in the gRNA. To ensure good U6 polymerase III expression and not introduce a mismatch within the gRNA spacer, all target sequences contained a G at their 5’ end. Two of these locations, one near liguleless1 (LIG1) and another within the 5th exon of the maize fertility gene Ms26, were previously mutated in maize by custom-designed homing endonucleases named LIG3:4 (Gao et al., 2010) and Ems26 (Djukanovic et al., 2013), respectively. To compare the efficiency of generating DSBs by Cas9-gRNA with that of these homing endonucleases, maize immature embryos were bombarded with DNA vectors containing constitutively expressed meganucleases (LIG3:4 and Ems26) or Cas9 components (S. pyogenes Cas9 and gRNA) and developmental genes (maize OVULE DEVELOPMENTAL PROTEIN2 [ODP2] and maize WUSCHEL [WUS]) and then analyzed by amplicon deep sequencing for the presence of mutations at the target sites. Embryos bombarded with either Cas9 or gRNA DNA expression cassettes served as controls. Embryos were harvested 7 d after bombardment, total genomic DNA was extracted, and fragments surrounding the targeted sequences were amplified by PCR. Barcoded PCR amplicons were sequenced to an approximate depth of 400,000 to 800,000 reads, aligned to their reference sequence, and analyzed. It is important to emphasize that, during bombardment, only a small percentage of embryo cells receive vectors with Cas9 and gRNA expression cassettes, whereas the majority of the cells were not transformed and would be expected to contain unmodified DNA sequences. As shown in Table 1, in contrast to controls, Cas9-gRNA-treated embryos yielded a high frequency of mutations. With the exception of one gRNA (MS45-CRISPR RNA-1 [CR-1]), the majority of the gRNAs tested yielded mutation frequencies greater than 1.3%. Notably, embryos transformed with gRNAs that targeted the same sequences as LIG3:4 and Ems26 endonucleases yielded 10- to 20-fold higher mutation frequencies (3.9% versus 0.2% and 1.75% versus 0.13%, respectively). Ten of the most prevalent
mutations generated by either the LIG-CR3 gRNA or the LIG3:4 homing endonuclease are shown in Supplemental Figure S1. In contrast to the predominance of various size deletions present in the sequences derived from the LIG3:4 endonuclease-treated embryos and in agreement with previous reports (Feng et al., 2014), the most common types of mutations promoted by LIG-CR3-guided Cas9 were single-nucleotide insertions and single-nucleotide deletions. Similar results were observed for the majority of gRNAs used in this study (data not shown).

Maize embryos, stably transformed by co-bombardment with Cas9, gRNA, and a selectable and visible marker gene (MoPAT-DsRED), were resistant to treatment with bialaphos because of the presence of the maize codon-optimized phosphinotricin acetyltransferase gene, MoPAT, and displayed red fluorescence. These traits allowed selection and growth of transformed calli, regeneration of fertile plants, and evaluation of inheritance of the Cas9-gRNA-induced mutations described above in subsequent generations. As shown in Table II, mutations were recovered at all targeted loci when multiple gRNA expression cassettes were introduced, either in duplex or triplex, with mutant read frequencies similar to those when gRNAs were delivered individually. These results show that the maize codon-optimized RNA-guided Cas9 can simultaneously introduce mutations at multiple loci in a single-transformation experiment. The frequency of stable event recovery has been reported to be an indicator of cytotoxicity caused by off-target recognition for ZFNs in human cell lines and plants (Szczepik et al., 2007; Comu et al., 2008; Townsend et al., 2009). In this multiplex experiment, the frequency of establishing callus sectors growing on selection was very similar (more than one event per embryo when developmental genes are co-bombarded) to the frequency in control experiments (no gRNA delivery). Normal event recovery suggests that, for the gRNAs used in this experiment, off-target activity was either absent or below a level sufficient to confer detectable cytotoxicity.

Red-fluorescing calli, indicative of a stably integrated DsRED gene, were used to regenerate plants from these multiplex gRNA experiments; 30 T0 plants (25 duplex and 5 triplex) were selected for mutation detection at the target site by copy number determination using quantitative PCR (qPCR) and direct sequencing of the targeted alleles. qPCR analysis revealed that all 30 plants were mutated at the corresponding target sites with a high percentage of plants containing biallelic mutations (LIGCas3, 91%; MS26Cas2, 77%; and MS45Cas2, 100%; Supplemental Table S2). To corroborate the results generated by qPCR, DNA fragments spanning these three target sites were amplified by PCR, cloned, and sequenced from selected plants. Sequencing data were in agreement with the qPCR results, confirming the high frequency of mutations at all three sites (Table III). As expected, T0 plants containing biallelic mutations at either Ms26 or Ms45 were male sterile (Cigan et al., 2001; Djukanovic et al., 2013). Plants were also screened by Cas9 and gRNA-specific PCR (Supplemental Table S3) for the presence of stably integrated copies of Cas9 and gRNA DNA expression cassettes. As shown in Table III, plant 5 did not contain detectable Cas9 and gRNA expression cassettes, suggesting that transient delivery of DNA vectors was sufficient to confer biallelic mutations in this experiment. Because of this observation, this plant was

Figure 1. Five genomic locations, LIG upstream of the LIG1 gene (A), coding regions of male fertility genes MS26 (B) and MS45 (C), and two ALS genes (ALS1 and ALS2; D), targeted for cleavage using Cas9-gRNA and meganuclease systems. Target sites for 12 different gRNAs are indicated by black lines. Underlined three-nucleotide sequences indicate the corresponding PAMs. Also shown are target sites for two meganucleases LIG3:4 and Ems26 (dashed lines in A and B). Two underlined nucleotides in the ALS sequence (D) indicate SNP positions in ALS1 sequence (D) indicate SNP positions in ALS1.
selected for transmission studies. This plant was fertilized using pollen from wild-type high type II (Hi-II) plants to produce T1 progeny. Examination of these T1 plants by qPCR and DNA sequencing showed sexual transmission of parental mutations with expected segregation ratios for all three sites tested (LIGCas3, 16:15 [wild type:+T]; MS26Cas2, 14:12 [wild type/+A: wild type/+G]; and MS45Cas2, 14:13 [wild type/+T: wild type/deletion]). Additional plants, pollinated with wild-type pollen and containing multiple biallelic gene mutations in the first generation, were observed to transmit these mutant alleles to progeny in an expected Mendelian fashion (data not shown). These results show that RNA-guided Cas9 can simultaneously cleave multiple chromosomal loci and sexually transmit these mutations to progeny.

