Targeted Mutagenesis of Duplicated Genes in Soybean with Zinc-Finger Nucleases

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We performed targeted mutagenesis of a transgene and nine endogenous soybean (*Glycine max*) genes using zinc-finger nucleases (ZFNs). A suite of ZFNs were engineered by the recently described context-dependent assembly platform—a rapid, open-source method for generating zinc-finger arrays. Specific ZFNs targeting DICER-LIKE (DCL) genes and other genes involved in RNA silencing were cloned into a vector under an estrogen-inducible promoter. A hairy-root transformation system was employed to investigate the efficiency of ZFN mutagenesis at each target locus. Transgenic roots exhibited somatic mutations localized at the ZFN target sites for seven out of nine targeted genes. We next introduced a ZFN into soybean via whole-plant transformation and generated independent mutations in the paralogous genes DCL4a and DCL4b. The dcl4b mutation showed efficient heritable transmission of the ZFN-induced mutation in the subsequent generation. These findings indicate that ZFN-based mutagenesis provides an efficient method for making mutations in duplicate genes that are otherwise difficult to study due to redundancy. We also developed a publicly accessible Web-based tool to identify sites suitable for engineering context-dependent assembly ZFNs in the soybean genome.

Soybean (*Glycine max*) is an ancient polyploid and major agricultural legume crop providing nutritional protein and oil that can be processed into a variety of feed and food products. Several genetic bottlenecks throughout its domestication and more recent intensive selection and breeding practices have greatly reduced the genetic variability of soybean germplasm (Hyten et al., 2006). Current efforts to expand genetic tools for breeding and gene discovery include random mutagenesis and RNAi-based approaches. Several published and ongoing studies have utilized chemical mutagens including ethyl methanosulfonate for TILLING (Cooper et al., 2008), radiation mutagens such as fast neutrons (Men et al., 2002), and transposable elements (Mathieu et al., 2009). However, random mutagenesis approaches in a highly duplicated genome such as soybean often result in many lines with no phenotype due to complementation by redundant genes. This can sometimes be circumvented by remutating single-homeolog mutant lines to obtain the required bona fide double-homeolog mutants. Another approach is to identify and combine mutations by genetic crossing (Pham et al., 2010) but this can be time consuming. RNAi-based approaches such as post-transcriptional gene silencing either by hairpin or virus-induced gene silencing vectors suffer from the opposite problem, namely that it is difficult to silence individual gene copies, and rather entire gene families are often silenced (Kachroo et al., 2008; Meyer et al., 2009).

An ideal mutagenesis approach for a highly duplicated genome like soybean would allow for the simultaneous recovery of plants with single or multiple mutations in each member of a gene family of interest without disruption to the rest of the genetic back-
Targeted Mutagenesis of Gene Duplicates

RESULTS AND DISCUSSION

ZFN-Induced Mutagenesis of a GFP Transgene in Soybean Hairy-Root Tissues

To test the ZFN-mutagenesis system on endogenous soybean genes, we used the recently described CoDA platform to engineer eight ZFNs that target endogenous soybean genes involved in various aspects of RNA silencing (Table I; Supplemental Table S1). Based on homology to known genes in Arabidopsis, the targeted genes include Dicer-Like (DCL), RNA-Dependent RNA Polymerase (RDR), and HUA Enhancer1 (HEN1) family members (Margis et al., 2006; Wassenegger and Krczal, 2006; Yang et al., 2006). Soybean is a highly duplicated paleopolyploid plant (Schmutz et al., 2010), therefore some of the ZFNs
recognize duplicate gene copies. Three ZFN constructs were developed to simultaneously target two paralogous gene copies (two constructs targeting \textit{DCL1a}/\textit{DCL1b} and one construct targeting \textit{DCL4a}/\textit{DCL4b}) and five constructs were developed to independently target individual genes (\textit{DCL2a}, \textit{DCL2b}, \textit{RDR6a}, \textit{RDR6b}, and \textit{HEN1a}; Table I; Supplemental Table S1).

The hairy-root transformation method was used to evaluate ZFN mutagenesis of the nine endogenous soybean genes. As with the GFP ZFN, the CoDA ZFNs were driven by an estrogen-responsive promoter, and expression of each ZFN was induced by the introduction of estrogen in the tissue culture media. DNA from transgenic root tissues was screened via the enrichment PCR method described above to determine whether ZFN activity generated site-specific mutations. Briefly, DNA samples were digested by an appropriate restriction enzyme that recognizes the spacer sequence in the wild-type target site (Fig. 2A) to enrich for the mutated sequences and then PCR amplified. The resulting PCR products were subsequently digested and then visualized by agarose gel electrophoresis (Fig. 2B). If the site was mutated in some cells, then the PCR product would fail to digest a portion of the sample. Undigested DNA fragments were observed for five ZFN-transformed lines, indicating putative mutations in a total of seven gene targets (Table I; Fig. 2B). The undigested PCR products were cloned and sequenced and several distinct mutated alleles consisting of small insertions or deletions ranging from 1 to 20 bp were recovered (Fig. 3). We failed to recover mutations from the remaining three ZFN constructs (Supplemental Table S1).

To assess whether CoDA ZFNs can discriminate between closely related DNA sequences, we constructed two ZFNs that independently target the \textit{RDR6} homeo-

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**Table I.** The gene target accessions, target sequence, and RHs of CoDA ZFNs that generated mutations in the target genes

| Gene Name | Accession No. | Target Site | Spacer | RH (F1) | RH (F2) | RH (F3) |
|-----------|---------------|-------------|--------|---------|---------|---------|
| \textit{DCL1a} | Glyma03g42290 | cAGCAACCTCTTATAAGAGGGCGTGGg | 6 | TKQILGR | HKSSLTR | RHDQLTR |
| \textit{DCL1b} | Glyma19g45060 | cAGCAACCTCTTATAAGAGGGCGTGGg | 6 | TKQILGR | HKSSLTR | RHDQLTR |
| \textit{DCL4a} | Glyma17g11240 | iTGCTTCATCACAATGGAGATGATt | 5 | RGQELRR | QQTNLTR | VGSNLTR |
| \textit{DCL4b} | Glyma13g22450 | iTGCTTCATCACAATGGAGATGATt | 5 | RGQELRR | QQTNLTR | VGSNLTR |
| \textit{RDR6a} | Glyma04g07150 | aGGCAACGACATCAGAGGAGTGGAAa | 6 | LKDLRRL | HKSSLTR | DRPLQR |
| \textit{RDR6b} | Glyma06g07250 | aGGCAACGACATCAGAGGAGTGGAAa | 6 | LKDLRRL | HKSSLTR | DRPLQR |
| \textit{HEN1a} | Glyma08g08650 | aGCACCGCCACCCGCTGACTGACTCa | 5 | RSRLNL | RTDRLAR | ESGALRR |

\(^a\)Gene names are based on homology to previously characterized genes in Arabidopsis. \(^b\)Measured in nucleotides. \(^c\)Represents two duplicate copies ("a" and "b") that both perfectly match the ZFN target site.
These ZFNs differ only by the subsite bound by F3 of the right ZFA, which should enable them to discriminate between the 2 bp distinguishing the RDR6a and RDR6b target sites (Table I). Enrichment PCR using primers common to both genes recovered ZFN-induced mutations for the predicted ZFN/homeolog combinations. Importantly, no evidence of ZFN activity was observed at noncognate homeologous sites among 16 clones sequenced, indicating specificity of the ZFNs to their respective targets. PCR enrichment assays specific to each homeolog were performed to further validate the specificity of the respective ZFNs (Fig. 4). The results indicate that the targeted gene copy was mutagenized at a much higher frequency than the off-target copy. We cannot, however, rule out the possibility that some mutations may be occurring at the off-target homeologous gene, albeit at a much lower frequency than the targeted gene.

Taken together, we have shown that CoDA ZFNs are exceptionally potent and selective mutagenic agents. We have also shown that hair-root transformation is a rapid and reliable method for testing the function of CoDA ZFNs prior to the arduous task of soybean whole-plant transformation.

**Mutagenesis and Heritability of a Duplicated Gene Pair in Soybean**

Whole-plant transformation was attempted on approximately 100 explants to introduce the ZFN targeting both paralogous copies of the DCL4 gene (DCL4a and DCL4b) into soybean. We recovered three ZFN-transformed T0 seedlings from the hormone-treated explants. Additionally, a control group of explants was transformed without hormone induction to gauge potential ZFN toxicity or hormone effects on transformation (Supplemental Table S2).

DNA from true and unifoliate leaves from the three hormone-treated plants were screened by the enrichment PCR method to identify ZFN-directed mutations. Restriction enzyme-resistant PCR fragments were recovered from two of the three plants. The undigested PCR products were subsequently cloned and sequenced, revealing one T0 seedling with an adenine base insertion at the ZFN target site in the DCL4a locus (Supplemental Fig. S1A) and the other T0 seedling with a two-base thymine and adenine insertion at the DCL4b locus (Fig. 5A). The presence of unmutated DCL4a and DCL4b sequences suggested that only one allele from both DCL4a and DCL4b had been mutated, thus both plants were likely either heterozygous (DCL4a/dcl4a and DCL4b/dcl4b) or chimeric.