Table I. Percentage of mutant reads at five different target sites 7 d posttransformation

<table>
<thead>
<tr>
<th>Target and DSB Reagents</th>
<th>Total Reads</th>
<th>Mutant Target Gene Reads</th>
<th>Mutant Reads in Target Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>LIG (chromosome 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIG3.4 meganuclease</td>
<td>616,536</td>
<td>1,211</td>
<td>0.20</td>
</tr>
<tr>
<td>LIG-CR1 gRNA + Cas9</td>
<td>716,854</td>
<td>33,050</td>
<td>4.61</td>
</tr>
<tr>
<td>LIG-CR2 gRNA + Cas9</td>
<td>711,047</td>
<td>16,675</td>
<td>2.35</td>
</tr>
<tr>
<td>LIG-CR3 gRNA + Cas9</td>
<td>713,183</td>
<td>27,959</td>
<td>3.92</td>
</tr>
<tr>
<td>MS26 (chromosome 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ems26 meganuclease</td>
<td>512,784</td>
<td>642</td>
<td>0.13</td>
</tr>
<tr>
<td>MS26-CR1 gRNA + Cas9</td>
<td>575,671</td>
<td>10,073</td>
<td>1.75</td>
</tr>
<tr>
<td>MS26-CR2 gRNA + Cas9</td>
<td>543,856</td>
<td>16,930</td>
<td>3.11</td>
</tr>
<tr>
<td>MS26-CR3 gRNA + Cas9</td>
<td>538,141</td>
<td>13,879</td>
<td>2.58</td>
</tr>
<tr>
<td>MS45 (chromosome 9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS45-CR1 gRNA + Cas9</td>
<td>812,644</td>
<td>3,795</td>
<td>0.47</td>
</tr>
<tr>
<td>MS45-CR2 gRNA + Cas9</td>
<td>785,183</td>
<td>14,704</td>
<td>1.87</td>
</tr>
<tr>
<td>MS45-CR3 gRNA + Cas9</td>
<td>728,023</td>
<td>9,203</td>
<td>1.26</td>
</tr>
<tr>
<td>ALS1 (chromosome 4) and ALS2 (chromosome 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALS-CR1 gRNA + Cas9</td>
<td>434,452</td>
<td>9,669</td>
<td>2.23</td>
</tr>
<tr>
<td>ALS-CR2 gRNA + Cas9</td>
<td>472,351</td>
<td>6,352</td>
<td>1.35</td>
</tr>
<tr>
<td>ALS-CR3 gRNA + Cas9</td>
<td>497,786</td>
<td>8,535</td>
<td>1.72</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
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<td></td>
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<tr>
<td>Cas9 only</td>
<td>640,063</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>LIG-CR1 gRNA only</td>
<td>646,774</td>
<td>1</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Transient gRNA Delivery into Cells with Preintegrated Cas9 Generates Mutations in Maize

As described above, codelivery of DNA vectors containing Cas9 and gRNA expression cassettes into maize embryos yielded heritable mutations. However, it has been shown in Arabidopsis (Feng et al., 2014) and wheat (Wang et al., 2014) that progeny plants often do not follow expected Mendelian (1:1) segregation. In this study, segregation distortion was also observed in progeny of two T0 plants (data not shown). One possibility is that stable integration and constitutive expression of gRNAs and Cas9 endonuclease in T0 and T1 plants leads to somatic mutations and consequently, chimeric plants. To overcome the potential problems associated with stable integration of DNA vectors containing Cas9-gRNA components, gRNA in the form

Table II. Read counts and percentage of mutant reads at maize target sites in multiplexed Cas9-gRNA experiments 7 d posttransformation

<table>
<thead>
<tr>
<th>Target Site and Cotransformed gRNAs</th>
<th>Total Reads</th>
<th>Mutant Target Gene Reads</th>
<th>Mutant Reads in Target Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>LIGCas3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIGCas3</td>
<td>645,107</td>
<td>12,631</td>
<td>1.96</td>
</tr>
<tr>
<td>LIGCas3 and MS26Cas2</td>
<td>579,992</td>
<td>10,348</td>
<td>1.78</td>
</tr>
<tr>
<td>LIGCas3, MS26Cas2, and MS45Cas2</td>
<td>648,901</td>
<td>12,094</td>
<td>1.86</td>
</tr>
<tr>
<td>MS26Cas2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS26Cas2</td>
<td>699,154</td>
<td>17,247</td>
<td>2.47</td>
</tr>
<tr>
<td>MS26Cas2, LIGCas3</td>
<td>717,158</td>
<td>10,256</td>
<td>1.43</td>
</tr>
<tr>
<td>MS26Cas2 and MS45Cas2</td>
<td>613,431</td>
<td>9,931</td>
<td>1.62</td>
</tr>
<tr>
<td>MS26Cas2, MS45Cas2, and LIGCas3</td>
<td>471,890</td>
<td>7,311</td>
<td>1.55</td>
</tr>
<tr>
<td>MS45Cas2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS45Cas2</td>
<td>503,423</td>
<td>10,034</td>
<td>1.99</td>
</tr>
<tr>
<td>MS45Cas2 and MS26Cas2</td>
<td>480,178</td>
<td>8,008</td>
<td>1.67</td>
</tr>
<tr>
<td>MS45Cas2, MS26Cas2, and LIGCas3</td>
<td>416,711</td>
<td>7,190</td>
<td>1.73</td>
</tr>
</tbody>
</table>
of in vitro synthesized RNA molecules was cobombarded with the Cas9 DNA vector and analyzed for mutations by amplicon deep sequencing 7 d posttransformation. As shown in Table IV, in contrast to the delivery of both Cas9 and gRNA expressed from DNA vectors, the mutation frequencies observed when LIG-CR3 gRNA delivered as RNA were approximately 100-fold lower (2.6% versus 0.02%, respectively).

One possible explanation for this difference may be that the requirement for coincident function of Cas9 and gRNA was not met when gRNA was delivered as RNA and Cas9 was delivered as a DNA vector. To test this idea, two Agrobacterium tumefaciens vectors (Supplemental Fig. S2) containing maize codon-optimized Cas9 under the transcriptional control of a constitutive (maize Ubiquitin [UBI]) or a temperature-regulated (maize mannitol dehydrogenase gene [MDH]) promoter were introduced into Hi-II embryo cells to establish lines containing preintegrated genomic copies of Cas9 endonuclease. These A. tumefaciens transfer DNAs (T-DNAs) also contained an aleuron-preferred endosperm promoter regulating the expression of a blue fluorescence gene (Anemonia majano Cyan fluorescence gene [AmCYAN]) as a visible marker and an interrupted copy of the DsRED gene (encoding the red fluorescence protein [RFP]) transcriptionally regulated by a maize Histone 2B promoter. Part of the DsRED sequence was duplicated in a direct orientation

Table III. Mutation analysis at maize target sites in multiplexed experiments

| Target Sites and T0 Plant qPCR Results | LIG3/4 TS | | | MS26 TS | | | MS45 TS | | | Stable Integration |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Allele 1 | Allele 2 | Allele 1 | Allele 2 | Allele 1 | Allele 2 | Allele 1 | Allele 2 | Cas9 | gRNA |
| LIGCas3/MS26Cas2 | Null/null | 1-bp Del | 2-bp Del + 1-bp ins | 1-bp Ins (A) | 19-bp Del | — | — | Yes | Yes |
| MS26Cas2/MS45Cas2 | Null/null | 1-bp Ins (T) | 1-bp Del | 1-bp Ins (A) | 1-bp Ins (G) | — | — | Yes | Yes |
| LIGCas3/MS26Cas2/MS45Cas2 | Het/null | — | — | 1-bp Ins (C) | Wild type | 2-bp Del | 65-bp Del | Yes | No |
| 4 | Null/null/null | 1-bp Ins (T) | Large mut* | 1-bp Ins (T) | 1-bp Del | 15-bp Del | Large mut* | Yes | Yes |
| 5 | Het/null/null | 1-bp Ins (T) | Wild type | 1-bp Ins (A) | 1-bp Ins (G) | 1-bp Ins (T) | Large mut* | No | No |

*Because of the size and nature of the mutation (likely the transformation vector insertion), it could not be resolved by the methods used in the analysis.

Table IV. Percentage of mutant reads at maize LIG and MS26 target sites produced in transient gRNA delivery into wild-type embryos and embryos with preintegrated Cas9 under constitutive (UBI) or regulated (MDH) promoters

<table>
<thead>
<tr>
<th>Target Site and Hi-II Embryos</th>
<th>Transformation</th>
<th>Mutant Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 d Posttransformation</td>
<td>7 d Posttransformation</td>
</tr>
<tr>
<td>LIG</td>
<td>Wild type</td>
<td>Cas9 (DNA) + gRNA (DNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cas9 (DNA) + gRNA (RNA)</td>
</tr>
<tr>
<td></td>
<td>UBI:Cas9</td>
<td>gRNA (DNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gRNA (RNA)</td>
</tr>
<tr>
<td></td>
<td>MDH:Cas9</td>
<td>gRNA (DNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gRNA (RNA)</td>
</tr>
<tr>
<td></td>
<td>MDH:Cas9</td>
<td>gRNA (DNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gRNA (RNA)</td>
</tr>
<tr>
<td></td>
<td>MDH:Cas9</td>
<td>gRNA (DNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gRNA (RNA)</td>
</tr>
<tr>
<td></td>
<td>Event 3</td>
<td>gRNA (DNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gRNA (RNA)</td>
</tr>
</tbody>
</table>

*In these experiments, analysis was performed on embryos collected 7 d after transformation, whereas in the experiments with preintegrated Cas9, analysis was done on embryos collected 2 d after transformation. The additional 5 d allows constitutively expressed Cas9 endonuclease to continue DNA cleavage, thus accounting for the difference in the percentage of mutant reads in the DNA delivery experiments.
Editing Maize Acetolactate Synthase2

Sulfonylurea herbicides prevent branched amino acid biosynthesis in plants because of the inhibition of the enzyme acetolactate synthase (ALS; Haughn et al., 1988; Lee et al., 1988; Yu and Powles, 2014). Resistance to one of these herbicides, chlorsulfuron, has been described as a result of single-amino acid changes in the ALS protein at position 197 in Arabidopsis (Pro to Ser; Haughn et al., 1988) and tobacco (Pro to Gln or Ala; Lee et al., 1988) and at a corresponding location, position 178, in soybean (Pro to Ser; Walter et al., 2014).

ALS was chosen for gene editing to show that RNA-guided Cas9 can facilitate specific DNA sequence changes in a native maize gene. There are two ALS genes in maize, ALS1 and ALS2, located on chromosomes 4 and 5, respectively (Burr and Burr, 1991), with 94% sequence identity at the DNA level. In the first experiments, gRNA-expressing DNA vectors targeting three different sites within both ALS1 and ALS2 genes were tested (Fig. 1D; Supplemental Table S1). Because these gRNAs were not gene specific, both ALS1 and ALS2 were mutated with approximately the same efficiency (data not shown) and resulted in low stable event recovery. Because ALS is a critical enzyme for cell function in plants, callus events containing simultaneous biallelic knockouts of ALS1 and ALS2 genes would not be expected to survive. It is likely that the rare events recovered in this experiment did not receive gRNA or Cas9 necessary to generate mutations and therefore, contained one or more functional ALS alleles. Alternatively, alleles may have been mutated but still retained wild-type function. To overcome this problem, an ALS2-specific ALS-CR4 gRNA (Fig. 2A) was designed based on the polymorphisms between ALS1 and ALS2 nucleotide sequences and tested. As shown in Figure 2B, amplicon sequence analysis revealed that ALS2 was highly preferred over ALS1 when ALS-CR4 gRNA was used. One important difference contributing to this result was that ALS1 lacks an NGG PAM sequence required for DNA cleavage by the S. pyogenes Cas9 endonuclease.

To generate ALS2-edited alleles, a 794-bp fragment of homology was cloned into a plasmid vector, and two 127-nucleotide single-stranded DNA oligos were tested as repair templates (Fig. 2C). The 794-bp fragment had the same sequence modifications as Oligo1. Repair templates contained several nucleotide changes compared with the native sequence (Fig. 2C; Supplemental Text S2). Single-stranded Oligo1 and the 794-bp repair templates included a single-nucleotide change that would direct editing of DNA sequences corresponding to the Pro at amino acid position 165 to a Ser (P165S) as well as three additional changes within the ALS-CR4 target site and PAM sequence. Modification of the PAM sequence within the repair template altered the Met codon (AUG) to Ile (AUU), which naturally occurs in the ALS1 gene (Fig. 2, A and C). A second 127-nt single-stranded oligo repair template (Oligo2) was also tested that preserved the Met at position 157 but contained three additional single-nucleotide changes in the sequence, which would influence base pairing with the ALS-CR4 gRNA (Fig. 2C).

Approximately 1,000 immature embryos per treatment were bombarded with the two oligo or single-plasmid repair templates, Cas9, ALS-CR4 gRNA, and MoPAT-DsRED in DNA expression cassettes and placed on media to select for bialaphos resistance conferred by the phosphinotricin acetyl transferase gene (PAT). Five weeks posttransformation, 200 (per treatment) randomly selected independent young callus sectors growing on selective media were separated from the embryos and transferred to fresh bialaphos plates. The remaining embryos (>800 per treatment) with developing callus events were transferred to the plates containing 100 μg L−1 chlorsulfuron as direct selection for an edited ALS2 gene. A month later, 384 randomly picked callus sectors growing on bialaphos (approximately 130 events for each repair template) and 7 callus sectors that continued growing on media
with chlorsulfuron were analyzed by PCR amplification and sequencing. Edited ALS2 alleles were detected in nine callus sectors: two derived from the callus sectors growing on bialaphos and generated using the 794-bp repair DNA template and the remaining seven derived from chlorsulfuron-resistant callus sectors edited using the 127-nt single-stranded oligos (three by Oligo1 and four by Oligo2; Fig. 2D; Supplemental Table S4). The second ALS2 allele in these callus sectors was mutated as a result of NHEJ repair (data not shown).