Both T0 plants were grown to maturity and seed harvested. While the plant with the dcl4b mutation appeared normal, the plant with the dcl4a mutation exhibited a severe developmental phenotype with large bulbous internodes and mostly undeveloped and aborted seeds. It is unclear whether this phenotype was due to the dcl4a mutation, insertional mutagenesis of the ZFN construct into a dosage-sensitive...
gene, off-target mutagenesis by the ZFN, or somaclonal variation induced by tissue culture. Only two viable seeds were harvested from the \( DCL4a/dcl4a \) T0 plant, neither of which showed compelling evidence for transmission of the \( dcl4a \) mutation. The detailed analyses of these plants are shown in Supplemental Figure S1.

The \( DCL4b/dcl4b \) plant produced approximately 500 seeds and these progeny were used to study the heritability of the ZFN-induced mutation. To test the germinal transmission of the \( dcl4b \) mutation, 24 T1 seedlings were grown and genotyped. A PCR assay using \( DCL4b \)-specific primers was carried out to determine if the mutation was heritable (Fig. 5B). The \( dcl4b \) mutation segregated exactly 1:2:1 in the T1 progeny, with six seedlings being homozygous for the mutation \( dcl4b/dcl4b \), 12 seedlings heterozygous \( DCL4b/dcl4b \), and six seedlings homozygous wild-type \( DCL4b/DCL4b \) (Fig. 5C). To confirm the genotyping, PCR was performed using \( DCL4b \)-specific primers on the genomic DNA of a T1 individual putatively homozygous for the mutation. The PCR product was cloned and 16 colonies were sequenced. Sequence data confirmed all colonies had the expected 2-bp insertion for \( dcl4b \) at the target site (Supplemental Fig. S2) and no wild-type allele was recovered from this assay. There was some evidence for increased lateral shoot growth in the \( dcl4b/dcl4b \) individuals, however no striking phenotypic alterations were observed. Further experimental replications with more detailed measurements will need to be performed to confirm and quantify the lateral shoot growth phenotype.

In using ZFNs as mutagens, it may be advantageous to remove the ZFN transgene from subsequent generations to minimize potential toxicity or additional rounds of mutagenesis. Transgene removal could be accomplished by normal genetic segregation. To look for this, the 24 T1 plants were PCR scored for the ZFN transgene. Only one of the T1 plants, a heterozygous

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**Figure 3.** Sequences of induced ZFN mutations in soybean hairy-root tissue. The recovered mutated alleles from seven soybean endogenous genes are shown below their respective wild-type sequence. The bold and underlined sequences represent the ZFN target sites of each wild type. Deletions and insertions are indicated by dashes or lowercase letters, respectively. Single roots often produced multiple independent mutations. The numbers to the side indicate the type of mutation and how many nucleotides were involved.

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**Figure 4.** The mutagenic specificity of the RDR6a and RDR6b ZFN transgenes was assessed by performing PCR enrichment assays with gene-specific primers for each homeolog. The ZFN target sites of this gene pair differ by only two nucleotides, so this experiment was important to measure whether the gene-specific ZFNs could discriminate between the homeologous targets (Table I provides details on the target site differences of the two ZFN transgenes). The PCR enrichment assays are analogous to those shown in Figure 2. A sample of estradiol-induced hairy roots was targeted for mutagenesis using the ZFN transgene targeting \( RDR6a \) (roots 1–4) and the ZFN transgene targeting \( RDR6b \) (roots 5–8). Primer sets differing at a single nucleotide were designed to allow for homeolog-specific PCR amplification in these samples (the polymorphic nucleotide is underlined in the reverse primer sequences). The top section shows the PCR enrichment results when testing for mutagenesis of gene \( RDR6a \) and the bottom section shows the PCR enrichment results when testing for mutagenesis of gene \( RDR6b \). In either case, an undigested top band indicates a mutation within the hairy-root sample. A digested nontransgenic root sample (WT/D) and an undigested nontransgenic root sample (WT/U) serve as controls. Seven out of the eight targeted hairy roots show the presence of the putative mutated top band; root 4 does not show this band, indicating that this sample either failed to transform or the ZFN failed to mutagenize any cells in this root. Faint top band shadows are observed in some samples for the nontargeted gene. Therefore, we cannot rule out the possibility that some mutations may have occurred at the nontargeted homeologous gene, albeit at a much lower frequency than the targeted gene.
The Identification of ZFN Target Sites in Soybean Using a Web-Based Tool

To aid in the identification of potential sites for ZFN engineering by CoDA, a version of ZFNGenome was implemented for soybean. ZFNGenome is a GBrowse-based (Stein et al., 2002) tool for identifying and visualizing potential target sites for CoDA ZFNs (Reyon et al., 2011). ZFNGenome provides researchers with information about each potential ZFN target site, including its chromosomal location, position relative to transcription initiation site(s), and frequency of occurrence within the genome. Users can query ZFNGenome using several different criteria (e.g. gene ID, transcript ID, or target site sequence). Targets identified using ZFNGenome can be visualized at multiple scales within the flexible GBrowse 1.7 environment and can be imported as annotations into other genome browsers. ZFNGenome is dynamically linked to the Zinc Finger Database (Fu et al., 2009), allowing users access to all available information about zinc-finger reagents, such as the effectiveness of a given ZFN in creating double-stranded breaks.