Analysis of the ALS1 gene revealed only wild-type sequence, confirming high specificity of the ALS-CR4 gRNA.

Plants were regenerated from seven of nine callus sectors containing edited ALS2 alleles for additional molecular analysis and progeny testing. DNA sequence analysis of ALS2 alleles confirmed the presence of the P165S modifications as well as the other nucleotide changes associated with the respective repair templates. T1 and T2 progeny of two T0 plants generated from different callus events (794-bp repair DNA and Oligo2) were analyzed to evaluate the inheritance of the edited ALS2 alleles; progeny plants derived from crosses using pollen from wild-type HI-II plants were analyzed by sequencing and showed sexual transmission of the edited alleles observed in the parent plant with expected 1:1 segregation ratio (57:56 and 47:49, respectively). To test whether the edited ALS sequence confers herbicide resistance, selected 4-week-old segregating T1 plants with edited, and wild-type ALS2 alleles were sprayed with four different concentrations of chlorsulfuron (50, 100 [1\footnotesize{\textup{3}}], 200, and 400 mg L^{-1}). Three weeks after treatment, plants with an edited allele showed normal phenotype, whereas plants with only wild-type alleles showed strong signs of senescence (Fig. 3A). In addition, embryos isolated from seed that had been pollinated with wild-type HI-II pollen were germinated on media with 100 mg L^{-1} chlorsulfuron. Fourteen days after germination, plants with edited alleles showed normal height and a well-developed root system, whereas plants with wild-type alleles were short and did not develop roots (Fig. 3B).

These experiments show that Cas9-gRNA can stimulate HDR-dependent targeted sequence modifications in maize, resulting in plants with an edited endogenous gene that properly transmits to subsequent generations.

HDR-Mediated Gene Insertion

In contrast to Agrobacterium- or biolistic-mediated delivery of trait genes, which can often result in random insertions and potential disruption of native gene function, DSBs using site-directed nucleases would allow for precise gene integration at a desired genomic location. In this experiment, given the differences in mutation frequencies observed between RNA-guided Cas9 and meganuclease described above, the ability of these two DSB technologies to promote site-specific gene insertion was compared in maize. Because LIG-CR3-guided Cas9 cleaves genomic DNA at the same position as LIG3:4 meganuclease, these DSB reagents were selected for this study. The DNA donor repair template contained the constitutively expressed PAT gene (UBI:MoPAT) flanked by approximately 1.0 kb of DNA fragments homologous to genomic sequences immediately adjacent to the LIG cleavage site (Supplemental Text S3; Supplemental Fig. S4). This
donor vector was cobombarded with Cas9 and LIG-CR3 gRNA expression cassettes or LIG3:4 homing endonuclease vector. In a separate experiment, Cas9, gRNA, and a modified donor DNA template containing UBI-regulated MoPAT-DsRED fusion flanked with the same regions of homology were delivered on the same vector (Supplemental Fig. S4). To compare different delivery systems, this vector was moved into A. tumefaciens strain LBA4044 and used in an Agrobacterium-mediated transformation experiment.

Because delivery of the donor DNA would result in both random and targeted insertions, approximately 2-month-old callus sectors growing on bialaphos-containing media were screened for mutations and gene insertions at the LIG target site. In contrast to sectors derived from biolistic-transformed LIG3:4 meganuclease, qPCR copy number analysis revealed that Cas9-gRNA yielded a higher target site mutation frequency (>83% versus 6% mutant reads; Table V). Notably, for the Cas9-gRNA system, the mutation frequency was independent of the delivery method (bombardment or Agrobacterium-mediated transformation). Callus events that contained site-specific gene insertions were identified by junction PCR using primer pairs shown in Figure 4A. Amplification products from positive integration events were verified by subcloning and sequencing. As shown in Table V, 2 of the 288 callus events in the meganuclease experiment yielded amplicons across both junctions (0.7% HDR frequency), whereas biolistic delivery of Cas9-gRNA and donor DNA resulted in frequencies of 2.5% and 4% when used either separately or as a single vector, respectively. Screening of the 192 callus events from Agrobacterium-mediated delivery did not produce amplicons indicative of HDR-mediated gene insertions (Table V), suggesting that biolistic transformation is the preferred delivery method to generate gene insertions at the LIG site in maize.

T0 plants regenerated from seven independent HDR-positive events generated in the experiment when donor DNA, Cas9, and gRNA were delivered separately were used for additional molecular characterization. DNA-blot hybridization analysis of regenerated plants was conducted using genomic- and donor DNA-specific probes shown in Figure 4A. These analyses in addition to sequencing across the entire inserted fragment (Supplemental Text S3) showed that plants regenerated from two events (events 1 and 2) contained a single copy of an intact MoPAT gene indicative of homology-directed gene insertion at the LIG target site (Fig. 4). Plants regenerated from the remaining five events contained extra, rearranged, and randomly integrated copies of MoPAT. It should be noted that, upon further molecular examination of events 1 and 2

**Table V. Summary of gene integration at LIG target site**

<table>
<thead>
<tr>
<th>System/Experiment</th>
<th>Events Analyzed</th>
<th>Mutation Frequency LIG Target Site</th>
<th>Two Junction PCR-Positive Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIG3:4 meganuclease + donor DNA, separate vectors, and particle bombardment</td>
<td>288</td>
<td>6</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>LIG-CR3 gRNA + Cas9 + donor DNA, three separate vectors, and particle bombardment</td>
<td>480</td>
<td>83</td>
<td>11 (2.5)</td>
</tr>
<tr>
<td>LIG-CR3 gRNA + Cas9 + donor DNA, all in a single vector, and particle bombardment</td>
<td>336</td>
<td>86</td>
<td>14 (4.1)</td>
</tr>
<tr>
<td>LIG-CR3 gRNA + Cas9 + donor DNA and A. tumefaciens-mediated delivery</td>
<td>192</td>
<td>84</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 3.** T1 maize plants with edited ALS2 allele (left) and the wild type (right) tested for resistance to chlorsulfuron. A, Four-week-old plants sprayed with chlorsulfuron (100 mg L\(^{-1}\)) 3 weeks after spraying. B, T1 generation embryos germinated on media with chlorsulfuron (100 μg L\(^{-1}\)) 14 d after germination.
by PCR analysis, the Cas9 and gRNA DNA vectors were not detected in plants, suggesting that transient expression of these components was sufficient to stimulate gene integration.

To examine transmission and segregation of MoPAT, T0 plants from events 1 and 2 were fertilized with wild-type pollen to produce T1 seeds; 96 T1 progeny plants from event 1 and 64 plants from event 2 were analyzed by junction PCR and gel electrophoresis and showed expected Mendelian segregation of the gene integrated at the LIG target site (48:48 and 31:33, respectively). DNA hybridization analysis performed on 20 random junction PCR-positive T1 plants from both events using DNA probes described above confirmed nuclear stability and integrity of MoPAT in progeny plants (data not shown).

Together, these genetic and molecular data show that maize-optimized RNA-guided Cas9 can be used to stimulate HDR for the insertion of genes at specific locations in maize chromosomes. Moreover, in these experiments, the frequency of site-specific trait integration depended upon the endonuclease target site cleavage efficiency and repair template delivery method.

**DISCUSSION**

During the past decade, several technologies, ZFNs, meganucleases, TALENs, and recently, Cas9-gRNA systems, were developed to generate targeted DSBs that can be used for genome editing by NHEJ or HDR. DSB technologies have three major applications in plant biotechnology: gene mutagenesis resulting in gene knockouts, gene editing allowing modification of a gene product or metabolic pathway in a desired way, and site-specific trait insertion, which would allow gene stacking in plant breeding programs. Here, we reported successful application of the Cas9-gRNA technology to generate multiple gene knockouts, ALS2 gene editing resulting in plants resistant to chlorsulfuron, and site-specific gene integration in an important crop species: maize.

**gRNA Evaluation by Amplicon Deep Sequencing**

Maize transformation is a time-consuming process, often taking 3 to 4 months from DNA delivery into immature embryo cells to plant regeneration. Thus,
testing the DSB reagents is an important step in developing proper tools for genome editing experiments. The rapid method based on amplicon deep sequencing described here allows for evaluation of mutation frequencies at any chromosomal site targeted by different gRNAs within 2 weeks after transformation. Analysis of multiple gRNAs (including ones not presented in this report) indicated that >90% of the gRNAs tested led to a high frequency of mutations at the corresponding target sites. Additional studies will be needed to determine if the inability of certain gRNAs that did not promote high frequencies of mutations in planta can be attributed to such factors as target site accessibility, secondary structure of gRNA molecules, and/or high frequency of flawless DSB repair at some target sites.

NHEJ-Mediated Gene Mutagenesis

Selected gRNAs with high mutation frequencies were used in multiplexed (duplexed and triplexed) targeted mutagenesis experiments. All genes were targeted with 100% success rate, and more than 80% of the regenerated T0 plants contained biallelic mutations. These results, along with previous publications, illustrate that the Cas9-gRNA technology can be used to efficiently promote the alteration of individual or multiple gene sequences (for review, see Kumar and Jain, 2015), gene families, or homologous genes in polyploid species (Wang et al., 2014) and delete chromosomal segments of various sizes (Brooks et al., 2014; Zhou et al., 2014; Gao et al., 2015). In this report, analysis of T0 plants and T1 segregating populations indicated that stably integrated Cas9 and gRNA expression cassettes remain active, often resulting in chimeric T0 plants and segregation distortions. Similar observations have been previously reported in several plant species (Brooks et al., 2014; Feng et al., 2014; Jiang et al., 2014; Wang et al., 2014; Zhang et al., 2014). At the same time, T0 plants regenerated from callus events without integrated Cas9-gRNA components were not chimeric and exhibited proper sexual transmission of the mutated alleles to the next generation. These results suggest that transient delivery of Cas9 and gRNA is sufficient to mutagenize multiple alleles and can speed the discovery and breeding process. In mammalian systems, both Cas9 and gRNA can be delivered transiently in the form of RNA-protein complexes providing high mutation frequencies (Kim et al., 2014; Lin et al., 2014; Zuris et al., 2015). However, this approach has limited application among plant species because of the currently available transformation methods. Therefore, one method to limit the Cas9-gRNA system to transient activity is to deliver gRNA in the form of RNA molecules rather than DNA expression cassettes. In this report, simultaneous delivery of gRNA in the form of RNA and a DNA vector containing a Cas9 expression cassette yielded about 100-fold lower frequencies of mutations compared with the control, in which both components were delivered as DNA vectors. Because Cas9-gRNA is a two-component system, both gRNA and Cas9 protein must be present in a cell at the same time. In the experiment in which gRNA and a Cas9 gene were delivered simultaneously, this condition likely was not met because of the predicted rapid turnover of RNA molecules and the delayed and therefore, noncoincident expression of functional Cas9 protein encoded on a DNA vector. To overcome this challenge, maize lines containing constitutively or conditionally expressed preintegrated Cas9 were generated. Amplicon sequence analysis performed on embryos 2 d after transformation showed that transient gRNA delivery into the maize embryo cells with preintegrated Cas9 resulted in mutation frequencies comparable with delivery of Cas9 and gRNA as DNA vectors. It is likely that the frequency of mutations in T0 plants would be reduced compared with the experiments when DNA vectors were used to deliver gRNAs, because expression cassettes often stably integrate into the genome and as the result, the Cas9-gRNA system continues to be active. Nevertheless, delivery of gRNA as RNA may not limit successful gene editing and site-specific gene integration, because gene insertion events were recovered without stable integration of either Cas9 or gRNA expression vectors. Therefore, gRNA delivery in the form of RNA can be a viable alternative to gRNA delivery as DNA vectors for plant gene and genome editing.

HDR-Mediated Gene Editing and Targeted Gene Insertion

Although gene mutagenesis can be the result of the error-prone process of DNA DSB repair through NHEJ, gene editing and targeted gene integration rely on the HDR repair pathway. Cas9-gRNA has been well documented in different plant species for targeted gene mutagenesis, both single and multiplexed (for review, see Kumar and Jain, 2015); however, examples of HDR-mediated gene editing and site-specific gene insertion have been limited to model systems (Li et al., 2013; Shan et al., 2013). Until now, delivery of the gene of interest and its targeted insertion by HDR in plants remains a serious challenge (Feng et al., 2013; Voytas and Gao, 2014).