The ZFNGenome tool for soybean indicates that 36,714 out of 55,582 (approximately 66%) protein-encoding transcripts can be targeted by CoDA ZFNs. There is an average of 2.93 ZFN targets per coding transcript (107,665 target sites among the 36,714 coding transcripts).

ZFNGenome is freely available at http://bindr.gdcb.iastate.edu/ZFNGenome. The interface for the soybean genome is found at http://bindr.gdcb.iastate.edu/ZFNGenome/Soybean/.

CONCLUSION

In conclusion, we describe a rapid and highly specific method for generating gene mutations in a genetically redundant paleopolyploid crop species. Our data indicate that the CoDA-designed ZFN pairs have a high rate of success as mutagens, and their ease of construction should facilitate the development of additional applications in soybean, for example, to create targeted gene insertions or allelic replacements, both of which have been accomplished in other plant species (Shukla et al., 2009; Townsend et al., 2009). We anticipate that the CoDA platform will be widely adopted as an efficient and powerful functional genomics tool for soybean and other nonmodel plant species with highly duplicated genomes. Similar site-directed approaches, such as the transcription activator-like effector nuclease system (Christian et al., 2010; Li et al., 2011; Miller et al., 2011), may also be successful at simultaneously targeting single and paralogous loci in such genomes.

MATERIALS AND METHODS

Construction of OPEN and CoDA ZFAs and ZFN Expression Vectors

The GFP ZFN nuclease was engineered by the OPEN platform, and has been previously reported (Maeder et al., 2008). Endogenous soybean (Glycine

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**Figure 5.** ZFN mutagenesis and heritability in whole-plant soybean. A, Genomic structure of the DCL4b gene in soybean. The target site is highlighted by dashed lines with the box indicating the ZFN-induced dcl4b mutation (2-bp insertion indicated in bold) relative to the wild type. B, A schematic of the strategy used to determine the segregation frequency of the homozygous and heterozygous mutations in the T1 progeny. C, A gel depicting the segregation frequency of the homozygous and heterozygous mutations in 14 T1 plants is shown (PCR results for the remaining 10 plants are not shown). The top section shows the DCL4b genotype (+/+ indicates DCL4b/DCL4b, /-- indicates dcl4b/dcl4b, and +/- indicates heterozygous DCL4b/dcl4b). The middle section shows the genotyping result for the ZFN transgene (BAR amplicon) and the bottom section shows the PCR positive control (Actin amplicon). The induced dcl4b mutation segregated as expected in the 1:2:1 ratio. PCR confirmed that all T1 plants, with the exception of the wild-type control and one heterozygous plant (lane nine), harbored the ZFN transgene.
Hairy-Root and Whole-Plant Transformation of ZFNs in Soybeans and Screening for Mutations

Each ZFN binary construct was independently transformed into Agrobacterium rhizogenes strain K599 for hairy-root transformation. Soybean cotyledons were inoculated with the transformed K599 strain using a previously reported protocol (Govindaraju et al., 2008) to introduce the ZFN transgene into the hairy-root progenitor cells. For a more detailed hairy-root transformation protocol, see Supplemental Materials and Methods S1. The ZFN transgene was driven by an estrogen-inducible expression system (Zuo et al., 2000). Inoculated cotyledons were incubated on Murashige and Skoog medium plates treated with 10 μM of 17β-estradiol (Sigma-Aldrich). Approximately 1 to 2 weeks after transformation roots were randomly selected for DNA extraction using either a DNeasy (Qiagen) or a hexadecyltrimethylammonium bromide protocol (Curtin et al., 2008). Confirmation of the ZFN transgene in hairy roots was performed using PCR with a transgene-specific primer set (Supplemental Table S3). Mutations introduced by the ZFN disrupt the restriction site by insertion or deletion of DNA at the target sequence. PCR using primers designed to span the target site was carried out on pre-digested genomic DNA. Amplicons were then digested with a restriction enzyme that recognizes the restriction site by insertion or deletion of DNA at the target sequence. PCR

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