Genes and genomes in many important crop species are often present in multiple copies, because rearrangements, duplications, and/or polyploidization. In some instances, all copies of a gene may need to be mutagenized to result in complete inactivation. However, in some cases, only one copy of a gene family would require editing to produce a desired phenotype. In this study, the ALS gene was chosen to test the ability of the Cas9-gRNA system to facilitate endogenous gene editing by HDR in maize immature embryo cells transformed by particle bombardment. ALS is a well-characterized gene family consisting of two genes, ALS1 and ALS2, which can be edited to confer plant resistance to sulfonylurea class herbicides. Several gRNAs targeting both genes simultaneously or ALS2
uniquely were tested. Delivery of a gRNA targeting both ALS1 and ALS2 leads to low recovery of stable events, likely because of the high frequency of biallelic knockout mutations and the important nature of the gene. However, when an ALS2-specific gRNA was tested, typical frequencies of event recovery were observed. To edit ALS2, three different repair templates (double-stranded vector DNA and two single-stranded oligos) were tested. All three experimental designs resulted in editing of ALS2, indicating that small single-stranded DNA oligonucleotides were sufficient for gene editing experiments in maize. Although all regenerated plants contained integrated and presumably, functional copies of Cas9 and gRNA expression cassettes, only wild-type alleles of ALS1 were observed, suggesting high targeting specificity by the ALS2-CR4 gRNA. This experiment showed that, by carefully choosing target sites and corresponding gRNAs, multiple or individual genes within a family may be targeted if sufficient nucleotide polymorphisms are present between the family members.

Targeted gene insertion allows clustering of multiple genes in a genomic location, which would allow for segregation as a single locus, significantly simplifying the breeding process. Previously, gene insertion at a specific target site was shown using ZFNs in tobacco (Cai et al., 2009) and maize (Shukla et al., 2009; Ainley et al., 2013) and meganucleases in cotton (Gossypium hirsutum; D’Halluin et al., 2013). The Cas9-gRNA system was tested for its ability to facilitate targeted gene insertion in maize immature embryo cells compared with a meganuclease targeting the same genomic location. Two different delivery systems, biolistic and A. tumefaciens-mediated transformation, were tested. Only particle bombardment experiments resulted in events with a gene inserted into the target site through an HDR process. Delivery of the donor DNA on the same plasmid with Cas9-gRNA components yielded approximately 2 times higher frequency of integration events compared with experiments in which components were delivered as separate vectors. In this experiment, Cas9-gRNA yielded approximately 5 times more integration events than LIG3:4 meganuclease. This observed difference was likely caused by the higher activity of Cas9-LIG-CR-3 gRNA as determined by the mutation frequencies measured at the target site. The inability to recover integration events in the A. tumefaciens-mediated experiment may be explained by the low number of copies of the T-DNA delivered into the plant cell compared with particle bombardment, which delivers multiple copies of all DNA molecules. However, this does not necessarily mean that Agrobacterium transformation cannot result in HDR-mediated gene integration but likely, indicates lower frequency of such events (S. Svitashhev, unpublished data). Therefore, selection of a delivery method is very important and should be considered based on the goal of individual experiments.

The efficiency of gene editing and targeted gene insertions has typically been associated with the activity of a nuclease and therefore, frequency of mutations at a given target site. Although the ability of a nuclease to generate DSB is the most important condition, this is not the only requirement and can sometimes be misleading in predicting frequency of HDR at the target site. Factors, such as efficiency of DSB repair, target site accessibility, presence of repetitive sequences close to the target site, and yet to be discovered factors, may significantly affect frequencies of gene editing and targeted gene insertions. Our unpublished data (S. Svitashev, H. Gao, and A.M. Cigan) indicate that, although similar mutation rates can be observed at different target sites, gene editing and gene insertion frequencies can vary from less than 1% to as high as 15%.

CONCLUSION

Only 3 years after the first publications introduced the Cas-gRNA system, it has become the DSB technology of choice for many laboratories working in the field of genome editing. The advantages of the system lay in not only its simplicity and activity but also, its versatility to more fully address problems associated with DSB technology applications in plant biology. These challenges include the inability to generate DSBs at a specific genomic location and lack of complete specificity that can result in off-target DNA cleavage. In the not too distant future, increasing the density of target sites may be achieved by the discovery and utilization of new Cas9 proteins with different PAM recognition sequences. PAM diversity would be particularly useful in plant species with high AT nucleotide content, whereas longer PAM sequences may increase the proportion of unique and highly specific target sites. Testing of S. pyogenes Cas9-gRNA in a variety of plant species has resulted in extremely low frequency of nonspecific DNA cleavage and therefore, high specificity of the system (Feng et al., 2013; Shan et al., 2013; Brooks et al., 2014; Zhang et al., 2014). Although targeting various sites may yield different results, incorporation of the Cas9 D10A nickase (Gasiunas et al., 2012) may further improve gene editing options for Cas9, because the nickase version has been successfully applied to improve specificity (Mali et al., 2013; Cho et al., 2014; Fauser et al., 2014). Furthermore, transient delivery of purified Cas9 protein and gRNA (as a ribonucleoprotein complex) into human culture cells has shown high frequencies of targeted mutagenesis and gene editing while reducing off-target mutations (Kim et al., 2014; Lin et al., 2014). These combined approaches may further improve the precision and accuracy of genome modification in plants. Thus, targeted mutagenesis or editing of specific genes or gene families to yield unique allelic variants will no longer be a limiting step for the discovery of gene function and establishment of their relationship to complex biological pathways. Adoption of robust genome modification methods to introduce genetic variation aided by inexpensive sequencing and novel plant transformation methods.
will accelerate the continued development of new breeding techniques to address challenges of modern agriculture.

**MATERIALS AND METHODS**

**Plasmids and Reagents Used for Plant Transformation**

Standard DNA techniques as described in Sambrook et al., 1989 were used for vector construction. The Cas9 sequence from Streptococcus pyogenes (SF370) was maize (Zea mays) codon optimized and designed to contain the potato (Solanum tuberosum) ST-LS1 intron (NCBI GenBank accession no. X04753; nucleotides 2,837–2,892 at nucleotide positions +402–591). To facilitate nuclear localization of the Cas9 protein in maize cells, the SV40 (MAPKKKRV) and the Agrobacterium tumefaciens VirD2 (KRPDRDHDELGKKRKR) nuclear localization signals were added at the amino and carboxyl termini of the Cas9 open reading frame, respectively (Supplemental Text S4). The chemically synthesized Cas9 gene contained on a 4,335-kb Ncol-HpaI DNA fragment was subcloned into a Ubiquitin promoter-containing vector (Sabelli et al., 2009) and digested with Ncol-HpaI, which links Cas9 to a maize constitutive Ubiquitin1 promoter including a 1.5-kb intron (chr8:–899,710,992; Christensen et al., 1992), whereas transcription is terminated by the addition of the 3' sequences from the potato proteasome inhibitor II gene (nucleotides 2–310; An et al., 1989) to generate UBI:Cas9 vector. Single gRNAs were designed using the methods described by Mali et al. (2013). A maize U6 polymerase III promoter and terminator residing on chromosome 8 (nucleotides 165,535,024–165,536,023 Maize [B73] Public Genome Assembly [ACP_v3.8]) were isolated and used to direct initiation and termination of gRNAs, respectively. Two BbsI restriction endonuclease sites were introduced in an inverted tandem orientation with cleavage oriented in an outward direction as described in Cong et al., 2013 to facilitate the rapid introduction of maize genomic DNA target sequences into the gRNA expression constructs (Supplemental Text S5). Only target sequences starting with a G nucleotide were used to promote favorable polymerase III expression of the gRNA. The gRNA expression cassettes contained on a 1.1-kb XmnI EcoRI DNA fragment were subcloned into Bluescript SK vectors and used for bombardment and subsequent vector construction.

Two A. tumefaciens transformation vectors were designed and introduced into maize to enable stably integrated Cas9 expression cassettes. To generate constitutively expressed Cas9, the UBI:Cas9 vector was digested with HindIII-EcoRI to release a 6.6-kb HindIII-EcoRI fragment (UBI:Cas9), and this fragment was subcloned into HindIII-EcoRI-digested plasmid pSB11 (Ishida et al., 1996). To generate the UBI:Cas9 T-DNA vector, the pSB11:UBI:Cas9 vector was digested with NolI, and a 5.8-kb NolI fragment containing the blue fluorescence gene (AmCYAN1; Clontech Laboratories Inc.) regulated by the maize endosperm promoter (chromosome 8; nucleotides 171,120,325–171,119,383 Maize [B73]) Public Genome Assembly [ACP_v3.8]; a visible marker for the identification of transformed embryos) and a red fluorescence gene (pDsRed Express; product no. 632412; Clontech Laboratories Inc.) regulated by the maize Histone 2B promoter (chromosome 3; nucleotides 16,509,805–16,571,259 Maize [B73]) Public Genome Assembly [ACP_v3.8]) was subcloned downstream of UBI:Cas9. A portion of DsRed gene in this vector was duplicated (nucleotides 148–516) in a direct orientation with 369 bp of overlap. The duplicated fragments were separated by a 347-bp spacer and contained sequences compatible for recognition and targeting by gRNAs and the LIG3:G4 meganuclease (Supplemental Fig. S2). To generate a conditionally expressed Cas9 (MDH: Cas9), a 1,048-bp fragment of temperature-regulated promoter from the maize MDH (chromosome 2; nucleotides 8,783,228–8,784,275 Maize [B73]) Public Genome Assembly [ACP_v3.2]) was modified to contain an Rca I restriction site at the 3' end of the sequence (Cigan and Unger-Wallace, 2013) to accommodate transpose fusion to the maize-optimized Cas9 to generate MDH:Cas9 expression cassette. The 6.6-kb HindIII-EcoRI DNA fragment in UBI:Cas9 T-DNA vector was replaced with the MDH:Cas9 DNA contained on a 5.7-kb HindIII-EcoRI DNA fragment to generate the MDH:Cas9 T-DNA vector. These plasmids were introduced into A. tumefaciens strain LBA4404 (Komari et al., 1996) by electroporation using a Gene Pulser II (Bio-Rad) as described by Gao et al. (2010).

To generate gRNA in the form of RNA molecules, the maize-optimized U6 polymerase III gRNA expression cassettes were amplified by PCR using a 5' oligonucleotide primer that also contained the sequence of the T7 polymerase promoter and transcriptional initiation signal just 5' of the spacer to gene. T7 in vitro transcription was carried out with the AmpliScribe T7-Flash Kit (Epicentre) according to the manufacturer's recommendations, and products were purified using NucAway Spin Columns (Invitrogen; Life Technologies Inc.) followed by ethanol precipitation.

The LIG3:4 and the Ems26 endonucleases vectors were previously described (Gao et al., 2010 and Djukanovic et al., 2013, respectively). Plasmids containing cell division-promoting polyptides ZmOD2P and ZmWus and selectable and visible marker MoPAT-DsRED (a translational fusion of the bialophos resistance gene, phosphaethrin-N-acetyl-transferase, and the red fluorescent protein DsRed) were previously described in Ananiev et al., 2009.

For ALS2 gene editing, single-stranded 127-nt-long oligos (sense and antisense strands) homologous to the fragment spanning the ALSCas4 target site with four and seven single-nucleotide changes (Fig. 2C) were synthesized (Integrated DNA Technologies). Single-nucleotide polymorphisms (SNPs) within gRNA target site and PAM sequence were introduced to prevent the donor DNA from cleavage by Cas9 protein and SNPs: c. T to G, T to and G to C replacements downstream from the cleavage site to change Pro to Ser at position 165 in the ALS2 gene. These oligos (sense and antisense strands) were annealed to generate double-stranded DNA, digested with BsuDI, ligated to PCR-amplified genomic DNA fragments upstream and downstream from the oligo sequence with primers 5'-ACAGCCGGCCACCATGCAGCACTGG-3' (forward) and 5'-ACCAATGCCCAATCCGAACGTA-3' (reverse) and 5'-ATAAGAGTGT-TGTGACCCGGGA-3' (forward) and 5'-CTGCCTACAATCCTGCTGGC-3' (reverse), respectively, also digested with BsuDI to extend the total homology region to 794 bp, and cloned into the pUC9 vector.

Vectors for HDR-mediated targeted integration were generated by flanking a UBI:MoPAT or UBI:MoPAT-DsRED fusion with 1,099- and 1,035-bp maize genomic DNA fragments homologous to the sequences immediately upstream and downstream from the cleavage site of the LjCas3 targets and named Homology Region. HDRU and HDR2, respectively (Fig. 4A). Vector containing the MoPAT-DsRED fusion was retrofitted with either UBI:Cas9 and U6gRNA or LIG3:4 meganuclease expression cassettes (Supplemental Fig. S4). For A. tumefaciens-mediated transformation, vector containing the expression cassettes shown in Supplemental Figure S4B was moved into the A. tumefaciens strain LBA4404 by electroporation using a Gene Pulser II (Bio-Rad).

**Plant Material**

Publicly available maize hybrid Hi-II line (Armstrong and Green, 1985) was obtained from internal Pioneer sources.

To generate plants with either constitutive or regulated expression of Cas9 protein, constructs described above and shown in Supplemental Figure S2 were used to transform Hi-II maize embryos using A. tumefaciens-mediated transformation. Regenerated T0 plants were screened by qPCR and DNA-blot hybridization analysis for single-copy T-DNA insertions. Independent UBI:Cas9 (4) and MDH:Cas9 (11) T-DNA-containing plants were identified; T0 plants were fertilized with wild-type Hi-II pollen, and blue-fluorescing embryos were used for further biolistic transformation experiments.

**Maize Transformation**

Biolistic-mediated transformation of maize immature embryos was performed as described in Ananiev et al., 2009) with slight modification. Briefly, the DNA was precipitated onto 0.6-μm (average diameter) gold microprojectiles using a water-soluble cationic lipid TransIT-2020 (Mirus) as follows: 50 μL of gold particles (water solution of 10 mg mL−1) and 1 μL of TransIT-2020 water solution were added to the premixed DNA constructs (DNA and RNA amounts used in each experiment are shown in Supplemental Table S5), mixed gently, and incubated on ice for 10 min. DNA-coated gold particles were then centrifuged at 10,000 rpm for 1 min. The pellet was rinsed with 100 μL of absolute alcohol and resuspended by a brief sonication. Immediately after sonication, DNA-coated gold particles were loaded onto the center of a macrocarrier (10 μL of each) and allowed to air dry. Immature maize embryos 8 to 10 d after pollination were bombarded using a PDS-1000/He Gun (Bio-Rad) with a rupture pressure of 425 pounds per square inch. Postbombardment culture, selection, and plant regeneration were performed as previously described (Gordon-Kamm et al., 2002). A. tumefaciens-mediated transformation followed the detailed procedures previously described in Gao et al., 2010. Regenerated plants were moved to soil, where they were sampled and grown to maturity in greenhouse conditions.

**PCR, qPCR Analysis, and Amplicon Sequencing**

For PCR analysis, DNA was extracted from a small amount (0.5 cm in diameter) of callus tissue or two leaf punches as described in Gao et al., 2010. PCR
was performed using REDExtract-N-Amp PCR ReadyMix (Sigma) or Phusion High Fidelity PCR Master Mix (NEB) according to the manufacturer’s recommendations. PCR-amplified fragments were cloned using the pCR2.1-TOPO cloning vector (Life Technologies Inc.). The primers used in PCR and qPCR reactions are shown in Supplemental Table S3. qPCR analysis was performed as in Wu et al., 2014. Because mutations within a population of individual callus sectors may differ from single (single-base pair insertions or deletions) to complex (deletions and additions) sequence modifications, target site copy number, as determined by qPCR analysis, was used to as a semiqualitative method to determine mutation frequencies. In general, mutation frequency was measured based on target site allele copy number: both alleles are defined to be intact if target site copy number is two, and the event would be called the wild type. Events for which qPCR revealed target site copy number reduced to one would be scored as one mutated allele and one wild-type allele. Finally, if both alleles of the target site are modified, the copy number is zero, and the event would be called null.

For deep sequencing analysis, 60 to 90 Hi-II maize immature embryos were co-hybridized with the maize-optimized Cas9 endonuclease and gRNA expression cassettes or I-Crel-modified endonuclease vectors and screenable marker UBL/MoPAT-DsRED and cell division-promoting genes, UBL/ZmODF2 and IN2/ZmWUS2. Seven days after bombardment, 20 to 30 of the most uniformly transformed embryos (based on transient expression of DsRED) from each treatment were pooled, and total genomic DNA was extracted. The DNA region surrounding the intended target site was amplified by PCR using Phusion High Fidelity PCR Master Mix (NEB), adding the ampiclon-specific barcodes and Illumina sequencing through two rounds of PCR. The primers used in the primary PCR reaction are shown in Supplemental Table S3, and the primers used in the secondary PCR reaction were 5'-AACATACTTGCCAGACACCGCCG-3' (forward) and 5'-CAAGAGACGGACCCGCTGAAGC-3' (reverse). The resulting PCR amplifications were purified with a Qiagen PCR Purification Spin Column (Qiagen), concentration was measured with a Hoechst dye-based fluorometric assay combined in an equinolar ratio, and single-read 100-nucleotide-length amplicon deep sequencing was performed on Illumina’s MiSeq Personal Sequencer with a 30% to 40% (v/v) spike of PhiX control v3 (FC-110-3001; Illumina) to offset sequence bias. Only those reads with a ≥1 nuclease INDEL arising within the 10-nucleotide window centered over the expected site of cleavage and not found in the negative controls were classified as NHEJ mutations. NHEJ mutant reads with the same mutation were counted and collapsed into a single read, and the top 10 most prevalent mutations were visually confirmed as arising within the expected site of cleavage. The total numbers of visually confirmed mutations were then used to calculate the percentage of mutant reads based on the total number of reads of an appropriate length containing a perfect match to the barcode and forward primer.

DNA Isolation and Blot Hybridization Analysis

For DNA isolation and blot hybridization analyses, maize leaf tissue (approximately 2.5 g of fresh weight) from transformed and control plants was freeze dried and ground. Total genomic DNA was extracted as described in Cigan et al., 2001. Five micrograms of DNA from each sample was digested overnight with HindIII, and the DNA fragments were separated on a 0.8% (w/v) agarose gel run at 23 V overnight. DNA was transferred to a nylon membrane (Roche) using standard protocol (Sambrook et al., 1989) and the barcode and forward primer.

was done by placing membranes in 1 X Detection Buffer (DIG Wash and Block Buffer Set) for 5 min, removing to a clean tray, pipetting CDP-STAR Ready to Use (Roche) onto the membranes, and then, exposing to x-ray film (Kodak).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Ten of the most prevalent mutations generated by either the Cas9-gRNA system or the LIG34 homing endonuclease identified by deep sequencing of the PCR amplicons across the corresponding target site.

Supplemental Figure S2. Agro vector for stable integration of the ZmUBI: Cas9 into the maize genome.

Supplemental Figure S3. Repair of the RF-FP gene by intramolecular homologous recombination.

Supplemental Figure S4. Constructs used for HDR-mediated targeted gene insertion at the LIG1 region using Cas9-gRNA system and LIG34 meganuclease.

Supplemental Table S1. Maize genomic sites targeted by Cas9-gRNA system.

Supplemental Table S2. To plant analysis by qPCR for mutations at maize target loci of 30 T0 plants produced in the multiplexed Cas9-gRNA experiments.

Supplemental Table S3. List of primers used in the study.

Supplemental Table S4. Edited ALS2 events recovered using different templates and selection.

Supplemental Table S5. DNA and RNA amounts used in bombardment experiments (single shot).

Supplemental Text S1. Nucleotide sequence and description of the RF-FP repair cassette.

Supplemental Text S2. DNA sequences of ALS repair templates.

Supplemental Text S3. Nucleotide sequence and description of the donor DNA in the integration experiment.


Supplemental Text S5. Nucleotide sequence of LIG-CR3 gRNA expression cassette.

ACKNOWLEDGMENTS

We thank Brian Lenderts, Meizhu Yang, Shan Zhao, Wally Marsh, Susan Wagner, and Min Zeng for technical and maize transformation support; DuPont Pioneer Controlled Environment Group for managing plants; DuPont Pioneer Genomics Group for DNA sequencing; Brooke Peterson-Burch for bioinformatics support; and Wayne Crismani, Jeff Sander, Doane Chilcoat, Philippe Horvath, and the anonymous Plant Physiology reviewers for thoughtful comments and suggestions.

Received May 28, 2015; accepted August 7, 2015; published August 12, 2015.

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